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Biocompatibility Assessment of Fabricated Melt-Derived 45S5 Bioactive Glass on Dental Cells Proliferation

Abstract—This study aim to characterize melt-derived bioactive glass and to determine the bioactive glass (BG) suitability for dental usage through proliferative activity assessment of stem cells from human exfoliated deciduous teeth (SHED) when exposed to bioactive glass conditioned medium. Bioglass 45S5 in mole percentages (46.13% SiO_2, 26.91% CaO, 24.35% Na_2O and 2.60% P_2O_5) was synthesized through melt-derived and characterized using X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) to confirm and identify its properties. SHED were used to evaluate the biocompatibility of 45S5 by exposing the cells to various concentration of BG-conditioned medium (1-10 mg/ml) using alamarBlue assay. The BG produced has an amorphous structure as shown by XRD analysis. The Si-O-Si bending, asymmetric Si-O stretching and asymmetric Si-O-Si stretching bands were observed in the BG structure supporting the presence of silicate network. For alamarBlue assay, SHED cultured in BG-conditioned medium showed high proliferation rate when subjected to minimal powder content in the DMEM cell culture medium. Hence, it can be concluded that SHED cultured in lower powder content of the BG-conditioned media showed high proliferative activity suggesting the potential of the BG for dental usage.

***Keywords*: alamarBlue, bioactive glass, proliferation, SHED.**

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1 INTRODUCTION

Currently, many people are suffering from trauma or bone diseases [1]. Hard tissue regeneration is one of the options to replace missing tissue and is currently the best option for promoting new bone formation within the body [2]. Bone tissue engineering (BTE) aimed to restore damaged tissue to its original condition.

However, problem exists in the bone tissue engineering due to the lack of availability of replacement materials which is able to act as templates for cell attachment. Implant made from stainless steel and titanium is commonly used for bone fixation. However, they do not possess natural characteristics of human bone. Hence, problem such as corrosion may predispose the implant for removal from the body [3].

Many types of biomaterials are available nowadays to promote hard and soft tissue growth. Biomaterials are defined as materials which promoted interfacial bonding between implantable materials and the host [4]. Biomaterial is classified into Class A and B [5]. Material which promotes new bone formation away from the material and bone interphase which bonds to hard and soft tissues is classified as Class A which normally has osteoconductive and osteoinductive properties. While materials that bind only to the hard tissues and promotes new tissue formation along its surface is classified as Class B.

Bioactive glass falls under Class A category [6]. The 45S5 also known as Bioglass® were first developed by Hench consisting of 45% silica (SiO_2), 24.5% calcium oxide (CaO), 24.5% sodium oxide (Na_2O), and 6% phosphorous pentoxide (P_2O_5) in weight percentages (wt.%) which corresponds to normal bone composition [5]. Changes in the original BG composition lead to deviations in bioglass properties when compared to standard 45S5 [7].

Studies have shown that dissolution products of BG are able to promote bone cells proliferation and differentiation [8]. Various forms of bioglass are available for human use including BG particle, disk and other three dimensional shapes. Bioglass forms HA layer upon exposure to simulated body fluid (SBF) where this layer helps to create strong bonds between the bioglass and human tissue. It has been shown that this HA layer

contain similar compositions in relation to bone. The mechanism of HA layer formation which contain few stages results in the strong bond between the host and the implant [9]. Conventional bioglass fabrications method include melt-derived, melt-annealed and sol-gel. The current project produces 45S5 BG powder through melt-derived method with composition 46.13% SiO_2, 26.91% CaO, 24.35% Na_2O and 2.60% P_2O_5 in mole percentages (mole %).

The fabrication of BG utilizes high temperature to ensure proper glass melting which is followed by direct quenching into water for obtaining frits [9]. Rapid quenching is important in order to prevent crystallization within the glass structure [10]. HA layer is formed on the BG surface at much faster rate at low pH due to faster ion exchange. In contrast, higher pH may delayed a HA formation [11].

