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In vivo performance of different scaffolds for dental pulp stem cells induced for odontogenic differentiation

Cigdem ATALAYIN[a]
Huseyin TEZEL[a]
Taner DAGCI[b]
Nefise Ulku KARABAY YAVASOGLU[c]
Gulperi OKTEM[d]
Timur KOSE[e]

[a]Ege University, School of Dentistry, Department of Restorative Dentistry, Izmir, Turkey.

[b]Ege University, School of Medicine, Department of Physiology, Izmir, Turkey.

[c]Ege University, Faculty of Science, Department of Biology, Izmir, Turkey.

[d]Ege University, School of Medicine, Department of Histology and Embryology, Izmir, Turkey.

[e]Ege University, School of Medicine, Department of Biostatistics and Medical Informatics, Izmir, Turkey.

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Corresponding Author:
Cigdem Atalayin
E-mail: dtcatalayin@gmail.com

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Abstract: This study was designed to determine the *in vivo* performance of three different materials as scaffolds for dental pulp stem cells (DPSC) undergoing induced odontogenic differentiation. An odontogenic medium modified by the addition of recombinant human bone morphogenetic protein 2 was used in the experimental groups to induce differentiation. Mesenchymal stem cell medium was used in the control groups. DPSC were transplanted onto the backs of mice via three scaffolds: copolymer of L-lactide and DL-lactide (PLDL), copolymer of DL-lactide (PDL) and hydroxyapatite tricalcium phosphate (HA/TCP). The expression levels of *dentin sialo-phosphoprotein (DSPP), dentin matrix protein-1 (DMP1), enamelysin/matrix metalloproteinase 20 (MMP20)* and *phosphate-regulating gene with homologies to endopeptidases on X chromosome (PHEX)* were analysed using RT-PCR. The expressions in the experimental groups were compared to those in the control groups. The transcript expressions at 6 and 12 weeks were significantly different for all scaffolds ($p < 0.05$), except for the expression of *DSPP* in the PLDL group with regard to the time variable. Although there was a decrease in the expression of enamelysin/*MMP20* in PLDL and HA/TCP at 12 weeks, all other expressions increased and reached their highest level at 12 weeks. The highest DSPP expression was in the PDL group ($p < 0.05$). The highest expression of DMP1 was detected in the HA/TCP group ($p < 0.05$). The highest expression of PHEX was in the PLDL group ($p < 0.05$). Consequently, PLDL and PDL seemed to be promising scaffold candidates for odontogenic regeneration at least as HA-TCP, when they were applied with the DPSC induced for odontogenic differentiation.

Keywords: Dentistry; Regeneration; Dental Pulp.

Introduction

The current dental treatments are unable to restore full biological function, including the mechanical properties of the lost or damaged tissue. The 'regenerative dentistry' concept of biological tooth repair appears to be a promising alternative for the future of dentistry.[1] Although different stem cells, morphogens and scaffolds can be combined to simulate natural odontogenic regeneration, it is crucial to determine which options most closely replicate the characteristics of human dental tissues.

Dental pulp stem cells (DPSC) seem to be the most appropriate cell group for the regenerative approach.[2] They have a higher odontogenic

differentiation capacity compared to other cell groups due to their content of specific progenitor cells.[3] Odontogenic differentiation of DPSC can be induced using various factors; for example, dexamethasone has been reported as the main inductive factor in previous DPSC studies.[4,5,6] Bone morphogenetic proteins (BMP), known as active components of odontogenic differentiation and tooth development, have been used as a growth factor in an appropriate medium.[7,8] This combination of inductive factors has been suggested as being more effective in enhancing differentiation.[4,9,10] Hence, it may be useful to investigate different medium modifications to create effective combinations of factors for inducing odontogenic differentiation.

Scaffolds imitate the conditions in the tissue.[11] The properties of a scaffold material are important. It must simulate the biological matrix and provide the desired tissue formation.[12] Many types of natural and synthetic materials have been tested as scaffolds in regenerative dentistry; nevertheless, there is still a need for new materials and designs. Both ceramics and synthetic polymers can provide optimal scaffold structures.[13] Hydroxyapatite tricalcium phosphate (HA-TCP) has been reported to be an appropriate scaffold material for DPSC and odontogenic regeneration.[6,14,15] Synthetic polymers are increasingly in demand as scaffold materials due to their properties of biocompatibility, biodegradability and the incorporation of bioactive molecules.[13] Copolymers, including a poly-L/DL lactide that is used in medical applications, may also be an option to consider when devising new scaffold materials.

