Effect of Hydroxyapatite and Polycaprolactone Scaffolds Geometry in Inducing Osteogenic Differentiation and Augmenting Maxillary Bone Regeneration in Rats

Nur Atmaliya Luchman[1], Rohaya Megat Abdul Wahab[1], Shahrul Hisham Zainal Ariffin[2], Nurrul Shaqinah Nasruddin[3], Seng Fong Lau[4]

[1] Department of Family Oral Health, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia
[2] Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia
[3] Department of Craniofacial Diagnostics and Biosciences, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia
[4] Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Corresponding Author:
Farinawati Yazid[1]
Universiti Kebangsaan Malaysia, Jalan Raja Muda Aziz, 50300 Kuala Lumpur, Malaysia
Email address: drfarinawati@ukm.edu.my

Abstract

Background. The geometry of scaffolds for bone regeneration has recently been shown to remarkably influence the cellular response and the rate of new bone formation. The usage of scaffolds with different geometry and their effect on the osteogenic performance of cells is not well studied and this can affect the selection of suitable scaffolds for transplantation. Hence, this study aimed to investigate the comparative ability of two different synthetic scaffolds mainly hydroxyapatite (HA) and polycaprolactone (PCL) scaffolds in promoting *in vitro* and *in vivo* bone regeneration.

Method. *In vitro* cell viability, morphology, and alkaline phosphatase (ALP) activity of MC3T3-E1 cells on HA and PCL scaffolds were determined in comparison to the accepted model outlined for two-dimensional systems. *In vivo* study involve transplantation of MC3T3-E1 cells with scaffolds into an artificial bone defect of 4 mm length and 1.5 mm depth in the rat's left maxilla. Three-dimensional analysis using micro-computed tomography (micro-CT) and histological evaluations were performed after six weeks of transplantation.

Results. MC3T3-E1 cells on HA scaffold showed the highest cell viability. The cell viability on both scaffolds decreased after 14 days of culture, which reflects the dominant occurrence of osteoblast differentiation. An early sign of osteoblast differentiation can be detected on PCL

scaffold. However, cells on HA scaffold showed more prominent results with intense mineralized nodules and significantly ($p < 0.05$) high levels of ALP activity with prolonged osteoblast induction. Micro-CT and histology analyses confirmed *in vitro* results with bone formation was significantly ($p < 0.05$) greater in HA scaffold.
Conclusion. HA and PCL scaffold geometry might have influenced the bone regeneration ability of MC3T3-E1. Regardless, *in vitro* and *in vivo* bone regeneration was better in the HA scaffold which indicates its great potential for application in bone regeneration.

Introduction

Bone tissue has the ability to spontaneously heal through bone deposition and remodeling (Fernandez-Yague et al., 2015). However in a larger bone defect as a result of trauma, surgical treatment of tumor and craniofacial defect such as cleft palate, bone repair can only be done by bone graft (Fishero et al., 2015; Robey et al., 2015). Bone graft in cleft palate repair is important for tooth eruption and orthodontic tooth movement (Wahab et al., 2020). Craniofacial defect repair by surgeons often requires sophisticated treatment strategies and multidisciplinary input with ideal situations using autologous bone. However, this option is limited by a finite supply of available bone, potential donor site morbidity, particular attention to growing patients, prolonged surgeries in 'hostile defect' that may associate with free flap loss, anesthetics/patient-related risks, and contour deformities (Lee et al., 2013). In the event of autologous bone is impractical or not feasible, application of tissue engineering can be a promising concept within craniofacial surgery field utilizing the engineered materials with a combination of cells to improve or replace biological functions.

Tissue regeneration aims to help the body heal naturally by implanting a scaffold to serve as a temporary matrix that would degrade over time while allowing the regeneration of the host tissue at the implant site. Cellular response and osteoblast differentiation can be affected by morphology, size, surface topography, surface chemistry, porosity, interconnected structure, and fibrous pore wall of the scaffold (Tavakol et al., 2012). Therefore, the selection of scaffold plays a crucial role in ensuring the success of bone regeneration. The chosen scaffold must allow the cells to migrate, proliferate, and differentiate into osteoblasts for the correct development of the bone tissue (Bose et al., 2012). Synthetic scaffolds haves advantages over natural scaffolds as theyit can be manufactured under controlled conditions that allow large scale production with uniform size and design as well as exhibiting reproducible physical and chemical properties (Farinawati et al., 2020). ~~Hydroxyapatite and polycaprolactone are scaffolds with these properties.~~

Hydroxyapatite (HA) is known for its excellent biocompatibility due to its similarity in composition to the apatite found in natural bone. In biological systems, HA occurs as the inorganic constituent for normal calcification such as on bone, teeth, fish enameloid, some species of shell, and in pathological calcification such as on dental and urinary calculus or stone (Hench & Thompson 2010; Kattimani et al., 2016). Natural occurring HA appears to be brown, yellow, or green in coloration while pure or synthetic HA appears in white coloration. HA

contains only calcium and phosphate ions and therefore no adverse local or systemic toxicity has been reported in any study (Kattimani et al., 2016). Biocompatibility, bioactivity, osteoinductivity, and osteoconductivity are good properties of HA that make them extensively being used as a scaffold for bone regeneration. Moreover, The different forms of HA scaffold actively being used include granules, paste, cement, coatings, porous and dense blocks (Beachley & Wen 2010; Liu & Webster 2007; Mendonça et al., 2008; Wang 2003). Nonetheless, concerns have been raised regarding the brittleness and limited degradation properties of HA including the slow degradation rate.

Polycaprolactone (PCL) is much preferred in terms of degradation. PCL is a synthetic polymer that can undergo degradation by hydrolysis of ester bonds in physiological conditions. PCL is an aliphatic semi-crystalline polymer with a melting temperature above body temperature. Hence, at physiological temperature, PCL attains a rubbery state resulting in its high toughness and superior mechanical properties (Dwivedi et al., 2020). PCL appears to be non-toxic and tissue compatible which makes it suitable as scaffolds for bone regeneration. Dwivedi et al., (2020) believed that PCL has easy availability, relatively inexpensive, and can be modified to adjust its chemical and biological properties, physiochemical state, degradability, and mechanical strength. PCL exhibits a degradation time of approximately two to three years and it can be degraded by microorganisms or under physiological conditions (Anderson & Shive 1997). Its degradation time makes it appropriate for the replacement of hard and load-bearing tissues by enhancing stiffness while for soft tissues by decreasing its molecular weight and degradation time. However, several reports have shown that PCL is lack of osteoconductive property due to its poor hydrophilic nature (Hajiali et al., 2018; Torres et al., 2017; Zhao et al., 2015).

