Human Umbilical Cord Mesenchymal Stem Cells attachment in the Hydroxyapatite-Tricalcium Phosphate Scaffold *in vitro*

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Abstract

Bone grafts in prosthodontics is required, particularly for socket preservation during denture fabrication. The hydroxyapatite-tricalcium phosphate (HA-TCP) scaffold is one of the materials that may be utilized to preserve sockets because it is employed as a bone regeneration substitute material. This scaffold has the features of being biocompatible, degradable, and non-toxic. To achieve biocompatibility, the scaffold must be readily linked to cells containing growth factors. Human Umbilical Cord Mesenchymal Stem Cells were used to seed the scaffold (HUCMSCs). In bone defect instances, a combination of HA-TCP scaffold material and hUCMSCs is likely to be used as bone remodeling treatment for socket preservation. Objective: this study aimed to investigate the attachment of hUCMSCs in the HA-TCP scaffold using a confocal microscope.

The scaffold HA-TCP samples were separated into four groups, and the control group was divided into two, namely HA-TCP 24 hours and HA-TCP 72 hours. The treatment group's sample was separated into two seeding times, 24 hours (P1) and 72 hours (P2) (P2). The sample was subsequently treated with bromodeoxyuridine (BrDU), and the quantity of hUCMSCs attachment was measured using a 400x magnification confocal lightning scanning microscope (CLSM). The Man-Whitney test was used with Kruskal-Wallis analysis to determine the difference between groups ($p<0.05$).

After seeding for 24 hours, hUCMSCS may be attached to the HA-TCP scaffold with a significant difference between the groups.

There is a substantial difference between the treatment and control groups, with 24 hours being the optimal shedding period.

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Introduction

Bone quality and quantity are critical elements in prosthetic therapy, particularly for implant osseointegration and early stability.[1] The damage induced by periodontal disease is one of the features of alveolar bone loss.[2] Occlusal trauma is the most common cause of bone injury.[3] Infectious microorganisms, mechanical stress, medicine, systemic metabolic disorder and pathological condition causes can all induce alveolar bone loss.[4-7] One of the elements that contribute to the effectiveness of implant therapy is an adequate volume of remaining alveolar bone. If an alveolar bone deficiency prevents implant therapy from succeeding, a person's quality of life will suffer owing to limits in speaking, mastication, and cosmetic processes.[8,9]

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Alveolar bone defects can be efficiently repaired with osteoconductive materials, which activate osteoprogenitor cells capable of creating bone.[10] The osteoconductive substance employed is a tissue engineering application idea that is still being researched as an active component capable of stimulating the integration and regeneration of the original bone tissue in the defect location, namely bone graft.[11] One concept of bone tissue engineering is the use of scaffolds that must have material biocompatibility properties in order to stimulate bone regeneration that supports the attachment, migration, proliferation, and differentiation of osteoprogenitor cells and osteoblasts or migrates from the periosteum around the tissue *in vivo*.[12]

The three fundamental components of bone tissue engineering are (1) an osteoconductive scaffold that stimulates host cell attachment, migration, and proliferation at the implant site; (2) osteoinductive proteins or growth factors capable of stimulating osteoprogenitor cells to differentiate and synthesize bone matrix minerals; and (3) osteogenic cells (osteoblasts) capable of synthesizing bone tissue.[13,14]

Van Meekeren created the first synthetic substance in 1892 when he used calcium sulfate to cure bone deformities.[15] Since then, bioceramics materials have been widely employed in humans as bone transplant alternatives. Hydroxyapatite (HA), which has a chemical composition and crystalline structure identical to human bone, is the most extensively utilized bioceramic material for bone transplant in humans.[16,17]

HA and numerous other calcium-based ceramic compounds may be thought of as bioactive chemicals that help bone tissue develop. However, HA is a non-resorbable and inert substance with high mechanical strength but low digestibility. Because HA is more crystalline than bone mineral, it is more stable and resistant to deteriorate when used in implants. Because of the complexity of this degrading process, bone abnormalities occur and the risk of bone fracture increases.[17,18]

