Article published online: 2021-11-01

Original Article

Dentinogenic Differentiation Potential of Fast Set White Portland Cements of a Different Origin on Dental Pulp Stem Cells

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Abstract

Background: Advances in endodontic biomaterials are at the forefront of endodontic research. **Purpose:** This study aimed to compare the dentinogenic differentiation potential of extracts of two formulations (normal and fast set [FS] by the addition of calcium chloride dihydrate ($CaCl_2.2H_2O$) of white Portland cements (WPCs) of a different origin (Aalborg, Malaysia, and Egypt) on dental pulp stem cells (DPSCs). **Materials and Methods:** The material extracts at 12.5 mg/ml were applied on DPSCs cultured in 96-well plates. After 1, 3, 7 and 14 days of incubation, the RNA was extracted, cDNA was prepared, and the expression of four dentinogenic gene markers (bone gamma-carboxyglutamate protein, dentin sialophosphoprotein, runt-related transcription factor 2, and secreted phosphoprotein 1 [*SPP1*]) was examined using the real-time polymerase chain reaction. One-way analysis of variance was used for statistical analysis, and the level of significance was set at 0.05 ($P = 0.05$). **Results:** Significant differences were observed between Malaysian WPC (MAWPC) and Egyptian WPC (EGWPC) and FS MAWPC), FS EGWPC in 7 out of 15 and 6 out of 10 comparisons, respectively. While more expressions in EGWPC group were observed in four comparisons and three for MAWPC, all FS formulations showed higher expressions for FS MAWPC compared to FS EGWPC ($P < 0.05$). The addition of $CaCl_2.2H_2O$ to MAWPC and EGWPC increased the upregulation of *SPP1* gene at all-day intervals, which was not observed with other genes. **Conclusions:** The dentinogenic differentiation of DPSCs shows different expression profiles to extracts of normal and fast formulations of WPC. Extracts of WPC of different origin have different dentinogenic differentiation potential on DPSCs.

Keywords: Calcium chloride dihydrate, dentinogenic differentiation, Egypt, Malaysia, real-time polymerase chain reaction, white Portland cement

Introduction

Gray mineral trioxide aggregate was first introduced as a root-end filling material,[1] but a white mineral trioxide aggregate (WMTA) has become available to fulfill esthetic concerns. Studies demonstrated the favorable biological profile of WMTA.[2,3] However, prolonged setting time and high cost continue to be its main drawbacks.[4,5] The reported similarity in the properties of WMTA and white Portland cement (WPC) has generated interest in the evaluation of WPC as an alternative to WMTA.[6] Investigations continue to support the favorable biological properties of WPC.[7-9]

Calcium chloride ($CaCl_2$) is the most common setting accelerator investigated as a potential additive to WPC.[5,10] Studies showed favorable biological properties of WPC/$CaCl_2$ combination.[11,12] However, a recent study found that WPC of different origins may show differences in chemical and biological properties, and different human cell types may react differently toward different formulations of WPCs.[13]

In vitro dentinogenic differentiation potential of WPC-based materials has been a subject of research studies. Two studies[14,15] examined the dentinogenic potential of Portland cement (PC) and other formulations on human dental pulp

Access this article online

Quick Response Code:

Website: www.ejgd.org

DOI: 10.4103/ejgd.ejgd_42_17

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How to cite this article: Ahmed HM, Luddin N, Kannan TP, Mokhtar KI, Ahmad A. Dentinogenic differentiation potential of fast set white portland cements of a different origin on dental pulp stem cells. Eur J Gen Dent 2017;6:115-22.

cells (HDPCs), and results supported the potential application of PC as a pulp capping material. Investigators[16] found that the addition of simvastatin and Emdogain improved cell growth and the differentiation of a PC formulation in HDPCs. Others[17] found that a PC-based material (WMTA) can stimulate the odontogenic differentiation of dental pulp stem cells (DPSCs). However, the response of DPSCs to fast set (FS) formulations of PC of different origins has not been reported.

This study aimed to compare the dentinogenic differentiation potential of extracts of WPC of different origins (Malaysian WPC [MAWPC] and Egyptian WPC [EGWPC]) and their FS formulations (addition of calcium chloride dihydrate [$CaCl_2.2H_2O$] – FS MAWPC and FS EGWPC) on DPSCs. The research hypotheses were that normal and FS WPC formulations show comparable dentinogenic differentiation potential on DPSCs.

