Effects of multi-components released from S-PRG filler on the activities of human dental pulp-derived stem cells

Hiroshi ISHIGURE¹, Harumi KAWAKI², Kohei SHINTANI³, Kyohei UENO², Masako MIZUNO-KAMIYA⁴, Eiji TAKAYAMA², Masato HOTTA⁵, Nobuo KONDOH² and Toru NIKAI¹

¹ Department of Operative Dentistry, Division of Oral Functional Science and Rehabilitation, Asahi University School of Dentistry, 1851-1 Hozumi, Mizuho, Gifu 501-0296, Japan

² Department of Oral Biochemistry, Division of Oral Structure, Function, and Development, Asahi University School of Dentistry, 1851-1 Hozumi, Mizuho, Gifu 501-0296, Japan

³ Department of Dental Materials Science, Division of Oral Functional Science and Rehabilitation, Asahi University School of Dentistry, 1851-1 Hozumi, Mizuho, Gifu 501-0296, Japan

⁴ Chemistry Laboratory, Department of Management and Information studies, Asahi University School of Business Administration, 1851-1 Hozumi, Mizuho, Gifu 501-0296, Japan

⁵ Asahi University, 1851-1 Hozumi, Mizuho, Gifu 501-0296, Japan

Corresponding author, Harumi KAWAKI; E-mail: kawaki@dent.asahi-u.ac.jp

Numerous studies have shown that the sustained release of ions from dental restorative materials have acid buffering capacity, prevents tooth enamel demineralization, and inhibits bacterial adhesion. Herein, the release behavior and bioresponsiveness of ions released from surface pre-reacted glass-ionomer (S-PRG) fillers were investigated in different types of media based on human dental pulp-derived stem cell (hDPSC) responses. The hDPSCs were cultured for 1–7 days in S-PRG eluates diluted with varying amounts of cell culture media. S-PRG released several types of ions, such as F^- , Sr^{2+} , Na^+ , Al^{3+} , BO_3^{3-} , and SiO_3^{2-} . The balance of eluted ions differed depending on the dilution and solvent, which in turn affected the cytotoxicity, cell morphology, cell proliferation, and alkaline phosphatase activity of hDPSCs, among other properties. The results suggest that tailored S-PRG filler eluates could be designed and prepared for application in dental practice.

Keywords: Multi-ion, Stem cell, Boron, Strontium, Fluoride

INTRODUCTION

Restorative materials and cements containing fluoride have been used for many years to suppress the occurrence and severity of secondary dental caries¹. Materials containing fluoroaluminosilicate glass, such as glass-ionomer cements, are common adhesive restorative materials with sustained fluoride release². In recent years, various materials have been developed to further improve the functionality of restorative materials. For example, a surface pre-reacted glass-ionomer (S-PRG) filler was developed based on glass-ionomer cement technology³. This filler contains a glass-ionomer formed *via* an acid–base reaction between fluoroboroaluminosilicate glass and polycarboxylic acid, and releases several types of ions such as fluoride (F^-), strontium (Sr^{2+}), sodium (Na^+), aluminum (Al^{3+}), boric acid (BO_3^{3-}), and silicic acid (SiO_3^{2-})^{4–6}. Analytical studies on the effects of different dental materials containing S-PRG fillers have reported that the sustained release of ions prevents tooth enamel demineralization^{7,8}, and acid buffering capacity^{8,9}, and inhibits bacterial adhesion^{10–14}. Hence, much attention has been paid to these materials as bioactive fillers. The ion release behavior of S-PRG fillers in distilled water (DW) is dependent on the concentration of the filler in the DW¹⁵.

However, although several studies have been conducted on the bioresponse of dental materials containing S-PRG fillers^{8–14}, few studies have assessed the release behavior and bioresponsiveness of the ions released from the filler^{7,15–17}. To improve our understanding of the release behavior and bioresponsiveness of ions released from S-PRG fillers, we examined whether the ratio of each ion released in the S-PRG filler eluate affected the responses of hDPSC.

In this study, eluates with varying amounts of DW or other media were prepared to provide for various media, which were used as cell culture media for hDPSCs. The cell responses such as cytotoxicity, cell proliferation, and cell morphology were observed. The null hypothesis tested was that multiple ions released from the S-PRG filler did not affect the activity of hDPSCs.

MATERIALS AND METHODS

Preparation of the S-PRG filler eluates

S-PRG filler (Lot 041501) was provided by Shofu (Kyoto, Japan). Shofu Inc. also supplied two S-PRG filler eluates (Lot 041211 and Lot 121501) prepared by stirring the filler in DW (1/1 volume ratio) at room temperature for 24 h. Based on this procedure, we prepared modified eluates with different filler/solvent volume ratios using two solvents (DW or α -modified Eagle's minimum essential medium (α -MEM)) (Table 1). The α -MEM is

Color figures can be viewed in the online issue, which is available at J-STAGE.