The aim of this study was to determine the *in vivo* performance of different scaffold materials, *i.e.*, a copolymer of L-lactide and DL-lactide (PLDL), a copolymer of DL-lactide (PDL) and hydroxyapatite tricalcium phosphate (HA/TCP) for DPSC induced for odontogenic differentiation by medium modification.

Methodology

Cell culture

Human impacted third molar teeth (n = 15) that had been surgically extracted were used as the cell source. The study protocol was approved by the Human Ethical Committee of Ege University (Research no. 11-5.1/8), and informed consent was obtained from the patients. A previously reported isolation and culture method was used.[5,13,14]

The cells were analysed with flow cytometry using a FACSCalibur (BD Biosciences, CA, USA), and the data were analysed using the Cell Quest software (BD Biosciences, CA, USA) to determine the phenotypic characteristics. Stem cells that were negative for CD34 (hematopoietic progenitor cell antigen; APC) and CD45 (leucocyte common antigen/cell marker of hematopoietic origin; APC-H) but positive for CD73 (NTES'-nucleotidase; PE) and CD90 (thymus cell antigen/Thy-1/Thy-1.1; FITC) were selected for this study. The rate of mesenchymal clusters of differentiation (CD73+CD90) was 98.3%.

In the control (-) groups, a mesenchymal stem cell medium[13] containing an alpha modification of Eagle's medium (Gibco Invitrogen, Grand Island, USA) supplemented with 15% fetal bovine serum (FBS) (Gibco Invitrogen, Grand Island, USA), 0.1 mM L-ascorbic acid phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 100 units/ml penicillin/streptomycin (Gibco Invitrogen, Grand Island, USA) was used during the culture period. In the experimental (+) groups, the medium was modified by adding 0.01 µM dexamethasone sodium phosphate, 1.8 mM monopotassium phosphate (KH_2PO_4) and 50 ng/ml recombinant human bone morphogenetic protein 2 (rhBMP2) (ProSpec-Tany TechnoGene Ltd., Israel) to induce odontogenic differentiation.[8,13] The cells were cultured at 37°C in 5% CO_2 and the medium was changed every two days.

Preparation of scaffolds

Three different scaffold materials (40 mg) were used as follows;

- **a.** PLDL in a 80/20 molar ratio with an inherent viscosity midpoint of 5.8 dl/g (Purasorb PLDL, Purac, Holland)
- **b.** PDL with an inherent viscosity midpoint of 2.0 dl/g. (Purasorb PDL, Purac, Holland)
- **c.** HA-TCP ceramic powder (Zimmer, Warsaw, Indiana, USA)

The polymer scaffolds, PLDL and PDL, were dissolved in dioxane before being mixed with the

cells. The cell suspensions, containing ~1x10^6 DPSC cultured to the third passage, were mixed with the appropriate scaffold material, and transplantation was performed as described below.

Transplantation

All transplantation procedures were performed under sterile conditions and in accordance with the guidelines approved by the Animal Ethical Committee of Ege University (Research no. 2010-21). Fifty mg/kg Ketamine HCl (Alfamine®), 5 mg/kg Xylazine HCl (Alfazyne®) and 1 mg/kg acepromazine maleate were intraperitoneally administered to each of the immunocompromised mice. The dorsal surface of each mouse was shaved and cleaned, and one midsagittal incision was made. A mixture of cell suspension and scaffold (n = 3 for each group) was then placed into a pocket that was prepared subcutaneously to the dorsal surface of each immunocompromised mouse. The pockets and incision lines were closed. The animals were euthanized with 100 mg/kg thiopental sodium at 6 or at 12 weeks; the samples were removed 6 or 12 weeks, respectively, after the transplantation, and the structure of the formed tissues was investigated using Real Time-Polymerase Chain Reaction (RT-PCR).

Gene expression analysis

The expressions of the following human enamel and dentin specific transcripts in the formed tissues were evaluated using RT-PCR: *dentin sialo-phosphoprotein (DSPP), dentin matrix protein-1 (DMP1), enamelysin/matrix metalloproteinase 20 (MMP20)* and the *phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX).* The total RNAs of the tissue were isolated at 6 and 12 weeks using the TriPure Isolation Reagent (Roche, Cat No 11 667 157 001) following the manufacturer's instructions. First-strand cDNAs were synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Cat No 04 379 012 001) using the total RNA. Specific primers were purchased as follows;