There is still a need to investigate the biological performance of HA and PCL scaffolds in terms of bone integration between the implanted scaffold and surrounding host tissues and the difference of bone retention in the defect between these scaffolds. In addition to that, the question arises whether different types of scaffold geometry will affect the cellular and osteogenic potential of the transplanted cells. Effect after transplantation such as tissue rejection and bone viability also need to be considered in determining the success of bone regeneration. Therefore, this study assesses the potential of HA and PCL scaffolds in supporting *in vitro* cell viability, attachment, morphology, and osteoblast differentiation in comparison to the accepted model outlined for two-dimensional (2D) systems. Preosteoblast MC3T3-E1 cells were being used as cell sources and cultured on scaffolds and 2D culture plate. Moreover, comparison during *in vivo* bone regeneration of HA and PCL scaffolds was also been assessed in this study by using rat model with a maxillary bone defect.

Materials & Methods

Scaffolds preparation

HA scaffold was obtained from GranuMaS® (Granulab, Malaysia) with a granule size range from 0.2-1.0 mm. Meanwhile, PCL scaffold was obtained from Osteopore™ (Singapore) with a

Açıklamalı [P1]: Please place citations right after the correct correspondant. For ex: include granules (Beachkey et al.), paste, cement (Wang),…. etc.

Açıklamalı [P2]: If this statement were made by other researchers pls place a citation and if it's your opinion please make that clearer that it is your saying.

size range from 3 mm x 1.5 mm (diameter by height). Both HA and PCL scaffolds were sterilized using 75% (v/v) ethanol for 30 minutes, washed three times in sterile phosphate buffer saline (PBS) (Gibco, USA), and exposed to 15 minutes of ultraviolet radiation for each side of scaffolds. Sterilized scaffolds were immersed in α-Minimum Essential Medium (α-MEM) (Gibco, USA) overnight prior to cell culture.

Cell culture

Mouse MC3T3-E1 subclone 14 preosteoblast cells (ATCC No: CRL-2594™) were cultured in a complete medium consist of α-MEM supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 1 mM sodium pyruvate (Sigma, USA) and 1% (v/v) penicillin-streptomycin (Gibco, USA). The 2D culture of MC3T3-E1 was conducted as previously done by Yazid et al., (2019). Scaffolds and 96-well plates were seeded with 50,000 MC3T3-E1 cells suspended in complete medium and incubated for overnight to permit cell attachment. MC3T3-E1 cells-seeded scaffolds were then transferred to a new 96-well plate to prevent a false positive result from cells attached at the bottom of wells. All cultured cells were maintained in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C and~~with~~ the medium changed every three days.

For osteoblast differentiation, MC3T3-E1 cells on HA scaffolds, PCL scaffolds, and 2D culture plates were cultured in complete media supplemented with osteoblast differentiation factors of 50 µg/mL ascorbic acid (Sigma, USA) and 10 mM β-glycerophosphate (Sigma, USA). Meanwhile, MC3T3-E1 cells on scaffolds and 2D culture plate in a complete medium without differentiation factors were used as a negative control for osteoblast differentiation. The differentiation and complete medium were changed every three days.

***In vitro* analysis**

MTT assay for cell viability

The viability of MC3T3-E1 cells on scaffolds and 2D culture plate were evaluated on days 0, 7, 14, and 21 of culture by using the substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) which was reduced to formazan that accumulated in the cytoplasm of viable cells. Briefly, MTT solution (5 mg/mL) and complete medium at a ratio of 1:9 were added to each well containing MC3T3-E1 cells. Cells with MTT solution were incubated at 37 °C in a humidified atmosphere for 4 hours. After incubation, the MTT solution was removed and a buffer solution containing dimethyl sulfoxide (Sigma, USA) was added to dissolve formazan salts produced by the enzymatic reaction. After 10 minutes of agitation, each solution was measured for optical density using a microplate reader (Varioskan Flash Model 680, Thermo, USA) at 570 nm. As control, increasing number of cells from 100, 500, 1,000, 5,000, 10,000. 50,000, 100,000, 500,000 and 1,000,000 of MC3T3-E1 cells were directly cultured and assessed as well. MTT optical density was normalized to the number of cells in scaffolds and 2D culture plate.

Cell attachment and morphology

MC3T3-E1 cells attachment and morphology on HA and PCL scaffolds were examined using Field Emission Electron Microscope (FESEM) (Supra 55VP, Zeiss). EDX spectroscopy was conducted to analyzed elemental composition on HA and PCL scaffold after 21 days of osteoblast differentiation. At osteoblast differentiation culture intervals of 0, 7, 14, and 21 days, cell-seeded scaffolds were fixed overnight in 2.5% (v/v) glutaraldehyde (Polysciences, Inc., USA) in PBS and stored at 4°C. The fixed samples were then washed with PBS three times and subjected to sequential dehydration for 10 minutes in a graded ethanol series (30% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), and 100% (v/v)). Samples of HA scaffold were dried using critical point drying while samples of PCL scaffold were allowed to dry in air for 24 hours at room temperature. Both samples were sputter coated (Quorum, Q150RS) for 30 seconds with gold and observed under FESEM, at 3-15 kV accelerating voltage.

Alkaline phosphatase specific activity and total protein content

The ALP specific activity was measured by Sensolyte® pNPP alkaline phosphatase assay kit (AnaSpec, USA) according to the manufacturer's protocol. Briefly, cells were homogenized in the lysis buffer provided in the kit. Lysate produced was centrifuged for 10 minutes at 2,500 g at 4 °C (Yazid et al., 2018). Supernatant was collected and incubated with p-nitrophenyl phosphate (pNPP) at 37°C for 30 minutes. Stop solution was added after 30 minutes of incubation and absorbance measurement was taken at a wavelength of 405 nm by using a microplate reader. Total protein content was measured by Bradford assay with bovine serum albumin used as standard (Kruger 2009). ALP activity results were normalized to total protein content and were represented as U/mg.

***In vivo* analysis**

Cells and scaffolds preparation

MC3T3-E1 cells were cultured *in vitro* on HA and PCL scaffolds for a period of 14 days before transplantation. Cells on scaffolds were cultured in complete media supplemented with osteoblast differentiation factors. Medium was changed every 3 days. After 14 days of in vitro culture~~d~~, cells-seeded scaffolds were transplanted into rats with a surgically made maxillary bone defect.

Animals

A total of 24 mature female *Sprague dawley* rats (age: 6 to 8 weeks, body weight: 200-300 g) were used in this study. The housing, care, and experimental protocol were approved by the Universiti Kebangsaan Malaysia Animal Ethical Committee with approval number FD/2018/ROHAYA/26-SEPT.-2018-JUNE-2019. The animal study was reported according to the ARRIVE guidelines with respect to relevant items. The rats were obtained from Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. Prior to the

transplantation's surgery, rats' health was monitored for a week. All of the rats were kept in pairs per cage at the animal house of Faculty of Health Science, Universiti Kebangsaan Malaysia, with 12 hours light-dark cycle at 21-25 °C~~,~~ and were fed with food pellets and water was supplied on *ad libitum* basis. The activity of rats were observed once daily throughout the study~~ periods~~.