Received Oct 30, 2020; Accepted Mar 17, 2021

doi:10.4012/dmj.2020-390 JOI JST.JSTAGE/dmj/2020-390

Table 1 Eluting conditions of preparation of S-PRG filler eluates in various

		Filler/Solvent ratio (w/v)			
		I	II	III	IV
Solvent	DW (W)	1/1	1/10	1/100	1/1000
	W-I	W-I	W-II	W-III	W-IV
	α -MEM (M)	—	—	M-III	M-IV

a medium based on minimum essential Eagle medium (MEM) published in 1971 by Stanners *et al.*¹⁸⁾. It contains more non-essential amino acids, sodium pyruvate, and vitamins (ascorbic acid, biotin, and cyanocobalamin) compared with MEM. This α -MEM is recommended and used for a wide variety of cell culture applications including hDPSC¹⁹⁾ and MC3T3-E1²⁰⁾ cell culture. The modified eluates in DW were prepared at filler/solvent volume ratios of 1/1 (denoted as W-I; supplied by Shofu Inc.), 1/10 (W-II), 1/100 (W-III), and 1/1000 (W-IV); while those in α -MEM were prepared at filler/solvent volume ratios of 1/100 (M-III) and 1/1000 (M-IV). The eluates were centrifuged at 6,300×g for 15 min to remove the filler, and then sterilized by filtering with a MILLEX® GV filter (Merck Millipore, Darmstadt, Germany) with a pore size of 0.22 μ m. Initially, we also prepared M-I (filler/solvent volume ratios of 1/1) and M-II (1/10), when comparing these diluted media with W-I and W-II diluted media. As a result of comparing M-I and M-II diluted media with W-I and W-II diluted media, there was no remarkable difference in the ratio of eluted ions, therefore they were not used in the experiments.

Preparation of experimental media containing S-PRG filler eluates

The S-PRG filler eluates were diluted with α -MEM to produce serially diluted media containing various concentrations of the components that were released from the S-PRG filler. First, the S-PRG filler eluates were diluted according to the following procedure. Powdered α -MEM was dissolved in 1/2 amount of DW described in the supplier's instruction manual, and then mixed with the W-I eluate at a 1/1 volume ratio. The pH was adjusted to 7.4 by adding an aqueous solution of 10% sodium bicarbonate. Fetal bovine serum (FBS; Thermo Fisher Scientific, Yokohama, Japan) was added to a final concentration of 2% with 1/100 (v/v) antibiotic penicillin–streptomycin liquid (containing 5,000 U penicillin and 5 mg/mL streptomycin) (Thermo Fisher Scientific Japan, Yokohama, Japan). Separately, α -MEM was prepared according to the supplier's instruction at a predetermined concentration (control α -MEM), and FBS and antibiotics were added in a similar manner. Then, the α -MEM containing W-I eluate was sequentially diluted with control α -MEM to 1/2^N (N=1–14) to produce serially diluted media.

Next, an experimental medium containing 1/500 (v/v) of the W-I eluate was prepared (denoted as E(W-I)), and then serially diluted to 1/2, 1/5, and 1/10 with

α -MEM. Additionally, a medium prepared by dissolving powdered α -MEM into the W-IV eluate was produced (denoted as E(W-IV)), and serially diluted to 1/2, 1/5, and 1/10 with α -MEM.

Based on the results of the cell culture experiments using the media described above, five experimental media were prepared as follows. E1; W-I diluted to 1/500 with α -MEM, E2; W-II diluted to 1/100, E3; M-III diluted to 1/10, E4; M-IV without dilution, and E5; M-IV diluted to 1/2. To avoid the complexity of providing for the media, M-III and M-IV were prepared using adjusted α -MEM (Fujifilm Wako Pure Chemical, Osaka, Japan). For each experimental medium, FBS and antibiotics were added in a similar manner described above.

Quantification of components derived from the S-PRG filler

Among the components in the prepared S-PRG filler eluates and eluate-containing media, B, Na, Al, Si, and Sr were measured using inductively coupled plasma (ICP) emission spectrometry (ICPS-8000, Shimadzu, Tokyo, Japan). In addition, F⁻ ions were also measured using an ion meter equipped with ion electrodes (Fluoride electrode, Model 9609BN; pH/ion meter, Model 720A; Orion Research, Beverly, MA, USA). Each experimental S-PRG filler eluate was measured three times and the average value was reported.

Cell culture

The hDPSCs (Allcells, Emeryville, CA, USA) were cultured in α -MEM containing 10% FBS and 1/100 volume of antibiotic liquid at 37°C and 5% CO₂. When the cells reached subconfluence, the cells were harvested using a 0.25% trypsin solution containing 0.02% EDTA and subcultured according to a conventional method. The hDPSCs subcultured 12–14 times were used in the experiment.

Toxicity evaluation of media containing S-PRG filler eluate at various concentrations

The hDPSCs were seeded at a density of 4,000 cells/well in 96-well plates. Six hours after seeding, the medium was replaced with a serially diluted medium containing several components derived from S-PRG filler. Next, the cells were cultured for 24 h, and the activity of lactate dehydrogenase (LDH), which leaks out of the cell due to cell membrane damage, was measured as an indicator of cytotoxicity. An α -MEM solution without S-PRG filler-derived components was used as a control.

