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N-acetyl cysteine prevents pain and hypersensitivity of bleaching agents without affecting their aesthetic appeal; evidence from in vitro to animal studies and to human clinical trials

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Abstract

Background: Tooth hypersensitivity and pain are undesirable side effects of bleaching agents in humans. The aim of this study is to implement strategies to counter such side effects, and to demonstrate the efficacy and mechanisms of action of N-acetyl cysteine (NAC) in countering the side effects of clinically used bleaching agents.

Methods: In a series of in vitro experiments, animal model studies, and human clinical trials, we demonstrate that NAC protects oral mucosa and teeth from damage induced by bleaching agents.

Results: The addition of NAC along with clinically used bleaching agents to dental pulp stromal/stem cells (DPSCs), stem-cells of apical papillae (SCAP) and oral epithelial cells, inhibited cell death mediated by bleaching agents in several in vitro assays. In addition, rat teeth, when treated with chair-side or over-the-counter bleaching agents, exerted adverse side effects to pulpal and gingival tissues as evidenced by the white lesions of gingivae as well as decreased survival and function of DPSCs. These side effects of bleaching agents were greatly mitigated by the application of NAC to the surfaces of the teeth and to the oral mucosa. NAC protected the surface topography and the appearance of the tissues after bleaching using scanning electron microscopic (SEM) analysis. Finally, application of NAC prior to the bleaching demonstrated significant translational benefit for the patients since it ameliorated pain and hypersensitivity and protected gingivae from bleaching induced white lesions and improved inflammatory index in the oral mucosa in human clinical trials.

Conclusions: Therefore, application of NAC to the surfaces of the teeth and oral mucosa prior to the use of bleaching materials is beneficial for countering adverse side effects of bleaching in patients and decreases pain, sensitivity, and potential damage to the dentition and oral mucosa associated with bleaching.

Trial registration: NCT03534115 (NAC Prevents Side-Effects of Teeth Bleaching). Registration 4 December 2014.

Keywords: NAC, Bleaching agents, Dental pulp stromal cells (DPSCs), Apoptosis

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Background

Teeth-bleaching is a widely used procedure in general aesthetic dentistry and it has become significantly more popular in recent years. To achieve whitening, highly oxidant compounds, such as carbamide and hydrogen peroxides are applied to teeth as bleaching agents [1]. The application of these whitening agents on the outer layer of the tooth, the enamel, leads to a significant decrease in calcium and phosphorous content and increase in surface roughness and porosity. The new surface topography allows the whitening agents to penetrate the tooth and promote the oxidation and consequent breakdown of staining compounds, which with time accumulate in the teeth [2]. There are two main methods of whitening: in-office peroxide-containing gels and white strips for application at home. In-office whitening gels contain higher concentrations of hydrogen peroxide (between 25 and 40%) and can be applied through deep bleaching in a single visit of 1 hour treatment or through the usage of take-home personalized trays containing peroxide gels. In-home white strips are composed of 3 to 15% hydrogen peroxide or carbamide peroxide, and are required to be used twice a day for 2 weeks to reach optimal efficacy [3].

Over the past couple of decades, bleaching has become one of the most patient-requested cosmetic dental procedures [4]. In the 1800's, dentists used internal bleaching techniques to reverse dental discoloration that was caused by trauma or in previously endodontically-treated teeth, and in the 1870's, oxalic acid was used for external bleaching to improve esthetics. It wasn't until the dawn of the nineteenth century that dentists began to modernize various bleaching techniques [5, 6]. In recent years, whitening systems used by patients at home have become increasingly popular [7]. Although these home kits had comparable esthetic results to chair-side bleaching, there were also potential side effects to the hard tissues [8–10] and soft tissues [3, 11]. Therefore, there is a need for effective strategies to prevent the side-effects of teeth whitening.

Reported problems associated with bleaching procedures include sensitivity in vital teeth [12], cervical root resorption after internal bleaching [13] and decrease in the hardness of the intertubular dentin [14]. In addition, bleaching products may also irritate the gingiva causing gingival ulcers and contribute to tooth hypersensitivity [15]. This form of gingival irritation is likely caused by the leaching of the bleaching materials onto the surfaces of the gingival tissues. Moreover, the application of acrylic resins that is used as part of the chair-side bleaching procedure in combination with the bleaching agents may cause additional toxicities [16, 17]. We have previously shown that resin materials induce significant apoptosis in various cell types including gingival fibroblasts [16]. In hopes of maintaining the aesthetic advantages of bleaching agents, we strive to design strategies to incorporate materials that would safely counter the side effects of the bleaching materials without affecting their esthetic benefits by the application of NAC.

NAC inhibits cell death partly through its anti-oxidant activity [18–20]. It is shown to act directly as a reducing agent and indirectly by stimulating the synthesis of other anti-oxidant enzymes such as glutathiones (GSH) [21, 22]. As a precursor for GSH synthesis, NAC is an intracellular antioxidant that easily penetrates red blood cells where it is deacetylated to form L-cysteine [23]. Previous reports suggest other mechanisms for the inhibitory function of NAC on cell toxicity such as thiol reduction devoid of toxicity and establishment of adherens junctions [24, 25]. NAC is reported to be an effective supplement for protecting oral tissues against blue light irradiation-induced oxidative damage in rat model [26]. Blue light significantly accelerated oxidative stress and increased the oxidized glutathione levels in gingival tissue of rats, but these effects were inhibited by NAC pre-administration [26]. We have previously shown that NAC-induced differentiation of DPSCs provided the basis for their increased protection from functional loss and cell death. The increased differentiation triggered by NAC was paralleled with an increased induction of NFκB activity, a transcription factor that is responsible for DNA transcription, cytokine secretion and cell survival [27, 28].

In this paper, we demonstrate that the use of topical NAC prior to the application of bleaching materials on teeth protects the pulp and oral tissues from adverse side effects induced by bleaching agents in the rat model as well as in randomized human clinical trials. Human clinical trials also show significant reduction in reported hypersensitivity and pain in patients with the application of NAC.

Materials and methods

Culture media and reagents

Dulbecco's Modified Eagle Medium (DMEM) (Fisher Scientific, PA, USA) was supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, CA, USA), 1% non-essential amino-acids, 1% sodium pyruvate, 1% streptomycin and 1% L-glutamine (Gemini Bio-Products, CA, USA) and used as the complete medium for cultures of the rat dental pulp and gingival cells. DMEM complete medium supplemented with Na-β-glycerophosphate (10 mM) and ascorbic acid (50 μg/ml) (Sigma Aldrich, St. Louis, MO) was used for the cultures of the dental pulp stromal/stem cells (DPSCs) and stem cells of the apical papilla (SCAP). Propidium iodide (PI), N-acetyl-cysteine (NAC-S), sodium hydroxide and hydroxyethyl-piperazineethane-sulfonic acid (HEPES)

were all purchased from Sigma, St. Louis, MO. NAC-N was purchased from Nutri*Vita*, CA. Annexin V for flow cytometry was purchased form Thermo-Fisher Sc. CA. The bleaching agents used for the experiments were purchased from Oral B (South Boston, MA), Opalescence, and Ultradent (South Jordan, UT). Enzyme-linked immunosorbent assay (ELISA) kits and reagents were purchased from R&D (Minneapolis, MN) and used according to the manufacturers suggestions.

Bleaching method for in-vivo studies

8-week old male Sprague-Dawley rats were divided into 3 groups. No bleaching agents were applied to the first group of rats, which served as the control group. The bleaching agent (Oral-B Rembrant Whitening system) was applied to the upper and lower incisors of the second group of rats as suggested by the manufacturer, which served as bleaching agent group. Finally, NAC at a concentration of 20 mM [29] was applied to the incisors (4 times for 5 min) before the application of the bleaching agent to the third group of rats, which served as bleaching agent+NAC group. After 4 h of bleaching, the teeth were extensively washed with water before they were extracted, and dental pulps were harvested.