This study aim to synthesize BG through melt-derived method and to determine its compatibility towards stem cells from human exfoliated deciduous tooth (SHED). BG powders in different weight ratio to the culture medium were prepared and the BG-conditioned media was used to culture SHED [12]. Moreover, SHED has higher proliferation rate when compared to dental pulp stem cell (DPSC) and is a suitable source for stem cell-based therapy compared to DPSC and BMMSCs [13]. According to Nakamura et al., the proliferation rate of SHED was significantly higher compare to DPSCs and BMMSCs. In addition to a higher proliferation capability, SHED also has the advantages of being abundant upon harvesting from the pulp tissue and painless stem cell collection with minimal invasion [13]. SHED was also shown to possess higher multipotent stem cells compared to adult tooth which is more immature thus having higher proliferative capacity [14]. Thus, SHED could be a desirable option as a cell source for potential therapeutic applications.

2 MATERIALS AND METHODS

2.1 Preparation of Bioactive Glasses

Briefly, the 45S5 BG powder with composition of silica (SiO_2) 46.13%, calcium oxide (CaO) 26.91%, sodium oxide (Na_2O) 24.35% and phosphorus pentoxide (P_2O_5) 2.60% (in mole percentages) were produced by a melt-quench route as previously described [9]. BG powder was prepared by mixing reagent grade Na_2CO_3, $CaCO_3$, SiO_2 and P_2O_5, raw materials (without further purification) in a sealed polyethylene bottle for overnight about 50 g per batch. Briefly, the weighted batches were melted in alumina crucibles in an electric furnace at a temperature of 1400 ± 10°C with soaking time of 3 hours and then rapidly quenched in distilled water. Next, the glass frit was dried in an oven at 110°C overnight. Then, the glass frit was milled at 500 rpm for 10 min and then followed by 30 min sieving in a mechanical shaker to obtain glass powder with particles size less than 38 µm.

2.2 Physical Analysis

2.2.1 X-ray Diffraction (XRD) Analysis

XRD analysis was carried out to identify the crystalline phases of the BG sample. The BG powder was subjected to phase analysis using an X-ray diffractometer, adopting Ni filter and Cu target with voltage of 40 KV and a current of 25 mA. The XRD patterns were recorded in a 2θ range of 20 to 80° and XRD patterns obtained were interpreted using the JCPDS - International Centre for Diffraction Data Cards as a reference data in the present work.

2.2.2 Fourier Transform Infrared (FTIR) spectroscopy

FTIR in transmission mode was used to identify the chemical groups in the BG structures. Approximately, 800 mg of grounded spectroscopic grade KBr with 10 mg of the powdered sample were mixed and pressed to make a transparent KBr pellet. BRUKER VECTOR 33 spectrometer was used to measure the IR spectra in the range of 400 cm^{-1} to 4000 cm^{-1} at a resolution of 2 cm^{-1}.

2.3 Bioglass-based medium preparation

The prepared 45S5 BG powder was weighted on a weighing boat. Then, the BG powder was incubated with culture medium at different concentrations from 1, 2, 4, 8 to 10 mg/ml and placed in an incubator shaker at 37 °C for 4 hours. Prior to keeping the BG-conditioned medium in a 4 °C fridge, the conditioned medium was filtered using 0.22 µm syringe filter. Then, the conditioned-medium was supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic/antimycotic (A/A) before being incubated in a CO_2 incubator at 37 °C.

