Ultrastructural morphological changes of VEGF treated-stem cells from human exfoliated deciduous teeth cultured on human amniotic membrane

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ABSTRACT
A Scanning Electron Microscope (SEM) is used for viewing the surface and morphological details of materials. The most crucial step in preserving their ultrastructure is the fixation protocol, which depends on their conductivity properties. In this study, stem cells from human exfoliated deciduous teeth (SHED) were cultured on a human amniotic membrane (HAM) scaffold. Since these two biological samples were non-conductive, this study aims to identify the coating type with suitable voltage required to distinguish SHED ultrastructural morphological changes. SHED were treated with vascular endothelial growth factor (VEGF) for endothelial differentiation. The samples were fixed with paraformaldehyde. Gold sputtered was used because the non-coated sample, when exposed to acceleration voltage of 5 kV, resulted in a dark image, while a decrease in acceleration voltage, 2 kV, reduced the clarity of the ultrastructure. The gold-sputtered samples showed remarkable ultrastructure of the cells and scaffold. We observed ultrastructural changes of VEGF treated-SHED, presumably indicating cell differentiation. In conclusion, gold-sputtered with 5 kV voltage exposure improved the SEM images of VEGF-treated SHED cultured on HAM.

Keywords: Gold-sputtered; human amniotic membrane; SHED; Scanning Electron Microscopy; scaffold; endothelial.

Cambios morfológicos ultraestructurales de células madre tratadas con VEGF de dientes deciduos exfoliados humanos cultivados en membrana amniótica humana

RESUMEN
El Microscopio Electrónico de Barrido (MEB) se utiliza para observar la superficie y detalles morfológicos de los materiales. El paso más crucial para preservar la ultraestructura de un material es el protocolo de fijación, el cual depende de las propiedades de conductividad del material de estudio. En la presente investigación, se cultivaron células madre de dientes deciduos exfoliados humanos (denominadas SHED, por sus siglas en inglés) en un andamio de membrana amniótica humana. Tomando en cuenta que estas dos muestras biológicas no eran conductoras, el objetivo del presente estudio fue identificar el tipo de recubrimiento con el voltaje adecuado para distinguir los cambios morfológicos ultraestructurales de las SHED. Las células madre de dientes deciduos exfoliados humanos fueron tratadas con factor de crecimiento endotelial vascular (VEGF, por sus siglas en inglés) para la diferenciación endotelial. Las muestras se fijaron con paraformaldehído. Se utilizó la pulveización catódica con oro, ya que la muestra no recubierta, al ser expuesta a un voltaje de aceleración de 5 kV, dio como resultado una imagen oscura, mientras que una disminución en el voltaje de aceleración, 2 kV, redujo la claridad de la ultraestructura. Las muestras con pulverización con oro mostraron una ultraestructura notable de las células y el andamio. Se observaron cambios ultraestructurales de SHED tratadas con VEGF, lo que presumiblemente indica diferenciación celular. En conclusion, el recubrimiento obtenido mediante la pulverización con oro junto con la exposición a un voltaje de 5 kV mejoró las imágenes por microscopía electrónica de las SHED tratadas con VEGF cultivado en membrana amniótica humana.

Palabras clave: Pulverización catódica con oro, membrana amniótica humana, SHED, Microscopio Electrónico de Barrido, andamio.

INTRODUCTION

Scanning Electron Microscope (SEM) is generally used for viewing the surface morphology of various types of materials. SEM microscopic observation can be affected by the type of material characterized as conductive or non-conductive [1]. The SEM sample preparation is critical because erroneous can distort the image due to vacuum exposure during the viewing process [2], leading to

artifacts [3, 4].

Before examining conductive materials, the metal coating is not usually required as long as the samples are thoroughly dried [1]. However, non-conductive biological samples need chemical fixation using either glutaraldehyde, formaldehyde, or paraformaldehyde, along with the metal coating. The steps stabilize the electron beam produced on the material's surface and improve the image contrast [2].

