Attachment Potential of Human Gingival Fibroblasts on Tooth-Colored Restorative Materials

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Abstract

Objectives To evaluate the attachment potential of the human gingival fibroblast (HGF) on tooth-colored restorative materials.

Materials and methods The specimens of five tooth-colored restorative materials (Fuji IX™ GP EXTRA, Fuji II™ LC, Beautifil® Flow plus, G-ænial™ Universal Flo and Premise™) were prepared and then primary cultures of HGF 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. The cell morphology was determined by SEM.

Results HGF cell attachment increased as time elapsed for all materials. The Fuji IX™ GP EXTRA demonstrated statistically significant the lowest cell attachment rate at every time point. The G-ænial™ Universal Flo had the highest attachment rate at the end of culture period.

Conclusion HGF attached on all tested materials in different potentials, which were all lower than the control. Chemical compositions and surface characteristics of materials affected attachment cells. G-ænial™ Universal Flo, a non-bis-GMA material, has the best cell attachment.

Keywords: *attachment potential; biocompatibility; human gingival fibroblasts; fluoride-releasing restorative materials*

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Introduction

Non-carious cervical lesions (NCCLs) are common occurrence in dental practice and were founded in all age groups. The NCCLs have a multifactorial etiology such as improper tooth brushing technique, abrasive dentifrice, non-axial occlusal force and chemical degradation by extrinsic and intrinsic origin[1, 2]. Because of the complex interaction of these various mechanisms, the lesions may occur alone or in combination[1]. These lesions can affect tooth sensitivity, plaque retention, increase caries risk and esthetic problem[2, 3]. Moreover, the NCCLs may occur with gingival recession result in lesion involved both the crown and the exposed root.

The common treatment of the NCCL is restorative therapy. But in case of extensive gingival recession combined with the cervical lesion, the restoration alone may not solve esthetic problem caused by excessive length of the tooth and non-harmonized gingiva. Consequently, combined restorative and periodontal treatment, in which the restorative therapy is completed before periodontal plastic surgery to achieve both esthetic and physical characteristics of tooth[4, 5]. After the healing period, part of the restoration was covered by the soft tissue.

Recently, numerous dental restorative materials have been developed. In addition to physical properties, restorative material that used to restore the NCCLs before gingival coverage should have good biocompatibility and no toxicity to gingival fibroblast cells. Recent studies shown successful root coverage treatment with resin composite and fluoride-releasing restorative materials[6, 7].

Cytotoxicity can be determined by the use of several methods such as cell attachments on tested materials and measurement of proliferation rate[8]. Attachment of human gingival fibroblast (HGF) on restorative material may lead to the regeneration of periodontal tissue resulted in successful treatment[9].

The aim of this study is to study attachment potential of the HGF on different tooth-colored restorative materials.

Materials and Methods

The five tooth-colored restorative materials shade Vita A3 were used in this study. The three fluoride-releasing restorative materials were: GIC (Fuji IX™ GP EXTRA - FIX (GC Corporation, Tokyo, Japan)), RMGIC (Fuji II™ LC capsule - FII (GC Corporation, Tokyo, Japan)) and Giomer (Beautifil® Flow plus - B (Shofu INC., Kyoto, Japan)). The two resin composite were G-ænial™ Universal Flo - G (GC Corporation, Tokyo, Japan)) and Premise™ - P (Kerr, Orange, CA, USA). (Table 1).

Specimen preparation

A black PVC mold with a centered hole 6.0 mm in diameter and 0.5 mm deep was prepared for specimen preparation[10]. Sixteen specimens per material were prepared. After polymerization, the specimens were stored in an incubator at 37˚C for approximately 24 hours and packed in sealed packages and sterilized using ethylene oxide gas.

For FIX group, the material 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 2.50 minutes. The

specimen was then removed from the mold.

For FII group, the material 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 and then light activated using an LED light-curing unit (Demi (Kerr, Orange, CA, USA)) with an irradiance of 1450 mW/cm^2 for 40 seconds, in contact with the glass cover slip. The intensity of 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.