2.4 Cell viability using alamarBlue® assay

The alamarBlue® (AB) assay was performed following instructions from the manufacturer. Once the SHED reached 80% confluence, the cells were trypsinized and counted using haemocytometer followed by seeding in 96-well plates (5×10^3 cells/cm^2). SHED has been harvested from previous project (304/CIPPT/6311045) under an

ethical approval of USMKK/PPP/JEPeM [233.3.3(09)] obtained from Internal Review Board and was donated for use in this study. The cells were exposed with BG-based conditioned media and incubated in a CO_2 incubator with 5 % CO_2 at 37 °C. For each time points (1, 2, 4 and 7 days), the cell-culture media from the well plates were taken out and then followed by washing steps using 100 µl of DPBS per well. Then, approximately 150 µl AB dye (10 % v/v alamarBlue® in DMEM with no phenol red, Gibco, USA) was added into each well (including one with no cells to be used as blank) and the well plates were further incubated in an incubator at 37 ºC for 2 hours. After 2 hours incubation period, 100 µl of the reaction product was then transferred to a black Costar 96-well plate. The fluorescence of Alamar Blue was measured using microplate reader (FLUOstar Omega, BMG Labtech) at 544 nm excitation and emission of 590 nm.

3 RESULTS

3.1 X-ray Diffraction Analysis

XRD result for 45S5 bioactive glass is shown in Figure 1. Based on Figure 1, the 45S5 BG structure was in amorphous phase since no crystalline peaks were detected. It has been proved by the presence of broad peak between 33° until 40°.

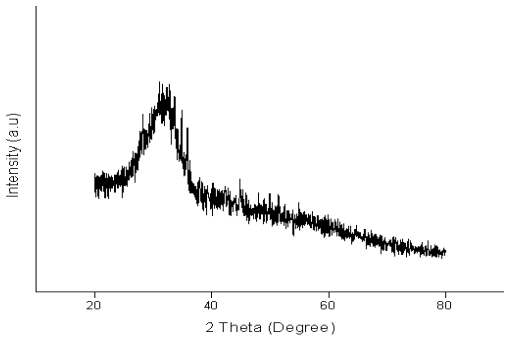

Figure 1: XRD result for bioactive glass

3.2 Fourier Transform Infrared spectroscopy

Figure 2 showed the FTIR reflectance spectra of 45S5 bioactive glass between 400 until 4000 cm^{-1}. The 45S5 bioactive glass was sampled from bioactive glass that was melted in an alumina crucible. Functional group Si-O-Si bending mode is present in the 45S5 bioactive glass which was located between 450 until 480 cm^{-1} in Figure 2. The functional group of Si-O-Si symmetric stretching vibration is detected in the range of 725 to 800 cm^{-1} in the 45S5 bioactive glass. Furthermore, presence of Si–O–Si asymmetric stretching vibration is observed between 1000 until 1200 cm^{-1}.

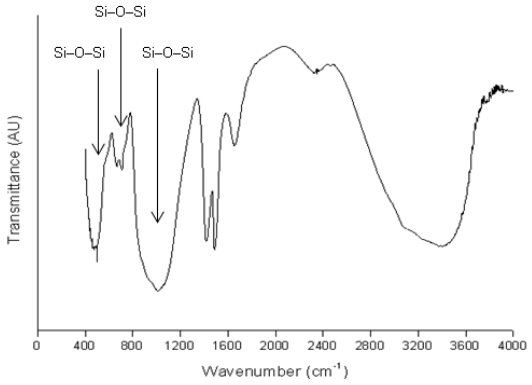

Figure 2 : FTIR reflectance spectra of 45S5 bioactive glass

3.3 Response of SHED towards BG-conditioned medium

SHED showed higher proliferation rate when exposed to BG-conditioned medium with the powder and liquid concentration was between 1 to 2mg/ml as shown in Figure 3.

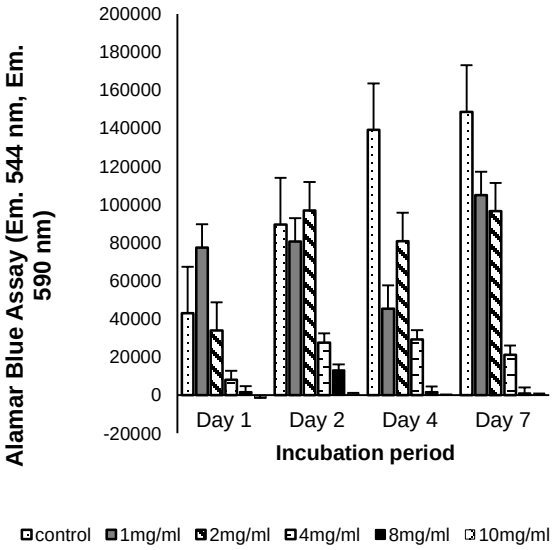

Figure 3: Proliferation rate of SHED when exposed to BG-conditioned medium.