To study the effect of bleaching strips (Treswhite, Opalescence-Ultradent) rats were divided in 2 groups. The group of rats that did not receive any treatments was used as controls whereas for the second group the bleaching strips were left on the upper and lower incisors for 9 h. After 9 h, the teeth were washed extensively with water before they were extracted, and dental pulps were harvested.

Scanning electron microscopy (SEM)

Rat palatal tissues from control, bleaching agent, and bleaching agent + NAC groups were excised after 5 h of treatment and the tissues were washed twice with distilled water and fixed with 2.5% gluteraldehyde for 30 min. They were then washed 6 times with distilled water and dehydrated in a graded series of ethanol (50–100%). Surface morphology of the specimens was then examined using SEM.

Cell death assays using chromium-51 release assay, PI staining, and fluorescent imaging

For ^{51}Cr release assay cells were treated with ^{51}Cr and incubated for 1 h, after which the cells were washed twice using DMEM media in order to wash the excess ^{51}Cr. Cells were then transferred to 96 well culture plates (10,000cells/100 μl media), and were treated with different concentrations of H_2O_2 in the presence and absence of NAC for 4 h. After a 4-h incubation period the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cell death was calculated as follows:

$$\%\text{cell death} = \frac{\text{Experimental cpm-spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

After treatment of cells with and without bleaching agents in the presence and absence of NAC, cells were stained with propidium iodide (PI) as described previously [30–32]. Briefly, the cells were washed twice with ice-cold PBS containing 1% BSA before 1×10^4 cells in 50 μl of cold-BSA were stained with 8 μg/ml propidium iodide (PI) and they were incubated on ice for 10 mins and brought to 500 μl with PBS-BSA. For Annexin V and PI staining, the cells were washed twice with ice-cold PBS containing 1% BSA before 1×10^4 cells in 50 μl of cold-BSA were stained with 5 μl of Annexin V and 8 μg/ml propidium iodide, and cells were incubated on ice for 5 mins and brought to 500 μl with PBS-BSA. Flow cytometric analysis was then performed using Beckman Coulter Epics XL cytometer (Brea, CA).

For fluorescent imaging the cells were stained with Target Cell Visualization Assay (TVA™) dye obtained from Cellular Technology Limited, OH and was used as suggested by the manufacturer. The TVA™ utilizes direct imaging of fluorescence-labeled cells. Labeled cells are cultured with different concentrations of H_2O_2 in the presence and absence of NAC for 4 h. Cells that are induced to undergo cell death lose their fluorescent signal whereas those remaining viable are visualized and counted which represent the proportions of surviving cells. The CTL S6 Ultimate Fluorescent Analyzer is used to analyze the surviving cells.

NAC application before bleaching in human clinical trials

NAC at 20 mM was prepared using sterilized distilled water and HEPES at pH 7–7.2 and applied to gums and teeth before bleaching. Sterilized distilled water in the absence of NAC was used as control. The 25% hydrogen peroxide ZOOM whitening gel and ZOOM kits used for the treatments were purchased from Discus Dental (Culver City, CA) and used accordingly to the manufacturer's suggestions.

Bleaching method during clinical trials and saliva collection

A single center, randomized controlled single-blinded clinical trial (NCT03534115) was conducted using human subjects ($n = 41$) to determine the protective role of NAC on chair-side bleaching procedures (NCT03534115). Exclusion criteria included pregnant human subjects as well as any prospective patients with significant enamel translucency, unstable posterior occlusion, missing anterior teeth, current placement of anterior crowns or

orthodontic devices. Informed consent was obtained based on the IRB protocol and the subjects were randomly assigned and were blinded to control ($n = 20$) and experimental ($n = 21$) groups. Patients were informed to refrain from eating 2 h prior to the bleaching procedure. The NAC and control solutions were prepared 1 h before every chair-side bleaching procedure by the same individual who did not participate in the patient treatments. For bleaching, the suggested ZOOM protocol was used. Patients with a randomly pre-assigned group were given applications of either topical NAC or H2O on the soft and hard dental tissues for 5 min prior to the placement of the liquid dam. The NAC or H2O was also applied on the teeth for 5 min after cycle 1 and cycle 2 of the suggested Zoom protocol. The same clinician was used for every procedure. Pre-operative and post-operative pictures of the gingiva and teeth were taken, and the shades of the teeth were recorded using VitaShade Classical, VitaShade Bleach and Portrait Bleach guides. Saliva was collected prior to the procedure, after the first application of NAC or H2O and after the removal of the liquid dam for cytokine studies. The levels of pain and hypersensitivity were recorded during and after chair-side bleaching with and without NAC application using a standard questionnaire. The clinician also recorded any signs of patient discomfort or inability to talk. Throughout the procedures, patients were asked to communicate with the clinician by writing on a notepad and raising their hands.

All of the patients were advised to take over-the-counter Tylenol or the prescribed medication (Norco) for pain management specifically and were further notified to refrain from consuming any colored sustenance within 24–72 h in order to prevent teeth discoloration. They were also informed to collect saliva samples every 2 h for 12 h for the first day and thereafter once daily for 5 days, freeze the samples and transport them on ice. For every saliva sample collected, the patients were instructed to record their at-home pain and sensitivity levels and number of drugs taken. After 6 days from the procedure, the patients were instructed to transport the frozen saliva samples on ice along with their recorded responses. They were then interviewed on their overall experience. The interview included questions regarding their level of satisfaction, overall resting pain and sensitivity experience to cold and hot drinks.

Elisa

ELISAs were performed as described previously [32] . Human IL-8 DuoSet ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA) and the procedures were conducted as suggested by the manufacturer. Analysis was performed using the Star Station software to analyze and obtain the chemokine concentrations of IL-8. Standard curves were generated using two-fold dilution of recombinant cytokines provided by the manufacturer. Standard curves were constructed using a 5-parameter logistic curve to ensure optimal sensitivity at lower readings. IL-8 saliva samples were diluted 1:10 prior to ELISA analyses. Values that fall below the lower detection limit (62.5 pg/ml for IL-8) were deemed inaccurate and assigned the midpoint between 0 and the lower detection limit.

Pain and cold sensitivity ratings

Pain was defined as a generalized throbbing and burning sensation of the gums. Patients' pain and sensitivity levels were recorded during each of the three cycles, every 2 h post-procedure for 10 h total, and thereafter every day for 5 days. The mean of patient responses was compared between NAC or H2O across all time points. A Wilcoxon Rank Sum Test (Mann-Whitney U Test) was performed for each surveyed time point to compare differences in patient responses. Sensitivity to cold was defined as a localized sharp, shooting and fleeting sensation due to cold air stimulus.

Statistical analysis

All data were analyzed for the significant differences of the mean using a two-tailed unpaired T-test. One-way ANOVA using Prism-7 software was used to compare different groups. (n) denotes the number of mice for in-vivo experiments, and number of patients for human clinical studies. The following symbols represent the levels of statistical significance within each analysis, ***(p-value < 0.001), **(p-value 0.001–0.01), *(p-value 0.01–0.05).