The addition of β-tricalcium phosphate (β-TCP) has excellent biodegradability and osteoconductive capabilities, particularly osteoconductive qualities, and has the potential to be used as a bone tissue engineering scaffold material. The inclusion of β-TCP has the potential to serve as a carrier for cells and growth factors required for bone healing.[19] Unlike HA, β-TCP is quickly absorbed and creates new bone. Because its solubility is similar to that of bone mineral, β-TCP does not dissolve under physiological conditions but is absorbed by cells.[18,19]

Scaffold made from blood clam shells (*Anadara Granosa*) is one natural biomaterial that has the potential to be utilized as a candidate bone substitute material.[20] Scaffolding is a three-dimensional structure utilized as a buffer media to aid in the formation and creation of new regenerate tissue.[21] The HA-TCP hybrid scaffold was created utilizing blood clam shells (*A. granosa*) and a variety of hydrothermal synthesis techniques.[20]

Coccuses of Blood Blood clams (*A. granosa*) have the greatest calcium concentration of any shellfish, reaching 97 percent, and have the potential to be employed as a source of hydroxyapatite production.[20] Many blood clams are produced in Asia, one of which is in Indonesia, therefore this raw material is easily accessible. For bone formation, the scaffold must have linked and integrated pores, as well as neovascularization. Scaffolds should ideally be biocompatible, biodegradable, and non-toxic.[20,22] One of the ideal conditions for clinical application of a biomaterial is its non-toxicity.[23]

Human umbilical cord mesenchymal stem cells (HUCMSCs) are an alternative source of mesenchymal stem cells (MSCs) to bone marrow mesenchymal stem cells (BMMSCs) derived from umbilical cord tissue that surrounds blood vessels.[24,25] In addition to being simple to get, vast in quantity, able to multiply fast and having strong immunocompatibility, HUC-MSCs are a biological waste that is abandoned or underutilized.[26,27]

HUCMSCs have more basic cells than bone marrow, allowing them to develop into multipotent, non-hematopoietic cells that can repair themselves and differentiate into other cells such as osteoblasts, adipose tissue, and chondroblasts.[26,27] These HUCMSCs exhibit a fibroblast-like appearance. Despite being obtained from several umbilical compartments, all of these cells are multipotent MSCs. These cells have the potential to proliferate rapidly and have a longer doubling time than adult stem cells as a source of extraembryonic cells.[28] The viability of HUCMSCs culture further demonstrates that HUCMSCs may be cultured in

gelatine and its media. HUCMSCs with scaffold can regenerate the osteoporotic bone *in vivo.*[29-31]

Toxicity testing is a preliminary step in the biocompatibility process. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) test revealed that hUCMSCs can be used to investigate the biocompability of biomaterial.[32,33] Biocompatibility is the ability of a material to be utilised in the body. Biocompatibility can be tested in terms of local and systemic toxicity, as well as the capacity to trigger allergies and carcinogens. The biocompatibility test performed on this material is a main test in which the substance's cytotoxicity is evaluated using cell culture, one of which contains 5-bromo-2'-deoxyuridine (BrDu).[23,34,35]

BrdU is one of the materials that may be used to observe cell proliferation and can be incorporated into DNA during the S-phase of the cell cycle.[36] MSCs might be used as a cell source for cell-based treatment. Several cell labeling approaches were used to demonstrate MSC survival and migration, one of which was BrdU.[34] Proliferating cells have the ability to split asymmetric chromosomes during the self-renewal process, such that the old DNA strands become stem cells and the young cells create new strands to be separated into differentiated cells. Stem cells also operate to retain DNA labels by employing BrdU, which can split asymmetric chromosomes or divide slowly.[35]

Based on the foregoing, the hypothesis of this study is that there is an attachment property of scaffold HA-TCP in various stages of shedding to HUCMSCs *in vitro* using confocal microscope analysis. Furthermore, the purpose of this work was to investigate the attachment of HUCMSCs to HA-TCP utilizing a Confocal Laser Scanning Microscope (CLSM) and BrDU staining.

Materials and methods

This research is a laboratory experimental research with post test only control group design with simple random sampling. A total of 16 HA-TCP scaffold samples were divided into 4 groups, namely Control Group 1 (K1) namely HA-TCP only for 24 hours incubation, Control Group 2 (K2) namely HA-TCP only for 72 hours incubation, Treatment Group (KP1) namely HA-TCP by seeding UCMSCs during the 24 hours incubation, and Treatment Group 2 (KP2) which is seeding UCMSCs during the 72 hours incubation. This study protocol was obtained the research permission from Airlangga University Health Research Committee with approval number: 010/HRECC.FODM/1/2020.