Materials and Methods

Preparation of materials

All materials were prepared as described in our previous work.[13] WPC manufactured by one company (Aalborg) but in two different countries (Sinai, Egypt and Perak, and Malaysia) were used in this study, and the materials were divided into the following groups:

- Group 1: 1 g of MAWPC mixed with 300 mL of sterile distilled water
- Group 2: 1 g of EGWPC mixed with 300 mL of sterile distilled water
- Group 3: 1 g of MAWPC mixed with 10% $CaCl_2.2H_2O$ (Merck, Germany) and 250 mL of sterile distilled water (FS MAWPC)
- Group 4: One gram of EGWPC mixed with 10% $CaCl_2.2H_2O$ and 250 mL of sterile distilled water (FS EGWPC).

Cells culture

Human DPSCs cell line isolated from third molars (adult) and characterized was purchased from AllCells (MD, USA) and was used in this study. The complete growth medium was prepared by supplementing 500 ml of basal cell growth medium (AllCells), with 50 ml of human mesenchymal stem cell stimulatory supplements (AllCells). DPSCs were then thawed and cultured.

Dentinogenic differentiation potential using real-time polymerase chain reaction

The aim of this experiment was to examine the expression of bone gamma-carboxyglutamate (gla) protein (*BGLAP*), dentin sialophosphoprotein (*DSPP*), runt-related transcription factor 2 (*RUNX2*), and secreted phosphoprotein 1 (*SPP1*) [Table 1] in DPSCs after incubation in extracts of MAWPC, EGWPC, FS MAWPC, and FS EGWPC after 1, 3, 7, and 14 days.

Preparation of the extracts

After mixing, placement in acrylic molds, and setting, the study samples were retrieved, weighed, and sterilized using ultraviolet light. DPSCs cell line from passage 7 was used for this study. Based on the results of methylthiazolyldiphenyl-tetrazolium assay demonstrated in our previous study on DPSCs after 1 and 3 days,[13] extracts at 12.5 mg/ml concentration presented the best noncytotoxic cell viability values for both formulations.[13] Therefore, this concentration was selected for examining the dentinogenic differentiation potential on DPSCs cultured in 25-T flasks (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). The amount of extracts needed was determined based on the consumable amounts for every 25-T flask at every time interval (each flask consumes 5 ml), given that the extracts should be exchanged every 3rd day.

Table 1: Gene names and symbols used for evaluation of the dentinogenic differentiation potential of the test materials

Gene name	Gene symbol/s
Bone gamma-carboxyglutamate (gla) protein	*BGLAP* (*OCN*) Hs587814_g1
Dentin sialophosphoprotein	*DSPP* (*DMP3*) Hs00171962_m1
Runt-related transcription factor 2	(*RUNX2*) Hs00231692_m1
Secreted phosphoprotein 1	*SPP1* (*OPN*) Hs959010_m1
Beta actin (housekeeping gene)	(*ACTB*) Hs01060665_g1

Due to different incubation times (1, 3, 7, and 14 days), DPSCs were applied in the culture flasks at different numbers to prevent overconfluence. Flasks scheduled for 1 and 3 days of incubation were loaded with about 170,000 cells. This number was selected because it is approximately the amount required for confluence (80%–100%) of DPSCs after 3–4 days. Flasks scheduled for 7 and 14 days were loaded with 125,000 and 80,000 cells, respectively. After the cells were applied in the labeled flasks, 5 ml of each extract was added after 1 day of incubation to allow for cell attachment. The extracts were changed every 3rd day. A control group (normal prepared medium) was designed for every time interval.

Extraction, measuring the concentration, and purity of RNA

After application of the extracts and at each time interval, the RNA was extracted from the DPSCs. The protocol for RNA extraction designed by Ambion kit (Life technologies, CA, USA) was followed. The concentration of RNA was measured using a spectrophotometer (Eppendorf, Hamburg, Germany). After this, RNA electrophoresis on denaturing agarose gel was also performed for double-checking the quality of RNA. The overall quality of RNA preparation was assessed using electrophoresis on a denaturing agarose gel. Electrophoresis showed successful isolation of the RNA (28S and 18S ribosomal RNA bands were identified).

