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ORIGINAL ARTICLE

Biological effects of silk fibroin 3D scaffolds on stem cells from human exfoliated deciduous teeth (SHEDs)

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Abstract The aim is to investigate in vitro biological effects of silk fibroin 3D scaffolds on stem cells from human exfoliated deciduous teeth (SHEDs) in terms of proliferation, morphological appearance, cell viability, and expression of mesenchymal stem cell markers. Silk fibroin 3D scaffolding materials may represent promising suitable scaffolds for their application in regenerative endodontic therapy approaches. SHEDs were cultured in silk fibroin 3D scaffolds. Then, cell numbers were counted and the Alamar blue colorimetric assay was used to analyse cell proliferation after 24, 48, 72, and 168 h of culture. The morphological features of SHEDs cultured on silk fibroin scaffolds were evaluated by scanning electron microscopy (SEM). Finally, cell viability and the expression of mesenchymal stem cell markers were analysed by flow cytometry. One-way analysis of variance (ANOVA) followed by a Bonferroni post-test was performed ($P < 0.05$). At 24 and 48 h of culture, SHED proliferation on scaffolds was modest compared to the control although still significant ($p < 0.05$). However, cell proliferation progressively increased from 72 to 168 h compared with the control ($p < 0.001$; $p < 0.01$). In addition, flow cytometry analysis showed that the culture of SHEDs on silk fibroin scaffolds did not significantly alter the level of expression of the mesenchymal markers CD73, CD90, or CD105 up to 168 h; in addition, cell viability in silk fibroin was similar to than obtained in plastic. Moreover, SEM studies revealed a suitable degree of proliferation, cell spreading, and attachment, especially after 168 h of culture. The findings from the current study suggest that silk fibroin 3D scaffolds had a favourable effect on the biological responses of SHEDs. Further in vivo investigations are required to confirm these results.

Keywords Scaffold · Silk fibroin · Cytotoxicity · SHEDs · Human dental stem cells

Introduction

Stem cell-based therapy (SC-BT) in regenerative endodontics is one of the most promising therapeutic strategies, which would extend the longevity of teeth and improve the quality of life of patients [1, 2]. SC-BT includes stem cells and the use of scaffolds and growth factors that are involved in the regeneration of the pulp-dentin complex [3]. In a typical SC-BT approach of dental pulp regeneration, a regenerated pulp graft is prepared by culturing mesenchymal stem cells (MSCs) in a porous scaffold for a given time and then implanting the graft into the damaged region [4]. Several studies have previously demonstrated that MSCs can be isolated from multiple tissues such as bone marrow, peripheral blood, umbilical cord blood, adult connective tissue, placenta, amniotic

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membrane, and dental tissues [5, 6]. Among these tissues, dental pulp is considered as a rich source of mesenchymal stem cells (MSCs) suitable for tissue engineering approaches [7].

Stem cells from human exfoliated deciduous teeth (SHEDs), which are obtained from dental pulp explants or by the digestion of the dental pulp tissue from exfoliated deciduous teeth, have immunosuppressive properties [8, 9]. SHEDs have a higher proliferation rate than bone-marrow mesenchymal stem cells (BMMSCs) and dental pulp stem cells (DPSCs) and express Oct4, CD13, CD29, CD44, CD73, CD90, CD105, CD146, and CD166, but not express CD14, CD34, or CD45 [10, 11]. These postnatal stem cells are important in the regeneration and repair of craniofacial defects, tooth loss, and bones because of their capability to proliferate and differentiate [12, 13]. Indeed, it has been reported that these mesenchymal stem cells may be appropriate for regenerative endodontic therapy [14].

For its part, silk fibroin is a protein obtained from the domesticated mulberry silkworm *Bombyx mori*. It is a natural polymeric protein composed of three protein components: the heavy chain fibroin (H-chain, 350 kDa), the light chain fibroin (L-chain, 25 kDa), and the P25 protein [15, 16]. This protein, which is commonly used in the textile industry, represents a highly attractive scaffolding material suitable for tissue regeneration [17]. The main advantages of silk fibroin as biomaterial are its easy processability, superior mechanical properties, controlled degradability, oxygen and water permeability, as well as its excellent cell biocompatibility, making it a suitable material for different biological applications [18]. Moreover, silk fibroin is less immunogenic and inflammatory than other reported biomaterials such as poly(lactide-*co*-glycolide) (PLGA) copolymers or collagen-based scaffolds [19]. Several configurations have been designed using silk fibroin for biomedical applications, including fibres [20, 21], films [22], sponges, and hydrogels [23, 24].

Importantly, preliminary studies using DPSCs have demonstrated the biocompatibility of silk fibroin [25] and the good mineralization potential of DPSCs seeded onto porous silk fibroin scaffolds in a mechanically dynamic environment provided by spinner flask bioreactors [26]. However, there is no information regarding the behaviour of SHED cultured in silk fibroin scaffolds, an important aspect to be considered in stem cell-based approaches. In this regard, the aim of this study was to investigate the biological effects of silk fibroin 3D scaffolds on stem cells from human exfoliated deciduous teeth (SHEDs) in terms of proliferation, morphological appearance, cell viability, and expression of mesenchymal stem cell markers.

