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RESEARCH ARTICLE

Comparative Analysis of Different Fixation and Dehydration Methods for Cells Seeded on Gel-Based Scaffolds using Scanning Electron Microscopy

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Abstract Matrigel™, a complex mixture of ECM proteins, and Collagen type 1 (Col-1), a major component of the natural extracellular matrix, are widely utilised in both cell culture and tissue engineering. High magnification by scanning electron microscopy allows detailed microstructural analysis of the cells and scaffolds. However, the efficiency of the techniques may be directly affected by different fixation and dehydration methods. Optimisation of the fixation and dehydration protocol for cell growth on scaffold is important as it influences the morphological integrity and structural preservation of cells within gel-based scaffolds. This study compared the osmium tetroxide hexamethyldisilane (HMDS) and critical point drying (CPD) method to fix and dehydrate cells which were seeded on Col-1 and Matrigel™ scaffolds by SEM analysis. Col-1 and Matrigel™ scaffolds were prepared and seeded with dental pulp stem cells (DPSC) for 3 days; followed by fixation using with McDowell-Trump fixative, osmium tetroxide hexamethyldisilane (HMDS) or the critical point drying (CPD) method and analysed by SEM. Both HMDS and CPD displayed good compatibility with cells and scaffolds for SEM analysis. No significant differences were observed on the morphology of DPSC prepared using either HMDS or CPD (Mann–Whitney U test, p = 0.10 for both). However, each of the fixation methods substantially provided different preservative effects on Col-1 and Matrigel™ microstructures. In conclusion, both HMDS and CPD methods were found suitable for fixation and dehydration of DPSC plated on Col-1 and Matrigel™ scaffolds for analysis by SEM.

Keywords: Scanning electron microscopy, scaffold, collagen type 1, Matrigel™, dental pulp stem cells.

Introduction

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Tissue engineering (TE) is a branch of regenerative medicine dedicated to developing viable biological tissues to repair or replace damaged or diseased tissues in the body. The interdisciplinary field combines principles from basic biology, engineering, and medicine to develop functional replacements for tissues, and even whole organs, using scaffolds, cells, and biologically active molecules. One of the key players in TE is stem cells, a population of undifferentiated cells which possess long-term self-renewal capacity and multilineage differentiation potential. Dental pulp stem cells are known to play a significant role in regenerative medicine and disease therapy by activating specific signalling pathways, which induce cellular migration and differentiation into various type of committed cells, to support new tissue formation [1].

Extracellular matrix (ECM) is the extracellular components of natural tissue which give structural support to the cells and tissues, to regulate cell attachment and interaction, as well as direct cell proliferation and differentiation. Mimicking the ECM structure is the primary aim in the designing of scaffolds for tissue engineering. Matrigel™ is an extract derived from Engelbreth–Holm–Swarm (EHS) mouse tumours which closely mimics the natural environment of cells *in vivo.* Matrigel™'s rich matrix can influence stem

cell fate and offers a valuable tool for studying stem cell behaviours in a controlled environment [2], [3]. Type I collagen constitutes ~90% of the organic bone matrix and is a major structural protein of connective tissues. The distinct properties of Col-1 such as low antigenicity, excellent biocompatibility, and biodegradability, make it a suitable biomaterial for scaffold fabrication [4], [5]. Col-1 can also stimulate cell adhesion via receptors that recognise specific peptides on the cells surface. Collectively, data gathered from previous research show that Col-1 provide structural pathways that affect cells migration, which is particularly important in processes such as wound healing and tissue regeneration [6]. Col-1 can activate various signalling pathways that promote cell proliferation and influence cell fate decisions. For example, the interaction between Col-1 and integrin receptors can activate pathways like ERK and Akt, which are involved in cell cycle regulation and proliferation [7].