Surgery and transplantation

To create the bone defect at rat's left maxilla, each rat was first anesthetized with an intravenous injection of 80 mg/kg ketamine (TROY Laboratories PTY Limited, Glendenning) combined with 7.5 mg/kg xylazine (Indian Immunological Limited, India) and 12 mg/kg tramadol (Y.S.P, Malaysia). A buccal sulcular incision was made to expose the maxillary bone. A bone defect with 4 mm length and 1.5 mm depth was created~~s~~ in the anterior part of left maxilla using a trephine bur under constant irrigation. Constant irrigation with cooled sterile PBS was performed to prevent overheating of bone. The rats were randomly divided into four groups each containing six animals (n = 6). Group 1 consists of rats with surgically-made maxillary bone defects who received implantation of HA scaffold. Group 2 consists of rats with surgically-made maxillary bone defects who received implantation of PCL scaffold. Groups 1 and 2 serve as controls. Meanwhile, group 3 consists of rats with surgically-made maxillary bone defects who received transplantation of MC3T3-E1 cells-seeded HA scaffold. In group 3, rats with surgically-made maxillary bone defects received transplantation of MC3T3-E1 cells-seeded PCL scaffold.

After the transplantation, the mucosal flaps were closed with a simple interrupted suture pattern using 4-0 non-absorbable black silk suture. Postoperatively, each rat received a subcutaneous injection of 2 mg/kg dexamethasone (Duopharma, Malaysia) and 20 mg/kg amoxicillin (Duopharma, Malaysia) for a week to prevent tissue rejection and perioperative infection. Rats were maintained on a soft high-glucose diet for a week. A regular diet of ground pellet food and water *ad libitum* was resumed 1 week postoperatively. Rats were monitored daily by visual observation for signs of infection, inflammation, lack of food and water intake, and lethargy.~~ Veterinarian would be contacted or the rat would be euthanized if any of these adverse signs were observed.~~

Euthanasia and maxilla sample collection

All of the surviving animals were sacrificed six weeks after transplantation surgery. Rats were sacrificed via drug overdosing using a commercial euthanasia solution of 390 mg sodium pentobarbital (Vetoquinol SA, France) and 50 mg/ml sodium phenytoin (Duopharma, Malaysia) administered intravenously at the lateral vein. Maxilla with intact surrounding tissues from all the rats was dissected. Maxilla was cleaned from surrounding soft tissue and immediately placed in 10% (v/v) neutral buffered formalin (R&M Chemical, UK) for 24 hours before being rinsed with PBS. Samples were maintained in a buffer solution consists of PBS with penicillin and streptomycin at 4 °C until further use.

Micro-computed tomographic analysis

Açıklamalı [P3]: Please indicate the anesteshia protocol prior to euthanasia

In vivo high-resolution micro-computed tomography (Skyscan 1176; Skyscan, Belgium) was used to evaluate bone regeneration of HA and PCL scaffolds in the rat model. The harvested samples were scanned by a camera with a pixel size of 12.32 µm, and a frame was achieved using a 1 mm aluminum filter, with a rotation step of 0.7° and a rotation angle of 360°. The X-ray was operated at a voltage of 70 kV, a current of 142 µA, and an exposure time of 580 ms. Approximately 400 scan slices per sample with an image pixel size of 17.56 µm were taken, and serial coronal-oriented tomograms were reconstructed from the raw images in the CTAnalyser Skyscan software. A region of interest (ROI) was precisely positioned over each defect for quantitative analysis, encompassing all new bones within the defect site. Six samples for each group were measured and percentages of new bone volume (%), bone surface (mm^2), and bone surface density (mm^{-1}) of newly formed bone within each ROI were measured by assigning a threshold for total bone content (including trabecular and cortical bone). The results were assessed by a trained examiner, who was blinded to the experimental groups (L.S.F.).

Histology analysis

After micro-CT scanning, tissue samples were decalcified in 10% (v/w) buffered ethylene diamine tetraacetic acid (Sigma, USA) (pH 7.4) for 5-6 weeks then dehydrated with graded series of increasing ethanol concentrations (from 70% to 100%) and embedded in paraffin. Serial 5 µm-thick sections were cut using a microtome and sections were stained with hematoxylin and eosin (H&E) following standard protocols. The stained section was observed under light microscope (BX51; Olympus, Tokyo, Japan) and a digital image was obtained using CellB software. Results of new bone formation within the defect area were taken under 40x and 100x magnifications.

Histological level of new bone formation was assessed using a seven-point scale outline by Salkeld et al., (2001): 0 = no incorporation and no new bone formation, 1 = some incorporation and small amount of new bone, 2 = some incorporation and moderate amount of new bone formation, 3 = some corporation with new bone formation continuous with host bone and early remodeling changes in new bone, 4 = good graft incorporation and ample new bone, 5 = good graft incorporation of graft and new bone with host and ample new bone, 6 = excellent incorporation and advanced remodeling of new bone with graft and host. The analysis was validated by an experienced pathologist blinded to the study groups (N.S.N.).

Statistical analysis

Multiple comparisons for *in vitro* cell viability and osteoblast differentiation potential of scaffolds as well as *in vivo* bone morphometric and histology grading analysis were evaluated using Bonferroni-corrected by one-way analysis of variance (ANOVA). Values of p less than 0.05 were~~as~~ considered to be statistically significant. The data were expressed as the mean ± standard deviation (SD) of five independent experiments for *in vitro* study while a repeated experiment using six rats were used as *in vivo* biological replicates. Statistical analyses were performed using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA).

Ethical approval

This research was carried out according to the ethical and legal requirements of Universiti Kebangsaan Malaysia Animal Ethical Committee (UKMAEC). This permission allowed us to use rats as experimental animals while abiding to legal and ethical guidelines. Experiments utilized rats were performed humanely throughout this research. The euthanasia method was performed in accordance with the guideline from American Veterinary Medical Association (Underwood & Anthony 2020). All described experimental protocols involving rats were designed and performed according to the animal ethics guidelines approved by the UKMAEC with approval reference number FD/2018/ROHAYA/26-SEPT./944-SEPT.-2018-JUNE-2019.

Results

***In vitro* analysis**

Cell viability

MC3T3-E1 cells viability on HA and PCL scaffolds as well as 2D culture plate were measured by the increased number of viable cells throughout the culture period of 21 days. Cells grown in all culture conditions showed a continuous increase with culture time, reflecting good cell viability (*Fig. 1*). The number of viable MC3T3-E1 cells on scaffolds was markedly higher than the control 2D culture plate, significantly during the initial day of culture (days 0) between MC3T3-E1-HA and MC3T3-E1-2D ($p = 0.034$). Meanwhile, MC3T3-E1-HA ($p = 0.000$) and MC3T3-E1-PCL ($p = 0.0004$) showed significant higher number of cell viable on day 7 compared to MC3T3-E1-2D (*Table 1*). Interestingly, MC3T3-E1 cells grown in HA and PCL scaffolds continued to grow for up to 14 days but this growth decreased at 21 days. MC3T3-E1 cells showed an increased number of viable cells on HA scaffold compared to PCL scaffold significantly on days 7 ($p = 0.000$) and 14 ($p = 0.002$) (*Table 1* and *Fig. 1*).

