Assessment and Quantification of Noncollagenic Matrix Proteins Released from Human Dentin Powder Incorporated into a Silated Hydroxypropylmethylcellulose Biomedical Hydrogel

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Abstract

Introduction: The dentin extracellular matrix is a reservoir of bioactive molecules sequestered into dentin during dental initial development. They can be released under pathological conditions but also by controlled demineralization with bioactive materials. The purpose of this study was to investigate the ability of a biomedical hydrogel to extract and release these proteins from smashed dentin. Methods: Smashed dentin was obtained with 2 different kinds of grinders: a blade mill and a zirconia mortar grinder. The particle size was measured by scanning electron microscopy. Dentin powder was incorporated into a silated hydroxypropylmethylcellulose hydrogel. Several types of mixtures with variable parameters were tested. The mixtures were immersed into phosphate-buffered saline. The supernatants were collected, and the total released proteins were quantified by gel shift migration and Coomassie staining. The presence of transforming growth factor beta 1 using ELISA. Results: The mixture dentin powder/hydrogel released proteins (from 49.1 mg/mL–137.9 μg/mL according to the mixtures). The release kinetics was growing and started from the first day until stabilization at 14 days. The quantity of released proteins was directly related to the size of the particles and the weight of the powder incorporated into the hydrogel. Gel shift with direct revelation by ultraviolet and Western blot analyses confirmed the presence of transforming growth factor beta 1 using ELISA. Conclusions: We showed that silated hydroxypropylmethylcellulose hydrogel was able to extract dentin matrix proteins from smashed dentin powder. This mixture could be considered a new way of dental treatment for the dentin-pulp complex and bone regeneration. (J Endod 2016;42:1371–1376)

Key Words
Dentin powder, drug delivery, hydrogel, tissue engineering, transforming growth factor β1

The main objective of conservative dental treatment is to maintain pulp vitality and its functions. The dentin-pulp complex is able to answer and adapt itself to various stimuli. The dentin is a mineralized connective tissue composed of collagen type I and bioactive proteins sequestered into this matrix during initial dental development. These proteins can be released under pathological conditions but also by controlled demineralization thanks to bioactive materials (1, 2). These proteins, once secreted, diffuse to the pulp through the tubuli up and induce tertiary dentin formation (3). This allows protection of the pulp by increasing the thickness of hard tissue. Among these proteins, members of the transforming growth factor (TGF) beta family, involved in many biological processes like tissue repair, cell differentiation, and anti-inflammatory effects, have been detected (4, 5).

Regenerative dentistry is based on tissue engineering principles. The introduction of growth factors for regeneration of missing tissue is commonly used in oral surgery, implantology, and periodontology for bone regrowth. Recombinant proteins or human tissue bank–derived material have been the main sources of growth factors used for clinical applications to date. Nevertheless, it has been shown that growth factors can also be extracted from patients’ dentin (eg, by etching the mineralized tissue with chelating solutions such as EDTA) (6).

Because extracted teeth are generally discarded, it may be interesting to use such tissue as a source of growth factors for autoimplantation. Our hypothesis is that growth

Significance
A novel biomaterial made of a mix of hydrogel and dentin powder could provide an autologous source of growth factors for capping an exposed pulp tissue or promoting tissue regeneration to fill an empty root canal.
factors sequestered in dentin could be released from dentin powder after mixing with a highly basic hydrogel, which could be readily injected into the missing tissue space. If this hypothesis is confirmed, such a novel biomaterial could provide an autologous source of growth factors for promoting bone growth to fill a socket after dental extraction, capping an exposed pulp tissue, or promoting tissue regeneration to fill an empty root canal.

Numerous investigations have been performed on the use of biomedical scaffolds to maintain cells or molecules onto the site of interest and to control drug delivery. It is commonly accepted that polymeric hydrogels could be potential scaffolds for 3-dimensional culture of cells (7, 8). They possess appropriate biological properties, which make them suitable for biomedical applications. In the present study, silated hydroxypropylmethylcellulose (Si-HPMC) was used as a hydrogel scaffold. The self-hardening principle of the hydrogel is based on the silanes grafted along the hydroxypropylmethylcellulose chains. The dissolution of the material takes place in a strongly basic medium, which results in ionization of the silane into sodium silanolate. The limit pH of sodium silanolate stabilization is approximately 12.4. By decreasing the pH, sodium silanolate transforms into silanols. Gel formation is based on the condensation between the silanol groups by decreasing the pH (9). The solution is transformed into Si-HPMC hydrogel with the formation of a tridimensional network. The kinetics of the hydrogel cross-linking is linked to the pH value. Once cross-linked, the hydrogel shows a nanoporous macromolecular structure. Pores have an average diameter of 10 nm, which is greater than the diameter of the proteins. This structure allows the liberation of proteins and growth factors.