Higher powder to liquid ratio resulted in lower SHED proliferation rate. The relative proliferative activity upon exposure of cells towards BG-conditioned medium are expressed in relation to the control medium (0 mg/ml BG). Following 24 hours (Day 1), SHED incubated with 1 mg/ml BG-conditioned medium showed the highest proliferative activity. However, SHED exposed to 2 mg/ml BG-conditioned medium showed the highest proliferative activity at Day 2. Meanwhile,

the control sample showed the highest proliferative activity at Day 4 and also followed by Day 7.

In addition, we concluded that a higher dose of powder to liquid ratio for the BG-conditioned medium (8 to 10 mg/ml concentration) is not compatible for cell growth but minimal dose of BG-conditioned medium (1 to 2 mg/ml) concentration promote cell growth based on Figure 3. Meanwhile, Figure 4 shows a photographic images of SHED exposed to the BG conditioned medium from Days 2 to 7. There was an increase in the proliferative activity of SHED upon exposure to the BG-conditioned medium from Days 2 to 7 indicating the suitability of this bioactive glass for biomedical applications.

4 DISCUSSION

A typical XRD pattern with no crystalline peaks was detected from the powder obtained from 45S5 bioactive glass indicating the amorphous phase (Figure 1). This is in concordance as reported previously [15].

According to Fan et al., [16], because of the amorphous nature of the glass, a broad band in the range of 20° until 40° has been seen as no diffraction maxima being detected. As expected, the 45S5 bioactive glass that was produced from our study through melt-derived method showed amorphous phase and is free from any significant crystalline phase based on the XRD result.

As shown in Figure 2, the band located for 45S5 bioactive glass showed that the Si–O–Si bending mode is detected between 450 until 480 cm^{-1}. This phenomenon was also reported previously by El-Kady [17]. In addition, our 45S5 BG powder showed the presence of Si-O-Si bending in the range of 400 until 500 cm^{-1} for FTIR reflectance spectra of bioactive glasses and glass – ceramics. This finding is in accordance with the study of Srivastava [18].

Hence, the Si-O-Si bending mode is present in the bioactive glass 45S5 which has been showed between those ranges in Figure 2. Moreover, the Si-O-Si symmetric stretching vibration band is normally located between 725 until 800 cm^{-1}, while the Si–O–Si asymmetric stretching vibration band is identified between 1000 until 1200 cm^{-1} as reported previously [17].

In Figure 2, the 45S5 bioactive glass showed the presence of Si-O-Si bending, Si-O-Si symmetric stretching vibration and Si–O–Si asymmetric stretching vibration that appeared between those ranges.

Nowadays, the potential application of biomaterials as scaffolds, the addition of growth factors and the presence of stem cells from various sources are the triads commonly advocated in tissue engineering towards the repair and regeneration of dental structures.

SHED response to different BG concentrations varies and is dose-dependent. The lower powder to liquid ratio from 1 to 2 mg/ml promoted SHED proliferation rate and vice-versa for higher powder to liquid ratio from 4 until 8 mg/ml. This finding may suggest that there is a suitable dose of BG for dental related stem cells and requires further exploration.

For BG to be used as cavity liners or injectable materials for root canal therapy and also implantable materials in stimulating hard tissues regeneration, the dose-dependent effect must also be further explored.

Studies have shown that similarities exist in the morphological characteristic of human dental pulp tissues with the tissues produced from dental stem cells seeded in tooth slice/scaffolds and transplanted into mice after transplantation for 21-28 days [19].