Results

NAC protects DPSCs, SCAP and oral epithelial cells from cell death induced by clinical grade bleaching agent containing hydrogen peroxide (H_2O_2)

To determine the effect of clinical grade bleaching agent containing hydrogen peroxide on DPSCs, SCAP and oral squamous cell carcinoma cells (OSCCs) several distinct assays were performed. We first treated DPSCs and SCAPs with different concentrations of clinical grade hydrogen peroxide (Fig. 1) or bleaching agent used to whiten patients' teeth (Additional file 1: Figure S1) in the presence and absence of NAC, and determined the levels of cell death induced by these agents and their protection by NAC as measured by ^{51}Cr release assay, propidium iodide (PI) staining, and fluorescent imaging. Cell death induced by dose dependent concentrations of hydrogen peroxide (Fig. 1a, b), and bleaching agent (Additional file 1: Figure S1) was significantly inhibited by NAC. Similarly, treatment of oral squamous cell carcinoma cells (OSCCs) with bleaching agent also mediated dose dependent cell death which was neutralized by

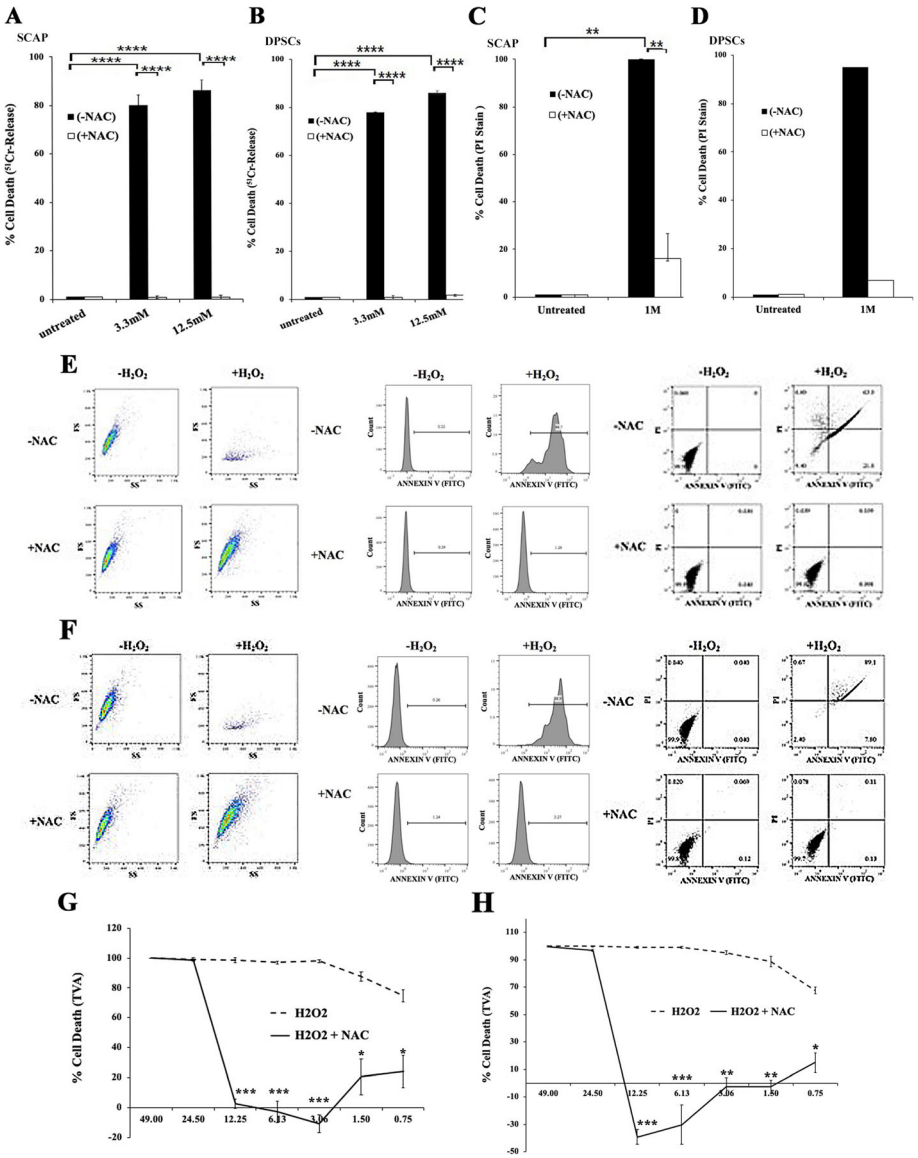

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 NAC protects DPSCs and SCAP cells from cell death induced by different concentrations of bleaching agents, and clinical grade hydrogen peroxide. Human SCAP cells were treated with ^{51}Cr and incubated for 1 h, after which the cells were washed twice to remove excess ^{51}Cr, followed by their treatment with different concentrations of H_2O_2 in triplicates in the presence and absence of NAC. After 4-h incubation the supernatants were harvested and counted for released radioactivity using standard 4-h ^{51}Cr release assay as described in Materials and Methods (**a**). DPSCs obtained from human teeth were treated with ^{51}Cr and incubated for 1 h, after which the cells were washed twice to remove the excess ^{51}Cr, followed by their treatment with different concentrations of H_2O_2 in triplicates in the presence and absence of NAC. After 4-h of incubation the supernatants were harvested and counted for released radioactivity using standard 4-h ^{51}Cr release assay as described in Materials and Methods (**b**). Human SCAP cells were cultured (0.2 million/ ml) overnight in 12 well plates before they were treated with bleaching agents containing hydrogen peroxide in the presence and absence of NAC (20 mM) in triplicates for 24 h. Afterwards SCAP cells were detached and their viability was determined using propidium iodide (PI) staining followed by flow cytometric analysis (**c**). DPSCs obtained from human teeth were cultured (0.2 million/ ml) overnight in 12 well plates, before they were treated with bleaching agents containing hydrogen peroxide in the presence and absence of NAC (20 mM) in triplicates for 24 h. Afterwards DPSCs were detached and their viability was determined using propidium iodide (PI) staining followed by flow cytometric analysis (**d**). DPSCs obtained from human teeth were cultured (0.2 million/ml) overnight in 12 well plates, before they were treated with clinical grade hydrogen peroxide (5 mM) in the presence and absence of NAC (20 mM) for 24 h. Afterwards DPSCs were detached and their viability was determined by flow cytometric analysis of forward and side scatter (left two panels), Annexin V staining alone (middle two panels), or PI and Annexin V staining (right two panels). One of 3 representative experiments is shown in this Fig. (**e**). Human SCAP cells were cultured (0.2 million/ ml) overnight in 12 well plates, before they were treated with clinical grade hydrogen peroxide (5 mM) in the presence and absence of NAC (20 mM) for 24 h. Afterwards SCAP cells were detached and their viability was determined by flow cytometric analysis of forward and side scatter (left two panels), Annexin V staining (middle two panels), or PI and Annexin V staining (right two panels). One of 3 representative experiments is shown in this Fig. (**f**). DPSCs obtained from human teeth were stained with TVA™ dye as described in Materials and Methods section before they were treated with clinical grade hydrogen peroxide at the concentrations indicated in the figure in the presence and absence of NAC (20 mM) for 4 h. Afterwards CTL S6 Ultimate Fluorescent Analyzer was used to analyze the surviving cells as described in Materials and Methods section. One of 3 representative experiments is shown in this Fig. (**g**). Human SCAP cells were stained with TVA™ dye as described in Materials and Methods section before they were treated with clinical grade hydrogen peroxide at the concentrations indicated in the figure in the presence and absence of NAC (20 mM) for 4 h. Afterwards CTL S6 Ultimate Fluorescent Analyzer was used to analyze the surviving cells as described in Materials and Methods section. One of 3 representative experiments is shown in this Fig. (**h**)

two different batches of NAC purchased from two different companies (Additional file 1: Figure S1). When clinical grade H_2O_2 was used to assess cell death in both DPSCs (Fig. 1d and e) and SCAP cells (Fig. 1c, f), both types of cells were susceptible to H_2O_2 mediated cell death as evidenced by decreased forward and side scatter (Fig. 1e, f (left panels)) and increased Annexin V and/or PI staining (Fig. 1e, f (middle and right panels)). NAC completely inhibited cell death induced by H_2O_2. Finally, loss of cell viability by H_2O_2 was also detected by staining with TVA™ dye and measured by fluorescent imaging (Figs. 1g, h). There was dose dependent induction of cell death by H_2O_2 and inhibition by NAC in both DPSCs (Fig. 1g) and SCAPs (Fig. 1h).

