Effects of Fluoride-Releasing Restorative Materials on Cultured Human Periodontal Ligament Fibroblast Cell Behavior

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***Abstract*-Fluoride-releasing materials are commonly used to restore cervical lesions and in reconstructive periodontal surgery. The attachment and proliferation of various materials on human periodontal ligament fibroblast (HPDL) cells were evaluated in this study. Four fluoride-releasing restorative materials were tested: GIC (F9), RMGIC (F2), Giomer (B), and resin composite (G); while resin composite (P) was used as a negative control. The specimens were prepared according to the standard protocol, and then primary cultures of HPDL cells were seeded on specimens and control glass cover slips. The attached cells were counted at 1, 3, 24, and 72 h after cell seeding. Cell proliferation was determined using MTT assay at 1, 3, 5, and 7 days after cell seeding. The cell morphology was determined by SEM. Cell attachment increased as time elapsed for all materials. After initial attachment, Phad the best cell attachment profile when compared to the other groups. G had the greatest amount of cell attachment at the end of cell culture time, while F9 showed the least. The cell proliferation profile of G was the highest, while F9 and F2 were the least ($p < 0.05$) among all groups. SEM evaluation determined that HPDL cells on the materials were round or oval at the initial time of cell culture. As time elapsed, HPDL cells presented a variety of cell morphologies in the development of cytoplasmic processes.**

Keywords- Attachment Potential; Fluoride-Releasing Restorative Materials; Periodontal Fibroblast; Proliferation Potential

I. INTRODUCTION

Gingival recession, associated with cervical/root caries or non-carious cervical lesions (NCCL), is commonly observed in dental practice. Surgical root coverage and restoration of the lesions can be used to treat these lesions [1-3].

Root perforation is a common complication of endodontic treatment or post preparation [4]. Several procedures are currently used to treat this complication including the reparation of the root perforation with specific materials.

Resin composite and fluoride-releasing restorative materials, i.e. conventional glass ionomer cement (GIC), resin-modified glass ionomer cement (RMGIC), and compomer, are recommended to restore cervical lesions and root perforations [4, 5]. There are many materials that have been developed for this treatment, each with different compositions and properties. Apart from the physical and mechanical properties, these materials should be compatible with periodontal connective tissue and present no cytotoxicity [6, 7].

Human periodontal ligament fibroblast (HPDL) cells play a major role in wound healing of periodontal tissue. Attachment and proliferation of these cells on restorative materials may lead to cell differentiation, which is an important process of wound healing and the regeneration of periodontal tissues [8].

The aim of this study was to investigate the attachment and proliferation potential of HPDL cells on different fluoride-releasing restorative materials.

II. MATERIALS AND METHODS

A. Specimen Preparation

The four fluoride-releasing restorative materials used in this study included Fuji IX ™ GP EXTRA (F9), Fuji II™ LC (F2), Beautifil® Flow plus (B), and G-ænial™ Universal Flo (G). A resin composite, Premise™ (P) used as a negative control (see Table 1). A black PVC mold with a centered hole 6.0 mm in diameter and 0.5 mm deep was prepared for specimen preparation [9].

The F9 specimen was prepared and mixed according to the manufacturer instructions and placed into the mold. A glass cover slip (0.04 mm thick) was placed above the mold and allowed to set for 2.5 minutes. The specimen was then removed from the mold.

The F2 was prepared and mixed according to the manufacturer instructions and placed into the mold. A glass cover slip was placed above the mold and the material. The material was then light activated using an LED light-curing unit (Demi (Kerr, Orange, CA, USA)) with an irradiance of 1,450 mW/cm^2 for 40 seconds, in contact with the glass cover slip. The intensity of

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the light-curing unit was measured using a hand-held radiometer (L.E.D. radiometer by Demitron (Kerr, Orange, CA, USA)), which was recalibrated after 10 times of usage. After polymerization, the specimen was removed from the mold.

