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EFFECTS OF 3D CHITOSAN NANOFIBROUS SCAFFOLD AND TGFB1 ON DENTAL PULP STEM CELLS (DPSCS) DIFFERENTIATION

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ABSTRACT

Objective: The goal of this work is to investigate and analyze the impact of chitosan (CHT) nanofibrous scaffold in the presence of recombinant transforming growth factor-beta 1 (TGF-β1) growth factor on dental pulp stem cells (DPSCs) odontoblastic differentiation for dentin pulp regeneration.

Materials: Dental pulp stem cells (DPSCs) were seeded as a control 2d culture or as a 3d culture on CHT scaffold alone or on CHT scaffold with TGF-β1 growth factor treatment for 21 days. Scanning electron microscopy (SEM) was used to evaluate the morphological features of DPSCs on the CHT scaffold and in the presence of TGF-β1. MTT cytotoxicity assay was applied to test the cell viability on the scaffold. Moreover, Alizarin Red mineralization test was used to detect the calcium deposits in cells seeded as 2d with no treatment or 3d with CHT scaffold and TGF-β1.

Results: Scanning electron microscopy showed DPSCs were able to attach to and multiply on a CHT nanofibrous scaffold surface with more cells extended and long cytoplasmic prolongations with TGF-β1 treatment. CHT scaffold showed a significant increase in the cell viability compared to the control group after 3, 6, and 9 days ($p < 0.05$). Calcium deposition was highly observed in DPSCs seeded on CHT scaffold alone or with TGF-β1 treatment than the control group.

Conclusion: Growth factor TGF-β1 played a pivotal role in the differentiation of DPSCs in vitro. It can stimulate odontoblastic differentiation of DPSCs in 3D culture with CHT nanofibrous scaffolds.

KEYWORD: Chitosan, DPSC, TGF-β, Tissue engineering

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INTRODUCTION

The development and manipulation of cells, tissues, or organs with the purpose of restoring, maintaining, or supporting the function of injured tissues is performed through the multidisciplinary field of research known as tissue engineering[1] Scaffolds are biological substitutes that facilitate the development and repair of tissues by acting as a temporary extracellular matrix (ECM) made of a porous three-dimensional structure[2,3]. For structures to be created that serve various purposes in tissue regeneration, in-depth research on the selection of a biomaterial or biocomposite that forms the scaffolds is essential [4]. Recently with the development of tissue engineering, regenerative endodontics has arisen as a new dental specialty.

Stem cells, bioactive signaling molecules, and biocompatible scaffolds are the three key components that need to be interact together for successful endodontic tissue regeneration [5]. The discovery of human dental pulp stem cells (hDPSCs), which have the capability to develop into dentin-like odontoblastic cell lineages, has made the therapeutic possibility of pulp regeneration a more realistic prospect [6]. In addition, a number of studies have demonstrated that DPSCs hold a significant potential for the engineering of dental tissues as a result of their capacity to differentiate into functional odontoblasts in vitro and to form dentin-pulp complex structures in vivo when transplanted in conjunction with scaffolds [7] Hence, employing these cells in conjunction with an appropriate scaffold may have the potential to make a significant contribution to the field of regenerative endodontics.

A regenerative procedure that is based on a scaffold utilizes a constructed substrate in order to entice a population of stem cells to a particular site. The scaffold used in regenerative endodontics must have tissue-forming properties and be able to express such properties at the appropriate time and concentration [8] They are chosen based on the characteristics of the interactions between cells and their supports. The attachment, proliferation, and differentiation of cells on a scaffold matrix are all influenced by this interaction.

Polymers have a great deal of design flexibility due to the fact that both their composition and their structure can be tailored to the specific needs. As a result, polymers have been the subject of extensive research in a variety of tissue engineering applications, including tooth tissue engineering. Polycaprolactone, also known as PCL, is one example of the types of polymers that are being used for the regeneration of both soft and hard tissues. Nevertheless, its limited cell attachment and proliferation as well as its very hydrophobic nature prevent its widespread application. Therefore, combining PCL with other hydrophilic material is suggested [9].

Chitosan (CHT) is utilized in the field of tissue engineering as a scaffold due to its biodegradability, biocompatibility, osteoinductivity and the products of its decomposition are not toxic or allergic. It is possible for the cytocompatibility of chitosan to change depending on the characteristics of the target cells and the surface of the scaffold. Chitosan was first considered as a potential scaffold for dental pulp cells after previous studies showed that chitosan monomers could assist in the regeneration of dental pulp wounds [10].