DSPP forward: 5'AACATCACAGCAAATGGCATC
DSPP reverse: 5'CTTCCAGCTACTTGAGGTCCA
DMP1 forward: 5'AGACAGTGCCCAAGATACCACC
DMP1 reverse: 5'ATTCCCTCATCGTCCAACTCG
MMP20 forward: 5'ACAAGCAGCCTCTAACTGGATC
MMP20 reverse: 5'GATTTCGCATAAAGTTGCCCAT
PHEX forward: 5'AACTTTGCTGCCTCAATGGGA
PHEX reverse: 5' GTCAATAAAGGCCCAGCGAAC
β-actin forward: 5' AGCCTCGCCTTTGCCGA
β-actin reverse: 5' CTGGTGCCTGGGGCG

A total of 0.3 ml of each primer (0.5 mM) was mixed with 10.5 ml of LightCycler FastStart DNA Master SYBR Green I (Roche, Cat no: 03 003 230 001) for a final volume of 18 ml. The standard cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 62°C for 8 s and 72°C for 13 s. The comparative Ct method (2–$^{\Delta\Delta Ct}$ method) was used to analyse the data.[16] The expression of genes was normalized using the glucose *β-actin* gene as a control housekeeping gene. The relative changes in the expression levels of the analysed genes in the experimental group were compared to those in the control group. The details of the analysis were as follows:

Ct: Threshold cycle, HKG: Housekeeping gene *(β-actin*), GOI: Experimental gene

ΔCt(Control): Ct(GOI) – Ct(HKG)

ΔCt(Experimental): Ct(GOI) – Ct(HKG)

ΔΔCt: ΔCt (Experimental) – ΔCt (Control)

Fold change: 2 $^{-\Delta\Delta Ct}$

IBM SPSS (Version 20.0) was used for the statistical analysis. The variables were summarized by means of mean ± standard deviation. First, the scaffold type (repeated factor) and time effect (independent factor) were analysed using a repeated measures ANOVA. Because the scaffold x time interaction was found to be significant, the results at 6 and 12 weeks were measured and tested separately based on the scaffold type (PLDL x PDL x HA-TCP) by means of repeated measures ANOVA followed by a Bonferroni correction. Finally, 6th week x 12th week comparisons for each scaffold were analysed via a two independent samples t-test. All hypothesis tests were performed at the $\alpha = 0.05$ significance level.

Results

The fold change rates were obtained for each time point. The expression of human enamel-dentin specific transcripts in the experimental (+) groups compared to the control (-) groups was determined. There were differences in transcript expressions with regard to both the groups and the time variables ($p < 0.05$).

The differences between the groups are presented in Table. The fold changes in gene expression are shown in Figure. The transcript expressions at 6 and 12 weeks were significantly different for all scaffold materials ($p < 0.05$), except for a lack of difference in the expression of *DSPP* in the PLDL group with regard to the time variable ($p > 0.05$). Although there was a decrease in the expression of enamelysin/*MMP20* in PLDL and HA/TCP at 12 weeks, all other expressions were observed to have increased and reached their highest level at 12 weeks.

There was no significant difference between the PLDL, PDL and HA/TCP groups for *DSPP* expression at 6 weeks ($p > 0.05$). The highest *DSPP* expression

Table. The fold change rates of *DSPP, DMP1, MMP20* and *PHEX* (mean ± std). The superscripts indicate statistically significant differences between the groups ($p < 0.05$).

Group	Time	DSPP	DMP1	MMP20	PHEX
PLDL	6th week	1.027 ± 0.441	10.153 ± 0.318^{a,b,*}	4.911 ± 0.544^{a,*}	0.119 ± 0.009^{a,b,*}
	12th week	1.526 ± 0.127^{a}	12.622 ± 1.236^{a,b}	1.359 ± 0.413^{b}	17.867 ± 1.973^{a,b}
PDL	6th week	0.004 ± 0.001*	0.001 ± 0^{c,*}	0.120 ± 0.049^{c,*}	0^{c,*}
	12th week	6.495 ± 1.053^{c}	8.430 ± 1.335^{c}	2.183 ± 0.170^{c}	11.201 ± 1.210^{c}
HA/TCP	6th week	0.014 ± 0.009*	6.119 ± 0.065*	4.649 ± 0.081*	0.648 ± 0.007*
	12th week	1.031 ± 0.093	360.417 ± 7.170	0.964 ± 0.355	3.756 ± 0.265

DSPP: dentin sialo-phosphoprotein; DMP1: dentin matrix protein-1; MMP20: enamelysin/matrix metalloproteinase 20; PHEX: the phosphate-regulating gene with homologies to endopeptidases on the X chromosome; ^{a,b,c} Repeated measure ANOVA, Bonferroni. PLDL x PDL: a, PLDL x HA-TCP: b, PDL x HA-TCP: c; * t-test, 6th week x 12th week.