NAC protects rat DPSCs and gingival fibroblasts from growth inhibition induced by the application of bleaching agents

To determine the protective effect of NAC on bleaching, rat teeth were bleached with the chair side bleaching agent (Rembrandt bleaching gel) in the presence and absence of NAC as described in the Materials and Methods section. As shown in Additional file 1: Figure S2A, application of NAC inhibited the side effects of bleaching agents on DPSCs. Restoration of cell growth and function of DPSCs in the presence of NAC is evident when either the numbers of cells were determined (Additional file 1: Figure S2B) or ALP staining was measured (Additional file 1: Figure S2C). Therefore, the DPSCs obtained from teeth that were bleached in the presence of NAC grew as well as those obtained from unbleached teeth of control rats.

Palatal tissues from rats were treated with bleaching gel (Rembrandt bleaching gel) in the presence and absence of NAC as described in the Materials and Methods section. As shown in Additional file 1: Figure S3A, NAC inhibited the side effects of the bleaching agents on the palatal cells. Restoration of cell growth of palatal cells treated with bleaching agents in the presence of NAC is evident when the numbers of cells were determined and compared to those treated with bleaching agents in the absence of NAC (Additional file 1: Figure S3B). Examination of the palatal tissues by scanning electron microscopy (SEM) demonstrated a change from the usual topographical view seen in the unbleached tissue to relatively flat and smooth view observed in bleached tissues. The SEM images obtained from tissues treated with the bleaching agents in the presence of NAC demonstrated similar profiles to those seen with control unbleached, healthy tissues (Fig. 2a).

Similarly, DPSCs isolated from rat teeth which were exposed to the over-the-counter bleaching strips demonstrated less cell growth when compared to unbleached teeth (Additional file 1: Figure S4A). However, the growth rate of DPSCs treated with over-the-counter bleaching strips was still relatively higher than those obtained by the chair side application of bleaching agents (Additional file 1: Figures S4A and S2A, B). Accordingly,

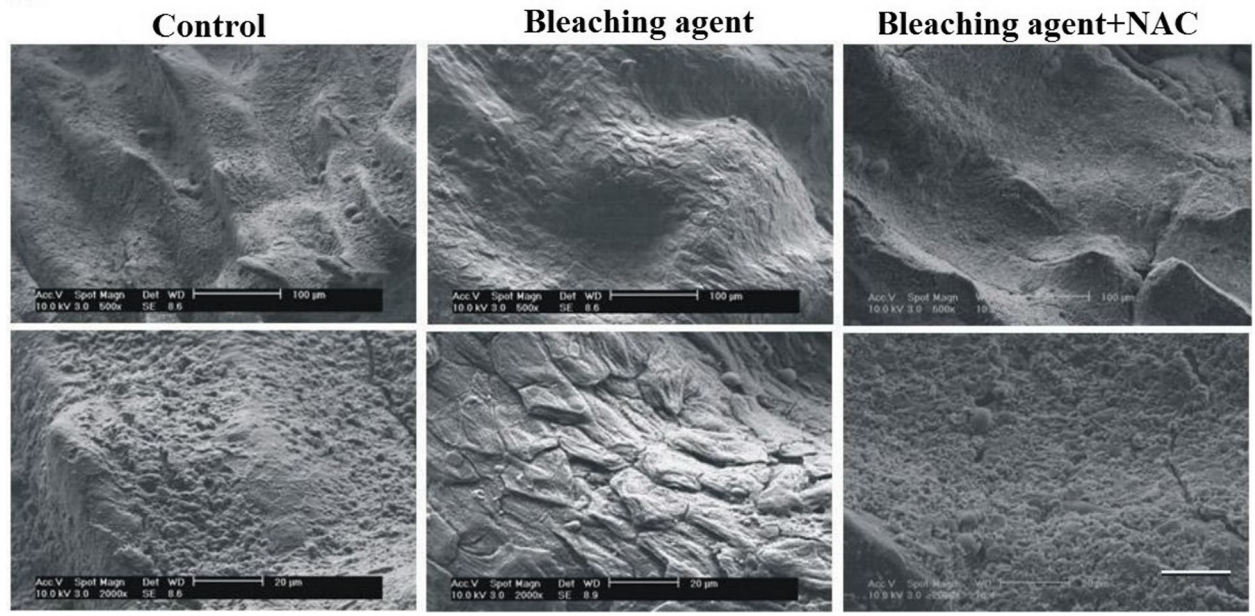

Fig. 2 NAC protects the gingival cells from undergoing cell death in the presence of bleaching agents in rats. 8-week old male rats were divided into three groups; No bleaching agent- control (left panel) ($n = 3$), bleaching agent only (middle panel) ($n = 3$) and bleaching agent with NAC (right panel) ($n = 3$). Four hours after application, the palatal tissues were extensively washed with sterilized saline, and the tissues were removed surgically and treated with 0.25% trypsin-EDTA and 0.1% collagenase to prepare single cell suspensions as described in the Materials and Methods section. The surface topography was analyzed using Scanning Electron Microscopy, magnification 500X and bar = 100 μm for the upper panels and, magnification 2000X and bar = 20 μm for the lower panels (**A**)

there was a reduction in the ALP staining of DPSCs obtained from the pulp tissues of bleached teeth as compared to the healthy control teeth (Additional file 1: Figure S4B). Therefore, over the counter bleaching strips can also exert adverse effect on pulp tissues.

Application of NAC with bleaching agents is as effective in whitening of rat teeth as bleaching alone

To determine whether NAC interferes with the whitening capacity of the bleaching agents, we applied NAC in the presence of the bleaching agents to the rat teeth. Animals which received no treatment served as controls whereas those that received the bleaching agents in the absence of NAC or the presence of NAC as described in Material and Methods section served as the experimental groups. As shown in Fig. 3a, the application of NAC prior to the bleaching of the rat teeth was as effective in lightening the shade of the tooth color as the application of bleaching agent in the absence of NAC. Therefore, no visual shade differences could be seen between the groups that had received NAC when compared to those without NAC (Fig. 3a). In addition, when the effects of bleaching on the soft tissue were assessed, a substantial area of gingival whitening/irritation could be seen when the bleaching agent was applied in the absence of NAC, whereas those with NAC exhibited considerably less gingival whitening/irritation, demonstrating the healthy pink appearance of the gingival tissues (Fig. 3b).