Although the use of polymeric-based scaffolds has been met with some success, improving the quality of those scaffolds by incorporating temporally-controlled bioactive molecules to promote new tissue formation is recommended [8]. Many studies have looked into pulp and dentin regeneration techniques, utilizing a wide variety of materials and bioactive molecules (BM) to promote and sustain new tissue growth [11]. According to recent research, one of the most significant growth factors in dentin-pulp regeneration has been identified as a member of the transforming growth factor (TGF)-b superfamily. TGF-s are expressed by maturing odontoblasts, which leads to their sequestration in

the dentin matrix [11]. Earlier studies indicated that dentin matrices store TGF-s, and alterations in the matrices due to caries or trauma could trigger the production of a reservoir of TGF-s present in the matrix. [12, 13].

Based on the above mentioned, it seems like it could be a good concept for future applications in managing damaged pulp-dentin complex to combine DPSCs with appropriate scaffold and suitable growth factor. Therefore, the purpose of the present study was to analyze the impact of CHT scaffold in the presence of TGFβ-1 growth factor for dentin pulp regeneration.

MATERIALS AND METHODS

Preparation of Chitosan Scaffold

Two aqueous polymeric solutions were prepared; 7.5% w/v of PEO, and 3% w/v of CHT using 1% acetic acid at room temperature. Then, the electrospinning solution was prepared by mixing equal volumes of the polymer solutions. Parameters for electrospinning were optimised at a flow rate of 0.6 mL/h, an applied voltage of 15 kV, and a distance of 15 cm between the spinneret tip and the ground stationary collector. The sandwich structure was constructed by the depositing of the CHT/PEO nanofibers layer on the PCL NFs layer from both sides. The process was done in conditions of 30–35% humidity. Then, to get rid of the extra solvent, the pieces were dried in a desiccator for 48 hours [14] . The study was approved by the Faculty of Dentistry's Ethical Committee, Mansoura University, Egypt (m17091019).

Dental stem cells culturing

The Poietics™ Dental pulp stem cells (DPSCs) was supported from the LONZA company. The cell line was extracted from adult third molars that were taken after a donor's wisdom teeth removed. DPSCs were revival and cultured aseptically in accordance to LONZA guidelines. For culture, we employed the DPSC BulletKit™ Medium (PT-3005, Lonza), which contains the essential nutrients for human DPSC development as well as the DPSC basal media (Cat.no. PT-3927, Lonza, Walkersville, MD, USA). The required supplements are included in the Single Quots TM Kit (Cat. No. PT-4516, Lonza, Walkersville, MD, USA), which also includes L-glutamine, MCGS, GA-1000, and ascorbic acid (Cat.no. PT-4517Q). Once the cells had reached 80% confluence, they were passaged using Trypsin/EDTA (Cat.no. CC-3232, Lonza, Walkersville, MD, USA). Cell washing was done with phosphate buffer saline (PBS) (Catalog no. 10010023, GibcoTM, Thermo Scientific, Life Technologies Corp, USA) [10].

Scan electron microscopy (SEM) analysis

To test cell adhesion on CHT nanofibers scaffolds, 5x 10^4 cell were seeded on scaffolds in 24 well plate as following; Group I (DPSCs+CHT): DPSCs were cultured on a CHT scaffold. Group II (DPSCs+CHT+TGFβ1): 5 ng/ml of human recombinant TGF-1β was added to DPSCs that cultured on a CHT scaffold and treated with. After 7 days, samples were rinsed by PBS then fixed with glutaraldehyde solution (2.5 %) for two hours at 4°C. Then were dried using hexamethyldisilane, after dehydration in a series of ethanol. SEM [JEOL JSEM-6510LV; JEOL, Tokyo, Japan] at faculty of Agriculture, Mansoura University was used to evaluate the scaffolds for cell adhesion after gold coating.

Cell viability assay

The cell viability experiment was performed using the Methyl-Thiazoltetrazolium (MTT) (MTT assay kit; Abcam). A 5x10^3 cells per well were seeded on PCL and CHT for 3, 6, and 9 in a 24 well plate. Then, the cells were incubated in 5% CO_2 and 37°C to evaluate cell viability at each time point. After each time point, 5mg/mL of methylthiazolydiphenyl-tetrazoliumbromide (MTT) was added to the cells (Sigma, St. Louis, MO) and incubated for 4 hours. The supernatants were decanted, and the formazan particles were dissolved with 100 μL/well DMSO

(Sigma) for 10 minutes. It caused the insoluble Formosan crystal to dissolve, resulting in a purple color. Spectrophotometric analysis was carried out at wavelength of 570 nm using Infinite F50 plate reader (Tecan Trading AG, Switzerland) to detect the quantity of formazan.