Analysis on surface morphology of biological samples and biomaterials are usually performed using scanning electron microscopy (SEM) as it provides high resolution of the detained images. However, the efficiency of SEM analysis relies on sample preparation, which includes the fixation and dehydration process. Chemical fixation or lyophilisation techniques can be used to maintain the structural integrity of biological samples and biomaterials by arresting the degenerative processes such as autolysis. Glutaraldehyde is commonly used for cell fixation in biomedical research for chromatin core particles fixation, where it forms crosslinking in protein. Meanwhile, formaldehyde can fix both protein and DNA core particles [8]. Hence, glutaraldehyde-formaldehyde fixative can produce higher percentage of revealed shrinkage with proper cytoplasm and organelles distortion [9]. Post-fixation with osmium tetroxide (OsO_4) could better preserve the cell morphology and enhance the contrast of SEM images [10], [11]. Osmium tetroxide will react with lipids contained in the biological sample and progressively blackened the lipid content into black, increase the density and contrast to the SEM image. Osmium tetroxide penetrates stabilized tissue at a relatively fast rate, with an immersion time of 60 to 90 minutes typically being adequate for most samples [12].

The final step in SEM sample preparation is drying, which ensures the complete removal of any remaining intermediate solvents or dehydrating agents from the fixed cells and tissues. Common drying techniques for SEM sample preparation include critical point drying (CPD) and air drying. CPD, which utilizes liquid carbon dioxide (CO_2), eliminates moisture from tissues while preventing surface tension damage by transitioning from liquid to gas at the critical point where both phases have equal density and no interface is present. Despite its higher cost and longer processing time, CPD is generally preferred due to its effectiveness in preserving sample structure. Recently, a low-cost hexamethyldisilazane (HMDS) has been introduced as an alternative to CPD. The samples undergo dehydration using a graded ethanol series, then are immersed in HMDS and left to air dry. The lower surface tension of HMDS helps stabilize the samples during drying and may minimize the risk of fractures in collapsed biological tissues. Of note, SEM imaging prepared using HMDS demonstrated identical quality of preservation with CPD [12]. Previous research comparing hexamethyldisilazane (HMDS) and critical point drying (CPD) has mainly explored non-gel systems such as porous scaffold and fibroblast cultures [13], [14]. The novelty of this study lies in the direct comparison of CPD and HMDS fixation methods applied to dental pulp stem cells (DPSCs) cultured on gel-based scaffolds. This is a major improvement over earlier research that mostly concentrated on non-mammalian or basic cell systems like fibroblasts and insect tissues [15], [16]. This work expands the use of these fixation methods to complicated, soft three-dimensional scaffolds that are important for research on stem cells and tissue engineering.

This study aimed to assess the effectiveness of various fixation and dehydration techniques for dental pulp stem cells (DPSC) cultured on gel-based scaffolds, utilizing SEM imaging for analysis. The preparation methods examined are CPD and HMDS techniques. This study specifically focused on evaluating the morphology and attachment of DPSC plated on Col-1 and Matrigel™ scaffolds, as well as comparing the microstructural integrity of these scaffolds when prepared with distinct fixation and dehydration approaches.

Materials and Methods

The biomaterials used for plating the DPSC are Matrigel™ (Sigma-Aldrich), Collagen type 1 (Thermo), phosphate-buffered saline (PBS, pH 7.4) without CaCl2 and $MgCl_2$ (Gibco/Invitrogen Life Technologies), alpha modified Eagle's medium (αMEM) (Gibco/Invitrogen Life Technologies), fetal bovine serum (FBS) (Gibco/Invitrogen Life Technologies) and trypsin-EDTA solution (Sigma-Aldrich).

Fabrication of Matrigel™ Scaffold

The Matrigel™ bottle was thawed overnight at 4°C in a refrigerator until fully liquefied. It was then divided into 300 µl aliquots and stored at −20°C until needed. For plate coating, a 1% Matrigel™ stock solution

was made by mixing a 300 µl aliquot with 29 ml of complete culture medium. This stock was further diluted with phosphate-buffered saline to create working solutions at concentrations of 0.5%, 0.25%, 0.1%, 0.02%, and 0.01%. The working solutions were applied to 12-well plates (1 ml per well) or 24-well plates (0.5 ml per well) to fully coat the surfaces. Plates were then incubated at 37°C for 15 minutes in a cell culture incubator. After incubation, the excess Matrigel™ was aspirated, and the wells were rinsed once with complete culture medium.