NAC decreases chemically-induced white lesions of the gingiva and decreases substantially pain and sensitivity associated with teeth bleaching in clinical trials of human subjects

Clinical trials (NCT03534115) were conducted as described in the Materials and Methods section. The average age of the patients who satisfied our inclusion criteria was 36.5 years old with a male:female ratio of 1.4:1. Teeth and gingiva from patients with bleaching and NAC exhibited teeth whitening with minimal to no gingival irritation as assessed by VitaShade Classical, Vita Bleach and Portrait Bleach shade guides when compared to those with bleaching in the absence of NAC application (Figs. 4a, b). No significant differences can be seen in shade improvement increments between the two groups of patients (Fig. 4c). In addition, patients using bleaching with NAC showed significantly less difficulty in speaking during and after the procedure due to the discomfort of the procedure when compared to those with bleaching alone (Fig. 4d). Overall assessment of gingival whitening across all patients was much less in

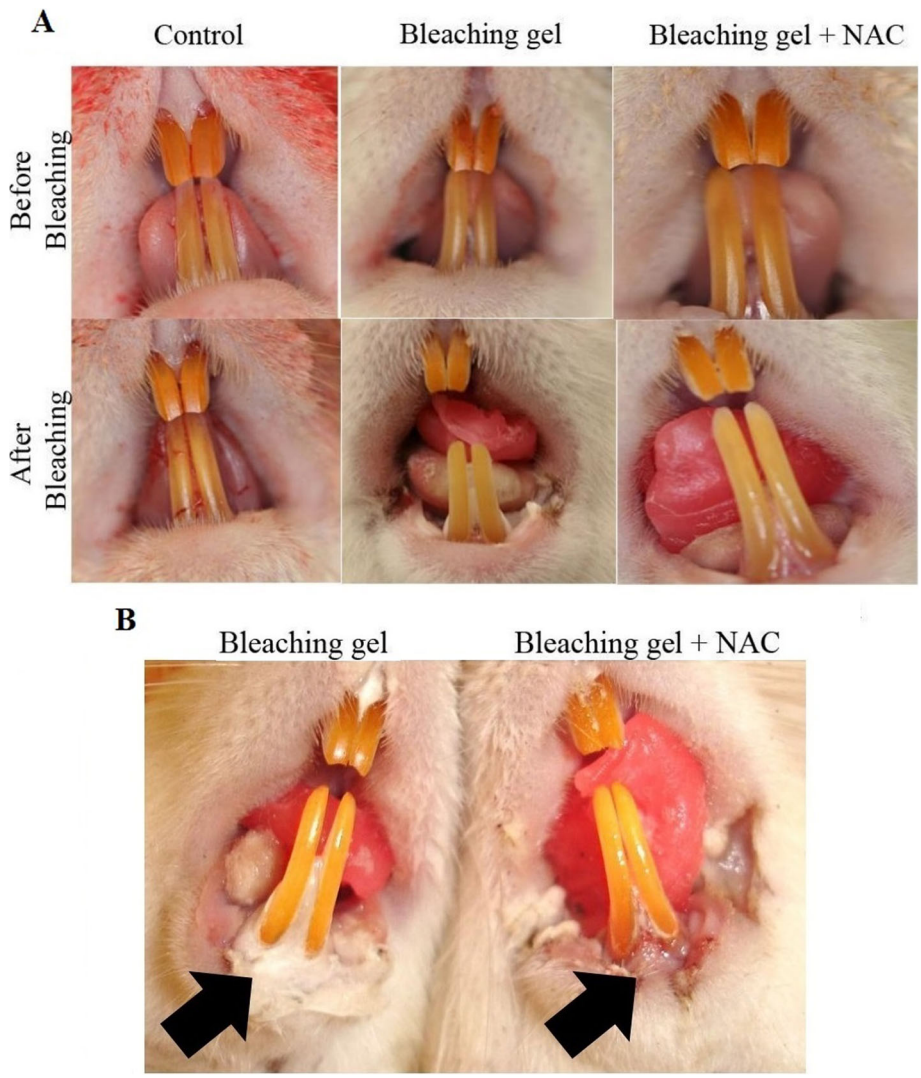

Fig. 3 NAC does not interfere with the teeth whitening ability of the bleaching agents. 8-week old male rats were divided into three groups; No bleaching agent- control (left panel) ($n = 3$), bleaching agent only (middle panel) ($n = 3$) and bleaching agent with NAC (20 mM) (right panel) ($n = 3$). After 4 h of treatment as described in Materials and Methods, the teeth were washed with sterilized water and photographs were taken to demonstrate differences in the color and shade of the teeth within the three groups before and after bleaching (**a**). Rat gingival tissues were treated with bleaching gel in the presence and absence of NAC for 4 h after which the tissues were washed with sterilized water and photographs were taken to demonstrate the differences in tissues between the two groups. The arrows point to the significant white lesions in the gingival tissues of the rats treated with bleaching gel alone and much less in rats which were treated with the bleaching gel and NAC (**b**)

patients receiving bleaching with NAC when compared to bleaching alone (Fig. 4d). Pain and sensitivity were analyzed using Wilcoxon Rank Sum Test (Mann-Whitney U Test) due to the nonparametric nature of the measurements. Patients gave their at-home responses to pain on a 0–10 pain scale with 10 being the highest for mean pain levels and were interviewed for their overall resting pain experience on a 0–5 pain scale with 5 being the highest for resting pain levels. Results indicated that the mean at-home pain levels and overall resting pain (Fig. 5a, b) and cold sensitivity (Fig. 5c) were significantly less in NAC and bleaching treated patients in comparison to bleaching in the absence of NAC. Patients' satisfaction with esthetics of bleaching was not statistically significant in those receiving bleaching in the absence of NAC versus those receiving NAC before bleaching application (Fig. 5d). As some patients had difficulty differentiating pain versus sensitivity, an aggregated response was measured for the pain and sensitivity ratings exhibiting less aggregated levels in those receiving NAC before the bleaching application when compared to those receiving bleaching in the absence of NAC (data not shown).

The amounts of IL-8 secreted in saliva were determined as a surrogate for the levels and extent of