Materials and methods

Silk fibroin processing

The silk fibroin processing and the production of biomaterials were performed using previously reported protocols [15, 16]. Briefly, silk fibroin was obtained from *Bombyx mori* silkworms reared in the sericulture facilities of IMIDA (Murcia, Spain). The cocoons of silkworms were sliced into 4–5 pieces and boiled in 0.02 M Na_2CO_3 for 30 min to remove glue-like sericin proteins. Then, raw silk fibroin was rinsed thoroughly with water and dried at room temperature (*r/t*) for 3 days. The extracted silk fibroin was dissolved in 9.3 M LiBr (across organics) for 3 h at 60 °C to generate a 20% w/v solution, which was after dialyzed against distilled water for 3 days (Snakeskin Dialysis Tubing 3.5 kDa MWCO, Thermo Scientific) with eight total changes of water. The resultant 7–8% w/v silk fibroin dissolution was recovered, filtered, and stored at 4 °C for no longer than 30 days (Fig. 1a). Silk fibroin sponges were made as a model of a three-dimensional (3D) scaffold for tissue engineering applications in combination with SHED. Polystyrene Petri dishes (9.5 cm in diameter) were used as moulds over which 15 g of sodium chloride particles (from 400 to 600 μm) were homogeneously distributed. Then, 8 mL of silk fibroin aqueous solution (7.5% w/v) was slowly dropped over the surface of the plate covered by the salt. The mixture was allowed to gel for 48 h at room temperature and annealed by incubation in absolute methanol for 1 h to increase the β-sheet content and strengthen the structure of the scaffold. After this step, a punch (15 mm in diameter) was used to make silk fibroin disks (2–3 mm thick) containing the salt, which was subsequently removed by transferring the disks to a beaker with distilled water that was changed 4 times per day for 2 days with stirring (Fig. 1b). Then, silk fibroin sponges were sterilized twice with 70% (v/v) ethanol for 10 min and rinsed three times with sterile ultrapure water. After these steps, they were washed with sterile PBS 1X and stored at 4 °C until use in the cell culture experiments (Fig. 1c). 3D scaffolds displayed a very rough surface mimetic with the bone extracellular matrix and highly porous. A lot of trabecular-like structures were observed during the microscopic visualization of the 3D scaffolds as well as a highly interconnected structure with pores, whose diameter ranged from 1 to 300 μm (average pore size 44.2 ± 55.3 μm).

Isolation and characterization of SHEDs

Normal exfoliated human deciduous teeth were collected from 6- to 9-year-old children ($n = 8$) in Murcia dental

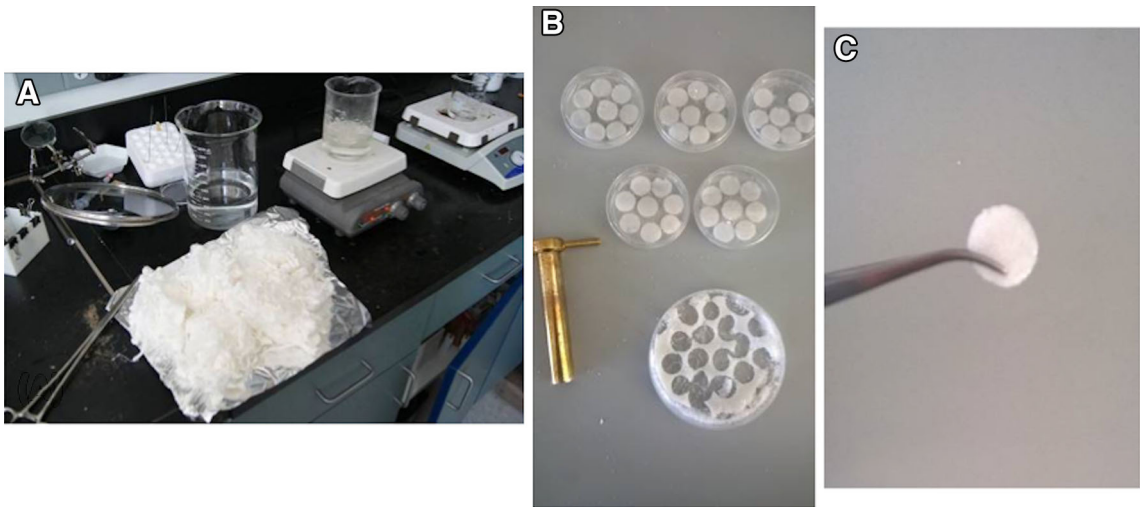

Fig. 1 Preparation of silk fibroin scaffolds. The cocoons of silkworms were sliced into 4–5 pieces and boiled in 0.02 M Na_2CO_3 for 30 min to remove glue-like sericin proteins (**a**); a punch was used to make silk fibroin disks (2–3 mm thick) containing the salt, which was subsequently removed by transferring the disks to a beaker with distilled water that was changed 4 times per day for 2 days with stirring (**b**); and macroscopic view of silk fibroin scaffold (**c**)