Many studies also showed that BG powder on its own promoted hard tissue regeneration [7, 9]. However, newer studies showed that BG nanoparticles could be combined with other materials such polymeric materials, ceramic and carbon nanotubes [20]. These researches supported the use of the BG for potentials used in orthopaedic application along with the implementation of BG scaffolds for tissue engineering and regenerative medicine [20].

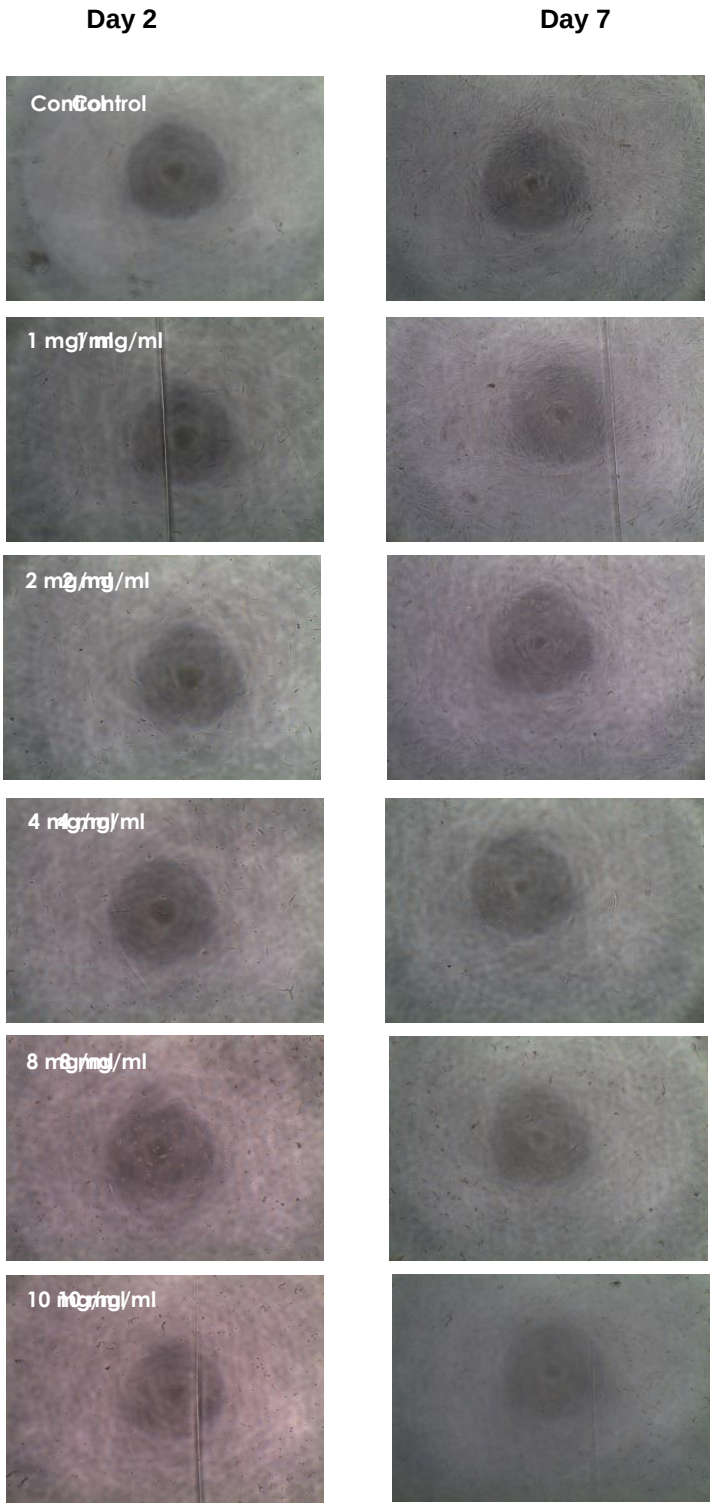

Figure 4: SHED exposed to BG conditioned medium from Days 2 to 7

5 CONCLUSION

The synthesis and characterization of 45S5 BG using melt-derived method was achieved and the BG powder sample produced showed typical characteristics of amorphous structure and was free from crystalline phase based on the XRD result. Identification of Si-O-Si bending, symmetric Si-O-Si stretching and asymmetric Si-O-Si stretching bands in the BG supported the notion that these bands is the main characteristic features of silicate network in the BG as showed by FTIR analysis. In relation to the SHED cell culture, the suitable concentration was 1 mg/ml of the 45S5 BG-conditioned medium based on the SHED proliferative activity using the alamarBlue assay. This study provides additional information that the 45S5 BG fabricated in the current project is biocompatible and has the potential to be used for future dental hard tissue regeneration.

CONFLICTS OF INTEREST

The authors have no conflicts of interest that are directly relevant to the content of this manuscript.

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DECLARATION

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REFERENCES

[1] Yamada, Y., Ito, K., Nakamura, S., Ueda, M. and Nagasaka T. (2011, July). Promising cell-based therapy for bone regeneration using stem cells from deciduous teeth, dental pulp, and bone marrow. *Cell transplantation*. [Online]. *20(7)*, pp.1003-1013. Available: http://www.ingentaconnect.com/content/cog/ct/2011/00000020/00000007/art00002

[2] Rahaman, M.N., Day, D.E., Bal, B.S., Fu, Q., Jung, S.B., Bonewald, L.F. and Tomsia, A.P. (2011, June). Bioactive glass in tissue engineering. *Acta biomaterialia*. [Online]. *7(6)*, pp.2355-2373. Available: http://www.sciencedirect.com/science/article/pii/S1742706111001280

