Dental Pulp Stem Cells Behaviors Cultured on Collagen-coated Polyether Ether Ketone and Porous Tantalum

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Introduction: Reconstruction of large bone defects at the load bearing sites like spinal cord are still associating with major challenges. Although, currently two commercially available implants like porous tantalum and polyether-ether-ketone (PEEK) cage are employed for spinal fusion surgery, however, both PEEK and tantalum are suffered a key property of bioinert surface. By coating the surface of implants with collagen type 1, it is expected surface wettability and bioactivity of implants be increased and consequently effects on cells behavior. The aim of this study was to compare the attachment, proliferation and osteogenic differentiation capability of human dental pulp derived stem cells (DPSCs) cultured on either collagen coated polyether ether ketone (PEEK) and tantalum and bare ones. **Materials and Methods:** Porous tantalum (Zimmer Company, USA) and PEEK (Abbott Spine, France) fragmented samples were treated by oxygen plasma irradiation following coated with collagen type 1 and seeded with dental pulp derived stem cells (DPSCs). Cells adhesion, proliferation, and osteogenic differentiation of DPSCs were evaluated on these both coated and non-coated scaffolds for up to two weeks by scanning electron microscopy (SEM) imaging, Alamar blue assay and alkaline phosphate (ALP) activity measuring respectively. **Results:** Alamar blue assay was performed and results over 5 and 7 days of culture showed that cell viability for collagen coated porous tantalum group at those days was higher than other ones. Likewise, ALP activity of cells which were seeded on scaffolds measured and interestingly, collagen coated tantalum group had highest amount over 7 and 14 days of culture. The adherence of DPSCs on the scaffolds was recorded after 3 days of culture by scanning electron microscopy (SEM) imaging. According to the SEM images, cells colonies and cells sheets were formed at the collagen coated porous tantalum and collagen coated PEEK surfaces respectively, whereas no cell attachments was seen at the surface of either bare tantalum or bare PEEK. **Conclusion:** Our results, therefore, show that the collagen coated porous tantalum in comparison to coated PEEK or bare ones, possess better cells attachments and biocompatibility and could be considered for using as cell seeded scaffold for orthopedic in vivo studies.

Keywords: Tantalum; Polyether ether ketone (PEEK); Collagen coating; Dental pulp stem cells; Spinal fusion; Bone regeneration

Introduction

Reconstruction of large bone defects at the load bearing sites, especially spinal cord, is still being a major challenges for orthopedic surgeons (1). Although autologous bone is considered as gold standard for defect site filling, but there are some obstacle such as low autograft bone sources and donor site morbidity through bone harvesting (2, 3). Allograft bone is another choice but associating with infections transmission risks (4). To tackle these limitations, synthetic biomaterials including polymers and metals are being applied. Specifically, biomaterials for load bearing orthopedic cases must possess acceptable mechanical and physicochemical properties to promote osseointegration between scaffold and host tissue (5-7). Materials such as porous tantalum and poly-ether-ether-ketone (PEEK) are two commercially available examples which are commonly using in spinal interbody fusion surgery(8, 9). Porous tantalum which is introduced as Trabecular Metal™ by Zimmer Company, exhibits corrosion resistant properties with the tailorable elastic modulus between 2 and 20 GPa, according to the percentage of porosity, similar to that of human cortical bone modulus, 14 to 18 GPa, which have potential to reduce stress shielding and related problems accompanied with load-bearing metallic implants (10).

On the other side, polyether-ether-ketone (PEEK) is a bioinert polymer with tailorable mechanical properties which

has drawn widespread acceptance for spinal and cervical fusions applications (11). Due to its low elastic modulus of 3 to 4 GPa which is closed to that of native cortical bone, the shielding effect is being reduced and makes it favorable for load bearing defects implantation (12). Apart from good mechanical properties and biocompatibility of PEEK implants, some drawbacks including surface hydrophobicity and consequently poor cell adhesion is still remaining and inevitable (13).

Currently, spine surgeons used cancellous bone graft to fill caged devices to enhance implants bioactivity and osseointegration (14). Due to the aforementioned challenges which relate to bone harvesting from other sites of body, stem or differentiated cells would be promising as alternative biological elements to promote implant-host tissue integrity. Todays, one of the most acceptable type of cells for bone regeneration applications is mesenchymal stem cells (MSCs). Mesenchymal stem cells are multipotent stem cells with ability to differentiate into 3 major cell types including adipocytes, chondrocytes and osteocytes. These stem cells can be harvested from a variety of tissues such as bone marrow and fat with the high scale proliferation potency (15). Besides, mesenchymal stem cells are immune privileged and possess immunosuppressive effects, which facilitates the allogeneic transplantation and could be use as immunomodulator elements in combination with scaffolds (16-18).