The B, G, and P specimens were placed into the mold and covered with a glass cover slip, and then were light activated using the same method as for specimen F2.

The specimens were stored in an incubator at 37℃ for approximately 24 hours and packed in sealed packages and sterilized using ethylene oxide gas.

TABLE 1 MATERIALS USED IN THIS STUDY AND THEIR COMPOSITIONS

Materials	Type of materials	Manufacturer	Composition provided by manufacturer
Fuji IX™ GP EXTRA	Conventional glass ionomer cement	GC Corporation, Tokyo, Japan	Powder: Fluoroaluminosilicate glass, Polyacrylic acid Liquid: Distilled water, Polyacrylic acid
Fuji II™ LC Capsule	Resin-modified glass ionomer cement	GC Corporation, Tokyo, Japan	Powder: Fluoroaluminosilicate glass Liquid: Distilled water, Polyacrylic acid, 2-Hydroxyethylmethacrylate, Urethanedimethacrylate, Camphorqunone
Beautifil® Flow plus	Giomer	Shofu INC., Kyoto, Japan	Bis-GMA, Triethylenglycoldimethacrylate, Aluminofluoro-borosilicate glass, Al2O3, DL-Camphorquinone
G-ænial™ Universal Flo	Nanohybrid resin composite	GC Corporation, Tokyo, Japan	Strontium glass, Urethane dimethacrylate, Bis-MEPP, Triethyleneglycoldimethacrylate, Silicon dioxide (fumed/amorphous)
Premise™	Nanohybrid resin composite	Kerr, Orange, CA, USA	Prepolymerized filler, Barium glass, Silica filler, Bisphenol A diglycidyl ether methacrylate, Triethylene glycol dimethacrylate, Light-cure initiators

B. Cell Isolation and Cultures

The HPDL cells were obtained from freshly-extracted teeth of three systemically- and periodontally-healthy and non-smoking subjects (two females and one male) aged 25±0.33 years, which had been referred to the Department of Oral Surgery, Faculty of Dentistry, Prince of Songkla University, for extraction of the sound teeth for orthodontic reasons. After extraction, the tooth from each patient was kept separately in DMEM (Gibco-BRL, Rockville, MD, USA), supplemented with antibiotics: 100 unit/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin-B (Gibco-BRL, Rockville, MD, USA) and transferred to the laboratory. The teeth were rinsed several times with DMEM. The periodontal ligaments were scraped from the middle one-third of the roots using a sterile scalpel. These tissues were immediately cultured in DMEM supplemented with 10% FBS (HyClone™(Thermo Fisher Scientific Inc., Waltham, MA, USA)) and antibiotics. The culture was maintained at 37℃ in an incubator equilibrated at 5% CO_2 and approximately 100% relative humidity. After reaching 80% confluence, the outgrowth cells on the culture dish were trypsinized with 0.25% trypsin/0.02% ethylene diamino tetraacetic acid (EDTA). The storage media was changed every 3 or 4 days. The cells from passages 3-5 were used.

C. Attachment Assay

The specimens were divided into 5 groups: F9, F2, B, G, and P (negative control). A glass cover slip was used as the positive control (C). Four samples from each group were fixed to the bottom of a 35x10 mm tissue culture plate (Costar® (Sigma-Aldrich Corp, Saint Louis, MO, USA)) using double-sided adhesive tape. Then, the tissue culture dishes were sterilized for 24 hours using UV light.

The dishes were rinsed with PBS (pH 7.4) and incubated withPBS at 37℃ in a humid atmosphere for 1 hour. Then the PBS was removed. The dishes were plated with 2 ml HPDL cells cultured in DMEM, supplemented with 10% FBS and antibiotics at a density of $5x10^4$ cells/ml and incubated at 37℃ in a humid atmosphere containing 5% CO_2.