For B, G and P groups, the material was placed into the mold and covered with a glass cover slip, and then were light activated as described above.

Table 1 Compositions of materials used in this study

Materials	Type of materials	Manufacturer	Composition provided by manufacturer
Fuji IX^{TM} GP EXTRA	Conventional glass ionomer cement	GC Corporation, Tokyo, Japan	Powder: Fluoroaluminosilicate glass, Polyacrylic acid Liquid: Distilled water, Polyacrylic acid
Fuji II^{TM} LC Capsule	Resin-modified glass ionomer cement	GC Corporation, Tokyo, Japan	Powder: Fluoroaluminosilicate glass Liquid: Distilled water, Polyacrylic acid, 2-Hydroxyethylmethacrylate, Urethane dimethacrylate, Camphorqunone
Beautifil® Flow plus	Giomer	Shofu INC., Kyoto, Japan	Bis-GMA, Triethylene glycol dimethacrylate, Aluminofluoro-borosilicate glass, Al_2O_3, DL-Camphorquinone
G-ænial^{TM} Universal Flo	Nanohybrid resin composite	GC Corporation, Tokyo, Japan	Strontium glass, Urethane dimethacrylate, Bis-MEPP, Triethylene glycol dimethacrylate, Silicon dioxide (fumed/amorphous)
Premise^{TM}	Nanohybrid resin composite	Kerr, Orange, CA, USA	Prepolymerized filler, Barium glass, Silica filler, Bisphenol A diglycidyl ether methacrylate, Triethylene glycol dimethacrylate , Light-cure initiators

Bis-GMA = Bisphenol A-glycidyl methacrylate, Bis-MEPP = Bisphenol Aethoxylte di methacrylate

Cell isolation and cultures

The HGF 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 teeth from each patient were separately kept 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 HGF were harvested from gingival epithelium 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˚C 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 diaminotetraacetic acid (EDTA). The storage media was changed every 3 or 4 days. The cells from passages 3-5 were used.

Attachment assay

Twenty-four experimental groups comprised of 5 materials (FIX, FII, B, G and P) and a glass cover slip group (positive control - C) that have been treated at 1, 3, 24 and 72 hr. Four specimens 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 exposed to PBS at 37°C in a humid atmosphere for 1 hour, then the PBS was then removed. The dishes were plated with 2 ml of HGF cultured in DMEM, supplemented with 10% FBS and antibiotics at a density of $5x10^4$ cells/ml and incubated at 37°C 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 HGF 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 (pH7.2).

The quantitative examinations of the attached HGF were obtained from 3 specimens from each group. The specimens 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 9 predetermined areas on each specimen. The number of cells in a unit area of 0.25 mm^2 was counted using an ocular micrometer at a magnification of x400. The experiments were repeated 3 times and were triplicated by using cells from three patients.

The remaining one specimen from each group was prepared for morphological study. 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 1000-5000.

Statistical analysis

SPSS software, version 16.0, was used to analyze the results at a 0.05 significance level ($P< 0.05$). The mean numbers of attached cells from the attachment assay were subjected to two-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.

Results

The data obtained from the 3 patients had similar profiles. The number of HGF cells attached and proliferated for each sample group for each patient were pooled and analyzed to obtain a representative data sample.

Table 2 and Figure 1show the numbers of cells attached on the specimens or glass cover slips in a unit area of 0.25 mm^2. At every time point, attachment rate of control group was statistically significant increased and was higher than other groups. At 1 hour after cell seeding, HGF cells on FIX demonstrated statistically significantly the lowest initial cell attachment and still had the lowest rate of cell attachment at every time point. In group B, the attachment rate at 1 to 3 hours was higher than other materials and then the rate was decreased. At 3 to 24 hours, P had the highest attachment rate when compared to the other materials. The G had the highest attachment rate at 24 to 72 hours after cell seeding. At 72 hours, the number of cells on G was approximately 2 times when compared with FIX and B.