A hydrogel containing endogenous growth factors and morphogens native to dentin is likely an important adjuvant in clinical regenerative endodontics. Therefore, the goal of this study was to develop a new bioactive material able to release noncollagenic proteins contained in the extracellular dentin matrix. This study investigated the ability of a Si-HPMC hydrogel to extract and release these proteins from smashed dentin.

### Materials and Methods

#### Preparation of Dentin Powder

Freshly extracted teeth were collected at the dental surgery department of the dental hospital of the University of Nantes (approval number: DC-2011-1399). The teeth were treated with a solution of sodium azide 15 mmol/L. They were then cut with an Isomet Precision Cutter (Buehler, Lake Bluff, IL). Enamel, cementum, and pulp were removed to keep slices of dentin. The slices were then crushed with 2 different grinders: a blade mill (Smart Dentin Grinder; KometTio, Holon, Israel) and a zirconia mortar grinder (RM 100; Retsch, Haan, Germany). The dentin powder was sieved and analyzed using scanning electron microscopy to obtain the most homogeneous and finest powder.

#### Positive Control: Powder and EDTA 10%

After scanning electron microscopic (SEM) analysis, the powder obtained with the zirconia mortar grinder was used for this positive control because of its homogeneity and fine particle size. This powder was mixed with a solution of EDTA 10% (pH = 7.2) in order to demineralize and release proteins contained in the dentin powder. Two particle sizes (>50 μm and <50 μm) of powder, obtained after using a sieve of 50 μm, were tested to analyze the effect of particle size on protein discharge. The powders (100 mg) were immersed into 400 μL EDTA solution with 1 μL protease inhibitors. The EDTA solution of the 2 powders was renewed every day up to 12 days. The EDTA solution supernatants, collected each day, were conserved at 4°C. Twenty-four supernatant samples of EDTA solution were obtained on day 12 for the analysis. A negative control was performed by mixing dentin powder with phosphate-buffered saline (PBS) solution. A total protein assay (Pierce Coomassie Plus [Bradford] Assay Kit; Thermo Fisher Scientific, Waltham, MA) was achieved on the 24 EDTA solution samples to quantify total proteins.

#### Dentin Powder/Hydrogel Mixtures and Total Protein Assay

The powder from the zirconia mortar grinder, with a particle size of less than 50 μm, was incorporated into the Si-HPMC hydrogel. This powder was selected based on the SEM analysis and positive control experiments. It was the most homogeneous powder, and it released more proteins than that with a particle size >50 μm. Several types of mixtures with variable parameters were tested including the quantity of powder (50 mg, 100 mg, and 150 mg), time of reticulation before incorporation of the powder, the order of elements incorporation, and the pH of the hydrogel (Table 1). In all the mixtures we used 0.6 mL Si-HPMC and 0.3 mL acid buffer, making a final volume of 0.9 mL. All 12 mixtures were laid out into 2-mL tubes and then immersed with 0.9 mL PBS solution. Total proteins assays were achieved on 70 μL supernatants of PBS taken at 1, 2, 7, 14, and 30 days.

#### Gel Shift

Three mixtures of dentin powder/Si-HPMC hydrogels, among the 12 presented in Table 1 and releasing the greatest amount of proteins, were achieved and laid out in a culture plate of 6 wells. Mixture 1