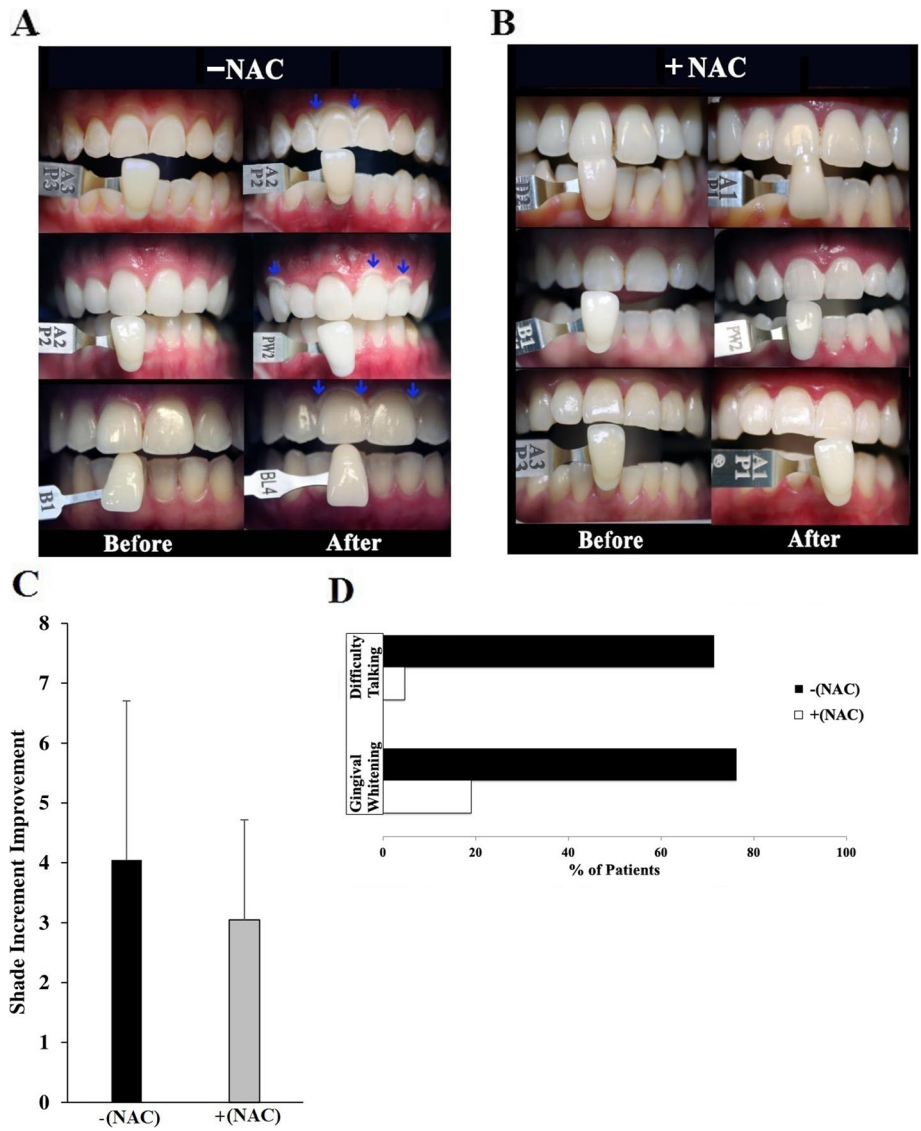

Fig. 4 NAC improves the extent of teeth whitening and prevents mucosal chemical burns of chair-side bleaching agents in patients. Clinical photographs of teeth after bleaching from patients in the control group (without NAC application) demonstrating VitaShade Classical, Vita Bleach and Portrait Bleach shade guides to match with the color of the teeth were taken. Arrows point to the white lesions in the gingival margins around teeth, one of several representative experiments is shown in this picture (**a**). Clinical photographs of teeth after bleaching from patients in the NAC group demonstrating VitaShade Classical, Vita Bleach and Portrait Bleach shade guides to match with the color of the teeth were taken. No or much lower levels of white lesions in the gingival margins around the teeth can be seen, one of several representative experiments is shown in this picture (**b**). VitaShade Classical, Vita Bleach and Portrait Bleach Guide are placed in a value-oriented order and given a score in increasing order from light to dark. Shade increment improvement scores for teeth color were determined by finding the differences of the before and after shade guide score. The scores indicated in the table below were used based on the shade guide. The average score was determined in control and NAC-treated groups and found to have no statistical significance ($p = 0.3276$) (**c**) Clinical observations were recorded after bleach whitening treatment of patients in control (H2O) or NAC groups. Assessments were made based on patient's ability to speak after the procedure due to discomfort, and the extents of whitening of the gingival tissues around the teeth after bleaching (**d**)

inflammatory responses during bleaching and it was found to be higher in patients who received bleaching alone as compared to those with bleaching and NAC (Figs. 6a, b). Statistical significance of the differences between bleaching in the absence or presence of NAC application was calculated for each time-point using a one-tailed T-test due to the parametric distribution of samples. Homogeneity of sample variance was tested by using an F-Test of Variance. Patients that received bleaching after NAC application overall secreted less IL-

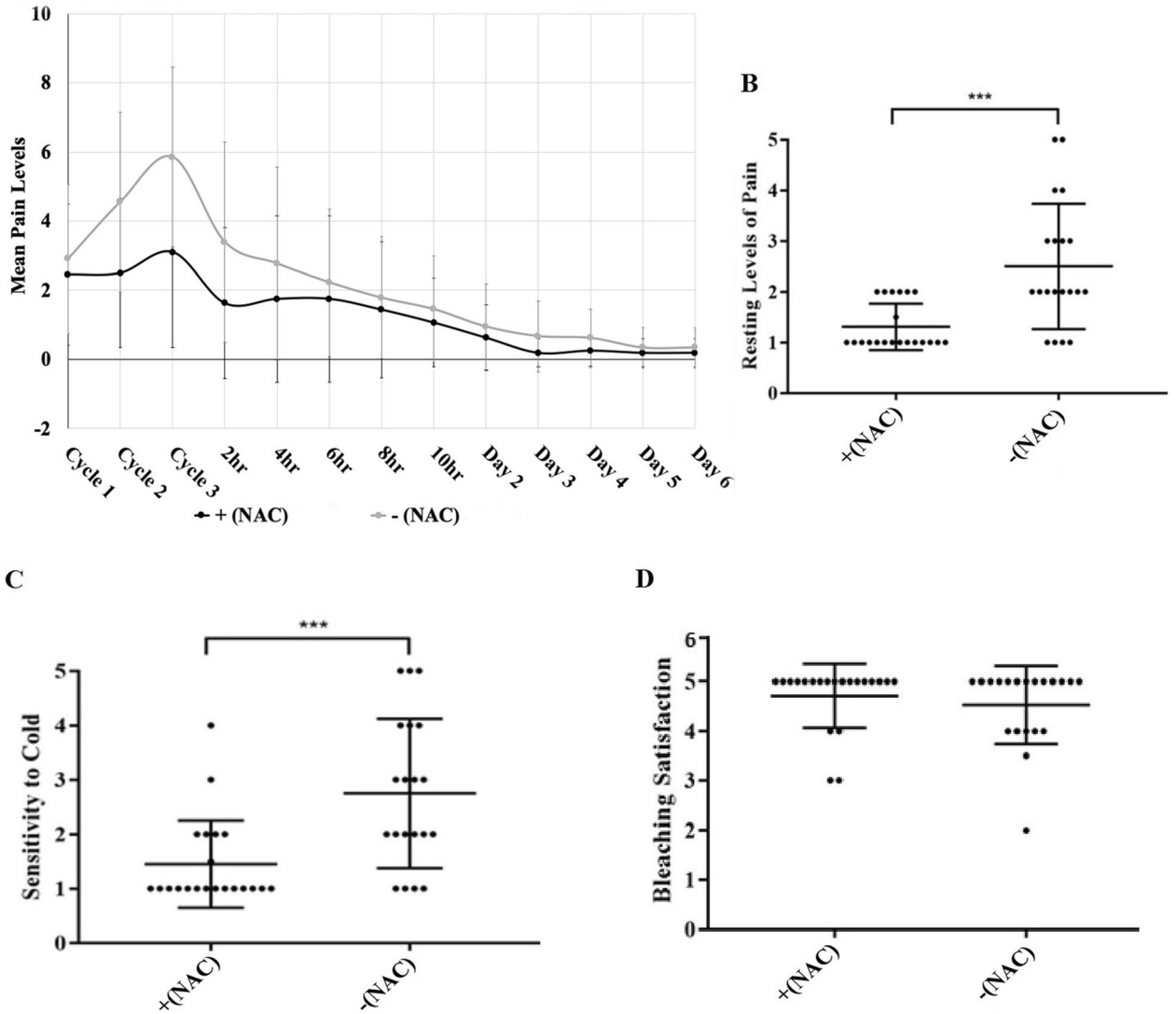

Fig. 5 NAC-treated group experienced lower levels of pain, decreased sensitivity to cold and similar degrees of satisfaction of the bleaching outcome based on their exit interview responses. The levels of pain measured at a scale of 0–10, 10 being the most painful, in patients were collected after each of the three bleaching cycles and every 2 hours post-procedure for the first 10 h, and thereafter on a daily basis for 5 days. The mean values for each time point was plotted for both H2O and NAC-treated patients. A Wilcoxon Rank Sum Test (Mann-Whitney U Test) was performed for each time point to compare significant differences in patient responses. Higher pain levels were noted in H2O group with significant differences in cycle 2 ($p = 0.012$), cycle 3 ($p = 0.005$), and 2-h post-treatment ($p = 0.048$) (**a**) Exit interview response for overall levels of pain experienced by patients were recorded on a scale of 0–5 with 5 being the most painful, and plotted for both H2O and NAC groups. A Wilcoxon Rank Sum Test (Mann-Whitney U Test) was used to calculate statistical significance. Patients receiving bleaching and NAC experienced a lower resting pain level with a *P*-value of 0.0002 ($n = 21$) (**b**). Exit interview response was obtained for overall experience to sensitivity to cold on a scale of 0–5, with 5 being the most sensitivity to cold stimulus. A two-tailed, unpaired t-test showed a significantly higher sensitivity to cold in control patients, with a P-value of 0.0006 ($n = 20$) (**c**). Exit interview response was obtained for post-procedure satisfaction of bleach whitening esthetics according to a 0–5 scale, with 5 being the most satisfied. A two-tailed t-test showed that NAC patients were equally satisfied with their bleaching cosmetic outcome, with a P = value of 0.35 ($n = 21$) (**d**)

