BUKTI KORESPONDENSI
JURNAL NASIONAL BEREPUTASI

Judul Artikel : **(C16)** Bone remodeling using a three-dimensional chitosan-hydroxyapatite scaffold seeded with hypoxic conditioned human amnion mesenchymal stem cellse

Jurnal : Dental Journal (Majalah Kedokteran Gigi)
Penulis : ***Michael Josef Kridanto Kamadjaja****

No	Perihal	FEBRUARI Tanggal	Halaman
1	Bukti submit dan artikel yang disubmit	02 November 2020, 16 November 2020	1-3
2	Bukti Revisi 1	21 Januari 2022	4-46
3	Bukti Revisi 2	11 Februari 2021	47-59
4	Bukti Revisi 3	28 Februari 2021	60-72
5	Decision 1		
6	Decision 2		
7	Decision 3		
8	Decision 4		
9	Bukti accepted	29 Maret 2021	73-78
10	Bukti published		

Subject: Keterangan submit naskah
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Bone regeneration mechanism using *a three dimensional chitosan - hydroxyapatite scaffold* with human amnion mesenchymal stem cells.
Authors: Michael Josef Kridanto Kamadjaja

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Bone regeneration mechanismusing *a three dimensional chitosan - hydroxyapatite scaffold* with human amnion mesenchymal stem cells
***Authors*: Michael Josef Kridanto Kamadjaja**

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Form Penilaian naskah_Bone regeneration mechanism using a three dimensional chitosan.docx	34,3 KB
naskah_Bone regeneration mechanism using a three dimensional chitosan.docx	8,0 MB
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Kamadjaja.MJK_ContempClinDent94592-6438463_014718.pdf	2,1 MB
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review ke-1 naskah Josef_Bone regeneration using a three dimensional chitosan (PA1)-.pdf	916 KB
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FORMAT PENILAIAN NASKAH DENTAL JOURNAL HASIL PENELITIAN (untuk Penyunting Ahli)

Judul Naskah: **Bone regeneration using a three dimensional chitosan - hydroxyapatite scaffold seeded with human amnion mesenchymal stem cells**

Tanggal Kirim : 5 Januari 2021 Tanggal Kembali ke Redaksi : 11 Januari 2021

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Surabaya., 11 Januari 2021

Penyunting Ahli,

Bone regeneration using a three dimensional chitosan - hydroxyapatite scaffold seeded with human amnion mesenchymal stem cells

Abstract

Background: Bone regeneration studies involving the use of chitosan–hydroxyapatite scaffold seeded with human amnion mesenchymal stem cells have largely incorporated tissue engineering experiments. However, at the time of writing, the results of such investigations remain unclear. **Purpose**: The aim of this study was to explain the osteogenic differentiation of the scaffold chitosan–hydroxyapatite that is seeded with human amnion mesenchymal stem cells (hAMSC) in the regeneration of calvaria bone defect. **Materials and Methods**: Chitosan-hydroxyapatite (Ch-HA) scaffold was created by lyophilization method. 20 male Wistar rat subjects were randomly divided into two groups: control and treatment. Defects were created in the calvarial bone of the control and treatment group subjects, but a scaffold was subsequently implanted only in the treatment group members. After observation lasting 1 and 8 weeks, the subjects were terminated and examined histologically and immunohistochemically. **Result:** Angiogenesis; expression of vascular endothelial growth factor (VEGF); bone morphogenetic protein (BMP); RunX-2; alkaline phosphatase (ALP); type-1 collagen; osteocalcin and the area of new trabecular bone were all significantly greater in the tissue-engineered group compared to the control group. Statistical analysis was significant according to the results of an ANOVA test using SPSS software version 15.0. **Conclusion**: A combination of hAMSCs with Ch-HA scaffold demonstrated osteogenic differentiation in the re-generation of calvaria bone defects and, consequently, can serve as an alternative for the bone regeneration process.

Keywords: *human amniotic mesenchymal stem cells, chitosan-hydroxy apatite, scaffold, SEM images, bone tissue engineering.*

Introduction

Tissue engineering has been undertaken to remedy many medical conditions, for instance: complications arising from wound healing, bone defects, immune system responses, and donor-transmitted disease. Three dimensional scaffolds were created to provide adequate support forming an extracellular matrix that enables cells to proliferate and differentiate. Scaffold made from Chitosan – Carbonate Apatite (Ch-CA) has been reported as producing a robust, interconnected three-dimensional (3D) porous structure which could support the proliferation and differentiation of osteoblast during osteogenic differentiation.[1,2] The biocompatibility of hydroxyapatite (HA) and the resemblance of its mineral composition to bone has rendered it an ideal material for bone tissue engineering (BTE). The development of HA into a 3-dimensional (3D) scaffold or a

support to mesenchymal stem cells (MSC) *in vitro* has also been extensively explored. HA scaffolds offer massive advantages within the field of BTE. [3,4]

Human amniotic mesenchymal stem cells (hAMSCs) derived from human placentas are known for their pluripotent properties; ability to differentiate into three forms of germ layer; and efficacy in reducing both potential inflammation and immune reaction[5,6]. A combination of Chitosan-Hidroxy-apatite scaffold and hAMSCs was expected to intensify osteogenesis. The aim of this study was to observe the effect of Ch-HA scaffold seeding with hAMSCs within tissue engineering techniques.

Materials and Methods

Isolation and culture of human amniotic mesenchymal stem cells.

The isolation and culture procedure was performed following the securing of approval from the faculty's research ethics committee (No. 378/Panke. KKE/VII/2015). Material from a newly-formed amnion was peeled from the chorion and rinsed using phosphate-buffered saline (PBS). The amnion was then soaked in Ringer's lactate (RL) containing 2.5 µg/mL gentamycin and 1000 U/mL amphotericin which had been obtained from Gibco ™ Amphotericin B, New York, USA.

The isolation and culture of hAMSCs using a modified Soncino's protocol.Small, fine pieces of amniotic membrane were treated with 0.25% trypsin in order to remove the epithelial cells. Centrifugation of five minutes duration at 2,000 rpm was carried with the supernatant subsequently being removed. This procedure was then repeated. The supernatant was washed using PBS containing 0.075 mg/ml DNase 1 (Takara Bio, Shiga, Japan) and 0.75 mb/ml type IV collagenase (Sigma-Aldrich, St. Louis, MO, USA). Incubation of the amnion was performed at 37^0C for 60 minutes. Filtration and centrifugation lasting five minutes were performed to obtain cells. Single cells culture were created then using collagen–coated discs. The medium for the cells consisted of Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) at a ratio of 1:1, added to fetal bovine serum and 10 ng/mL human leukemia inhibitory factor (Gibco BRL, Gaithersburg, MD, USA). This medium was replaced every three days. Once the cell growth had reached a confluent stage (80%), the cell was split using trypsin. The laboratory stem cell protocol was implemented as the isolation procedure.

The chitosan-hydroxy apatite scaffold preparation

Chitosan-hydroxy apatite scaffold was prepared by dissolving 200mg of medium-molecular weight Ch powder (Sigma-Aldrich, St. Louis, MO, USA) into 5ml of ethanoic acid at room temperature and mixing them for 15 minutes. 15ml of sodium hydroxide solution was used for neutralizing purposes in obtaining chitosan gel. Furthermore, samples of the chitosan gel were mixed homogenously with 200mg of HA prior to centrifuging at 1,500 rpm for ten minutes. After extraction of excess water, the solution gel was placed into the specific mold to produce scaffolds. Before being transferred to a drying machine, the gel was frozen for two hours at -80^0 C.

Cell Proliferation measurement in Ch-HA scaffold

Human Amniotic Mesenchymal Stem Cells were deposited onto a 96-well cell culture plate (M96) at a density of 5×10^4 cell/well and incubated at 37^0C for 24 hours with CO_2 5% concentration. Once the cell proliferation population had reached 80%, Ch-HA scaffold was added together with 100 µL of growth medium. The cells underwent a second incubation at 37^0C for 20 hours with 5% CO_2. After the addition of 5 mg/mL MTT reagent (25 µL/well), the cells were incubated a second time for four hours before being observed under an inverted microscope. The scaffold and medium were removed and added to 200 µL/well DMSO. A 595 nm wave length ELISA reader was employed to read the absorbance, while the living cells were counted by means of a Cell Counting Kit.

SEM imaging

2% glutaraldehyde was used to fixate hAMSC-seeded on Ch-HA scaffold at 40^0C for 2-3 hours. The subsequent stage in the procedure consisted of washing with PBS solution three times every five minutes. After exposure to osmic acid 1% for 1-2 hours, the cells were washed again with PBS. A 15-20 minute dehydration procedure using alcohol at varying concentrations (30% - 100%) was also completed for each concentration. The scaffold was dehydrated using a critical point drying (CPD) device, attached to a stud pad with specific adhesive, and coated with pure gold. The scaffold was examined under a scanning microscope and photographed by means of a scanning electron microscope (JEOL JSM-T100, Japan).

Preparation of experimental subjects

20 male Wistar rats were used as the animal subjects of the experiment. The inclusion criteria applied were as follows: aged 8-12 weeks old and weighing 100-150 grams. The subjects were randomly divided into a control group and a treatment group of equal size which were observed during weeks 1 and 8.

Chitosan-hydroxy apatite scaffold implantation procedure in the calvarial bone of rats

An anaesthetic procedure was performed 4-6 hours after the subjects were denied further food and water. 20mg of Ketamin HCL (Ketalar, Ireland) per kg of body weight and 3mg of Xylazine (Xyla,Ireland) per kg of body weight were injected intramuscularly. A mid-longitudinal skin incision was then made on the cranium dorsal surface after an aseptic procedure had been completed. The periosteum of the cranium was separated from the surface in order to produce a flap. A 2 mm diameter, circular, low speed bur (NSK, Japan) was used to create the bone defect 5 mm in diameter. The scaffold was implanted and sutured in order to re-attach the wound area but only in the treatment group.

Termination of animal models and collection of research specimens.

The subjects were sacrificed during weeks 1 and 8 in order to obtain the required specimens. The implantation region was decalcified and embedded in paraffin to produce microscopic specimens. In order to highlight the angiogenesis and trabecular bone area, the specimens were stained with Hematoxylin and Eosin, while post-scaffold implantation immunohistochemical staining (using BMP2, RunX-2, Alkaline phosphatase, Type-1 collagen, Osteocalcin, and VEGF) of the specimens of cranium calvarial preparations was carried out. The Remmele Scale Index was used to measure the raw data. A Nikon H600L (Tokyo, Japan) light microscope and a DS Fi2 300-megapixel digital camera with image processing software (Nikon Image System) were respectively employed to examine the specimens and observe the tissue.

Statistical analysis

Results were presented as mean, values, and standard deviation. SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data by means of an ANOVA test and $p < 0.05$ was considered statistically significant.

Result

The chitosan–hydroxyapatite was a solid 3D scaffold 5mm in diameter and 2mm thick (Figure 1). Toxicity tests incorporating the use of MTT Assay indicated that Ch-HA scaffold was not harmful to the hAMSC culture. The percentage of viable cells found in the Ch-HA scaffold was 79.42. SEM imaging showed that cells were able to attach themselves to the Ch-HA scaffold's porous surface that was embedded in the calvaria bone defect (Figure 2). SEM imaging of seeded cells in the scaffold are shown on Figure 3. All groups were examined for expression of RunX2, alkaline phosphatase (ALP); collagen type 1; osteocalcin; angiogenesis; and bone trabecular formation after 8 weeks. All treatment groups possessed a higher mean value than the control group (Table 1).

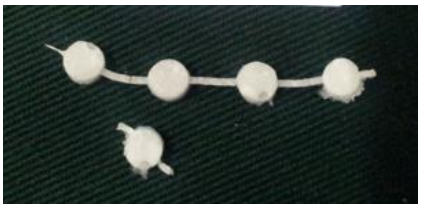

Figure 1:
Ch-HA scaffold

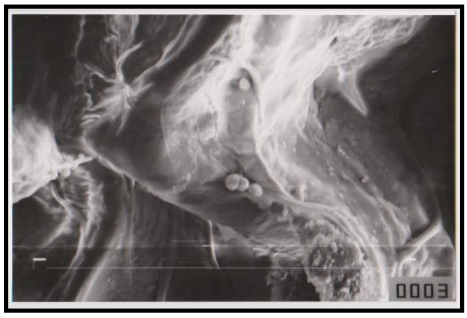

Figure 2:
SEM image of cells attached and proliferated into the scaffold pores
(SEM, 1000x magnification)

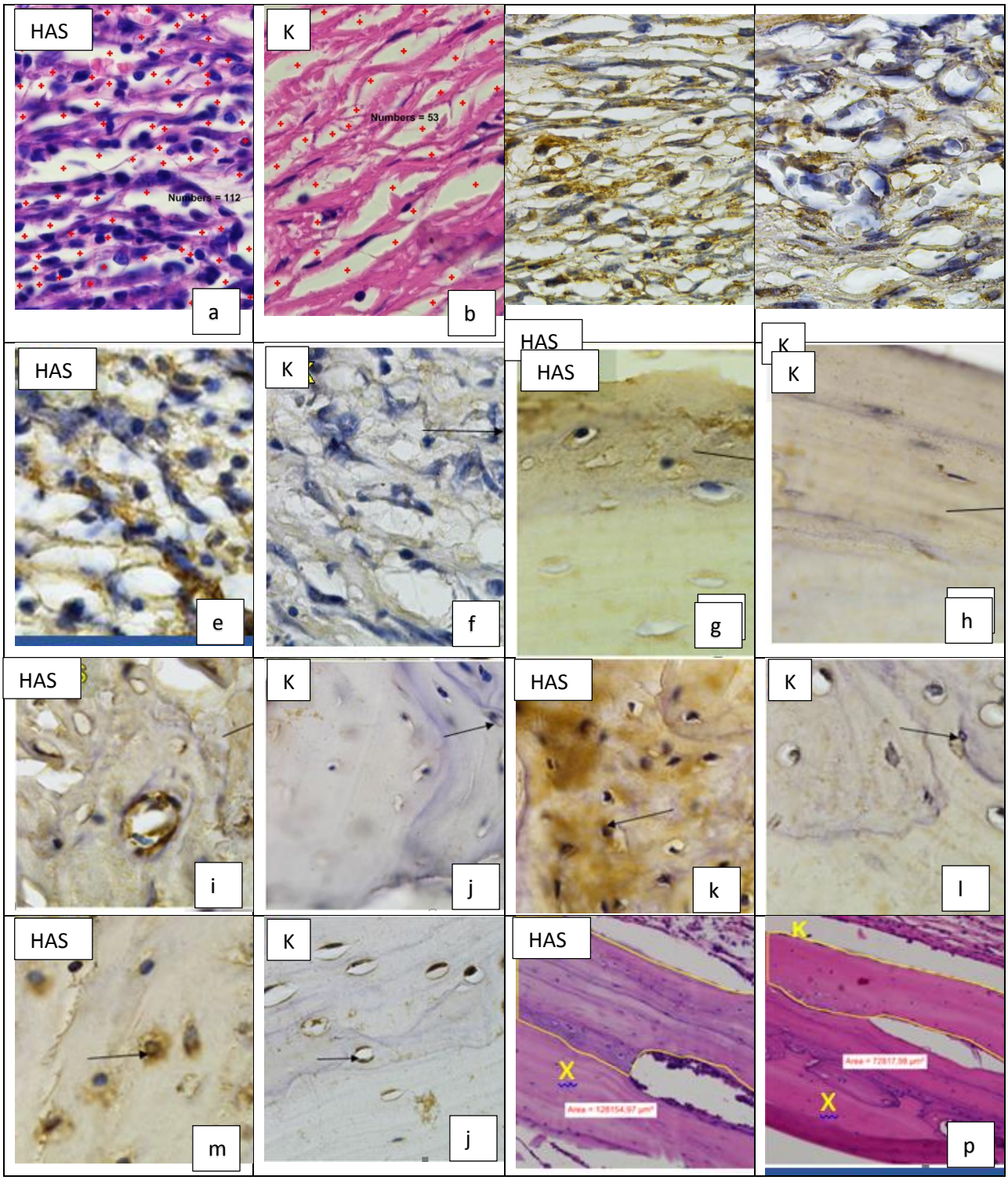

Figure 3: Angiogenesis (a and b), VEGF (c and d), BMP2 (e and f), RunX-2 (g and h), akaline phosphatase (i and j), type-1 collagen (k and l), osteocalcin (m and n) and trabecular bone area (o and p). HAS: hydroxy-apatite-chitosan scaffold group. K: control group.

