Brief Report

In Vitro Cultivation, Characterization and Osteogenic Differentiation of Stem Cells from Human Exfoliated Deciduous Teeth on 3D Printed Polylactic Acid Scaffolds

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Abstract

Background: Tissue engineering mainly focuses on creating appropriate conditions for the regeneration of tissues. Scaffolds, signal molecules, and stem cells interact with each other and compose the essential components of this field.

Objectives: This study aimed at investigating the osteogenic induction ability of PLA Poly Lactic Acid (PLA) scaffolds and comparing the osteogenic differentiation behavior of Stem Cells from Human Exfoliated Deciduous Teeth (hSHEDs) in standard culture medium and on PLA scaffolds.

Methods: The current clinical experimental study was conducted between April 2016 and October 2016 at the Near East University cell culture laboratory located in North Cyprus. The pulp tissues of deciduous teeth (non-decayed and in the absence of abscess, fistula or periapical lesion) were sampled from 10 healthy children aged between 6 and 11 years. The isolated hSHEDs were divided to 4 groups. The control group/Group1 consisted of cells, which were cultivated in standard culture medium, and Group2 cells were differentiated into an osteogenic lineage using osteogenic differentiation medium. Group 3 represented the non-differentiated group, which was transferred onto three dimensional (3D) printed PLA scaffolds and Group 4 cells were differentiated to the osteogenic lineage and transferred onto 3D printed PLA scaffolds. All groups were analyzed immunohistochemically and by immune-labeling, and were evaluated semi-quantitatively using the HSCORE.

Results: Cultivation of hSHEDS on PLA scaffolds was assessed for 14 and 21 days; osteogenic differentiation was detected both histochemically and immunohistochemically. Generally, Osteocalcin (OCN) immunoreactivities were higher than Osteonectin (ON) immunoreactions in all groups. Despite higher OCN immunoreactivities, the intensities of OCN between 14 days and 21 days in group 4 (497.3 ± 0.57% and 486.7 ± 5.77%, respectively) were similar (P > 0.05). While the intensity of ON was 280.0 ± 10% in group 4, in group 2 the intensity of ON was 206.7 ± 5.77%, and on the 14th day the results were statistically significant (P < 0.0001).

Conclusions: Poly lactic acid is a suitable scaffold material for osteogenic induction of the hSHEDs. The expression patterns of both markers showed that a 14-day cultivation period is adequate for hSHEDs with/without PLA scaffolds to differentiate into osteoblasts.

Keywords: Cell Differentiation, Dentistry, Stem Cell, Tissue Engineering

1. Background

Dental tissue loss due to trauma, disease or congenital abnormalities generates a major health issue worldwide (1).

Regenerative medicine is a rapidly developing field and newly emerging fields such as tissue engineering, material science, cell and molecular biology have been developed, some of which incorporate the use of biotechnologies to provide tissue regeneration (2). Tissue engineering mainly focuses on creating appropriate conditions for the regeneration of tissues. Scaffolds, signal molecules, and stem cells interact with each other and compose the essential components of this field (3).

Stem cells were quickly adopted as key elements of tissue engineering by means of colonization of self-renewable progenitor cells to constitute one or more cell types. Currently, stem cell contents from dental structures have been examined by various research groups. These areas are pulpal structures of primary and permanent teeth, periodontal ligaments, apical papilla, and dental follicles (4, 5).

Three-dimensional (3D) printing is used as a method

to construct three dimensional structures to hold living cells and mimic an extracellular matrix. Scaffolds fabricated by 3D printing were shown to provide a micro-environment for cells to adhere, proliferate, and differentiate. At the same time, 3D printed scaffolds could supply bio-absorbable physical networks for damaged tissue and may serve as customized "tissue constructs" such as dental pulp tissue in human or mice root canals (6-8).

The necessity of dental tissue regeneration with natural pathways requires multiple differentiation capacities and therapeutic effects of dental stem cells (9, 10). Therefore, in the current study, in order to cater for natural healing in osteogenic destructed dental areas, osteogenic differentiation potential of stem cells from human exfoliated deciduous teeth (hSHEDs) were combined with poly lactic acid (PLA) scaffolds in vitro.

The aim of this study was to investigate the osteogenic induction ability of PLA scaffolds and compare the osteogenic differentiation behavior of hSHEDs in standard culture medium and on PLA scaffolds.