hospital (Spain) with written informed consent letters signed by their parents. The use of the teeth in this study was approved by the Bioethics Committee of the University of Murcia (1417/2016 UM). The teeth were mechanically broken with a pincer to expose the soft pulp tissue, which was minced in sterile glass Petri dish and digested with 3 mg/mL collagenase IV (Sigma-Aldrich) for 1 h at 37 °C. Cells obtained after enzymatic digestion were seeded at 1.5×10^5 cells/cm^2 in tissue culture flasks (Falcon®, Corning, New York, USA) in DMEM medium (Gibco Invitrogen) supplemented with penicillin/streptomycin (PAA laboratories, Pasching, Austria), L-glutamine (PAA laboratories), and 10% fetal bovine serum (FBS) (Gibco Invitrogen) (complete medium) and cultured at 37 °C and 5% CO_2 for 3 days. The adherent cells were grown to 80% confluence and were defined as passage zero (P0). For subsequent passaging, cells were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) (Gibco Invitrogen) and detached by incubating with 0.25% trypsin–EDTA solution (Gibco Invitrogen) for 2–5 min at 37 °C. Culture medium was added to inactivate the trypsin activity. Finally, SHEDs were centrifuged at 1200 rpm for 5 min and plated at a density of 5×10^3 cells/cm^2.

Before evaluating the silk fibroin scaffolds, SHEDs were characterized by immunofluorescence using specific antibodies for CD90 (1:250) (Becton–Dickinson, San Jose, CA, USA), CD73 (1:200) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), and CD105 (1:100) (Abcam, Cambridge, UK). After three washes with PBS, cells were incubated in the dark for 1 h with anti-mouse Alexa Fluor® 488-conjugated secondary antibody (1:500), (Molecular Probes, Invitrogen, Eugene, OR, USA). Microscope slides were mounted with anti-fade solution (Vecta shield mounting medium, Vector Laboratories, Hercules, CA, USA) containing 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, 0.2 mg/mL in PBS) and examined under a fluorescence microscope (Leica DMI 4000B, Wetzlar, Germany).

Scaffold seeding

Silk fibroin scaffolds were previously incubated with 100 µL of FBS for 1 h at 37 °C, and then seeded with SHEDs (5 scaffolds per group) at a density of 7×10^3 cells per scaffold in 500 µL of complete medium. To encourage the attachment of the SHEDs to the scaffolds, they were cultured for 90 min in a humidified incubator at 37 °C. Thereafter, 500 µL of culture medium per well (in a 48-well plate) was added and the scaffolds were incubated for an additional 24 h. For samples still exposed to SHEDs, the culture medium was replaced in every 3 days.

SHED proliferation on silk fibroin 3D scaffolds

SHED proliferation on silk fibroin 3D scaffolds was assessed 24, 48, 72, and 168 h after culture using the Alamar Blue assay. This method is based on a colorimetric endpoint resulting from an oxidation–reduction reaction from cellular metabolic activity/proliferation [27, 28]. Alcohol/UV-sterilized scaffolds were placed in a 6-well plate, preincubated with complete medium for 6 h, and seeded with 1×10^4 cells/well. After the desired time intervals, scaffolds were transferred to fresh wells in phenol red-free serum-free medium, and Alamar Blue was

added and incubated overnight. The optical density at 570 nm was measured in a microplate spectrophotometer (Perkin ElmerModel 2030 Explorer), with reference wavelength setting at 600 nm.

Flow cytometry analysis of expression of mesenchymal stem cell surface markers on SHEDs exposed to silk fibroin 3D scaffolds

The expression of mesenchymal stem cell surface markers was analysed on SHEDs cultured on silk fibroin 3D scaffolds by flow cytometry. Briefly, cells were seeded at a density of 3.0×10^4 cells/cm^2 on silk fibroin scaffolds and cultured for 72 h at 37 °C. Then, cells were detached using Accutase (Thermo Fisher Scientific) and washed by adding PBS and incubated in the dark at 4 °C for 30 min with fluorescence-conjugated specific monoclonal antibodies. Cell concentration, antibody dilution, and incubation conditions were used as recommended in the antibody datasheet. The fluorochrome-conjugated antibodies were CD90-PE, Anti-CD105-APC, and Anti-CD73-PE (Miltenyi Biotec), which are recommended by the International Society of Cellular Therapy (ISCT) as essential for confirming the mesenchymal phenotype of the cells [29, 30]. Lack of expression of the hematopoietic markers CD14, CD20, CD34, and CD45 was also analysed. Non-specific fluorescence was measured using specific isotype monoclonal antibodies. Sample acquisitions and analysis were done with a BD FACS Canto flow cytometer (BD Biosciences, San José, CA, USA) and Kaluza analysis software (Beckman Coulter, Inc., Brea, CA, USA), respectively.

Determination of cell viability (Annexin-V/7-AAD staining)

Cells were cultured on the silk fibroin scaffolds for up to 24, 72, and 168 h. After culture, cell viability was detected using double staining with FITC-conjugated Annexin-V and 7-AAD (Immunostep, Salamanca, Spain) according to the manufacturer's instructions. Stained cells were analysed using a flow cytometer (BD Biosciences, Heidelberg, Germany) and the data obtained were analysed using the FACSCanto II (Fluorescence Activated Cell Sorting) software.

