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Cytotoxicity of ammonium hexafluorosilicate on human gingival fibroblasts

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ABSTRACT

Ammonium hexafluorosilicate (SiF), which is claimed to significantly improve occlusion of dentinal tubules, was proposed as a novel desensitizer for dentine hypersensitivity (DH). However, the cytotoxicity of SiF on oral cells is lacking. The purpose of this study was to investigate the cytotoxicity of SiF on human gingival fibroblasts (hGFs) under different dosages (0.001%, 0.01%, 0.1%, and 1%) and treatment durations (1, 5, 10, and 30 min). Cell proliferation, mitochondrial membrane potential (MMP) and cell cycle were tested by MTT assay, JC-1 staining and flow cytometry, respectively. Glutathione (GSH) depletion was analyzed to further investigate the underlying mechanism of SiF-induced cytotoxicity. MTT assay showed that there was significantly lower number of viable cells when the hGFs were treated with 0.01% (10 min), 0.1% (10 and 30 min) and 1% (5, 10, and 30 min) SiF than the control group ($p < 0.05$). MMP decreased and GSH depletion increased dramatically along with higher concentrations (0.1% and 1% SiF) and prolonged times (10 and 30 min). DNA synthesis [S (%)] of cells treated with 0.1% and 1% SiF (5, 10, and 30 min) was significantly lower than the control group ($p < 0.05$). Our results indicate exposure to up to 0.01% SiF for less than 5 min causes low or no cytotoxicity *in vitro*.

1. Introduction

Dentine hypersensitivity (DH) is characterized by short, sharp pain arising from the exposed dentin in response to evaporative, thermal, tactile, osmotic, or chemical stimuli, which cannot be ascribed to any other form of dental defect or pathology (Holland et al., 1997). DH has long been a troublesome symptom annoying many patients, with a high prevalence in 20–69 years old Chinese urban adults (29.7%) (Rong et al., 2010). Occlusal wear, abrasion, dietary erosion, gingival recession, abfraction, tooth abnormally positioned in the arch, periodontal surgery, and incorrect toothbrushing habits are the main factors that may contribute to the exposure of dentinal tubules (Cornelius and Temitope, 2011; Scherman and Jacobsen, 1992).

The most widely accepted theory that explains the mechanism of DH is the hydrodynamic theory, which proposes that certain stimuli induce pressure changes across the dentin. As a result of the pressure changes, fluids shift rapidly within the dentinal tubules, followed by the excitation of sensory nerves in the pulp dentin border (Brännström, 1962). Therefore, the effective treatment of DH is either physically blocking the exposed dentinal tubules or reducing the excitability of the relevant sensory nerves (Porto et al., 2009).

The conventional conservative approach to deal with DH is to apply chemical agents. Fluorides such as diamine silver fluoride [AgF: $(NH_3)_2AgF$], sodium fluoride (NaF), and stannous fluoride (SnF_2) have long been used in clinics or in home-care (Porto et al., 2009). However, AgF blackens the teeth due to silver precipitation (Suge et al., 2008) and the longevity of other fluorides' desensitizing effect is not satisfactory (Porto et al., 2009). Professionals have been trying to find a better desensitizer that has fewer drawbacks for a long time.

Recently, researchers have drawn their attention to ammonium hexafluorosilicate [SiF: $(NH_4)_2SiF_6$], which does not change tooth color and induces apatite formation and mineralization by silicate (Dong et al., 2011). Dentinal tubules were occluded homogeneously and completely with silica–calcium phosphate precipitation after SiF solutions treatment, and it had a continuous effect on dentinal tubule occlusion under a simulated oral environment (Suge et al., 2008, 2010). SiF was also effective in preventing dental caries from progressing, although the acid resistance of the

Abbreviations: DH, dentine hypersensitivity; FBS, fetal bovine serum; FCM, flow cytometry; GSH, Glutathione; hGFs, human gingival fibroblasts; JC-1, 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolyl-carbocyanine iodide; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazoli-umbromide; PBS, phosphate buffered saline; PI, Propidium Iodide; SiF, ammonium hexafluorosilicate.

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SiF-treated teeth was inferior to that of AgF-treated teeth (Kawasaki et al., 2005). Therefore, SiF seems to be a promising desensitizing agent. However, no studies have been performed on the cytotoxicity and biocompatibility evaluation of SiF.

The present study was designed to investigate the cytotoxicity of SiF on human gingival fibroblasts (hGFs) under different concentrations (0.001%, 0.01%, 0.1%, and 1%) and different treatment durations (1, 5, 10, and 30 min).

2. Materials and methods

2.1. Materials and sample preparation

SiF used in this study was purchased from SSS Reagent Co., Ltd., Shanghai, China. It was directly dissolved in double distilled water to obtain a 10% solution, which was then sterilized by filtration through a 0.22-μm filter (Millipore, Ireland) and then stored at 4 °C. For each experiment, different concentrations of the SiF (0.001%, 0.01%, 0.1%, and 1%) were serially freshly diluted using Dulbecco's modified Eagle's medium (DMEM) without fetal bovine serum (FBS) from the stored solution (Rajabalian et al., 2009).