Recently, dental pulp derived mesenchymal stem cells (DPSCs) is emerged as an attractive cell source in tissue engineering, specially bone tissue engineering, due to easy harvesting and high rate of proliferation(19, 20). There are also lots of studies have shown that the osteogenic differentiation potency of dental pulp stem cells (DPSCs) is comparable with bone marrow derived stem cells and in some cases was better (21, 22). Since the behavior of cells which are combined with a scaffolds are strongly depended on physicochemical properties of scaffolds and by considering to the bioinert behavior of both porous tantalum and PEEK, surface modification is suggested(23, 24). There are lots of strategies for surface modifications and increasing cells adhesion and proliferations, including insertion of some additives into scaffold compound, plasma irradiation, chemical etching or coating with bioactive materials like collagen or fibronectin (25-27). Among all above strategies, collagen coating is more favorable due to best mimic the ECM (28). Collagen is a natural protein and one of the main component of ECM which is exist in a variety forms like sheets, filaments and fibrils and different types (29). The most staple type of collagen in the body is type 1 collagen which exhibits osteocunductive property (30). Through the literature, scaffolds or tissue cultures coating with collagen type 1 has been reported frequently (31, 32).

This study sought to compare the attachment, proliferation and osteogenic differentiation capability of human dental pulp derived stem cells (DPSCs) cultured on either collagen coated polyether ether ketone (PEEK) and tantalum and bare ones as two commercially available scaffolds for spinal injury fusion.

Materials and Methods

Materials:

Dental pulp derived stem cell (DPSC), Dulbecco's modified Eagle's medium (DMEM), DMEM-F12 medium, Fetal Bovine Serum (FBS), Penicillin Streptomycin (pen/strep), Amphotericin B and Trypsin/EDTA were purchased from Thermo Fisher Scientific, Cell Lysis Reagent (C3228), Alkaline Phosphatase Yellow (pNPP) Liquid Substrate (P7998), β-Glycerol phosphate disodium salt pentahydrate, acid ascorbic, dexamethasone. Porous tantalum and PEEK interbody fusion device from Zimmer Biomet, Rat tail collagen (Sigma)

Sample preparation:

Both Tantalum and PEEK implants are fragmented into 2*3*4* mm in diameter. Twenty one pieces of each group are selected for plasma treatment and collagen coating. To enhance coating efficiency and increase surface hydrophilicity, air plasma treatment for each surface of samples was carried out by an inductive coupled radio frequency glow discharge plasma cleaner (HARRICK PLASMA PDC-002, USA) for 6 minutes with power of 30 W.

Collagen coating:

Before collagen coating and in order to collagen cross linking, 25 mg N-Hydroxysuccinimide (NHS; Sigma, USA) and 25 mg of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; Sigma, USA) were separately dissolved in 5 ml distilled water and then mixed together. Then the plasma-treated samples were immersed into the 96 well-plate which was filled with NHS+EDC solution and incubated at 4°C for 2 hours. After that, solution of each well removed and then the collagen solution in phosphate buffer saline (PBS; Gibco, USA) with a concentration of 1mg/ml was added to fill each well and samples were incubated at 4°C overnight. Finally, coated samples were washed with PBS for three times and dried under room temperature.

Sample sterilization:
In order to sterilization of scaffolds before cell seeding, all samples including collagen coated and non-coated ones, were immersed in 96 well plate that filled with 70% filtered ethanol for 4 hours, and then ethanol was removed by aspiration and each scaffold was rinsed five times by PBS containing 5X antibiotic (pen-strep) during five minutes intervals following incubation under basal culture medium containing 5X antibiotic for 16 hours.

Cell cultures:
Third passaged DPSCs were cultured under complete stem cell culture medium comprising a mixture of Dulbecco's modified Eagle's medium high glucose (DMEM-HG) (Thermo Fisher Scientific, United States) supplemented with 15% fetal bovine serum (FBS) (Thermo Fisher Scientific, United States) and 1% penicillin/streptomycin in the T-75 culture flask and maintained at 37°C and in 5% CO2 atmosphere. Cell culture medium was refreshed every three days and at the 85% confluency, cells were detached by trypsinizing and counted then passed in to new flask.