2. Methods

The current clinical experimental study was conducted between from April 2016 to October 2016 at the Near East University cell culture laboratory located in North Cyprus and was approved by the Near East University, institutional review board (YDU/2015/34-243). The pulp tissues of deciduous teeth included in this study were sampled from 10 healthy children aged between 6 and 11 years. The choice of pulp tissue samples was based on a selection criteria, which ensured that the samples came from non-decayed deciduous teeth without any abscess, fistula or periapical lesions.

All equipment (centrifugal machine, incubator, optical microscope, biological safety cabinet, and refrigerator) used in the current study were serviced and calibrated prior to use.

Three unbiased observers also evaluated each section independently.

2.1. Isolation and Cultivation of hSHEDS

Extirpated dental pulps of exfoliated deciduous teeth were cut into 1 mm^3 pieces and digested enzymatically with 1 mg/ml collagenase type 1 (Sigma Aldrich, Saint Louis, USA) for 1 hour at 370C with 5% CO2. After enzymatic digestion, single cell suspensions were centrifuged for 5 minutes at 1000 RPM. Following centrifugation, the cells were transferred to a 6-well plate and cultured in α - MEM (Biochrom, Berlin, Germany) supplemented with 15% Fetal Bovine Serum (FBS, EURX, South America, Brazil), 1%

L-glutamine (Capricorn Scientific, Ebsdorfergrund, Germany), 1% Penicillin-Streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany), 1% Gentamycin (Gibco, New York, USA) and Amphotericin-B (Gibco, New York, USA).

The standard culture medium of the hSHEDS was refreshed every 2 days. After 80% confluent growth, cells were trypsinized with Trypsin-EDTA (Capricorn Scientific, Ebsdorfergrund, Germany) for 10 minutes at 37°C in 5 % CO_2, during the passage procedure. The hSHEDS were extended through 3 passages and the cells from the third passage (P3) were used in the study. The cells from first passages (P1) and second passages (P2) were stored at -80°C.

2.2. Three-Dimensional Printing of Polylactic Acid (PLA) Scaffolds

Polylactic Acid scaffolds were designed and 3D printed in NEU3D Laboratories. Designs were made using the Solidworks Software. The scaffolds comprised of columns that intersect in a perpendicular manner forming a three-dimensional porous structure. The overall dimension of one scaffold was 5 $\times$ 5 $\times$ 2 mm^3, the column thickness was 0.4 mm, and the space between columns was 0.4 mm. Three-dimensional printing of scaffolds was performed in fused deposition modeling (FDM) 3D Printer (5th Generation, Makerbot Industries LLC) using 1.75 mm PLA filaments. The scaffolds were sterilized with vaporized hydrogen peroxide prior to cultivation.

2.3. Determination of Groups

The hSHEDs were divided to 4 groups. Group 1 represented the control group, where the cells were only cultivated in standard culture medium, Group 2 was differentiated to an osteogenic lineage using osteogenic differentiation medium, while Group 3 was the non-differentiated group, which was transferred onto 3D printed PLA scaffolds, and Group 4 was differentiated into an osteogenic lineage and was transferred onto 3D printed PLA scaffolds.

2.4. Characterization of hSHEDS with Immunohistochemistry

The cells were fixed with 4% paraformaldehyde (Invitrogen, Frederick, USA) in phosphate buffered saline (PBS pH: 7.3, Medicago, Uppsala, Sweden) for 30 minutes at room temperature. After fixation, they were washed three times with PBS, 5 minutes each. The cells were permeabilized with 0.1% Tween – 20 (Sigma Aldrich, Saint Louis, USA) in PBS for 15 minutes on ice. After washing with PBS, 3 % hydrogen peroxide (H_2O_2) was used to block endogenous peroxidase. After three additional PBS washes, the cells were incubated in blocking buffer solution (Invitrogen, Paisley, UK) for 1 hour at room temperature, the cells

were then incubated overnight with anti-CD90 (1:50; Abcam, Cambridge, USA) and anti-CD45 (1:50; Abcam, Cambridge, USA) primary antibodies at 40°C. Following three additional PBS washes, the cells were subsequently incubated with a broad spectrum secondary antibody (Invitrogen, Frederick, USA), according to the manufacturer's instructions. After washing with PBS, the intensity of immunoreactivities was identified with DAB (diaminobenzidine, ScyTek, Utah, USA) solution and Mayer's hematoxylin eosin (Sigma Aldrich, Saint Louis, USA). The stained cells were observed under an optical microscope (Olympus IX53). Immunolabeling was evaluated semi-quantitatively using the HSCORE method $[\sum$ Pi (I+1), where "i" stands for the intensity of staining with a value of 1, 2 or 3 (mild, moderate, or strong, respectively) and "P" is the percentage of positively stained cells with the intensity varying from 0 to 100%.]

