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Human dental pulp stem cells differentiation into odontoblast and osteoblast-like cells on scaffolds contain bioactive materials

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Abstract

Epigenetic change has been found to play an important role in cell differentiation and regulation and the dental pulp stem cell in tissue engineering is gaining attention due to the ability of cells to differentiate into odontoblast and other cells. This study evaluated the influence of poly L- lactic acid with hydroxyapatite-coated with polyaniline scaffold (PLLA/HA/PANI) on dental pulp stem cell (DPSC) proliferation and differentiation. After scaffold preparation and DPSCs seeding, the cells proliferation and differentiation were evaluated by immunocytochemistry assay and cell viability was measured by cytotoxicity / MTT assay. The results showed (PLLA/HA/PANI) scaffold facilitates DPSC proliferation and differentiation with gene expression. This finding underscores the promise of this biomaterial combination as a scaffold for dental tissue regeneration and application.

Keywords: Poly L- lactic acid, Dental pulp stem cell, Scaffold, Hydroxyapatite, Polyaniline.

1. Introduction

Over the past decade, the field of tissue engineering has experienced substantial growth. This is attributed to three fundamental elements crucial for successful tissue engineering: scaffolds, growth factors, and stem cells [1]. Scaffolds play a vital role in providing structural support and a framework for cell attachment, proliferation, and differentiation. Diverse materials, including polymers like PLLA, have been explored to create scaffolds that mimic the natural extracellular matrix, promoting tissue regeneration. Stem cells offer remarkable potential in tissue engineering. Their multipotency and ability to differentiate into various cell types make them valuable for regenerating specific tissues [1, 2].

Poly(L-lactic acid) (PLLA) scaffolds have gained significant attention in tissue engineering due to their biocompatibility and biodegradability 1. PLLA scaffolds have been widely used in various tissue engineering applications, including bone tissue engineering and dental tissue engineering [3]. The mechanical properties of PLLA nanofiber scaffolds can be enhanced by the incorporation of hydroxyapatite (HA) onto PLLA scaffolds has been shown to improve their bioactivity, making them suitable for bone tissue engineering [4]. There are several types of conductive polymers such as polyacetylene, polypyrene and polyaniline, and each of these polymers contains a unique conductivity system. Among conductive polymers, using polyaniline is increasing nowadays in tissue regeneration and engineering. When any type of scaffold is coated with polyaniline all the cells communicate with each other through electrical signals [5].

Dental pulp stem cells (DPSCs) have emerged as a promising source for tissue engineering due to their multipotency and angiogenic capacity 1. DPSCs have been investigated for their potential in dental pulp tissue engineering and regenerative endodontics [1, 6]. Additionally, DPSCs have been found to be suitable for applications in bone tissue engineering [2].

The use of DPSCs in conjunction with PLLA scaffolds holds great promise for tissue engineering applications. The combination of DPSCs with PLLA scaffolds can potentially lead to the development of advanced strategies for dental pulp and bone tissue regeneration. The biocompatibility of PLLA scaffolds makes them an ideal substrate for supporting the growth and differentiation of DPSCs, while the mechanical reinforcement provided by HA enhances the structural integrity of the scaffolds. These advancements in scaffold design and stem cell utilization represent

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a significant step forward in the field of tissue engineering.

The aim of this study to evaluate the influence of PLLA scaffold's pore on the proliferation and differentiation of DPSC by Cytotoxicity/ MTT assay and immunocytochemistry assay.

2. Material and Methods

Materials used for scaffolds preparation and formation were purchased from Sigma Aldrich company; which are Poly-L-lactic acid (PLLA) (99% purity), hydroxyapatite nanopowder (>50 nm, 90% purity) and polyaniline (99.5% purity).

