Synthetic Clay–based Hypoxia Mimetic Hydrogel for Pulp Regeneration: The Impact on Cell Activity and Release Kinetics Based on Dental Pulp–derived Cells *In Vitro*

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Abstract

Introduction: Thixotropic synthetic clays have been successfully used for tissue engineering in regenerative medicine. The impact of these clays on the dental pulp, in particular in combination with hypoxia-based approaches using hypoxia mimetic agents (HMAs), is unknown. Our aim was to reveal the response of dental pulp–derived cells (DPCs) to a synthetic clay–based hydrogel and evaluate the release of HMAs. **Methods:** Using resazurin-based toxicity assays, live-dead staining, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining, the viability of human DPCs seeded onto a synthetic clay–based hydrogel of 5%–0.15% as well as onto the hydrogels loaded with the HMAs dimethyloxalylglycine (DMOG), desferrioxamine, L-mimosine, and $CoCl_2$ was evaluated. Furthermore, supernatant of the hydrogels loaded with HMAs were generated. Vascular endothelial growth factor (VEGF) production of DPCs in response to the supernatant was measured to reveal the cellular response to the HMAs. **Results:** We found that the synthetic clay–based hydrogel did not impair the viability of DPCs. Cell monolayer and cell cluster formations were observed on the hydrogel. No significant increase of VEGF levels was observed in the supernatant when DPCs were cultured on hydrogels loaded with HMAs. Supernatant of DMOG-loaded hydrogels stimulated VEGF production in DPCs in the first hour, whereas the effect of desferrioxamine, L-mimosine, and $CoCl_2$ did not reach a level of significance. **Conclusions:** The synthetic clay–based hydrogel represents a promising biomaterial that does not induce prominent toxic effects in DPCs. It can be loaded with DMOG to induce hypoxia mimetic activity. Overall, we provided first insights into the impact of synthetic clays on DPCs for tissue engineering purposes in regenerative endodontics. *(J Endod 2018;■:1–7)*

Key Words

Clay, dental pulp, hypoxia, hypoxia mimetic agents, prolyl hydroxylase inhibitors, prolyl hydroxylases, regeneration

Significance

Synthetic clays represent a novel thixotropic material. We provide first insights into the effect of synthetic clays on dental pulp–derived cells. Our results are highly relevant for hypoxia-based approaches in cell therapy and tissue engineering for regenerative endodontics.

Tissue engineering approaches for regenerative endodontics rely on 3 key elements: cells, growth factors, and scaffold materials (1). Because of the anatomic structure and the limited access to the pulp chamber, injectable scaffold materials have been advocated (2–4). These include collagen and fibrin, which have been applied for various tissue engineering approaches in the field of regenerative medicine (3, 4). However, the ideal scaffold material has not been revealed so far. Synthetic clays are novel scaffold materials, such as LAPONITE (BYK Additives Ltd, Wesel, Germany), a synthetic clay with 59.5% SiO_2, 27.5% MgO, 2.8% Na_2O, and 0.8% Li_2O (5–7). These hydrogels are injectable and thixotropic and can adsorb proteins (5, 6). These properties make synthetic clay–based hydrogels promising candidates for tissue engineering approaches. Synthetic clays have been used as carriers for growth and differentiation factors such as bone morphogenetic proteins and vascular endothelial growth factor (VEGF) (5, 6). Also, the use of synthetic clays as carriers for the controlled delivery of pharmaceuticals such as dexamethasone was advocated (8). Soaking collagen sponges with synthetic clay improved the capacity to adsorb VEGF and led to a prolonged release of the proangiogenic factor (5). Although the application of synthetic clays has been granted patents in other fields concerning dentistry, it has not been tested as a hydrogel for regenerative approaches in endodontics (9, 10).

Both cell-free and cell-based approaches for regenerative endodontics can be considered in combination with synthetic clay. Cell-free approaches rely on the effect of cell homing. Hypoxic conditions lead to a chemotactic effect on mesenchymal stem cells (11). Cells that have been preconditioned in a hypoxic environment have higher survival rates as well as an increased proangiogenic potential (12). Furthermore, cell-based strategies have the advantage that they

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can be applied both for *de novo* synthesis of the whole pulp and partial pulp regeneration (13). It was recently shown in a study on minipigs that transplanted cells are not sufficient for dental pulp regeneration (14). Transplanted pulp cells proliferated, but a functional mesenchyme was not produced. Thus, other factors seem to play a major role such as insufficient blood supply as suggested by others (14).

Hypoxia-based strategies are novel approaches in regenerative endodontics (15). Among these, there is the application of hypoxia mimetic agents (HMAs) that target the cellular oxygen sensors to boost angiogenesis and stimulate regeneration (12, 15–19). Furthermore, preclinical studies in rodents showed that compromised healing such as in diabetes and osteonecrosis can be rescued by the application of HMAs (20–22). Cells of the dental pulp have been shown to be target cells for HMAs. An increase in VEGF, interleukin 8, and angiogenin was described (12, 16, 18). Also, an increase in the repair ability of dental pulp cells was described upon treatment with HMAs (19). HMAs could also induce a proangiogenic response in dental pulp tissue as shown in tooth slice organ cultures (17).

