N-acetylcysteine Regulates Dental Follicle Stem Cell Osteogenesis and Oral Bone Repair via ROS scavenging

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Abstract

Background

Dental follicle stem cells (DFSCs) show mesenchymal stem cell properties with the potential for oral bone regeneration. Stem cell properties can be impaired by reactive oxygen species (ROS), prompting us to examine the importance of scavenging ROS for stem cell-based tissue regeneration. This study aimed to investigate the effect and mechanism of N-acetylcysteine (NAC), a promising antioxidant, on the properties of DFSCs and DFSC-based oral bone regeneration.

Methods

DFSCs were cultured in media supplemented with different concentrations of NAC (0–10 mM). Cytologic experiments, RNA-sequencing and antioxidant assays were performed in vitro. Rat maxillary first molar extraction models were constructed, histological and radiological examinations were performed at day 7 post-surgery to investigate oral bone regeneration in tooth extraction sockets after local transplantation of NAC, DFSCs or NAC-treated DFSCs.

Results

5 mM NAC-treated DFSCs exhibited better proliferation, less senescent rate, higher stem cell-specific marker and immune-related factor expression with the strongest osteogenic differentiation; 10 mM NAC was not beneficial for maintaining stem cell properties. RNA-sequencing identified 803 differentially expressed genes between DFSCs with and without 5 mM NAC. “Developmental process (GO:0032502)” was prominent, bioinformatic analysis of 394 involved genes revealed functional and pathway enrichment of ossification and PI3K/AKT pathway, respectively. Furthermore, after NAC treatment, the reduction of ROS levels (ROS, superoxide, hydrogen peroxide), the induction of antioxidant levels (glutathione, catalase, superoxide dismutase), the upregulation of PI3K/AKT signaling (PI3K-p110, PI3K-p85, AKT, phosphorylated-PI3K-p85, phosphorylated-AKT) and the rebound of ROS level upon PI3K/AKT inhibition were showed. Local transplantation of NAC, DFSCs or NAC-treated DFSCs were safe and promoted oral socket bone formation after tooth extraction, with application of NAC-treated DFSCs possessing the best effect.

Conclusions

The proper concentration of NAC enhances DFSC properties, especially osteogenesis, via PI3K/AKT/ROS signaling, and offers clinical potential for stem cell-based oral bone regeneration.

Background

Stem cells derived from human dental tissues with properties of self-renewal, multilineage differentiation and immunomodulation have been intensively investigated for stem cell-based regenerative medicine (1, 2). Compared to other dental stem cells, dental follicle stem cells (DFSCs) are mainly isolated from human dental follicles within the developing wisdom teeth germ, so their plasticity is much better with the advantages of easier clinical access and less ethical controversy (3, 4). DFSCs are differentiated naturally into osteoblasts and cementoblasts, therefore they are a viable candidate for tissue engineering and regenerative medicine, particularly in oral bone tissue (5, 6).

Stem cell properties rely on various intrinsic and extrinsic factors (7–9). Accumulative reactive oxygen species (ROS) produced from endogenous and exogenous sources accompany decline in oxidative defense to cause cellular oxidative stress, impair stem cell properties and significantly reduce the efficacy of stem cells (10–12). Therefore, scavenging accumulative ROS is a beneficial strategy for the preservation of stem cell properties and the feasibility of stem cell-based therapy (13, 14).

A sophisticated antioxidant system comprising of endogenous and exogenous antioxidants is required for maintain cellular redox homeostasis and functions. Glutathione (GSH) is the most important endogenous antioxidant and supplementation of exogenous GSH has been proven to preserve stem cell properties by inhibiting ROS generation *in vitro* (13, 15). Whereas, GSH administration is not favorable *in vivo* due to its rapid degradation or oxidation (16, 17). Since GSH cannot be administered directly, the creative notion of precursors has been proposed to mimic physiologic and pharmacologic effects of GSH.

N-acetylcysteine (NAC) which has a proven role in specific clinical settings is a precursor for GSH synthesis and is considered as a modulator of the intracellular redox state (18). NAC is an amino acid derivative with small molecular weight presented in healthy cells at millimolar concentrations and has strong antioxidant capacity. NAC is not only rapidly deacetylated to promote intracellular GSH synthesis, but also eliminates electrophilic groups of free radicals directly through free thiol side-chain (19) (Additional file 1: Fig. S1A). Recent studies have highlighted a significant role of NAC in preserving the properties of stem cells through antioxidative effects, including bone marrow stem cells (20, 21) and adipose-derived stem cells (22). Furthermore, the synergistic effect of NAC and stem cells has been proved to yield superior therapeutic efficacy than monotherapy in bone injury(20), preclinical interstitial cystitis (23) and bioroot engineering (24). NAC is a life-saving drug with low toxicity and few side effects, which greatly facilitates its clinical research. Relationship between overdose and therapy seems to be the only apparent limitation in its efficacy (18). Moreover, accumulating evidence indicate that ROS-dependent signaling pathways play a crucial role in bio-modulation effects of exogenous antioxidants (25–27). Unfortunately, a comprehensive overview of physiological and pharmacological evidence of NAC on dental stem cells is insufficient. Nor is the underlying mechanism known.