Fabrication of Collagen Type-1 Scaffold

A working solution at a concentration of 2 µg/ml was prepared by diluting the stock solution with α-MEM (Gibco/Invitrogen Life Technologies). This solution was added to 12-well plates (1 ml per well) or 24-well plates (0.5 ml per well) and incubated for 1 hour at 37°C in a cell culture incubator. After incubation, the excess solution was removed, and the plates were rinsed twice with PBS.

Cell Line

Dental pulp stem cells (DPSC) (AllCells, USA) were purchased and revived according to the manufacturer's protocol. DPSC taken from passage 6 to 7 were used for the cell culture on gel-based scaffolds.

Cell Maintenance on Gel-Based Scaffold

DPSC were cultured in complete culture medium consisting of αMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin. DPSC was kept at 37°C in a 5% CO_2 humidified incubator and the cells were passaged when reaching 80%, confluency.

Cell Plating

The cell plating density was evaluated by calculating the number of cells using a cell haemocytometer with trypan blue staining. Cells were stained with 0.4% Trypan Blue (1:3 ratio) for 3 minutes at room temperature, and viable (unstained) and non-viable (blue-stained) cells were counted using a hemocytometer to determine cell viability. The cells were plated at a density of 5,000 cells per ml in a Col-1 and Matrigel™ coated 24-well culture plate (1ml/well) and incubated at 37°C in a 5% CO_2 humidified incubator. The medium was changed after 48 hours. Cells were allowed to grow for three days, before the fixation and dehydration process.

Hexamethyldisilane (HMDS) Protocol

DPSC plated on Col-1 and Matrigel™ scaffolds were fixed with McDowell Trump fixative for 2 hours and 1% osmium tetroxide solution (Sigma-Aldrich) for 1 hour. All the samples were dehydrated in a graded acetone solution (50%, 75%, 95%, and 100% each for 10 minutes, and 100% two times for 10 minutes) before applying 100% HMDS two times for 10 minutes for complete dehydration. The samples were air dried for 24 hours.

Critical Point Drying (CPD) Protocol

DPSC plated on Col-1 and Matrigel™ scaffolds were fixed with McDowell Trump fixative for 2 hours and 1% osmium tetroxide solution (EMS, United States) for 1 hour. All the samples were dehydrated in a graded acetone solution (50%, 75%, 95%, and 100% each for 10 minutes, and 100% twice for 10 minutes). After removing the acetone, the samples were placed on a sample holder for critical point drying in a critical point dryer.

Scanning Electron Microscopy (SEM)

The samples were sputter gold coated using Leica EM QSG100 sputter coater. SEM (FEI, QuantaTM 450 FEG) with an acceleration voltage of 15kV in a pressure of 1 x 10-5 Torr was utilised to observe the morphology of cells on the gel-based scaffolds. The DPSC morphology plated on Col-1 and MatrigelTM scaffolds were observed by SEM. Each sample were analysed triplicate with n=3 using imageJ and statistical analysis using Mann-Whitney U test, GraphPad Prism 8. SEM images were captured at three magnifications: 2,500× for cell-scaffold interaction, 5,000× for surface morphology, and 20,000× for fine visualization of micro and nanoscale characteristics.

Results and Discussion

Morphological Analysis of DPSC Cultured on Col-1 and Matrigel™ Followed by Different Fixation and Dehydration Method

The SEM images of DPSC fixed and dehydrated using CPD and HMDS methods was shown in Figure 1. The morphology of DPSC were compared under 2500X and 5000X magnifications. DPSC seeded on Col-1 (Figure 1a, b, c, d) and Matrigel™ (Figure 1e, f, g, h) exhibit a typical spindle-shaped, fibroblast-like morphology of mesenchymal stem cells, but show distinct differences in cellular protrusions based on the scaffold used.