Cell attachment and morphology

FESEM results indicated that both types of scaffolds allowed the attachment and spreading of the cells while maintaining a normal cellular morphology. As it can be seen in *Fig. 2A* and *B*, MC3T3-E1 cells on day 0 (a day after osteoblast induction) were already well attached and spread to the surface of both scaffolds, presenting a round shape configuration in HA scaffolds while a cluster of cells with extended cytoplasm in all directions observed in PCL scaffold. After 7 days of culture, cells on both scaffolds showed a typical morphology presenting a flat configuration with more lamellopodia connecting to neighboring cells and starting to form a continuous cell layer (*Fig. 2C, D*). Mineralized nodules can be observed as early as day 7 in the PCL scaffold. At day 14 of culture, MC3T3-E1 cells on HA scaffold start to aggregate and formed a monolayer of connected cells while a dense cell layer can be seen covering the surface of the PCL scaffold (*Fig. 2E, F*). At 21 days of culture, a dense cell layer could be observed on both scaffolds with some mineralized nodules appear in between the cell layer especially on HA

scaffold (arrows in *Fig. 2G, H*). During an early culture day, the density of the attached cells is higher in the PCL scaffold compare to the HA scaffold. However, as days of culture increase to day 21, the FESEM image showed that more cells were attached, and an intense appearance of mineralization nodule was observed over HA scaffold compare to PCL scaffold.

Energy dispersive x-ray spectroscopy (EDX) elemental analysis

EDX spectroscopy results showed the presence of a higher level of calcium and phosphorus after 21 days of MC3T3-E1 cultured on HA and PCL scaffolds (*Fig. 3*). HA scaffold had a higher ratio of calcium /~~ and~~ phosphorus (Ca/P) level from 2.29 to 2.4. Meanwhile, PCL scaffold showed an increase in Ca/P ratio from 2.22 to 2.36. Both scaffolds showed a slightly higher Ca/P ratio than the theoretical pure hydroxyapatite which is 2.15 (Venkatasubbu et al., 2011). Oxygen and carbon peaks present on the EDX indicate by-products excreted during the extracellular matrix production of MC3T3-E1 cells on the scaffold. The concentration of oxygen and carbon on both scaffolds were observed to be lower than calcium and phosphorus throughout 21 days of osteoblast differentiation.

ALP specific activity

The ALP specific activity was evaluated at days 0, 7, 14, and 21 ~~days~~ after MC3T3-E1was cultured in an osteogenic medium (*Fig. 4*). A trend of ALP specific activity was increased up to 21 days of osteoblast differentiation in all culture conditions. There were no statistically significant differences ($p > 0.05$) between MC3T3-E1 cells on scaffolds and 2D culture plate at day 0. The result also showed that the ALP specific activity of MC3T3-E1 cells on PCL scaffold (0.19 ± 0.03 U/mg) on day 7 was approximately 1.24 and 3.91 times higher than HA scaffold (0.15 ± 0.02 U/mg; $p = 0.001$) and 2D culture plate (0.05 ± 0.03 U/mg; $p = 0.000$). Interestingly, prolonged osteoblast induction of MC3T3-E1 cells on HA scaffold results in a significantly higher ALP specific activity compared to 2D culture plate especially on days 14 ($p = 0.000$) and 21 ($p = 0.000$) as well as on day 21 ($p = 0.019$) in comparison to PCL scaffold.

***In vivo* analysis**

Macroscopic analysis

After six weeks of transplantation, there were no significant complications such as dehiscence or fistula in the area of the maxilla defect. The maxillary bone defects were healing well without the presence of necrosis or obvious inflammation detected in any fresh maxilla specimen. A stable scaffold fixation with no migration was observed in all rat samples. HA and PCL scaffolds remained after six weeks of transplantation. Some parts of the HA and PCL scaffolds surface were covered by callus (*Fig. 5*).

Micro-computed tomographic analysis

Bone regeneration potential of HA and PCL scaffolds were also investigated using rat's maxillary bone defect. Surrounding tissues with scaffolds were imaged and analyzed using high resolution micro-computed tomographic (micro-CT), and 2D imaged were reconstructed (*Fig. 6A*). Limited to no bony bridge could be observed on empty HA (groups 1) and PCL (group 2) control groups. As shown in 2D reconstructed image, treatment of rat maxillary bone defect with MC3T3-E1-HA (group 3) and MC3T3-E1-PCL (group 4) demonstrated bony bridges with an increased amount of filled new bone compared to the control groups. Bridging of the defects with new bone occurred extensively in group 3. Some of the HA granules have consolidated and its radio density has increased. Group 4 showed minimal new bone formation and lower radio-density. Defects in group 4 showed a translucent bony bridge at six weeks postoperatively.

Furthermore, bone morphometric analysis of the micro-CT images was used to quantify the percentage of total new bone volume, new bone surface area, and the surface density of newly formed bone as presented in *Fig. 6B*. Rats on treatment group 3 (42.74% ± 9.45%) showed significantly increased new bone volume compared to group 4 (5.43% ± 1.82%; $p = 0.002$), HA scaffold control group (12.8% ± 7.08%; $p = 0.012$) and PCL scaffold control group (2.22% ± 0.36%; $p = 0.001$). There is no significant difference ($p > 0.05$) observed in the increment of bone surface area between treatment groups. While the new bone surface density level of group 3 (7.91 ± 1.07 mm^{-1}) was increased significantly compare to group 4 (1.69 ± 0.29 mm^{-1}; $p = 0.001$), HA scaffold control group (3.45 ± 1.43 mm^{-1}; $p = 0.014$) and PCL scaffold control group (0.9 ± 0.1 mm^{-1}; $p = 0.000$).

Microscopic analysis

New bone formation and biocompatibility of the chosen scaffolds in rat's maxillary bone defect model were further evaluated histologically using H&E staining. Defects treated with HA and PCL scaffolds showed cell and tissue infiltration with new bone formation through six weeks of transplantation period (*Fig.7*). The newly formed bone in the defect area of MC3T3-E1-HA (groups 3) and MC3T3-E1-PCL (group 4) was higher compared to the empty scaffold control groups. The connective tissue within the bone bridge in the group 3 and 4 was less prominent than in empty scaffold control groups.