The current study aimed to study the effects of NAC on the properties of DFSCs, explore possible molecular mechanisms utilizing RNA sequencing and investigate its use as a potential therapeutic agent for dental stem cell-based oral bone augmentation using a rodent animal model.

Methods

NAC preparation

NAC powder (Aladdin Chemical, Shanghai, China) was dissolved in HEPES buffer (Solarbio, Beijing, China) for an NAC stock solution (500 mM stock, pH = 7.0) stored at 4℃ and protected from light.

DFSC isolation and culture

The sample contained 5 fresh human dental follicles from 5 individuals who were selected to volunteer in this study according to the inclusion criteria: age between 13 and 22 years, healthy subjects, with good oral health conditions, referred for third mandibular molar extraction due to orthodontic or embed-impacted reasons in Tianjin Medical University Stomatology Hospital, unerupted teeth with low density of the dental follicle tissues around crowns observed on panoramic radiographs (Additional file 1: Fig. S1B and C). For this purpose, approval from the Ethics Committee of the Affiliated Stomatological Hospital of Tianjin Medical University (permission no. TMUSH-hMEC2016082) and written consent from each patient were obtained. Isolation and characterization of human DFSCs were performed according to our previous study (28). Cells were cultured in minimum essential medium-α (α-MEM; HAKATA, Shanghai, China) containing 10% heat-inactivated fetal bovine serum (FBS; HAKATA, Shanghai, China) and 1% penicillin/streptomycin (Solarbio, Beijing, China) in a humidified atmosphere of 5% CO_2 at 37℃. When adherent cells reached approximately 80% confluent, they were washed with phosphate buffer solution (PBS; Solarbio, Beijing, China), detached using 0.25% trypsin with 1 mM EDTA-4Na (Gibco, MA, USA) and seeded for passage culture. For NAC treatment, each culture medium supplemented with or without NAC stock solutions (final concentrations of 0, 2.5, 5, or 10 mM) was added immediately after seeding and renewed every 2 days. For PI3K inhibition, cells were treated with LY294002 (10 μM; Beyotime, Shanghai, China) for 2 days. Cells from passage 6 to 12 were used for all experiments. This study was conducted according to World Medical Association Declaration of Helsinki, ethical principles for medical research involving human subjects.

Oxidant and antioxidant assays

DFSCs were cultured as above for 2–4 days before assays. According to a ROS assay kit (Beyotime, Shanghai, China), after washed three times with PBS, DFSCs were incubated in 10 μM DCFH-DA solution at 37ºC for 20 minutes and observed by laser confocal microscope (Zeiss, Oberkochen, Germany) at the wavelength of 488/525 (excitation/emission). For quantitation, DFSCs were dissociated by 0.25% trypsin-EDTA and incubated as described above and the relative mean fluorescence intensity (MFI) was analyzed and normalized to the control using flow cytometry (BD, NY, USA) and FlowJo (BD, NY, USA). A superoxide assay kit (Beyotime, Shanghai, China), a hydrogen peroxide (H_2O_2) assay kit (Solarbio, Beijing, China), a total glutathione assay kit (Beyotime, Shanghai, China), a total superoxide dismutase (SOD) assay kit (Beyotime, Shanghai, China) and a catalase (CAT) assay kit (Beyotime, Shanghai, China) were used respectively. Absorbance was measured by a microplate reader (Tecan, Männedorf, Switzerland). Protein

concentration of each sample was determined at A280 using Nanodrop2000 (Thermo Scientific, MA, USA).

Cell viability assays

DFSCs were seeded immediately at a density of $4*10^3$/well in a 96-well plate and incubated with NAC at 37℃ and 5% CO_2. At defined end points (24, 48, 72 hours). cells were rinsed with PBS three times to remove residual NAC and cell viability was quantified using a cell counting kit-8 (CCK-8; Solarbio, Beijing, China) following the manufacture's protocol for 90 minutes. A microplate reader (Tecan, Männedorf, Switzerland) was used to measure absorbance at 450 nm. Cells were observed at 72 hours using an inverted microscope (Zeiss, Oberkochen, Germany).