At 1, 3, 24, and 72 hours after cell seeding, a morphological and quantitative examination of the HPDL cells attached to the specimens or glass cover slips occurred. At each time point, the specimens were rinsed with PBS and fixed for 48 hours using 4% paraformaldehyde/1.25% glutaraldehyde in PBS + 4% sucrose (pH 7.2). Attachment assay was performed as previously described by Takata, et al. [10] with slight modifications.

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1) Morphological Study of the Attached HPDL Cells:

One sample from each group was transferred to another dish, washed in buffer (PBS + 4% sucrose) and post fixed in 1% osmium tetroxide (OsO_4) + PBS for 30 min. The samples were dehydrated in 70%, 90%, and 95% ethanol for 15 min each, and finally in 100% ethanol for 1 min. The samples were then carefully removed from the dish and exposed to a critical point drying process. The samples were mounted onto SEM stubs and coated with a carbon/gold alloy. The samples were evaluated under a scanning electron microscope (JOEL/JSM-5910L (JEOL Ltd., Tokyo, Japan)) at an accelerating voltage of 15 kV and magnification of 1000-5000x.

2) Quantitative Examination of the Attached HPDL Fibroblasts:

The remaining 3 samples from each group were further washed in washing buffer (PBS + 4% sucrose) and stained with haematoxylin, followed by washing in PBS to remove excess stain. Cell counting was performed in nine predetermined areas on each sample. The number of cells in a unit area of 0.25 mm^2 was counted using an ocular micrometer at a magnification of 400x. The experiments were repeated 3 times and were triplicated by using cells from three patients.

D. Proliferation Assay

The material specimens were divided into 5 groups on the basis of the attachment assay. A polystyrene 96-well tissue culture plate was used as a positive control (C) group. The process was performed as previously described by Zhang, et al. [11] and Shin, et al. [12].

For the proliferation assay, methyl thiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) was used as previously described [11, 12].

The specimens were fixed to 96-well tissue culture plates using double-sided adhesive tape, and then the plates were sterilized for 24 hours using UV light. An amount of 100 microliters of cultured HPDL cells in DMEM, supplemented with 10% FBS and antibiotics at an initial density of $1x10^5$ cells/ml was placed in each well. All samples were incubated at 37℃ in a humid atmosphere containing 5% CO_2.

At 1, 3, 5, and 7 days after cell seeding, the samples were rinsed with fresh medium to remove the unattached cells. MTT was dissolved in PBS at 5 mg/ml and passed through the filter. An amount of 100 microliters of the MTT solution was added to each well. The plates were then incubated at 37℃ in an incubator for 4 hours in an atmosphere of 5% CO_2.

After incubation, the plates were vibrated for 15 minutes and then the MTT solution was removed. An amount of 200microlitersof dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals from the tetrazolium salts. The plate was covered with tin foil, vibrated for 15 min, and then kept dark for 12 hours. The absorbance or optical density (OD) of the colored complex that formed was measured in each well, including controls, on a microtiter plate reader at a wavelength of 570 nm with a reference filter of 630 nm using isopropanol as a blank. The experiments were done in quadruplicate for each experimental set and were triplicated by using cells from three patients.

The morphology of the attached cells was examined by SEM. The attached cells were fixed, stained, dehydrated, and examined as previously described.

E. Statistical Analysis

SPSS software version 16.0 was used to analyze the results at a 0.05 significance level ($P < 0.05$). The mean number of attached cells from the attachment assay and mean absorbance from the MTT proliferation assay were subjected to three-way ANOVA to determine significant differences between groups. The one-way ANOVA and Dunnett's T3 multiple comparison test were used to compare the amount of cell attachment on each material at different times and to compare the amount of cells attached on different materials at each time. A p-value of <0.05 was considered statistically significant.

III. RESULTS

The data obtained from the patients had similar profiles. The number of HPDL cells attached and proliferated for each sample group for each patient were pooled and analyzed to obtain a representative data sample.