2.1. Scaffold preparation

preparation of poly L. lactic acid (PLLA) nanofiber scaffold with Hydroxyapatite by elecrtospan Firstly prepare of polymer solution with hydroxyapatite (HA) to ensure good dispersion of hydroxyapatite (HA) in the polymer matrix before electrospinning, the PLLA and HA powder were dried in an oven at 60 C for 24 hours. then 12 g of PLLA dissolved 100 mL of chloroform at room temperature for 3 hours under magnetic stirring, then 0.1 g nanosized (HA) powder with particle size 50nm was dispersed in the polymer solution under continuous stirring for 3 hours at room temperature, after this, the two solutions were mixed and stirred for 3 more hours [7]. Then the electrospinning unit (full option lab 2 ESI -II from Bagdad Center (BPC) that is located in Baghdad -Iraq) was used to prepare elecrtospun fibers. The electrospinning unit consists of two syringes connected to a -21-gauge needle via an extension tube. the polymer solution was loaded into 5 mL/plastic ring and pumped at speed of 0.5 ml/ hr. The rotating drum collector was located 25 mm from the needle tip, covered with aluminum foil for deposition of fiber, this is near needle tip to collector distance 25 mm. The resulting nano-fibers were collected on the aluminum foils and kept at room temperature under ambient conditions [8].

2.2. Coating PLLA nanofibers with polyaniline

A 1 molar solution of HCL was prepared by dissolving an appropriate amount of HCl in 100 ml of distilled water. Subsequently, 0.01 mol of polyaniline was added to the prepared HCl solution. The previously synthesized PLLA /HA nanofibers were then immersed in this solution and subjected to ultrasonic treatment for a duration of 20 minutes. This step aimed to promote the interaction between the PLLA/ HA nanofibers and the aniline solution.

Following the ultrasonic treatment, a separate solution consisting of 100 ml of 1 molar HCl and containing 0.0125 mol of ammonium persulfate (APS) was prepared. The addition of the APS solution to the aniline solution was performed slowly, with a controlled rate of approximately 1 ml per minute, using a burette. The polymerization process took place at a temperature of zero ^{o}C. To maintain this temperature, the beaker containing the solution was carefully placed in a container filled with a mixture of ice and water, and the temperature was continuously monitored using a thermometer. The polymerization reaction proceeded for 4 hours while stirring the solution, the PLLA color was changed from white to green.

After the completion of the polymerization process, the polymer fibers were carefully removed from the solution and subjected to thorough washing with distilled water. This washing step was repeated several times to remove any residual reactants or by-products. Finally, the polymer fibers were placed at room temperature to dry.

2.3. Cellular protocol and seeding

2.3.1. Preparation and proliferation of DPSC cells (Human Dental Pulp Stem Cells)

DPSC cells (IBRC C10265) were purchased from the Iranian Biological Research Center in Iran. The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) medium containing (CATNO*:* GIBCO ™ 41965039) 10% FBS (CATNO: 10082147*,* GIBCO-USA) and placed in a cell incubator for the culture to reach the appropriate density. Medium change of the cells was done every three days.

2.3.2. Cell counting and seeding

To quantify cell numbers, the cells were trypsinized, centrifuged, and resuspended in one milliliter of culture medium. Cell counting was conducted by adding 10 µl of the homogenized cell suspension to a 1.5 ml microtube containing 80 µl of culture medium. Subsequently, 10 µl of trypan blue dye was introduced. After pipetting, 10 µl of the solution was transferred to a hemocytometer slide. The number of viable cells in four squares of the slide was enumerated under a microscope and the number of cells per millilitre was calculated using the following formula:

Number of cells/mL = (The sum of the cells of 4 squares/4) × the inverse of the dilution factor× 10^4

Explanation: The dilution factor of the cells in this experiment was 1 to 10 or one-tenth, which is the inverse of 10.

2.3.3. Cytotoxicity and MTT assay

MTT (Cat No.: DMA100) was provided at a concentration of 5 mg/ml in PBS (Cat No.: A-0018). The scaffolds were placed in the culture medium and the cells were cultured on the scaffolds in a number of about 5000 cells in each well of a 96-well plate in a three-dimensional condition and triplicate. In order to check the toxicity of the scaffolds, the plate was placed in an incubator (37° C) for 3 days, and then the supernatant was removed.

To conduct the test, 100 microliters of MTT solution, with a dilution ratio of 1:10 (MTT initial stock to culture media), was added to each well. The plates were then incubated at 37°C for 3 to 4 hours, during which the medium turned purple due to formazan production. Following incubation, the cell supernatant was aspirated, and 100 microliters of Dimethylsulfoxide (DMSO) (CATNO: BP231-100) were added to each well to dissolve the formed crystals. The plates were then incubated for an additional 15 minutes. After solubilizing the DMSO solvent, the scaffolds were removed from the wells, and the absorbance was measured at a wavelength of 570 nm using a spectrophotometer.