Preparation of cDNA from the extracted RNA and gene expression using real-time polymerase chain reaction

The amount of RNA per 30 μl reaction was adjusted at 75 ng. Nine microliters having 50 ng RNA and nuclease-free water was added into 10 μl of reverse transcription (RT)

buffer (TaqMan, Applied biosystems, CA, USA) and 1 µl RT enzyme mix (TaqMan) in a polymerase chain reaction (PCR) tube. The thermocycler (Eppendorf, Hamburg, Germany) was set at standard mode, and the steps were adjusted according to the manufacturer instructions (Step 1: 37°C for 60 min, Step 2: 95°C for 5 min, and finally step 3: 4°C). After the cycle was finished, the tubes were stored at −20°C.

Each group had four genes to be examined in triplicates. Three additional wells were added for the housekeeping gene (Beta actin). One 96-well plate was prepared for each time interval. The PCR reaction mix was prepared for each group according to the manufacturer's instructions. In addition, nine wells were added as no template control to exclude the possibility of contamination. The plate was then covered by a clear transparent film (Applied Biosystems, USA), centrifuged at 3000 rpm for 2 min, and then introduced into the real-time PCR machine (7500 software, Applied Biosystems, CA, USA). After the run was finished, the cycle threshold (cT) values were determined, and the level of gene expression was classified according to Thompson *et al.*[18] After that, the delta cT (mean cT of test gene−mean cT of beta actin) and delta delta cT (mean delta cT untreated−mean delta cT treated) values were calculated.

Statistical analyses

One-way analysis of variance followed by *post hoc* (Tukey's honestly significant difference) was performed to analyze the data collected (Statistical Package for the Social Sciences (SPSS) version 20, Chicago, IL, USA). The level of significance was set at 0.05 ($P = 0.05$).

Results

Multicomponent plot and classification of gene expression using the cycle threshold values

Results showed that the expression of *RUNX2* gene was the highest in all groups [Table 2]. The expression of *SPP1* gene was higher than *BGLAP* and *DSPP* genes. The latter two genes basically showed low and very low expressions [Table 2]. The expression of *DSPP* gene was undetermined in FS EGWPC (day 1 and 3), EGWPC (day 7), and control (day 14) [Table 2]. At day 14, the RNA extraction from FS MAWPC was insufficient because the cells were very few. Same finding was observed with FS EGWPC despite the successful extraction of the RNA. Data obtained from this group were excluded because of the cytotoxic effect at day 14.

Relative quantification using delta cycle threshold values (comparison of the expression of genes)

Table 3 shows the mean values, standard deviation, and statistical analysis after 1, 3, 7, and 14 days. Intergroup comparisons are listed in Table 4.

Significant differences were observed between MAWPC and EGWPC, FS MAWPC, and FS EGWPC in 7 out of

Table 2: Expression levels of genes in dental pulp stem cells treated with extracts of normal set formulations

Gene	Groups	Expression			
		Day 1	Day 3	Day 7	Day 14
BGLAP	MAWPC	Low	Low	Low	Low
	EGWPC	Low	Low	Very low	Low
	FS MAWPC	Low	Low	Low	X
	FS EGWPC	Low	Low	Low	XX
	Control	Low	Low	Low	Low
DSPP	MAWPC	Very low	Very low	Very low	Very low
	EGWPC	Very low	Very low	EL/NE	Very low
	FS MAWPC	Very low	EL/NE	Very low	X
	FS EGWPC	EL/NE	EL/NE	Very low	XX
	Control	Very low	Very low	Very low	EL/NE
RUNX2	MAWPC	High	High	High	High
	EGWPC	High	High	High	High
	FS MAWPC	High	High	High	X
	FS EGWPC	High	High	High	XX
	Control	High	High	High	High
SPP1	MAWPC	Low	Low	Low	Low
	EGWPC	Low	Low	Moderate	High
	FS MAWPC	Moderate	Moderate	Moderate	X
	FS EGWPC	Low	Moderate	Moderate	XX
	Control	Low	Low	Very low	Very low

20-23 – Extremely high, 23-26 – Very high, 26-29 – High, 29-32 – Moderate, 32-35 – Low, 35-38 – Very low, >38 – Extremely low/not expressed. X – No enough cells for RNA extraction, XX – Results were excluded because of the toxic effect of the extract. MAWPC – Malaysia white Portland cement, EGWPC – Egypt white Portland cement, FS – Fast set, EL/NE – Extremely Low/Not Expressed, *RUNX2* – Runt-related transcription factor 2, *BGLAP* – Bone gamma-carboxyglutamate (gla) protein, *DSPP* – Dentin sialophosphoprotein, *SPP1* – Secreted phosphoprotein 1