### Table 1. Mixtures of Dentin Powder and Si-HPMC Hydrogel (n = 12) According to Various Conditions

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Quantity of dentin powder</th>
<th>Mixture 1</th>
<th>Time before incorporation of the third component</th>
<th>Third component added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 mg</td>
<td>Si-HPMC + powder</td>
<td>8 min</td>
<td>Buffer</td>
</tr>
<tr>
<td>2</td>
<td>50 mg</td>
<td>Si-HPMC + buffer</td>
<td>10 min</td>
<td>Powder</td>
</tr>
<tr>
<td>3</td>
<td>50 mg</td>
<td>Buffer + powder</td>
<td>20 min</td>
<td>Si-HPMC</td>
</tr>
<tr>
<td>4</td>
<td>100 mg</td>
<td>Si-HPMC + powder</td>
<td>8 min</td>
<td>Buffer</td>
</tr>
<tr>
<td>5</td>
<td>100 mg</td>
<td>Si-HPMC + buffer</td>
<td>10 min</td>
<td>Powder</td>
</tr>
<tr>
<td>6</td>
<td>100 mg</td>
<td>Buffer + powder</td>
<td>20 min</td>
<td>Si-HPMC</td>
</tr>
<tr>
<td>7</td>
<td>100 mg</td>
<td>Powder + 0.2 mL EDTA</td>
<td>20 min</td>
<td>Si-HPMC + Buffer</td>
</tr>
<tr>
<td>8</td>
<td>100 mg</td>
<td>Powder + 0.5 mL EDTA</td>
<td>20 min</td>
<td>Si-HPMC + Buffer</td>
</tr>
<tr>
<td>9</td>
<td>100 mg</td>
<td>Si-HPMC + powder</td>
<td>8 min</td>
<td>Buffer</td>
</tr>
<tr>
<td>10</td>
<td>150 mg</td>
<td>Si-HPMC + powder</td>
<td>10 min</td>
<td>Powder</td>
</tr>
<tr>
<td>11</td>
<td>150 mg</td>
<td>Si-HPMC + buffer</td>
<td>20 min</td>
<td>Si-HPMC</td>
</tr>
<tr>
<td>12</td>
<td>150 mg</td>
<td>Buffer + powder</td>
<td>20 min</td>
<td>Si-HPMC</td>
</tr>
</tbody>
</table>

Si-HPMC, silated hydroxypropylmethylcellulose.
contained 0.6 mL Si-HPMC and 0.3 mL acid buffer; 100 mg powder was then incorporated after 10 minutes. Mixture 2 contained 100 mg powder with 0.2 mL EDTA 10%. This mixture was incorporated after 20 minutes in 0.6 mL Si-HPMC with 0.3 mL acid buffer. Mixture 3 contained 0.6 mL Si-HPMC and 100 mg powder without acid buffer.

The mixtures were immersed with 1.2 mL PBS solution. The supernatants of the 3 mixtures were withdrawn at 7 and 14 days to achieve gel shift (Mini Protean TGX Stain Free Gels; Bio-Rad, Plano, TX). After migration, the revelation of proteins contained in the gel was achieved with ultraviolet imaging (Chemidoc MP System, Bio-Rad).

Western Blot of the Active and Inactive Form of TGF-β1

Mixtures of 100 mg, 125 mg, and 150 mg dentin powder with 0.6 mL Si-HPMC were achieved. The supernatants (25 μL) of each sample, taken after 14 days of salting out, were used for Western blot analysis. A human latency-associated peptide (LAP) TGF-β1 antibody (Antigen Affinity-purified Polyclonal Goat IgG; R&D Systems, France, Lille) was used for the inactive form of the growth factor, and a TGF-β1/1.2 antibody (Polyclonal Chicken IgY, R&D Systems) was used for the active form.

Quantification of TGF-β1 Release by ELISA

Several mixtures of 150 mg dentin powder with 0.6 mL Si-HPMC were achieved. Three samples were prepared for each day of quantification (n = 3); 1.2 mL supernatant was collected at 1, 3, 5, and 14 days and stored at −80°C. After completion of the 12 samples, they were thawed and subjected to growth factor quantification by using an immunosorbent assay test system for TGF-β1 (Human TGF-β1 Quantikine ELISA Kit, R&D Systems). A total protein assay was also performed to estimate the proportion of TGF-β by report of total proteins.

Statistical Analysis

First, the statistical analysis was performed with SYSTAT 10.5 software (Systat Software, San Jose, CA). Normal distribution of the variables was evaluated with a Lilliefors test. Friedman tests were achieved to analyze the reproducibility of our results and the influence of time and amount of proteins, with a significance level of 5%.