Alizarin Red mineralization assay

A 6-well plate containing $2x10^5$ DPSCs were seeded on CHT scaffolds then incubated at 37°C and 5% CO_2. After differentiation, the DPSCs were subjected to the Alizarin red mineralization test (Cat. no. 2003999, EMD Millipore Corp., USA) to detect the calcium deposits. The Alizarin Red S staining solution was generated according to the manufacturer's instructions. The cells were rinsed with Dulbecco's PBS, w/o Ca++/Mg++ and then submerged with 10% formalin. After 30 minutes, the formalin was removed, and the cells were washed with distilled water. In an appropriate quantity, the Alizarin Red S staining solution was added to the cells then they were incubated for 45 minutes at room temperature in the dark. The Alizarin Red S staining solution was removed, and then the cells were washed four times with 1 ml distilled water. The cells were covered by PBS, after water removing. Cells with extracellular calcium deposits were somewhat reddish, but cells without extracellular calcium deposits showed brilliant orange red. Elisa reader at 570 nm using Infinite F50 plate reader (Tecan Trading AG, Switzerland) was used to quantify calcium deposits.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyse the data, followed by a least significant difference (LSD) analysis to determine the significance between the groups. The data were expressed in mean and standard deviation (as mean SD). The results were considered significant when $p \leq 0.05$.

RESULTS

DPSC culture

Dental pulp stem cells (DPSCs) were cultured in BulletKit™ media at 37°c 5% CO2, the cells attached to the tissue culture flask and the spindle shape started to appear in the cells. While after 2 weeks, the cells extended and increased with confluence 80% (Fig. 1).

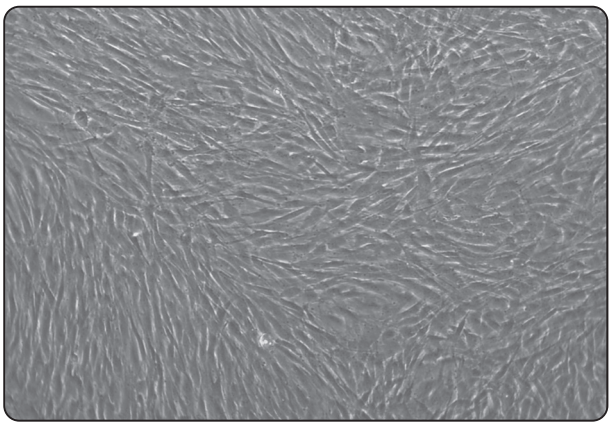

Fig. (1) Microscope images of DPSCs showing the cells spindle shape after 2 weeks, X:100.

Cellular viability after culturing CHT nanofiber for 3, 6 and 9 days

The viability of cells seeded on CHT scaffolds were detected after 3, 6, and 9 days (Table 1 and Fig.2). Compared with the 2d negative control cells, the cells seeded on CHT manifested a significant increase in the cell viability compared to the control cells ($p < 0.001$), suggesting that CHT scaffold has a high compatibility and cell attachment.

TABLE (1): The optical density of DPSCs attachment on CHT scaffolds for 3, 6 and 9 days

	NC	CHT
3DAYS	0.167±0.01	0.27±0.06*
6 DAYS	0.225±0.01	0.324±0.05*
9 DAYS	0.273±0.04	0.41±0.04*

Significant difference compared to the control group at p* ≤0.05 NC: negative control, CHT; Chitosan scaffold.

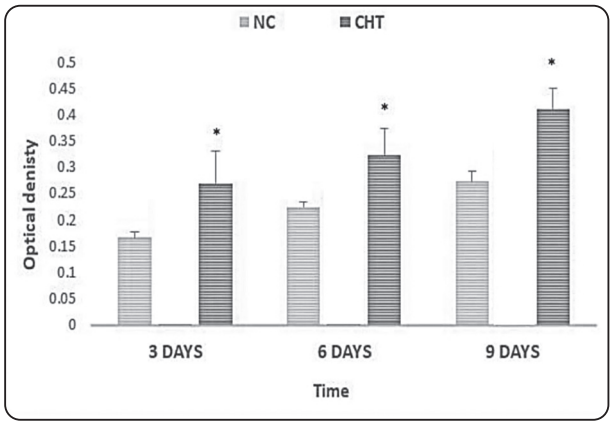

Fig. (2) Optical density of DPSCs attachment on CHT scaffold for 3, 6 and 9 days.