Figure 2 shows the attached cells morphology. At 1 hour after cell seeding, HGF cells on all materials and the control glass cover slip were round or oval. In the control groups, the cells were flattened with well-dispersed cytoplasmic processes. In the other groups, the cells with limited or no cytoplasmic process were loosely attached to the material surfaces. At 3 hours after cell seeding, all cells were more flattened and elongated. In C, well-dispersed star-shaped cells with abundant cytoplasmic extensions 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 shape with poor spread cytoplasmic processes.

Discussion

Restoring cervical lesions and repairing endodontic perforations both require biocompatibility of the restorative materials that are in contact with the periodontal tissue. The one important property of restorative materials is the biocompatibility with the periodontal connective tissue attachment apparatus. This property minimizes the negative effects on periodontal tissue induced by direct contact with restorative material[11]. Fibroblasts were account for most connective tissue cells and also play a major role in normal connective tissue turnover, as well as in wound healing, repair and regeneration[12, 13]. HGF cells are periodontal cells that play a crucial role in the maintenance of periodontal tissue and in the wound healing process. These cell behaviors, such as cell growth, attachment and proliferation play an important role in periodontal wound healing and tissue regeneration[14]. Thus this cell type was chosen due to its availability and culturing characteristics[15, 16].

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 glass cover slip (control group), suggesting that these materials have cell cytotoxicity on HGF cells. Several studies have indicated that all materials were cytotoxic to the fibroblast cells by inhibiting cell attachment and proliferation[17].

Table 2 Number of attached cells at different periods after cell seeding

Study groups	Mean cell number per unit area			
	Time (Hours)			
	1	3	24	72
C	86.00 ± 16.92^{Aa}	92.58 ± 12.58^{Ba}	130.92 ± 19.03^{Ca}	140.50 ± 21.75^{Da}
FIX	11.58 ± 4.01^{Ab}	17.83 ± 3.17^{Bb}	33.99 ± 4.76^{Cb}	44.00 ± 12.27^{Db}
FII	19.00 ± 5.50^{Ac}	23.17 ± 7.41^{Bc}	47.58 ± 10.40^{Cc}	76.33 ± 15.54^{Dc}
B	21.00 ± 2.67^{Ad}	33.25 ± 5.49^{Bd}	40.50 ± 6.47^{Cd}	54.25 ± 10.99^{Dd}
G	17.00 ± 4.08^{Ae}	28.58 ± 5.49^{Be}	35.25 ± 7.03^{Cb}	97.25 ± 14.27^{De}
P	16.75 ± 4.77^{Ae}	19.75 ± 7.41^{Bb}	53.00 ± 4.34^{Ce}	71.00 ± 6.92^{Df}

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

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

Figure 1 Number of HGF cells on different fluoride-releasing material at different periods after cell seeding