Empty HA scaffold group showed the defect was surrounded by fibrous connective tissue and the new bone formation was growing from the edge of cavity toward the center. A moderate amount of bone islands with numerous osteoblast cells as well as a small number of osteocytes in the irregular lacuna could be observed at the edge of the defect. Group 3 showed considerably enhanced new bone formation compared to the empty HA control group. Effective scaffolding property of HA in osteoconduction could be seen with new bone ingrowth that is well developed throughout the pore channels of the HA scaffold. This can be observed with connective tissue migrated within the HA scaffold. Significant deposition of osteoblast at the marginalized parts of the new bone surrounding the periphery of the HA scaffold could also be observed extensively on group 3 than empty HA control group.

Empty PCL group showed small new bone formation in the middle of the defect areas with the appearance of extensive fibrous connective tissue ingrowth. Group 4 showed a presence of new bone that was extended into the scaffolds from the edge of defect toward the center. A moderate amount of bone islands could be observed peripherally. This confirmed that bone formation started at the periphery of the PCL scaffold. However, intense fibrous and connective tissues still present in the defect region treated with PCL scaffold compared to HA scaffold. Granulation tissue and neovascularization were visible at defect areas treated with both scaffolds. Both scaffolds demonstrated good biocompatibility with no significant inflammatory reaction.

Histological grading for new bone formation in the defect area treated with MC3T3T-E1-HA and MC3T3T-E1-PCL were higher compared to the empty scaffold control groups. In comparison to their respective controls, MC3T3-E1-PCL scaffold showed the highest new bone formation with 1.64 fold increase while only 1.25 fold increase was observed on MC3T3-E1-HA scaffold. Defect treated with HA scaffold showed the highest histological grading of new bone formation compared to their corresponding PCL scaffold. However, the mean difference between scaffold treatment groups was not statistically significant ($p > 0.05$) (*Table 2*).

Discussion

In this study, MC3T3-E1 cell viability on HA scaffold, PCL scaffold, and 2D culture plate was measured and compared to confirm the ability of chosen scaffolds to support the growth of cells during 3D *in vitro* culture. Through MTT assay, we identified that the viability of MC3T3-E1 cells on HA scaffold was significantly increased with a peak number of viable cells observed on day 14. The cellular response and behavior of cells can be influenced by the characteristic of the material surface (Seebach et al., 2010; Shamsuddin et al., 2017). It has been proved that the surface topography of rough-texture~~d~~ is capable to enhance the cellular adhesion and production of more mineralized matrix during osteoblast differentiation of cells. The HA scaffold used in this study exhibits a rough surface that may enhance cell attachment and subsequently promote cell growth. A previous study by Ling et al., (2015) demonstrated that cells-seeded on the HA-composite scaffold had higher cell proliferation and attachment compare to the β-tricalcium phosphate-composite scaffold due to the rough texture present on the surface of HA. This report is consistent with our finding that HA scaffold promotes higher cell growth during the initial period. However, it significantly decreased with a prolonged culture which reflects the dominant occurrence of osteogenic differentiation. This pattern has also been observed in cell viability on PCL scaffold. The obvious reduction in cell viability on HA and PCL scaffolds in comparison with control 2D culture plate may be due to the transition of the cells from a proliferative phase into the differentiation phase induced by direct interactions with the scaffold. This finding suggests that HA and PCL scaffolds are cytocompatible as it support and enhance MC3T3-E1 cell viability.

MC3T3-E1 cells differentiation toward osteoblast was observed by FESEM image, EDX analysis, and ALP specific activity. FESEM image showed intense attachment, well-spread morphology and extensive growth of osteoblast differentiated MC3T3-E1 cells on both scaffolds.

Açıklamalı [P4]: cellular proliferation? To evaluate cellular adhesion, other technics should be taken into consideration. If you're making this statement depending upon your electron microscopy imaging results, please refer this analysis in the sentence. Otherwise, it feels like your MTT results provided this knowledge of better adhesion.

Açıklamalı [P5]: Changing this as "enhance the adhesion of cells and mineralization of the matrix." could provide a more clear statement.

These results reflect those of Seebach et al., (2010) and Jamal et al., (2018) who also found strong attachment, growth, and proliferation of mesenchymal stem cells on HA and PCL scaffolds. Although HA has the potential to naturally induced osteoblast differentiation, MC3T3-E1 cells on PCL scaffold able to show signs of osteoblast differentiation as early as day 7 of induction. On day 7 of osteoblast differentiation, mineralization nodules were detected on PCL scaffold while it was not present on HA scaffold. Cell to cell contact is assumed to influence this phenomenon. It is widely believed that when cells become in close contact with each other, they can be activated to differentiate, and once they form multilayers they start mineralization (Yu et al., 2017). In this study, MC3T3-E1 cells on PCL scaffold at day 7 became close to each other as more cells can be observed attached to the scaffold compared to HA scaffold. These results are in agreement with those obtained by Jamal et al., (2018) which demonstrate early osteoblast differentiation of dental pulp stem cells on PCL scaffold was influenced by an intense cell to cell contact. However, mineralized nodules can be observed more prominently on HA scaffold with prolonged osteoblast differentiation. These results are likely to be related to the rough texture present on the surface of HA that coherently supports extensive MC3T3-E1 cell growth. An increased number of MC3T3-E1 cells may permit more cell to cell contact which directly induced osteoblast differentiation and mineralization. This finding raises the possibility that the surface characteristic of the scaffold could provide a suitable microenvironment for cell attachment, cellular interaction, and osteoblast differentiation.

These results were further supported by EDX analysis which allowed assessment and quantification of the presence of different bone types based on elemental analysis of calcium, phosphorus, and nitrogen (Prati et al., 2020). Therefore, EDX spectroscopy was utilized to detect the amount of elemental calcium and phosphorus produced by cells grown on HA and PCL scaffolds. EDX results demonstrate that MC3T3-E1 cells were able to attach and grow on HA and PCL scaffolds and form mineralized nodules or tissue which consists of calcium and phosphorus deposits. HA scaffold showed the higher ratio of calcium to phosphorus after 21 days of osteoblast differentiation which indicates a higher quantity of minerals.

In addition to the initial evaluation of MC3T3-E1 cell morphology using FESEM following osteoblast differentiation, an increase in ALP specific activity of these cells on scaffolds is evidence of successful osteoblast formation. ALP is one of the generally recognized biochemical markers for bone cell activity and is considered to play a role in bone mineralization (Megat Abdul Wahab et al., 2020). Scaffolds for bone regeneration application must support the differentiation of cells to functional bone tissue. Our results showed an overall increase of ALP specific activity throughout 21 days of osteoblast differentiation for all culture conditions. Higher ALP specific activity of MC3T3-E1 cells has been observed on HA and PCL in comparison to the control 2D culture plate. Both scaffolds were able to support osteoblast differentiation of MC3T3-E1 cells. The results of this study also showed that MC3T3-E1 cells on PCL scaffold required a shorter time to undergo osteoblast differentiation with high ALP specific activity can be detected as early as day 7. However, as the day of osteoblast differentiation increase, ALP specific activity of MC3T3-E1 cells on HA scaffold showed a more prominent

Açıklamalı [P6]: In this article Yu et al mentions double layered cell transfer method.There's no mentioning of multilayered cells start mineralization.