2.5. In Vitro Osteogenic Differentiation of hSHEDS

Before osteogenic differentiation and morphologic analysis, PLA scaffolds were placed onto 24-well plates overnight. After reaching 80% to 90% confluence, the hSHEDS from the P3 were collected at a concentration of 3.75×10^5 cells/mL after trypsinization and were seeded into 3D printed PLA scaffolds. Osteogenic groups were induced using an osteogenic differentiation kit (Gibco, StemPRO, Grand Island, USA). The osteogenic differentiation period was planned as 14 days (2 weeks) and 21 days (3 weeks).

2.6. Light Microscopy Analyses of Scaffolds with and without Differentiated hSHEDS

The PLA scaffolds with and without differentiated hSHEDs were incubated for 14 days in borax-sodium carbonate buffer (0.01 M Na_2CO_3 and 0.3 mm $Na_2B_4O_7$, pH: 11) to induce rapid softening of PLA (9).

They were then embedded in paraffin and 5 μm sections were prepared. For histochemical analyses, sections were stained with hematoxylin eosin. Immunohistochemical distribution analyses of ON (1:50; Abcam, Cambridge, USA) or OCN (1:50; Abcam, Cambridge, USA) were evaluated using standard protocol of indirect immunoperoxidase staining as explained above.

2.7. Statistical Analysis

Initially, Kolmogorov Smirnov was used to test the normality for checking the parametric assumptions. Since all distributions showed normality, the researchers applied the parametric statistical analysis method. Comparable data groups were evaluated using one-way analysis of variance (ANOVA). In case of significance, Tukey post hoc test was applied to see pairwise differences. Graphpad Prism

software (version 7.02) and SPSS program (version 20.0) was used for analyses. The level of significance was accepted at 0.05 for the entire statistical analysis.

3. Results

3.1. Culture of hSHEDS

Immunohistochemical staining of isolated hSHEDS demonstrated positive CD90 (Figure 1A) and negative CD45 (Figure 1B).

3.2. Osteogenic Differentiation of hSHEDS

Immunoreactivities of ON and OCN were positive in osteogenic-induced groups (Group 2) when compared with the control group after 14 and 21 days.

### 3.3. The PLA Scaffold Column Thickness Changes

After 21 days of cultivation of PLA scaffolds, the column thickness decreased and ranged from 12.5% to 30%.

3.4. Analyses of Osteogenic Inducted hSHEDs on PLA Scaffolds

After seeding and cultivation of hSHEDS on PLA scaffolds for 14 and 21 days, osteogenic differentiation was detected both histochemically and immunohistochemically. The cells were detected both on the surface and inner parts of the PLA scaffolds in all culture periods. Immunoreactivity of ON was detected in all groups. The intensity of ON was higher in group 4 than in group 2 after 14 days of culture (HSCORES: 280.0 ± 10% and 206.7 ± 5.774%, respectively, P < 0.0001). The ON immunoreactivity in group 3 was higher than in group 1 (P = 0, 0352). In group 4 the intensity of ON was initially 280.0 ±10% at 14 days with a decrease to 213.3 ± 5.77% at the end of the 21 days (P < 0.0001). The intensity of ON in group 2 was initially 206.7 ± 5.77% at 14 days increasing to 263.3 ± 15.28% at 21 days (P < 0.0001). The difference between ON intensities in group 2 (263.3 ± 15.28 %) and group 4 (213.3 ± 5.77 %) after 21 days was also significant (P < 0.0001).