Results

Dentin Powder and Particle Size Selection: Scanning Electron Microscopy and Positive Control with EDTA 10%

After SEM analysis, the finest and the most homogeneous powder was obtained with the zirconia mortar grinder (Fig. 1A–D). SEM analysis confirmed that the size of particles was smaller than 5 μm in diameter with a higher quantity of particles between 0.6 μm and 0.8 μm (12% in number). The powder obtained with the Smart Dentin Grinder was generally homogeneous, but the particle size was bigger than with the zirconia mortar grinder.

The 2 powders treated with the EDTA solution released proteins. The protein discharge was maximal on the first day, with 189.1 mg proteins for the powder <50 μm and 144.5 mg for the powder >50 μm. This discharge decreased until day 12 (Fig. 2). The amount of released proteins by powder was more important when particle size was smaller than 50 μm. The amount of released proteins was correlated to the particle size (Friedman test for reproducibility, P = .117). The finer the powder was the higher the amount of released proteins. The negative control did not release any proteins.

Protein Characterization of Si-HPMC/Dentin Powder Release

All 12 samples released total proteins (Fig. 3). We noticed that the salting out of proteins started on day 1 until stabilization at 2 weeks.
Si-HPMC hydrogel was able to release the proteins from the dentin powder (Friedman test, \(P = .000\)). The mixture of dentin powder/Si-HPMC hydrogel without acid buffer showed a release of proteins higher than the other mixtures (sample 9, Fig. 3), with a maximal concentration of 198.6 \(\mu\)g/mL on day 30. The amount of released proteins in sample 9 is statistically more important than for the others (\(P = .000\)). The dentin powder alone could cross-link the hydrogel by decreasing the pH sufficiently. In the mixtures containing acid buffer, the more important the amount of powder incorporated in the hydrogel was the more important the amount of released proteins.

Gel shift, with a direct revelation by ultraviolet analysis, showed bands whose molecular weights were close to 95 kd and 24 kd. In the literature, the molecular weight of dimeric TGF-\(\beta\)1 is 25 kd, and the molecular weight of small latent complex of TGF-\(\beta\)1 is 105 kd.

Western blots of the active form and the inactive form of TGF-\(\beta\)1 (LAP TGF-\(\beta\)1) showed bands at 20 kd, 35 kd, and 60 kd (Fig. 4A–C).
corresponding to the molecular weights of the different forms of TGF-β1.

Enzyme-linked immunosorbent assay measurements of TGF-β1 released from the mixture dentin powder/Si-HPMC hydrogel showed a time-dependent profile (Fig. 4). The Si-HPMC hydrogel was able to release TGF-β1 contained in dentin powder. This release started on day 1 for 14 days with an average grade of 96.81 pg/mL growth factors at 14 days. Measurements were reproducible for each sample (Friedman test, *P* = .368). In all the samples, TGF-β1 represents 0.03% of the total proteins.

**Discussion**

The objective of current regenerative therapies is to promote tertiary dentin formation during deep caries lesions (10) to maintain pulp vitality. Nowadays, medical research is more and more interested in the development of new bioactive scaffolds to maintain and release bioactive molecules on the site of interest.

In our study, we tested the potential of a Si-HPMC hydrogel to release noncollagenic matrix proteins from dentin powder. We tested different parameters like the time of reticulation, the amount of powder incorporated into the hydrogel, and the order of element incorporation. The objective was to find the mixture that releases the biggest amount of proteins. The results of our study confirm the fact that Si-HPMC hydrogel is able to release the extracellular matrix proteins of the dentin, particularly TGF-β1. The mixture dentin powder/Si-HPMC hydrogel without acid buffer released the most proteins. This facilitates the manipulations by using only 2 elements. The environment created by the dentin powder/hydrogel mixture at a basic pH showed the highest rate of release proteins, without necessarily being able to explain the molecular mechanisms. Some authors, like Graham et al (11), have shown that calcium hydroxide, a material with a basic pH, can solubilize bioactive dentine matrix components like TGF. These observations show that a basic medium can also release dentin matrix components, as in acidic environments.