2.3.4. Spectrophotometric data analysis and determination of cell viability

After the addition of DMSO, the cells began to change color. Control samples exhibited a purple hue, indicating cell viability. A lighter color in the well suggests a higher death rate. Subsequently, the optical absorbance of the solution at a wavelength of 570 nm was measured using a spectrophotometer. The survival percentage of the cells

was then calculated using the following formula.

(Absorption of treatment sample /absorption of control sample) × 100 = survival percentage of each treatment sample.

The groups were designed as follows and incubated for 3 days in a 37°C incubator.

- Cell: Control
- Cell+ Scaffolds 1 (PLLA-HA PANi)
- Cell+ Scaffolds 2 (PLGA-HA-PANi-PDA)

2.3.5. Immunocytochemistry assay by using immunofluorescence stain

Human dental pulp stem cells (DPSCs) were cultured on PLLA-HA PANi scaffolds and as a monolayer in 24-well plates at a density of 1 × 10^5 cells per well for 7 and 21 days compared to control by using ICC assay. On the day of the assay, the media were removed, and the cells were washed with PBS before fixation with 4% paraformaldehyde for 20 minutes. After washing with PBS, the cells were permeabilized with 0.3% Triton X-100 in PBS. Subsequently, they were washed with PBS three times for 5 minutes each and then blocked with a solution of 1% BSA in PBST (PBS + 0.1% Triton X-100) at room temperature for 1 hour. The cells were then incubated with primary antibodies against Oct4, OCN, and DSPP (diluted 1:100 in 1% BSA in PBST) overnight at 4°C. The following day, after washing with PBS to remove unbound primary antibodies, the cells were incubated with FITC-conjugated anti-rabbit secondary antibody (diluted 1:100 in 1% BSA in PBST) at room temperature for 45 minutes in the dark. After washing with PBS three times for 5 minutes each, the cells were stained with DAPI solution (0.5 µg/ml) for 2 minutes to visualize nuclei, followed by another wash with PBS. Finally, the cells were examined under a fluorescent microscope equipped with DAPI and FITC filters.

2.4. Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences Software (SPSS) version 25 (IBM, Armonk, NY, USA). Data were presented as mean and standard deviation (SD) for each group. One-way Analysis of Variance (ANOVA) was utilized to analyze the differences among the groups and determine its significance. P-values < 0.05 was considered as statistically significant, then Tukey's post hoc test was utilized to evaluate the significance of each individual difference.

3. Results

3.1. Immunocytochemistry assay by using immunofluorescence stain

To start with, test of normality with Shapiro-Wilks as well as test of homogeneity (Levene Variance Test) assumptions were assessed and with reference to the results shown in Table 1 and Figure 1, it was reported the variables were parametric and hence independent sample test, paired t-test and One-Way ANOVA were utilized, and adjusted Bonferroni techniques was implemented for pairwise comparison between the genes.

3.2. Immunofluorescence stain analysis

Results of Immunofluorescence from Table 1 show useful information about stem cell growth and the presence of certain proteins in two experimental groups, "Control" and "PLLA-HA-PANI," using immunofluorescence analysis at two different time points. The study focuses on three genes: OCT4, OCN, and DSPP, which are associated with

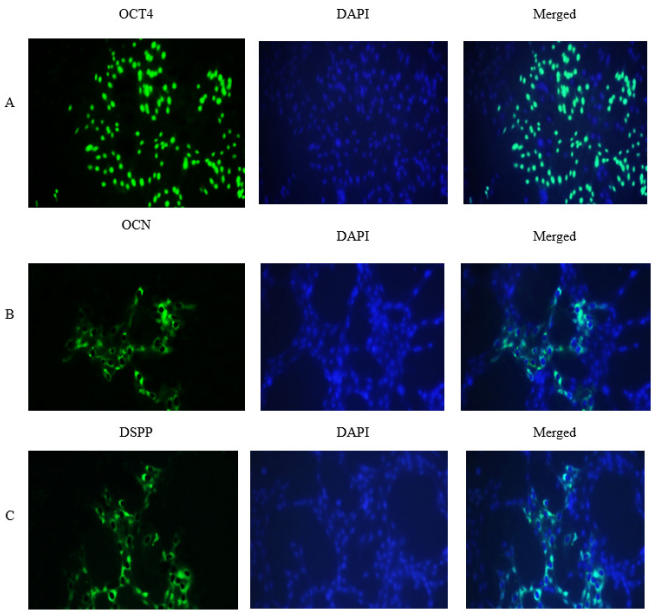

Fig. 1. 7 days gene expression Immunofluorescence staining and quantitative analysis (A) OCT4; (B) OCN; (C) DSPP.