Table 2 Concentration of eluted components in two types of experimental medium containing the S-PRG filler eluate

		Al	B	Si	Sr	F
E (W-I)	(ppm)	0.11	2.67	0.06	0.53	0.37
	(μ M)	4.08	247.00	2.14	6.05	19.47
E (W-IV)	(ppm)	0.44	6.79	2.36	28.44	11.90
	(μ M)	16.31	628.12	84.01	324.58	626.32

E (W-I): containing 1/500 (v/v) of W-I, E (X-IV): dissolved powder of α -medium with W-IV

The cells were treated according to the manufacturer's instructions using a cytotoxicity detection kit (Takara Bio, Otsu, Japan). The media were collected in new 96-well plates and the absorbance at 492 nm was measured with a plate reader (Infinite Pro-200, TECAN, Tokyo, Japan). Six wells per experimental group were used for measurement.

Evaluation of hDPSC proliferation using WST-1 reagent

The hDPSCs were seeded at a density of 4,000 cells/well in 96-well plates. Six hours after seeding, these were replaced with the serially diluted media as above. After the hDPSCs were cultured further 24 or 72 h, the cells were treated with WST-1 reagent (Cell Counting Kit, Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. Following this, the culture medium containing the lysed cells was collected in new 96-well plates, and the absorbance at 450 nm was measured with a plate reader to obtain the hDPSC proliferation index. Six wells per experimental group were used for evaluation.

Evaluation of hDPSC proliferation using AlamarBlue®

AlamarBlue® (Thermo Fisher Scientific Japan) was used to evaluate hDPSC proliferation over time. The hDPSCs were seeded at a density of 12,000 cells/well in 48-well plates. Six hours after seeding, these were replaced with E (W-I) and E (W-IV) dilution series experimental media shown in Table 2.

These were replaced with the experimental media (E(W-I) and E(W-IV)). After the hDPSCs were cultured further 48 h, the cells were treated with AlamarBlue® reagent according to the manufacturer's instructions. After reacting with AlamarBlue® reagent for 1 h, 200 μ L of the medium was collected from each well using 96-well black plates (Corning Costar). The fluorescence intensity was measured at an excitation wavelength of 560 nm and emission wavelength of 590 nm and used as an index of hDPSC proliferation. For comparison, α -MEM without S-PRG filler-derived components was used as a control. After collecting the medium containing AlamarBlue®, the medium was replaced, and the hDPSC culture was continued. Six wells per experimental group were used for evaluation.

Alkaline phosphatase (ALP) activity

The cells used in the AlamarBlue® proliferation

evaluations were cultured in experimental medium for further 7 days. In particular, the concentrations of serum in the five experimental media E1-E5 were increased to 10%. The cells were then fixed using 4% paraformaldehyde (PFA) (Nacalai Tesque, Kyoto, Japan) phosphate buffered saline (PBS). After washing twice with 25 mM tris-buffered saline (TBS) (pH 7.4) (Bio-Rad Laboratories, Hercules, CA, USA) and further washing with 25 mM TBS (pH 10.0), ALP activity staining was performed using the NBT/BCIP reagent kit (Nacalai Tesque). Three wells per experimental group were stained in each group and presented one result from each group.

Statistical analysis

Statistical analyses were performed using KaleidaGraph statistical analysis software (version 4.1.1, Hulinks, Tokyo, Japan) and one-way analysis of variance (ANOVA). Significance ($p < 0.05$) was determined using a multiple comparison test (Scheffé's method).

RESULTS

Quantification of components eluted from S-PRG filler in various S-PRG eluates and experimental media

Table 3 shows the concentrations of elements in the S-PRG filler eluates, as measured by ICP emission spectrometry and F⁻ ion concentration analysis. W-I contained approximately 1,300–1,500 ppm boron and 500 ppm sodium. The concentrations of other eluted elements were less than one third of that of boron. For eluting conditions II–IV, the ratio of other components to boron was increased. In particular, a greater amount of strontium and fluorine were present in W-IV. There were no significant differences in the concentrations of eluted elements in the S-PRG eluates using different solvents (DW or α -MEM).

Next, two experimental media (E(W-I), prepared by diluting the W-I eluate to 1/500 (approx. 1/2⁹), and E(W-IV), prepared from W-IV eluate, were employed for the purpose of understanding how to increase the amount of eluted components other than boron. Table 2 shows the "ppm" concentrations and "molar" concentrations calculated from the atomic weights of each component in these experimental media. E(W-I) contained 247 μ M boron and small amounts of other components. In contrast, E(W-IV) contained much more strontium and

Table 3 Concentration (ppm) of eluted components in each type of S-PRG filler eluate

	Al	B	Na	Si	Sr	F
W-I ^b	53.80	1,333.40	474.40	31.40	264.00	185.00
W-I ^c	21.51	1,520.72	535.57	10.94	224.64	153.50
W-II	2.12	247.28	129.70	3.60	93.68	99.50
W-III	1.57	39.74	23.65	3.52	66.98	41.20
W-IV	0.44	6.79	4.57	2.36	28.44	11.90
M-III ^a	N.D.	45.77	3,457.47	4.90	161.38	38.70
M-IV ^a	N.D.	2.44	3,516.43	0.47	20.71	8.50

^aLot 041501, ^bLot 041211, ^cLot 121501, N.D.: Not detected

Table 4 Concentration of eluted components in five types of experimental medium containing the S-PRG filler eluate