Table 1: The mean value and standard deviation for several parameters

Parameter	K	HAS	P
Angiogenesis	190,20 ± 36,670[a]	569,60 ± 79,773	0.015
VEGF	5.4400±3.73915	7.6800±2.59461	0.443
BMP2	3.4400±0.74027	7.6800±3.49457	0.051
RunX-2	2.88±1.689	5.28±2.335	0.039
ALP	1.32 ± 0,228	4.72 ± 2.637	0.009
Type-1 collagen	2.92 ± 1.501	9.04 ± 1.486	0.004
Osteocalcin	4.52 ± 3.196	6.84 ± 2.321	0.005
Trabecular bone area	58,279.99 ± 5,769.328	105,138.85 ± 22,459.330	0.036

P<0.05 showed statistically significant.
K : control group; HAS: Hidroxy – apatite – chitosan scaffold group

Tambahkan grafik

Discussion

In this study, an attmept was made to combine chitosan with hidroxy-apatite in order to create Ch-HA scaffold. 200mg Chitosan and 200mg hidroxy-apatite were mixed using a lyophilization technique. Alkaline salt ions were eliminated through a process of desalination. In the experiment conducted, the scaffold characteristics included; retentiveness without brittleness, and a sponge-like rather than solid structure. SEM imaging showed that the Ch-HA scaffold had a three dimensional structure with a porous surface. hAMSC could attach to and proliferate effectively within the scaffold's porosity. The interconnected interstices of the scaffold were highly retentive and an excellent niche for osteoblast proliferation and differentiation. The ideal pore structure for tissue engineering scaffold ranged from 40-300 μm since this enabled vascular tissue migration and tissue growth[(2,7)].

The investigation was conducted to observe the interaction between scaffold and human osteoblast-like SaOS2 cells. SaOS2 cells were capable of adhering, proliferating and migrating to the surface of the scaffold. Furthermore, the number of living cells was higher on porous scaffold than dense HA[(9)]

The potential of hAMSCs to act as a form of xenogenic MSCs during bone tissue engineering procedures has been thoroughly investigated. Several studies utilizing xenogenic hAMSCs transplantation in various organs of rats confirmed a less intense immune reaction that could affect the tissue healing process. [(10-12)]

The first three days post-implantation consists the inflammatory phase, that is, the initial bone healing stage. During this phase, the hypoxic condition of the Ch-HA scaffold and the degranulation of platelets resulting from hematoma trigger increased VEGF expression that, in turn, induces angiogenesis which is essential in early healing processes. Functional capillary tissues provide nutritional intake, essential bioactive molecules, and adequate oxygen tension [13] Angiogenesis plays an important role in the healing process in bone defects because it ensures cell survival in the scaffold.[14] Mesenchymal stem cells placed in hypoxic conditions enhance the expression of angiogenic factors, mainly VEGF [15] Which methods showed that msc was placed in hypoxic condition? Should be added tin materials and methods

During the early stages of the regeneration process, the proliferation of MSC was followed by the differentiation of osteoblas. External signals produced by MSC and osteoblasts, particularly BMP2 protein, influence this regeneration process. In later stages, activation of transcription factor RunX-2 led by BMP2 helped induce MSC differentiation of preosteoblast and osteoprogenitors, which, in turn, continued to form a collagen and non-collagen bone matrix. [16]

The bone matrix maturation level was shown by the expression of type 1 collagen fibers. Mineralization within the bone matrix maturation process will be influenced by type 1 collagen in previous stages. If the maturation level of bone matrix increases, type 1 collagen fibers will also be thicker.

In this experiment, matured osteoblast marker was identified by osteocalcin. Osteoblast specifically expressed osteocalcin that is a non-collagen protein present in bone matrix. [17,18] In the treatment group the area of trabecular bone at the end of eight weeks was significantly higher compared to that of the control group, leading to the conclusion that new bone formation in the treatment group rate was higher than that in the control group.

The process of osteogenesis indicated by the expressions of ALP, type-1 collagen, and osteocalcin produced a better result in the treatment group compared to the control group. Therefore, the maturation level of bone matrix in the treatment group at the end of eight weeks was higher when compared to that of the control group. Mesenchymal stem cells could undergo differentiation to become osteoblasts, thereby producing the apropriate environment or stimulus. During osteogenic differentiation, several genes such as ALP, type 1 collagen, and osteocalcin

were expressed by MSCs. At the time, when osteoblasts turn into osteocytes, ALP activity decreases. The latest marker of mature osteoblasts expressed by osteocytes was osteocalcin.

In this study, certain limitations occurred, including lack of systematic complication. The purpose of this research was to focus on regeneration of calvarial bone defects using hAMSC and chitosan–hidroxyapatite scaffol. The study reported here should be continued to include research on their clinical application for bone augmentation.

Conclusion

Bone regeneration was more effective in the treatment group compared to the control group. The new trabecular bone area of treatment group members were found more solid than was the case with their control group counterparts. Combining Ch-HA scaffold and hAMSCs could be used as an alternative bone tissue engineering method in order to escalate the clinical use of bone formation.

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FORMAT PENILAIAN NASKAH DENTAL JOURNAL HASIL PENELITIAN (untuk Penyunting Ahli)

Judul Naskah: **Bone regeneration using a three-dimensional chitosan-hydroxyapatite scaffold seeded with human amnion mesenchymal stem cells**

Tanggal Kirim: Januari 2021 Tanggal Kembali ke Redaksi: 21 Januari 2021

HAL YANG DISUNTING	YA*	TIDAK*
1. Apakah naskah ini pernah dimuat pada media lain? **Keterangan:** naskah ini belum pernah dimuat pada media lain		V
2. Apakah judul tepat, singkat, jelas, dan menggambarkan kontribusi pengembangan keilmuan? (maksimal 10 kata, melingkupi variabel yang diteliti) **Keterangan:** Judul tepat, singkat, jelas, dan menggambarkan kontribusi pengembangan keilmuan, terdiri dari 14 kata dan melingkupi variabel yang diteliti). **Mohon dipertimbangkan untuk merubah judul (contoh judul dapat dilihat pada naskah)**	V	
3. Apakah pada naskah hasil penelitian:		
a) Pendahuluan mencakup latar belakang secara jelas? **Keterangan:** Pendahuluan masih belum mencakup latar belakang secara jelas		
b) Tujuan cukup jelas? **Keterangan:** Tujuan sudah cukup jelas		
c) Metode dan rancangan penelitian sesuai dengan tujuan penelitian? **Keterangan:** Metode dan rancangan penelitian sudah sesuai dengan tujuan penelitian,		
d) Prosedur penelitian diuraikan secara tepat dan rinci, sehingga menjamin validitas internal/ eksternal? **Keterangan:** Prosedur penelitian sudah diuraikan secara tepat dan rinci, sehingga menjamin validitas internal/ eksternal. Akan tetapi ada prosedur penelitian yang dilakukan tetapi tidak tertuang dalam abstrak.		
e) Hasil penelitian dapat menjawab *research question*?		

Keterangan: Hasil penelitian kurang dapat menjawab *research question* oleh karena tidak ada keterangan terkait satuan yang digunakan. Ada kerancuan dari hasil penelitian. (mohon dilihat pada naskah)		
f) Pembahasan • Apakah pembahasan tidak mengulang hasil? • Selaras dengan lingkup penelitian dan dibandingkan dengan hasil penelitian sejenis? • Menerangkan makna hasil penelitian dalam menjawab permasalahan? **Keterangan:** Pembahasan tidak mengulang hasil, cukup selaras dengan lingkup penelitian, kurang ada perbandingan dengan hasil penelitian sejenis serta kurang menerangkan makna hasil penelitian dalam menjawab permasalahan		
g) Acuan selaras dengan materi penelitian dan menggunakan literatur 10 tahun terakhir? **Keterangan:** Acuan selaras dengan materi penelitian dan beberapa acuan menggunakan literatur lebih dari 10 tahun terakhir		
HAL YANG DISUNTING	**YA***	**TIDAK***
h) Kesimpulan • Kesimpulan sesuai dengan judul, permasalahan? • Hasil penelitian memberi kontribusi untuk pengembangan Ilmu kedokteran gigi? • Melakukan sintesis berdasar hasil penelitian sejenis yang mendahului **Keterangan:** Kesimpulan tidak sesuai dengan judul serta permasalahan yang dikemukakan. Hasil penelitian dapat memberi kontribusi untuk pengembangan Ilmu kedokteran gigi. Tidak Melakukan sintesis berdasar hasil penelitian sejenis yang mendahului		
i) Pustaka perlu ditambahi/ dikurangi**)? **Keterangan:** Pustaka perlu ditambahi oleh karena masih terdapat acuan lebih dari 10 tahun		
4. Apakah ada bagian yang perlu ditambahi/ diringkas**)? **Keterangan:** Mohon dilihat pada naskah		

Catatan:
1. *) Bubuhkan tanda tilik (√), **) Coret yang tidak perlu
2. Koreksi dapat dilakukan langsung pada naskah
3. Apabila form keterangan kurang, mohon ditulis pada lembar tambahan

REKOMENDASI untuk KETUA PENYUNTING

[.....] 1. Naskah dapat dimuat tanpa perubahan.

[V] 2. Naskah dapat dimuat dengan perbaikan sesuai dengan arahan penyunting Ahli (saran perbaikan mohon ditulis langsung pada naskah)

Keterangan:

[…] 3. Naskah tidak dapat dimuat

Alasan: ……

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Surabaya, 21 Januari 2021

Penyunting Ahli,

Bone regeneration using a three-dimensional chitosan-hydroxyapatite scaffold seeded with human amnion mesenchymal stem cells

Abstract

Background: Bone regeneration studies involving the use of chitosan–hydroxyapatite scaffold seeded with human amnion mesenchymal stem cells have largely incorporated tissue engineering experiments. However, at the time of writing, the results of such investigations remain unclear. **Purpose**: The aim of this study was to explain the osteogenic differentiation of the scaffold chitosan–hydroxyapatite that is seeded with human amnion mesenchymal stem cells (hAMSC) in the regeneration of calvaria bone defect. **Materials and Methods**: Chitosan-hydroxyapatite (Ch-HA) scaffold was created by lyophilization method. 20 male Wistar rat subjects were randomly divided into two groups: control and treatment. Defects were created in the calvarial bone of the control and treatment group subjects, but a scaffold was subsequently implanted only in the treatment group members. After observation lasting 1 and 8 weeks, the subjects were terminated and examined histologically and immunohistochemically. **Result:** Angiogenesis; expression of vascular endothelial growth factor (VEGF); bone morphogenetic protein (BMP); RunX-2; alkaline phosphatase (ALP); type-1 collagen; osteocalcin and the area of new trabecular bone were all significantly greater in the tissue-engineered group compared to the control group. Statistical analysis was significant according to the results of an ANOVA test using SPSS software version 15.0. **Conclusion**: A combination of hAMSCs with Ch-HA scaffold demonstrated osteogenic differentiation in the re-generation of calvaria bone defects and, consequently, can serve as an alternative for the bone regeneration process.

Keywords: *human amniotic mesenchymal stem cells, chitosan-hydroxy apatite, scaffold, SEM images, bone tissue engineering.*

Introduction

Tissue engineering has been undertaken to remedy many medical conditions, for instance: complications arising from wound healing, bone defects, immune system responses, and donor-transmitted disease. Three dimensional scaffolds were created to provide adequate support forming an extracellular matrix that enables cells to proliferate and differentiate. Scaffold made from Chitosan – Carbonate Apatite (Ch-CA) has been reported as producing a robust, interconnected three-dimensional (3D) porous structure which could support the proliferation and differentiation of osteoblast during osteogenic differentiation. [1,2] The biocompatibility of hydroxyapatite (HA) and the resemblance of its mineral composition to bone has rendered it an ideal material for bone tissue engineering (BTE). The development of HA into a 3-dimensional (3D) scaffold or a support to

Commented [A1]: Change the title!
Example: Osteogenic differentiation of the chitosan–hydroxyapatite scaffold seeded with human amnion mesenchymal stem cells (hAMSC) of calvaria bone defect

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Commented [A4]: Age and weight of Wistar rat?

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mesenchymal stem cells (MSC) *in vitro* has also been extensively explored. HA scaffolds offer massive advantages within the field of BTE. [3,4]

Human amniotic mesenchymal stem cells (hAMSCs) derived from human placentas are known for their pluripotent properties; ability to differentiate into three forms of germ layer; and efficacy in reducing both potential inflammation and immune reaction [5,6]. A combination of Chitosan-Hidroxy-apatite scaffold and hAMSCs was expected to intensify osteogenesis. The aim of this study was to observe the effect of Ch-HA scaffold seeding with hAMSCs within tissue engineering techniques.

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Materials and Methods

Isolation and culture of human amniotic mesenchymal stem cells.

The isolation and culture procedure was performed following the securing of approval from the faculty's research ethics committee (No. 378/Panke. KKE/VII/2015). Material from a newly-formed amnion was peeled from the chorion and rinsed using phosphate-buffered saline (PBS). The amnion was then soaked in Ringer's lactate (RL) containing 2.5 µg/mL gentamycin and 1000 U/mL amphotericin which had been obtained from Gibco ™ Amphotericin B, New York, USA.

The isolation and culture of hAMSCs using a modified Soncino's protocol. Small, fine pieces of amniotic membrane were treated with 0.25% trypsin in order to remove the epithelial cells. Centrifugation of five minutes duration at 2,000 rpm was carried with the supernatant subsequently being removed. This procedure was then repeated. The supernatant was washed using PBS containing 0.075 mg/ml DNase 1 (Takara Bio, Shiga, Japan) and 0.75 mb/ml type IV collagenase (Sigma-Aldrich, St. Louis, MO, USA). Incubation of the amnion was performed at 37^0C for 60 minutes. Filtration and centrifugation lasting five minutes were performed to obtain cells. Single cells culture was created then using collagen–coated discs. The medium for the cells consisted of Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) at a ratio of 1:1, added to fetal bovine serum and 10 ng/mL human leukemia inhibitory factor (Gibco BRL, Gaithersburg, MD, USA). This medium was replaced every three days. Once the cell growth had reached a confluent stage (80%), the cell was split using trypsin. The laboratory stem cell protocol was implemented as the isolation procedure.

The chitosan-hydroxy apatite scaffold preparation

Chitosan-hydroxy apatite scaffold was prepared by dissolving 200mg of medium-molecular weight Ch powder (Sigma-Aldrich, St. Louis, MO, USA) into 5ml of ethanoic acid at room temperature and mixing them for 15 minutes. 15ml of sodium hydroxide solution was used for neutralizing purposes in obtaining chitosan gel. Furthermore, samples of the chitosan gel were mixed homogenously with 200mg of HA prior to centrifuging at 1,500 rpm for ten minutes. After extraction of excess water, the solution gel was placed into the specific mold to produce scaffolds. Before being transferred to a drying machine, the gel was frozen for two hours at -80^0 C.

Cell Proliferation measurement in Ch-HA scaffold

Human Amniotic Mesenchymal Stem Cells were deposited onto a 96-well cell culture plate (M96) at a density of 5 x 10^4 cell/well and incubated at 37^{0}C for 24 hours with CO_2 5% concentration. Once the cell proliferation population had reached 80%, Ch-HA scaffold was added together with 100 µL of growth medium. The cells underwent a second incubation at 37^{0}C for 20 hours with 5% CO_2. After the addition of 5 mg/mL MTT reagent (25 µL/well), the cells were incubated a second time for four hours before being observed under an inverted microscope. The scaffold and medium were removed and added to 200 µL/well DMSO. A 595 nm wave length ELISA reader was employed to read the absorbance, while the living cells were counted by means of a Cell Counting Kit.

SEM imaging

2% glutaraldehyde was used to fixate hAMSC-seeded on Ch-HA scaffold at 40^{0}C for 2-3 hours. The subsequent stage in the procedure consisted of washing with PBS solution three times every five minutes. After exposure to osmic acid 1% for 1-2 hours, the cells were washed again with PBS. A 15–20-minute dehydration procedure using alcohol at varying concentrations (30% - 100%) was also completed for each concentration. The scaffold was dehydrated using a critical point drying (CPD) device, attached to a stud pad with specific adhesive, and coated with pure gold. The scaffold was examined under a scanning microscope and photographed by means of a scanning electron microscope (JEOL JSM-T100, Japan).

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Preparation of experimental subjects

20 male Wistar rats were used as the animal subjects of the experiment. The inclusion criteria applied were as follows: aged 8-12 weeks old and weighing 100-150 grams. The subjects were randomly divided into a control group and a treatment group of equal size which were observed during weeks 1 and 8.

Chitosan-hydroxy apatite scaffold implantation procedure in the calvarial bone of rats

An anesthetic procedure was performed 4-6 hours after the subjects were denied further food and water. 20mg of Ketamin HCL (Ketalar, Ireland) per kg of body weight and 3mg of Xylazine (Xyla, Ireland) per kg of body weight were injected intramuscularly. A mid-longitudinal skin incision was then made on the cranium dorsal surface after an aseptic procedure had been completed. The periosteum of the cranium was separated from the surface in order to produce a flap. A 2 mm diameter, circular, low speed bur (NSK, Japan) was used to create the bone defect 5 mm in diameter. The scaffold was implanted and sutured in order to re-attach the wound area but only in the treatment group.