Osteo-differentiation analysis

The osteogenic-inducing medium was α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, varying concentrations of NAC, 10 mM Na-β-glycerophosphate (Solarbio, Beijing, China), 100 nM dexamethason (Sigma-Aldrich, MO, USA) and 50 μg/mL vitamin C (Solarbio, Beijing, China). Alkaline phosphatase (ALP) assays were conducted at day 7 of osteogenic induction. ALP staining was carried out using a BCIP/NBT alkaline phosphatase color development kit (Beyotime, Shanghai, China). ALP activity was performed using alkaline phosphatase assay kit (Beyotime, Shanghai, China). Protein concentration of each sample was as described above. Alizarin red S (ARS) staining was performed at day 17 of osteogenic induction using 0.2% ARS (Solarbio, Beijing, China) at 37℃ for 30 minutes. For semi-quantitive analysis, PBS containing 10% cetylpyridine chloride (Sigma-Aldrich, MO, USA) was added into each well to dissolve precipitation after staining and the absorbance was measured using a microplate reader (Tecan, Männedorf, Switzerland) at 542 nm. Cells were observed using an inverted microscope (Zeiss, Oberkochen, Germany).

Detection of apoptosis

The percentage of apoptotic cells was examined after culture for 4 days using an Annexin V-FITC apoptosis detection kit (KeyGEN, Jiangsu, China). Briefly, cells were harvested, washed three times in cold PBS, resuspended in binding buffer at a concentration of $1*10^5$ cells/mL and incubated with 5 μL Annexin V-FITC and 5 μL PI for 15 minutes away from light at room temperature. Viable cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V-/PI+) and necrotic cells (Annexin V+/PI+) were analyzed using flow cytometry (BD, NY, USA) and FlowJo (BD, NY, USA).

Cell cycle and aneuploidy assay

With a cell cycle detection kit (KeyGEN, Jiangsu, China), cells reached 70% confluence were used for the detection of cell cycle and cells reached 90% confluence were used for the evaluation of aneuploidy. After trypsin digestion and centrifugation, precipitates were washed twice and resuspended with PBS into a single-cell suspension. Cold absolute alcohol was quickly added in the cell suspension and fixed cells at 4℃ for 24 hours (final alcohol concentration of 70%). Finally, fixed cells were rinsed twice with PBS and

stained with PI following instructions. Cell cycle and DNA aneuploidy were analyzed using flow cytometry (BD, NY, USA) and Modfit LT (VSH, USA).

Cell senescence assay

The activity of senescence-associated β-galactosidase (SA-β-Gal) was evaluated using a cell senescence detection kit (Beyotime, Shanghai, China). DFSCs (from passage 9 to passage 12) with or without NAC were cultured in 6-well plates and stained following the manufacturer's protocol. Positive cells were observed in blue and calculated from 5 randomly selected fields using an inverted microscope (Zeiss, Oberkochen, Germany). Thereafter, the percentage of positive cells to whole cell number was calculated.

Surface marker expression analysis

After culture for 4 days, DFSCs were washed 3 times with PBS and collected using 0.25% trypsin with 1 mM EDTA at passage 6. Cell suspension (100,000 cells) was mixed with 5 μL of FITC-anti-CD44 (cat#338803, BioLegend, CA, USA) and PE-anti-CD90 (cat#328109, BioLegend, CA, USA) according to the manufacturer's instructions. After incubation for 30 minutes in the dark, the cells were washed 3 times with PBS and then acquired by flow cytometry (BD, NY, USA) and the MFI was analyzed by FlowJo (BD, NY, USA).

RNA extraction and qRT-PCR

After culture for 4 days, total RNA was extracted with TRIzol reagent (TransGen, Beijing, China) and a RNA kit (TransGen, Beijing, China). cDNA was synthesized with a First-Strand cDNA Synthesis Kit (TransGen, Beijing, China). The qPCR was carried on the 7500 Real Time PCR system (Thermo Fisher, MA, USA), using a green qPCR supermix (TranGen, Beijing, China). Results were analyzed and normalized to the housekeeping gene GAPDH using the ΔΔCt method. All primers (Sangon, Shanghai, China) were listed in Additional file 3: Tab. S1.

Transcriptome analysis

After culture for 4 days, total RNA was extracted with TRIzol reagent (TransGen, Beijing, China), RNA sequencing was performed in collaboration with BGI-shenzhen following the standard operational procedure (https://www.genomics.cn/). The expression of human genes was performed by transforming mapped transcript reads to TPM. The significant levels were corrected by Q value with a threshold (Q value < 0.05) by Bonferroni. Differential expression analysis was performed using the DESeq2 (v1.4.5), Dr. Tom (BGI, Shenzhen, China) and R language (v4.0.3) ComplexHeatmap package (v2.4.3) with Q value < 0.05 and fold change > 1.5. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed by Dr. Tom (BGI, Shenzhen, China) and R language TopGO package (v2.42.0) and ClusterProfiler package (v3.18.1).