Different scaffolds were utilized and re-engineered in tissue engineering to meet the demand required for clinical usage, especially in burnt and wound skin management. Meanwhile, in dentistry, a scaffold is crucial for intra- and extra-oral application [5]. Certain materials are naturally derived, such as chitosan from seashells [6] and extracellular matrix membrane derived from tissue [7]. Some materials are synthesized, such as coumarin [8] and hydrogel [9]. In cell biology, the selected scaffold must provide a microenvironment compatible with the cells' interaction to enable cell growth and differentiation [10]. Previously, a study has been performed where the cell viability improves significantly when cultured on the natural scaffold compared to a modified synthetic scaffold [11].

Therefore, we used the human amniotic membrane (HAM) as a scaffold for stem cells from human exfoliated deciduous teeth (SHED). HAM is a biological tissue characterized by translucent structure [12] and elastic capability [13]. It is used in research related to wound healing and regenerative medicine [14, 15]. HAM was selected due to its extracellular-rich properties. It is abundantly available and raises less ethical concern as it is typically discarded. The ability of HAM to serve as a promising scaffold for cell growth and differentiation was previously proven [16, 17]. Like other biological samples, HAM required a different technique to preserve the structure for SEM observation [7]. This is because biological samples are generally good insulators, which caused imaging artifacts as a result of the excessive charging in SEM [4]. Due to its nature, the typical recommended acceleration voltages value for non-coated biological samples ranges from 3 to 5 kV [18]. Depending on material types, the range can be as high as 15 kV to 20 kV [19]. However, high acceleration voltage will penetrate deeper into a sample and disrupt the morphological view [20].

Meanwhile, the ability of SHED to grow and differentiate on HAM has been described elsewhere [7, 21, 22]. As reported previously, it can also differentiate into many cells [23, 24]. The original shape of SHED, spindle-shaped, might change to another kind of cell morphology. In this study, we cultured stem cells SHED on a HAM scaffold and treated them with vascular endothelial growth factor (VEGF) to induce SHED endothelial differentiation [7]. The aim was to highlight the combination of metal-coating and voltage exposure that enhanced the SHED ultrastructural changes caused by VEGF and might indicate cell differentiation. Initially, we attempted to use the uncoated samples but were unsuccessful. However, the morphological differences of the cells on HAM can be distinguished in the end.

MATERIALS AND METHODS

HAM Preparation for SEM.

Gamma-irradiated glycerol-preserved HAM was obtained from Tissue Bank Unit, School of Medical Sciences, Universiti Sains Malaysia. As mentioned previously, the removal of epithelial cells at the basement side was carried out [7]. HAM was prepared to 2x2 cm and used for cell culture.

Preparation of SHED Cultured on HAM with VEGF Treatment.

SHED (AllCells, USA) were cultured in a complete medium made of the Minimum Essential Medium (MEM) Alpha Medium (α-MEM) (Gibco, USA), supplemented with 15% of fetal bovine serum (FBS) (Gibco, USA) and 50 U/ml of penicillin-streptomycin (Gibco, USA). SHED with cell densities of $2x10^5$ was cultured on the stromal

sides of 2x2 cm glycerol-preserved HAM in a culture dish for 24 hours. HAM without cells was used as a control. VEGF treatment has been described previously [7]. The slow-release delivery system used for the VEGF treatment in this study is fibrin sealant [TISSEEL Kit (Baxter AG, Austria)]. The release of VEGF was determined in the lab (unpublished data), and it was conducted according to a study reported previously [25]. SHED cultured on HAM was treated with VEGF for 24 hours.

Sample Fixation for SEM.

The fixation method of the samples has been described [12]. Briefly, HAM scaffolds with and without cells were washed with PBS before being fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer) for 2 hours at room temperature. The HAM samples were rewashed with PBS and incubated for 2 days in 8% of formaldehyde at 4ºC. This was followed with serial ascending diluted alcohol fixation (20, 30, 40, 50, 60, 70, 80, 90, and 100%) for 10 min, respectively. The sample was incubated in hexamethyldisilazane (HMDS) for the drying process for 10 min, followed by 10 min in a desiccator. The sample was gold-coated with sputter coating machine SCD 0005 (Legaci, USA) and viewed by SEM FEG 450 (Quanta, Czech) at an accelerating voltage of 5 kV. Meanwhile, uncoated samples were considered at an accelerating voltage of 2 kV and 5 kV.