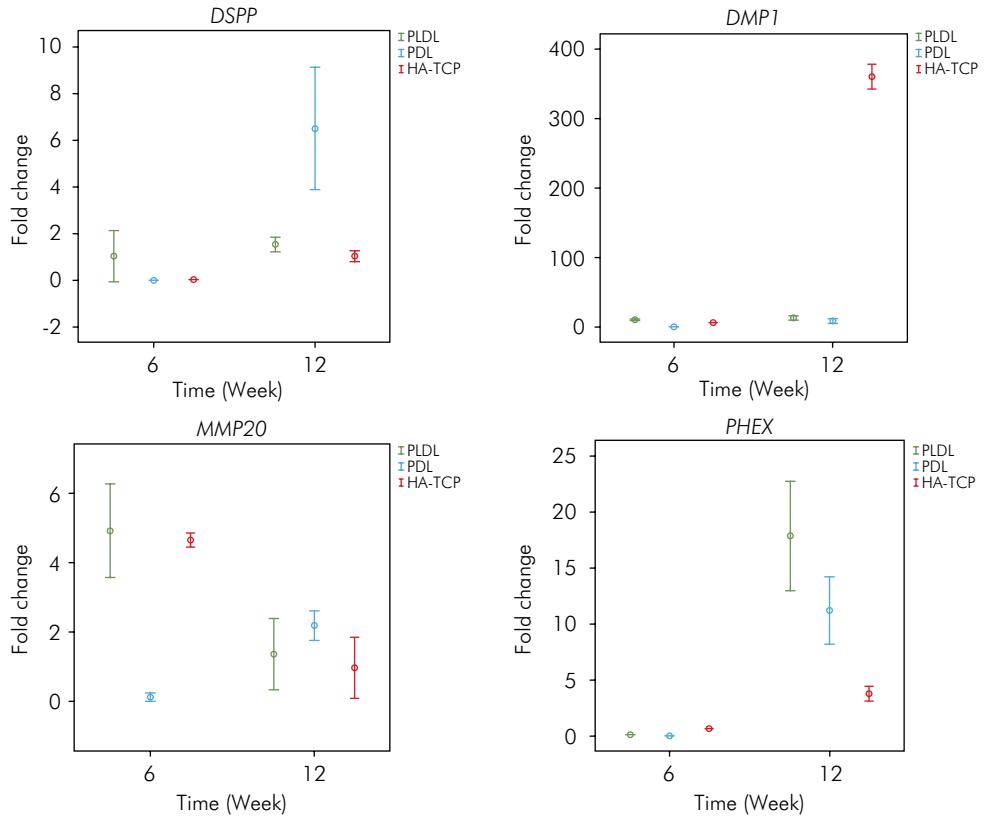

Figure. The fold change rates of *DSPP, DMP1, MMP20* and *PHEX* in the experimental (+) groups compared to the control (-) groups for three scaffold materials, 6 and 12 weeks after transplantation.

was determined in the PDL group at 12 weeks and was significantly different from those of the other groups ($p < 0.05$). The *DSPP* levels were observed to be similar in the PLDL and HA/TCP groups at 12 weeks ($p > 0.05$).

The highest *DMP1* expression was found in the PLDL group at 6 weeks ($p < 0.05$). There was an increase in the expression of *DMP1* in all groups consistent with the time period ($p < 0.05$). The highest expression of *DMP1* was shown in the HA/TCP group at 12 weeks ($p < 0.05$).

The expression of *MMP20* was similar in the PLDL and HA/TCP groups at 6 weeks ($p > 0.05$). Although the expression of *MMP20* was lowest in the PDL group at 6 weeks, it was the highest at 12 weeks ($p < 0.05$). No significant difference was observed between the PLDL and PDL groups for *MMP20* expression at 12 weeks ($p > 0.05$).

The highest *PHEX* expression was observed in the HA/TCP group at 6 weeks ($p < 0.05$). Although there was an increase in *PHEX* expression in all groups at 12 weeks, the highest expression was found in the PLDL group ($p < 0.05$).

Discussion

Cells, morphogens and scaffolds are the key elements for cellular regeneration. It is important to understand the relationships among these elements to develop successful models for odontogenic regeneration. A model using DPSC, medium modification via the addition of rhBMP2 and three different scaffold materials (PLDL, PDL and HA-TCP) was tested in this study.