A. Attachment Assay

1) Number of Cells Attached on Specimens/Glass Cover Slips:

During the experimental period, the HPDL cells attached and grew on all the samples. The numbers of cells attached on the specimens or glass cover slips in a unit area of 0.25 mm^2 are shown in Table 2 and Fig. 1.

HPDL cells on F9, F2, and B at 1 to 72 hours after cell seeding demonstrated a linear proliferation. The number of HPDL cells on P and G demonstrated a high rate of proliferation at 3 to 24 and after 24 hours of cell seeding, respectively. At every time point, the number of cells attached on every material and on the glass cover slips was statistically significant increased. At 72 hours after cell seeding, the number of cells on G was approximately 2 times more when compared to F9 and B. F9

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presented the statistically significantly lowest cell attachment profile at every time point.

TABLE 2 NUMBER OF CELLS AT DIFFERENT PERIODS AFTER CELL SEEDING

Materials	Mean cell number per unit area			
	Time (Hours)			
	1	3	24	72
Control	78.08 ±21.23^{Aa}	86.99 ±15.58^{Ba}	113.00 ±21.13^{Ca}	148.00 ±21.35^{Da}
Fuji IX™ GP EXTRA	11.91 ±1.5^{Ab}	13.75 ±3.25^{Bb}	21.67 ±4.15^{Cb}	43.67 ±4.83^{Db}
Fuji II™ LC Capsule	21.50 ±5.76^{Acd}	26.25 ±5.45^{Bc}	33.42 ±10.96^{Cc}	66.58 ±10.56^{Dc}
Beautifil® Flow plus	20.58 ±10.56^{Acd}	34.00 ±10.59^{Bd}	37.17 ±6.33^{Cd}	48.08 ±9.05^{Dd}
G-ænial™ Universal Flo	20.08 ±4.33^{Ac}	22.08 ±5.48^{Be}	32.25 ±20.39^{Ccd}	102.75 ±24.74^{De}
Premise™	22.92 ±4.72^{Ad}	23.08 ±8.07^{Bf}	63.58 ±11.38^{Ce}	75.91 ±8.88^{Df}

*Different uppercase letters in the same row indicate significant difference ($P < 0.05$) among time in each material.

*Different lowercase letters in the same column indicate significant difference ($P < 0.05$) among materials in each time.

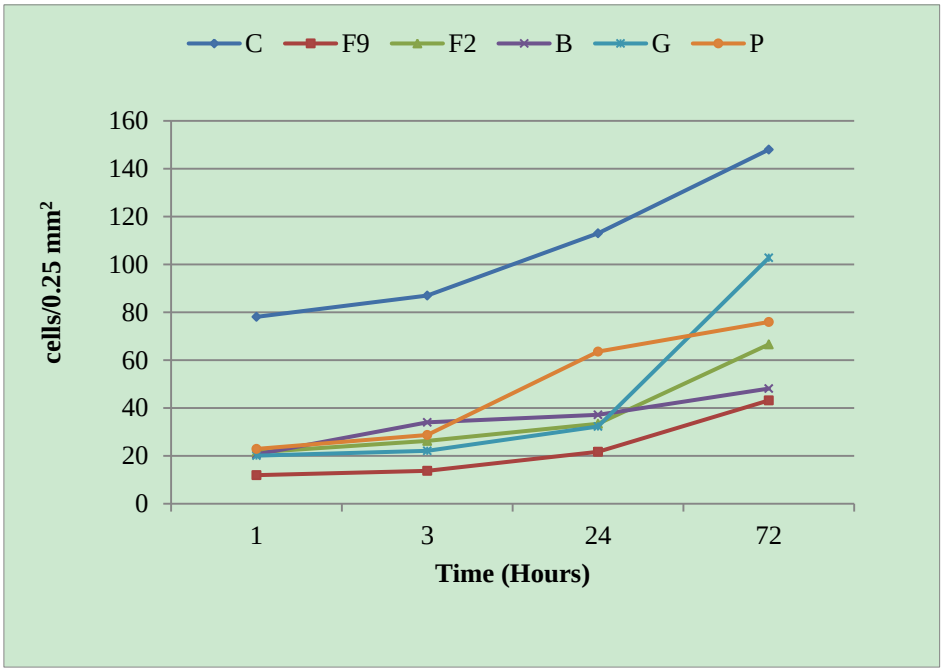

Fig. 1 Number of HPDL cells on different fluoride-releasing material at different periods after cell seeding

