N-acetylcysteine promotes osteogenic differentiation in periodontal ligament stem cells under cyclic mechanical stretch

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Abstract

Background

Biomechanical forces are vital for the regulation of skeletal tissue. Mechanical stretch plays a vital role in osteogenic differentiation of periodontal ligament stem cells (PDLSCs) during orthodontic treatment. Cyclic mechanical stretch may trigger the up-regulated production of reactive oxygen species (ROS). ROS has a critical effect on bone cell function and the pathophysiology of bone diseases. N-acetylcysteine (NAC), a ROS scavenger, possesses powerful antioxidant capacity. The aim of this study was to determine the role of ROS and NAC in PDLSCs during osteogenic differentiation under cyclic mechanical stretch. We further investigated that the therapeutic potential of NAC to improve the changes of the microstructure of alveolar bone during orthodontic tooth movement in rats by micro-CT system.

Methods

The expression of COL1 (collagen type I), RUNX2 (runt-related transcription factor 2) and OPN (osteopontin) by qRT-PCR and Western blot experiments, and alkaline phosphatase (ALP) staining as well as ALP activity tests were used to examine osteogenic differentiation tendency of PDLSCs subjected to cyclic mechanical stretch of 10% and 0.5Hz deformation induced by the Flexcell tension system. ROS production in PDLSCs were measured under cyclic mechanical stretch by Flow Cytometry. The levels of reduced glutathione (GSH), oxidized GSH (GSSG) and the GSH/GSSG ratio with or without NAC treatment were analyzed. And we evaluated the changes of the microstructure of alveolar bone during orthodontic tooth movement in rats employing micro-CT system.

Results

NAC treatment could promote the osteogenic differentiation of PDLSCs under cyclic mechanical stretch. Down-regulated ROS generation and the up-regulated level of GSH and the ratio of GSH/GSSG in PDLSCs treated with NAC were observed in response to cyclic mechanical stretch. NAC improved the microstructure of alveolar bone, including BV/TV (bone volume/total volume), Tb.Th (trabecular thickness), Tb.Sp (trabecular separation) and SMI (microstructure model index), during orthodontic tooth movement in rats.

Conclusion

These results revealed that NAC might be a potential therapeutic approach for the remodeling of the alveolar bone during orthodontic tooth movement.

Introduction

Biomechanical forces are vital for the regulation of skeletal tissue. The biological basis of orthodontic tooth movement (OTM) is the alveolar bone remodeling under the biomechanical stimuli caused by the orthodontic appliances [1]. Bone formation by osteoblasts at the tension side and bone resorption by osteoclasts at the pressure side are involved in the alveolar bone remodeling during orthodontic tooth movement. Osteogenic differentiation in periodontal ligament stem cells (PDLSCs) introduced by mechanical stretch is the crucial component in alveolar bone remodeling at the tension side. However, some unwanted iatrogenic sequelae, including alveolar bone loss, gingival recession and dehiscence in orthodontic patients, remain to be resolved [2, 3]. It is pivotal to elucidate the molecular mechanism underlying osteogenic differentiation of PDLSCs in response to mechanical stretch in orthodontics, which may contribute to the development of orthodontic therapeutic approach to cause minimum tissue damage to the patients and improve the remodeling of the alveolar bone during orthodontic tooth movement.

Cyclic mechanical stretch is a known inducer of the up-regulated level of reactive oxygen species (ROS) [4, 5]. ROS, as intracellular signaling molecules, can be essential for regulation of cell physiological functions, while excessive accumulation of ROS can disrupt physiological functions and cause numerous pathophysiological processes [6]. Numerous studies confirmed that ROS had a critical role in bone cell function and the pathophysiology of bone diseases. The heightened levels of ROS are involved in bone remodeling and disrupt bone homeostasis, leading to more bone resorption caused by osteoclast differentiation while restraining osteoblastic differentiation and osteogenesis [7, 8]. The elevated intracellular ROS and downregulation of antioxidant defenses were correlated with osteoporosis [9]. The excessive ROS alters bone remodeling process contributing to an unbalance between osteoclast and osteoblast function, which can be implicated in bone diseases including osteoporosis [10]. Antioxidant administration has a vital effect on protecting bone health and preventing bone loss as well as restoring the physiological bone remodeling [10].