Western blot analysis

After culture for 4 days, cells were lysed in RIPA lysis buffer (Solarbio, Beijing, China) and 1 mM PMSF (Solarbio, Beijing, China) on ice. The concentration of protein was metered using the BCA Protein Assay

Kit (Thermo Scientific, MA, USA) and heat denaturation of protein in protein sample loading buffer (EpiZyme, Shanghai, China) at 100℃ for 10 min. Equal quantities of protein were separated on 10% SDS-PAGE gels by electrophoresis, transferred onto 0.45 um polyvinylidene-fluoride membranes (PVDF) (Immobilon, MA, USA). Membranes were blocked with 5% (w/v) non-fat milk in TBST buffer pH 7.5 for 1 hour at room temperature, incubated with the first antibody. Anti-GAPDH, PI3K p85, PI3K p110, AKT, phosphorylated-PI3K p85 and phosphorylated-AKT were used as primary antibodies. All antibodies were listed in Additional file 4: Tab. S2. After washed with TBST 10 minutes for 3 times and followed by incubation with secondary antibodies at room temperature for 1 hour. All uncropped blots were visualized by ECL reagents (TransGen, Beijing, China).

Rat tooth extraction model

The experimental protocol was approved by the Medical Ethics Committees of Stemmatological Hospital, Tianjin Medical University. The Unit for Laboratory Animal Medicine facilitated animal care at the Tianjin Hospital of ITCWM. This study conformed with the ARRIVE guidelines 2.0. Isolation and characterization of rat DFSCs were performed according to our previous study (29) and NAC treatment was the same as before. Under anesthesia by intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg), the right maxillary first molars were extracted on 20 Sprague-Dawley rats (male, 6-week-old) purchased from Beijing SPF Biotechnology. The sockets were formed using an 1# small ball drill to remove residue roots and bone pieces as much as possible, absorbable gelatin sponges (Xiang'en, Jiangxi, China) absorbed with PBS vehicle (15 μL), 5 mM NAC solution (15 μL), rat DFSC suspension (10^6 cells, 15 μL) or 5 mM NAC-treated rat DFSC suspension (10^6 cells, 15 μL) were placed into tooth extraction sockets depending on the randomized allocation of the animals, the sockets were finally sealed with tissue adhesive (n-butyl cyanoacrylate; 3M, MN, USA) (Fig. 5A). A few animals with unsuccessful extraction were excluded. Only the experimenter who filled the sponges was aware of the group allocation. Based on different fillers, rats were divided into four groups (n = 4/group): CON (PBS vehicle), NAC (5 mM), CELL (rat DFSCs), NAC + CELL (5-mM-NAC-treated rat DFSCs). Four groups were always housed separately for the quality assurance of the treatment with the same housing condition and no diet modifications. Rats were humanely sacrificed by an overdose of anesthetics on postoperative day 7.

Histologic assays

Right maxillary samples were fixed with 4% paraformaldehyde (Servicebio, Hubei, China) for 2 days at 4°C, decalcified with EDTA solution (Servicebio, Hubei, China) for 21 days at room temperature, embedded in paraffin (Leica, Wetzlar, Germany) and cut into 4-μm-thick slices using a HistoCore AUTOCUT (Leica, Wetzlar, Germany). Extraction socket sections were stained with haematoxylin-eosin (HE) and masson trichrome following the manufacturer's instructions (Servicebio, Hubei, China). Optical microscope (Nikon, Tokyo, Japan) was used to scan sections.

Micro-computed tomography assessment

Right maxillary samples were fixed with 4% paraformaldehyde (Servicebio, Hubei, China) for 2 days at 4°C, transferred to 70% alcohol, scanned using a SkyScan1276 system (BRUKER, MA, USA) at 10-μm scaled image pixel size with an energy level of 55 kV. The scans were reconstructed to produce 3-dimensional images by CTvox (BRUKER, MA, USA) and 2-dimensional section images by DataViewer (BRUKER, MA, USA). The extraction socket of the first molar was isolated by manual contouring and analyzed with regard to bone volume/tissue volume, bone mineral density and trabecular parameter (trabecular thickness, trabecular number, trabecular separation) using CTan software (BRUKER, MA, USA).