Table 3: Mean, standard deviation, and one-way analysis of variance statistical analysis for the delta cycle threshold values after 1, 3, 7, and 14 days of incubation

Gene	Material ($n=3$)	Mean (SD) Day 1	SE Day 1	*P*	Mean (SD) Day 3	SE Day 3	*P*	Mean (SD) Day 7	SE Day 7	*P*	Mean (SD) Day 14	SE Day 14	*P*
BGLAP	MAWPC	16.193 (0.131)	0.076	<0.001	16.835 (0.194)	0.112	0.047	15.725 (0.391)	0.226	<0.001	13.697 (0.090)	0.052	<0.001
	EGWPC	15.994 (0.086)	0.050		16.860 (0.230)	0.133		17.836 (0.255)	0.147		12.617 (0.165)	0.096	
	FS MAWPC	15.324 (0.147)	0.085		16.535 (0.307)	0.177		16.733 (0.251)	0.145		X	X	
	FS EGWPC	16.165 (0.005)	0.003		16.298 (0.198)	0.114		16.578 (0.480)	0.277		XX	XX	
	Control	15.688 (0.119)	0.069		16.169 (0.429)	0.248		16.269 (0.191)	0.110		14.181 (0.429)	0.248	
DSPP	MAWPC	17.732 (0.301)	0.175	<0.001	18.293 (0.378)	0.218	0.192	19.998 (0.743)	0.429	<0.001	15.437 (0.593)	0.342	0.653
	EGWPC	18.126 (1.162)	0.671		18.648 (0.418)	0.241		Und	Und		15.647 (0.321)	0.185	
	FS MAWPC	18.690 (1.254)	0.724		Und	Und		18.158 (0.419)	0.242		X	X	
	FS EGWPC	Und	Und		Und	Und		18.020 (0.348)	0.201		XX	XX	
	Control	17.438 (0.002)	0.001		18.071 (0.171)	0.099		17.780 (0.234)	0.135		Und	Und	
RUNX2	MAWPC	9.369 (0.229)	0.132	<0.001	8.771 (0.183)	0.105	<0.001	8.792 (0.029)	0.017	0.001	6.919 (0.092)	0.053	<0.001
	EGWPC	9.050 (0.149)	0.086		8.996 (0.184)	0.106		9.587 (0.281)	0.162		7.034 (0.111)	0.064	
	FS MAWPC	8.376 (0.234)	0.135		8.790 (0.102)	0.059		8.867 (0.190)	0.110		X	X	
	FS EGWPC	9.406 (0.047)	0.027		9.386 (0.082)	0.047		9.280 (0.098)	0.057		XX	XX	
	Control	9.313 (0.151)	0.087		8.704 (0.026)	0.015		9.035 (0.063)	0.036		7.724 (0.051)	0.029	
SPP1	MAWPC	14.552 (0.200)	0.115	<0.001	16.102 (0.199)	0.115	<0.001	15.495 (0.234)	0.135	<0.001	14.952 (0.212)	0.123	<0.001
	EGWPC	14.476 (0.049)	0.028		15.355 (0.199)	0.115		13.171 (0.130)	0.075		8.720 (0.179)	0.103	
	FS MAWPC	12.907 (0.132)	0.076		12.663 (0.158)	0.091		12.535 (0.137)	0.079		X	X	
	FS EGWPC	14.199 (0.022)	0.012		13.182 (0.117)	0.068		13.032 (0.025)	0.014		XX	XX	
	Control	15.635 (0.150)	0.086		16.907 (0.069)	0.040		15.179 (0.111)	0.064		16.77 (0.501)	0.289	

Und - Undetermined, X – No enough cells for RNA extraction, XX – Results were excluded because of the toxic effect of the extract. SE – Standard error, SD – Standard deviation, MAWPC – Malaysia white Portland cement, EGWPC – Egypt white Portland cement, FS – Fast set, *RUNX2* – Runt-related transcription factor 2, *BGLAP* – Bone gamma-carboxyglutamate (gla) protein, *DSPP* – Dentin sialophosphoprotein, *SPP1* – Secreted phosphoprotein 1

15 and 6 out of 10 comparisons, respectively. While more expressions in EGWPC group were observed in four comparisons and three for MAWPC, all FS formulations showed higher expressions for FS MAWPC compared to FS EGWPC ($P < 0.05$) [Table 4].