Table 1. Descriptive statistics, normality and homogeneity tests for immunofluorescence findings.

Groups	Time Points	Genes	N	Mean	SD	Minimum	Maximum	Shapiro-Wilks (P-Value)	Levene Test
Control	7 Days	OCT4	5	60.138	4.034	55.200	64.330	0.472	1.367 (0.292)
		OCN	5	9.672	2.878	6.640	14.250	0.622	
		DSPP	5	9.022	2.002	6.020	11.130	0.741	
	21 Days	OCT4	5	63.572	4.433	58.110	69.130	0.858	0.612 (0.558)
		OCN	5	13.444	3.454	10.200	18.890	0.477	
		DSPP	5	15.462	3.149	11.180	19.750	0.993	
PLLA-HA-PANI	7 Days	OCT4	5	51.884	4.110	46.170	57.230	0.996	1.051 (0.380)
		OCN	5	34.048	4.075	30.170	39.600	0.472	
		DSPP	5	36.182	2.176	33.140	38.930	0.996	
	21 Days	OCT4	5	31.530	4.599	25.110	37.940	0.854	0.076 (0.927)
		OCN	5	54.046	3.895	50.130	59.880	0.687	
		DSPP	5	58.516	3.312	53.100	61.320	0.237	

stem cell behaviour and protein expression. For example, in the "Control" group at "7 Days," the average expression of OCT4 is 60.138 (SD = 4.034), indicating active stem cell development. Furthermore, the presence of OCN (mean =9.672, SD = 2.878) and DSPP (mean = 9.022, SD = 2.002) at this time point indicates that certain proteins associated with stem cell functions are expressed. Fig. 1 shows the immunofluorescence stain of OCT4, OCN, DSPP after 7 days.

As the study advances to "21 Days," there are significant fluctuations in mean expressions for these genes, indicating dynamic changes in stem cell function and indicating stem cell growth by small number of changes with 63.572, 13.44 and 15.462 for OCT4, OCN and DSPP respectively. Fig. 2 showed immunofluorescence of OCT4, OCN, and DSPP gene expression after 21 days.

On the other hand, the "PLLA-HA-PANI" scaffold shows unique patterns, with changes in growth and protein expression profiles at both time points compared to the "Control" group except for OCT4 gene where a sharp decrease was recorded over time. This thorough study provides important insights into the complicated relationship between gene expression, stem cell development, and protein presence in immunofluorescence experiments. In this group, different patterns were found, with OCT4 at 7 days recording a mean of 51.884 (SD = 4.110), OCN at 34.048 (SD = 4.075), and DSPP at 36.182 (SD = 2.176).

At "21 Days," mean expressions of OCT4 fall down to 31.530, whereas at OCN, and DSPP higher mean values were noticed with 54.046 and 58.516 correspondingly. This comprehensive study provides important insights into the complex link between gene expression, stem cell development, and protein presence in immunofluorescence experiments for both experimental groups. Fig. 3.

Table 2 displays paired sample t-test between 7 days and 21 days for each gene in each experimental group and statistically significant differences occurred for all genes and from the mean difference column, it confirmed that over time, the presence of proteins was larger except in OCT4 at PLLA-HA-PANI. Figures 3 and 4 display the plot illustration for each gene.

Further investigations were applied and according to Table 3, the findings indicated significant differences between the groups at each time point for all three genes where all p-values were less than significance level 0.05. Figure 4 visualizes their distributions and the differences can be noticed clearly.