Subsequently, the percentages of each population were calculated. All determinations were performed in triplicate.

Morphological analysis of SHEDs cultured on silk fibroin 3D scaffolds by SEM

Scanning electron microscopy (SEM) was used to analyse cell morphology on the silk fibroin 3D scaffolds. To remove possible toxic subproducts, the 15 scaffolds prepared for this experiment were first incubated at 37 °C in 24-well culture plates containing 1 mL of distilled water which was changed daily for 5 days. Then, SHEDs were directly seeded onto each disk at a density of 5×10^4 cells/mL. After 24, 72, and 168 h of culture, the samples seeded with SHEDs were removed from the culture wells, rinsed with PBS, and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer for 1.5 h at 4 °C. Then, they were rinsed again and post-fixed in osmium tetroxide for 1 h before being dehydrated in a series of graded ethanol solutions (30, 50, 70, and 90% v/v). Drying was performed by the critical-point method (CPDO2 Balzers Union). Finally, gold-coated specimens were examined by SEM.

Statistical analysis

Statistical analyses were performed using the SPSS version 15.0 statistical software (SPSS, Inc., Chicago, IL, USA). Comparisons of the groups were performed using one-way analysis of variance (ANOVA). Data were considered statistically significant at $p < 0.05$. When there were significant differences ($p < 0.05$), comparisons between the groups were further assessed with a Bonferroni multiple-comparison test.

Results

Isolation and characterization of SHEDs

Before evaluation of the behaviour of SHEDs cultured on silk fibroin 3D scaffolds, they were characterized by immunofluorescence techniques using specific antibodies for CD73, CD90, and CD105, which showed more than 95% positive expression for these markers (Fig. 2).

In vitro proliferation of SHEDs on silk fibroin 3D scaffolds

The proliferation of SHEDs cultured on silk fibroin scaffolds for up to 24, 48, and 72 h was measured by Alamar Blue assay (Fig. 3). SHEDs cultured in the absence of the scaffold were considered as a positive control (cells on surface), whereas scaffolds without cells served as negative control. The significant increase of Alamar blue reduction detected at 72 h compared with the reduction at 24 h indicated that SHEDs could survive and proliferate on the silk fibroin scaffolds ($p < 0.01$). At 168 h, cell proliferation on the scaffolds was similar to that seen for the positive control.

Expression of mesenchymal stem cell surface markers after culture on silk fibroin 3D scaffolds

To determine possible phenotypic changes of SHEDs after culture on the silk fibroin scaffolds, flow cytometry studies