		Al	B	Si	Sr	F
E1	(ppm)	0.04	3.04	0.02	0.45	0.31
	(μ M)	1.48	281.22	0.71	5.14	16.32
E2	(ppm)	0.02	2.48	0.04	0.94	1.00
	(μ M)	0.74	229.41	1.42	10.73	52.63
E3	(ppm)	N.D.	4.58	0.49	16.14	3.87
	(μ M)	N.D.	423.68	17.44	184.20	203.68
E4	(ppm)	N.D.	2.44	0.47	20.71	8.50
	(μ M)	N.D.	225.72	16.73	236.36	447.37
E5	(ppm)	N.D.	1.22	0.24	10.36	4.25
	(μ M)	N.D.	112.86	8.54	118.24	223.68

E1: W-I diluted to 1/500 with α -MEM, E2: W-II diluted 1/100 with α -MEM, E3: M-III diluted 1/10 with α -MEM, E4: M-IV, E5: E4 diluted 1/2 with α -MEM, N.D.: Not detected

fluorine than E(W-I), and about 2.5 times as much boron. In this experiment, sodium was excluded because of the large amount of sodium (approximately 141 mM) in the α -MEM.

Using the modified S-PRG filler eluates listed in Table 1, five types of experimental media (E1–E5) with varying contents and weight ratios of S-PRG filler-derived components were prepared. Each component was quantified or calculated (Table 4) based on Table 3. The results showed that boron was contained in a large amount in E1 and E2 media compared to other elements, although strontium and fluorine were detected in a large amount in E3, E4, and E5 media.

Cytotoxicity of media containing S-PRG filler eluate W-I to hDPSCs

Figure 1 shows the results of the cytotoxicity evaluation after 1 day of culture in media obtained by serially diluting eluate W-I. Significant cytotoxicity was observed in the media containing $\geq 1/16$ W-I (N=1–4) when compared with the control (α -MEM). No significant

Fig. 1 Cytotoxic evaluation of the media containing S-PRG filler eluate at gradual concentrations to hDPSCs after 48 h of culture. Data are mean $\pm$ SD ($n=6$). The letters a–c indicate a significant difference between the results ($p<0.05$), and the error bars indicate standard error. N ($1/2^N$ =Concentration of S-PRG eluate W-I in the experimental medium)

Fig. 2 Evaluation of cell morphology in media containing S-PRG filler eluate at gradual concentrations. Representative example of hDPSCs in each experimental medium observed using a phase-contrast microscope (A, control; B, N=1; C, N=3; D, N=6; E, N=9; F, N=12). N ; $1/2^N$ =Concentration of S-PRG eluate W-I in the experimental medium. Scale bar: 200 μ m.

cytotoxicity was observed in the medium diluted more than 1/32 ($N>5$).

Cell morphology of hDPSCs in serially diluted media containing W-I eluate

Figure 2 shows the results of observation of the cell morphology of hDPSC, which were cultured under the same conditions as in the cytotoxicity evaluation, using a phase contrast microscope. No extended cells were observed in the medium with $N=1$ (Fig. 2B), where remarkable cytotoxicity was observed compared with the control (Fig. 2A). The cells in the medium with $N=3$ showed only slightly extended cells, and most of these cells were round and retracted (Fig. 2C). On the other hand, in the medium with $N=6$, where no cytotoxicity was observed (Fig. 1), the number of extended cells was increased (Fig. 2D), but no sufficient expansion was observed (Fig. 3A). The cells in the medium with $N=9$, most of the cells were extended (Fig. 2E), although the degree of expansion was slightly lower than that in the control (Fig. 3A). In addition, the cells in the medium with $N=12$, extended cells with the similar morphology as the control were observed (Fig. 2F).

Fig. 3 Evaluation of hDPSC proliferation in media containing S-PRG filler eluate at gradual concentrations.

The graphs show the cell proliferation after 1 day (A) and 3 days (B) of culture. Data are mean $\pm$ SD ($n=6$). The letters a–c indicate a significant difference between the results ($p<0.05$), and the error bars indicate standard error. N ($1/2^N$ =Concentration of S-PRG eluate W-I in the experimental medium)

Proliferation of hDPSCs in serially diluted W-I eluate media

The cell proliferation of the hDPSCs was evaluated (Fig. 3) under the same conditions as the cytotoxicity evaluation. After 1 day of culture, cell proliferation was significantly inhibited in the medium containing 1/2 to 1/64 (v/v) of W-I eluate ($N=1-6$) (Fig. 3A). After 3 days, the cell proliferation was significantly suppressed even in the 1/128 diluted medium ($N=7$) (Fig. 3B).

Proliferation of hDPSC in two types of eluate series media

The cell proliferation was assessed in two types of media shown in Figs. 4A and 4B with different eluted component ratios (Table 2), namely, the experimental E(W-I) medium and its dilution media, and the experimental E(W-IV) medium and its dilution media. After 2 days of culture, there was no change in cell proliferation in the E(W-I) series media. However, in the E(W-IV) series media, the medium diluted to 1/2 significantly

Fig. 4 Evaluation of hDPSC activities in 2 series of experimental media containing S-PRG filler-eluted components.