Scanning electron observation of CHT nanofibers scaffold with DPSCs

Scanning electron microscopy (SEM) was used to evaluate the surface morphology and DPSC density on CHT scaffold with/without TGFβ1 treatment seven days after seeding. DPSCs displayed cortical cell morphologies and were able to adhere and spread uniformly on the surface of CHT with good cytocompatibility and interaction between stem cells and CHT nanofibrous scaffolds (Fig. 3a). Moreover, treatment with TGFβ1 showed more expanded cells with elongated cytoplasm (Fig. 3b).

Alizarin Red assay for CHT with DPSCs

The capability of CHT nanofibrous scaffold with TGFβ1 to induce DPSCs odontoblastic differentiation was detected by Alizarin red staining. Compared to the negative control group, different pattern of calcium calcifications was observed in all treated groups (Fig. 3). As shown in Table 2; Figure. 4, mean absorbance of alizarin red stain was measured in all treated groups. DPSCs+CHT+TGFβ1 groups illustrated a significant increase in the calcium concentration compared to the control, and DPSCs+CHT group ($p < 0.05$).

TABLE (2): Alizarin red absorbance after culturing of DPSCs on CHT scaffolds for 21 days

Groups	Optical Density
NC	1.03±0.06
DPSCs+CHT	1.58±0.04[a]
DPSCs+CHT+TGFβ1	2.23±0.07[ab]

Significant difference compared to corresponding acontrol, and bDPSCs+CHT, at p≤0.05. NC: negative control, CHT; Chitosan scaffold, DPSCs: dental pulp stem cells.

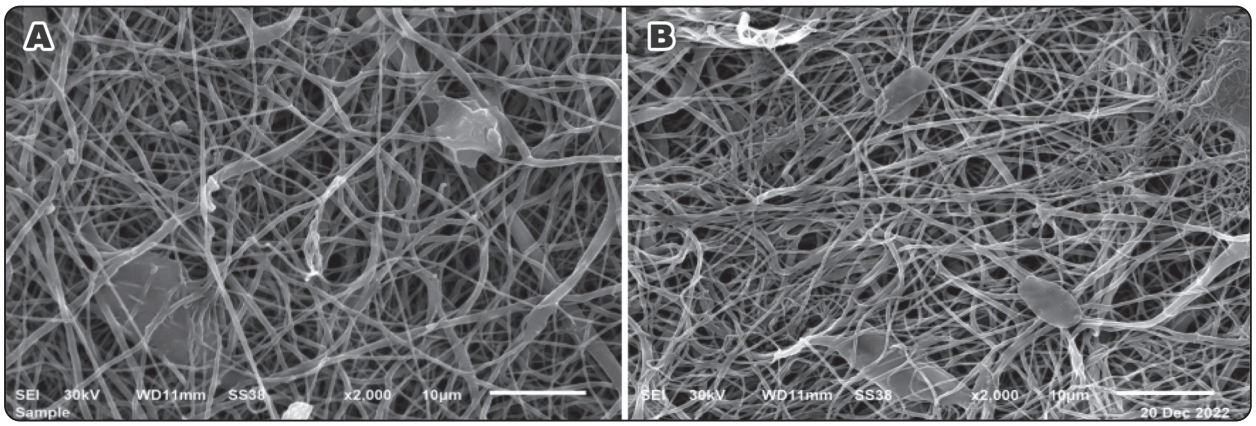

Fig. (3) Scanning electron microscopy for CHT nanofibrous scaffold showing; a) DPSCs+ CHT and d) DPSCs+ CHT+ TGFβ1.