RESULTS AND DISCUSSION

Gold-coated Sputtering of Biological Sample, SHED Cultured on HAM.

The result showed that when SHED cultured on HAM sample exposed at accelerating voltage of 5 kV, the image turned out to be as figure 1. The 5 kV, in this case, was conducted based on a previous study for non-coated biological material [18]. The effect was observed probably because, without a proper coating, a negative charge will accumulate on the sample surface and lead to a dark image [26]. The suggestion of Ushiki et al. [13] was suitable if we used glutaraldehyde instead of paraformaldehyde for fixation. Glutaraldehyde is widely applied for the fixation of HAM scaffold [12, 27-30].

Most studies opt for glutaraldehyde as it provides better cross-linking of protein [31, 32]. The usage of paraformaldehyde is less reported [33]. The penetration of tissue made up of small molecules is faster when using formaldehyde [32]. We choose paraformaldehyde as an alternative to avoid osmium tetroxide usage because it can damage the protein structure due to its highly toxic compound [34]. Besides, prolonged fixation causes oxidation and might wash away the cells on tissue samples [35].

Some researchers used HMDS as an alternative for osmium tetroxide because it does not distort the sample [36]. However, the usage of HMDS in our study was not a replacement method for post-fixative osmium tetroxide, but it was required for the critical drying step.

Then, we tried to decrease the accelerating voltage to 2 kV, hoping that it could improve the viewing. The result of figure 2 was an image of SHED cultured on HAM without gold-sputtered. It showed that the cells were hardly identified, probably because both cells and scaffold were exhibited at the same level of brightness on the HAM surface [19].

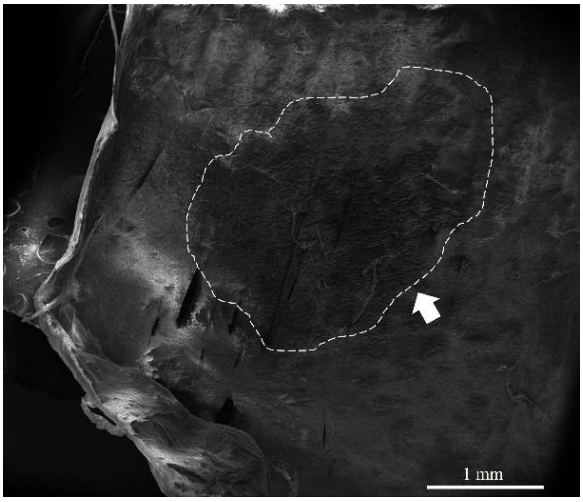

Fig. 1. Image of SHED cultured on HAM with recommended accelerating voltage (5 kV) for non-coated SEM sample. The burnt effect was observed—magnification at 60x.

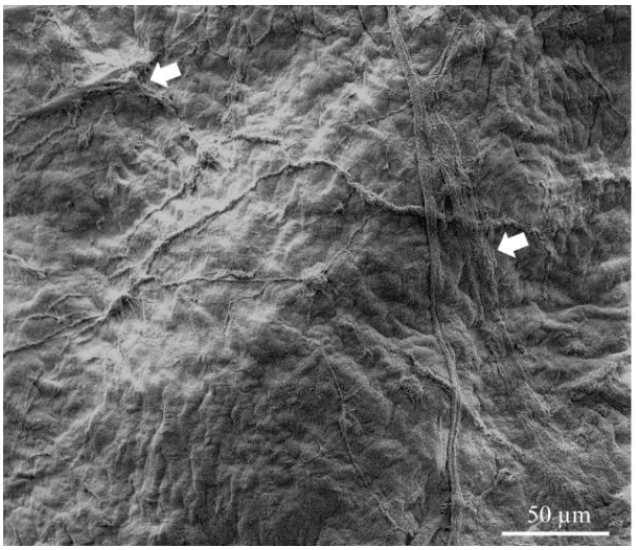

Fig. 2. Image of SHED cultured on HAM at 2 kV accelerating voltage without gold-sputtered. The cell morphology was undetermined—magnification of 1000x.