Cell viability:
All samples including coated and non-coated PEEK and porous tantalum were placed in 96 well plate seeded with amount of 3×10^3 cells and incubated under standard cell culture medium at 37 °C and in 5% CO_2 atmosphere for 24 hours. Cells viability and proliferations on both porous tantalum and PEEK (coated and non-coated) was studied by using the Alamar Blue metabolic activity assay (Thermo Fisher Scientific, USA). Briefly, after predetermined time points (24h, 5 and 7 days of cell seeding) of culture, medium was removed and 130 µL of assay medium comprising culture medium, 10% FBS and 10%Alamar blue was inserted to each well. After 3hours of incubation, 100 µL assay medium was transferred into empty 96-well plates subjected to flourometric measurement at 570 and 595 nm. Finally, cell viability (CV) was calculated according to the following equation:

$$CV = 100 \times \left(\frac{FDs - FDb}{FDc - FDb}\right)$$

Where FD_s, FD_b, and FD_c are the fluorescence density of the AB for the sample (S), blank (B) (culture medium without cells), and control (C). The assay was performed in replicates (n=4)

Osteogenic differentiation:
Similar to the cell viability procedures, PEEK and porous tantalum (coated and non-coated) samples were placed in 96 well plate and amount of 3×10^3 cells were seeded on them. To minimize the possible error of osteogenic capability due to cell number differences, cells were subjected to the standard culture medium for first 24hour. After that, standard medium was removed and cells were incubated under osteogenic inductive medium for 14 days. Inductive medium comprising DMEM supplemented with 10% FBS, 1% penicillin (100 U/mL) and streptomycin sulphate (100 mg/mL), 100 nM dexamethasone (Sigma-Aldrich), 50 µg/mL ascorbic acid (Sigma-Aldrich), and 10 mM β-glycerophosphate sodium (Sigma-Aldrich). Likewise standard cell culture, osteogenic inductive medium was refreshed every three days.

Cell proliferation was evaluated using the Alamar blue reduction assay (11). For this, culture medium was removed and 1 ml of assay medium containing a-MEM, 10% FBS and 10% Alamar blue (Invitrogen) was added to each well. After 2h incubation, 100 lL assay medium was loaded into 96-well plates and optical density was read at 570 and 595 nm. Alamar blue reduction was calculated using the formula provided by the manufacturer (Invitrogen).

To confirm results of Alamar blue reduction, a cell counting-based method was performed to obtain num-bers of viable dissociated cells, as described in the litera-ture (13). In particular, DFSCs were seeded in T-25 flasksat density of 104 ce lls/flask and cultured for 5 days. They were then trypsinized and collected by centrifugation. After re-suspension in 5 ml fresh medium, numbers of dissoci-ated cells were counted for each treatment, using a Cellome-ter Auto T4 cell counter (Nexcelom Bioscience, Lawrence, MA, USA). Results were reported as number of dissociated cells/ml.

ALP activity assay:
The osteogenic differentiation capability of DPSCs on both porous tantalum and PEEK (coated and non-coated group) was characterized by ALP activity assay after 7 and 14 days of culture under osteogenic inductive medium. Briefly, after washing the cells seeded scaffolds by PBS, they were homogenized in lysis buffer (Sigma-Aldrich, St. Louis, Missouri, United States) and then the resulting mixture was centrifuged at 12500 rpm for 10 minutes at 4°C. ALP activity was determined by measuring the amount of enzymatic conversion of p-nitrophenyl phosphate to the p-nitrophenol using ALP assay kit (Sigma-Aldrich, St. Louis, Missouri,

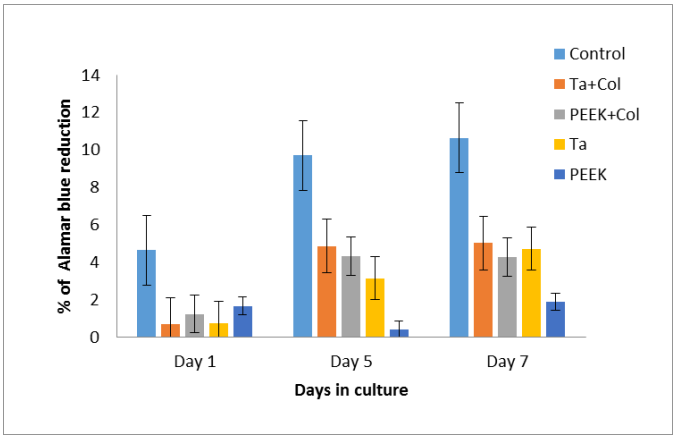

Figure 1. Proliferation of DPSCs at both porous tantalum and PEEK (coated and non-coated) samples was continuously monitored using Alamar blue reduction assay after 1, 5 and 7 days incubation