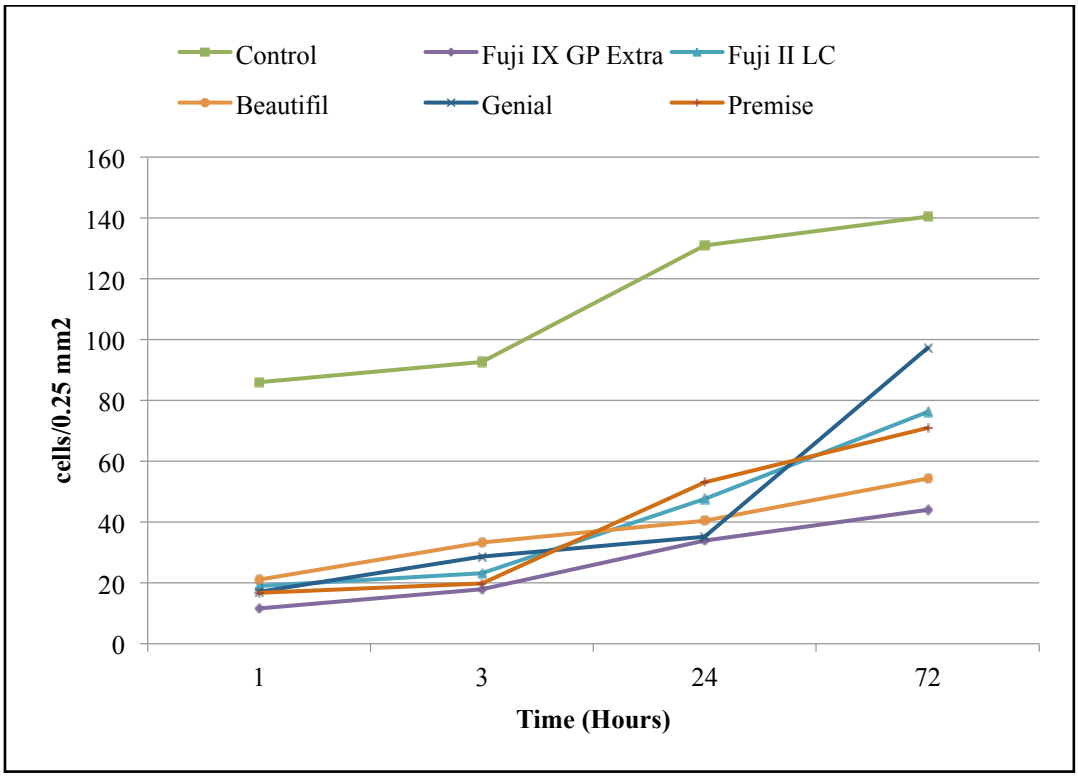
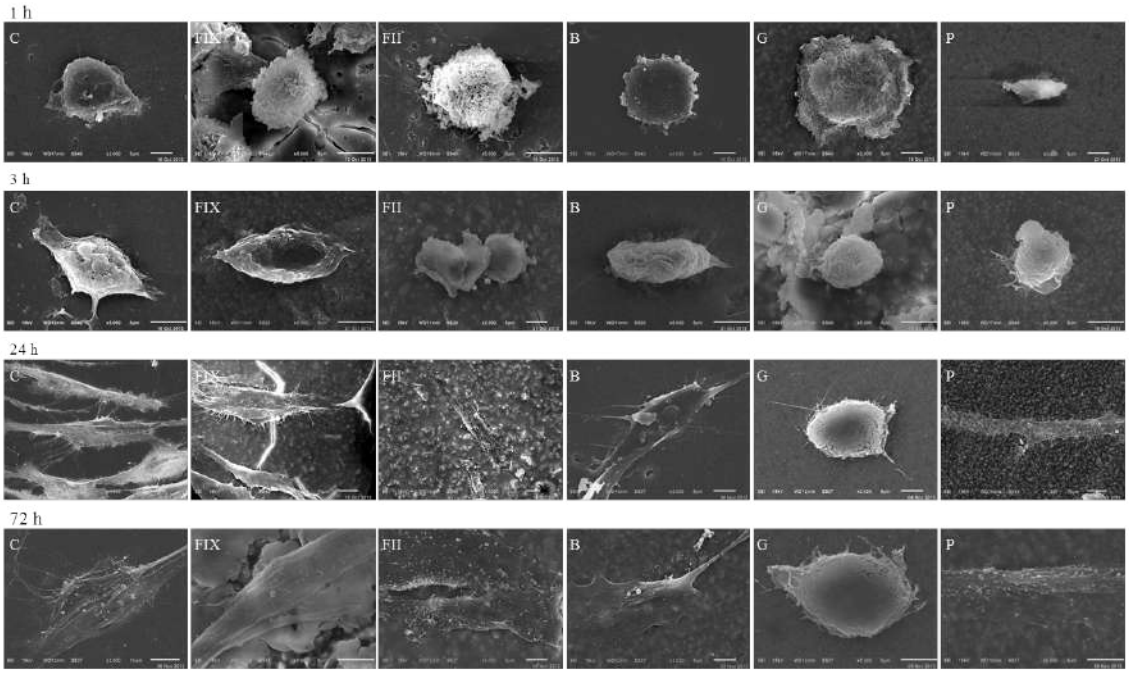