8 with significant differences at the 6 and 8 h time points ($P = 0.01$) (Fig. 6)a. When overall levels of IL-8 secretion were determined between the two groups significantly less IL-8 was secreted in the saliva of patients who received bleaching with NAC when compared to bleaching alone (Fig. 6b). Similar results to IL-8 were also seen

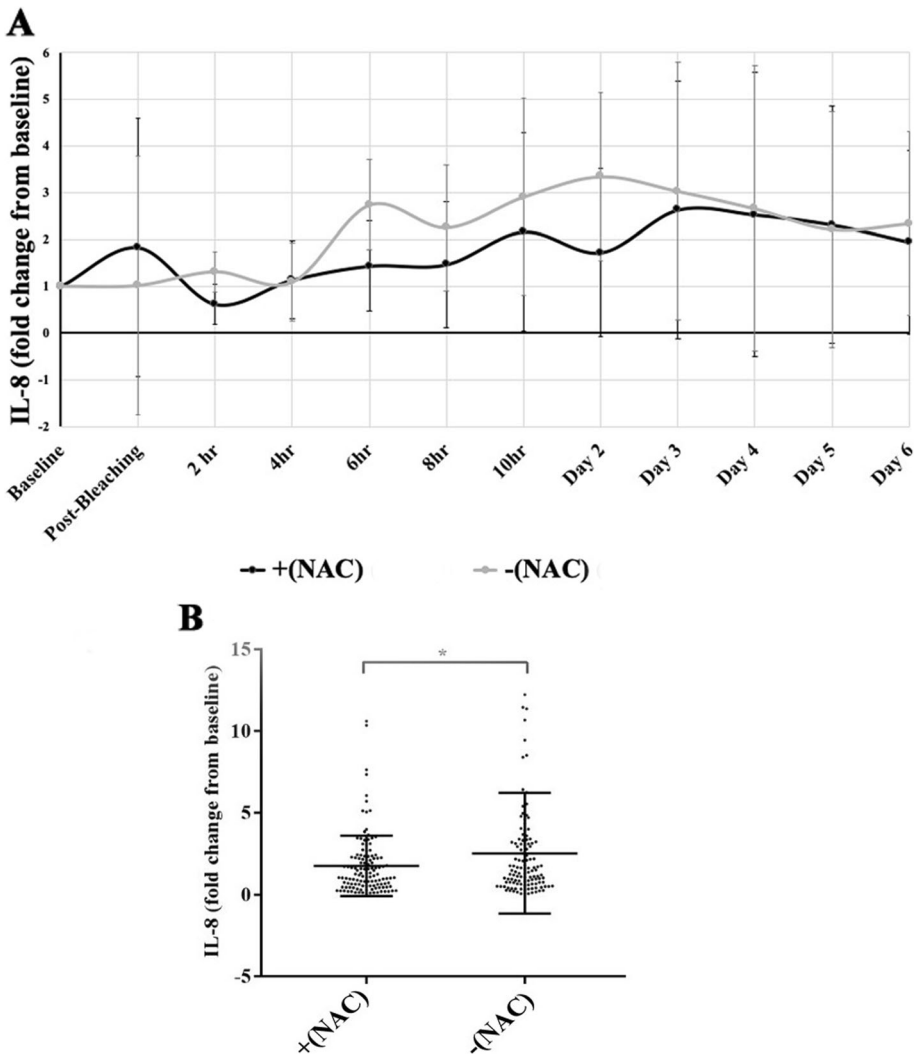

Fig. 6 NAC resulted in decreased levels of IL-8 secretion in saliva after bleaching in patients. Patients' saliva samples were collected immediately before and after the bleaching procedures, as well as every two hours post-procedure for the first 10 h, and on daily bases for 5 days. The levels of IL-8 obtained at the pre-bleaching saliva samples were used as the baseline to calculate the fold-change of IL-8 secretion in subsequent time points. The mean fold-change values were plotted for both H2O and NAC-treated patients across all time points (**a**). A combined scatter plot, along with mean and standard deviation, from the 4-h post bleaching to day 3 post-bleaching was generated to examine the distribution of IL-8 changes. Control patients ($n = 20$) demonstrated higher IL-8 fold change values compared to NAC patients ($n = 20$), with a P-value of 0.038 (**b**)

when IL-6 was measured in patients who received bleaching in the absence or presence of NAC application; however, the differences in IL-6 levels did not reach statistical significance between the two groups (data not shown).

When comparing the peak increase in pain and sensitivity and IL-8 secretion, important differences can be seen (Figs. 5 and 6). The levels of pain and sensitivity increased significantly in the group receiving bleaching in the absence of NAC immediately after bleaching was initiated and it leveled off at 4–6 h post-procedure. However, the levels of IL-8 secretion started to rise at 4–6 h and they leveled off on day 3.

Discussion

Dental bleaching is a simple procedure for aesthetic restoration of vital and discolored teeth. This procedure may exert significant adverse effects in oral cavity since it was previously shown to affect the ratio of Ca:P of enamel and dentin in teeth. In addition bleaching agents were shown to cause DNA strand breaks in cells [33]. With the exception of a few in vitro studies which demonstrated significant demineralization of enamel following bleaching with 35% carbamide peroxide, very little progress has been made to demonstrate and establish the adverse effects of these agents on dental pulp and the surrounding tissues [34], nor have there been

significant attempts to design strategies to counter such effects in cosmetic dentistry previously.

Our data demonstrates that chair-side bleaching agents have adverse effects on the pulpal and gingival tissues. Rat teeth that were treated with routinely used chair-side bleaching agents demonstrated decreased cell growth and function. Not only was there a decrease in the numbers of dental pulp stromal/stem cell (DPSC) growth when bleaching agents were applied to teeth (Additional file 1: Figure S2A, B) but also there was a decline in the function of the cells as assessed by the staining of alkaline phosphatase, which is one of the important functions of DPSCs (Additional file 1: Figure S2C). Previous studies from our laboratory demonstrated that DPSCs in the absence of NAC demonstrated no or low levels of differentiation gene expression whereas treatment with NAC or its addition to the other differentiation agents substantially increased gene expression for osteopontin (OPN), osteocalcin (OCN), and dentin sialoprotein (DSP) in DPSCs [29]. In order to find novel strategies to minimize and prevent adverse effects of bleaching materials in the oral tissues, we identified NAC as a chemo-protectant capable of decreasing unwanted effects of the bleaching materials while preserving their aesthetic benefits. As shown in Additional file 1: Figure S3A, cells grown from the rat palatal cells treated with the bleaching agents exhibited decreased growth as compared to those treated with the combination of the bleaching agents and NAC. SEM analysis also demonstrated a substantial change in the surface topography of the rat palatal tissues treated with the bleaching agent alone (Fig. 2). In addition, gingival tissues treated with bleaching agents in the absence of NAC demonstrated a blanched appearance in comparison to the healthy tissues seen in the presence of bleaching and NAC (Fig. 3b). Decreased cell growth seen in Additional file 1: Figure S3A and S3B when bleaching agents are applied in the absence of NAC is also indicative of the adverse effects of bleaching agents on the tissues. The results obtained in this study demonstrate that NAC protects the surface topography and the integrity of the oral tissues, while maintaining the quality of the whitening of the bleaching on teeth. Changes in enamel morphology after bleaching were also observed under SEM and it was concluded that the bleaching agents were able to alter the microhardness, roughness and morphology of dental enamel surfaces [35, 36]. Thus, when tooth bleaching was carried out in the presence of NAC in vivo, the viability along with the appearance of cells and tissues closely resembled those obtained by control unbleached teeth and gingival tissues in the rat model (Figs. 2, 3, Additional file 1: S2 and S3).