HUCMSCs Cell Culture

The umbilical cord was taken from the baby's placenta which was cut 10 cm long, the piece was placed into a drum that had been coated with a sterile cloth. The umbilical cord was washed with a solution of Phosphate-Buffered Saline (PBS) to remove any adhering blood. The isolation procedure was carried out at the stem cell laboratory, Institute for Tropical Diseases, Airlangga University. Cut the umbilical cord to about 1 cm, to get Wharton's Jelly of 1 mm^3. Wharton's Jelly was carried out by trypsinase to separate the supernatant and the pellet. The pellets were taken after undergoing a centrifugation process to obtain cells. The pellet was added with Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) medium and resuspended. The medium containing single cells was then transferred to a petri disk and incubated at 37°C and 5% CO_2.[29]

The cells obtained should always be observed using a microscope. The time taken for cell confluence to occur is recorded. Medium is replaced every 3 days. Cells that were 80% confluent were in passage by removing the medium and then adding trypsin and then incubating for 5 minutes until the cells were released, then adding the growth medium and resuspended. The results of the resuspension were centrifuged for 5 minutes. After being centrifuged, the supernatant was discarded and the pellet was planted and incubated with 5% CO2 at 37°C. The cells used are cells in passage 4, which have been confirmed to be positive for CD73, CD90, CD105, negative for CD45 and CD34. Confirmed cells were evaluated using a Nikon TMS inverted microscope (Nikon, Minato, Japan) and were ready for further treatment.[30,31]

Scaffold HA-TCP

HA+TCP is produced from the synthesis of Anadara Granosa blood clam shell which is formed into a scaffold. Scaffolding begins with making powder graft from the shell of Anadara Granosa's blood clam. The shells of Anadara Granosa blood clams are cleaned, washed and cleaned first and then boiled for 30 minutes. The next stage is the shells are mashed using a mortar and pastle until smooth, then filtered using fine filter paper with a size of 100 mesh to get

finer particle formations. The blood clam shell powder that has been refined as much as 1 M is dissolved in 0.6 M $NH_4H_2PO_4$ liquid then stirred using a magnetic stirrer for 30 minutes then put into the reactor. Then the reactor is put into an electric oven and heated to a temperature of 200°C for 12 hours, after which the reactor is cooled at room temperature. The heated powder was then washed using aqua distillate in a magnetic stirrer again until the results were separated between the water and the powder, the indication was that the pH of the liquid returned to normal to 7. The final washing of the powder used methanol to form into HA particles during the drying process. The powder sample was then dried again using an electric oven at 50°C for 4 hours. Then the sample was sintered at 900°C for 3 hours to remove impurities and increase crystallinization.[20] The HA-TCP scaffold was then cut into pieces and then weighed each with a size of 1.5 micrograms. The HA-TCP scaffold was then shedding into HUCMSCs passage 6 cells as much as $1x10^5$ in 100µl, for 24 hours and 72 hours.

Confocal Laser Scanning Microscope (CLSM) examination

$1x10^5$ HUCMSCs passage 6 cells in 100 l of -MEM media that had been shed on the HA-TCP scaffold then added 10 µl of BrdU and then incubated for 24 hours and 72 hours in an incubator 37º and 5% CO_2. After incubation, the microplate containing the HA-TCP scaffold that had been shed in HUCMSCs cells was washed with 300 l/well PBS. After washing the PBS, anti-BrdU was added which had been dissolved in 300 l/well BSA, then incubated for 1 hour in an incubator at 37º and 5% CO_2.

After incubation, the HUCMSCs cells and the scaffold in the microplate were washed again with 300 l/well PBS. Then the cells were fixed with 4% paraformaldehyde (PFA) at 300 l/well for 5 minutes, then washed again with PBS and repeated twice. The cover slip in the bottom of the well is taken, then placed on a cover slip measuring 24x60 mm and then observed with a confocal lightning scanning microscope (Olympus FV1000, Tokyo, Japan) with 400x magnification, the number of cell attachments to the HA-TCP scaffold that has been calculated

Statistical Analysis

This research sample used various scaffolds HA-TCP doses with four samples in each group. Data being counted based on average and standard deviation. Statistical analysis was conducted by means of statistical package for social science (SPSS) 20.0 version (IBM corporation, Illinois, Chicago, USA). Man-Whitney continued with Kruskal-Wallis analysis was used to analyze the difference between groups ($p<0.05$).