A and B: The graph shows cell proliferation after 2 days (A) and 7 days (B) of culture. Data are mean $\pm$ SD ($n=6$). The letters a–c indicate a significant difference between the results ($p<0.05$), and the error bars indicate standard error. C: Phase contrast microscopic images of ALP activity staining after 7 days of culture in media containing S-PRG filler eluate, control MEM (a), E(W-I)(b), and 1/2E(W-IV)(c). Scale bar: 200 μ m.

promoted hDPSC proliferation (Fig. 4A). After 7 days, cell proliferation was significantly suppressed in the E(W-I) series media. In the E(W-IV) series media, the medium diluted to 1/2 significantly promoted hDPSC proliferation, but there was no significant difference in the other three media (Fig. 4B).

Changes in ALP activity of hDPSC in two types of eluate series media

ALP activity staining was performed after the proliferation assessment (using the same cells) on the 7th day of culture (Fig. 4C). The results revealed that there was little ALP activity staining in the control (Fig. 4Ca), or 1/2E(W-IV) which promoted hDPSC proliferation (Fig. 4Cc). ALP activity was remarkably increased in the hDPSCs cultured in E(W-I) media (Fig. 4Cb).

Fig. 5 Effects of multi-components eluted from S-PRG filler on the hDPSC activities.

A: Cell proliferation in five types of experimental media containing S-PRG filler eluate after 2 days of culture. Data are mean $\pm$ SD ($n=6$). The letters a–c indicate a significant difference between the results ($p<0.05$), and the error bars indicate standard error. B: Bright field microscopic images of hDPSCs stained based on ALP activity after 7 days of culture; control (a), E1 (b), and E4 (c). Three wells were used for evaluation and the results are similar within the group, thus presenting one result from each group. Scale bar: 200 μ m.

Proliferation of hDPSC in five types of experimental media

hDPSC was cultured using five types of experimental media with different contents of S-PRG filler-derived components. The concentration of eluted components in the experimental media are listed in Table 4. The cell proliferation was evaluated at two days after seeded (Fig. 5A). The results revealed that there was no change in the cell proliferation in the E1 and E2 media compared with control medium, which both contained a large amount of boron and little amounts of other components. Cell proliferation was significantly promoted in experimental media E3 to E5, which contained large amounts of strontium and fluorine as well as boron. The promotion of cell proliferation was particularly remarkable in E4, which contained the largest amount of strontium and fluorine.

Changes in the ALP activity of hDPSCs in five types of experimental media

Figure 5B shows the results of ALP activity staining performed on the 7th day of culture by continuously culturing the cells evaluated for proliferation shown in Fig. 5A. The ALP activity remarkably increased in E1

(Fig. 5Bb) compared to that in the control media (Fig. 5Ba) and E4 (Fig. 5Bc). In addition, because the serum concentration was increased, ALP activity was enhanced compared to the results in Fig. 4C.

DISCUSSION

Bioactive glass has been widely studied in the field of regenerative engineering²¹⁻²³. In 1975, Hench²¹ reported that $\text{Na}_2\text{O}-\text{CaO}-\text{SiO}_2-\text{P}_2\text{O}_5$ glass (45S5) chemically binds to bone *in vivo* and has osteoconductive ability. Since then, bioactive materials have been widely used in clinical practice and research²³⁻²⁵. The S-PRG filler used in this study has also attracted attention as a bioactive glass^{3,6,16,17}. It has been reported to suppress the demineralization of enamel and dentin^{8,9}, induce hard tissue conductivity^{19,26,27}, and suppress plaque formation^{12,13,28}, among other effects. Shiiya *et al.*²⁹ conducted a study on resin-based fillers containing S-PRG and reported that 10% or more filler can suppress the demineralization of the adjacent dentin. S-PRG fillers have been applied to various dental materials such as restorative materials¹⁹, pulp capping materials²⁷, root canal sealers⁴, sealants³⁰, and tooth-coating materials²⁶. In addition, some reports showed the evaluation of the amounts and the actions of ions eluted from these materials^{3-6,8-11,13,14,31}. Ito *et al.*⁶ reported the concentration of ions eluted from the experimental resin containing the S-PRG filler. Their results of ion concentrations were not remarkably different from the results of the S-PRG eluate that we used. On the other hand, Kawashima *et al.*³¹ reported that mouse osteoblasts Kusa-A1 were cultured in a medium with ions eluted from a sealer containing S-PRG filler, osteoblastic differentiation was promoted. The concentration of various ions eluted in the medium was approximately 1/100 of our result of eluting the ions directly from the filler, and their experimental medium diluted to 1/16 was effective.

Thus, studies on the physiological activity of ions released from S-PRG fillers have been widely conducted. For example, fluorine ions enhance the resistance of teeth to acid exposure by forming fluorapatite^{30,32}; boron, silicon, and strontium promote bone formation by inducing osteoblast differentiation of undifferentiated mesenchymal cells³³; boron promotes the expression of human dental germ stem cell tooth or bone differentiation markers³⁴; and boron-containing poly(lactic-co-glycolic acid) (PLGA) promotes the healing of bone defects *in vivo*³⁵. Although several types of ions are released from S-PRG fillers, only a few studies have focused on the amount of each eluted component and its effect on cellular functional activity. In previous reports, the amount of released ions was expressed in parts per million (ppm). There was no discussion of the biological activity expressed with different atomic weights of each component.