Statistical analysis

Data represented mean ± standard error (SEM) of independent samples. Statistical analysis was performed by Student's t-test using GraphPad Prism 8 (GraphPad Software, CA, USA). Statistically significant differences between groups were determined by $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

Results

Cytotoxic effects of NAC on DFSCs

DFSCs exhibited a mesenchymal-like shape with a polygonal morphology which was not altered by NAC, cells treated with 2.5- or 5-mM NAC showed better viability than non-treated cells at 24 hours and there was no distinct difference at 72 hours, while addition of 10 mM NAC had no effect at 24 hours but thereafter reduced viability (Fig. 1A). Flow cytometry showed decreased G1/S ratio and untriggered apoptosis in 2.5- or 5-mM NAC treatment, however, 10 mM NAC caused an increase in both G1/S ratio and apoptotic rate (Fig. 1B and C). Moreover, addition of 2.5- or 5-mM NAC decreased SA-β-gal-positive cell rate and 10 mM NAC increased the rate (Fig. 1D). Despite that relative mRNA levels of the tumor suppressor genes (p53, p21) decreased in all concentrations (Fig. 1E), only 10 mM NAC slightly increased the aneuploid cell rate (Fig. 1F).

NAC enhances the properties of DFSCs

The stem cell properties were assessed by stem cell-specific markers, osteogenic differentiation and immunomodulatory factors. The intensities of stem cell surface markers (CD44, CD90) (Fig. 2A) and the expression of DFSC-specific factor (Notch-1) (Fig. 2B) were enhanced in NAC-treated DFSCs. ALP (Fig. 2C and D) and ARS assays (Fig. 2E and F) showed that NAC increased osteogenic differentiation and calcium deposition; notably, the effect of 5 mM NAC was the best. Further, in 5 mM NAC-treated DFSCs, anti-inflammatory IL-4 receptor (IL-4r), IL-16, TGF-β3, TGF-β2 receptor (TGF-β2r), TGF-β3 receptor (TGF-β3r) and TGF-β induced (TGF-βi) were fairly upregulated (Fig. 2G) while pro-inflammatory IL-6 receptor (IL-6r) and IL-18 were downregulated (Fig. 2H).

Transcriptomic analysis and biological interpretation

Considering that 5 mM NAC-treated DFSCs exhibited better performance in enhancing stem cell properties, we performed transcriptomic profiling between DFSCs cultured with and without 5 mM NAC (SRA data: PRJNA780260) to understand the mechanistic insight into genetic alterations. 803 differentially expressed genes (DEGs) were observed in total, with 448 genes upregulated and 355 genes downregulated (Fig. 3A and B), and expression patterns of genes mentioned above were consistent with our transcriptomic data (Additional file 5: Tab. S3). 226 GO terms of biological process were significantly overrepresented (Additional file 6: Tab. S4) and the 20 most enriched GO terms were showed (Fig. 3C). The directed acyclic graph (DAG) further compared the membership of the first 10 GO terms with that of branches and pointed to the high hierarchical regulatory role of “developmental process (GO: 0032502)” (Fig. 3D).

Enrichment analysis of “developmental process (GO: 0032502)”

Developmental potential is a key feature for the therapeutic purpose of a stem cell lineage (30–32), thus we screened 394 DEGs involved in “developmental process (GO: 0032502)” (Fig. 3E) and performed enrichment analysis based on Q value, gene number and rich ratio. GO enrichment analysis showed NAC could potentiate development of multiple tissues, especially bone tissue development (Fig. 3F). KEGG pathway analysis revealed a significant role of PI3K/AKT signal pathway in NAC-driven developmental events (Fig. 3G).

NAC activates PI3K/AKT/ROS pathway

To investigate underlying mechanisms, oxidant and antioxidant systems and PI3K/AKT signaling pathway were evaluated. After NAC incubation, ROS (Fig. 4A and B), superoxide (Fig. 4C) and H_2O_2 (Fig. 4D) were significantly decreased comparing with those in control passaged DFSCs. Notably, the ROS level in 10 mM NAC-treated cells was the lowest (Fig. 4A and B). NAC replenished intracellular GSH contents (Fig. 4E) and increased the activities of two antioxidant enzymes, SOD (Fig. 4F) and CAT (Fig. 4G). Protein levels of PI3K-p85 (PI3K-regulatory subunit), PI3K-p110 (PI3K-catalytic subunit), AKT, phosphory-lated-PI3K-p85 and phosphorylated-AKT increased in DFSCs treated with 5 mM NAC (Fig. 4H, Additional file 2: Fig. S2A), consistent to KEGG enrichment (Fig. 3G) and TPM results (Additional file 5: Tab. S3). We then used LY294002, a PI3K functional inhibitor to suppress PI3K/AKT pathway and reevaluated ROS. LY294002 treatment caused the rebound of ROS in both control group and 5 mM NAC-treated group (Fig. 4I and J) due to the suppression of PI3K/AKT pathway (Additional file 2: Fig. S2B and C). ROS levels were decreased in NAC groups with and without LY294002 (Fig. 4I and J). These data indicate that NAC activates PI3K/AKT pathway to reinforce antioxidant defense and scavenge ROS.