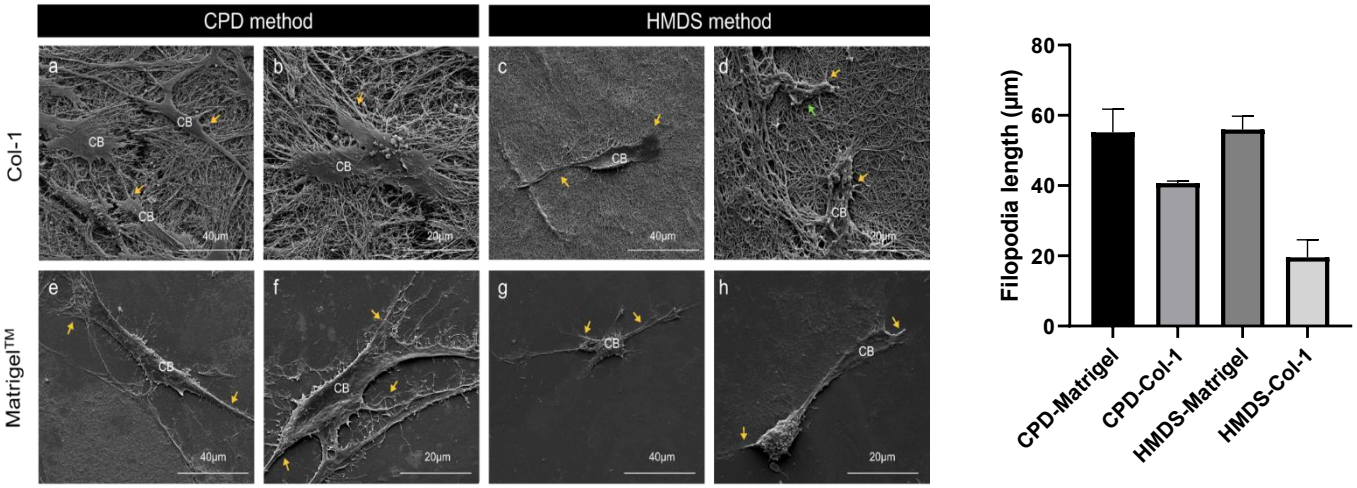

Figure 1. SEM images of Col-1 (a, b, c, d) and Matrigel™ (e, f, g, h) showed the different effect of fixation and dehydration process on cell morphology in two different magnification levels. (a, c, e, g) Magnification 2500X. (b, d, f, h) Magnification 5000X. Filopodia are indicated with yellow arrows. Average number of filopodia on Matrigel™ appeared more distinct and better defined. Green arrows are indicative of cell aggregation. CB= cellular body of DPSC, Data are presented as median ± range (n = 3). Statistical analysis using the Mann–Whitney U test (GraphPad Prism 8) showed no significant difference between groups (p = 0.10 for both fixation methods)

When seeded on Col-1, DPSC showed flat and spindle-like morphology, they developed short filopodia from their lamellipodia at the apical poles, a characteristic typical of fibroblast-like cells. In contrast, DPSC seeded on Matrigel™ not only form similar short filopodia but also extend long, thin protrusions directly from the cellular body, which are much more pronounced than those observed on Col-1. However, the differences were not statistically significant (Mann–Whitney U test, CPD: p = 0.10; HMDS: p = 0.10). Both CPD and HMDS methods demonstrated comparable efficiency in preserving the structural integrity of DPSC, underscoring the effectiveness of these techniques in detailed morphological studies of stem cells on different substrates.

Assessment of DPSC Attachment to Col-1 and Matrigel™ Following CPD and HMDS Methods

DPSC attachment to Col-1 and Matrigel™, following the CPD and HMDS methods was assessed, as shown in Figure 2. Both Col-1 and Matrigel™, scaffolds provide suitable surface area for cell attachment and spreading. However, a notable finding was recorded with the development of long, thin protrusions from DPSC plated on Matrigel™, a feature scarcely visible on Col-1.

