Hydrogel Arrays and Choroidal Neovascularization Models for Evaluation of Angiogenic Activity of Vital Pulp Therapy Biomaterials

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Abstract

Introduction: This study intended to evaluate the angiogenic properties of vital pulp therapy materials including white mineral trioxide aggregate (WMTA), calcium hydroxide ($Ca[OH]_2$), Geristore (Den-Mat, Santa Maria, CA), and nano WMTA biomaterials. **Methods:** WMTA, $Ca(OH)_2$, Geristore, and nano WMTA disks were prepared, dispersed into 2 mL Milli-Q (Millipore, ThermoFisher, Hanover Park, IL) distilled water, and centrifuged to obtain 2 mL supernatant elution. Thirty-five wells of polyethylene glycol hydrogel arrays were prepared and divided into 5 groups of 7 ($n = 7$). Mice molar endothelial cells (ECs) were placed on hydrogel arrays. The elution prepared from each sample was diluted in growth medium (1:3) and added to the hydrogel arrays. The EC medium alone was used for the control. For the choroidal neovascularization (CNV) model, thirty-five 6-week-old female mice were lasered and divided into 5 groups, and elution from each sample (2 μL) or saline (control) was delivered by intravitreal injection on the day of the laser treatment and 1 week later. The mean number of nodes, the total length of the branches in the hydrogel arrays, and the mean area of CNV were calculated using ImageJ software (National Institutes of Health, Bethesda, MD) and analyzed by 1-way analysis of variance and post hoc Tukey honest significant difference tests. **Results:** The comparison of results regarding the number of nodes showed the values of control > Geristore > nano WMTA > WMTA > $Ca(OH)_2$. Regarding the total branch length and the CNV area, the comparison of results showed values of Geristore > control > nano WMTA > WMTA > $Ca(OH)_2$. **Conclusions:** All tested materials showed minimal antiangiogenic activity, whereas Geristore and nano WMTA showed a higher proangiogenic activity than WMTA and $Ca(OH)_2$. *(J Endod 2018;■:1–7)*

Key Words

Calcium hydroxide, endothelial cells, Geristore, nano white mineral trioxide aggregate, tubulogenesis, white mineral trioxide aggregate

Significance

Regarding the angiogenic properties of dental pulp capping materials, only Geristore showed enhanced angiogenic activity, whereas the calcium silicate-based cements tested showed a diminished angiogenic activity. Based on our findings, Geristore can be regarded as a proangiogenic dental pulp capping material, which can be used in cases in which the enhancement of angiogenesis events is required at the applied area.

Pulpotomy is defined as the surgical removal of the inflamed coronal part of the dental pulp in order to protect and save the remaining healthy dental pulp tissue (1). The success rate of vital pulp therapy is strictly related to maintaining pulp vitality and homeostatic functions by restoration of the integrity of the vascular network through angiogenesis. This is a determinant component that guides the regenerative procedures toward the survival or necrosis of pulp tissue.

Human dental pulp is a highly vascularized tissue, which because of its vascular network and progenitor or postnatal dental pulp stem cells (DPSCs) has a great naturally inherent regenerative capacity (2). Although this issue might seem very simple in theory, the success of this procedure is closely related to the reestablishment of the homeostasis of dental pulp (3). Thus, maintaining the dental pulp blood supply, through the up- or down-regulation of pro- or antiangiogenic growth factors, is a key determinant component for the regeneration of dental pulp tissue (4). In a recently reported study, the authors showed that the application of proangiogenic agents such as iloprost

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during vital pulp therapy procedures can induce angiogenesis and the formation of tertiary dentin. These authors showed that the promotion in the expression of angiogenic factors can enhance tertiary dentin formation *in vivo* (5).

Many inorganic elements are recognized as being essential for tissue regeneration (6–8). Calcium hydroxide ($Ca[OH]_2$) is able to release Ca ions and promote the migration of DPSCs and enhance the production of biomolecules such as bone morphogenic proteins and fibronectin (9). White mineral trioxide aggregate (WMTA), because of several advantages over $Ca(OH)_2$, has become the most commonly used dental material in vital pulp therapy procedures (10). Geristore is a resin-modified glass ionomer with a composition of resin-based fluoroaluminosilicate glass (11). It was previously reported that glass ionomer cements might have an inducing effect on proliferative activity in dental pulp such as $Ca(OH)_2$ (1). Nano WMTA is a nano-modification of WMTA, which is composed of nanoparticles ranging in size from 40–100 nm with several additives compared with WMTA. Nano WMTA exhibits remarkable advantages in physiochemical properties including setting time, microhardness (12), compressive strength (13), and resistance in acidic environments (14). However, the inherent angiogenic properties of these cements have not been previously determined.

There a few studies regarding the angiogenic properties of calcium silicate–based cements. Huang et al (15) indicated that WMTA is able to activate the p38 pathway in human DPSCs (15). Yun et al (16) evaluated the combination effect of WMTA and growth hormone (GH) and indicated that this combination can promote angiogenesis events in human DPSCs. In a similar study, Chang et al (17) reported that the combination of WMTA and human placental extract can promote angiogenesis in rat dental pulp. However, the inherent angiogenic properties of these cements have not been previously determined. The present study was undertaken to investigate the angiogenic properties of WMTA, $Ca(OH)_2$, Geristore, and nano WMTA biomaterials. The hypothesis tested here is that these biomaterials exhibit different inherent angiogenic capacities that could impact their reparative potential.