Termination of animal models and collection of research specimens.

The subjects were sacrificed during weeks 1 and 8 in order to obtain the required specimens. The implantation region was decalcified and embedded in paraffin to produce microscopic specimens. In order to highlight the angiogenesis and trabecular bone area, the specimens were stained with Hematoxylin and Eosin, while post-scaffold implantation immunohistochemical staining (using BMP2, RunX-2, Alkaline phosphatase, Type-1 collagen, Osteocalcin, and VEGF) of the specimens of cranium calvarial preparations was carried out. The Remmele Scale Index was used to measure the raw data. A Nikon H600L (Tokyo, Japan) light microscope and a DS Fi2 300-megapixel digital camera with image processing software (Nikon Image System) were respectively employed to examine the specimens and observe the tissue.

Statistical analysis

Results were presented as mean, values, and standard deviation. SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data by means of an ANOVA test and $p < 0.05$ was considered statistically significant.

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Result

The chitosan–hydroxyapatite was a solid 3D scaffold 5mm in diameter and 2mm thick (Figure 1). Toxicity tests incorporating the use of MTT Assay indicated that Ch-HA scaffold was not harmful to the hAMSC culture. The percentage of viable cells found in the Ch-HA scaffold was 79.42. SEM imaging showed that cells were able to attach themselves to the Ch-HA scaffold's porous surface that was embedded in the calvaria bone defect (Figure 2). SEM imaging of seeded cells in the scaffold are shown on Figure 3. All groups were examined for expression of RunX2, alkaline phosphatase (ALP); collagen type 1; osteocalcin; angiogenesis; and bone trabecular formation after 8 weeks. All treatment groups possessed a higher mean value than the control group (Table 1).

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Commented [A23]: bone trabecular formation or trabecular bone area? Which one is correct?

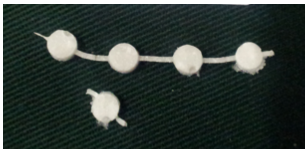

Figure 1:
Ch-HA scaffold

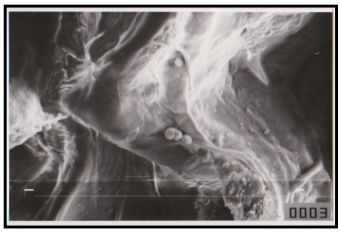

Figure 2:
SEM image of cells attached and proliferated into the scaffold pores (SEM, 1000x magnification)

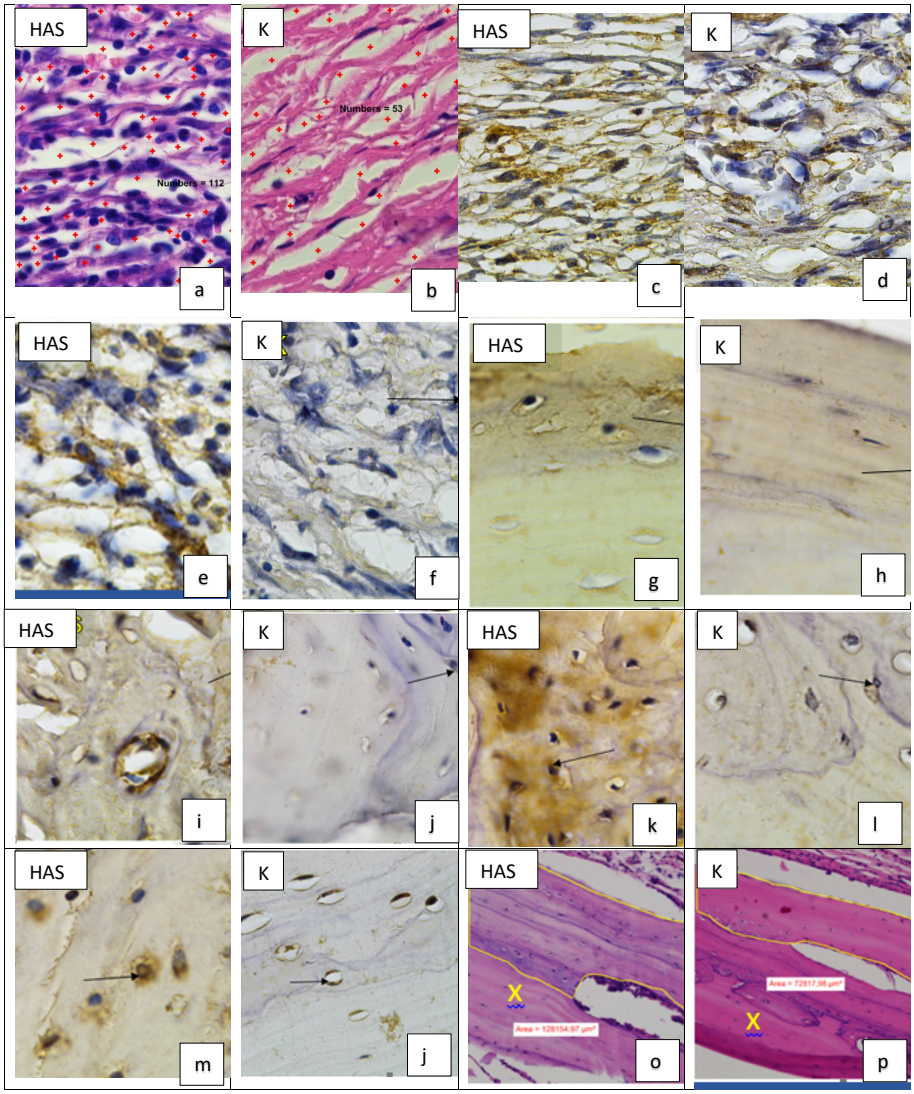

Figure 3: Angiogenesis (a and b), VEGF (c and d), BMP2 (e and f), RunX-2 (g and h), akaline phosphatase (i and j), type-1 collagen (k and l), osteocalcin (m and n) and trabecular bone area (o and p). HAS: hydroxy-apatite-chitosan scaffold group. K: control group.

Table 1: The mean value and standard deviation for several parameters

Parameter	K	HAS	P
Angiogenesis	190,20 ± 36,670[a]	569,60 ± 79,773	0.015
VEGF	5.4400±3.73915	7.6800±2.59461	0.443
BMP2	3.4400±0.74027	7.6800±3.49457	0.051
RunX-2	2.88±1.689	5.28±2.335	0.039
ALP	1.32 ± 0,228	4.72 ± 2.637	0.009
Type-1 collagen	2.92 ± 1.501	9.04 ± 1.486	0.004
Osteocalcin	4.52 ± 3.196	6.84 ± 2.321	0.005
Trabecular bone area	58,279.99 ± 5,769.328	105,138.85 ± 22,459.330	0.036

P<0.05 showed statistically significant.
K : control group; HAS: Hidroxy – apatite – chitosan scaffold group

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Discussion

In this study, an attmept was made to combine chitosan with hidroxy-apatite in order to create Ch-HA scaffold. 200mg Chitosan and 200mg hidroxy-apatite were mixed using a lyophilization technique. Alkaline salt ions were eliminated through a process of desalination. In the experiment conducted, the scaffold characteristics included; retentiveness without brittleness, and a sponge-like rather than solid structure. SEM imaging showed that the Ch-HA scaffold had a three dimensional structure with a porous surface. hAMSC could attach to and proliferate effectively within the scaffold's porosity. The interconnected interstices of the scaffold were highly retentive and an excellent niche for osteoblast proliferation and differentiation. The ideal pore structure for tissue engineering scaffold ranged from 40-300 µm since this enabled vascular tissue migration and tissue growth[2,7].

~~The investigation was conducted to observe the interaction between scaffold and human osteoblast-like SaOS2 cells. SaOS2 cells were capable of adhering, proliferating and migrating to the surface of the scaffold. Furthermore, the number of living cells was higher on porous scaffold than dense HA[9]~~

The potential of hAMSCs to act as a form of xenogenic MSCs during bone tissue engineering procedures has been thoroughly investigated. Several studies utilizing xenogenic hAMSCs transplantation in various organs of rats confirmed a less intense immune reaction that could affect the tissue healing process. [10-12]

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hAMSCs is human amnion mesenchymal stem cell..then why you said that hAMSCs is Xenogenic?

The first three days post-implantation consists the inflammatory phase, that is, the initial bone healing stage. During this phase, the hypoxic condition of the Ch-HA scaffold and the degranulation of platelets resulting from hematoma trigger increased VEGF expression that, in turn, induces angiogenesis which is essential in early healing processes. Functional capillary tissues provide nutritional intake, essential bioactive molecules, and adequate oxygen tension [13] Angiogenesis plays an important role in the healing process in bone defects because it ensures cell survival in the scaffold.[14] Mesenchymal stem cells placed in hypoxic conditions enhance the expression of angiogenic factors, mainly VEGF [15]

During the early stages of the regeneration process, the proliferation of MSC was followed by the differentiation of osteoblas. External signals produced by MSC and osteoblasts, particularly BMP2 protein, influence this regeneration process. In later stages, activation of transcription factor RunX-2 led by BMP2 helped induce MSC differentiation of preosteoblast and osteoprogenitors, which, in turn, continued to form a collagen and non-collagen bone matrix. [16]

The bone matrix maturation level was shown by the expression of type 1 collagen fibers. Mineralization within the bone matrix maturation process will be influenced by type 1 collagen in previous stages. If the maturation level of bone matrix increases, type 1 collagen fibers will also be thicker.

In this experiment, matured osteoblast marker was identified by osteocalcin. Osteoblast specifically expressed osteocalcin that is a non-collagen protein present in bone matrix. [17,18] In the treatment group the area of trabecular bone at the end of eight weeks was significantly higher compared to that of the control group, leading to the conclusion that new bone formation in the treatment group rate was higher than that in the control group.

The process of osteogenesis indicated by the expressions of ALP, type-1 collagen, and osteocalcin produced a better result in the treatment group compared to the control group. Therefore, the maturation level of bone matrix in the treatment group at the end of eight weeks was higher when compared to that of the control group. Mesenchymal stem cells could undergo differentiation to become osteoblasts, thereby producing the apropriate environment or stimulus. During osteogenic differentiation, several genes such as ALP, type 1 collagen, and osteocalcin were expressed by MSCs. At the time, when osteoblasts turn into osteocytes, ALP activity decreases. The latest marker of mature osteoblasts expressed by osteocytes was osteocalcin.

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In this study, certain limitations occurred, including lack of systematic complication. The purpose of this research was to focus on regeneration of calvarial bone defects using hAMSC and chitosan–hidroxyapatite scaffol. The study reported here should be continued to include research on their clinical application for bone augmentation.

Conclusion

~~Bone regeneration was more effective in the treatment group compared to the control group. The new trabecular bone area of treatment group members were found more solid than was the case with their control group counterparts~~. Combining Ch-HA scaffold and hAMSCs could be used as an alternative bone tissue engineering method in order to escalate the clinical use of bone formation.

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DAFTAR TILIK *MANAGING EDITOR*

Judul Naskah: **Bone regeneration mechanism using *a three dimensional chitosan - hydroxyapatite scaffold* with human amnion mesenchymal stem cells.**

Tanggal Kirim : …… Tanggal Kembali ke Redaksi : ……..

HAL YANG DISUNTING	KETERANGAN*)
FORMAT	
Apakah panjang naskah cukup memadai? ▪ (10-12 halaman, 1,5 spasi, dengan ukuran kertas HVS A4, *times new roman* ukuran font 12)	Ya/ Tidak
▪ Bagian-bagian isi naskah proporsional (Pembahasan lebih panjang dari Pendahuluan)	Ya/ Tidak
Judul	
▪ Sesuai dengan masalah, tujuan dan memuat variabel utama	Ya/ Tidak
▪ Tidak terlalu panjang (maksimal 10 kata) dengan huruf kecil diawali huruf kapital	Ya/ Tidak
Abstrak	
▪ Panjang < 250 kata, 1 spasi, terstruktur dalam 1 paragraf	Ya/ Tidak
▪ Kata kunci sesuai dengan variabel/konsep utama	Ya/ Tidak
▪ Kata kunci maksimal 5 kata/frase	Ya/ Tidak
▪ Abstrak terstruktur, satu paragraph, terdiri atas: latar belakang (*background*), tujuan (*purpose*), metode (*method*), hasil (*result*), kesimpulan (*conclusion*)	Ya/ Tidak
Acuan	
▪ Cara mengacu: sistem *Vancouver superscript*	Ya/ Tidak
Gambar dan tabel	
▪ Sesuai dengan gaya selingkung jurnal (kebenaran, kelengkapan judul dan keterangan/*legend*) dan dicantumkan acuannya	Ya/ Tidak
▪ Jumlah gambar/tabel pada research report dan *literature review* maksimal 4	Ya/ Tidak
▪ Jumlah gambar/tabel pada *case report* maksimal 8	Ya/ Tidak
▪ Gambar/tabel ditulis terpisah dengan teks	Ya/ Tidak

HAL YANG DISUNTING	KETERANGAN*)
Daftar Pustaka (Sistem *Vancouver superscript*)	
▪ Sesuai dengan gaya selingkung jurnal (sistem *Vancouver superscript*)	Ya/ Tidak
▪ Maksimal 10 tahun terakhir	Ya/ Tidak
▪ Acuan primer ± 70% (jurnal, buku, dokumen paten)	Ya/ Tidak
▪ Nomor / volume dan halaman jurnal sudah tercantum	Ya/ Tidak
▪ Edisi, penerbit, kota dan halaman buku sudah tercantum	Ya/ Tidak
▪ Urut pemunculan pada teks artikel	Ya/ Tidak
▪ Nama pengarang ditulis semua (tanpa et al)	Ya/ Tidak
▪ Konsistensi penyingkatan nama penulis	Ya/ Tidak
▪ Acuan dari internet cantumkan waktu pengacuan dan alamat *website*	Ya/ Tidak
▪ Cara menyingkat judul jurnal sesuai dengan indeks dental dan indeks medicus	Ya/ Tidak
BAHASA	
▪ Tidak enumeratif	Ya/ Tidak
▪ Tidak terjadi kesalahan ketik	Ya/ Tidak
▪ Ejaan baku	Ya/ Tidak
▪ Kalimat baku (subyek, predikat, obyek)	Ya/ Tidak
▪ Satu paragraf, satu pokok pikiran (>2 kalimat)	Ya/ Tidak
HASIL PENELITIAN	
FORMAT	
▪ Sistematika naskah hasil penelitian terdiri dari pendahuluan, bahan dan metode, hasil, pembahasan diakhiri kesimpulan, daftar pustaka.	Ya/ Tidak
Pendahuluan	
▪ Latar belakang empirik/teoritik	Ada/Tidak
▪ Masalah/tujuan	Ada/ Tidak
Bahan dan Metode	
▪ Rancangan (jenis, masa (waktu), tempat penelitian)	Ada/ Tidak
▪ Teknik pengambilan sampel	Ada/ Tidak

▪ Cara kerja penelitian	Ada/ Tidak
HAL YANG DISUNTING	**KETERANGAN*)**
▪ Analisa data	Ada/ Tidak
Hasil	
▪ Paparan data	Ada/ Tidak
▪ Analisa hasil	Ada/ Tidak
Pembahasan	
▪ Pembahasan tidak mengulang hasil	Ya/ Tidak
▪ Selaras dengan lingkup penelitian dan dibandingkan dengan hasil penelitian sejenis?	Ya/Tidak
▪ Menerangkan makna hasil penelitian dan menjawab permasalahan	Ya/Tidak
▪ Kesimpulan	Ada/Tidak
▪ Saran	Ada/Tidak
LAPORAN KASUS	
FORMAT	
▪ Sistematika naskah *Case Report* terdiri dari pendahuluan, tatalaksana kasus, pembahasan diakhiri kesimpulan, dan daftar pustaka. ▪ Abstrak terstruktur satu paragraf terdiri atas: latar belakang (*background*), tujuan (*purpose*), kasus (*case*), tatalaksana kasus (*case management),* kesimpulan (*conclusion*)	Ya/ Tidak

Catatan:

1. *) Coret yang tidak perlu
2. Apabila tidak ada kesesuaian antara penulis dan penyunting seyogyanya dipertemukan untuk mendapatkan solusi.

REKOMENDASI *MANAGING EDITOR* (PILIH SALAH SATU)

[.....] 1. Naskah dapat dimuat tanpa perbaikan oleh penulis

[.....] 2. Naskah dapat diproses dengan perbaikan oleh penulis, yaitu pada bagian :
(saran perbaikan mohon ditulis langsung pada naskah)

Bone regeneration mechanism using *a three dimensional chitosan - hydroxyapatite scaffold* with human amnion mesenchymal stem cells.