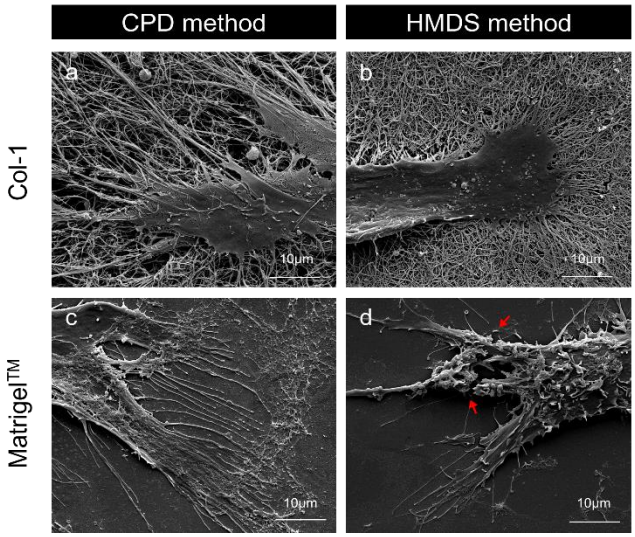

Figure 2. SEM images of Col-1 (a,b) and Matrigel™ (c,d) showed the different effect of fixation and dehydration process on cell attac hment. Magnification 10,000X. Red arrows are indicated ruptures present in cell

Based on figure 2(d), there was surface crack noted on sample prepared using HMDS drying method. Similar crack and hole formations have been attributed to preparation artifacts caused by HMDS-induced shrinkage rather than true structural defects [17]. Additionally, the CPD method exhibited better preservation capacity, enhancing cell adhesions more effectively compared to the HMDS method.

The Microstructural Analysis of Col-1 and Matrigel™ Scaffolds Following CPD and HMDS Methods

Both CPD and HMDS method demonstrated effective preservative capacities for the microstructures of Col-1, which exhibited a dense fibrillary network characterized by narrow, elongated, and crosslinked collagen fibers (Figure 3). Interestingly, the HMDS method led to the formation of clusters and aggregates of an unidentified naturally derived protein, indicated by blue arrows, within the Col-1 scaffold. On the other hand, the CPD method was superior in preserving the porosity of the Matrigel™ scaffold compared to the HMDS method, suggesting that CPD may be more suitable for applications requiring the maintenance of scaffold porosity and microarchitecture.

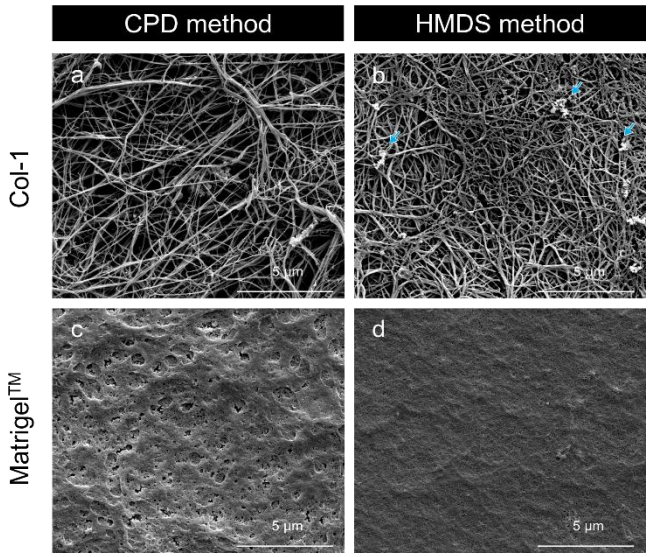

Figure 3. SEM images of Col-1 (a,b) and Matrigel™ (c,d) showed the different effect of fixation and dehydration process on scaffold microstructures at 20,000X magnification

The morphology of DPSC following sample preparation with HMDS drying method shows no significant differences with CPD method, which is the standard method for preparing highest quality SEM specimens. This observation is consistent with findings from previous research that examined CPD and HMDS across various biological samples, including soft insect tissue [18], mouse fibroblasts [19], cervical cells [17] and primary breast fibroblasts [20]. In our study, both methods effectively preserved the typical spindle-shaped and fibroblast-like morphology of DPSC, features crucial for the cell's motility and migration.

The use of Col-1 and Matrigel™ scaffolds has been particularly effective in supporting the attachment and spreading of DPSC, as these cells exhibit cytoplasmic extensions that signal strong adhesion to these scaffolds [21]. The main components of Matrigel™ include collagen type IV, laminin, entactin heparin sulfate, proteoglycan, and perlecan. Due to its composition, Matrigel™ has multiple application in 3D cell culture research, *in vivo* studies for analyzing cell differentiation, angiogenesis assays, tumor assay, drug screening, toxicology testing, and disease modelling [22], [23], [24], [25].