Açıklamalı [P7]: changing it to "successful induction of osteogenic differentiation." could be a better choice. There are several cellular steps throughout the osteogenic differentiation starting with mesenchymal stem cells to osteocytes and beginning from the early osteoprogenitor cells, these cells express ALP gene and produce ALP enzyme. Therefore, saying successful osteoblast formation is a bit assertive as these cells could be any cell type from an early progenitor to mature osteoblast.

result. A previous study by Deligianni et al., (2000) suggested that there was a delay in the expression of ALP activity on HA scaffold with rougher surfaces. These results are consistent with the FESEM images in which mineralized nodules of MC3T3-E1 cells on PCL can be observed as early as day 7 and an increase of mineralized nodules were observed with a prolonged culture on HA scaffold. An increase in ALP specific activity of MC3T3-E1 cells on HA scaffold at the end of osteoblast differentiation period was further enhanced by osteoinductive property present on the scaffold. Studies by Usui et al., (2010) and Wang et al., (2015) indicates the potential of calcium and phosphate ions released from HA could directly induce and up-regulates osteoblast differentiation which promotes bone formation through calcification. Taken together, these findings suggest the potential HA and PCL scaffolds in promoting *in vitro* osteoblast differentiation which can be observed more prominently in HA scaffold.

Nonetheless, since this study also aims to test different scaffolds geometry on enhancing *in vivo* bone regeneration, it is of note to consider that our animal model is suitable for scaffold placement in the maxillary bone defect treatment. Postoperatively, all rats showed immediate recovery as daily activities resumed within 24 hours. There were no complications such as dehiscence or fistula in the area of the transplantation. A stable scaffold fixation with no migration was observed in all rat samples. HA and PCL scaffolds remained after six weeks of transplantation. This indicates that both scaffolds have slow biodegradation under physiologic conditions with high mechanical resistance. A similar finding was also reported by Wongsupa et al., (2017) which suggests that slow biodegradation of PCL-biphasic calcium phosphate scaffold could have advantages for highly loaded areas that required long-term critical functional support.

In the presented study, *in vivo* bone regeneration of transplanted MC3T3-E1 cells on HA and PCL scaffolds were evaluated by micro-CT and histology analyses. The reconstructed micro-CT image revealed a radio-dense appearance that suggested bone ingrowth within HA and PCL scaffolds and subsequently filled the defect area. These results corroborate the ideas of Crovace et al., (2020) who showed that new bone formation could be observed by radio-dense aspect from X-ray images. Meanwhile, the bone morphometric analysis revealed that HA scaffold significantly enhanced new bone volume and surface density, although no significant differences were found on bone surface area in comparison to PCL scaffold. Our results are in accordance with a previous study by Jang et al., (2017) in which bone formation was indicated by higher bone volume present in granular and porous HA scaffolds. Low bone surface area occurs when trabecular thickness, bone surface density to tissue volume are at a high level (Kim et al., 2004). Micro-CT can be over-estimated depending on the characteristics of bone substitutes or scaffolds. Therefore, there are chances that an increase in bone formation is due to the inherent radio-opacity contain in HA scaffold (Jang et al., 2017). While the low value of bone volume, surface area, and surface density on PCL scaffold is due to the translucent property of PCL. This led us to use a qualitative histological approach to further evaluate the new bone formation.

The results from histological observation showed that HA and PCL scaffolds are capable of enhanced new bone formation with intensive vascularity at the defect area, indicating the bone vitality and beginning of new bone formation. Both scaffolds revealed good biocompatibility with no adverse inflammatory side effects. Furthermore, the defect area with transplanted HA scaffold showed extended bone formation on its surface with the infiltration of connective tissue present within HA particle. This showed that HA scaffold used in this study is a porous scaffold that allows cell migration and proliferation. The infiltration of connective tissue resulted from the interaction between MC3T3-E1 cells and the osteoconductive property of HA scaffold. These results are in agreement with those obtained by Sulaiman et al., (2013) which demonstrate bone formation for ceramic scaffold started on the surface and proceeded to the center of the pores. Meanwhile, for PCL scaffold, bone tissue originated from the surface of the PCL fibers containing differentiated cells that further grow in between several fibers and encapsulate them over time. The results from the present study indicate that HA and PCL scaffolds have the potential to repair rat's maxillary defects with properties applicable for bone tissue engineering. Moreover, it was concluded that transplantation of cells and HA scaffolds showed better *in vivo* bone regeneration potential with enhanced new bone volume, increment of bone surface density, and new bone formation as shown by micro-CT and histology analyses.

Açıklamalı [P8]: What are the evidences to support this statement? Connective tissue does not infiltrate into an area, in normal physiological healing, cells do migrate to defect area and they differentiate into fibrous tissue. Afterwards cells do condensate and form fibro-cartilage tissue parts which would enlarge and form a cartilage later on. Cells keep on differentiating and due to vascularization and ALP activity, osteoid structures occur.

Conclusions

In conclusion, HA and PCL scaffolds used in this study can support *in vitro* cell viability, attachment, morphology, and osteoblast differentiation following the accepted model of the two-dimensional system. Both scaffolds demonstrated good bone regeneration properties with no significant inflammatory reaction. However, HA scaffold with granule geometry showed better new bone formation when transplanted on the maxillary bone defect of rats compared to PCL scaffold with lattice geometry. This confirmed *in vitro* growth and osteoblast potential of HA scaffold. Based on the obtained result, it is suggested that HA scaffold could be considered as a potential and attractive scaffold for clinical use in maxillary bone regeneration.

Acknowledgements

The authors wish to thank Dr. Siti Khadijah Shuhaimy Basha for her kind help during the animal surgery and transplantation procedure.

References

Anderson JM, and Shive MS. 1997. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Advanced drug delivery reviews* 28:5-24.

Beachley V, and Wen X. 2010. Polymer nanofibrous structures: Fabrication, biofunctionalization, and cell interactions. *Progress in polymer science* 35:868-892.

Bose S, Roy M, and Bandyopadhyay A. 2012. Recent advances in bone tissue engineering scaffolds. *Trends in biotechnology* 30:546-554.