Abstract

Background : Experimental study of bone tissue engineering, an alternative to autogenous bone graft showed promising result, however its healing mechanisms and effectiveness has not been fully understood until today. **Purpose** :. The aim of this study is to describe the difference in bone healing mechanism and effectiveness between chitosan - hidroxy apatite seeding with hAMSC in the regeneration of calvaria bone defect. **Materials and Methods** : Chitosan-hydroxyapatite scaffold was created by lyophilization method. Twenty Whistar male rats were randomly divided into two groups : control and treatment.Defects were created in the calvarial bone of each rat in control and treatment group, but a scaffold was subsequently implanted only in treatment group. After 8 weeks, the rats were terminated for histology and immunochemistry examination. **Result:** Angiogenesis, expression of vascular endothelial growth factor, alkaline phosphatase, type-1 collagen, osteocalcin and new trabecular bone area were significantly greater in the tissue-engineered group than that in the control group. **Conclusion** : These result showed that combination of hAMSCs with Ch-HA scaffold could become one of the alternative for bone tissue engineering.

Keywords: *human amniotic mesenchymal stem cells, chitosan-hydroxy apatite, scaffold, SEM images, bone tissue engineering.*

Introduction

Tissue engineering technique has been attempted to solve many problems, such as complications in wound healing, insufficient supply of bone, immunogenic reaction and disease transmission from donor tissues and fluids. Three dimensional scaffolds have been created to provide adequate support as extracellular matrix that allows the cells to proliferate and differentiate. Chitosan-Carbonate Apatite (Ch-CA) scaffold is reported to have an interconnnected, non fragile three-dimensional (3D) porous structure and the ability to support proliferation and differentiation of osteoblast [1,2] The biocompatibility and resemblance of hydroxyapatite (HA) to the mineral composition of the bone has rendered HA a potential candidate in bone tissue engineering (BTE). Application of hydroxyapatite as a material to develop a 3-dimension scaffold or carrier to support mesenchymal stem cells in vitro has also been investigated. Thus, HA based scaffolds have tremendous potential in bone tissue engineering application [3,4]

Commented [HP1]: Apakah sesuai dengan isi artikel?? Apakah ada ttg bagaimana mekanisme dari scaffold yang dipakai dalam study ini terhadap bone regeneration????????

Commented [HP2]: Background harap diperbaiki. Dalam backround ini, penulis tdk menjelaskan permasalahan yang dihadapi yang sesuai dengan judul artikel.

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Dalam Tujuan ada point tentang "mekanisme", tapi apakah dalam kesimpulan, ada simpulan ttg mekanisme??????????????????????????????????????

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Human amniotic mesenchymal stem cells (hAMSCs) from human placenta have been known for their pluripotent properties, the ability to differentiate itself into three forms of germ layer whilst having anti inflammatory and low immunogenicity properties [5,6]. Combination of Chitosan-Hidroxy-apatite scaffold and hAMSCs were expected to increase the new bone formation. The aim of this study is to examine the effect of Ch-HA scaffold that seeding with hAMSCs in tissue engineering technique.

Materials and Methods

Isolation and Culture of human amniotic mesenchymal stem cells.

This procedure was performed with the approval of the health research ethics commitee (No. 378/Panke. KKE/VII/2015). The fresh amnion was mechanically skinned from the chorion and was washed three times with phosphate-buffered saline (PBS) to remove excess blood before being soaked in Ringer's lactate containing 2,5 µg/mL gentamycin (Gibco ™ Gentamicin, New York, USA) and 1000 U/mL amphotericin (Gibco™ Amphothericin B, New York, USA).

Isolation and culture of hAMSCs was performed at the Stem Cell Research and Development Center. A modified Soncini's protocol was employed for the isolation procedure. The amniotic membrane was cut into small fine pieces and subjected to 0,25% trypsin to remove epithelial cells. The supernatant was removed after 5 minutes of centrifugation at 2000 rpm., in which this protocol was done twice. The supernatant was washed with PBS containing 0,75 mg/mL of type IV collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 0,075 mg/ML Dnase 1 (Takara Bio, Shiga, Japan), incubated at 37 C for 60 minutes. The cells were obtained after filtration and 5 minute centrifugation. Single cells were then cultured on collagen-coated discs using Dulbecco's minimal Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) (1:1) medium (Gibco BRL, Gaithersburg, MD, USA), supplemented by human leukemia inhibitory factor (10 ng/mL) and fetal bovine serum (Gibco BRL). The medium was changed every 3 days and when the confluence reached 80% cell spliting was performed using trypsin. The isolation procedure used was according to the laboratory of stem cell protocol.

The chitosan-hydroxy apatite scaffold preparation

200 mg of medium-molecular weight Ch powder (Sigma-Aldrich, St.Louis, MO, USA) was dissolved in 5 ml acetic acid in room temperature, mixed for 15 min, neutralized using 15 ml NaOH solution to obtain chitosan gels. Then 200 mg of HA were homogenously mixed with the chitosan gels and centrifuged at 1500 rpm for 10 min. The excess water was extracted and an impression was made to produce a scaffold. The gel was frozen at -80^{0}C for 2 hours before transferred to a drying machine.

Cell Proliferation measurement in Ch-HA scaffold

Human Amniotic Mesenchymal Stem Cells were added into culture well 96 (M96) to a density of 5 x 10^4 cell/well and incubated for 24 hours in 37^{0}C and CO_2 5%. After getting 80% of cell proliferation, Ch-HA scaffold was put into the well and 100 µL of medium was added to each well. The cells were incubated again for 20 hours in 37^{0}C and CO_2 5%. 5mg/mL of MTT reagent (25 µL/well) was added to each well, incubated for 4 hours and was examined under inverted microscope. Scaffold and medium were removed and sDMSO was added to each well (200 µL/well). The absorbance was read by using Elisa reader at 595 nm and the number of living cells were determined utilizing MTT assay using Cell Counting Kit.

SEM Images of Ch-HA scaffold

Scaffold Ch-HA that has been seeded with hAMSC, fixated with glutaraldehyde 2% for 2-3 hours at 40^{0}C, washing by PBS solution 3 times for each 5 minutes. The solution subsequently was changed by osmic acid 1% for 1-2 hours and continued washing with PBS solution like before. Dehydration with multiple concentrated alcohol like 30%, 50%, 70%, 80%, 90% and absolute, 15-20 minute for every concentration. The following solution was Amyl acetate absolute as preservative solution until it becomes dry. Scaffold was dried with critical point drying (CPD) device. After sticking to pad stud (holder) with special glue and coating with pure gold with vacuum evaporator, scaffold was ready to examine and photo with scanning electron microscope (JEOL JSM-T100 Scanning Microscope, Japan)

Preparation of experimental animals

The research reported here had been received approval from the Health Ethics Committee of the Faculty of Veterinary Medicine No. 49-KE. The animal subjects of the experiment were twenty male Wistar rats, aged between 8 to 12 weeks and weighing 100-150 gram. The rats were

divided equally into two groups: the treatment group and the control group, for randomized treatments. Each group was subdivided into further two goups: the first group was performed for 1 week and the other performed for 8 weeks.

Chitosan-hydroxy apatite scaffold implantation procedure in rat's calvaria bone.

The animal subjects were fasted from food for 4-6 hours before the anesthetic procedure. Ketamine HCL (Ketalar, Ireland) at a dose of 20 mg/kg of body weight and xylazine premedication (Xyla, Ireland) at 3 mg/kg body weight was injected intramuscularly. An aseptic procedure and mid-longitudinal skin incision on the dorsal surface of the cranium were carried out. A flap was cut untill the periosteum was released from the cranium surface. The bone defect site of 5 mm diameter was created to every rats using a low-speed contra-angle handpiece (NSK, Japan) with a 2mm thick round burr. The scaffold implanted into the defect site only for the treatment group and suturing was done to reattach the wound area.

Termination of experimental animals and collection of research specimens.

The animal rats were sacrificed in 1 week and 8 weeks to obtain the required specimens. The area of bone around the implantation was separated from the surrounding soft tissue. Decalcification and embedding in paraffin were completed for the manufacture of microscopic specimens. Hematoxylin and Eosin staining was performed to highlight the angiogenesis and the bone trabecular area. A secong staining was performed for further examination, including immunohistochemical imaging using anti-rabbit vascular endothelial growth factor (VEGF) polyclonal antibody (ABIN, USA), anti-rabbit bone morphogenetic protein 2 (BMP2) polyclonal antobody (ABIN, USA), anti-human Runx-2 monoclonal antibody (Cruz Biotech, USA), anti-human osteocalcin monoclonal antibody (Novus Biological, USA), anti-human collagen type 1 monoclonal antibody (Novus Biological, USA), and anti-human alkaline-phosphatase monoclonal antibody (Novus Biological, USA) on the surface of cranium calvarial preparations post scaffold implantation. The raw data were measured using a Remmele scale index. The specimens were thwn inspected by means of a light microscope (Nikon H600 L, Tokyo,Japan) equipped with a digital camera DS Fi2 300 megapixel and image processing sofware Nikon Image System.

Statistical analysis

Data from the experiment described above were expressed as mean values ± deviation standard. Statistical significance was determined by means of ANOA using SPSS software version 15.0 (SPSS, Inc., Chicago, IL., USA) and $p<0,05$ was considered statistically significant.

Results

The scaffolds were solid 3D structures of 5 mm diameter and 2 mm thickness. Toxicity tests with MTT Assay showed that Ch-HA scaffold was not toxic to hAMSC culture. The number of viable cells in Ch-HA scaffold was 79,42% The result of examination and photo with SEM showed that cells could attached to the porosity surface of the scaffold Ch-HA (Figure 1) implanted into the injury model of the calvarial bone defect. After 8 weeks, the expressions of VEGF, BMP2, RUNX2, alkaline phosphatase (ALP), collagen type 1, osteocalcin, angiogenesis, and bone trabecular width were observed. Microscopic results of this study are shown in Figure 2.

In all treatment groups, the mean values were higher than in the control group. The results of statistical analysis are also shown in Table 1.

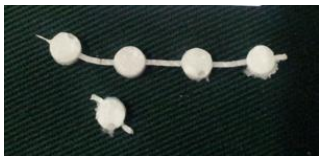

Figure 1:
Ch-HA scaffold

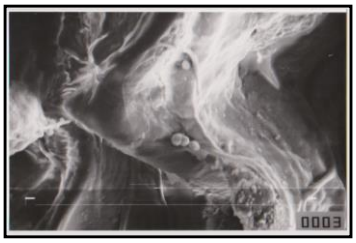

Figure 2:
Cell could attach and proliferate among the pores of the scaffold (SEM, 1000x magnification)

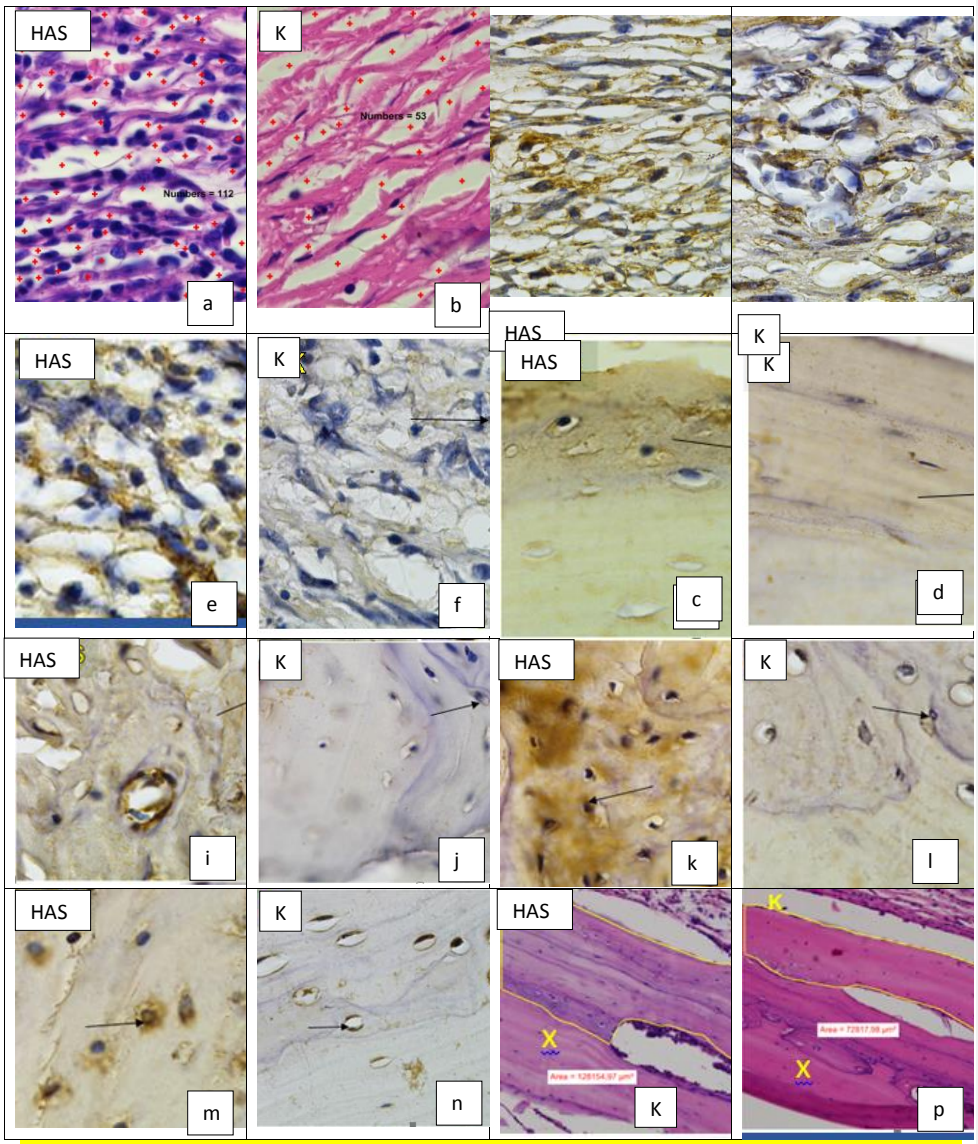

Figure 3 : Microscopic picture of sample at x 1000 magnification for angiogenesis (a and b), expression of Vascular Endothelial Growth Factor (c and d), Bone Morphogenetic Protein 2 /BMP 2 (e and f), RUNX2 (g and h), alkaline phosphatase (i and j), type 1 collagen (k and l), osteocalcin (m and n), and trabecular bone area (o and p). HAS is the hydroxy-apatite-chitosan scaffold treatment group and K is the control group.

o

Table 1 : The mean value and standard deviation of experiments on various parameters.

Parameter	K	HAS	P
Angiogenesis	190,20 ± 36,670	569,60 ± 79,773	0,000[a]
VEGF	5.44±3.79	7.68±2.59	0.443
BMP2	3.44±0.74	7.68±3.49	0,051
RUNX2	2.88±1.69	5.28±2.33	0,062
ALP	1.32±0.23	4.72±2.64	0,038[b]
Type 1 collagen	2.92±1.50	9.04±1.49	0,012[c]
Osteocalcin	4.52±3.19	6.84±2.32	0,026[d]
Trabecular bone area	58279,99 ± 5769,33	105138,85 ± 22459,33	0,002[e]

The $\rho<0,05$ is considered statistically significant[abcde]

HAS: Hidroxy-apatite-chitosan scaffold group; VEGF: Vascular endothelial growth factor; BMP2: Bone morphogenetic protein 2; ALP: Alkaline phosphatase

Discussion

In the present study, we attempted to combine chitosan with hidroxy-apatite in making Ch-HA scaffold. We mixed 200 mg Chitosan and 200 mg hidroxy-apatite based on lyophilization technique. Desalination was conducted to remove alkaline salt ions. The result was scaffold that had retentive form without brittleness, sponge-like and not solid. Based on SEM images it was showed that Ch-HA scaffold had three dimensional structure with many pores and hAMSC could attached and proliferated among the porosity of the scaffold. The interconnected porous structure of the scaffold had good retentive forms and ability to support the proliferation and differentiation of osteoblasts. Scaffolds used for tissue engineering ideally should have porous structure with pore size ranging from 40-300 µm allowing tissue in growth and vascular tissue migration [(2,7)].

The biological response of the scaffold was investigated using human osteoblast like SaOS2 cells. The results showed that SaOS2 cells were able to adhere, proliferate and migrate into pores of scaffold. Furthermore, the cell viability was found to increase on porous scaffold compared to dense HA[(9)]

The potential of hAMSCs as a form of xenogenic MSCs in bone tissue engineering procedure has been increasingly investigated. Several studies uitilizing xenogenic hAMSCs transplantation in may organs of rats revealed no significant immunologic response that could affect tissue healing processes.[(10-12)]

The early stage of healing process in bone defects begins with inflammatory phase, occuring within the first 3 days post implantation. In the inflammatory phase, the occurence of

platelet degranulation in the hematoma and hypoxic conditions within the Ch-HA scaffold triggers an increase in VEGF expression that induces angiogenesis. The occurrence of angiogenesis is essential in the early healing process, because functional capillary tissue will ensure adequate oxygen tension, nutritional intake and bioactive molecules [13]. It was found in this study, that an increase in VEGF expression and capillary numbers occured in the treatment group compare to the control group. Angiogenesis has an important role in cell survival in the scaffold as a bridge to the healing process in bone defects [14]. Previous research has also shown, that if MSC is in a hypoxic microenvironment, it will increase the production of angiogenic factors, especially VEGF [15]

During early stages of the regeneration process, MSC proliferation occurs followed by an osteoblastic differentiation process, which is influenced by external signals that produced by MSC and osteoblasts, particularly BMP2 protein. In the later stages, BMP2 leads to the activation of transcription factor RUNX2 to regulate MSC differentiation toward osteoprogenitor and preosteoblast, which serves to form a collagen and non collagen bone matrix [16].