NAC facilitates oral bone repair in the tooth extraction socket

After extraction of the first molars, local transplantation of NAC, DFSCs or NAC-treated DFSCs exhibited acceleratory trends of epithelium and bone repair without adverse signs (Fig. 5B). Masson trichrome staining showed similar trends (Fig. 5C). Three-dimensional reconstruction and sectional images also showed more osteoid formation in the sites filled with NAC, DFSCs and NAC-treated DFSCs than the control group (Fig. 5D). Quantitative assessment of first molar extraction sites confirmed that local transplantation of NAC, DFSCs or NAC-treated DFSCs promoted socket bone fill (15.89% vs 22.52% vs 23.72% vs 32.24%), with the highest potency of NAC-treated DFSC transplantation (Fig. 5E). BMD increased only in the NAC-treated DFSC group (6.623% vs 6.87% vs 6.86% vs 6.991%) (Fig. 5F). Additionally, NAC-treated DFSC transplantation expressed a significant elevation in the trabecular thickness and number, together with a reduction in the trabecular separation, compared to the control group (Fig. 5G-I). Collectively, these data demonstrate that NAC triggers PI3K/AKT signaling pathway that resulted in the reduction of ROS, leading to the enhancement of DFSC properties, while NAC facilitates oral bone repair in the tooth extraction socket and achieves better in treatment with DFSCs (Fig. 6).

Discussion

In this study, we have experimentally demonstrated that NAC regulates the properties of DFSCs via PI3K/AKT/ROS pathway. Additionally, we suggest a possibility that exogenous NAC is a putative contributing factor for oral bone repair in the field of dental stem cell-based therapy.

Studies often use H_2O_2 (20), high glucose (33) or hypoxia (34) to mimic oxidative stress and verify the antioxidative and cytoprotective effects of NAC. However, whether NAC would regulate stem cell properties by scavenging ROS accumulated during *in vitro* subculture merit further exploration. We observed that low concentrations of NAC (2.5- or 5-mM) promoted transient proliferation, but 10 mM NAC generated slight cytotoxicity which was consistent to previous studies (24, 35). Our results further indicated that cell cycle arrest and apoptosis could be the cause of cytotoxicity. In addition, studies reported that antioxidant application could delay senescence and prevent stem cell dysfunction (13). Indeed, we found that 2.5- or 5-mM NAC delayed senescence, but 10 mM NAC induced premature senescence. This result was similar to studies which illustrated premature senescence as one negative effect of high dose of antioxidants (35). Oncogenic transformation is another concerned issue of stem cell-based therapy (36). Downregulated p53 and p21 were observed after NAC treatment and 10 mM NAC increased aneuploid cell ratio, suggesting that DNA repair ability weakened with decreased ROS levels (37). Notably, low concentrations of NAC did not affect gene stability. Taken together, re-evaluating the benefits of high dosage of antioxidants on stem cells seems important.

During tooth development, dental follicle develops into cementum and alveolar bone, so DFSCs possess strong directional osteogenic potential. In this study, expression of CD44, CD90 and Notch-1 as stemness factors typical of DFSCs (38) increased with increasing NAC concentrations. Each NAC concentration promoted osteogenic differentiation in varying degrees, 5 mM showed the best osteogenic effect. Similarly, Masahiro Yamada also found 5 mM NAC was the most effective concentration for osteogenesis without apparent toxicity in bone marrow stem cells (21). Additionally, altered expression

levels of immune-related factors also indicated that 5 mM NAC contributed to the immunomodulatory properties of DFSCs to some extent (39, 40). The above results suggested that NAC improved the properties of DFSCs during *in vitro* culture.

Transcriptome analysis was used to study the functional interaction and signal transduction networks of NAC in DFSCs. Both GO term and DAG analysis revealed a significant association between NAC and developmental events. NAC has been reported as an osteogenesis-enhancing molecule for bone marrow stem cells (20, 21), our results confirm this point in DFSCs and indicate broader regenerative potentials including extracellular matrix organization, urogenital and renal system development which need further investigation. We further explored underlying mechanisms of these application. In DFSCs, NAC not only served as a source of GSH but also activated primary antioxidant enzymes, SOD and CAT (41), leading to enhancement of antioxidant system and reduction of ROS. KEGG enrichment shed light on the PI3K/AKT pathway as a key mechanism for NAC-mediated effects on DFSCs. The PI3K/AKT signaling is a critical ROS-dependent pathway and strongly links with stem cell properties (42, 43). Upregulations of PI3K, AKT and their phosphorylation forms after NAC treatment were confirmed by western blot. More importantly, ROS rebounded in both groups and NAC still exhibited the antioxidative effect following PI3K inhibition, suggesting that PI3K/AKT signaling contributed to the antioxidative mechanism of NAC and there were other, as yet unknown molecular mechanisms that remained to be examined. PI3K/AKT pathway was thought to be mediated by ROS (43), paradoxically we found the activation of PI3K/AKT pathway could inhibit ROS in turn. We speculated that there was a feedback loop between ROS and PI3K/AKT pathway in regulating stem cell properties. This needs to be further researched.