DPSC are postnatal somatic stem cells with high differentiation potential in response to environmental stimuli.[14] The BMP family plays an active role in the initiation, morphogenesis and cytodifferentiation phases of tooth development.[7] Bone morphogenetic protein 2 (BMP2) induces odontoblastic differentiation and dentin formation.[8,17] The stimulation of odontoblastic differentiation in a cell culture before implantation has been reported to exert a positive influence on dentin formation.[8] Accordingly, dexamethasone- and rhBMP2-supplemented odontogenic medium was used in the experimental (+) groups of this study. This supplementation promoted the odontoblastic differentiation of DPSC and enhanced the expression of odontogenic/odontoblastic markers (Figure). The results verify the crucial role of growth factor (rhBMP2 in this study) in the odontogenic differentiation of DPSC. Similar results were obtained by using a combination of dexamethasone and bone morphogenetic protein 7 in a previous study.[4] Medium modification via the addition of BMP2 has previously been reported to improve the *in vivo* performance of HA/TCP[18]. Our results reveal the positive contribution of the above-described induction when using not only HA/TCP but also PLDL and PDL scaffolds.

Odontogenic regeneration starts with odontoblastic differentiation. *DSPP*, which is known as a marker for odontoblastic differentiation, is responsible for encoding dentin phosphoprotein (DPP) and dentin sialoprotein (DSP). Another extracellular matrix protein, *DMP1*, has an active role during tooth development and odontoblastic differentiation.[19,20,21,22] The comparatively increased expressions of *DSPP* and *DMP1* in the experimental (+) groups (Figure) are evidence of the differentiation of DPSC into functional odontoblasts. In addition, the highest level of the dentin transcripts (which is similar to tertiary dentin formation) at 12 weeks supports our findings (Table).

Enamelysin/*MMP20* is responsible for the enamel matrix and is expressed during the secretory stage of dental enamel formation.[23,24,25] *PHEX* is responsible for phosphate homeostasis.[26] The increased expression of these transcripts are consistent with the time periods (Figure) and are related to the tissue organization in all three scaffold materials. Despite the absence of the fold change in *PHEX* expression in the PDL group at 6 weeks, the expression rate was consistent with those of the other scaffold materials at 12 weeks. The decrease of enamelysin/*MMP20* level in the PLDL and HA-TCP groups at 12 weeks may be related to transient inactivity or to the structure of the scaffold materials. This finding also suggests the potential difficulty of enamel regeneration with these scaffold materials. However, PDL may be a more suitable scaffold material for enamel when considering the Enamelysin/*MMP20* expression profile.

The complex structure of the tooth requires scaffold materials that have different tissue specifications

when using the regenerative dentistry concept. Three different materials were tried as a scaffold in this study. Among these materials, HA/TCP is a recommended scaffold for hard tissue regeneration, and it has been tested before.[6,14,15] This study is the first to report on two new polymers as scaffold materials. Our data suggest that PLDL and PDL copolymers may be appropriate scaffold materials for DPSC and odontogenic regeneration. The tested materials performed adequately in this research experiment, and each may have different advantages in future applications. It is difficult to evaluate one of them as being superior to another based on the RT-PCR results. The tested polymers seem to perform similarly to HA/TCP regarding the enamel-dentin transcript expressions. PLDL and PDL enhanced the odontogenic matrix formation within the applied experimental conditions. Further modifications will be necessary to improve polylactides as useful biomedical materials.[27] Therefore, there is a need for further studies in this field.

New scaffold designs will increase the probability of the future implementation of the regenerative dentistry concept; however, the development of the appropriate materials for each clinical application is crucial. Biocompatibility and adequate matrix formation properties are considered essential in clinical situations.[19] Biodegradation and the appropriate structural configuration to meet clinical needs are reported to be the desired features.[4] In this respect, the biocompatibility and biodegradability properties of PLDL and PDL arising from their copolymer structures are promising and may prove to be an advantage. New materials can also be designed by incorporating bioactive molecules into these copolymer structures.[13] Biodegradable/biocompatible scaffolds loaded with growth factors will help optimize the biomaterial design.[28] Thus, the site-specific pharmacological release of growth factors that have the desired kinetics and a crucial role in regeneration should be attempted by using PLDL and PDL scaffolds in further studies.

Conclusion

Within the limitations of this study, it can be concluded that medium modification by the addition of rhBMP2 expedites odontogenic differentiation. PLDL and PDL are promising scaffold candidates for odontogenic regeneration at least as HA-TCP, if they are applied with the DPSC induced for odontogenic differentiation.

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