Crovace AM, Lacitignola L, Forleo DM, Staffieri F, Francioso E, Di Meo A, Becerra J, Crovace A, and Santos-Ruiz L. 2020. 3D biomimetic porous titanium (Ti6Al4V ELI) scaffolds for

large bone critical defect reconstruction: an experimental study in sheep. *Animals* 10:1389.
Deligianni DD, Katsala ND, Koutsoukos PG, and Missirlis YF. 2000. Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength. *Biomaterials* 22:87-96.
Dwivedi R, Kumar S, Pandey R, Mahajan A, Nandana D, Katti DS, and Mehrotra D. 2020. Polycaprolactone as biomaterial for bone scaffolds: Review of literature. *Journal of oral biology and craniofacial research* 10:381-388.
Farinawati Y, Nurmimie A, Nur Atmaliya L, Shahrul Hisham ZA, and Rohaya MAW. 2020. Scaffold Selection for Tissue Engineering in Dentistry. *Med & Health* 15:34-53.
Fernandez-Yague MA, Abbah SA, McNamara L, Zeugolis DI, Pandit A, and Biggs MJ. 2015. Biomimetic approaches in bone tissue engineering: Integrating biological and physicomechanical strategies. *Advanced drug delivery reviews* 84:1-29.
Fishero B, Kohli N, Das A, Christophel J, and Cui Q. 2015. Current concepts of bone tissue engineering for craniofacial bone defect repair. *Craniomaxillofacial trauma & reconstruction* 8:23-30.
Hajiali F, Tajbakhsh S, and Shojaei A. 2018. Fabrication and properties of polycaprolactone composites containing calcium phosphate-based ceramics and bioactive glasses in bone tissue engineering: a review. *Polymer reviews* 58:164-207.
Hench LL, and Thompson I. 2010. Twenty-first century challenges for biomaterials. *Journal of the Royal Society Interface* 7:S379-S391.
Jamal M, Greish Y, Chogle S, Goodis H, and Karam SM. 2018. Growth and Differentiation of Dental Stem Cells of Apical Papilla on Polycaprolactone Scaffolds. *Novel Biomaterials for Regenerative Medicine*: Springer, 31-40.
Jang SJ, Kim SE, Han TS, Son JS, Kang SS, and Choi SH. 2017. Bone regeneration of hydroxyapatite with granular form or porous scaffold in canine alveolar sockets. *in vivo* 31:335-341.
Kattimani VS, Kondaka S, and Lingamaneni KP. 2016. Hydroxyapatite–-Past, present, and future in bone regeneration. *Bone and Tissue Regeneration Insights* 7:BTRI. S36138.
Kim D-G, Christopherson GT, Dong XN, Fyhrie DP, and Yeni YN. 2004. The effect of microcomputed tomography scanning and reconstruction voxel size on the accuracy of stereological measurements in human cancellous bone. *Bone* 35:1375-1382.
Kruger NJ. 2009. The Bradford method for protein quantitation. *The protein protocols handbook*:17-24.
Lee JC, Kleiber GM, Pelletier AT, Reid RR, and Gottlieb LJ. 2013. Autologous immediate cranioplasty with vascularized bone in high-risk composite cranial defects. *Plastic and reconstructive surgery* 132:967-975.
Ling LE, Feng L, Liu HC, Wang DS, Shi ZP, Wang JC, Luo W, and Lv Y. 2015. The effect of calcium phosphate composite scaffolds on the osteogenic differentiation of rabbit dental pulp stem cells. *Journal of Biomedical Materials Research Part A* 103:1732-1745.
Liu H, and Webster TJ. 2007. Nanomedicine for implants: a review of studies and necessary experimental tools. *Biomaterials* 28:354-369.
Megat Abdul Wahab R, Abdullah N, Zainal Ariffin SH, Che Abdullah CA, and Yazid F. 2020. Effects of the Sintering Process on Nacre-Derived Hydroxyapatite Scaffolds for Bone Engineering. *Molecules* 25:3129.

602 Mendonça G, Mendonça DB, Aragao FJ, and Cooper LF. 2008. Advancing dental implant
603 surface technology–from micron-to nanotopography. *Biomaterials* 29:3822-3835.
604 Prati C, Zamparini F, Botticelli D, Ferri M, Yonezawa D, Piattelli A, and Gandolfi MG. 2020.
605 The Use of ESEM-EDX as an Innovative Tool to Analyze the Mineral Structure of Peri-
606 Implant Human Bone. *Materials* 13:1671.
607 Robey PG, Kuznetsov SA, Ren J, Klein HG, Sabatino M, and Stroncek DF. 2015. Generation of
608 clinical grade human bone marrow stromal cells for use in bone regeneration. *Bone*
609 70:87-92.
610 Salkeld SL, Patron LP, Barrack RL, and Cook SD. 2001. The effect of osteogenic protein-1 on
611 the healing of segmental bone defects treated with autograft or allograft bone. *JBJS*
612 83:803-816.
613 Seebach C, Schultheiss J, Wilhelm K, Frank J, and Henrich D. 2010. Comparison of six bone-
614 graft substitutes regarding to cell seeding efficiency, metabolism and growth behaviour of
615 human mesenchymal stem cells (MSC) in vitro. *Injury* 41:731-738.
616 Shamsuddin SA, Ramli R, Razali M, Baharin B, Sulaiman S, Hwei Ng M, Low CK, Jabar MNA,
617 Nordin R, and Yahaya N. 2017. Guided bone regeneration using autologous plasma, bone
618 marrow cells and β-TCP/HA granules for experimental alveolar ridge reconstruction in
619 Macaca fascicularis. *Journal of Biomaterials and Tissue Engineering* 7:111-118.
620 Sulaiman SB, Keong TK, Cheng CH, Saim AB, and Idrus RBH. 2013. Tricalcium
621 phosphate/hydroxyapatite (TCP-HA) bone scaffold as potential candidate for the
622 formation of tissue engineered bone. *The Indian journal of medical research* 137:1093.
623 Tavakol S, Kashani IR, Azami M, Khoshzaban A, Tavakol B, Kharrazi S, Ebrahimi S, and
624 Sorkhabadi SMR. 2012. In vitro and in vivo investigations on bone regeneration potential
625 of laminated hydroxyapatite/gelatin nanocomposite scaffold along with DBM. *Journal of*
626 *Nanoparticle Research* 14:1-14.
627 Torres E, Fombuena V, Vallés-Lluch A, and Ellingham T. 2017. Improvement of mechanical
628 and biological properties of polycaprolactone loaded with hydroxyapatite and halloysite
629 nanotubes. *Materials Science and Engineering: C* 75:418-424.
630 Underwood W, and Anthony R. 2020. AVMA guidelines for the euthanasia of animals: 2020
631 edition. *Retrieved on March* 2013:2020-2021.
632 Usui Y, Uematsu T, Uchihashi T, Takahashi M, Takahashi M, Ishizuka M, Doto R, Tanaka H,
633 Komazaki Y, and Osawa M. 2010. Inorganic polyphosphate induces osteoblastic
634 differentiation. *Journal of dental research* 89:504-509.
635 Venkatasubbu GD, Ramasamy S, Ramakrishnan V, and Kumar J. 2011. Nanocrystalline
636 hydroxyapatite and zinc-doped hydroxyapatite as carrier material for controlled delivery
637 of ciprofloxacin. *3 Biotech* 1:173-186.
638 Wahab RMA, Ng WC, Yazid F, Luchman NA, and Ariffin SHZ. 2020. Orthodontic
639 Considerations in Bone Graft Selection for Alveolar Cleft Repair. *Sains Malaysiana*
640 49:349-356.
641 Wang M. 2003. Developing bioactive composite materials for tissue replacement. *Biomaterials*
642 24:2133-2151.
643 Wang T, Yang X, Qi X, and Jiang C. 2015. Osteoinduction and proliferation of bone-marrow
644 stromal cells in three-dimensional poly (ε-caprolactone)/hydroxyapatite/collagen
645 scaffolds. *Journal of translational medicine* 13:1-11.
646 Wongsupa N, Nuntanaranont T, Kamolmattayakul S, and Thuaksuban N. 2017. Assessment of
647 bone regeneration of a tissue-engineered bone complex using human dental pulp stem