In this study, to elucidate the effect of each component, S-PRG filler eluates with different solvent amounts were produced and their effects on cell activity were evaluated using hDPSC. First, screening was

performed at a dose suitable for cell culturing using the S-PRG filler eluate provided by the manufacturer (W-I). The eluate W-I contained 123 mM boron, 20 mM sodium, and trace amounts of other elements. As a result of evaluating the cytotoxicity, cell morphology, and cell proliferation by serially diluting the W-I media, it was found that media containing ≥ 1 mM of boron ($N=7$, diluted to 1/128) inhibited cell proliferation. In addition, cell proliferation was suppressed in the media containing 15–62 μM boron ($N=9-11$). The other media containing 25–250 μM boron were reevaluated taking the above results into account. In addition, the effects of the serially diluted W-IV eluate media were evaluated. By increasing the amount of solvent in the preparation of the S-PRG filler eluate, the amount of other eluted ions increased compared to the amount of boron. In particular, the elution amount of strontium and fluorine increased remarkably. The modified S-PRG filler eluate W-IV had a fluorine concentration of 626 μM and strontium concentration of 325 μM . In addition, a medium containing 628 μM boron, which is more than that in the media produced by diluting W-I, was also produced and evaluated. The results showed that the E(W-I) medium containing 247 μM boron but almost no other components did not affect the proliferation of hDPSC after culturing for two days. However, after seven days, the ALP activity was higher than that of the control, although proliferation was significantly suppressed. Hakki *et al.*³⁶ reported that the addition of 10 ng/mL (925 μM) of boron can promote calcification in the mouse osteoblast-like cell line MC3T3-E1. Nemoto *et al.*¹⁷ reported that adding 1/1000 (v/v) of S-PRG filler eluate to a medium containing 1.31 ppm of boron (121 μM) promotes the calcification of established bone marrow stem cells (UE7T-13). This suggests that the increase in ALP activity of hDPSC is caused by the release of boron from S-PRG fillers. On the other hand, hDPSC proliferation was promoted in the E(W-IV) series media based on the modified S-PRG filler eluate W-IV, which contains a larger amount of boron than this medium and is rich in fluorine and strontium. To verify these results, five types of culture media (E1–E5) were produced from the eluates of four S-PRG eluate with different filler/solvent ratios, and the hDPSC cultures were evaluated. No change in cell proliferation was observed after culturing for two days when using E1 and E2, which contained approximately 280 μM or 230 μM boron and almost no other components. The ALP activity of hDPSCs cultured in E1 increased remarkably. On the other hand, the proliferation of hDPSC was promoted in E3, E4, and E5, which contained several-hundred μM of strontium and fluorine. The ALP activity was similar to that of the control in E2, E3, E4, and E5. The effects of strontium on cell proliferation have been discussed previously. For instance, Okita *et al.*³⁷ reported that the addition of 1.5 mM of SrCl_2 promoted chondrocyte differentiation of dedifferentiated fat cells, and Cheng *et al.*³⁸ reported that the addition of 3 mM of SrCl_2 promoted the differentiation of the mouse osteoblast-like cell line MC3T3-E1. Strontium, like calcium, is a Group

2 element and has similar physicochemical properties. It is found in trace amounts in natural bone and teeth³⁹⁾ and is incorporated into hydroxyapatite⁴⁰⁾. The involvement of strontium in bone formation has been studied based on a calcium concentration of 2.5 mM in human serum^{37,38)}. In this study, cell proliferation was promoted in the medium containing strontium at about one-tenth of the concentration that promotes the calcification of osteoblasts, as reported previously. Regarding fluorine, Nakade *et al.*⁴¹⁾ reported that the addition of 50–100 μ M NaF promotes osteogenic differentiation and proliferation of hDPSCs. The amount of fluorine in the media that promoted hDPSC proliferation was higher than that reported by Nakade *et al.* However, the ALP activity, which is a differentiation marker of osteoblast-like cells, did not change. In addition, the medium with half as much of the strontium-containing medium showed a higher proliferation promotion effect than the medium with the highest strontium content (E(W-IV); 324 μ M). Therefore, it was suggested that an optimal level of strontium and fluorine may be effective in promoting the proliferation of hDPSC. Thus, the null hypothesis tested in this study that multiple ions released from the S-PRG filler did not affect the activity of hDPSCs was rejected. However, the action and optimal concentrations of ions eluted from S-PRG fillers, including our results, have not yet been fully identified. To verify this, we will remove specific components from the media by ion exchange in the future to confirm the action of individual ions. Furthermore, it is possible that two or more types of ions interact, causing a synergistic or suppression effect (canceling effect). We are conducting further analyses to explore this possibility.

CONCLUSION

The results of this study demonstrate that it is possible to prepare eluates containing S-PRG filler-derived components at various concentrations. Depending on the ratio of each component, a medium that promotes the proliferation of hDPSC and a medium that promotes ALP activity were obtained, suggesting the involvement of boron, fluorine, and strontium. The results suggest that tailored S-PRG filler eluates could be applied in dental practice.

ACKNOWLEDGMENTS

Authors thank all the staff at the Department of Operative Dentistry, Oral Biochemistry, and Dental Materials Science, Asahi University School of Dentistry.