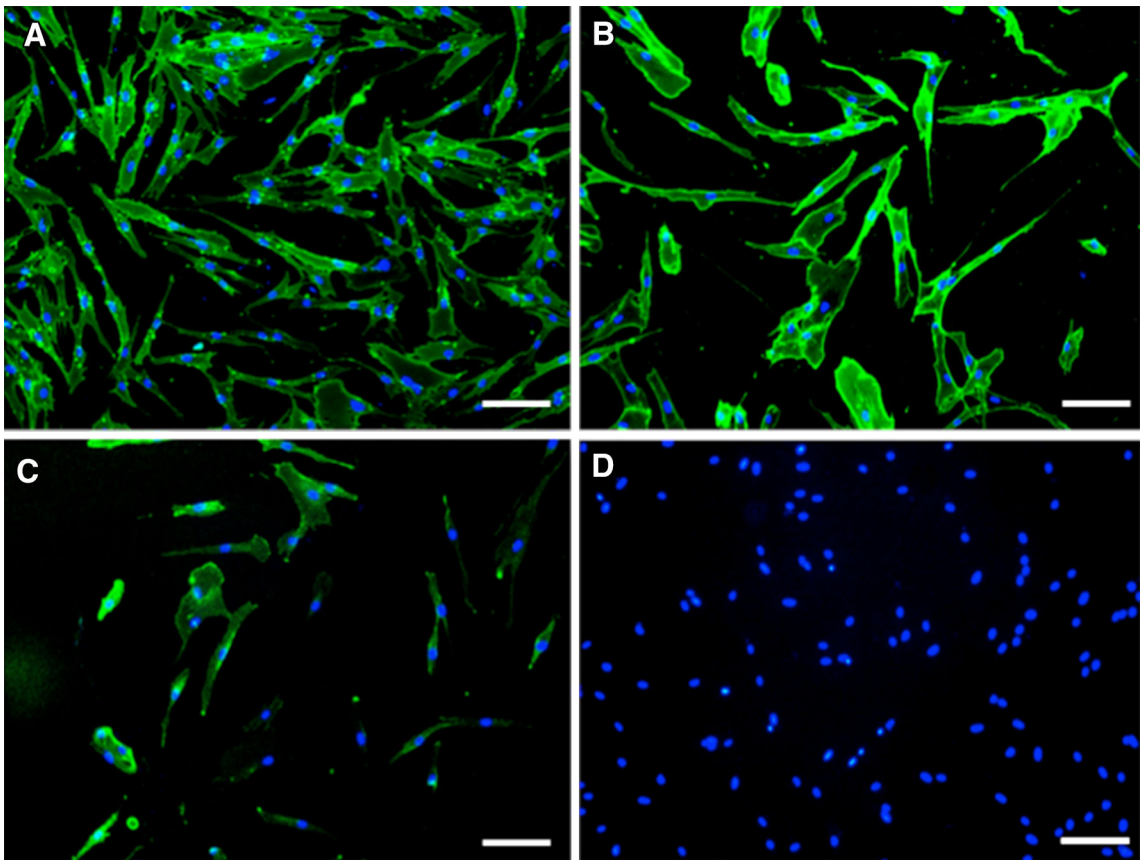

Fig. 2 Representative immunofluorescent images of stained SHEDs, using specific antibodies for CD73 (**a**), CD90 (**b**), CD105 (**c**), and an isotype negative control (**d**). Cell nuclei was labelled using DAPI (*blue*). Mesenchymal markers displayed a characteristic cytoplasmic pattern. *Scale bar* 100 µm

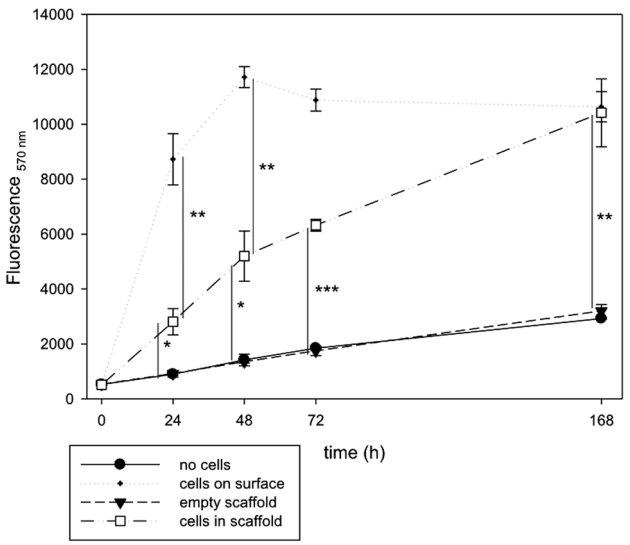

Fig. 3 Proliferation of SHEDs cultured on silk fibroin scaffolds for up to 24, 48, and 72 h was measured by Alamar Blue assay; at 168 h, cells on the scaffold or cells on the surface of plastic (positive control) pointed to a level of proliferation significantly higher than observed in the negative control or empty scaffold (**$p < 0.01$); (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

were carried out. In all the silk fibroin scaffolds tested, the mesenchymal stem cell surface molecules CD73, CD90, and CD105 were expressed at levels greater than 95%, whereas the expression of the hematopoietic markers CD34 and CD45 was lower than 5% (Fig. 4). Importantly, level of expression of all these markers was not significantly different compared to shown by SHEDs cultured on a plastic surface (control).

Cellular viability of SHEDs cultured on silk fibroin 3D scaffolds

Representative 2-dimensional dot plots of the distribution of live (Annexin-V^-/7-AAD^-), early apoptotic (Annexin-V^+/7-AAD^-), or late apoptotic/necrotic (Annexin-V^+/7-AAD^+ and Annexin-V^-/7-AAD^+) cells cultured on silk fibroin scaffolds are shown (Fig. 5). The percentages of live cells cultured for 168 h on silk fibroin scaffolds were higher than 93% and similar to the values obtained in cells cultured in the absence of the scaffold (0 h). These results suggested the biocompatibility of this silk fibroin 3D scaffold configuration.