The OCN immunoreactivity was detected to be higher in group 2 and 4 once compared with groups 1 and 3 both at 14 and 21 days of culture. The OCN intensity was 497.7 ± 1.16 % at 14 days and 493.3 ± 5.77% at 21 days in group 2. In group 4 the OCN intensity was 497.3 ± 0.57% at 14 and 486.7 ± 5.77% at 21 days (P > 0.05). The OCN intensity was higher in group 2 (497.7 ± 1.16% and 493.3 ± 5.77 5) when compared with group 1 (480.7 ± 2.08%, 393.3 ± 15.28%) after both 14 and 21 days of culture time. The same trend was detected between group 4 (497.3 ± 0.57% and 486.7 ± 5.77 %) and group 3 (211.0 ± 3.61% and 340.0 ± 10%).

The comparisons between ON and OCN and HSCORE details for all groups are presented in Figures 2 and 3 and Table 1, respectively.

Figure 1. Characterization of hSHEDs
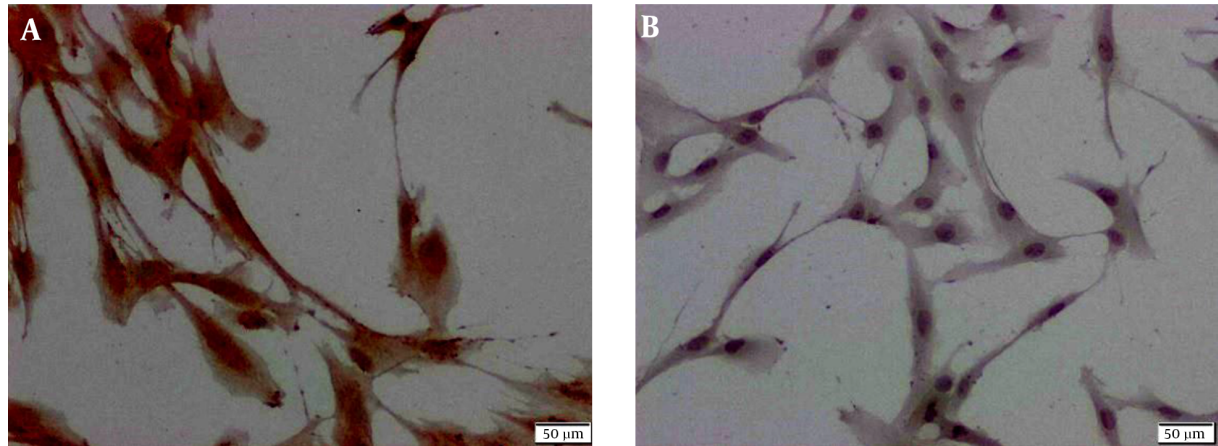
The cells expressed surface antigen CD90 (A), yet did not express CD45 surface antigen (C). All magnifications were 50 μm.

Table 1. Osteonectin (ON) and Osteocalcin (OCN) Intensities with HSCORE Analyses of Each Group[a]

HSCORE, %	PLA-C-2w	PLA-hSHEDs-2w	PLA-C-3w	PLA-hSHEDs-3w	C-2w	hSHEDs-2w	C-3w	hSHEDs-3w
Osteonectin	292.7 ± 2.08	280.0 ± 10	286,7 ± 5.77	213.3 ± 5.77	265.7 ± 9.29	206.7 ± 5.77	273.3 ± 11.55	263.3 ± 15.28
Osteocalcin	211.0 ± 3.61	497.3 ± 0.57	340.0 ± 10	486.7 ± 5.77	480.7 ± 2.08	497.7 ± 1.16	393.3 ± 15.28	493.3 ± 5.77

[a]Values are expressed as mean ± SD.

4. Discussion

The hSHEDs were first isolated by Miura et al. and are highly proliferative-clonogenic multipotent cells that have the potential of differentiating to functional osteoblasts, odontoblasts, chondroblasts, adipocytes, neural cells, and endothelial cells both in vitro and in vivo(4, 5, 11-14). The broad differentiation and proliferation capacity of hSHEDS makes these cells a good potential source for treatments of craniofacial defects and tooth loss and may also be used for bone regeneration (15).

The hSHEDS are being widely investigated for their differentiation ability to odontoblastic cells, osteoblasts, endothelial cells, neural cells, adipocyte cells, and myocyte cells for tissue engineering purposes, in conditions which include, muscular dystrophy, spinal cord injuries, and various skeletal defects (16-19).