Result

HUCMSCs attached in the HA-TCP scaffold was observed and counted with a confocal microscope at 400x magnification (Figure 1). The data above is said to be normally distributed using the Shapiro-Wilk test with a significance value of 0.025 ($p>0.05$) which was then continued with the homogeneity test. The results of the homogeneity test using Levene's Test showed a significance value of 0.000, meaning that the data was said to be inhomogeneous ($p< 0.05$).

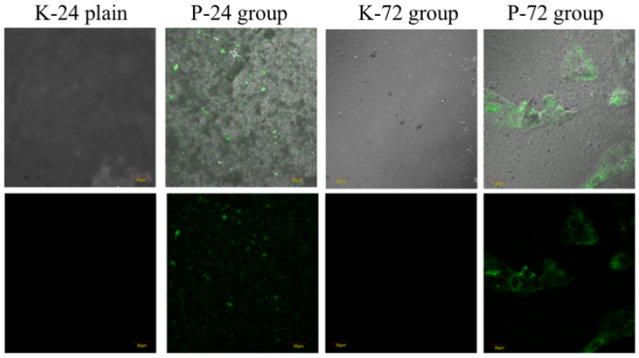

Figure 1. Attachment Cells on various sheeding.

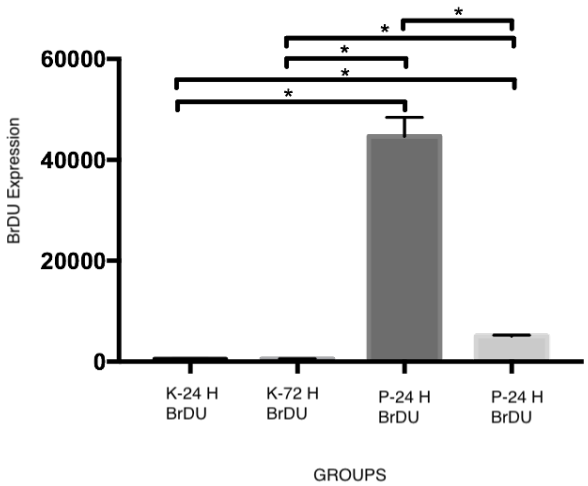

Figure 2. There is significant differences between the analyzed groups by means of kruskal wallis statistical analysis (*significant different between groups).

Groups (Time)	Mean±SD	Normality Test (*Shapiro-Wilk*)	Homogenity Test (Levene Test)	Mann-Whitney
K-24 H BrDU	499.75± 51.4	0.942		0.019**
K-72 H BrDU	509.00 ± 120.08	0.729		
P-24 H BrDU	44675.25 ± 3740.54	0.865	0.0001*	
P-72 H BrDU	5069.50 ± 213.15	0.913		

Table 1. The mean ± Standart Deviation (SD) and the Mann-Whitney test result attachment HUCMSCs to scaffold HA-TCP after 24 Hours and 72 Hours Seeding.
Information: *data was not homogenous revealed with Levene's test p<0.05; **significant different between groups p<0.05.

The inhomogeneous data was then retested using Kruskal-Wallis with a significance result > 0.05, it is said that between the 4 groups there was a significant difference (Table 1). There was a significant difference between the control group 24, 72 hours and the treatment group 24, and 72 hours significantly. The 24-hour HA-TCP scaffold group found a significant increase compared to the other groups (Figure 2).