REFERENCES

- 1) Kutuk ZB, Vural UK, Cakir FY, Miletic I, Gurgan S. Mechanical properties and water sorption of two experimental glass ionomer cements with hydroxyapatite or calcium fluorapatite formulation. *Dent Mater J* 2019; 38: 471-479.
- 2) Saad A, Inoue G, Nikaido T, Abdou AMA, Sayed M, Burrow MF, *et al.* Effect of dentin contamination with two hemostatic agents on bond strength of resin-modified glass ionomer

- cement with different conditioning. *Dent Mater J* 2019; 38: 257-263.
- 3) Ikemura K, Tay FR, Endo T, Pashley DH. A review of chemical-approach and ultramorphological studies on the development of fluoride-releasing dental adhesives comprising new pre-reacted glass ionomer (PRG) fillers. *Dent Mater J* 2008; 27: 315-339.
- 4) Han L, Okiji T. Evaluation of the ions release/incorporation of the prototype S-PRG filler containing endodontic sealer. *Dent Mater J* 2011; 30: 898-903.
- 5) Shimazu K, Ogata K, Karibe H. Evaluation of the ion-releasing and recharging abilities of a resin-based fissure sealant containing S-PRG filler. *Dent Mater J* 2011; 30: 923-927.
- 6) Ito S, Iijima M, Hashimoto M, Tsukamoto N, Saito T. Effects of surface pre-reacted glass-ionomer fillers on mineral induction by phosphoprotein. *J Dent* 2011; 39: 72-79.
- 7) Uo M, Wada T, Asakura K. Structural analysis of strontium in human teeth treated with surface pre-reacted glass-ionomer filler eluate by using extended X-ray absorption fine structure analysis. *Dent Mater J* 2017; 36: 214-221.
- 8) Nakamura K, Hamba H, Nakashima S, Sadr A, Nikaido T, Oikawa M, *et al.* Effects of experimental pastes containing surface pre-reacted glass ionomer fillers on inhibition of enamel demineralization. *Dent Mater J* 2017; 36: 482-490.
- 9) Mukai Y, Kamijo K, Fujino F, Hirata Y, Teranaka T, Ten Cate JM. Effect of denture base-resin with prereacted glass ionomer filler on dentin demineralization. *Eur J Oral Sci* 2009; 117: 750-754.
- 10) Nomura R, Morita Y, Matayoshi S, Nakano K. Inhibitory effect of surface pre-reacted glass-ionomer (S-PRG) eluate against adhesion and colonization by *Streptococcus mutans*. *Sci Rep* 2018; 8: 5056.
- 11) Miki S, Kitagawa H, Kitagawa R, Kiba W, Hayashi M, Imazato S. Antibacterial activity of resin composites containing surface pre-reacted glass-ionomer (S-PRG) filler. *Dent Mater* 2016; 32: 1095-1102.
- 12) Shimazu K, Oguchi R, Takahashi Y, Konishi K, Karibe H. Effects of surface reaction-type pre-reacted glass ionomer on oral biofilm formation of *Streptococcus gordonii*. *Odontology* 2016; 104: 310-317.
- 13) Hotta M, Morikawa T, Tamura D, Kusakabe S. Adherence of *Streptococcus sanguinis* and *Streptococcus mutans* to salivacoated S-PRG resin blocks. *Dent Mater J* 2014; 33: 261-267.
- 14) Daneshmehr L, Matin K, Nikaido T, Tagami J. Effects of root dentin surface coating with all-in-one adhesive materials on biofilm adherence. *J Dent* 2008; 36: 33-41.
- 15) Fujimoto Y, Iwasa M, Murayama R, Miyazaki M, Nagafuji A, Nakatsuka T. Detection of ions released from S-PRG fillers and their modulation effect. *Dent Mater J* 2010; 29: 392-397.
- 16) Ogawa A, Wada T, Mori Y, Uo M. Time dependence of multi-ion absorption into human enamel from surface prereacted glass-ionomer (S-PRG) filler eluate. *Dent Mater J* 2019; 38: 707-712.
- 17) Nemoto A, Chosa N, Kyakumoto S, Yokota S, Kamo M, Noda M, *et al.* Water-soluble factors eluated from surface pre-reacted glass-ionomer filler promote osteoblastic differentiation of human mesenchymal stem cells. *Mol Med Rep* 2018; 17: 3448-3454.
- 18) Stanners CP, Eliceiri GL, Green H. Two types of ribosome in mouse-hamster hybrid cells. *Nat New Biol* 1971; 230: 52-54.
- 19) Okamoto M, Ali M, Komichi S, Watanabe M, Huang H, Ito Y, *et al.* Surface pre-reacted glass filler contributes to tertiary dentin formation through a mechanism different than that of hydraulic calcium-silicate cement. *J Clin Med* 2019; 8: 1440.
- 20) Miyaji H, Mayumi K, Miyata S, Nishida E, Shitomi K, Hamamoto A, *et al.* Comparative biological assessments of endodontic root canal sealer containing surface pre-reacted