Moreover, the differences between the genes were also investigated for each experimental group at both time points separately, and the results from One-way ANOVA test confirmed the presence of statistically significant tests among the genes. The post hoc Bonferroni test revealed

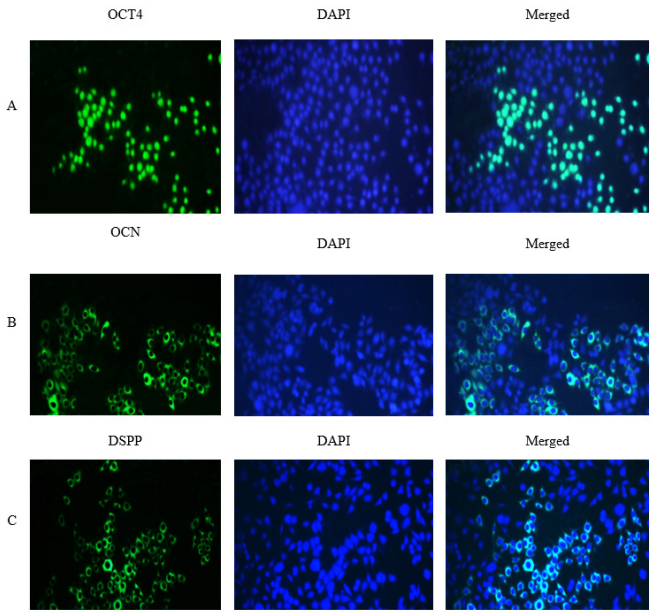

Fig. 2. 21 days gene expression Immunofluorescence staining and quantitative analysis (A) OCT4; (B) OCN; (C) DSPP.

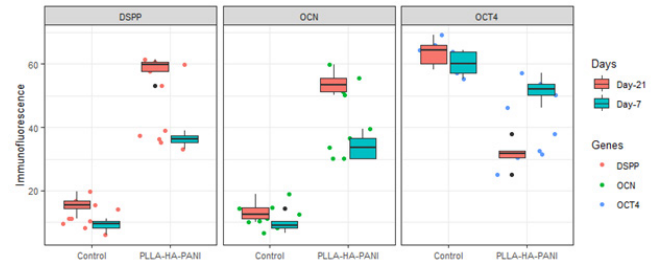

Fig. 3. Box-Plot Exploration of for each Genes under both experimental groups.

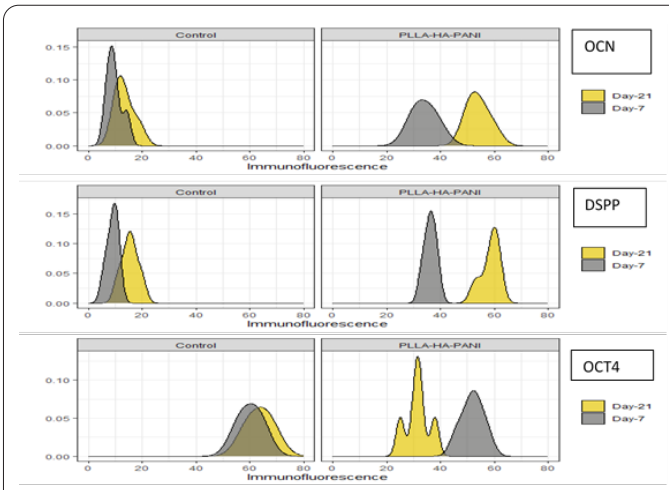

Fig. 4. Density Plot Illustration for each gene separately.

Table 2. Comparison test statistics by paired sample test over the two-time points.

Genes	Groups	Mean Difference	95% Confidence Interval of the Difference		Paired Test (P-value)
			Lower	Upper	
OCT4	Control	-3.434	-9.615	2.747	-1.281 (0.236)
	PLLA-HA-PANI	20.354	13.993	26.715	7.379 (0.000)
OCN	Control	-3.772	-8.409	0.865	-1.876 (0.098)
	PLLA-HA-PANI	-19.998	-25.811	-14.185	-7.933 (0.000)
DSPP	Control	-6.440	-10.288	-2.592	-3.860 (0.005)
	PLLA-HA-PANI	-22.334	-26.421	-18.247	-12.602 (0.000)

Table 3. Comparison Test Statistics by Independent sample test over the two experimental groups.

Genes	Time Points (Groups)	Mean Difference	95% Confidence Interval of the Difference		T-Test (P-value)
			Lower	Upper	
OCT4	7 Days (Control - PLLA-HA-PANI)	8.254	2.315	14.193	3.205 (0.013)
	21 Days (Control - PLLA-HA-PANI)	32.042	25.455	38.629	11.217 (0.000)
OCN	7 Days (Control - PLLA-HA-PANI)	-24.376	-29.521	-19.231	-10.925 (0.000)
	21 Days (Control - PLLA-HA-PANI)	-40.602	-45.971	-35.233	-17.439 (0.000)
DSPP	7 Days (Control - PLLA-HA-PANI)	-27.160	-30.209	-24.111	-20.542 (0.000)
	21 Days (Control - PLLA-HA-PANI)	-43.054	-47.767	-38.341	-21.067 (0.000)

Table 4. Post Hoc statistical test using Bonferroni adjusted test.