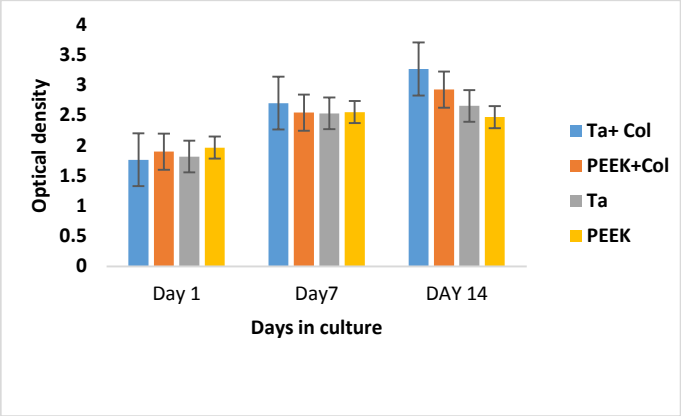

Figure 2. The results of ALP activity assay after 24 hours, 7 and 14 days culturing in osteogenic medium

United States). Absorbance was measured at 405 nm using microplate ELISA reader (Bio Tek, USA).

Scaffold morphology and cell attachment:

Scaffolds morphology and cells attachment on porous tantalum and PEEK (coated and non-coated) after 3 days of seeding was assessed by SEM (JEOL-T330A- Japan). In brief, all cultured samples were rinsed two times with phosphate-buffered saline (PBS) and then fixed with 3% glutaraldehyde solution and incubated at 4 ºC for 1 hour. In order to post fixation, samples were washed with PBS for three times again and dehydrated progressively with ethanol series 30%, 50%, 70%, 80%, 90% and 100% and finally dried overnight in desiccator. Dried samples were then adhered on copper sticky tape and coated with gold through sputtering process and viewed by scanning electron microscopy with voltage of 25 kv.

Results

Cell viability and proliferation:

To evaluate cells viability and proliferations in contact with scaffolds, Alamar blue assay was performed and results over 7 days of culture depicted in Figure 1. By increasing in the vital cell number, Alamar blue reduction goes higher and color changing is sharper. As this assay is a continuous cell monitoring method, more accuracy can be achieved. According to the results, cell numbers in all groups were increased by time. Interestingly, cells viability for collagen coated porous tantalum group at 5 and 7 days of culture was higher than other groups, albeit this amount was low at the initiate. Although cell viability for the coated PEEK at 5 days of culture was higher than bare PEEK and bare tantalum, but this story was reversed at 7th day and cells viability for bare tantalum was higher than coated PEEK and bare PEEK. Besides, cells proliferations at bare PEEK scaffolds in all times was lowest.

ALP activity:

One of the early markers of osteogenesis of stem cells is the production of ALP protein. In this regard, ALP activity of cultured cells on the each surface was evaluated after 7 and 14 days of culture. As it is showed in Figure 2, ALP activity of DPSCs on collagen coated porous tantalum is highest over 7 and 14 days of culture compare to other groups. Although the amount of ALP for other three groups, collagen coated PEEK, bare porous tantalum, and bare PEEK, for 7 days of culture is equal, these amounts had negative trend at 14 days of culture respectively.

Scanning electron microscopy:

As it can be observed with scanning electron microscope images were shown in Figure 3, DPSC which cultured on either coated porous tantalum and coated PEEK for three days under standard culture condition were well adhered at the surface of scaffolds in the forms of cells colonies and cells sheets respectively, whereas no cell attachments was seen at the surface of either bare tantalum and bare PEEK.

Alamar blue reduction assay was conduct ed to monitor cell proliferation. Increase in Alamar blue reduction indicates increase in cell number in the culture. Signifi-cantly higher Alamar blue reduction was seen when cells were incubated at 38–40°C compared to 37°C control over 3 days incubation (Figure 1A). In contrast, cells incubated at 41°C had lowest Alamar blue reduc-tion, which was lower than cells cultured at 37°C and other heat-stress temperatures (Figure 1A).

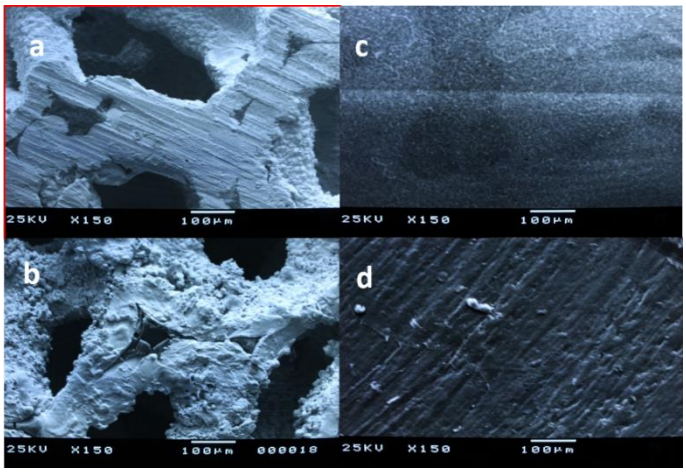

Figure 3. SEM images of attached DPSCs after 3 days of incubation: A) Bare porous tantalum; B) Collagen coated porous tantalum; C) Bare PEEK; D) Collagen coated PEEK