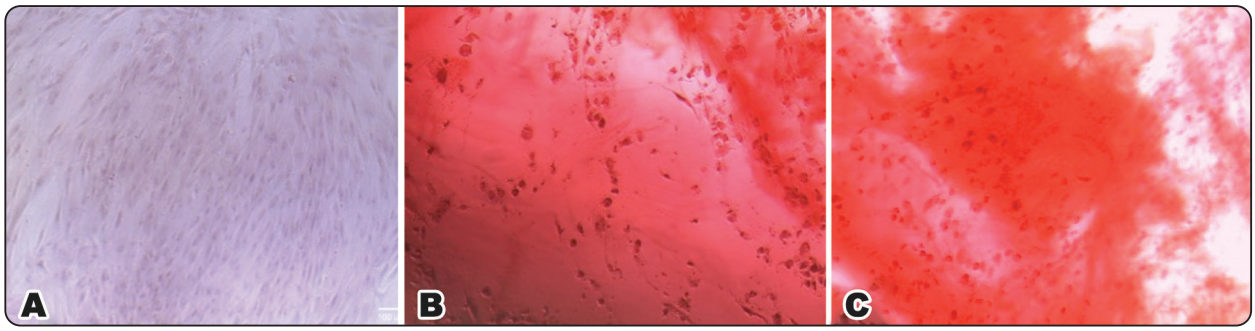

Fig. (4) Photomicrograph of Alizarin S Red staining at 21 days showing; a) negative control, B) DPSCs+ CHT, and C) DPSCs+ CHT +TGFβ1.

DISCUSSION

Endodontic specialists face a complicated clinical scenario when addressing pulp-dentin injury in immature teeth [15, 16] Although apexification is the therapy in these situations, it has some drawbacks. The barriers can be delicate to work with, and there is no guarantee that the root will continue to grow and close at the apex normally [17].

Regenerative endodontic procedures (REPs) are a relatively new form of dental treatment. REPs try to repair the dentin-pulp complex by regenerating lost connective tissue, blood vessels, and nerve endings after pulp damage [18, 19].

Stem cells, three-dimensional scaffolds, and growth factors are the cornerstones of tissue engineering that REPs are expected to contribute in order to achieve a positive outcome [20]. The discovery of DPSC has opened up a new area of study in dentistry, with potential applications in tissue regeneration and replacement in the dental tissues [21].

Chitosan (CHT) is a naturally occurring mucopolysaccharide that possesses the ideal and fundamental qualities necessary for tissue engineering. Chitin is the source of a promising biomaterial with desirable properties for medical use, including low immunogenicity, biodegradability, biocompatibility, nontoxicity, osteoinductivity, hemostasis, prolonged drug administration, and bacteriostaticity [22].

Some of the chemotactic factors that have been studied are TGF-1, it has been reported that microspheres loaded with TGF-β1 increase cell proliferation and migration in the dental pulp [21].

In the present study, CHT nanofibrous scaffolds was fabricated through an electrospinning method and evaluated for dental regenerative applications.

In this study, SEM was used to analyze the adhesion of cells on the scaffold surface. Cell showed good spreading on CHT scaffold treated with TGF-β1, and cells were placed in parallel and perpendicularly aligned to the cell nodule. This may provide good evidence about the synergic effect of TGF-β1 which can promote the functional differentiation of DPSCs in 3d culture in vitro. These morphological findings are in accordance with previous studies demonstrated the effect of TGF-β1 on dental cells differentiation to odontoblast like-cells in 3d cultures [23, 24].

The effect of CHT nanofibrous scaffolds on cell viability was compared to 2d cultured cells. Cells seeded on CHT scaffold showed a significant increase in the cell viability compared to the control cells after 3, 6, and 9 days, suggesting that CHT scaffold exhibit a high level of compatibility and cell attachment, as porous electrospun nanofibrous bioscaffolds allow enough 3d void space for the adhesion and proliferation of DPSCs and delivery oxygen and nutrients through the interconnected pores to the cells on the bioscaffolds. This finding

is in the same line with previous studies pointed the effect of the hydrophilic characteristics of CHT on cell attachment [25, 26].

Alizarin red assay was performed on DPSCs cells after 21 days. Cells treated with TGF-β1 showed mineral deposition compared to the untreated cells, while the higher intensity of red orange color on CHT nanofibrous scaffold surface treated with TGF-β1 compared to CHT scaffold without TGF-β1 treatment indicated the more mineral deposition and odontogenic differentiation of DPSCs. These findings are supported by previous studies indicated the role of CHT nanofibrous scaffold in osteogenic differentiation [26]. Moreover, effect of TGF-β1 on odontogenic differentiation was previously studied by Vahabi, Torshabi [27]

CONCLUSION

TGF-β1with 3D porous CHT scaffolds might act synergistically on the morphological and functional differentiation of DPSCs.This study provides useful information about the effects of signaling molecules on DPSCs based tissue engineering and pulp regeneration.

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