2) Morphology of Cells Attached on Different Fluoride Releasing Materials:

From the SEM study (Fig. 2), HPDL cells on all materials and the control glass cover slip at 1 hour after cell seeding were round or oval. In the control groups, the cells were flat with well-dispersed cytoplasmic processes. The cytoplasmic processes of cells on P appeared to merge with the material matrix. In the other groups, the cells with limited or no cytoplasmic processes were loosely attached to the material surfaces.

At 3 hours after cell seeding, the cells were more flattened and elongated. In F9 and F2, some cellular structures merged to the material matrix. In C, well-dispersed star-shaped cells with abundant cytoplasmic extension were seen.

At 24 and 72 hours after cell seeding, the cells on the control and all experimental materials, except on group B, were stellate, flattened, and elongated, with well-defined cytoplasmic extensions. For group B, the cells demonstrated round or oval shapes with poorly-spread cytoplasmic processes.

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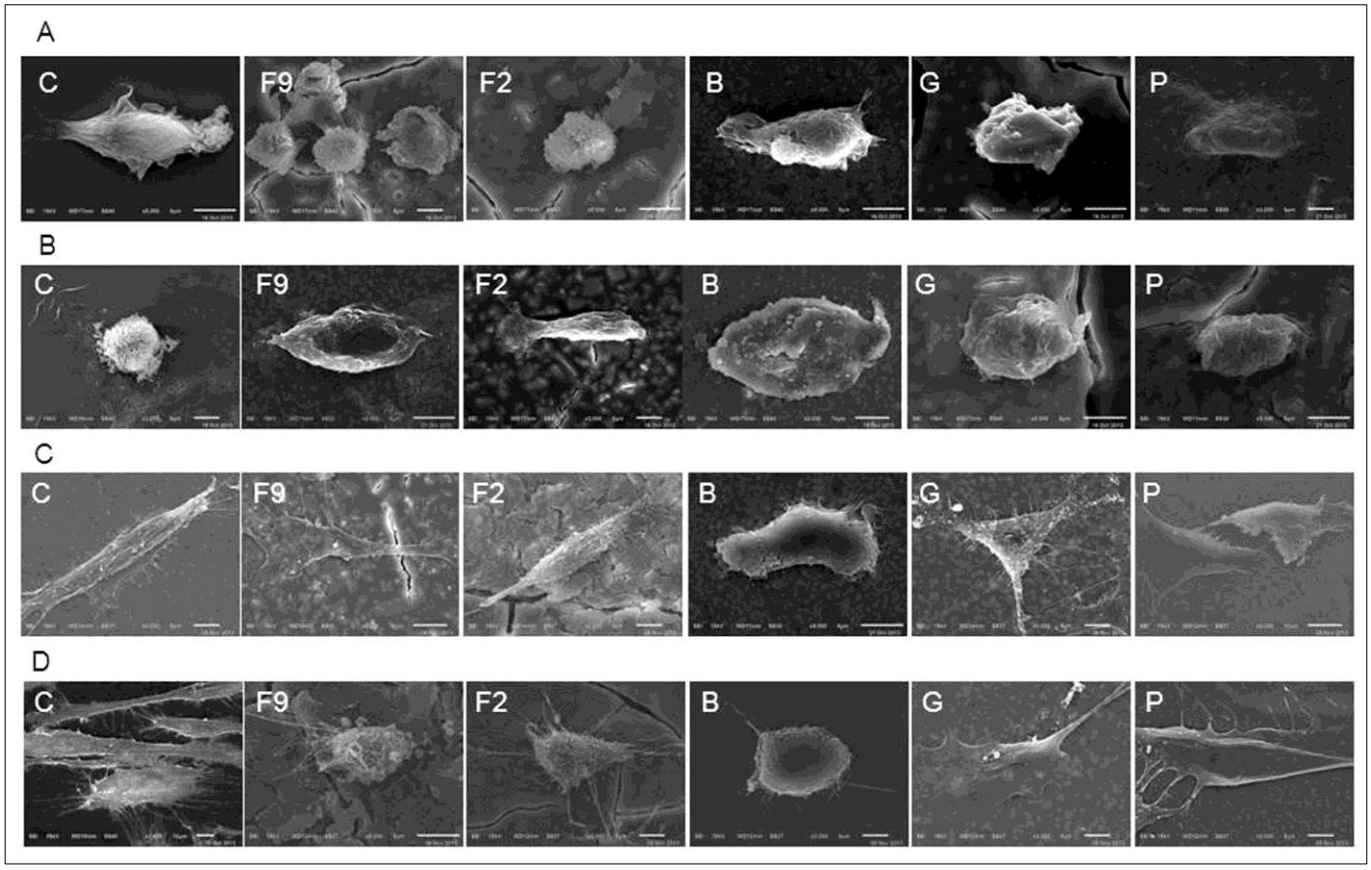