Discussion

Currently, spine surgeons used bone graft or caged devices which were filled with cancellous bone to augment spinal fractures. Due to many challenges of bone harvesting from other sites of body, stem or differentiated cells would be promising as alternative biological element to promote implant-host tissue integrity. Indeed, behavior of cells which are combined with a scaffolds are strongly depended on physicochemical properties of scaffolds including surface roughness and wettability. This study sought to compare the proliferation and osteogenic differentiation capability of human dental pulp derived stem cells (DPSCs) cultured on either collagen coated polyether ether ketone (PEEK) and porous tantalum and bare ones as two commercially available scaffolds for spinal fusion application. Both PEEK and Tantalum are bioinert materials which are usually incapable of cells attachments or bone fusing, and their integrities with bone grows weaker with time after implantation. To overcome this challenge, there are many strategies including surface modification by physically or chemically process like plasma irradiation or coating with bioactive materials.

Collagen has been widely applied as a source for surface coating on bioinert materials due to of its high wettability, osteoconductivity and non-toxicity. Coated scaffolds compare to bare ones have rough surfaces which provide extended interfacial contact area to promote cell adhesion. Beside, collagen coating and plasma treatment before coating process increase hydrophilicity of coated surface which lead to enhance cell attachment and consequently cell proliferation. These effects of surface modification was confirmed by cell viability assay and SEM images.

According to the Alamar Blue results that is depicted in Figure.1, both scaffolds (coated and non-coated) not cause severe cytotoxicity, however coated ones were more favorable for more proliferation. The rate of proliferation on all scaffold are progressive over time. Cells proliferations differences on the coated PEEKs and bare PEEKs were significant during 5 to 7 days of culture which could be referred to coating effect. In spite of the relatively poorer cells adhesion on bare PEEK compare to coated one according to SEM images, the DPSCs exhibited a higher Alamar blue reduction activity on bare PEEK at the first day of culture compared with coated PEEK which could be due to higher amount of cell were seeded. In the case of porous tantalum groups, at the 5 and 7 days of culture, cells on coated scaffolds exhibited higher proliferation rate than bare ones. However, at the first 24 hours, cells viability on both coated porous tantalum and bare tantalum were equal.

According to aforementioned results, the porous tantalum samples have superior biocompatibility over PEEK samples and this scenario is replicated intensively by collagen coating as well.

By analyzing the SEM images, it was found that collagen coating significantly improved cell attachments, on both porous tantalum and PEEK scaffold. Indeed, both PEEK and tantalum have hydrophobic surface which makes it difficult for cells or proteins to attach, leading to their low osseointegration in vivo. Like several other studies using collagen coating convert hydrophobic surface into hydrophilic one which is favorable for cells attachment (33). Besides, the uniform pore architecture of porous tantalum with rough surface provides a 3-D favorable structure with larger interfacial contact area that enhance cell attachment and consequently promote cell proliferation and differentiation in comparison with bare PEEK which has soft surface. However, cell sheets could be observed on coated PEEK scaffolds due to collagen effect which could change soft surface to rough ones.

The ALP activity results revealed that porous tantalum scaffolds stimulate cell osteogenic differentiation more than PEEK scaffolds due to surface roughness which significantly affects on the fate of stem cells (34).By coating the collagen onto both PEEK and tantalum, ALP activity at 14 days of osteogenic inductive culture increased than non-coated groups, however, this amount was higher for coated tantalum than other groups which might be related to synergism of surface roughness and collagen effects. Collagen coating moreover cells adhesion, increase stem cells osteogenic differentiation due to its osteocundivity property (35).

Conclusion

Our study shows that the porous tantalum scaffolds possess superior bioactivity in contact with DPSCs due to its 3D porous structure which made surface roughness and provides higher surface area for cell attachment and promote cell differentiation, and when it coated with collagen, its bioactivity goes higher than other groups due to synergism effect of collagen which could enhance cells adhesion and osteogenic differentiation as well.

Our results may provide useful information for combining MSCs with commercially implant instead of cancellous autograft bone, to enhance bioactivity of implants and implant to host tissue integration in spinal cord surgery.

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Conflict of Interest: 'None declared'.

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