Figure 2 SEM image of HGF cells at different periods after cell seeding



Attachment of HGF cells on tested materials

In this study, HGF cell attachment rates from 1 hour to 3 hours for all materials had quite similar profiles. After 3 hours, the attachment rates were varies among study groups until the end of culture period. In general, the initial or early attachment of fibroblast 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 a few hours) is essential for fibroblast adhesion and cytoskeleton reorganization. A previous study[18] indicated that resin-based materials were cytotoxic to gingival fibroblasts by inhibiting cell attachment and proliferation. During materials insertion and even after polymerization, unreacted monomer can be release from materials and may influence cytotoxicity[19, 20]. Ferracane and Condon[21] found that free monomers and additives were dissolved from resin base materials, especially during the first 24 hours. Bis-GMA, UDMA, TEGDMA and HEMA are most frequently used monomer. The cytotoxicity increased as follows: HEMA<TEGDMA <UDMA<BisGMA[22].

As well as the resin-based materials, in vitro assessment of GICs has shown that materials released chemical substances that were highly toxic to mammalian cells in the hours and days after mixing[23, 24]. Therefore, in the initial attachment phase, cell could attach on all tested materials in the similar tendency. These may be due to no or only little amount of cytotoxic substances release from tested materials. The attachment rates for all materials at the end of culture period were higher than that at initial time of culture period but lower than control group. This may possibly imply that the toxicity of leachable component of materials affected the rate of cell progression through cell cycle rather than cause cell death[25]. The G, a non-bis-GMA material, had the highest attachment rate at the end of culture period while B and P, which composed of Bis-GMA, had lower attachment rate. Then material that comprised of less toxic substances is possible explanation for the good cell attachment and proliferation[21]. However, G contained other methacrylate monomers (UDMA and Bis-MEPP) which did not affect attachment rate. It may possibly result from high degree of conversion of material or low concentration of these monomers.

In this study, the FIX demonstrated the lowest rate of HGF cell attachment. While FII, which contained HEMA, showed higher attachment rate. It may be explain by the level of fluoride released from these materials. The high levels of released fluoride correlated to the high cytotoxic effect of fluoride-releasing materials[26]. The maximum cumulative fluoride release after 21 days was the highest for GIC, followed by RMGIC and then giomer[27]. Furthermore, GIC has more solubility than RMGIC and releases more water-soluble molecule which induce more cytotoxicity[28].

In addition to the cytotoxicity of materials, the surface properties of the materials, such as surface hydrophilicity /hydrophobicity and surface roughness directly relate to adhesion and proliferation[29]. Li et al.[30] found that rougher surfaces are better in enhancing cell attachment and proliferation than smooth surfaces. A previous study[31] showed that the surface roughness value of resin composite was lower than that of the GIC. The number of attached cells on GIC should be more than those on resin composite. In contrast with this study, only G demonstrated statistically significant highest cell attachment at the end of cell seeding time point. This finding is consistent with study of Attia et al.[32], which reported that fibroblasts had good attachment and spread more quickly on a smooth surface than on a porous surface. The increased ability of a cell to attach to materials may be result of increasing their surface hydrophilicity[29].

Generally, 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[33, 34]. The difference in substances released from the material and the surface morphology of each material and may influence both the rate of cell attachment and proliferation.

SEM evaluation of HGF cell morphology

Cell morphology is the main regulators for cell proliferation. It was found that round or oval cells had a lower rate of cell proliferation than cells that were flat, spindle- or stellate-shaped with numerous cytoplasmic extensions in the matrix[35-37]. In the present study, at the initial cell seeding time, HGF cells on all materials present only short and small cytoplasmic processes, which loosely attached on the material surfaces. This result may have been due to the surface characteristics of the material as described above. At the late attachment period, the cytoplasmic processed of HGF cells on all materials were merged to material surfaces except HGF attached on B which loosely attached and poor cytoplasmic extensions.