Fig. 2 SEM image of HPDL cells at A (1 hour); B (3 hours); C (24 hours); and D (72 hours) after cell seeding

B. Proliferation Assay

1) The Mean Absorbance on Specimens/Glass Cover Slips:

The mean absorbance at different time points after cell seeding are shown in Table 3 and Fig. 3.

The mean absorbance of only G group was increased from day 1 to day 7. The mean absorbances of the other groups varied in proliferation tendencies. Only G and F2 showed an increase in mean absorbance when compared to initial attachment and 7 days after cell seeding. In comparison to C, all other materials demonstrated statistically significant lower mean absorbance values.

TABLE 3 MEAN ABSORBANCE AT DIFFERENT PERIODS AFTER CELL SEEDING

Materials	Mean cell number per unit area			
	Time (Days)			
	1	3	5	7
Control	0.16 ± 0.01^{Aa}	0.27 ± 0.01^{Ba}	0.41 ± 0.01^{Ca}	0.87 ± 0.12^{Da}
Fuji IX™ GP EXTRA	0.10 ± 0.01^{Ab}	0.15 ± 0.01^{Bb}	0.05 ± 0.01^{Cb}	0.06 ± 0.01^{Db}
Fuji II™ LC Capsule	0.10 ± 0.16^{Aabcde}	0.07 ± 0.01^{Ac}	0.09 ± 0.03^{ABc}	0.11 ± 0.01^{ACc}
Beautifil® Flow plus	0.06 ± 0.00^{Ac}	0.07 ± 0.01^{Bd}	0.05 ± 0.00^{Cb}	0.07 ± 0.01^{Bd}
G-ænial™ Universal Flo	0.13 ± 0.01^{Ad}	0.19 ± 0.01^{Be}	0.24 ± 0.01^{Cd}	0.33 ± 0.02^{De}
Premise™	0.16 ± 0.01^{Aae}	0.18 ± 0.00^{Bf}	0.17 ± 0.00^{Ce}	0.23 ± 0.01^{Df}

*Different uppercase letters in the same row indicate significant difference ($P < 0.05$) among time in each material.

*Different lowercase letters in the same column indicate significant difference ($P < 0.05$) among materials in each time.