Material and Methods

The dental material, including WMTA (ProRoot MTA; Dentsply, Tulsa, OK), $Ca(OH)_2$ (Calcium Hydroxide Powder USP; Dharma Research, Miami, FL), and Geristore (Den-Mat, Santa Maria, CA), were prepared according to the suppliers' directions. The angiogenic properties of these biomaterials were evaluated using an *in vitro* model of a polyethylene glycol (PEG) hydrogel array (0.4-kPa shear modulus) similar to Matrigel (BD Biosciences, Franklin Lakes, NJ) and endothelial cells (ECs) isolated from mouse molars. For *in vivo* experiments, the laser-induced mouse choroidal neovascularization (CNV) model was used to assess the angiogenic activity of various biomaterials.

Isolation of ECs from Dental Pulp

The isolation of dental pulp was performed by a method used by Saghiri et al (18, 19). Briefly, ECs were isolated from 1 litter (6 or 7 pups) of 4-week-old C57BL/6j immorto-mice. Harvested molars were broken at the cementoenamel junction, and the pulp tissues were collected under a dissecting microscope. Pulp tissues were cut into small pieces using a razor blade and then digested in 5 mL collagenase type I enzyme (Worthington, Lakewood, NJ; 1 mg/mL in serum-free Dulbecco-modified Eagle medium [DMEM]) and incubated at 37°C for 40 minutes. The digested tissues were washed with DMEM containing 10% fetal bovine serum (FBS) and centrifuged. The pellet was resuspended in 10 mL DMEM with 10% FBS and passed through nylon mesh with a pore size of 70 μm. The cells were pelleted and resuspended in 1 mL DMEM with 10% FBS and mixed with magnetic beads coated with platelet endothelial cell adhesion moleule-1/CD31 (PECAM-1) antibody prepared as previously described. The mixture allowed to rock at 4°C for 1 hour. After incubation, the beads were collected using a magnet and washed 6 times with 1 mL DMEM with 10% FBS. The beads were then resuspended in 0.5 mL EC growth medium and plated in a well of a 24-well plate coated with fibronectin (2 μg/mL in serum-free medium overnight) (20). The EC growth medium contained DMEM with 10% FBS, 2 mmol/L L-glutamine, 2 mmol/L sodium pyruvate, 20 mmol/L 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), 1% nonessential amino acids, 100 μg/mL streptomycin, 100 U/mL penicillin, freshly added heparin at 55 U/mL (Sigma-Aldrich, St Louis, MO), 100 μg/mL endothelial growth supplement (Sigma-Aldrich), and murine recombinant interferon gamma (R&D, Minneapolis, MN) at 44 U/mL. Cells were then passed to a 35-mm dish coated with 1% gelatin and subsequently expanded into gelatin-coated 60-mm dishes. The identity of ECs was confirmed by fluorescence-activated cell sorting (FACS) analysis for EC markers including CD31 and VE-cadherin. All cells were used before passage 15.

PEG Functionalization with Norbornene

Modification of PEG-OH molecules with terminal norbornene groups was performed using methods similar to previous studies (21). Briefly, PEG-OH (20-kd molecular weight, 8-arm, tripentaerythritol core; Jenkem USA, Allen, TX), dimethylaminopyridine, and pyridine (Sigma-Aldrich) were dissolved in anhydrous dichloromethane (Thermo Fisher Scientific, Waltham, MA). In a separate reaction vessel, N,N′-dicyclohexylcarbodiimide (Thermo Fisher Scientific) and norbornene carboxylic acid (Sigma-Aldrich) were dissolved in anhydrous dichloromethane and reacted for 30 minutes to activate the norbornene. Norbornene carboxylic acid was covalently coupled to PEG-OH through the carboxyl group by combining the PEG solution and norbornene solutions and stirring the reaction mixture overnight under anhydrous conditions. Urea by-product was removed from the reaction mixture using a fritted glass funnel, and the filtrate was precipitated in cold diethyl ether (Thermo Fisher Scientific) to extract the norbornene-functionalized PEG (PEGNB). The precipitated PEGNB was collected and dried overnight in a fritted ceramic filter. To remove impurities, the PEGNB was dissolved in chloroform (Sigma-Aldrich), precipitated in diethyl ether, and dried a second time in a Büchner funnel. To remove excess norbornene carboxylic acid, PEGNB was dissolved in deionized H_2O, dialyzed in deionized H_2O for 1 week, and filtered through a Millex 0.45-μm pore-size polyvinylidene difluoride syringe filter (Millipore, Darmstadt, Germany). The aqueous PEGNB solution was frozen using liquid nitrogen and lyophilized. Functionalization of PEG was quantified using proton nuclear magnetic resonance spectroscopy to detect protons of the norbornene-associated alkene groups located at 6.8–7.2 ppm. Functionalization efficiency for norbornene coupling to PEG-OH arms was above 90% for all PEGNB used in these experiments.

Formation of the PEG Hydrogels

Hydrogel substrates were formulated and characterized in a study by Nguyen et al (21), and hydrogel substrates were formed here using similar methods. Briefly, hydrogel solutions, containing 2 mmol/L of PEGNB; 8-arm norbornene-functionalized PEG; 0.125 mmol/L cyclized RGD peptide (RGDfC; Genscript, Piscataway, NJ); 4 mmol/L cross-linking peptide (KCGGPQGIWGQGCK, Genscript); and 0.2% I2959 photoinitiator (Ciba Specialty Chemicals, Tarrytown, NY) dissolved in 1× PBS were added as 100-μL volumes to 48-well plates; the droplets were cross-linked under 365 nm ultraviolet light for 2 minutes at a