Most previous literature reports that various NAC concentrations preserve stem cell function by inhibiting oxidative stress induced by ROS. In current study, the effects of NAC on DFSC properties are not always positive correlation with NAC concentrations. High concentrations (such as 10 mM) showed suboptimal osteogenic ability even with mild cytotoxicity, as mentioned in a few studies (21, 24). The presence of reductive stress may be the reason. Reduced ROS levels are critical for maintaining stem cell identity, meanwhile a persistent lack of ROS, or reductive stress, also impedes stem cell functioning (44–46). This may explain why 10 mM NAC minimizes the intracellular ROS level, but 5mM NAC shows better results. These findings imply that cellular antioxidant defenses are saturated when an antioxidant reaches a certain concentration, after exceeding this concentration stem cells generate reductive stress from overdosed antioxidants.

Finally, we use rodent tooth extraction model to investigate the pro-osteogenic effect of NAC by itself or with DFSCs. Ideal tooth extraction socket bone repair is necessary for any prosthodontic therapies. However, the oral cavity is exposed to ROS produced by many oxidizing agents, such as oral microflora and mechanical injuries (47–49), leading to delayed and undesirable bone healing in the sites after tooth extraction. In this study, exogenous supplementation of DFSCs or NAC at an optimal concentration promoted tooth extraction socket bone formation and NAC-treated DFSCs were superior. Therefore, NAC may be of therapeutic benefit in oral bone regeneration. To simulate the clinical situation, the current study uses topical administration carried by the gelatin sponge instead of systemic injection (50). The

gelatin sponge carrier and the tissue glue are designed to deliver drugs deep into the extraction socket, seal the wound and assist in blood clot formation, which may have a limited healing effect. Clearly, further studies are needed to explored the pharmacology of NAC and stem cells carried by gelatin sponge.

Conclusions

In summary, this study shows that the proper concentration of NAC enhances the properties of DFSCs, especially osteogenesis, via PI3K/AKT/ROS signaling, and promotes oral bone repair by itself or with DFSCs. We also provide evidence for the effect of the reductive stress in stem cells caused by excessive antioxidants. NAC as a safe and FDA-approved drug has multiple advantages in clinical applications of dentistry. Thus, it will be of great interest to study the role, mechanism and appropriate usage of NAC in dental stem cell biology and oral bone regenerative medicine.

Abbreviations

DFSCs: Dental follicle stem cells; NAC: N-acetylcysteine; ROS: Reactive oxygen species; GSH: Glutathione; RNA: Ribonucleic acid; HEPES: N-2-hydroxyethylpiperazine-N-ethane-sulphonicacid; α-MEM: Minimum essential medium-α; FBS: Fetal bovine serum; PBS: Phosphate buffer solution; EDTA: Ethylene diamine tetraacetic acid; PI3K: Phosphatidylinositol 3 kinase; DCFH-DA: 2', 7'-dichlorofluorescent yellow diacetate; MFI: Mean fluorescence intensity; H_2O_2: Hydrogen peroxide; SOD: Superoxide dismutase; CAT: Catalase; CCK-8: Cell counting kit-8; ALP: Alkaline phosphatase; ARS: Alizarin red S; SA-β-Gal: Senescence-associated β-galactosidase; FITC: Fluorescein isothiocyanate; PI: Propidium iodide; PE: Phycoerythrin; TPM: Transcripts per million; AKT: Protein kinase B; HE: Haematoxylin and eosin; IL, Interleukin; TGF-β, Transforming growth factor-β; DEGs, Differentially expressed genes; GO, Gene ontology; DAG, Directed acyclic graph; KEGG, Kyoto encyclopedia of genes and genomes; Micro-CT, Micro-computed tomography; BV/TV, Bone volume/tissue volume; BMD, Bone mineral density; Tb.Th, Trabecular thickness; Tb.N, Trabecular number; Tb.Sp, Trabecular separation.

Declarations

Supplementary Information

The online version contains supplementary material available at.

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Authors' contributions

ZM and JL conceived the ideas; ZM and JL designed and performed the study and were the main writers of the manuscript; LS and HL proofread this manuscript; JL, ZF, SG, MW, ZW and ZL guided the experiments and analysis; LS and HL reviewed the article. All the authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. SRA records of RNA-sequencing will be accessible with the following link after 2022-12-01: https://www.ncbi.nlm.nih.gov/sra/PRJNA780260.