In the present study, both Col-1 and Matrigel™ scaffolds allow for cells attachment and spreading, which provide an extensive space for cells to expand. The microstructure of Col-1 facilitates the process of cell attachment. This attachment is mediated through integrins and other receptors, providing both mechanical support and biochemical signals that influence cell behaviour [26]. Similarly, Matrigel™ has been used in angiogenesis assays to study the process of new blood vessel formation. Its composition promotes the formation of capillary-like structures by endothelial cells, which is crucial for studies in cancer research, wound healing, and vascular biology [26], [27]. The extensive proliferation of DPSC can sometimes obscure the surface of the scaffolds, highlighting the importance of scaffold porosity for enhancing cell surface contact and facilitating cellular communication and interaction [28]. It further justifies the shorter time used in this study to plate the cells and monitor cells attachment.

It appears that CPD has shown superior capability in preserving the gross morphology and microstructural integrity of DPSC on each Col-1 and Matrigel™ scaffolds, particularly for delicate and porous structures. Meanwhile, HMDS presents a faster, simpler, and more cost-effective alternative. Because of its hydrophobic properties, low surface tension, and volatility, HMDS has an impact on scaffold and cell shape. The high volatility of HMDS makes it easier for solvents to evaporate quickly during drying, which lessens capillary stress that could cause fragile biological structures to shrink or collapse [15]. Its low surface tension improves the preservation of tiny cellular characteristics by reducing mechanical damage to nanoscale structures like filopodia. However, during the drying process, HMDS may cause localized deformation or shrinkage in hydrophilic gel matrices [29], [30]. These combined effects assist to explain the minor differences in scaffold surface and cellular morphology seen when compared to CPD-treated samples. Although HMDS may not preserve microstructures as effectively as CPD, it does not require specialized equipment beyond a laboratory fume hood, making it a practical option for many applications [31]. This reagent is frequently employed in gas chromatography to create silyl ethers from compounds that include one or more reactive substances, including alcohols, sugars, and amino acids [16]. However, extreme precaution steps should be taken as it is dangerous and extremely volatile. Overall, both methods are found to be suitable for the fixation and dehydration of DPSC on Col-1 and Matrigel™ scaffolds for SEM analysis, each offering distinct advantages depending on the specific requirements of the study. This fact is supported by the findings that HMDS was considered more suitable for accurate SEM imaging of the cellular actin cortex due to its reduced artifacts and better preservation of cellular structures, whereas CPD, although widely used, is more prone to artifacts and structural distortions [15].

Conclusions

In conclusion, SEM has proven to be a valuable tool in elucidating the intricate details of DPSC morphology, including variations in cell shape, size, and the presence of cellular extensions such as filopodia and lamellipodia. Adhesion of DPSC is consistently apparent across all groups, marked by distinct cytoplasmic extensions. While the CPD method offers superior preservation of these structural details, it does require specialized equipment and entails longer processing times. Conversely, HMDS might introduce minor distortions but remains a faster and more accessible option for sample preparation. Ultimately, both CPD and HMDS methods have demonstrated their suitability for effectively fixing and dehydrating DPSC on both Col-1 and Matrigel™ scaffolds for SEM analysis, offering flexibility depending on specific research needs and available resources. For researchers creating similar gel-based scaffolds for SEM, both CPD and HMDS can be employed efficiently, but careful tuning is required. HMDS provides a simpler and more cost-effective option; however, progressive solvent exchange and controlled drying are required to reduce surface shrinkage and crack formation. To preserve fine cellular details like

filopodia without charging or collapse artifacts, use low accelerating voltage and intermediate magnifications (2,500-20,000x). Future studies should include additional scaffold types and stem cell lines to confirm the generality of these findings. Optimization of HMDS and CPD parameters, along with complementary imaging methods such as cryo-SEM, is recommended to minimize artifacts and validate true morphological features.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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