Examination of the expression of type 1 collagen fibers are performed to asses the maturation level of bone matrix. In later stages the type 1 collagen will undergo mineralization as part of bone matrix maturation process. The lower thickness of type 1 collagen fibers indicates the higher maturation level of bone matrix and vice versa.

Osteocalcin is a non collagen protein in bone matrix specifically expressed by osteoblasts, which in this case is used as a matured osteoblast marker [17,18]. The formation of new trabecular bone is the result of calvarial bone defect. The area of trabecular bone in the treatment group was significantly greater than that in the control group at the end of th 8^{th} weeks. This finding concludes that the rate of new bone formation in the tissue-engineered group is higher than that in the control group.

The analysis result of ALP, type-1 collagen and osteocalcin expressions, which represent an osteogenesis process, confirmed a higher increase in treatment group compared to the control group. This situation indicates that the maturation level of bone matrix in the control group was lower than in the treatment group at the end of 8^{th} weeks. MSC could differentiate into osteoblasts, given the apropriate environment or stimulus. MSCs would express several genes such as ALP, type 1 collagen and osteocalcin, while engaging in osteogenic differentiation. The

increase in those expressions indicated the occurrence of osteogenic differentiation. Once the osteoblast turns into osteocytes, ALP activity would decrease. Osteocalcin was thought to be the ultimate marker of mature osteoblasts that appear on osteocytes [19]. The result of this study was consistent with other studies, that showed which under certain induction condition, stem cells isolated from the human amnion membrane could exhibit osteoblast differentiation [20]

There are some limitations in this study, including the risk of complication systematically. This research only focused on the bone regeneration of the calvarial bone defect using hAMSC and Ch-HA scaffold. This study should be continued further included applied as future mandible augmentation.

Conclusion

Bone regeneration more effectively in the tissue-engineered group compare to the control group. The new trabecular bone area in the later stages of healing of the calvarial bone defects in the rats were greater in the treatment group than the control group. Combining application of Ch-HA sca ffold and hAMSCs could be suggested as a novel bone tissue engineering for provoking bone formation in clinical use.

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Subject: Pemberitahuan revisi ke-2
From: "Dental Journal (Majalah Kedokteran Gigi)" <dental_journal@fkg.unair.ac.id>
Date: 11/02/2021 18.30
To: josef_310563@yahoo.com

Kepada Dr. Josef

Berikut terlampir review ke-2 dari reviewer, mohon merevisi sesuai komentar dan memberi highlight warna pada perubahan yg dilakukan.
Revisi mohon dikirim kembali paling lambat tanggal 17 Feb 2021.
Terimakasih.

Salam,

Attachments:

[review 2] Josef Bone regeneration using a three dimensional chitosan.docx 8,2 MB

Bone remodeling using a three dimensional chitosan - hydroxyapatite scaffold seeded with hypoxic conditioned human amnion mesenchymal stem cells

Michael Josef Kridanto Kamadjaja
Department of Prosthodontics, Faculty of Dental Medicine, Universitas Airlangga, Surabaya – Indonesia

Abstract

Background: Bone regeneration studies involving the use of chitosan–hydroxyapatite scaffold seeded with human amnion mesenchymal stem cells have largely incorporated tissue engineering experiments. However, at the time of writing, the results of such investigations remain unclear. **Purpose**: The aim of this study was to determine the osteogenic differentiation of the scaffold chitosan–hydroxyapatite that is seeded with hypoxia conditioned human amnion mesenchymal stem cells (hAMSCs) in the regeneration of calvaria bone defect. **Methods**: hAMSCs were cultured in hypoxia environment (5% oxygen, 10% carbon dioxide, 15% nitrogen) and seeded on the scaffold. Cell proliferation measurement was also being observed. Chitosan-hydroxyapatite (Ch-HA) scaffold of 5 mm diameter and 2 mm height was created by lyophilization and desalination method. Twenty male Wistar rat subjects (8 – 10 weeks, 200 - 250 grams) were randomly divided into two groups: control and treatment. Defects (similar size to scaffold size) were created in the calvaria bone of the control and treatment group subjects, but a scaffold was subsequently implanted only in the treatment group members. Control group left without treatment. After observation lasting 1 and 8 weeks, the subjects were terminated and examined histologically and immunohistochemically. **Result:** Angiogenesis; expression of vascular endothelial growth factor (VEGF); bone morphogenetic protein (BMP); RunX-2; alkaline phosphatase (ALP); type-1 collagen; osteocalcin and the area of new trabecular bone were all significantly greater in the tissue-engineered group compared to the control group. Statistical analysis was significant according to the results of an ANOVA test ~~using SPSS software version 15.0.~~ **Conclusion**: All osteogenic marker and blood vessels marker were significantly increased in treatment group compared to control group. Therefore, a combination of hypoxia conditioned hAMSCs with Ch-HA scaffold demonstrated osteogenic differentiation in the re-generation of calvaria bone defects and, consequently, can serve as an alternative for the bone regeneration process.

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Keywords: *human amniotic mesenchymal stem cells, chitosan-hydroxy apatite scaffold, ~~SEM images~~, bone tissue engineering*

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Introduction

Tissue engineering has been undertaken to remedy many medical conditions, for instance: complications arising from wound healing, bone defects, immune system responses, and donor-transmitted disease. Three dimensional scaffolds were created to provide adequate support forming an extracellular matrix that enables cells to proliferate and differentiate. Scaffold made

from Chitosan – Carbonate Apatite (Ch-CA) has been reported as producing a robust, interconnected three-dimensional (3D) porous structure which could support the proliferation and differentiation of osteoblast during osteogenic differentiation.[1,2] By combining chitosan and carbonate apatite into scaffold, this material was expected to increase its mechanical strength and reduce the degradation time. The biocompatibility of hydroxyapatite (HA) and the resemblance of its mineral composition to bone has rendered it an ideal material for bone tissue engineering (BTE). The development of HA into a 3-dimensional (3D) scaffold or a support to mesenchymal stem cells (MSC) *in vitro* has also been extensively explored. HA scaffolds offer massive advantages within the field of BTE.[3,4]

Human amniotic mesenchymal stem cells (hAMSCs) derived from human placentas are known for their pluripotent properties; ability to differentiate into three forms of germ layer; and efficacy in reducing both potential inflammation and immune reaction.[5,6] ~~A combination of~~ Chitosan-Hydroxyapatite scaffold seeding with hAMSCs was expected to intensify osteogenesis. The aim of this study was to observe the effect of Ch-HA scaffold seeding with hAMSCs within tissue engineering techniques.

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Materials and Methods

Isolation and culture of human amniotic mesenchymal stem cells.

The isolation and culture procedure were performed following the securing of approval from the faculty's research ethics committee (No. 378/Panke. KKE/VII/2015). Material from a newly-formed amnion was peeled from the chorion and rinsed using phosphate-buffered saline (PBS). The amnion was then soaked in Ringer's lactate (RL) containing 2.5 µg/mL gentamycin and 1000 U/mL amphotericin which had been obtained from Gibco ™ Amphotericin B, New York, USA.

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37^0C for 60 minutes. Filtration and centrifugation lasting five minutes were performed to obtain cells. Single cells culture was created then using collagen–coated discs. The medium for the cells consisted of Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) at a ratio of 1:1, added to fetal bovine serum and 10 ng/mL human leukemia inhibitory factor (Gibco BRL, Gaithersburg, MD, USA). This medium was replaced every three days. Once the cell growth had reached a confluent stage (80%), the cell was split using trypsin. The laboratory stem cell protocol was implemented as the isolation procedure. hAMSCs were cultured in hypoxia chamber (1% oxygen, 5% carbon dioxide, and 94% nitrogen).

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Chitosan-hydroxy apatite scaffold was prepared by dissolving 200mg of medium-molecular weight Ch powder (Sigma-Aldrich, St. Louis, MO, USA) into 5ml of ethanoic acid at room temperature and mixing them for 15 minutes. 15ml of sodium hydroxide solution was used for neutralizing purposes in obtaining chitosan gel. Furthermore, samples of the chitosan gel were mixed homogenously with 200mg of HA prior to centrifuging at 1,500 rpm for ten minutes. After extraction of excess water, the solution gel was placed into the specific mold to produce scaffolds (5 mm diameter and 2 mm height). Before being transferred to a drying machine, the gel was frozen for two hours at -80^0 C.[1,2]

Cell Proliferation measurement in Ch-HA scaffold

Human Amniotic Mesenchymal Stem Cells were deposited onto a 96-well cell culture plate (M96) at a density of 5 x 10^4 cell/well and incubated at 37^0C for 24 hours with CO_2 5% concentration. Once the cell proliferation population had reached 80%, Ch-HA scaffold was added together with 100 µL of growth medium. The cells underwent a second incubation at 37^0C for 20 hours with 5% CO_2. After the addition of 5 mg/mL MTT reagent (25 µL/well), the cells were incubated a second time for four hours before being observed under an inverted microscope. The scaffold and medium were removed and added to 200 µL/well DMSO. A 595 nm wave length ELISA reader was employed to read the absorbance, while the living cells were counted by means of a Cell Counting Kit.[1,2]

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SEM imaging

2% glutaraldehyde was used to fixate hAMSCs-seeded on Ch-HA scaffold at 40^0C for 2-3 hours. The subsequent stage in the procedure consisted of washing with PBS solution three times every five minutes. After exposure to osmic acid 1% for 1-2 hours, the cells were washed again with PBS. A 15-minute dehydration procedure using alcohol at varying concentrations (30% - 100%) was also completed for each concentration. The scaffold was dehydrated using a critical point drying (CPD) device, attached to a stud pad with specific adhesive, and coated with pure gold. The scaffold was examined under a scanning microscope and photographed by means of a scanning electron microscope (JEOL JSM-T100, Japan).[1,2]

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Preparation of experimental subjects

20 male Wistar rats were used as the animal subjects of the experiment. The inclusion criteria applied were as follows: aged 8-12 weeks old and weighing 100-150 grams. The subjects were randomly divided into a control group and a treatment group of equal size which were observed during weeks 1 and 8.

Chitosan-hydroxy apatite scaffold implantation procedure in the calvaria bone of rats

An anaesthetic procedure was performed 4-6 hours after the subjects were denied further food and water. 20mg of Ketamin HCL (Ketalar, Ireland) per kg of body weight and 3mg of Xylazine (Xyla,Ireland) per kg of body weight were injected intramuscularly. A mid-longitudinal skin incision was then made on the cranium dorsal surface after an aseptic procedure had been completed. The periosteum of the cranium was separated from the surface in order to produce a flap. A 2 mm diameter, circular, low speed bur (NSK, Japan) was used to create the bone defect 5 mm in diameter. The scaffold was implanted and sutured in order to re-attach the wound area but only in the treatment group.[1,2]

Termination of animal models and collection of research specimens.

The subjects were sacrificed during weeks 1 and 8 in order to obtain the required specimens. The implantation region was decalcified and embedded in paraffin to produce microscopic specimens. In order to highlight the angiogenesis and trabecular bone area, the specimens were stained with Hematoxylin and Eosin, while post-scaffold implantation immunohistochemical staining (using BMP2, RunX-2, Alkaline phosphatase, Type-1 collagen, Osteocalcin, and VEGF) of the specimens of cranium calvaria preparations was carried out. The Remmele Scale Index was used to measure the raw data. A Nikon H600L (Tokyo, Japan) light microscope and a DS Fi2 300-megapixel digital camera with image processing software (Nikon Image System) were respectively employed to examine the specimens and observe the tissue.[2]

Statistical analysis

The data were presented as mean values, and standard deviation. SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data by means of an ANOVA test and $p < 0.05$ was considered statistically significant.

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Result

The chitosan–hydroxyapatite was a solid 3D scaffold 5mm in diameter and 2mm thick (Figure 1). Toxicity tests incorporating the use of MTT Assay indicated that Ch-HA scaffold was not harmful to the hAMSCs culture. The percentage of viable cells found in the Ch-HA scaffold was 79.42 %. SEM imaging showed that cells were able to attach themselves to the Ch-HA scaffold's porous surface that was embedded in the calvaria bone defect (Figure 2). SEM imaging of seeded cells in the scaffold are shown on Figure 3. All groups were examined for expression of RunX2, alkaline phosphatase (ALP); collagen type 1; osteocalcin; angiogenesis; and trabecular bone area after 8 weeks. All treatment groups possessed a higher mean value than the control group (Table 1).

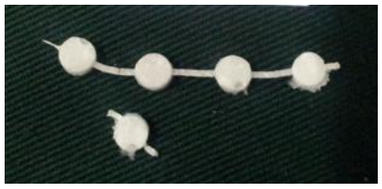


Figure 1:
Ch-HA scaffold

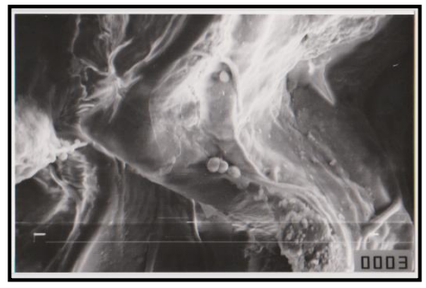
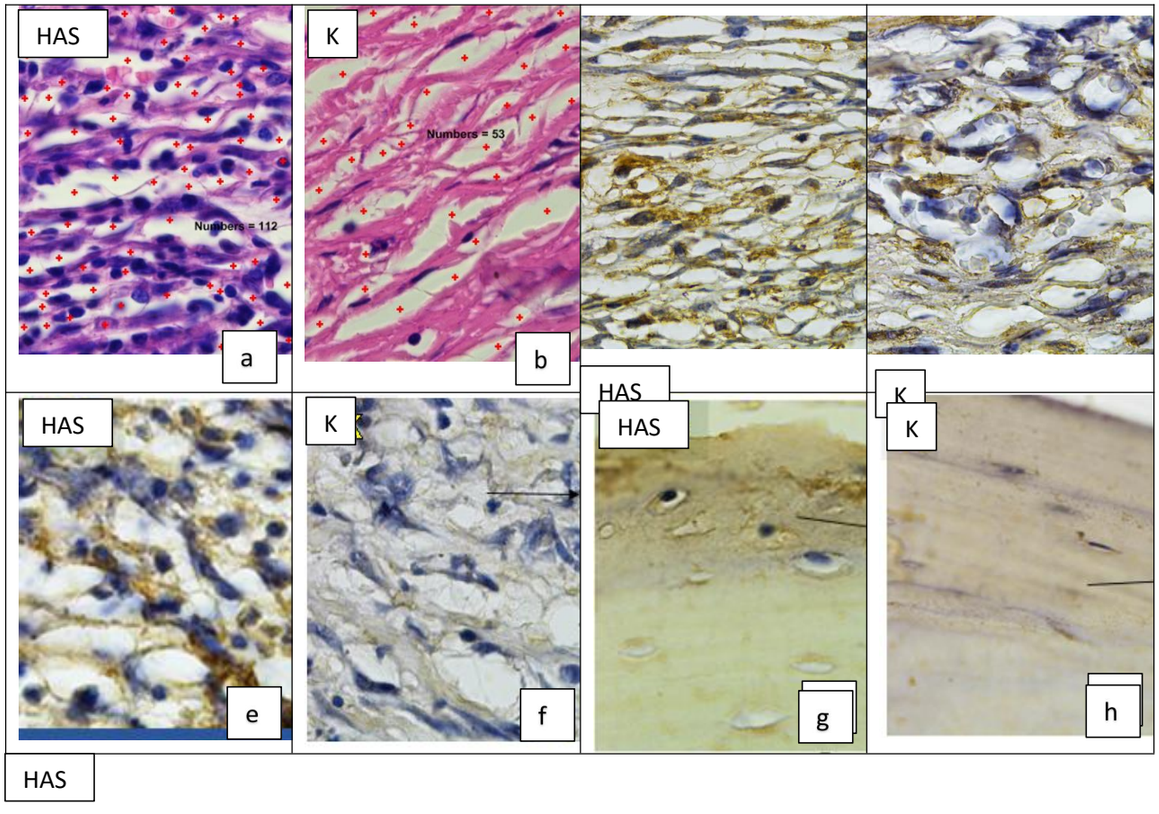


Figure 2:
SEM image of cells attached and proliferated into the scaffold pores
(SEM, 1000x magnification)