In the field of regenerative dentistry, hSHEDS have demonstrated their dental pulp-like tissue, tubular dentin, endothelium, and periodontal regeneration capacities in vivo (20-23). Dental pulp stem cells express a set of Mesenchymal Stem Cell (MSC) markers like positivity for CD90, CD73, CD105, CD146, and STRO-1 and negativity for CD34, CD45, CD11b/c and HLA-DR (3, 18, 24). The hSHEDS in the current study displayed a positively typical pattern for important MSC marker CD90 and showed a negative expression of CD45 hematopoietic marker.

The 3D printing has rapidly been adopted in the tissue engineering field for oral and maxillofacial regeneration (25). The 3D printed scaffolds may ensure structural integrity and allow for cell infiltration and proliferation, extracellular matrix production, and remodeling (8). Biocompatible materials like polylactic acid (PLA), polyglycolic acid (PGLA), polycaprolactone (PCL), and polyethylene glycol (PEG) are widely used for scaffold production. The PLA is a thermoplastic and biodegradable aliphatic polyester, which makes it very popular for biological and medical applications. Rosenzweig et al. reported that PLA scaffolds provide good mechanical stability for cells to grow and proliferate (26, 27). The authors preferred PLA in their study due to its biodegradability, recyclability, low immunogenic and toxic waste production properties.

In 2014, Su et al. investigated osteogenic differentiation and mineralization of hSHEDS, and reported an increase in gene expression of ON and OCN showed osteogenic induction on modified chitosan scaffolds in the 21-day period (28). Similarly, the expressions of ON and OCN in the current study were found to increase in PLA scaffolds, which demonstrates that PLA is a suitable scaffold
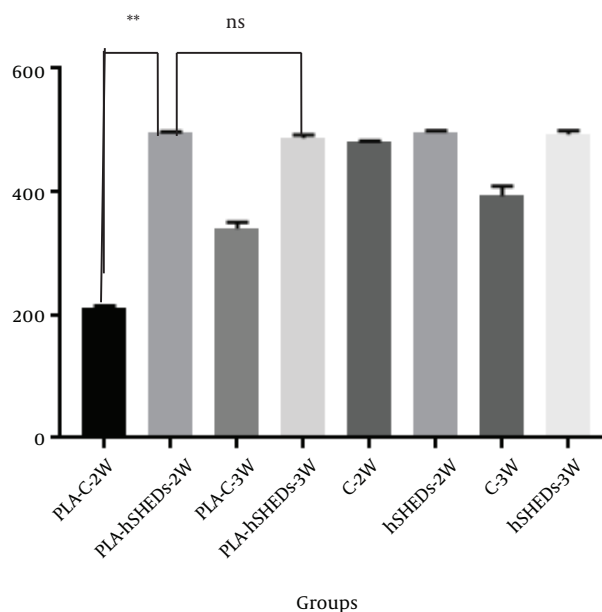
Figure 2. Osteonectin (ON) Gene Expression of Each Group
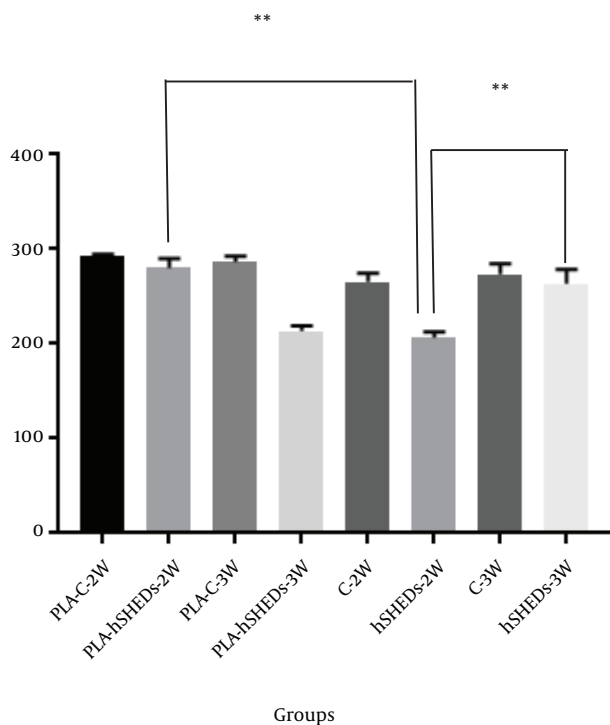
In group 4, intensity of ON was 280.0 ± 10% at 14 days, in contrast ON intensity was detected at 206.7 ± 5.77% at 14 days. In addition, the intensity of ON in group 2 was initially 206.7 ± 5.77% at 14 days and increased to 263.3 ± 15.28% at 21 days of culture time; the difference between the ON intensities in group 2 was also statistically significant (**P < 0.0001).