cells/poly (ε-caprolactone)-biphasic calcium phosphate scaffold constructs in rabbit calvarial defects. *Journal of Materials Science: Materials in Medicine* 28:77.

Yazid F, Kay ANM, Qin WY, Luchman NA, Wahab RMA, and Ariffin SHZ. 2019. Morphology and Osteogenic Capability of MC3T3-E1 Cells on Granular Hydroxyapatite Scaffold. *Journal of Biological Sciences* 19:201-209. 10.3923/jbs.2019.201.209

Yazid F, Luchman NA, Megat Abdul Wahab R, Zainal Ariffin SH, and Senafi S. 2018. Proliferation and osteoblast differentiation mice dental pulp stem cells between enzyme digestion and outgrowth method. *Sains Malaysiana* 47:691-698.

Yu M, Wang L, Ba P, Li L, Sun L, Duan X, Yang P, Yang C, and Sun Q. 2017. Osteoblast progenitors enhance osteogenic differentiation of periodontal ligament stem cells. *Journal of periodontology* 88:e159-e168.

Zhao X, Lui YS, Choo CKC, Sow WT, Huang CL, Ng KW, Tan LP, and Loo JSC. 2015. Calcium phosphate coated Keratin–PCL scaffolds for potential bone tissue regeneration. *Materials Science and Engineering: C* 49:746-753.

REVIEWER NOTES

1. The title of the manuscript is referring the effect of "scaffold geometry", but I believe the used methodology and obtained results are not sufficient to say anything about the effect of geometry. HA and PCL are entirely different materials and as it is mentioned in the manuscript, they have some different qualities effecting the osteogenesis. If the HA and PCL had the same geometry, could we expect to get similar results between the experimental groups? The geometry of scaffolds indeed proved have good amount of influence but scientists usually use different geometrical patterns with same type of material like using HA scaffolds in hexagonal geometry vs HA scaffolds in square geometry. Therefore, the title must be changed and all the statements related with scaffold geometry should be discarded.
2. Lines 82-84: Please place citations right after the correct correspondant. For ex: include granules (Beachkey et al.), paste, cement (Wang), …. etc.
3. Lines 84-85: If this statement were made by other researchers please place a citation and if it's your opinion please make that clearer that it is your saying.
4. Did you used penicillin streptomycin and/or dexamethasone in osteogenic differentiation medium? Some researchers prefer to use phenol red-free mediums for osteogenic differentiation studies as it has some osteoinductive effects. If you used phenol red-free medium please mention it.
5. What was the confluence of cells during 2D culture? The proliferation of the cells might be effected by the lack of availability of surface area to grow?

Biçimlendirdi: Yazı tipi: 26 nk, Kalın

Biçimlendirilmiş: Ortadan, Aralık Önce: 6 nk, Sonra: 6 nk

Biçimlendirdi: Yazı tipi: (Varsayılan) Times New Roman, 14 nk

Biçimlendirilmiş: Aralık Önce: 6 nk, Sonra: 6 nk

6. What could be the reason behind seeing dramatically different results in cell viability at the initial day? Are there any similar results available in previous studies?

7. Did you remove scaffolds from the wells before absorbance reading for MTT?

8. Did you perform any replicates for MTT and ALP Assay?

9 Why did you prefer female animals? Did you perform any synchronization for their oestrus cycles? As estradiol has a significant effect on osteogenesis, you might have higher standard deviations.

10. Please mention the use of anesthetics prior to euthanasia.

11. Have you lost any animals during the study? If yes, did you replace them or decrease the sample size?

12. Line 420: To evaluate cellular adhesion, other technics should be taken into consideration. If you're making this statement depending upon your electron microscopy imaging results, please refer this analysis in the sentence. Otherwise, it feels like your MTT results provided this knowledge of enhanced adhesion.

13. Line 420-421: Changing this as "enhance the adhesion of cells and mineralization of the matrix." could provide a more clear statement.

Biçimlendirilmiş: Aralık Sonra: 6 nk

14. Line 444: and once they form multilayers they start mineralization (Yu et al., 2017).

In this article Yu et al. mentions double-layered cell transfer method (https://www.nature.com/articles/srep33286). There's no mentioning of that the cells start mineralization when they become multilayered.

Biçimlendirilmiş: Aralık Önce: 6 nk, Sonra: 6 nk

15. Line 466: changing "successful osteoblast formation" to "successful induction of osteogenic differentiation." could be a better choice. There are several cellular steps throughout the osteogenic differentiation starting with mesenchymal stem cells to osteocytes and beginning from the early osteoprogenitor cells, those cells express ALP gene and produce ALP enzyme. Therefore, saying successful osteoblast formation is a bit assertive as these cells could be of any cell type from an early progenitor to mature osteoblast.

16. Authors are suggested to use marks like arrows and arrow heads in figure 7. For example in the high magnification PCL image, authors placed NB near to an osteoid structure, yet the writing is on connective tissue. Inexperienced readers may misunderstand this image therefore, putting marks could be beneficial.

17. Is it possible for authors to use better staining technics for paraffin sections if you still have unstained sections or blocks available? Staining techniques such as Masson Goldner trichrome staining, Movat's pentachrome staining, etc. are commonly used staining technics to identify tissue transitions and all tissue types relevant with the osteogenesis which would allow you to address different tissue types much better. For example, in the last image (high magnification MC3T3-PCL) of figure 7, some of the NB areas are probably consist of cartilage and there's a fibro-cartilage transition area as well but H&E staining is not providing enough details to fully identify these structures.

18. Line 522-523:"The infiltration of connective tissue resulted from the interaction between MC3T3-E1 cells and the osteoconductive property of HA scaffold"

What are the evidences of authors to support this statement? Connective tissue normally does not infiltrate into an area. In normal physiological healing, cells do migrate to defect area and they differentiate form a fibrous tissue. Afterwards cells do condensate and form fibro-cartilage tissue parts which would enlarge and form a cartilage later on. Cells keep on differentiating and due to vascularization and ALP activity, osteoid structures occur in the defect site.