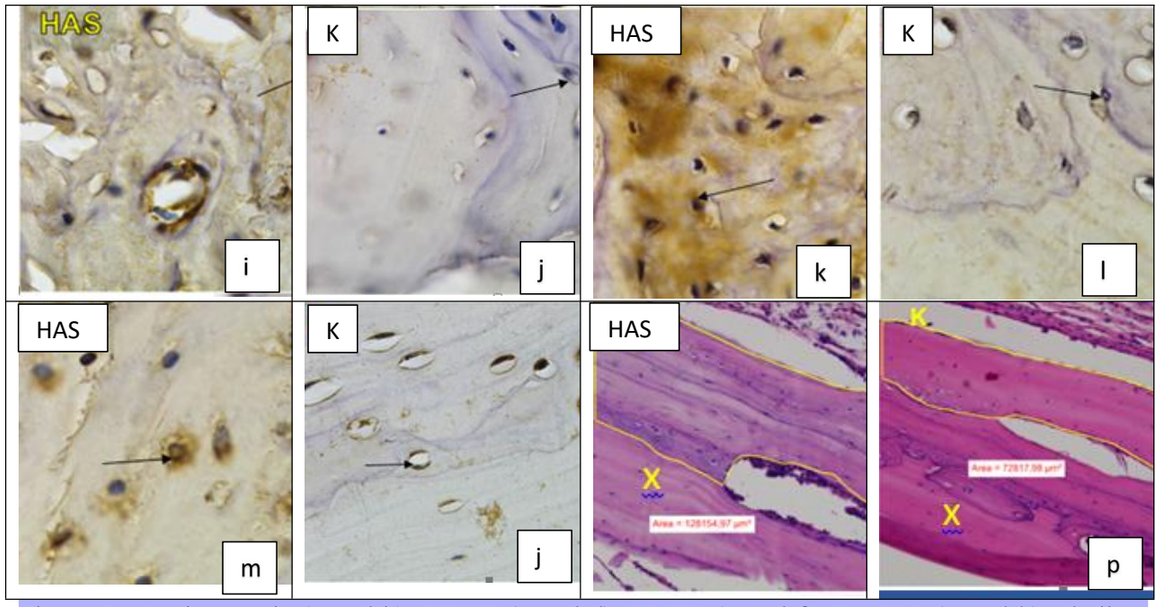
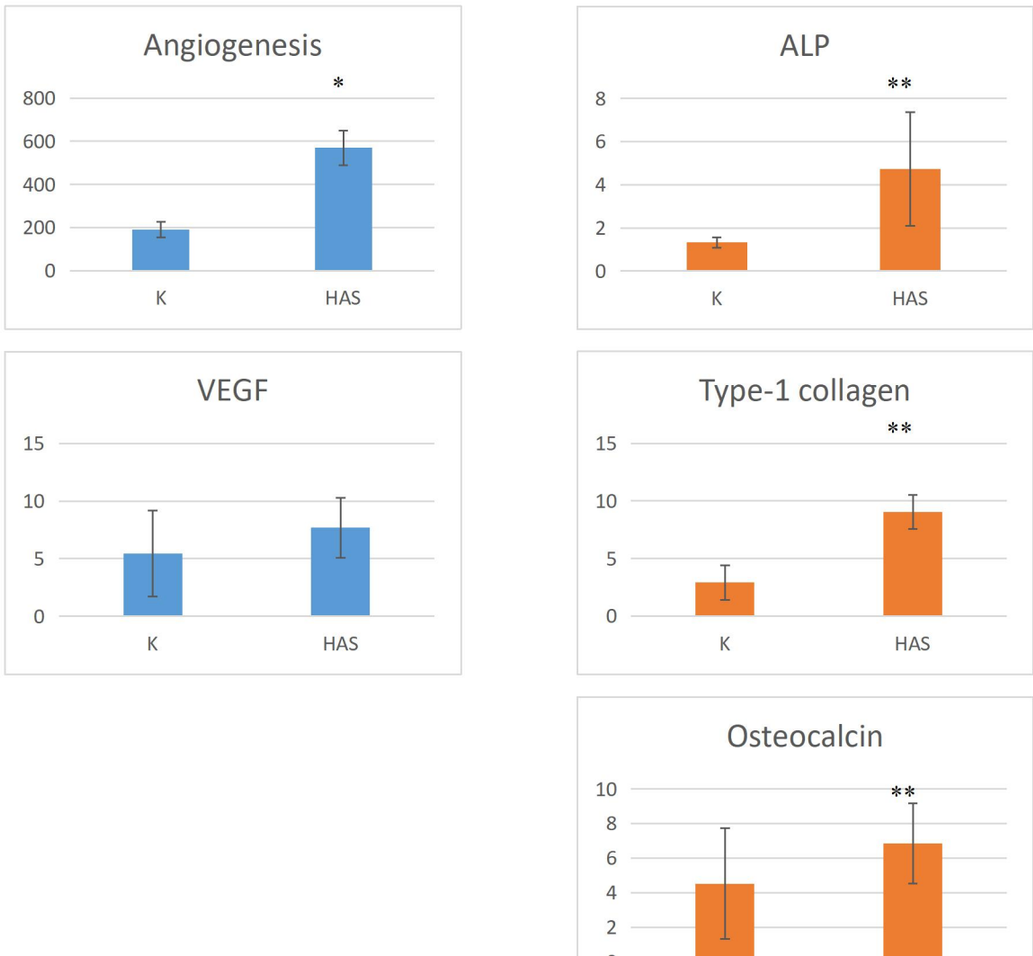
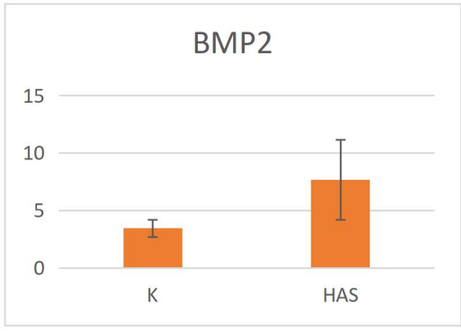
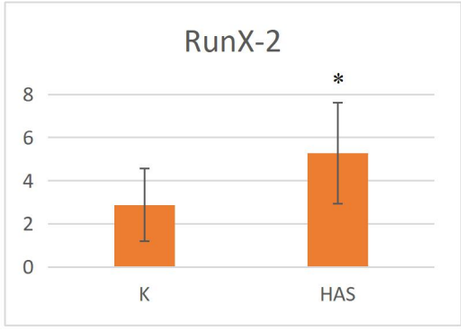


Figure 3: Angiogenesis (a and b), VEGF (c and d), BMP2 (e and f), RunX-2 (g and h), akaline phosphatase (i and j), type-1 collagen (k and l), osteocalcin (m and n) and trabecular bone area (o and p). HAS: hydroxy-apatite-chitosan scaffold group. K: control group.







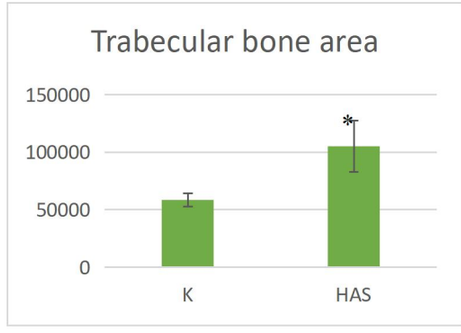


Figure 4. The mean value and standard deviation for several parameters observed after 8 weeks. K : control group; HAS: Hidroxy – apatite – chitosan scaffold. *: $p < 0.05$, **: $p < 0.01$ showed statistically significant.

Discussion

In this study, an attmept was made to combine chitosan with hidroxy-apatite in order to create Ch-HA scaffold. Chitosan was combined with hydroxyapatite to increase the mechanical strength of the scaffold and decrease the degradation time of the material. ~~200mg Chitosan and 200mg hidroxy-apatite were mixed using a lyophilization technique.~~ ~~Alkaline salt ions were eliminated through a process of desalination.~~ In the experiment conducted, the scaffold characteristics included; retentiveness without brittleness, and a sponge-like rather than solid structure. SEM imaging showed that the Ch-HA scaffold had a three dimensional structure with a porous surface. hAMSCs could attach to and proliferate effectively within the scaffold's porosity. The interconnected interstices of the scaffold were highly retentive and expected to be an excellent niche for osteoblast proliferation and differentiation. The ideal pore structure for

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tissue engineering scaffold ranged from 40-300 μm since this enabled vascular tissue migration and tissue growth.[2,7]

The potential of hAMSCs to act as a form of xenogenic MSCs during bone tissue engineering procedures has been thoroughly investigated. Several studies utilizing xenogenic hAMSCs transplantation in various organs of rats confirmed a less intense immune reaction that could affect the tissue healing process.[9–11]

The first three days post-implantation consists the inflammatory phase, that is, the initial bone healing stage. During this phase, the hypoxic condition of the Ch-HA scaffold and the degranulation of platelets resulting from hematoma trigger increased VEGF expression that, in turn, induces angiogenesis which is essential in early healing processes. Functional capillary tissues provide nutritional intake, essential bioactive molecules, and adequate oxygen tension. [12] Angiogenesis plays an important role in the healing process in bone defects because it ensures cell survival in the scaffold.[13] Mesenchymal stem cells placed in hypoxic conditions enhance the expression of angiogenic factors, mainly VEGF.[14]

During the early stages of the regeneration process, the proliferation of MSC was followed by the differentiation of osteoblast. External signals produced by MSC and osteoblasts, particularly BMP2 protein, influence this regeneration process. In later stages, activation of transcription factor RunX-2 led by BMP2 helped induce MSC differentiation of preosteoblast and osteoprogenitors, which, in turn, continued to form a collagen and non-collagen bone matrix.[15]

The bone matrix maturation level was shown by the expression of type 1 collagen fibers. Mineralization within the bone matrix maturation process will be influenced by type 1 collagen in previous stages. If the maturation level of bone matrix increases, type 1 collagen fibers will also be thicker.

In this experiment, matured osteoblast marker was identified by osteocalcin. Osteoblast specifically expressed osteocalcin that is a non-collagen protein present in bone matrix.[16,17] In the treatment group the area of trabecular bone at the end of eight weeks was significantly higher compared to that of the control group, leading to the conclusion that new bone formation in the treatment group rate was higher than that in the control group.

The process of osteogenesis indicated by the expressions of ALP, type-1 collagen, and osteocalcin produced a better result in the treatment group compared to the control group. Therefore, the maturation level of bone matrix in the treatment group at the end of eight weeks was higher when compared to that of the control group. Mesenchymal stem cells could undergo differentiation to become osteoblasts, thereby producing the apropriate environment or stimulus. During osteogenic differentiation, several markers such as ALP, type 1 collagen, and osteocalcin were expressed by MSCs. At the time, when osteoblasts turn into osteocytes, ALP activity decreases. The latest marker of mature osteoblasts expressed by osteocytes was osteocalcin.

In this study, certain limitations occurred, including lack of systematic complication. The purpose of this research was to focus on regeneration of calvaria bone defects using hAMSCs and chitosan–hidroxyapatite scaffold. The study reported here should be continued to include research on their clinical application for bone augmentation.

Conclusion

Combining Ch-HA scaffold and hAMSCs could be used as an alternative bone tissue engineering method in order to escalate the clinical use of bone formation.

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Subject: Pemberitahuan revisi ke-3 naskah
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Kepada Yth.
Dr. Michael Josef Kridanto Kamadjaja, drg.,M.Kes., Sp.Pros.
Departemen Prostodonsia
Fakultas Kedokteran Gigi
Universitas Airlangga

Bersama ini kami kirimkan hasil review ke-3 naskah dengan judul:

Bone remodeling using a three-dimensional chitosan - hydroxyapatite scaffold seeded with hypoxic conditioned human amnion mesenchymal stem cells
Authors: Michael Josef Kridanto Kamadjaja

Agar naskah tersebut dapat kami proses lebih lanjut, sejawat dimohon melakukan **perbaikan kembali** sesuai dengan catatan penyunting yang tertulis pada naskah.

Hasil perbaikan naskah mohon diberi *highlight* warna pada perubahan yang dilakukan serta dibuat tabel revisi (terlampir) dan dikirim kembali ke alamat E-mail: **dental_journal@fkg.unair.ac.id** selambat-lambatnya tanggal **5 Maret 2021**.

Apabila sampai batas waktu yang kami tentukan penulis belum mengirimkan kembali revisi naskah tersebut sesuai dengan masukan yang diberikan oleh Penyunting, maka kami anggap penulis telah **membatalkan** artikel tersebut untuk diterbitkan pada Dental Journal (Majalah Kedokteran Gigi). Penentuan penerimaan naskah berdasarkan hasil revisi yang dikirimkan.

Atas perhatiannya, kami ucapkan terima kasih.

Hormat Kami,
Ketua Penyunting Dental Journal (Majalah Kedokteran Gigi)

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[review 3] Josef Bone regeneration using a three dimensional chitosan (1 Maret 2021).docx	8,2 MB

Bone remodeling using a three-dimensional chitosan - hydroxyapatite scaffold seeded with hypoxic conditioned human amnion mesenchymal stem cells

Michael Josef Kridanto Kamadjaja
Department of Prosthodontics, Faculty of Dental Medicine, Universitas Airlangga, Surabaya – Indonesia

Abstract

Background: Bone regeneration studies involving the use of chitosan–hydroxyapatite (Ch-HA) scaffold seeded with human amnion mesenchymal stem cells (hAMSCs) have largely incorporated tissue engineering experiments. However, at the time of writing, the results of such investigations remain unclear. **Purpose**: The aim of this study was to determine the osteogenic differentiation of the scaffold Ch-HA that is seeded with hAMSCs in the regeneration of calvaria bone defect. **Methods**: Ch-HA scaffold of 5 mm diameter and 2 mm height was created by lyophilization and desalination method. hAMSCs were cultured in hypoxia environment (5% oxygen, 10% carbon dioxide, 15% nitrogen) and seeded on the scaffold. Twenty male Wistar rat subjects (8 – 10 weeks, 200 - 250 grams) were randomly divided into two groups: control and hydroxyapatite scaffold (HAS). Defects (similar size to scaffold size) were created in the calvaria bone of the all-group subjects, but a scaffold was subsequently implanted only in the treatment group members. Control group left without treatment. After observation lasting 1 and 8 weeks, the subjects were examined histologically and immunohistochemically. Statistical analysis was done using ANOVA test. **Result:** Angiogenesis; expression of vascular endothelial growth factor; bone morphogenetic protein; RunX-2; alkaline phosphatase; type-1 collagen; osteocalcin and the area of new trabecular bone were all significantly greater in the HAS group compared to the control group. **Conclusion**: The three-dimensional Ch-HA scaffold seeded with hypoxic hAMSCs induced bone remodeling in calvaria defect according to the expression of the osteogenic and angiogenic marker.

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Keywords: *human amniotic mesenchymal stem cells, chitosan-hydroxy apatite scaffold, hypoxia, bone tissue engineering*

Introduction

Tissue engineering has been undertaken to remedy many medical conditions, for instance: complications arising from wound healing, bone defects, immune system responses, and donor-transmitted disease. Three dimensional scaffolds were created to provide adequate support forming an extracellular matrix that enables cells to proliferate and differentiate. Chitosan alone as a scaffold suffers from its mechanical strength. Chitosan could easily break and therefore not able to create a suitable matrix for cell delivery.[1] Carbonate apatite also faces the similar problem, its brittle nature has limited its application as a scaffold. Therefore, combining both materials are

predicted to create stronger scaffold.[2] Scaffold made from Chitosan – Carbonate Apatite (Ch-CA) has been reported as producing a robust, interconnected three-dimensional (3D) porous structure which could support the proliferation and differentiation of osteoblast during osteogenic differentiation.[3,4] Hydroxyapatite has chemical structure that similar to human bone, therefore it has good affinity towards the bone and subsequently form chemical bond directly to the hard tissue. [5,6] By combining chitosan and carbonate apatite into scaffold, this material was expected to increase its mechanical strength and reduce the degradation time. The biocompatibility of hydroxyapatite (HA) and the resemblance of its mineral composition to bone has rendered it an ideal material for bone tissue engineering (BTE). The development of HA into a 3-dimensional (3D) scaffold or a support to mesenchymal stem cells (MSC) *in vitro* has also been extensively explored. HA scaffolds offer massive advantages within the field of BTE.[7,8] Human amniotic mesenchymal stem cells (hAMSCs) derived from human placentas are known for their pluripotent properties; ability to differentiate into three forms of germ layer; and efficacy in reducing both potential inflammation and immune reaction.[9,10] Chitosan-Hydroxyapatite scaffold seeded with hAMSCs was expected to intensify osteogenesis. The aim of this study was to observe the effect of Ch-HA scaffold seeded with hAMSCs within tissue engineering techniques.