- glass-ionomer (S-PRG) filler or silica filler. *Dent Mater J* 2020; 39: 287-294.
- 21) Hench LL. Ceramic implants. *Glass Ceram Bull* 1975; 22: 82-86.
 - 22) Gough JE, Nottingher I, Hench LL. Osteoblast attachment and mineralized nodule formation on rough and smooth 45S5 bioactive glass monoliths. *J Biomed Mater Res A* 2004; 68A: 640-650.
 - 23) Yamada M, Valanezhad A, Egoshi T, Tashima Y, Watanabe I, Murata H. Bioactive glass coating on zirconia by vacuum sol-dipping method. *Dent Mater J* 2019; 38: 663-670.
 - 24) Sauro S, Babbar A, Gharibi B, Feitosa VP, Carvalho RM, Azevedo Rodrigues LK, *et al.* Cellular differentiation, bioactive and mechanical properties of experimental light-curing pulp protection materials. *Dent Mater* 2018; 34: 868-878.
 - 25) Sepulveda P, Jones JR, Hench LL. Characterization of melt-derived 45S5 and sol-gel-derived 58S bioactive glasses. *J Biomed Mater Res* 2001; 58: 734-740.
 - 26) Ma S, Imazato S, Chen JH, Mayanagi G, Takahashi N, Ishimoto T, *et al.* Effects of a coating resin containing S-PRG filler to prevent demineralization of root surfaces. *Dent Mater J* 2012; 31: 909-915.
 - 27) Takahashi Y, Okamoto M, Komichi S, Imazato S, Nakatsuka T, Sakamoto S, *et al.* Application of a direct pulp capping cement containing S-PRG filler. *Clin Oral Investig* 2019; 23: 1723-1731.
 - 28) Suzuki N, Yoneda M, Haruna K, Masuo Y, Nishihara T, Nakanishi K, *et al.* Effects of S-PRG eluate on oral biofilm and oral malodor. *Arch Oral Biol* 2014; 59: 407-413.
 - 29) Shiiya T, Tomiyama K, Iizuka J, Hasegawa H, Kuramochi E, Fujino F, *et al.* Effect of the coating material on root dentin remineralization in vitro. *Am J Dent* 2014; 27: 258-262.
 - 30) Ntaoutidou S, Arhakis A, Tolidis K, Kotsanos N. Clinical evaluation of a surface pre-reacted glass (S-PRG) filler-containing dental sealant placed with a self-etching primer/adhesive. *Eur Arch Paediatr Dent* 2018; 19: 431-437.
 - 31) Kawashima N, Hashimoto K, Kuramoto M, Bakhit A, Wakabayashi Y, Okiji T. A novel bioactive endodontic sealer containing surface-reaction-type prereacted glass-ionomer filler induces osteoblast differentiation. *Materials (Basel)* 2020; 13(20): 4477.
 - 32) Iijima Y, Koulourides T. Fluoride incorporation into and retention in remineralized enamel. *J Dent Res* 1989; 68: 1289-1292.
 - 33) Movahedi Najafabadi BA, Abnosi MH. Boron induces early matrix mineralization via calcium deposition and elevation of alkaline phosphatase activity in differentiated rat bone marrow mesenchymal stem cells. *Cell J* 2016; 18: 62-73.
 - 34) Taşı PN, Doğan A, Demirci S, Şahin F. Boron enhances odontogenic and osteogenic differentiation of human tooth germ stem cells (hTGSCs) in vitro. *Biol Trace Elem Res* 2013; 153: 419-427.
 - 35) Doğan A, Demirci S, Bayir Y, Halici Z, Karakus E, Aydin A, *et al.* Boron containing poly-(lactide-co-glycolide) (PLGA) scaffolds for bone tissue engineering. *Mater Sci Eng C Mater Biol Appl* 2014; 44: 246-253.
 - 36) Hakki S, Bozkurt BS, Hakki EE. Boron regulates mineralized tissue-associated proteins in osteoblasts (MC3T3-E1). *J Trace Elem Med Biol* 2010; 24: 243-250.
 - 37) Okita N, Honda Y, Kishimoto N, Liao W, Azumi E, Hashimoto Y, *et al.* Supplementation of strontium to a chondrogenic medium promotes chondrogenic differentiation of human dedifferentiated fat cells. *Tissue Eng Part A* 2015; 21: 1695-1704.
 - 38) Cheng Y, Huang L, Wang Y, Huo Q, Shao Y, Bao H, *et al.* Strontium promotes osteogenic differentiation by activating autophagy via the AMPK/mTOR signaling pathway in MC3T3-E1 cells. *Int J Mol Med* 2019; 44: 652-660.
 - 39) Burton JH, Wright LE. Nonlinearity in the relationship between bone Sr/Ca and diet: paleodietary implications. *Am J Phys Anthropol* 1995; 96: 273-282.
 - 40) Featherstone JD, Shields CP, Khademazad B, Oldershaw MD. Acid reactivity of carbonated apatites with strontium and fluoride substitutions. *J Dent Res* 1983; 62: 1049-1053.
 - 41) Nakade O, Koyama H, Arai J, Aiji H, Takada J, Kaku T. Stimulation by low concentrations of fluoride of the proliferation and alkaline phosphatase activity of human dental pulp cells in vitro. *Arch Oral Biol* 1999; 44: 89-92.