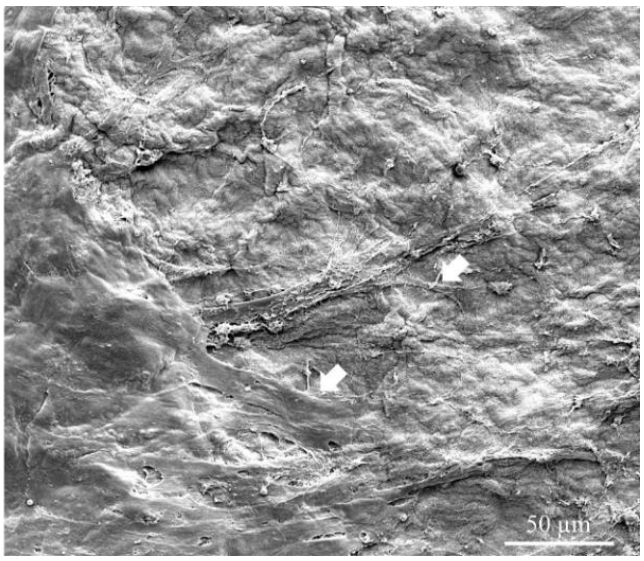

Fig. 3 Image of SHED cultured on HAM at 5 kV accelerating voltage with gold-sputtered. The cell morphological edge was identified—magnification of 1000x.

In general, metal coating improves the observation as the metal can stabilize the electron beam produced during observation of the non-conductive sample.

There are many types of metal coating, including gold (Au), gold/palladium (Au/Pd), platinum (Pt), iridium (Ir), tungsten (W), or carbon (C) [37]. Au, Au/Pd, and Pt are commonly used for low-resolution imaging [37]. Hence, we thought a better image would be produced by gold-sputtering the sample and increasing the accelerating voltage to 5 kV.

Figure 3 showed an image of a gold-coated SHED sample cultured on HAM treated. The lamellipodia of the cells on HAM could be observed. We decided to gold-sputtered the samples based on this image and used an acceleration voltage of 5 kV.

Although a previous study suggested that biological samples SEM images are better without gold-coating [21], it may be contributed by the technical aspect for stub preparation and model of SEM machine.

Morphology of SHED Cultured on HAM with and without VEGF Treatment.

We utilized the SEM images to display the effect of VEGF treatment on the morphological changes of SHED cultured on HAM [38]. Figure 4A showed the image of the stromal side of HAM without cells, with fibrous-like structures. Once we cultured the cells on HAM without VEGF, on day 1, all cells were attached to the membrane based on the cell flattened image (figure 4B). Figure 4C showed the image of SHED cultured on HAM with VEGF. Some cells were flattened, but some still showed the round cell structure. The structure observed probably was a cell undergoing a differentiation process and not attaching to the HAM surface. Another possibility is that the cell was an endothelial-like differentiated cell that showed ultrastructural changes from flattening to rounded-like. The ultrastructural changes of SHED cultured on HAM scaffold with VEGF addition were significantly observed and improved with gold-sputtered, which may confirm the endothelial formation. Based on a previous study, SEM observation can determine SHED underwent osteogenic differentiation when cultured on HAM [39].

Due to budget and time constraints in the current study, we could not use another microscopy device, Variable Pressure SEM (VPSEM), which is more suitable for uncoated material at low voltage. However, we will consider it for future applications.