In addition, our human clinical trials demonstrate that bleaching treatment with NAC reduces undesired gingival whitening/irritation (Fig. 4d). In general, interview responses from patients showed that bleaching with NAC exhibited significant reduction in overall resting pain, cold (Figs. 5a-c) and hot sensitivity (data not shown) while demonstrating no differences in the ability to whiten teeth or the extent of patients' satisfaction with the aesthetics of their tooth bleaching (Fig. 5d).

Blockade of cell death by NAC may relate to its inhibitory effect on the generation of Reactive Oxygen Species (ROS) and oxidative stress in bleached tooth since NAC was previously shown to have significant anti-oxidant effects [37–39]. Additionally, previous studies have shown that carbamide may decrease the levels of intracellular glutathione [40]. However, since well-known anti-oxidants such as Trolox and Ascorbates were unable to change the course of 2-Hydroxyethyl methacrylate (HEMA) mediated cell death, and under certain conditions contributed to cell apoptosis, it is conceivable that NAC may function via mechanisms which are distinct to those reported for Trolox and Ascorbates [41]. Indeed, we have reported previously that NAC is an important agent of differentiation and inducer of NFκB in epithelial cells and dental pulp stromal cells. Therefore, NAC may provide the protective mechanisms in part by increasing the anti-apoptotic proteins regulated by NFκB [42].

Even though the major portion of NAC's protective effect on cell death induced by toxic agents appears to be regulated by NFκB, a small but significant effect of NAC's protective effect seems to be NFκB-independent. This can be seen in our previous studies which demonstrated that NAC can protect NFκB knock down cells [42]. We have also identified several important candidate genes, which are modulated during NFκB independent protective effect of NAC such as MAPKK1/MKK1, JNK and proline oxidase 1 and are in the process of further characterization (data not shown) [42].

A significant number of our patients complained about tooth sensitivity and pain after application of bleaching agents in the absence of NAC. It is likely that these adverse events were caused by damage to the local microenvironment of the tooth and gingiva resulting in the potential recruitment of inflammatory cells to aid in tissue repair and establishment of homeostatic balance within the tissues after the application of bleaching agents. Indeed, hypersensitivity may be due to increased pulpal blood flow and possibly heightened nociceptors. Therefore, by inhibiting local damage to the tissues by NAC, we may be able to prevent subsequent insults to the pulp region. The levels and type of cell death induced by bleaching agents, namely apoptotic versus necrotic, should also contribute to the degree of hypersensitivity and inflammation [43, 44].

Although blood flow will certainly influence eventual clearance of both toxins and inflammatory mediators

from pulp after bleaching, the initial exposure of dental pulpal cells to the bleaching materials may be sufficient enough to initiate cell damage and loss of cellular function in the short term and contribute to the adverse effects of bleaching agents [15, 45] . To determine how chemical clearance through the vasculature may influence local concentration dynamics on pulp cells and how the inclusion of NAC in bleaching materials may positively affect long term effects of bleaching in patients, additional animal studies should be performed [46–48]. Therefore, presently it is not clear what the fate and long-term consequences on the dentition and oral mucosa may be if insults to pulp or gingiva is continuously delivered by the aesthetic procedures in individuals with repeated use of these agents [43, 44]. At minimum, pain, discomfort and hypersensitivity suffered by most, if not all, patients after bleaching at one end of the spectrum and tooth loss and gingival damage at the extreme end of the spectrum should provide the basis for intervention using NAC to not only protect the oral mucosa and teeth from short term effects but also avoid unforeseen long term dental adverse effects.

IL-8 secretion measured in human saliva samples from patients showed increased levels at post-bleaching (Fig. 6). The IL-8 levels were lower in NAC treated patients when compared to controls. IL-8 is a chemotactic factor that allows immune effectors to migrate to the site of infection or trauma, thus playing a major role in initiation and maintenance of inflammatory reactions. Decreased levels of IL-8 secretion in patients receiving NAC and bleaching may indicate less damage and subsequent lower requirement for the recruitment of immune effectors, co-relating with the decrease in discomfort observed in patients clinically.

Conclusion

There is substantial agreement between studies performed in the rat model and humans regarding the protective role of NAC in bleaching in our studies. In both models, NAC was able to substantially decrease the induction of chemically-induced white lesions of marginal gingivae which is a good indicator of the protective role of NAC on gingiva (Figs. 3b and 4d). However, the advantages of human studies allowed us to determine the levels of pain and sensitivity, which was not possible to ascertain in rat studies. In contrast, assessment of protective role of NAC on DPSCs cell growth, and inhibition of bleaching mediated cell death could mainly be assessed in in vitro tissue culture models and in animal studies and not in human patients. Therefore, several models employed in our study offered complementary systems to comprehensively evaluate the protective role of NAC in bleaching.

Although the studies performed with humans can be expanded to include a much larger cohort of patients, the clinical benefit observed with smaller cohort of patients treated with NAC in this study indicated safety, feasibility and efficacy of NAC in bleaching, and provided the basis for its potential inclusion in the bleaching protocol for the health and comfort of dental patients in aesthetic dentistry.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s41231-019-0048-1.

Additional file 1: Figure S1. NAC protects DPSCs and OSCCs from cell death induced by different concentrations of bleaching agents containing hydrogen peroxide. **Figure S2.** NAC protects DPSCs from growth inhibition after bleaching in rats. **Figure S3.** NAC protects the gingival cells from undergoing cell death in the presence of bleaching agents in rats.

Abbreviation

DMEM: Dulbecco's Modified Eagle Medium; DPSCs: Dental pulp stromal/stem cells; DSP: Dentin sialoprotein; ELISA: Enzyme-linked immunosorbent assay; FBS: Fetal bovine serum; GSH: Glutathiones; HEMA: 2-Hydroxyethyl methacrylate; HEPES: Hydroxyethyl-piperazineethane-sulfonic acid; NAC: N-acetyl cysteine; OCN: Osteocalcin; OPN: Osteopontin; PI: Propidium iodide:; ROS: Reactive Oxygen Species; SCAP: Stem-cells of apical papillae; SEM: Scanning electron microscopic; TVA™: Target Cell Visualization Assay

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Authors contributions

DW performed the clinical chair side bleaching, and assisted in the preparation of the manuscript. KK prepared the agents required for the chair side bleaching, oversaw and performed in-vitro experiments, performed data analysis and prepared the manuscript. AV performed in-vitro studies and assisted in the preparation of manuscript. EL, MW, and DS assisted DW and KK in performing the experiments and edited the manuscript. DH and ECS oversaw chair side bleaching and edited the manuscript. AJ oversaw the design of the experiments, data analysis and preparation, and writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics approval and consent to participate

A single center, randomized controlled single-blinded clinical trial (NCT03534115) was conducted using human subjects ($n = 41$) to determine the protective role of NAC on chair-side bleaching procedure. Exclusion criteria included pregnant human subjects as well as any prospective patients with significant enamel translucency, unstable posterior occlusion, missing anterior teeth, current placement of anterior crowns or orthodontic devices. Informed consent was obtained based on the IRB protocol and the subjects were randomly assigned and were blinded to control ($n = 20$) and experimental ($n = 21$) groups.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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