[3] Fu, Q., Saiz, E., Rahaman, M.N. and Tomsia, A.P. (2011, Oct.). Bioactive glass scaffolds for bone tissue engineering: state of the art and future perspectives. *Materials Science and Engineering.* [Online]. *31(7),* pp.1245-1256. Available: http://www.sciencedirect.com/science/article/pii/S0928493111001275

[4] Pirayesh, H. and Nychka, J.A. (2013, May). Sol–Gel Synthesis of Bioactive Glass-Ceramic 45S5 and its in vitro Dissolution and Mineralization Behavior. *Journal of the American Ceramic Society.* [Online]. *96(5)*, pp.1643-1650. Available: http://onlinelibrary.wiley.com/doi/10.1111/jace.12190/full

[5] Cao, W. and Hench, L.L. (1996,Jan.). Bioactive materials. *Ceramics international*. [Online]. *22(6),* pp.493-507. Available:http://www.sciencedirect.com/science/article/pii/0272884295001263

[6] Jones, J.R., Lee, P.D. and Hench, L.L. (2006, Jan.). Hierarchical porous materials for tissue engineering. *Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences*. [Online]. *364(1838)*, pp.263-281. Available: http://rsta.royalsocietypublishing.org/content/364/1838/263.short

[7] Wu, Z.Y., Hill, R.G. & Jones, (2010, Feb.). Optimizing the Processing of Porous Melt-Derived Bioactive Glass Scaffolds. *Bioceramics Development and Applications.* [Online]. *1*, pp.1–4. Available: http://www.omicsonline.com/open-access/optimizing-the-processing-of-porous-meltderived-bioactive-glass-scaffolds-2090-5025.110150.pdf

[8] Hench and L.L.. (2006, Nov.). The story of Bioglass®. *Journal of Materials Science: Materials in Medicine*. [Online]. *17(11)*, pp.967-978. Available: http://link.springer.com/article/10.1007/s10856-006-0432-z

[9] Xynos, I.D., Hukkanen, M.V.J., Batten, J.J., Buttery, L.D., Hench, L.L. and Polak, and J.M. (2000, Feb.). Bioglass® 45S5 stimulates osteoblast turnover and enhances bone formation in vitro: implications and applications for bone tissue engineering. *Calcified Tissue International*. [Online]. *67(4),* pp.321-329. Available: http://link.springer.com/article/10.1007/s002230001134