This result was in accordance with the cell attachment profile. As time elapsed, the SEM showed a few cells had detached from the material surface, suggesting loose attachment of cells on these materials[38]. At the later culture period, cytoplasmic processes of HGF 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. However, this study found that HGF had poor attached on B. It may result from different chemical substances such as Bis-GMA released from materials which may be effect to these cells.

Further study is to determine the monomer or chemical composition release from restorative materials that effect cell attachment behavior. Moreover, the clinical studies are necessary to improve the knowledge about materials biocompatibility in intraoral conditions.

Conclusion

HGF cells attached and proliferated on all tested materials in different potentials, which were all lower than the control, suggesting that all materials have different degrees of cytotoxicity. Both chemical substances released from restorative materials and surface characteristics of materials affected attachment cells on restorative materials. Within limitations of this study, G-ænial™Universal Flo which is a non-Bis-GMA material, has the best HGF cell attachment.

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แนวโน้มการยึดเกาะของเซลล์ไฟโบรบลาสต์จากเหงือกของมนุษย์ต่อวัสดุบูรณะสีเหมือนฟัน

พิมพ์มาดา เกษรักษ์ สุชาดา พันธุรักษ์***

บทคัดย่อ

วัตถุประสงค์ เพื่อศึกษาการยึดเกาะของเซลล์ไฟโบรบลาสต์จากเหงือกของมนุษย์ต่อวัสดุบูรณะสีเหมือนฟัน

วัสดุและวิธีการ เตรียมชิ้นตัวอย่างของวัสดุบูรณะสีเหมือนฟัน 5 (Fuji IX™ GP EXTRA, Fuji II™ LC, Beautifil® Flow plus, G-œnial™ Universal Flo and Premise™) จากนั้นเพาะเลี้ยงเซลล์ไฟโบรบลาสต์จากเหงือกของมนุษย์ บนผิววัสดุบูรณะฟันชนิดต่างๆ และแผ่นแก้วปิดสไลด์ จากนั้นทำการนับจำนวนเซลล์ที่ยึดเกาะบนชิ้นตัวอย่างที่ 1, 3, 24, และ 72 ชั่วโมง และศึกษารูปร่างของเซลล์ภายใต้กล้องจุลทรรศน์อิเลคตรอนแบบส่องกราด

ผลการศึกษา การยึดเกาะของเซลล์ไฟโบรบลาสต์จากเหงือกของมนุษย์มีแนวโน้มเพิ่มขึ้นเมื่อเวลาผ่านไป การยึดเกาะของเซลล์บนวัสดุ Fuji IX™ GP EXTRA มีอัตราต่ำที่สุดในทุกช่วงเวลา มีอัตราการยึดเกาะของเซลล์บนวัสดุ G-œnial™ Universal Flo มีอัตราสูงที่สุดเมื่อสิ้นสุดการเพาะเลี้ยงเซลล์

สรุป เซลล์ไฟโบรบลาสต์จากเหงือกของมนุษย์สามารถยึดเกาะกับวัสดุชนิดต่างๆ ในอัตราแตกต่างกัน และต่ำกว่ากลุ่ม ควบคุมอย่างมีนัยสำคัญทางสถิติ องค์ประกอบทางเคมีและลักษณะพื้นผิวของวัสดุมีผลต่อการยึดเกาะของเซลล์ G-œnial™ Universal Flo ซึ่งเป็นวัสดุที่ไม่มีส่วนประกอบของบิสจีเอ็มเอ มีการยึดเกาะของเซลล์ดีที่สุด

คำสำคัญ *ความเข้ากันได้ทางชีวภาพ; เซลล์ไฟโบรบลาสต์จากเหงือกของมนุษย์; แนวโน้มการยึดเกาะ; วัสดุบูรณะที่สามารถปลดปล่อยฟลูออไรด์ได้*

**ภาควิชาทันตกรรมอนุรักษ์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา*
***ภาควิชาปริทันตวิทยา คณะทันตแพทยศาสตร์ มหาวิทยาลัยเวสเทิร์น อำเภอลำลูกกา จังหวัดปทุมธานี*