Experimental Groups	Time	Genes		Mean Difference (I-J)	SE	P-value	95% Confidence Interval	
							Lower Bound	Upper Bound
Control	7 Days	OCT4	OCN	50.466*	1.952	0.000	45.042	55.890
			DSPP	51.116*	1.952	0.000	45.692	56.540
		OCN	DSPP	0.65000	1.952	1.000	-4.774	6.074
	21 Days	OCT4	OCN	50.128*	2.352	0.000	43.590	56.666
			DSPP	48.110*	2.352	0.000	41.572	54.648
		OCN	DSPP	-2.018	2.352	1.000	-8.556	4.520
PLLA-HA-PANI	7 Days	OCT4	OCN	17.836*	2.258	0.000	11.561	24.111
			DSPP	15.702*	2.258	0.000	9.427	21.977
		OCN	DSPP	-2.1340	2.258	1.000	-8.409	4.141
	21 Days	OCT4	OCN	-22.516*	2.511	0.000	-29.495	-15.537
			DSPP	-26.986*	2.511	0.000	-33.965	-20.007
		OCN	DSPP	-4.470	2.511	0.301	-11.449	2.509

Table 5. Descriptive statistics of MTT of cytotoxicity measurement.

	Control	PLLA-HA-PANI	Shapiro Wilk Test (P-value)	Mean Difference	T-test (P-value)
Mean	100	92.813	0.837 (0.158)	7.187	1.657 (0.136)
SD	6.48	5.796	0.970 (0.875)		

that no statistically significant differences were found between OCN and DSPP genes at both time points for the two experimental groups. Fig. 5. Table 4 represents the results for further reference.

3.3. Cytotoxicity and MTT assay result

In Table 5 where cytotoxicity assay figures are presented, the mean values reveal the average cell viability percentages for two distinct groups after 21 days: the control group and the group of (PLLA-HA-PANi) scaffold. The control group's mean of 100% indicates that, on average, all cells in this group maintained full viability. However, (PLLA-HA-PANi) scaffold group's mean of 92.81% suggests a slight reduction in cell viability compared to the control indicated non-toxicity.

In cytotoxicity assays, mean values are crucial indicators of the overall impact of a substance or treatment on cell viability. Researchers use these means to assess whether the scaffold has cytotoxic effects and to what extent, providing valuable information about the potential impact of the tested substances on cellular health.

Therefore, an independent sample t-test was carried out on the dataset since it was confirmed that the dataset was normally distributed with reference to Shapiro-Wilk test and Table 5, the comparison test turned out to be not statistically significant with p-value (0.136) greater than significant level (0.05).

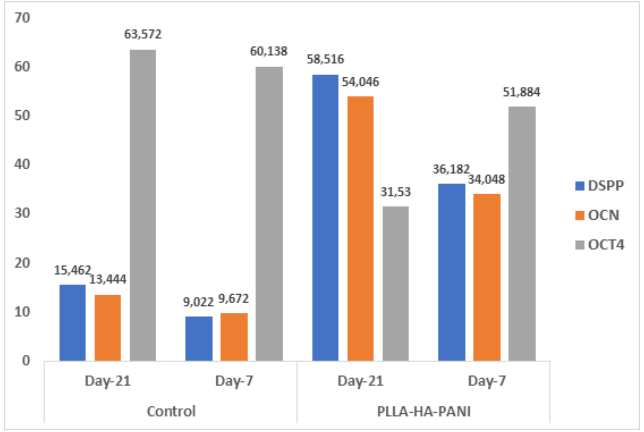

Fig. 5. Bar-chart Exploration of mean values.

4. Discussion

The combination of poly L lactic acid (PLLA) with hydroxyapatite scaffold has been shown to provide a conducive environment for dental pulp stem cell growth and differentiation [9] and monstrated the regeneration of dental pulp and dentin using stem/progenitor cell-based approaches and tissue engineering technologies, indicating the potential for regeneration of dental pulp with newly deposited continuous layers of dentin [9].