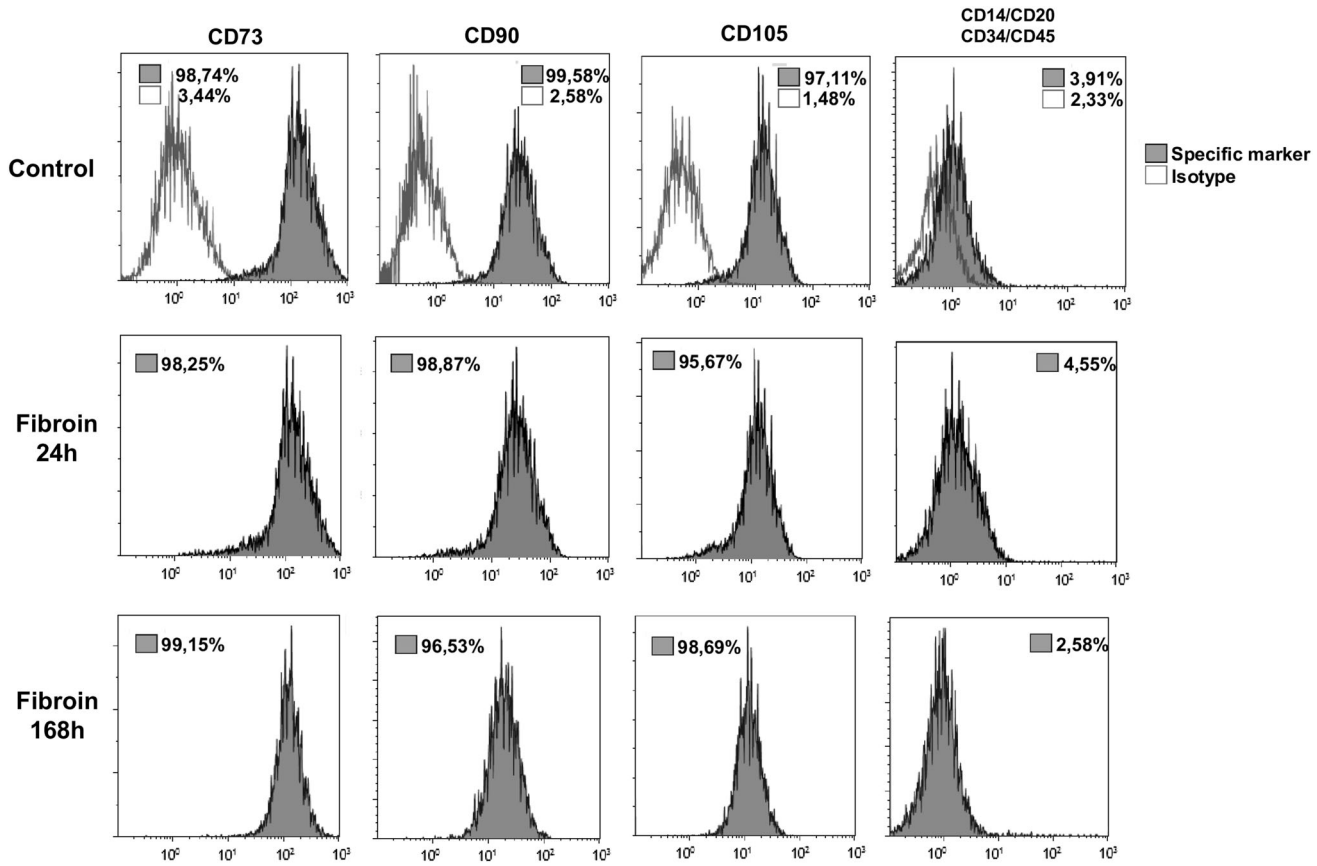

Fig. 4 Mesenchymal phenotype analysis of SHEDs after culture on silk fibroin scaffolds or plastic (*control*) by flow cytometry. After 24 and 168 h of culture, cells were detached and labelled with fluorescence-conjugated specific antibodies for the mesenchymal surface markers CD73, CD90, and CD105 and the hematopoietic markers CD14, CD20, CD34, and CD45. *Insert numbers* represent the percentage of viable positive cells. Histograms show representative flow cytometry results obtained after three independent experiments

SHED attachment to silk fibroin 3D scaffolds

The morphology of SHEDs and their adhesion to the surface of the silk fibroin 3D scaffold after culture for 24, 72, and 168 h are shown (Fig. 6). The results showed that a small amount of SHEDs were evenly attached to the silk fibroin scaffolds after 24 and 72 h of culture followed by a gradual increase in cell attachment up to 168 h. Importantly, SHEDs proliferated and covered the scaffold, adopting a spindle, polygonal, and flattened morphology, and showing multiple prolongations that anchored the cells to the biomaterial surface (Fig. 6b, c).

Discussion

The fabrication, composition, mechanical properties, and biocompatibility of scaffolds are important considerations, because these factors determine the capability of an engineered dental pulp to stimulate dynamic in vivo repairs [31]. Therefore, for endodontic purposes, scaffolds must provide an adequate porous structure with interconnected pores to allow pulp stem or progenitor cells to migrate and/or organize within a three-dimensional structure, be capable of supporting cell organization, vascularization, and to provide necessary nutritional support [32].

Fibroin-based biomaterials have been previously studied for several in vitro and preclinical applications [20–22], but they have never been tested in conjunction with mesenchymal stem cells isolated from human exfoliated deciduous teeth (SHED). SHEDs are an easily accessible source of multipotent cell populations [33]. Thus, the goal of this study was to investigate the effects of silk fibroin 3D scaffolds on mesenchymal stem cell phenotype, adhesion, proliferation rate, and viability of SHEDs. The biocompatibility of scaffolds is a pre-requisite for generating cell-biomaterial constructs and for their successful clinical application, and the initial attachment of SHEDs to the scaffolds is a critical step for achieving long-term stability and differentiation [3]. Thus, the scaffold's capacity for SHED proliferation was confirmed by following their metabolic activity using the Alamar Blue assay normalized to the amount of cells in