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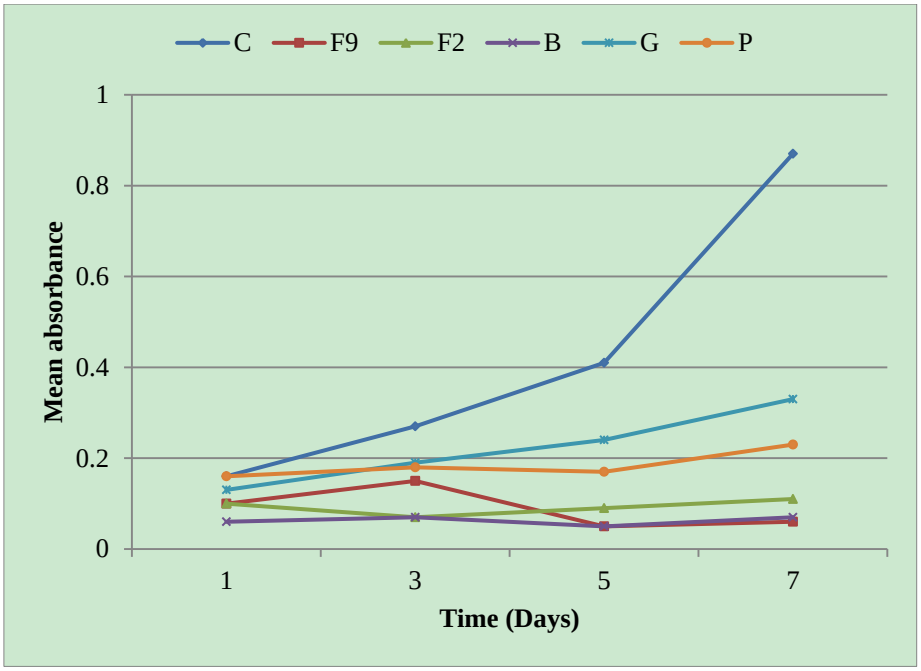

Fig. 3 Mean absorbance of different fluoride-releasing materials at different periods after HPDL cell seeding

2) Morphology of Cells Proliferated on Different Fluoride Releasing Materials:

In the SEM analysis (Fig. 4), cells were found on every material surface, but the cell counts were lower in number when compared to the control group. HPDL cells on all material groups were round or oval with poor cytoplasmic extensions.

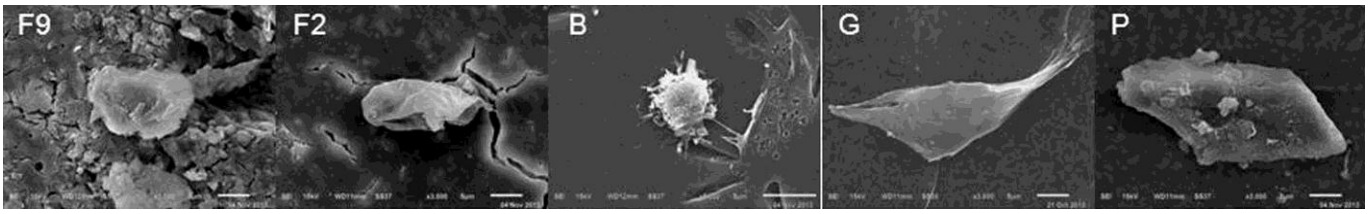

Fig. 4 Morphology of cells proliferated on different fluoride releasing materials

IV. DISCUSSIONS

Restoring cervical lesions and repairing endodontic perforations both require biocompatibility of the restorative materials that are in contact with the periodontal tissues. HPDL is a periodontal cell that plays a crucial role in the maintenance of periodontal tissue and in the wound-healing process. Periodontal ligament cell behaviors, such as cell growth, attachment, and proliferation, play an important role in periodontal wound-healing and tissue regeneration [13].