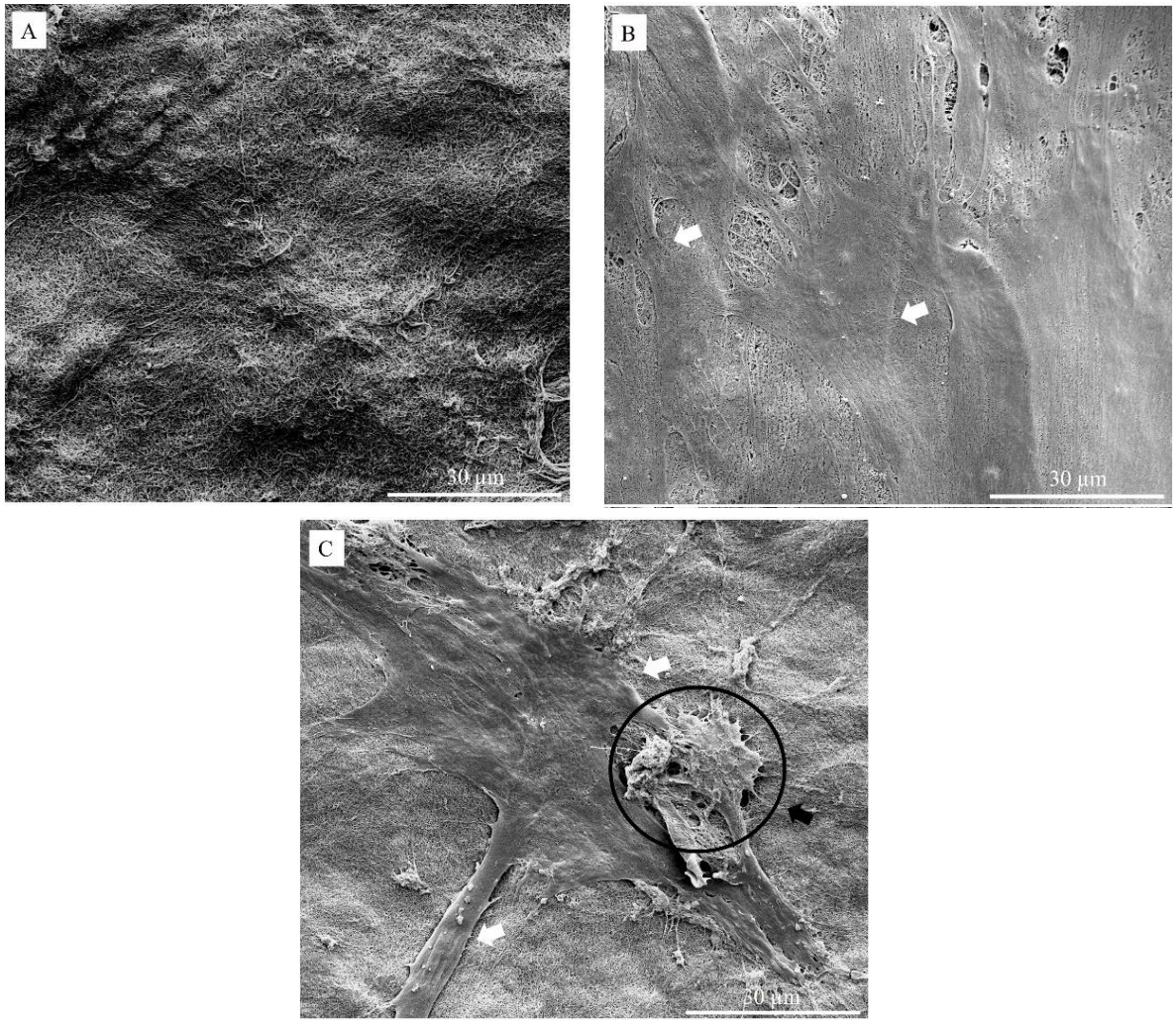

Fig. 4. Images of SHED cultured on HAM with and without VEGF at 5 kV accelerating voltage. The samples were treated with gold-coated sputtering: A) The stromal side of HAM without cells; B) SHED cultured on HAM without VEGF; C) SHED cultured on HAM treated with VEGF. The structural change was noticeable in the VEGF group—magnification at 3000x.

CONCLUSION

The synergistic action of gold-coating improved the ultrastructural morphology of biological samples of SHED and HAM at 5 kV. Therefore, we were able to distinguish endothelial-differentiated SHED when treated with VEGF.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL STATEMENT

This study was approved by the human ethics committee, known as the Research and Ethical Committee of USM (USM/JEPeM/14110477), for the usage of HAM from the

Tissue Bank Unit, School of Medical Sciences, Universiti Sains Malaysia.

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