Declarations

Ethics approval and consent to participate

This research was approved by the Ethics Committee of the Affiliated Stomatological Hospital of Tianjin Medical University (permission no. TMUSH-hMEC2016082). Human dental follicle samples were collected from freshly extracted third molars with the informed consents of the donors or their guardians.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Cytotoxicity of NAC at various concentrations in DFSCs. **(A)** Cell morphology at 72 hours and viability detected by CCK-8 assay at 24, 48 and 72 hours. **(B)** Cell cycle picture and distribution detected by flow cytometry. **(C)** Apoptosis picture and the distribution of early and late apoptotic and viable cells detected by flow cytometry. **(D)** Photomicrographs and the distribution of blue stained senescent cells of passage 12 using SA-β-Gal staining. Scale bars: 100 μm. **(E)** Relative mRNA expression of p21 and p53. **(F)** The distribution of aneuploid cells detected by flow cytometry.

Figure 2

Effects of various NAC concentrations on stem cell-specific markers, osteogenesis and immune-related factors of DFSCs. **(A)** MFI of CD44 and CD90 detected by flow cytometry. **(B)** Relative mRNA expression of Notch-1. **(C)** Photographs and micrographs depicting the osteogenic differentiation using ALP staining on day 7 after osteogenic induction. Scale bars: 250 μm. **(D)** Quantification of ALP activity. **(E)** Photographs and micrographs depicting the matrix mineralization using ARS staining on day 14 after osteogenic induction. Scale bars: 250 μm. **(F)** Semi-quantification of ARS staining. **(G)** Relative mRNA expression of anti-inflammation factors and receptors (IL-4r, IL-16, TGF-β3, TGF-β2r, TGF-β3r, TGF-βi). **(H)** Relative mRNA expression of pro-inflammation factors and receptors (IL-6r, IL-18).

Figure 3

Differences in transcriptome signatures between controls and DFSCs exposed to 5 mM NAC at day 4. DEGs visualized using a heatmap **(A)** and a volcano plot **(B)**. **(C)** Graph depicting the first 20 GO terms of DEGs and their significance. **(D)** Graph depicting the regulatory relationship between the first 10 GO terms using DAG analysis. **(E)** Heatmap of 394 involved DEGs in “developmental process (GO: 0032502)”. **(F)** GO enrichment of DEGs in “developmental process (GO: 0032502)”. **(G)** KEGG enrichment of DEGs in “developmental process (GO: 0032502)”.

Figure 4

Effects of various NAC concentrations on ROS, antioxidant enzymes and PI3K/AKT pathway. **(A)** Fluorescent imaging depicting the intracellular ROS intensity. Scale bars: 100 μm. **(B)** Relative MFI of ROS

intensity detected by flow cytometry. **(C)** Graph depicting the intracellular superoxide content. **(D)** Graph depicting the intracellular H_2O_2 content. **(E)** Graph depicting the intracellular GSH content. **(F)** Graph depicting the SOD activity. **(G)** Graph depicting the CAT activity. **(H)** Western blot depicting protein expression of PI3K-p85 (85 kD), PI3K-p110 (110 kD), AKT (60 kD), phosphorylated-PI3K-p85 (85 kD) and phosphorylated-AKT (60 kD) in DFSCs treated with 5 mM NAC or not. **(I)** Fluorescent imaging depicting the intracellular ROS intensity after LY294002 treatment. Scale bars: 50 μm. **(J)** Relative MFI of ROS intensity after LY294002 treatment detected by flow cytometry.

Figure 5

NAC-treated DFSCs promoted tooth extraction socket bone formation. **(A)** The right maxillary first molars were extracted and filled with gelatin sponges carried vehicle (15 μL, PBS), NAC (15 μL, 5 mM), rat DFSCs (15 μL, 1×10^6 cells) or NAC-treated DFSCs (15 μL, 5 mM NAC, 1×10^6 cells), then sealed with cyanoacrylate glue. **(B)** Representative photomicrographs of HE-stained tooth extraction sockets. Original magnification: 4×. **(C)** Representative photomicrographs of masson trichrome-stained tooth extraction sockets. Original magnification: 4×. **(D)** Three-dimensional reconstruction and section images of tooth extraction sockets using micro-CT. Micro-CT assessment of BV/TV **(E)**, BMD **(F)**, Tb.Th **(G)**, Tb.N **(H)**, Tb.Sp **(I)**.

Figure 6

Schematic diagram of the proposed mechanism. NAC triggered PI3K/AKT signaling pathway that resulted in the reduction of ROS, leading to the enhancement of DFSC properties, NAC-treated DFSCs or NAC facilitated oral bone repair in the tooth extraction socket, created with BioRender.com.

Supplementary Files

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