Significant differences were also observed between MAWPC and FS MAWPC, EGWPC, and FS EGWPC in 8 out of 11 and 3 out of 9 comparisons, respectively [Table 4]. While more expressions in FS MAWPC group were observed in six comparisons compared to only two for MAWPC, FS EGWPC showed higher expressions in two comparisons compared to only one for EGWPC ($P < 0.05$) [Table 4].

Relative quantification using delta delta cycle threshold values

The expression of each gene was normalized with the control group to determine the upregulation and downregulation of each gene [Figures 1 and 2]. A comparison of the up/downregulations between MAWPC and FS MAWPC showed that the addition of $CaCl_2.2H_2O$ to MAWPC resulted in an upregulation of *BGLAP* gene (day 1) and *RUNX2* gene (day 1). In addition, it increased the upregulation of *SPP1* gene at all day intervals though MAWPC induced an upregulation to *BGLAP* (day 7) [Figure 2]. The addition of $CaCl_2.2H_2O$ to EGWPC resulted in an increased upregulation of *SPP1* gene at all day intervals though EGWPC induced an upregulation to *RUNX2* (day 1) [Figure 2].

Discussion

The development of molecular biology is one of the greatest achievements in biological science. The technological innovation of real-time PCR has become increasingly important in research laboratories due to its capacity for generating quantitative results.[19] Advantages of real-time PCR include the ease of quantification, greater sensitivity, reproducibility and precision, rapid analysis, better control of quality in the process, and a lower risk of contamination.[19] Accordingly, real-time PCR was applied in this study to examine the dentinogenic differentiation potential of PC-based formulations on DPSCs.

BGLAP, DSPP, RUNX2, and *SPP1* were selected as target genes, and were examined at different time intervals (1, 3, 7, and 14 days) to evaluate the ability of extracts of normal and FS WPC formulations to induce early and/or late expression of these target genes in DPSCs. Harvesting the cells after each time interval was performed to extract the RNA. However, it was noted that the RNAs extracted from DPSCs incubated in extracts of FS MAWPC group for 14 days were not enough because the cells were very few in number compared to normal set formulations. A similar observation has been reported in another study when cementoblasts were examined with intermediate restorative material (IRM) and MTA, and the cells did not yield enough RNA with IRM due to its cytotoxic effect.[20]

During the experiment, it was observed that the DPSCs (P7) became reduced in number (day 14), especially for the FS

Table 4: *Post hoc* intergroup comparisons for all genes after 1, 3, 7, and 14 days of incubation