Concerning total protein release, some mixtures can release up to 252.5 μg/mL proteins. These total protein assays measure all the proteins contained in the dentin extracellular matrix (dentin sialo protein [DSP], dentin matrix protein [DMP], bone sialoproteins, osteopontin, etc). Although many other growth factors and proteins could have been analyzed in this study, TGF-β1 was chosen as the primary target because it is a well-recognized studied growth factor present in dentin (4, 12, 15). Even if TGF-β1 represents only a small quantity of proteins with regard to total proteins (0.03%), some authors showed that a low level of growth factor, in the order of the picogram, allowed them to obtain cellular answers, cellular proliferations, and mineralization (14, 15).

Many researchers are interested in TGF-β, particularly its chemotactic and anti-inflammatory properties (12, 13). In our study, we achieved many Western blots targeting TGF-β1. The molecular weights highlighted on the gels are not exactly similar to the molecular weights described in the literature. We cannot assert with certainty thanks to Western blots that TGF-β1 is really present, which is why we have performed a TGF-β1 quantification using the enzyme-linked immunosorbent assay to confirm the results obtained with the Western blots. Nevertheless, the differences concerning the molecular weights encountered in our study, regarding the literature, could be explained by various factors. First of all, TGF-β1 can be in a homodimeric form (25 kd) but also in a heterodimeric form, with TGF-β2 forming a complex with a molecular weight above 30 kd. In the same way, the molecular weight of active TGF-β is dependent on partial or total cleavage of the LAP form. TGF-β is found in 2 different latent forms before ending up in the extracellular medium (small latent complex or large latent complex). These 2 complexes are composed of a dimerization of an amino terminal portion (called LAP) and a carboxy terminal portion (active part of the TGF). The activation of these latent forms by the cleavage of the LAP portion by proteases leads to the release of active TGF-β. Then, during gel shift, the molecular weight can change because of phosphorylation or glycosylation reactions. Finally, the antibody used affects the results. Mozes et al (16) compared the specificity of 8 isoforms of anti-TGF antibodies. They found bands at 14 kd and 25 kd but also at 29 kd, 50 kd, and 67 kd that they do not explain. They concluded that there are a large number of antibodies, which are more or less sensitive and specific.

Many research teams focused on these new tissue bioengineering concepts and the development of hydrogels (17). Komabayashi’s team (18) focused on an injectable and photopolymerizable hydrogel able to release different kinds of molecules, like calcium. They showed that calcium was released in an increasing and controlled way, like in our study with total proteins. The current materials present several inconveniences like an uncontrolled release and an insufficient quantity of molecules reaching the site of interest. Hydrogels are biodegradable materials, with better quality compared with other materials, allowing a controlled release kinetics and a low risk of any overdose (19, 20). Among the numerous materials available today, hydrogel could be considered as the most adapted material to serve as a bioactive scaffold.

Hydrogels are already used in various medical disciplines including orthopedic surgery. On the other hand, teeth grinding is
also used in dental surgery and implantology thanks to a new machine commercialized for 2 years called the Smart Dentin Grinder. This device allows the use of dentin powder after tooth extraction to preserve bone volume or in case of sinus lift (21). The dentin has the same constituents and biological properties as bone actually used in autologous grafts. Authors have shown that the dentin powder grafted onto surgical sites and biological properties as bone actually used in autologous grafts. Our results confirm that Si-HPMC hydrogel is able to release proteins from the hydrogel, particularly TGF-β1. If its biological effects on pulp cells are confirmed, we can totally imagine using extracted teeth from the hydrogel, particularly TGF-β1, to grind and incorporate them into the hydrogel and to use this mixture as an autologous graft on another tooth to stimulate tertiary dentin formation in case of pulp capping or pulpotomy. So far, no scientific explanation is available, and results from observational studies remain empirical.

Conclusion

Our results confirm that Si-HPMC hydrogel is able to release noncollagenic matrix proteins from a smashed dentin powder, particularly TGF-β1. This mixture provides the ability, according to our results, to achieve an autologous contribution of growth factors. These results open new therapeutic perspectives in dentistry and in the field of tissue engineering. The next step will be to investigate its biological effect on pulp cells. The use of this dentin powder/hydrogel mixture on undifferentiated pulp cells and the study of cell differentiation markers could confirm its dentinogenic potential. If this is confirmed, this mixture could be considered as a new way of dental treatment for the dentin-pulp complex.

Acknowledgments

Fabienne Pérez and Stephane Simon contributed equally to this study. The authors deny any conflicts of interest related to this study.

References