In the current study, five restorative materials with different compositions were tested. The numbers of cells attached on each of these materials in the present study were lower than those on the glass cover slip (control group), suggesting that these materials have cell cytotoxicity, which also affects cell attachment to asolid surface. Several studies have indicated that all materials were cytotoxic to the PDL cells by inhibiting cell attachment and proliferation [14]. In this study, HPDL cell attachment for every material had similar profiles from 1 hour to 3 hours; after that, the rate of cell attachment was remarkably high only for P. In general, the initial or early attachment of PDL occurred at 30 minutes after cell seeding and the late attachment or cell spreading phase occurred later. A very short period after cell seeding (within 1-3 hours) is essential for fibroblast adhesion and cytoskeletal reorganization. An increasing rate of attachment in this step may result in an increase in subsequent proliferation [15].

At 24 hours to 72 hours after cell seeding, G had the highest rate of cell attachment, although it did not have the highest rate from previous culture periods. In general, the formation of the specialized contact phase starts about 24 hours after the culture period. This phase involves the formation of focal contacts and focal adhesion structures, the use of cell microfilaments and specialized attachment proteins [16, 17]. The surface morphology of each material and the difference in substances released from the material may influence the rate of cell attachment.

The reason G had the highest number of cell attachment and rate of proliferation at the end of the culture period may have been due to its organic matrix composition. For all tested materials, only G and F9 were non-bis-GMA materials. Previous studies have reported that bis-GMA is the most toxic substance used in organic matrices, followed by UDMA, TEGDMA, and

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HEMA [18, 19]. However, F9 had a lower proliferation profile than F2 and B. The level of fluoride released from these materials may result in specific proliferation profiles. A previous study demonstrated that high levels of released fluoride correlated with the high cytotoxic effect of fluoride-releasing materials [20]. Another study showed that the maximum cumulative fluoride release after 21 days was the highest for GIC, followed by RMGIC, and then giomer [21].

In addition to the cytotoxicity of the materials, the surface properties of the materials, such as surface hydrophilicity/hydrophobicity and surface roughness, directly relate to adhesion and proliferation [22]. The increased ability of a cell to attach to a material may be explained by its surface hydrophilicity [22]. The GIC presented more hydrophilicity than the resin composite [23]. Previous studies have suggested that cell numbers increase with increasing surface hydrophilicity [22, 24]. In contrast, this study found that cell attachment numbers on both types of resin composite were more than that of the glass ionomer cement, which may have resulted from lower roughness values of the resin composite when compared to the GIC [25]. The remarkable increasing rate of initial cell attachment in P may be explained by this phenomenon. Attia, et al. [26] reported that fibroblasts have good attachment on a smooth surface, spread more quickly, and have earlier formation of filopodia than on a porous surface. Previous studies have shown that the surface roughness values of RMGIC are more than those of GIC and compomer [27, 28], and F2 is assumed to have a rougher surface than F9. However, the current study found that the cell attachment numbers for F2 were more than those for F9. It was probably due to the level of fluoride release from the materials, as described.

Cell morphology is a main regulator for cell proliferation. It was found that round or oval cells had a lower rate of cell proliferation than cells that were flat, or spindle-or stellate-shaped with numerous cytoplasmic extensions in the matrix [29-31]. In the present study, only P presented cytoplasmic processes, which merged with the material matrix at the initial cell seeding time. This result may have been due to the surface characteristics of the material. At the late attachment period, the cytoplasmic processes had merged to all material surfaces except G, suggesting that some cells attached more firmly than others. This result was in accordance with the cell attachment profile. As time elapsed, SEM showed that a few cells had detached from the material surface, suggesting loose attachment of cells on these materials [32]. At the later culture period, cytoplasmic processes of HPDL cells were discovered on every material. These cell-material interactions implied that the materials promoted cell attachment and proliferation, although their values were lower in number and quality when compared to the control.

The results obtained in this study were different in some aspects from those of previous studies [22, 24, 26]. This difference may be due to material composition, tested cells, or the assay used.

V. CONCLUSIONS

HPDL cells attached and proliferated on all tested materials with different potentials, and were all lower than the control. Within the limitation of this study, all materials had different degrees of cytotoxicity.

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