Comparisons	Mean difference Day 1	*P*	Mean difference Day 3	*P*	Mean difference Day 7	*P*	Mean difference Day 14	*P*
BGLAP								
MAWPC/EGWPC	0.199	0.248	−0.025	1.000	−2.111	<0.001	1.080	0.006
MAWPC/control	0.505	0.002	0.667	0.097	−0.544	0.325	−0.485	0.295
EGWPC/control	0.306	0.042	0.692	0.083	1.567	0.001	−1.564	<0.001
FS MAWPC/FS EGWPC	−0.841	<0.001	0.237	0.842	0.155	0.976	-	-
FS MAWPC/control	−0.364	0.015	0.366	0.545	0.463	0.467	-	-
FS EGWPC/control	0.477	0.002	0.129	0.979	0.309	0.782	2.975	-
MAWPC/FS MAWPC	0.869	<0.001	0.300	0.704	−1.008	0.025	-	-
EGWPC/FS EGWPC	−0.171	0.372	0.562	0.189	1.258	0.006	-	-
DSPP								
MAWPC/EGWPC	−18.204	<0.001	−0.355	0.456	-	-	−0.210	0.889
MAWPC/control	0.294	0.974	0.222	0.716	20.133	<0/001	-	-
EGWPC/control	18.498	<0.001	0.577	0.175	-	-	-	-
FS MAWPC/FS EGWPC	-	-	-	-	0.138	0.983	-	-
FS MAWPC/control	19.062	<0.001	-	-	0.379	0.766	-	-
FS EGWPC/control	-	-	-	-	0.241	0.923	-	-
MAWPC/FS MAWPC	−18.768	<0.001	-	-	19.755	<0.001	-	-
EGWPC/FS EGWPC	-	-	-	-	-	-	-	-
RUNX2								
MAWPC/EGWPC	0.319	0.247	−0.224	0.288	−0.795	0.001	−0.114	0.779
MAWPC/control	0.056	0.994	0.068	0.965	−0.243	0.401	−0.805	<0.001
EGWPC/control	−0.263	0.409	0.292	0.115	0.552	0.012	−0.691	<0.001
FS MAWPC/FS EGWPC	−1.030	<0.001	−0.596	0.002	−0.413	0.063	-	-
FS MAWPC/control	−0.937	<0.001	0.086	0.922	−0.168	0.710	-	-
FS EGWPC/control	0.093	0.093	0.682	0.001	0.245	0.391	-	-
MAWPC/FS MAWPC	0.993	<0.001	−0.018	1.000	−0.075	0.976	-	-
EGWPC/FS EGWPC	−0.356	0.172	−0.390	0.028	0.307	0.211	-	-
SPP1								
MAWPC/EGWPC	0.076	0.946	0.747	0.001	2.324	<0.001	6.232	<0.001
MAWPC/control	−1.083	<0.001	−0.805	0.001	0.3167	0.124	−1.816	0.001
EGWPC/control	−1.159	<0.001	−1.552	<0.001	−2.008	<0.001	−8.048	<0.001
FS MAWPC/FS EGWPC	−1.292	<0.001	−0.519	0.015	−0.497	0.012	-	-
FS MAWPC/control	−2.728	<0.001	−4.244	<0.001	−2.644	<0.001	-	-
FS EGWPC/control	−1.436	<0.001	−3.725	<0.001	−2.147	<0.001	-	-
MAWPC/FS MAWPC	1.644	<0.001	2.920	<0.001	2.960	<0.001	-	-
EGWPC/FS EGWPC	0.277	0.136	2.173	<0.001	0.139	0.758	-	-

MAWPC – Malaysia white Portland cement, EGWPC – Egypt white Portland cement, FS – Fast set, *RUNX2* – Runt-related transcription factor 2, *BGLAP* – Bone gamma-carboxyglutamate (gla) protein, *DSPP* – Dentine sialophosphoprotein, *SPP1* – Secreted phosphoprotein 1

formulations, probably due to the cytotoxic effect of the continuous exposure to the extracts for long time intervals (14 days) despite the favorable cytotoxic profile of that concentration (6.25 mg/ml) after 1 and 3 days of incubation demonstrated in our previous study.[13] Notably, DPSCs behaved differently toward both FS MAWPC and FS EGWPC, and more cells were obtained from FS EGWPC. Therefore, the research hypothesis for this objective is rejected.

BGLAP is an intermediate/late osteogenic marker gene,[21-23] and it was selected in this study to examine the ability of extracts of PC-based materials to induce expression of this gene. Results showed that the expression of *BGLAP* in DPSCs was low at all-time intervals, and generally, was downregulated in both normal and FS formulations at days 1, 3, and 7. However, at day 14, the gene was upregulated in normal set formulations, and the difference was significantly higher in EGWPC than MAWPC and control (the difference between MAWPC and control was insignificant). This finding demonstrates that extracts of normal set WPCs can induce a late expression of *BGLAP*.

Results obtained from this study are quite consistent with the existing literature on the ability of normal set WPC-based materials to induce the expression of *BGLAP*, but some differences exist which might be attributed to the different cell types or materials used or the time interval chosen for examination. One study[24] found that the expression of *BGLAP* was significantly higher in MG63 osteosarcoma cell line after treatment with a PC-based material for 7 days compared to day