As shown in previous publications, the proangiogenic potential of dental pulp cells is increased in spheroids and does not hinder their formation. Preconditioning of dental pulp cells with HMAs before their transplantation into the root canal may lead to better survival (18, 23, 24).

The potential therapeutic principle underlying the use of HMAs relies on the stabilization of the labile transcription factor hypoxia-inducible factor (HIF)-1alpha, which, among other pathways, induces proangiogenic activity (20). However, the specific mode of action of the different HMAs varies. Dimethyloxalylglycine (DMOG) structurally mimics 2-oxoglutarate, therefore inhibiting all prolyl hydroxylase domain–containing enzymes (PHDs) and factor inhibiting HIF-1 (FIH-1). Desferrioxamine (DFO) chelates Fe^{2+} and thereby inhibits PHD 3 and 1 as well as FIH-1. L-mimosine (L-MIM), a nonproteogenic amino acid, blocks the active site of the oxygen sensors PHD and FIH and also chelates Fe^{2+}. The metal ion Co^{2+} substitutes for Fe^{2+} and by that confers hypoxia tolerance. When PHD and FIH-1 are inhibited by HMAs it leads to increased levels of HIF-1, and, consequently, the hypoxia responsive genes are activated (25). A combination of the properties of synthetic clay with hypoxia mimetic activity would be a promising approach for both cell-free and cell-based therapies in regenerative endodontics. Although synthetic clay has been successfully applied as a carrier for VEGF, the release kinetics of the different HMAs is unclear.

Here we evaluated the feasibility of a synthetic clay as carrier for HMAs. The effect of synthetic clay–based hydrogels on dental pulp–derived cells (DPCs) was evaluated as well as the hypoxia mimetic activity and release of HMAs from the hydrogels based on an established bioassay with DPCs.

Materials and Methods

Preparation of the Synthetic Clay Hydrogel

The synthetic clay LAPONITE XLG (BYK Additives Ltd, kindly provided by IMCD South East Europe GmbH, Vienna, Austria) hydrogels at 5%, 2.5%, 1.25%, 0.62%, 0.31%, and 0.15% were prepared by mixing synthetic clay into deionized water at the respective concentration. Furthermore, 2.5% synthetic clay–based hydrogels containing DMOG, DFO, L-MIM, and $CoCl_2$ were prepared at 1 mmol/L. All steps were performed at room temperature.

Cell Culture

We prepared human DPCs from extracted third molars after informed consent was given by the donors (Ethics Committee of the Medical University of Vienna, Vienna, Austria) based on a previously described protocol (16, 26). The donor age was 24–45 years. In brief, the dental pulp was exposed, and tissue was collected. We performed explant cultures in alpha-minimum essential medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (PAA Laboratories, Linz, Upper Austria, Austria) in Petri dishes and antibiotics at 37°C, 5% CO_2, and 95% atmospheric moisture. DPCs were further expanded in T175 flasks. For the experiments, DPCs were seeded at 50,000 cells/cm^2 on top of the indicated materials in 96-well plates and incubated for 24 hours. For the experiments, cells were used at passages 3 to 8.

Release of the HMAs from the Synthetic Clay–based Hydrogels

In 96-well plates, 100 μL LAPONITE XLG hydrogel per well with and without HMA were incubated with 100 μL medium. The supernatants were collected and the medium was replaced at 1, 3, 6, 24, 48, and 72 hours. To assess the release and bioactivity of the HMAs, DPCs seeded at 50,000 cells/cm^2 in 96-well cell culture plates without hydrogel were incubated with the supernatant of the hydrogels for 24 hours. The cell culture medium was then subjected to immunoassays for VEGF and resazurin-based toxicity assay.

Live-dead Staining

Cells were stained with the Live-Dead Cell Staining Kit (Enzo Life Sciences AG, Lausen, Switzerland) according to the instructions of the manufacturer. The cells were evaluated using fluorescence microscopy (Diaphot 300; Nikon, Vienna, Austria) for green and red with a B-2A filter (excitation filter wavelengths: 450–490 nm) at a 100× magnification. Vital cells appeared green, whereas dead cells would have appeared red. Images were taken.

MTT Staining

DPCs in 96-well culture plates were incubated with 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO) at 37°C for the last 2 hours of exposure. Formazan formation was observed under a light microscope (Eclipse TS100; Nikon, Vienna, Austria) at a 200× magnification.

Alkaline Phosphatase Staining

To reveal if DPCs maintain their capacity to differentiate into alkaline-positive cells in the presence of the hydrogel, we performed staining for alkaline phosphatase. The cells were cultured on the hydrogel at 2.5% as previously described in the presence of differentiation medium consisting of alpha-minimum essential medium supplemented with 10% fetal calf serum, antibiotics, 50 mmol/L L-ascorbic acid (Sigma-Aldrich), and 10 mmol/L b-glycerophosphate (Sigma-Aldrich). After 7 days of culture, alkaline phosphatase staining was performed. Cells were fixed with neutral buffered formalin and incubated with the substrate solution containing Naphthol AS-TR phosphate disodium salt (Sigma-Aldrich) and Fast Blue BB Salt (Sigma-Aldrich), and then images were taken.

Resazurin-based Toxicity Assay

A resazurin-based toxicity assay was performed according to the manufacturer's protocol. Resazurin dye solution (Sigma-Aldrich) in an amount equal to 10% of the culture medium was added to the DPCs in