Hydroxyapatite (HA) is a major inorganic component of hard tissues in the human body and can be used as a

suitable material for improving cell proliferation and differentiation [10]. This suggests that the combination of scaffold materials such as PLLA and hydroxyapatite can support the growth and differentiation of dental pulp stem cells.

Polyaniline (PANI) is one of the attractive conductive polymers due to it is unique properties including biocompatibility, thermal, antibacterial, ion exchangeable and electrical conductivity. Therefore, polyaniline was used in this study [5].

In the present study, a combined scaffold, composited from PLLA, HA, and PANI to investigate the combined effect of these three biocompatible scaffold materials on dental pulp stem cell proliferation and differentiation.

Dental pulp stem cells were used in this study due to the ability of this cell to differentiate into other cell types including odontogenic, osteogenic, chondrogenic and other cells. The broad spectrum of differentiation potential and relative ease of collection make the dental pulp stem cell a valuable cell source for tissue regeneration.

Furthermore, each protein gene used in this study serves specific purpose; dentin sialophosphoprotein (DSPP) had reported the development of tooth defects similar to human dentinogenesis imperfecta III in Dspp-null mice, highlighting the role of dentin sialophosphoprotein (DSPP) in dentin mineralization and tooth development [11, 13]. This underscores the significance of DSPP in dental tissue formation and the potential relevance of its gene expression in the context of dental pulp stem cell growth within PLLA/HA/PANI scaffolds.

The osteocalcin (OCN) which is a small (49-amino acid) noncollagenous protein hormone found in dentin and bone, is identified as a calcium-binding protein. Its production is restricted to cells responsible for mineralization such as osteoblast, odontoblast and cementoblast [15, 17].

Oct-4 is a homedomain transcription factor, Oct-4 exerts tight control over pluripotency regulator expression and protects embryonic stem cells in an undifferentiated state.

The result for immunochemistry demonstrated cell differentiation, DSPP is a marker of early odontogenic differentiation that functions during first stage of dentinogenesis and it is involved in dentin generation. This study compared DSPP expression between control and (PLLA/HA/PANI) scaffold and with other gene markers to determine the odontogenic differentiation of dental pulp stem cells. The percentage of DSPP gene expression in (PLLA/HA/PANI) scaffold increased after 7 days and more increased after 21 days if you compared it with control group and among all genes the expression of DSPP gene was more obvious, this means higher odontogenic differentiation. On the other hand, the OCN gene expression also increased after 7 and 21 days, OCN act as early osteoblastic and odontoblastic proliferation marker [13, 19, 21, 23] and it is a symbol of hard tissue engineering [11–13]. When the result showed OCN expression mean regulation of cell mineralization ability and formation of mineral nodules [14, 15]. Indeed, OCN expression was found to considerably increase when dental pulp stem cells underwent osteoinduction and dentinoinduction, especially after 5-day mark [10, 14, 16, 17].

OCT-4 increased after 7 days meaning the DPSC differentiated while after 21 days decreased if you compared it to DSPP and OCN genes this is due to the stem cell differentiation to odontoblast and osteoblast, in addition to that the OCT-4 is highly expressed in pluripotent cells and become silenced upon differentiation and proliferation of stem cells [18–20].

The cytotoxicity result showed a biocompatible scaffold that promotes cell differentiation and growth, according to the literature review, 90% of cell viability indicated non-toxicity, 60%-90% indicated mild, 30%-60% indicated moderate and less than 30% indicated severe toxicity. In this study 92% of cell viability was recorded, viability of cell after 21 days revealed (PLLA/HA/PANI) scaffold is biocompatible and biodegradable. Furthermore, may be coating the (PLLA/HA/PANI) scaffold with polyaniline polymer increasing cell viability due to antibacterial and electrical conductivity accompanied with ion exchangeability features.

5. Conclusion

PLLA with hydroxyapatite and polyaniline scaffold facilitates the growth and gene expression of dental pulp stem cells, particularly in relation to the expression of key gene proteins such as OCT4, OCN, and DSPP. These findings underscore the promise of this biomaterial combination for dental tissue engineering and regenerative applications.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Informed consent

The authors declare not to use any patients in this research.

Availability of data and material

The data of this study are available from the corresponding author upon reasonable request.

Authors' contributions

The authors contributed equally and did all the steps of the study work.

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