Figure 3. Osteocalcin (OCN) Gene Expression of the Four Groups

In group 4, the OCN intensity was 497.3 ± 0.57% at 14 days and 486.7 ± 5.77% at 21 days. This comparison was not found statistically significant (P > 0.05). In addition, OCN intensity was higher in group 4 than (497.3 ± 0.57%) in group 3 (211.0 ± 3.61%) at 14 days of culture time. This comparison was statistically significant (**P < 0.0001, ns: no significance).

material for the osteogenic induction of the hSHEDs.

Osteogenic differentiation and mineralization characterization of hMSCS on PCL/PLA scaffolds were also investigated and the outcomes showed that the differentiation and mineralization rates of hMSCS were influenced by matrix capabilities of PLA/PCL scaffolds. The authors stated that hydrodynamic stress and fluidodynamic features of the matrix induce perfusion and osteogenic differentiation during the dynamic culture (29).

Recently, Khojasteh et al. investigated osteogenic differentiation (7 and 14 days) of Human Dental Pulp Stem Cells (hDPSCS) similar to the cultivation periods used in the current study, however, the point of difference was the scaffolds used (SureOSS, Cerabone, poly-L-lactic acid (PLLA) and Osteon II Collagen). They reported that PLLA scaffolds supported adhesion, proliferation, and osteogenic differentiation of hDPSCS compared to other scaffolds. For the current study the expression patterns of both markers at 14 and 21 days showed that 14-day cultivation periods is adequate for hSHEDs with/without PLA scaffolds to differentiate into osteoblasts (30).

There have only been a limited number of studies, which use triple combination (stem cells, growth factors, and scaffolds) in host tissues, which ultimately increases the success rate of osteogenic regeneration, which is supported by the results of the current study (31-33).

Early period degradation pattern of PLA scaffolds was one of the outcomes that was investigated in the current study. Column thickness alterations in PLA scaffolds after cultivation varied between 12%, 5%, and 30%. It is difficult to state that PLA started to degrade in this short period because the measurements were taken after the researchers used borax-sodium carbonate buffer for softening of scaffolds prior to histologic evaluation. Pure PLA was shown to be degraded by nearly 50% in a 10-day period in highly alkaline borax-sodium carbonate buffer (34). However, in the current study the PLA scaffolds have undergone a glass transition process during 3D printing, which made it more solid than pure PLA fibers and probably decreased its degradability to some extent. In comparison with previous studies on the degradability of the PLA in vivo, it is obvious that this polymer is not degraded in a short period (35). Due to the requirement of prolonged mechanical integrity for osteogenic regeneration, the study did not accept this issue as a disadvantage.

Late term passage procedures can lead to cellular variations in these cells, and due to this limitation, isolated hSHEDS were passaged three times (early term passage) resulting in successful osteogenic differentiation and cellular attachments on PLA scaffolds. On the other hand, PLA scaffolds supported the osteogenic differentiation of hSHEDS. As a result, hSHEDS could be used in early term passages with combination PLA scaffolds for three dimensional osteogenic regeneration in vitro. This point of difference comprises the novel step of the current study.

The research findings demonstrated that PLA scaffolds provide a supporting medium for structural integrity, easy mobility of hSHEDS, and create a three-dimensional physical microenvironment. The isolated hSHEDS have survival ability, adhesion to the scaffold surface, and maintain their osteogenic differentiation on PLA scaffolds. Ultimately, the hSHEDS-PLA scaffold combination seems to be a potentially good osteogenic filling material for regenerative dentistry in the near future.

4.1. Limitations

- It is needed to include more differentiated cells in cell cultures, as they are only able to obtain small amounts of hSHEDS.

- In vivo experiments need to be conducted for better understanding of hSHEDS-PLA scaffold combined metabolism.

Acknowledgments

Footnote

Conflict of Interest: The authors reported no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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