[10] Peitl Filho, O., Latorre, G.P. and Hench, and L.L.. (1996, Aug.). Effect of crystallization on apatite-layer formation of bioactive glass 45%. *J Biomed Mater Res*. [Online]. *3,*pp.509-514. Available: http://www.lamav.ufscar.br/artpdf/jbmr30.pdf

[11] Bingel, L., Groh, D., Karpukhina, N. and Brauer, D.S. (2015, March). Influence of dissolution medium pH on ion release and apatite formation of Bioglass® 45S5. *Materials Letters*. [Online]. *143*, pp.279-282 Available: http://www.sciencedirect.com/science/article/pii/S0167577X14023015

[12] Bahniuk, M.S., Pirayesh, H., Singh, H.D., Nychka, J.A. and Unsworth, L.D. (2012, June). Bioactive glass 45S5 powders: effect of synthesis route and resultant surface chemistry and crystallinity on protein adsorption from human plasma. *Biointerphases*. [Online]. *7(1)*, p.41. Available: http://scitation.aip.org/content/avs/journal/bip/7/1/10.1007/s13758-012-0041-y

[13] Nakamura, S., Yamada, Y., Katagiri, W., Sugito, T., Ito, K. and Ueda, M. (2009, Nov.). Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene

expression profile from promising dental pulp. *Journal of endodontics*. [Online]. *35(11),* pp.1536-1542. Available: http://www.sciencedirect.com/science/article/pii/S0099239909006542

[14] Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L.W., Robey, P.G. and Shi, S., "SHED: stem cells from human exfoliated deciduous teeth," in *Proceedings of the National Academy of Sciences*, 2003, pp. 5807-5812

[15] Schickle, K., Zurlinden, K., Bergmann, C., Lindner, M., Kirsten, A., Laub, M., Telle, R., Jennissen, H. and Fischer, H. (2011, April). Synthesis of novel tricalcium phosphate-bioactive glass composite and functionalization with rhBMP-2. *Journal of Materials Science: Materials in Medicine*. [Online] *22(4)*, pp.763-771. Available: http://link.springer.com/article/10.1007/s10856-011-4252-4

[16] Fan, J.P., Kalia, P., Di Silvio, L. and Huang, J. (2014, March). In vitro response of human osteoblasts to multi-step sol–gel derived bioactive glass nanoparticles for bone tissue engineering. *Materials Science and Engineering: C*. [Online]. *36*, pp.206-214. Available: http://www.sciencedirect.com/science/article/pii/S0928493113006723

[17] El-Kady, A.M., Ali, A.F., Rizk, R.A. and Ahmed, M.M. (2012, Jan.). Synthesis, characterization and microbiological response of silver doped bioactive glass nanoparticles. *Ceramics International*. [Online]. *38(1)*, pp.177-188. Available: http://www.sciencedirect.com/science/article/pii/S0272884211005980

[18] Srivastava, A.K. and Pyare, R., (2012, Feb.). Characterization of ZnO Substituted 45S5 Bioactive glasses and glass-ceramics. *Journal of Materials Science Research*. [Online]. *1(2)*, p.207. Available: http://www.ccsenet.org/journal/html/jmsr/articles/14985.html

[19] Casagrande, L., Cordeiro, M.M., Nör, S.A. and Nör, J.E. (2011, Jan.). Dental pulp stem cells in regenerative dentistry. *Odontology*. [Online]. *99(1*), pp.1-7. Available: http://link.springer.com/article/10.1007/s10266-010-0154-z

[20] Boccaccini, A.R., Erol, M., Stark, W.J., Mohn, D., Hong, Z. and Mano, J.F. (2010, Nov.). Polymer/bioactive glass nanocomposites for biomedical applications: a review. *Composites science and technology*. [Online]. *70(13)*, pp.1764-1776. Available: http://www.sciencedirect.com/science/article/pii/S0266353810002289