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Materials and Methods

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length ELISA reader was employed to read the absorbance, while the living cells were counted by means of a Cell Counting Kit.[3,4]

SEM imaging

2% glutaraldehyde was used to fixate hAMSCs-seeded on Ch-HA scaffold at 40^0C for 2-3 hours. The subsequent stage in the procedure consisted of washing with PBS solution three times every five minutes. After exposure to osmic acid 1% for 1-2 hours, the cells were washed again with PBS. A 15-minute dehydration procedure using alcohol at varying concentrations (30% - 100%) was also completed for each concentration. The scaffold was dehydrated using a critical point drying (CPD) device, attached to a stud pad with specific adhesive, and coated with pure gold. The scaffold was examined under a scanning microscope and photographed by means of a scanning electron microscope (JEOL JSM-T100, Japan).[3,4]

Preparation of experimental subjects

20 male Wistar rats were used as the animal subjects of the experiment. The inclusion criteria applied were as follows: aged 8-12 weeks old and weighing 100-150 grams. The subjects were randomly divided into a control group and a treatment group of equal size which were observed during weeks 1 and 8.

Chitosan-hydroxy apatite scaffold implantation procedure in the calvaria bone of rats

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An anaesthetic procedure was performed 4-6 hours after the subjects were denied further food and water. 20mg of Ketamin HCL (Ketalar, Ireland) per kg of body weight and 3mg of Xylazine (Xyla,Ireland) per kg of body weight were injected intramuscularly. A mid-longitudinal skin incision was then made on the cranium dorsal surface after an aseptic procedure had been completed. The periosteum of the cranium was separated from the surface in order to produce a flap. A 2 mm diameter, circular, low speed bur (NSK, Japan) was used to create the bone defect 5 mm in diameter. The scaffold was implanted and sutured in order to re-attach the wound area

but only in the treatment group.[3,4] The defect was subsequently sutured with blue nylon 5-0 mono suture (Ailee Co. Ltd, Busan, Korea).

Termination of animal models and collection of research specimens.

The subjects were sacrificed during weeks 1 and 8 in order to obtain the required specimens. The implantation region was decalcified and embedded in paraffin to produce microscopic specimens. In order to highlight the angiogenesis and trabecular bone area, the specimens were stained with Hematoxylin and Eosin, while post-scaffold implantation immunohistochemical staining (using mouse anti-human monoclonal (Novus Biological, USA) and polyclonal (Thermo Scientific, USA) antibody: BMP2, RunX-2, Alkaline phosphatase, Type-1 collagen, Osteocalcin, and VEGF of the specimens of cranium calvaria preparations was carried out. The Remmele Scale Index was used to measure the raw data. A Nikon H600L (Tokyo, Japan) light microscope with 1000x magnification and a DS Fi2 300-megapixel digital camera with image processing software (Nikon Image System) were respectively employed to examine the specimens and observe the tissue.[4]

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Statistical analysis

The data were presented as mean values, and standard deviation. SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data by means of an ANOVA test and $p < 0.05$ was considered statistically significant.

Result

The chitosan–hydroxyapatite was a solid 3D scaffold 5mm in diameter and 2mm thick (Figure 1). Toxicity tests incorporating the use of MTT Assay indicated that Ch-HA scaffold was not harmful to the hAMSCs culture. The percentage of viable cells found in the Ch-HA scaffold was 79.42 %. SEM imaging showed that cells were able to attach themselves to the Ch-HA scaffold's porous surface that was embedded in the calvaria bone defect (Figure 2). Histological image of seeded cells in the scaffold are shown on Figure 3. All groups were examined for expression of

RunX2, BMP2, VEGF, alkaline phosphatase (ALP), collagen type 1, osteocalcin, angiogenesis and trabecular bone area after 8 weeks. All treatment groups possessed a higher mean value than the control group (Figure 4).

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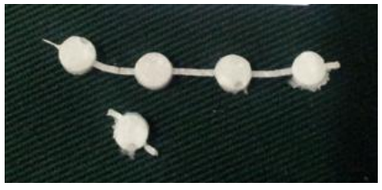


Figure 1:
Ch-HA scaffold

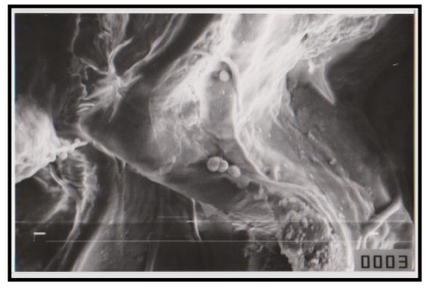
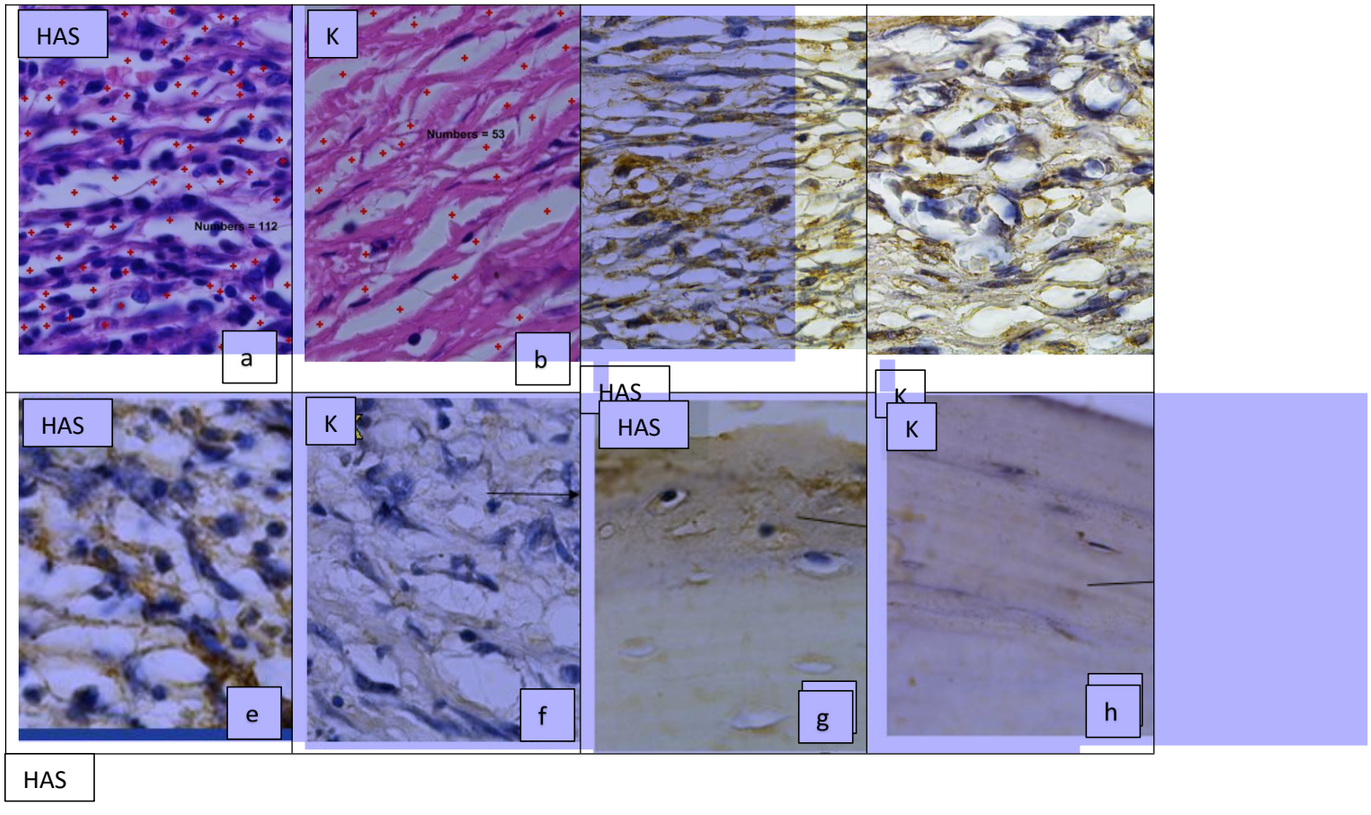


Figure 2:
SEM image of cells attached and proliferated into the scaffold pores
(SEM, 1000x magnification)



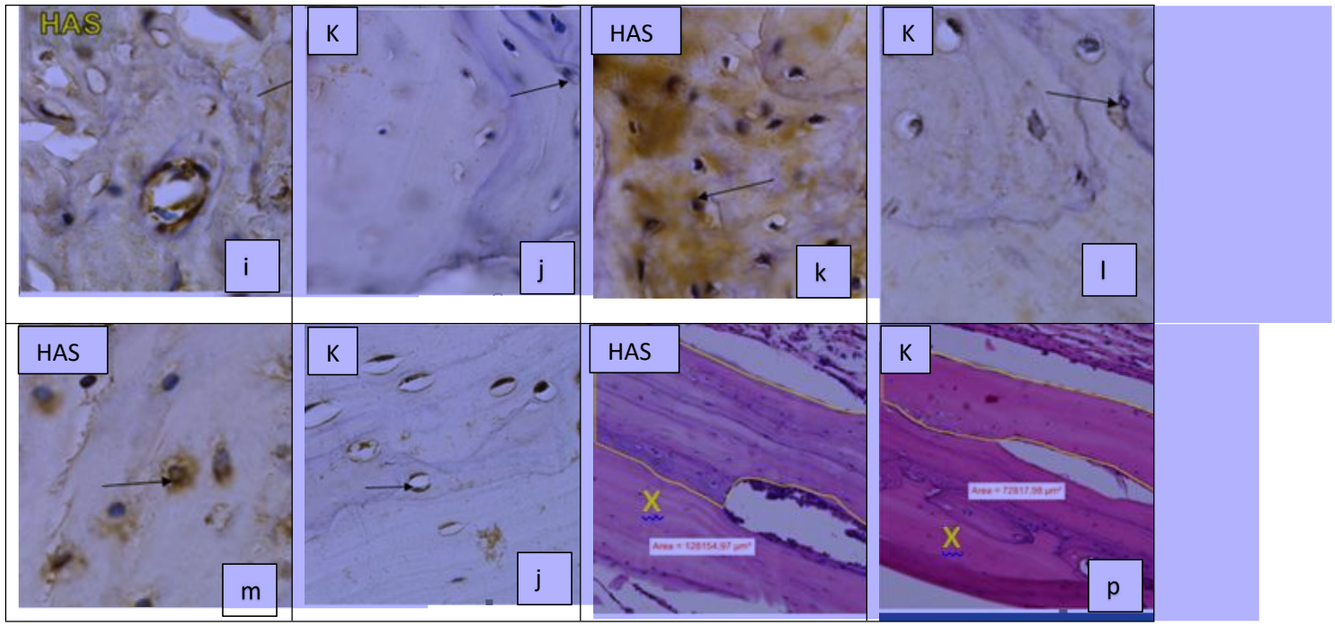
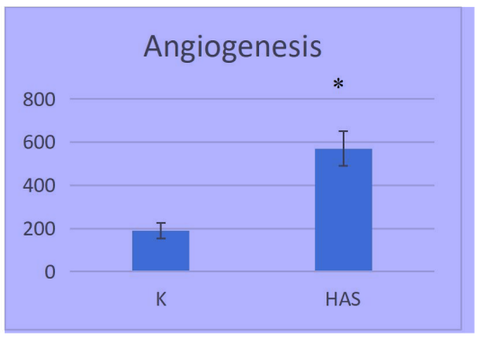
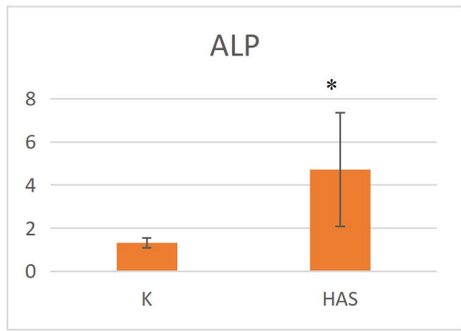
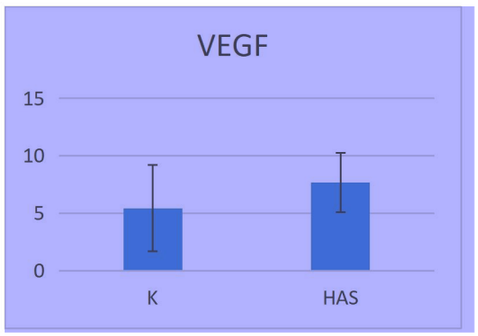
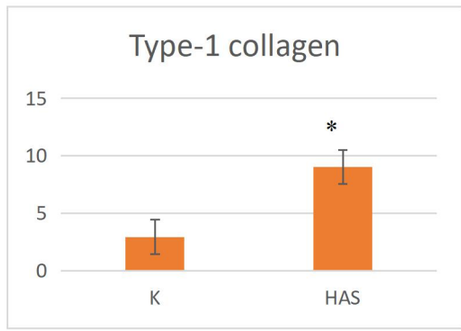


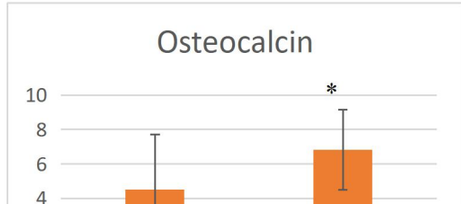
Figure 3: Angiogenesis (a and b), VEGF (c and d), BMP2 (e and f), RunX-2 (g and h), akaline phosphatase (i and j), type-1 collagen (k and l), osteocalcin (m and n) and trabecular bone area (o and p) with 1000x magnification. HAS: hydroxy-apatite-chitosan scaffold group. K: control group.

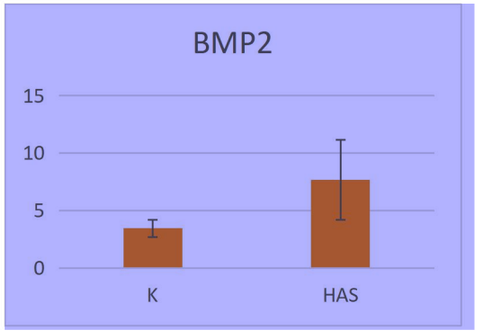


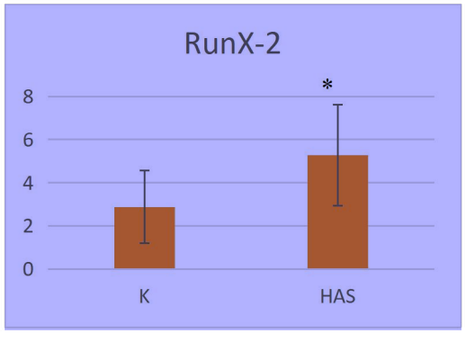












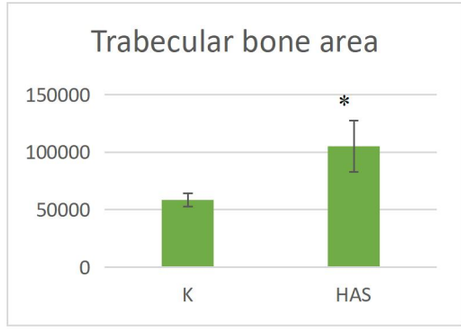


Figure 4. The mean value and standard deviation for several parameters observed after 8 weeks. K : control group; HAS: Hidroxy – apatite – chitosan scaffold. *: $p < 0.05$showed statistically significant.

Discussion

In this study, an attempt was made to combine chitosan with hidroxy-apatite in order to create Ch-HA scaffold. Chitosan was combined with hydroxyapatite to increase the mechanical strength of the scaffold and decrease the degradation time of the material. hAMSCs could attach to and proliferate effectively within the scaffold's porosity. The interconnected interstices of the scaffold were highly retentive and expected to be an excellent niche for osteoblast proliferation and differentiation. The ideal pore structure for tissue engineering scaffold ranged from 40-300 µm since this enabled vascular tissue migration and tissue growth.[4,11]

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The potential of hAMSCs to act as a form of xenogenic MSCs during bone tissue engineering procedures has been thoroughly investigated. Several studies utilizing xenogenic hAMSCs transplantation in various organs of rats confirmed a less intense immune reaction that could affect the tissue healing process.[12–14]

The first three days post scaffold-implantation consists the inflammatory phase, that is, the initial bone healing stage. During this phase, the hypoxic condition of the Ch-HA scaffold and the degranulation of platelets resulting from hematoma trigger increased VEGF expression that, in turn, induces angiogenesis which is essential in early healing processes. Functional capillary tissues provide nutritional intake, essential bioactive molecules, and adequate oxygen tension. [15] Angiogenesis plays an important role in the healing process in bone defects because it ensures cell survival in the scaffold.[16] Mesenchymal stem cells placed in hypoxic conditions enhance the expression of angiogenic factors, mainly VEGF.[17]

During the early stages of the regeneration process, the proliferation of MSC was followed by the differentiation of osteoblast. External signals produced by MSC and osteoblasts, particularly BMP2 protein, influence this regeneration process. In later stages, activation of transcription factor RunX-2 led by BMP2 helped induce MSC differentiation of preosteoblast and osteoprogenitors, which, in turn, continued to form a collagen and non-collagen bone matrix.[18]

The bone matrix maturation level was shown by the expression of type 1 collagen fibers. Mineralization within the bone matrix maturation process will be influenced by type 1 collagen in previous stages. If the maturation level of bone matrix increases, type 1 collagen fibers will also be thicker.[19]

In this experiment, matured osteoblast marker was identified by osteocalcin. Osteoblast specifically expressed osteocalcin that is a non-collagen protein present in bone matrix.[20,21] In the treatment group the area of trabecular bone at the end of eight weeks was significantly higher compared to that of the control group, leading to the conclusion that new bone formation in the treatment group rate was higher than that in the control group.