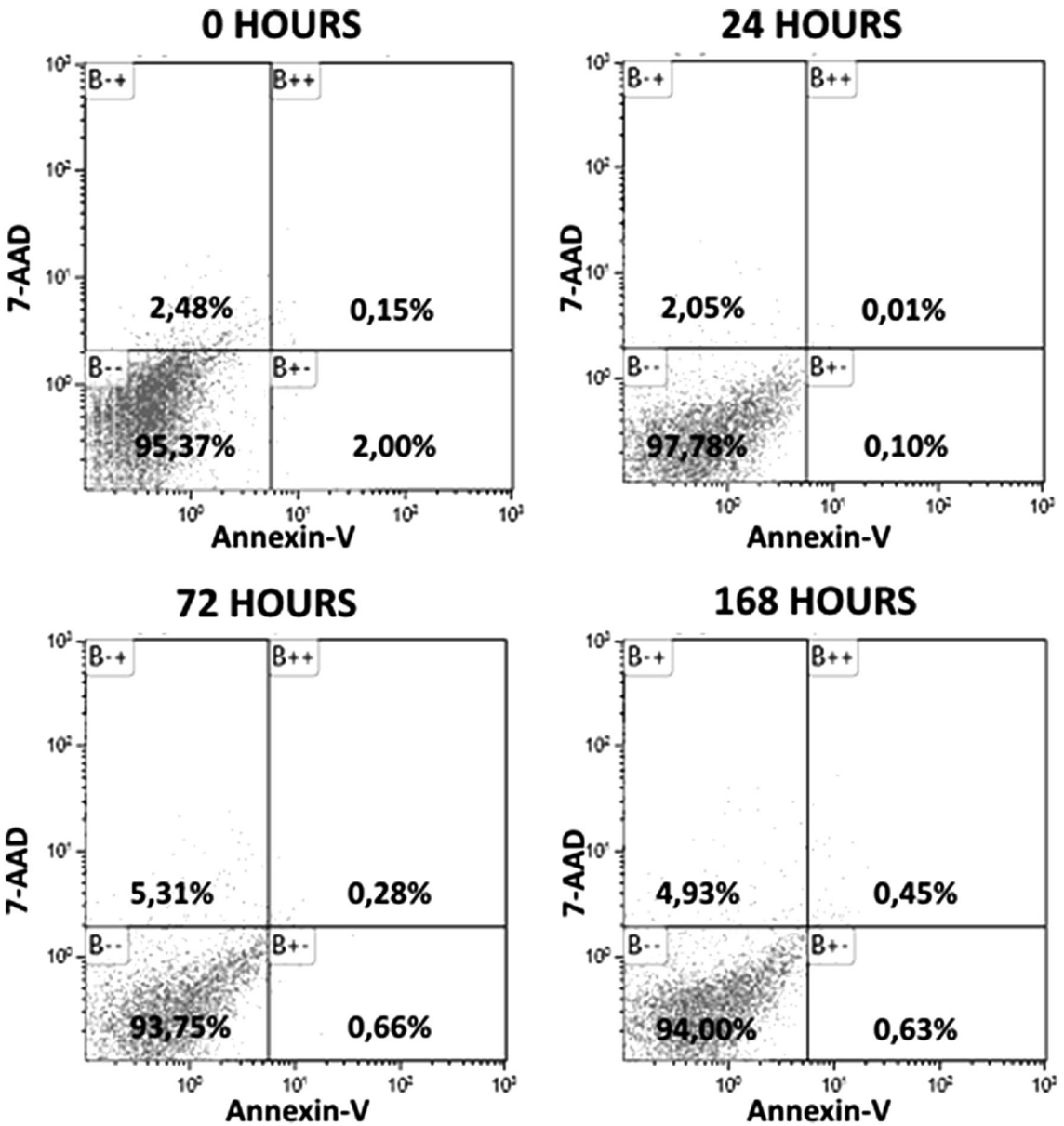

Fig. 5 SHEDs were cultured on silk fibroin scaffolds for 0 (plastic), 24, 72, and 168 h. After, SHEDs were labelled with Annexin-V-FITC and 7-AAD and analysed by flow cytometry. *Insert numbers* in the different quadrants represent the percentages of live (Annexin-V-/7-AAD-), early apoptotic (Annexin-V+/7-AAD-), or late apoptotic and necrotic (Annexin-V+/7-AAD+ and Annexin-V-/7-AAD+) cells. *Dot plots* show representative flow cytometry results obtained after three independent experiments

the respective sample. Cell viability is an important parameter in the evaluation of scaffold capacity to support the initial cell proliferation [34]. Importantly, the cell viability observed on the silk fibroin scaffolds was comparable to that obtained on tissue culture plates in the absence of the biomaterial (Fig. 5). A possible reason for this finding could be the optimal initial cell adhesion to the surface of the scaffold, allowing further attachment and cell spreading (Fig. 6). Previous studies using MTT assays demonstrated that cell viability and proliferation were promoted by silk fibroin [35, 36].

In addition, we investigated the cell death in SHEDs cultured on the silk fibroin biomaterials by measuring the binding of Annexin-V and 7-AAD, and two colour flow cytometry analyses commonly used to determine the cell apoptosis stage. This method allows three cell populations to be differentiated: live (Annexin-V^-/7-AAD^-), early apoptotic (Annexin-V^+/7-AAD^-), and both late and necrotic (Annexin-V^+/7-AAD^+ and Annexin-V^-/7-AAD^+). Our results point to the suitable biocompatibility of this silk fibroin configuration, which displayed more than 93% of viable cells after 7 days of culture. In a