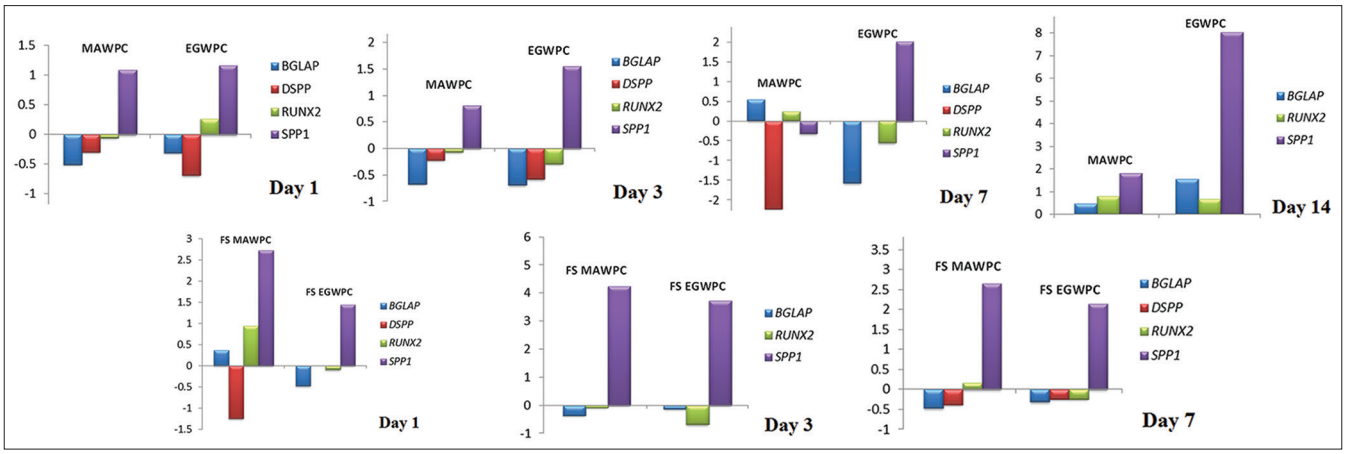

Figure 1: Relative quantification of the genes expressions (the gene expression of the control group was set at 0), after 1, 3, 7, and 14 days

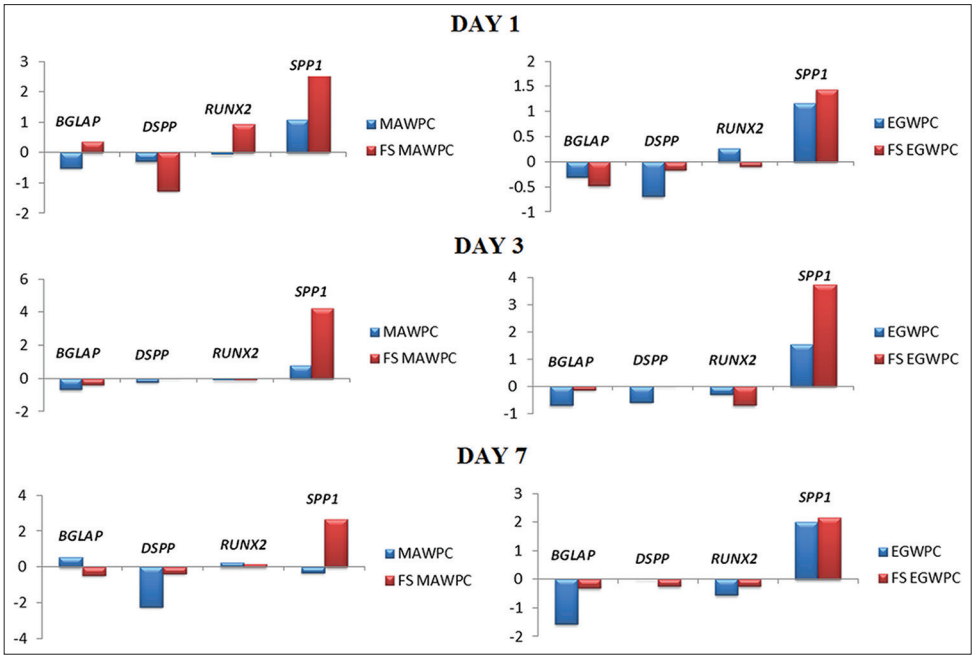

Figure 2: Relative quantification of the genes expressions of the fast set compared to the normal set formulations after 1, 3, and 7 days

14 with DPSCs found in our study. Conversely, two studies reported the upregulation of *BGLAP* as early as 1 day after treatment with PC-based materials on MC3T3-E1 osteoblast cells and HDPCs, respectively.[25,26]

DSPP is a late marker gene for odontoblast differentiation.[27] DPSCs have the capacity to differentiate into odontoblast-like cells.[28] Therefore, *DSPP* was selected in this study to examine the ability of extracts of PC-based formulations to induce the expression of *DSPP* in DPSCs. Results showed that the expression of *DSPP* in all groups was either very low or undetermined; however, for the normal set formulations, the DSPP was upregulated at day 14. Our findings are consistent with one study[14] which confirmed the ability of PC to induce the expression of *DSPP* in HDPCs after 7 days compared to 14 days reported in another study on the same cell type,[15] which is similar to the expression behavior of DPSCs examined in this study. In contrast, Schneider *et al.*[29] did not observe an increase in the expression of *DSPP* when stem cells from the apical papillae were treated with a WPC-based material (WMTA) for 7, 14, and 21 days.