The process of osteogenesis indicated by the expressions of ALP, type-1 collagen, and osteocalcin produced a better result in the treatment group compared to the control group. Therefore, the maturation level of bone matrix in the treatment group at the end of eight weeks was higher when compared to that of the control group. Mesenchymal stem cells could undergo differentiation to become osteoblasts, thereby producing the apropriate environment or stimulus. During osteogenic differentiation, several markers such as ALP, type 1 collagen, and osteocalcin were expressed by MSCs. At the time, when osteoblasts turn into osteocytes, ALP activity decreases. The latest marker of mature osteoblasts expressed by osteocytes was osteocalcin.

In this study, certain limitations occurred, including lack of systematic complication. The purpose of this research was to focus on regeneration of calvaria bone defects using hAMSCs and chitosan–hidroxyapatite scaffold. The study reported here should be continued to include research on their clinical application for bone augmentation.

Conclusion

Combining Ch-HA scaffold and hAMSCs could be used as an alternative bone tissue engineering method in order to escalate the clinical use of bone formation.

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Date: 29/03/2021 12.10
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We have reached a decision regarding your submission to Dental Journal (Majalah Kedokteran Gigi), "Bone remodeling using a three-dimensional chitosan - hydroxyapatite scaffold seeded with hypoxic conditioned human amnion mesenchymal stem cells".
Author: Michael Josef Kridanto Kamadjaja

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Rauf Shahbazov, Mazhar A. Kanak, Morihito Takita, Faisal Kunnathodi et al. "Essential phospholipids prevent islet damage induced by proinflammatory cytokines and hypoxic conditions", Diabetes/Metabolism Research and Reviews, 2016

Bone remodeling using a three-dimensional chitosan - hydroxyapatite scaffold seeded with hypoxic conditioned human amnion mesenchymal stem cells ABSTRACT Background: Bone regeneration studies involving the use of chitosan–hydroxyapatite (Ch-HA) scaffold seeded with human amnion mesenchymal stem cells (hAMSCs) have largely incorporated tissue engineering experiments. However, at the time of writing, the results of such investigations remain unclear. Purpose: The aim of this study was to determine the osteogenic differentiation of the scaffold Ch-HA that is seeded with hAMSCs in the regeneration of calvaria bone defect. Methods: Ch-HA scaffold of 5 mm diameter and 2 mm height was created by lyophilisation and desalination method. hAMSCs were cultured in hypoxia environment (5% oxygen, 10% carbon dioxide, 15% nitrogen) and seeded on the scaffold. Twenty male Wistar rat subjects (8 – 10 weeks, 200 - 250 grams) were randomly divided into two groups: control and hydroxyapatite scaffold (HAS). Defects (similar size to scaffold size) were created in the calvaria bone of the all-group subjects, but a scaffold was subsequently implanted only in the treatment group members. Control group left without treatment. After observation lasting 1 and 8 weeks, the subjects were examined histologically and immunohistochemically. Statistical analysis was done using ANOVA test. Result: Angiogenesis; expression of vascular endothelial growth factor; bone morphogenetic protein; RunX-2; alkaline phosphatase; type-1 collagen; osteocalcin and the area of new trabecular bone were all significantly greater in the HAS group compared to the control group. Conclusion: The three-dimensional Ch-HA scaffold seeded with hypoxic hAMSCs induced bone remodeling in calvaria defect according to the expression of the osteogenic and angiogenic marker. Keywords: bone tissue engineering; chitosan-hydroxyapatite scaffold; human amniotic mesenchymal stem cells; hypoxia INTRODUCTION Tissue engineering has been undertaken to remedy many medical conditions, for instance: complications arising from wound healing, bone defects, immune system responses, and donor-transmitted disease. Three dimensional scaffolds were created to provide adequate support forming an extracellular matrix that enables cells to proliferate and differentiate. Chitosan alone as a scaffold suffers from its mechanical strength. Chitosan could easily break and therefore not able to create a suitable matrix for cell delivery.1 Carbonate apatite also faces the similar problem, its brittle nature has limited its application as a scaffold. Therefore, combining both materials are predicted to create stronger scaffold.2 Scaffold made from Chitosan – Carbonate Apatite (Ch-CA) has been reported as producing a robust, interconnected three-dimensional (3D) porous structure which could support the proliferation and differentiation of osteoblast during osteogenic differentiation.3,4 Hydroxyapatite has chemical structure that similar to human bone, therefore it has good affinity towards the bone and subsequently form chemical bond directly to the hard tissue. 5,6 By combining chitosan and carbonate apatite into scaffold, this material was expected to increase its mechanical strength and reduce the degradation time. The biocompatibility of hydroxyapatite (HA) and the resemblance of its mineral composition to bone has rendered it an ideal material for bone tissue engineering (BTE). The development of HA into a 3- dimensional (3D) scaffold or a support to mesenchymal stem cells (MSC) in vitro has also been extensively explored. HA scaffolds offer massive advantages within the field of BTE.7,8 Human amniotic mesenchymal stem cells (hAMSCs) derived from human placentas are known for their pluripotent properties; ability to differentiate into three forms of germ layer; and efficacy in reducing both potential inflammation and immune reaction.9,10 Chitosan- Hydroxyapatite scaffold seeded with hAMSCs was expected to intensify osteogenesis. The aim of this study was to observe the effect of Ch-HA scaffold seeded with hAMSCs within tissue engineering techniques. MATERIALS AND METHODS The isolation and culture procedure were performed following the securing of approval from the faculty’s research ethics committee (No. 378/Panke. KKE/VII /2015). Material from a newly-formed amnion was peeled from the chorion and rinsed using phosphate-buffered saline (PBS). The amnion was then soaked in Ringer’ s lactate (RL) containing 2.5 µg/mL gentamycin and 1000 U/mL amphotericin which had been obtained from Gibco TM Amphotericin B, New York, USA. The isolation and culture of hAMSCs using a modified Soncini’s protocol. Small,

fine pieces of amniotic membrane were treated with 0.25% trypsin in order to remove the epithelial cells. Centrifugation of five minutes duration at 2,000 rpm was carried with the supernatant subsequently being removed. This procedure was then repeated. The supernatant was washed using PBS containing 0. 075 mg/ml DNase 1 (Takara Bio, Shiga, Japan) and 0.75 mb /ml type IV collagenase (Sigma-Aldrich, St. Louis, MO, USA). Incubation of the amnion was performed at 37°C for 60 minutes. Filtration and centrifugation lasting five minutes were performed to obtain cells. Single cells culture was created then using collagen–coated discs. The medium for the cells consisted of Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) at a ratio of 1:1, added to fetal bovine serum and 10 ng/mL human leukemia inhibitory factor (Gibco BRL, Gaithersburg, MD, USA). This medium was replaced every three days. Once the cell growth had reached a confluent stage (80%), the cell was split using trypsin. The laboratory stem cell protocol was implemented as the isolation procedure. hAMSCs were cultured in hypoxia chamber (1% oxygen, 5% carbon dioxide, and 94% nitrogen). Chitosan-hydroxy apatite scaffold was prepared by dissolving 200mg of medium- molecular weight Ch powder (Sigma-Aldrich, St. Louis, MO, USA) into 5ml of ethanoic acid at room temperature and mixing them for 15 minutes. 15ml of sodium hydroxide solution was used for neutralising purposes in obtaining chitosan gel. Furthermore, samples of the chitosan gel were mixed homogenously with 200mg of HA prior to centrifuging at 1,500 rpm for ten minutes. After extraction of excess water, the solution gel was placed into the specific mold to produce scaffolds (5 mm diameter and 2 mm height). Before being transferred to a drying machine, the gel was frozen for two hours at -80°C.3,4 Human amniotic mesenchymal stem cells were deposited onto a 96-well cell culture plate (M96) at a density of 5 x 104 cell/well and incubated at 37°C for 24 hours with CO2 5% concentration. Once the cell proliferation population had reached 80%, Ch-HA scaffold was added together with 100 µL of growth medium. The cells underwent a second incubation at 37°C for 20 hours with 5% CO2. After the addition of 5 mg/mL MTT reagent (25 µL/well), the cells were incubated a second time for four hours before being observed under an inverted microscope. The scaffold and medium were removed and added to 200 µL/well DMSO. A 595 nm wave length ELISA reader was employed to read the absorbance, while the living cells were counted by means of a Cell Counting Kit.3,4 2% glutaraldehyde was used to fixate hAMSCs-seeded on Ch-HA scaffold at 40°C for 2-3 hours. The subsequent stage in the procedure consisted of washing with PBS solution three times every five minutes. After exposure to osmic acid 1% for 1-2 hours, the cells were washed again with PBS. A 15-minute dehydration procedure using alcohol at varying concentrations (30-100%) was also completed for each concentration. The scaffold was dehydrated using a critical point drying (CPD) device, attached to a stud pad with specific adhesive, and coated with pure gold. The scaffold was examined under a scanning microscope and photographed by means of a scanning electron microscope (JEOL JSM-T100, Japan).3,4 20 male Wistar rats were used as the animal subjects of the experiment. The inclusion criteria applied were as follows: aged 8-12 weeks old and weighing 100-150 grams. The subjects were randomly divided into a control group and a treatment group of equal size which were observed during weeks 1 and 8. An anaesthetic procedure was performed 4-6 hours after the subjects were denied further food and water. 20mg of Ketamin HCL (Ketalar, Ireland) per kg of body weight and 3mg of Xylazine (Xyla,Ireland) per kg of body weight were injected intramuscularly. A mid-longitudinal skin incision was then made on the cranium dorsal surface after an aseptic procedure had been completed. The periosteum of the cranium was separated from the surface in order to produce a flap. A 2 mm diameter, circular, low speed bur (NSK, Japan) was used to create the bone defect 5 mm in diameter. The scaffold was implanted and sutured in order to re-attach the wound area but only in the treatment group.3,4 The defect was subsequently sutured with blue nylon 5-0 mono suture (Ailee Co. Ltd, Busan, Korea). The subjects were sacrificed during weeks 1 and 8 in order to obtain the required specimens. The implantation region was decalcified and embedded in paraffin to produce microscopic specimens. In order to highlight the angiogenesis and trabecular bone area, the specimens were stained with Hematoxylin and Eosin, while post-scaffold implantation immunohistochemical staining (using mouse anti-human monoclonal (Novus Biological, USA) and polyclonal (Thermo Scientific, USA) antibody: BMP2, RunX-2, Alkaline phosphatase, Type-1 collagen, Osteocalcin, and VEGF of the specimens of cranium calvaria preparations was carried out. The Remmele Scale Index was used

to measure the raw data. A Nikon H600L (Tokyo, Japan) light microscope with 1000x magnification and a DS Fi2 300- megapixel digital camera with image processing software (Nikon Image System) were respectively employed to examine the specimens and observe the tissue.4 The data were presented as mean values, and standard deviation. Statistical Package for Social Sciences (SPSS) software version 15.0 (SPSS Inc., Chicago, IL, USA) was used to analyse the data by means of an ANOVA test and $p < 0.05$ was considered statistically significant. RESULTS The chitosan–hydroxyapatite was a solid 3D scaffold 5mm in diameter and 2mm thick (Figure 1). Toxicity tests incorporating the use of MTT Assay indicated that Ch-HA scaffold was not harmful to the hAMSCs culture. The percentage of viable cells found in the Ch-HA scaffold was 79.42 %. SEM imaging showed that cells were able to attach themselves to the Ch-HA scaffold's porous surface that was embedded in the calvaria bone defect (Figure 2). Histological image of seeded cells in the scaffold are shown on Figure 3. All groups were examined for expression of RunX2, BMP2, VEGF, alkaline phosphatase (ALP), type 1 collagen, osteocalcin, angiogenesis and trabecular bone area after 8 weeks. All treatment groups possessed a higher mean value than the control group (Figure 4). Figure 1. Ch-HA scaffold. Figure 2. SEM image of cells attached and proliferated into the scaffold pores (SEM, 1000x magnification) Figure 3. Angiogenesis (a and b), VEGF (c and d), BMP2 (e and f), RunX-2 (g and h), akaline phosphatase (i and j), type-1 collagen (k and l), osteocalcin (m and n) and trabecular bone area (o and p) with 1000x magnification. HAS: hydroxy-apatite-chitosan scaffold group seeded with hAMSCs. K: control group. Figure 4. The mean value and standard deviation for several parameters observed after 8 weeks. K : control group; HAS: Hidroxy – apatite – chitosan scaffold. *: $p < 0.05$showed statistically significant. DISCUSSION In this study, an attempt was made to combine chitosan with hidroxy-apatite in order to create Ch-HA scaffold. Chitosan was combined with hydroxyapatite to increase the mechanical strength of the scaffold and decrease the degradation time of the material. Similar previous study shown that chitosan addition on a scaffold immersed in synthetic body fluid yielded a stronger mechanical strengh, greater strain, and more stable characteristic.11 Another similar study also shown thatchitosan-hydroxyapatite scaffold had good biocompatibility and provided enhanced strength.12 hAMSCs could attach to and proliferate effectively within the scaffold's porosity. The interconnected interstices of the scaffold were highly retentive and expected to be an excellent niche for osteoblast proliferation and differentiation. The ideal pore structure for tissue engineering scaffold ranged from 40-300 μm since this enabled vascular tissue migration and tissue growth.4,13 The potential of hAMSCs to act as a form of xenogenic MSCs during bone tissue engineering procedures has been thoroughly investigated. Several studies utilising xenogenic hAMSCs transplantation in various organs of rats confirmed a less intense immune reaction that could affect the tissue healing process.14–16 The first three days post scaffold-implantation consists the inflammatory phase, that is, the initial bone healing stage. During this phase, the hypoxic condition of the Ch-HA scaffold and the degranulation of platelets resulting from hematoma trigger increased VEGF expression that, in turn, induces angiogenesis which is essential in early healing processes. Functional capillary tissues provide nutritional intake, essential bioactive molecules, and adequate oxygen tension. 17 Angiogenesis plays an important role in the healing process in bone defects because it ensures cell survival in the scaffold.18 Mesenchymal stem cells placed in hypoxic conditions enhance the expression of angiogenic factors, mainly VEGF.19 During the early stages of the regeneration process, the proliferation of MSC was followed by the differentiation of osteoblast. External signals produced by MSC and osteoblasts, particularly BMP2 protein, influence this regeneration process. In later stages, activation of transcription factor RunX-2 led by BMP2 helped induce MSC differentiation of preosteoblast and osteoprogenitors, which, in turn, continued to form a collagen and non- collagen bone matrix.20 The bone matrix maturation level was shown by the expression of type 1 collagen fibers. Mineralisation within the bone matrix maturation process will be influenced by type 1 collagen in previous stages. If the maturation level of bone matrix increases, type 1 collagen fibers will also be thicker.21 In this experiment, matured osteoblast marker was identified by osteocalcin. Osteoblast specifically expressed osteocalcin that is a non-collagen protein present in bone matrix.22,23 In the treatment group the area of trabecular bone at the end of eight weeks was significantly higher compared to that of the control group, leading to the conclusion that new bone formation in the treatment group rate was higher than that in the control group. The process of

osteogenesis indicated by the expressions of ALP, type-1 collagen, and osteocalcin produced a better result in the treatment group compared to the control group. Therefore, the maturation level of bone matrix in the treatment group at the end of eight weeks was higher when compared to that of the control group. Mesenchymal stem cells could undergo differentiation to become osteoblasts, thereby producing the apropriate environment or stimulus. During osteogenic differentiation, several markers such as ALP, type 1 collagen, and osteocalcin were expressed by MSCs. At the time, when osteoblasts turn into osteocytes, ALP activity decreases. The latest marker of mature osteoblasts expressed by osteocytes was osteocalcin. In this study, certain limitations occurred, including lack of systematic complication. The purpose of this research was to focus on regeneration of calvaria bone defects using hAMSCs and chitosan–hidroxyapatite scaffold. The study reported here should be continued to include research on their clinical application for bone augmentation. In conclusion, combining Ch-HA scaffold and hAMSCs could be used as an alternative bone tissue engineering method in order to escalate the clinical use of bone formation.