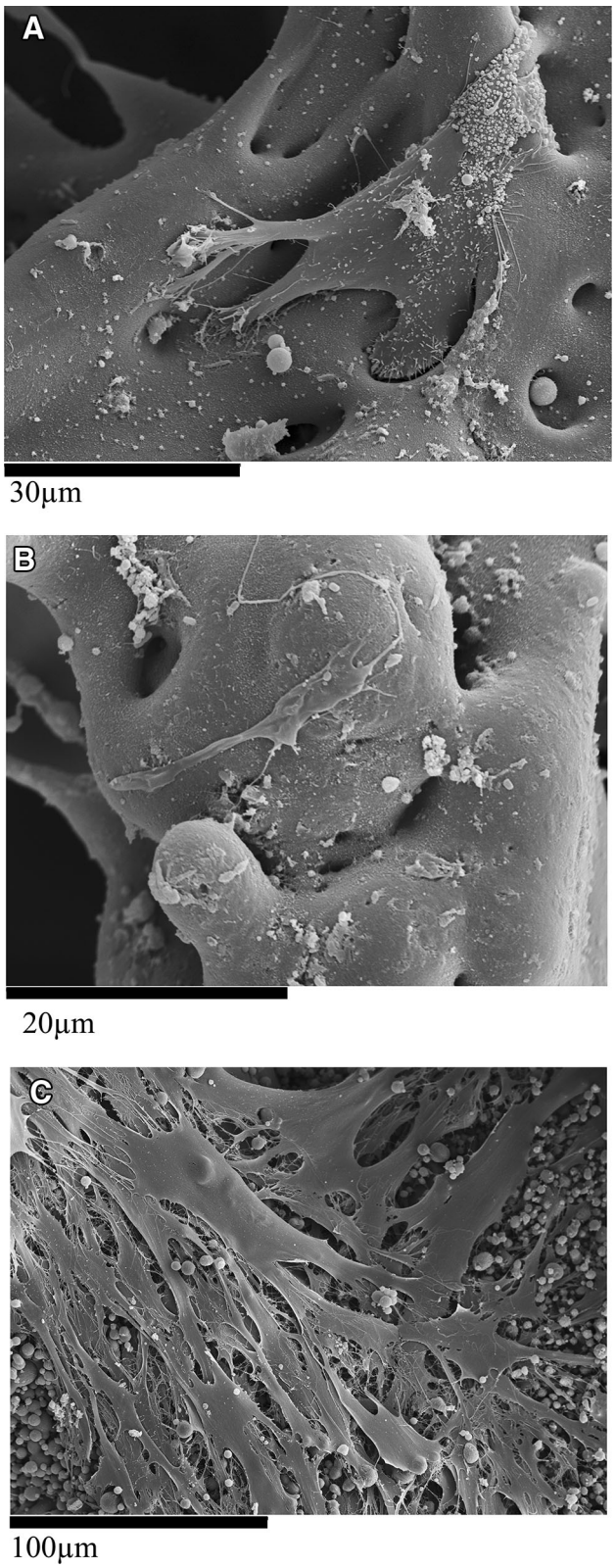

Fig. 6 SEM photomicrographs of silk fibroin 3D scaffold after SHED seeding. SHEDs were seeded on silk fibroin scaffolds and cultured for 24 h, (**a**) for 72 h (**b**), or for 168 h (**c**). *Scale bar* 100 µm

previous study using silk fibroin films, we determined that SHEDs cultured on silk fibroin, graphene oxide, or a combination graphene oxide/silk fibroin (ratio 1:3) showed only slight initial cell death, with more than 85% of cells viable after 7 days of culture on all biomaterials [22]. Therefore, silk fibroin 3D scaffolds seem to improve the number of living cells, an important pre-requisite for subsequent regenerative endodontics.

In addition, the expression of mesenchymal stem cell markers is an important aspect to be analysed in regenerative medicine [37]. The International Society for Cellular Therapy states that multipotent mesenchymal stromal cells must express CD105, CD73, and CD90 and should be devoid of the expression of hematopoietic markers such as CD45, CD34, CD14, or CD11b [29, 30]. Therefore, to evaluate the possible changes in the expression of mesenchymal surface markers, we characterized their surface molecule expression pattern by flow cytometry (Fig. 4). Culture of SHEDs on fibroin scaffold did not significantly alter the level of expression of CD73, CD90, or CD105 after 24 or 168 h compared to expression levels displayed by SHEDs cultured on tissue culture plates in the absence of the biomaterial. Thus, the scaffolds employed in this study were able to maintain the mesenchymal phenotype of SHEDs.

Scanning electron microscopy (SEM) is a useful tool to examine the morphology and cell attachment of primary cultured cells seeded on certain biomaterials [38]. Therefore, cell adhesion and attachment were analysed by SEM in our study. The morphology of these cells was considered to represent a typical mesenchymal morphology with numerous projections. At the first timepoint, cell adhesion was lower than the following timepoint (72 h). After 168 h of culture, large amounts of SHEDs adhered to the scaffolds were observed, appearing as multilayered cultures (Fig. 6). Moreover, numerous filamentous extracellular matrix components were detected on the surface of the cells. These findings suggest that there was a higher degree of cell growth, which correlated with the higher number of cells measured at this time using the Alamar blue assay. These results agreed with previous reports that confirmed good biocompatibility in terms of cell attachment and proliferation on different silk fibroin-based biomaterials [35, 39].

Conclusions

The findings from the current study suggest that silk fibroin 3D scaffolds had a favourable effect on the biological responses of SHEDs. Further in vivo investigations are required to confirm these results.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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