Discussion

HUCMSCs activity continued even after the cells were sheathed utilizing the HA-TCP scaffold, as seen by the strong cell attachment exhibited, particularly in the P-24h and P-72h groups as compared to the K-24h and K-72h control groups. When compared to the P-72h group, the P-24h group had significantly more cell attachment to the scaffold. This is because the scaffold has solubility properties, so even after 24 hours, the cells are still attached to the scaffold, and after several days, the scaffold is biodegradable, so the number of cells attached to the scaffold decreases.[22] Cell attachment to the scaffold that happened in the P-24 H and P-72 H groups was caused by the pores in the scaffold, which give a site for cells to connect and generate new tissues.[37] Scaffold can help cells adhere, migrate, proliferate, and differentiate as they construct a new matrix.[22,38]

Because of the potential of these two materials to affect and encourage the attachment of osteoprogenitors to sites where bone injury occurs, the ability of HUCMSCs to proliferate and adhere to the HA-TCP scaffold plays a significant role in this work. When supplied to damaged bone conditions, the HA-TCP scaffold stimulates an increase in the number of bone cells, allowing the process of bone creation or bone tissue regeneration to proceed more quickly. In the case of bone injury, it is envisaged that the use of these two materials would result in quicker bone tissue regeneration than normal circumstances, which take a long time.[18,19]

HUCMSCs are a source of mesenchymal stem cells that are easy to acquire, plentiful, and easily cultured.[29,30] These HUCMSCs have the potential to develop into diverse cells, are multipotent, non-hematopoietic, repair themselves, and differentiate into other cells such as osteoblasts.[31-33] MSCs may adhere to the scaffold well 24 hours after shedding due to stem cell properties that allow them to recruit main cell precursors, allowing cells to easily attach to the scaffold. In conditions lasting longer than 24 hours, the number of cells connected to the scaffold is substantially lower, because one of the scaffold's features is that it degrades easily, resulting in a lower number of cell attachments. Cells may live under medium circumstances for 24 hours without being harmed; during this period, the cells generate cytokines and growth factors that act as mediators of cellular communication and drive cell differentiation or cell maturity.[12,38,39]

Cell metabolic activity utilizing tetrazolium salts such as MTTcan only be read using a spectrophotometer and is simple to compute, however this approach is less exact, can cause toxicity to the cells, and cells must be cultivated beforehand.32,33 Because BrDU does not cause DNA damage, it can be used in flow cytometry and immunohistochemistry studies.[34,35,39]

MSCs are multipotent cells that may differentiate into a variety of tissues.[40-42] MSCs can be derived from the placenta (umbilical cord) or the umbilical cord.[30-33] MSCs also have immunosuppressive, anti-inflammatory, and neurotropic capabilities, and they start the regenerative treatment process.[42-44] Several studies have identified stem cells and phenotypic traits using labeling approaches, one of which is BrDU. The labeling procedure is completely safe and does not interfere with MSC attachment, proliferation, or the production of growth factors, cytokines, or other immunological capacities.[34]

One of the scaffold's morphological forms, one of which must feature holes or microporosity that has already been evaluated using SEM.[12] This microporosity has several

advantages, one of which is the potential to develop as much as possible into bone, and it can also enhance the attachment of osteoprogenitors to new bone during the bone regeneration process.[12] The interaction of proteins on the tissue surface may still be the foundation for osteoblast attachment to the HA scaffold. Therefore, the size of the porosity is one of the elements that determine the attachment to cells and bone tissue.[17] Scaffolding can be used effectively within the first 24 hours of installation.[12]

MSCs are a cell source that might be used in cell-based treatment. Proliferating cells have the ability to split asymmetric chromosomes during the self-renewal process, such that the old DNA strands become stem cells and the young cells create new strands to be separated into differentiated cells.[44] Stem cells can also preserve DNA labels by employing 5-bromo-2-deoxyuridine (BrdU), which has the ability to split asymmetric chromosomes or divide slowly.[34,35]

The combination of scaffolds and HUCMSCs for the treatment of bone defects in the field of prosthodontics is still being researched because it is hoped that by administering this therapy, the bone healing process will occur faster than under normal conditions, allowing for faster socket preservation for prosthodontic therapy needs. However, this study result is limited only to cell attachment in the HA-TCP scaffold *in vitro*, further study is still required to investigate its interaction with other bone cells *in vivo.*

Conclusions

These findings show that the HA-TCP scaffold shed with HUCMSCs cells for 24 hours exhibited the greatest cell adhesion compared to the scaffold shed for 72 hours. Suggestions for future study include conducting more studies on the injection of these two substances to enhance the number of bone cells in vitro. Further research is required to analyze the various time series and progress to the in vivo stage of molecular markers connected to bone.

Declaration of Interest

The authors declare there is no conflict of interest within study.

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