2.2. Cell culture and immunostaining for hGFs identification

hGFs were prepared from young healthy premolars extracted for orthodontic reasons or the third molars for impaction reasons (3 donors, 15–20 years old). The use of hGFs in this study was approved by West China Hospital of Stomatology Institute Review Board (WCHSIRB-D-2011-008), following informed consents taken from the patients. hGFs were cultivated from gingival connective tissue explants digested with collagenase I (Wang et al., 2005). Briefly, freshly extracted teeth were washed with phosphate buffered saline (PBS) three times and then immersed in an antibiotic solution (10^4 U/mL penicillin, 10^4 μg/mL streptomycin) for 5 min. Gingival tissue was dissected from the cervical tooth using a sharp surgical scalpel, minced, placed in a centrifugal tube with 0.2% collagenase I (Sigma Chemical Co., St. Louis, Missouri, USA) dissolved in PBS for 30 min at 37 °C water bath, and then centrifuged for 8 min at 1000 rpm. Afterward, it was resuspended in DMEM (Gibco, Grand Island, NY, USA) with FBS (Gibco, Grand Island, NY, USA), pipetted into 25 cm^2 flasks, kept in a medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/L streptomycin (Invitrogen, Carlsbad, CA, USA), and then cultured in a humidified 37 °C/5% CO_2 incubator. Cells grew out of the tissue and reached confluence. hGFs were then passaged, expanded, and identified for future use. Cells between the fourth and seventh passages were used in subsequent experiments.

hGFs of passage three were identified using immunocytochemical staining for vimentin and cytokeratin (Xia et al., 2011). Cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, permeabilized with 0.5% Triton X-100 for 10 min followed by 3% H_2O_2 treatment for 20 min, and then blocked with 1% bovine serum albumin for 30 min. Next, the cells were incubated with primary antibody to vimentin or cytokeratin overnight at 4 °C, followed by HRP-conjugated secondary antibody at 37 °C for 30 min. The diaminobenzidine (DAB) kit was used to develop the color, and the slides were counterstained with hematoxylin. The specimens incubated in the absence of primary antibodies were used as negative controls. All the reagents were purchased from ZSGB Bio., Beijing, China.

2.3. MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoli-umbromide (MTT) assay (Mosmann, 1983; Ulukaya et al., 2008; Wang et al., 2010), which is based on the ability of the mitochondrial enzyme succinate dehydrogenase to convert yellow water-soluble tetrazolium salt (MTT) into formazan crystals in metabolically active cells. This dark-blue product stored in the cytoplasm is made soluble by dimethyl sulphoxide (DMSO) afterwards, generating a blue color. The color intensity is directly proportional to the amount of viable cells.

hGFs were seeded in 96-well plates (200 μL per well of 2×10^4 cells/mL) and cultured in DMEM containing 10% FBS for 24 h until the cells attached properly. Cells were then treated with the culture medium (control) and SiF of different concentrations (0.001%, 0.01%, 0.1%, and 1%) for 1, 5, 10, and 30 min. Each group had five replicates. After the cells were incubated with 0.5 mg/mL MTT (Sigma Chemical Co., St. Louis, Missouri, USA) for another 3.5 h at 37 °C, the medium was removed and the crystal formazan was solubilized in 150 μL DMSO (Sigma Chemical Co., St. Louis, Missouri, USA). Absorbance was measured at 490 nm by a multiwell scanning spectrophotometer (Thermo Varioskan Flash, USA). The relative growth rate (RGR) of cells was calculated as follows: RGR = (average optical density of experiment group/average optical density of control group) × 100%.

2.4. Assessment of mitochondrial membrane potential (MMP)

The alteration of mitochondrial membrane potential (MMP) in treated and untreated cells was tested by 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (Beyotime Biotech, Shanghai, China). Cells were seeded in 6-well plates and cultured in DMEM containing 10% FBS until they attached, and then treated with SiF of different concentrations (0.001%, 0.01%, 0.1%, and 1%) for 1, 5, 10, and 30 min. Untreated cells worked as the control. Each group had three replicates. The cells were then transferred to 1 mL fresh medium and 1 mL JC-1 dyeing liquid following the kit's instruction. After incubation for 20 min, specimens were rinsed with 2 mL JC-1 buffer twice and placed onto the stage of a fluorescence microscope. Images were collected using 490/525 nm excitation wavelengths within 30 min. JC-1 is a cationic dye whose emitted fluorescence changes from red (J-aggregates) to green (JC-1 monomers) following a mitochondrial membrane depolarization.

2.5. Cell cycle analysis

hGFs were seeded in 6-well plates and cultured in DMEM containing 10% FBS until the cells grew to grew to 80–90% confluence. The cells were then treated with the culture medium (control) and SiF of different concentrations (0.001%, 0.01%, 0.1%, and 1%) for 1, 5, 10, and 30 min. The distribution of cell cycle phases was analyzed by flow cytometry (FCM) (Wang et al., 2010; Kim et al., 2004). Cells were harvested using 0.25% trypsin (KEYGEN, Nanjing, China), and aliquots of 1×10^6 were prepared for analysis. To avoid membrane damage as much as possible, the cells were observed under the inverted microscope after about 2 min of trypsin digestion, and then removed from the plates when most of the cell shapes changed from long spindle to round. The cells were then centrifuged and washed in phosphate buffered solution (PBS), fixed with prechilled 70% ethanol overnight, and then treated with 20 mg/L RNase A (KEYGEN, Nanjing, China) for 30 min. Propidium Iodide (PI) (KEYGEN, Nanjing, China) was added to a final concentration of 20 mg/L. DNA content of the samples was analyzed on a flow cytometer FC500 (Beckman-Coulter, Fullerton, CA), and the number of cells in every phase was calculated using Multicycle software (Phoenix Flow System, San Diego, CA). Each group had three replicates.