RUNX2 is one of the most common osteogenic markers. *RUNX2* plays an important role in the process of osteoblast differentiation and maturity, and it is the earliest and the most specific marker for bone formation.[30] Results showed that the expression of *RUNX2* is high in all groups at all-time intervals (early and late). The upregulation was fluctuating at days 1, 3, and 7. However, the upregulation was pronounced in the normal set formulation groups at day 14. Data presented in this investigation are consistent with one study[31] that compared the differentiation potential of dental pulp stromal cells of permanent teeth and deciduous teeth cultured on a PC-based material for 1, 2, 4, and 7 days. The authors found

that the expression of *RUNX2* increased at day 2 and then gradually decreased until day 7, which is consistent with this study; however, results of this study showed an upregulation for the expression of *RUNX2* after 14 days. This emphasizes the importance to extend the examination procedure to evaluate the dentinogenic differentiation potential for longer periods (more than 1 week).[24]

SPP1 is one of the intermediate osteogenic gene markers that plays an important role in osteogenesis.[23] Findings showed that the expression of *SPP1* was low in normal set formulations and control at days 1 and 3, compared to moderate expressions in the FS formulations (except FS EGWPC day 1). At day 7, the FS formulations continued to induce moderate expression. EGWPC showed moderate and high expressions at days 7 and 14, respectively, compared to low and very low expressions in MAWPC and control, respectively. The upregulation is observed in almost all groups at all-time intervals, consistent with one study,[24] but it was more pronounced with the FS formulations. Results indicate that the addition of $CaCl_2.2H_2O$ to WPC enhanced the expression of *SPP1* significantly. Notably, detectable variations in the expression of *SPP1* in MAWPC and EGWPC were observed, and the difference was significant.

Generally, it can be concluded that the expression of osteogenic/dentinogenic genes is upregulated with time, and this is consistent with the current literature. The addition of $CaCl_2.2H_2O$ to MAWPC and EGWPC resulted in (a) an upregulation of downregulated genes, namely, *BGLAP* (MAWPC - day 1), *RUNX2* (MAWPC - day 1), and *SPP1* (MAWPC - day 7) and (b) more upregulation of *SPP1* (all groups at all-time intervals). This observation indicates that FS formulations may induce DPSCs to express ostengenic/dentinogenic gene markers, especially *SPP1*, more than normal set counterparts, thus rejecting the research hypothesis. This might be attributed to the higher pH values obtained by this combination compared to pure cements.[10] It is worth noting that the dentinogenic differentiation potential of FS MAWPC was induced more in DPSCs compared to FS EGWPC. This might be attributed to the different chemical composition of both materials.[13]

Combining results for the osteogenic/dentinogenic differentiation potential of the FS formulations with results of our previous study on cytotoxic effects and cell attachment properties[13] indicate that the more cytotoxic effects of FS formulations on DPSCs are partially compensated with a higher induction of DPSCs for osteogenic/dentinogenic genes expression.

The dentinogenic differentiation potential of the test materials was evaluated using *in vitro* models, which is one limitation of this study. Experimental models may not typically simulate the clinical situation where the material is applied on vital tissues having different types of cells, blood, and interstitial fluids. The application of test materials in a biological biosystem may affect the response of related cell populations to the material. The evaluation of different types of radiopacifiers as potential additives to the normal and FS formulations of MAWPC and EGWPC could be an area of future research.

Conclusions

The dentinogenic differentiation of DPSCs shows different expression profiles to extracts of normal and fast formulations of WPC. Extracts of WPC of different origin have different dentinogenic differentiation potential on DPSCs.

Acknowledgment

We would like to express their gratitude to members of the craniofacial laboratory (School of Dental Sciences), Universiti Sains Malaysia (USM), for their technical support. This study was supported by the USM Research University Grant number 1001/PPSG/813056.

Financial support and sponsorship

This study was supported by the USM Research University Grant number 1001/PPSG/813056.

Conflicts of interest

There are no conflicts of interest.

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