N-acetylcysteine (NAC), a greatly applied antioxidant, possesses powerful antioxidant capacity by scavenging ROS and facilitating the generation of glutathione (GSH). NAC could enhance osteoblastic phenotypic expression in osteoblastic culture and accelerate bone healing in rat femoral critical size defects [11]. Supplementation of NAC played a protective role in orchiectomy-induced osteoporosis through the increase of osteoblastic bone formation and inhibition of the increased ROS and osteoclastic bone resorption [12]. These data revealed the potential clinical therapy of NAC as an osteogenic enhancer in bone forming and regeneration applications. The biological and pharmacological characteristics of NAC, including antioxidation, anti-inflammatory activity and antimicrobial activity, render it a potential therapeutic candidate in dental and oral disorders [13]. However, it is undefined the therapeutic potential of NAC in orthodontic tooth movement.

Given the role of ROS in osteogenic differentiation and the powerful antioxidation activity of NAC, the aim of this study was to determine the role of ROS and NAC in PDLSCs during osteogenic differentiation under cyclic mechanical stretch. We further attempted to evaluate the therapeutic potential of NAC to

improve the changes of the microstructure of alveolar bone during orthodontic tooth movement in rats by micro-CT system to investigate the effect of NAC on orthodontic tooth movement.

Materials And Methods

Culture and identification of PDLSCs

The experimental protocols and procedures were approved by the Medical Ethical Committee of the School of Stomatology, Shandong University (Protocol Number: 20201206). Human periodontal ligament (PDL) tissues were isolated and collected from premolars, extracted from systemically healthy participants (aged 12–16) for orthodontic reasons at the Stomatological Hospital of Shandong University. The informed consents were obtained from the subjects and subjects' guardians. After the extraction, the premolars were collected and kept in minimum essential medium-α (α-MEM) (BI, Beit Haemek, Israel) containing 5% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin, Sigma-Aldrich, St Louis, MO, USA), and washed with phosphate-buffered saline (PBS). PDL tissues at the middle third of the premolar root were scraped off and cut into small fragments (1-2 mm^3) by an aseptic scalpel, and then placed and attached to the bottom of a culture flask. The primary cells were cultured with the culture medium containing α-MEM, 20% fetal bovine serum (FBS) (BI) and 1% penicillin/streptomycin in incubators at 37 °C in 5% CO_2. The culture medium was replaced every 3 days. The cells were separated with 0.25% trypsin (Beijing Solarbio Science & Technology co., Ltd., Beijing, China) and passaged after reaching the 90% confluence in 10% FBS culture medium. The following experiments were performed using PDLSCs at passages 3-6.

Phenotype analysis of PDLSCs by flow cytometry

The surface markers of the PDLSCs at passage 5 were detected and analyzed by flow cytometry. The cells were trypsinized, collected and washed with PBS and then incubated with monoclonal antibodies specific for STRO-1, CD146, CD34, CD45 (Proteintech Group, Inc Rosement, IL 60018, USA) for 1 hour at 4°C in the darkness. Then the cells were washed with PBS three times and measured by BD Accuri™ C6 flow cytometer.

Multidirectional differentiation of PDLSCs

To evaluate the multilineage differentiation capacity of PDLSCs, the cells were cultivated in the osteogenic, adipogenic or chondrogenic induction medium and the induction medium was replaced with fresh induction medium every 3 days.

The osteogenic induction medium contained 10% FBS medium with 10 nM dexamethasone (Solarbio), 10 mM β-glycerophosphate (Solarbio) and 50 mg/l ascorbic acid (Solarbio). After osteogenic induction for three weeks, cells were performed by Alizarin Red staining (Sigma-Aldrich; Merck KGaA) to observe the formation of mineralized nodules.

The adipogenic induction medium contained 10% FBS medium with 1 μM dexamethasone (Solarbio), 0.2 mM indomethacin (Solarbio), 0.01 g/l insulin (Solarbio) and 0.5 mM isobutyl-methylxanthine (Solarbio). After adipogenic induction for three weeks, cells were performed by Oil Red O staining (Solarbio) to detect the formation of lipid droplets.

For chondrogenic induction, PDLSCs were cultured in the chondrogenic induction medium (Sigma Aldrich; Merck KGaA). After chondrogenic induction for three weeks, cells were performed by Alcian Blue staining (Solarbio) to detect the formation of cartilage.

Application of cyclic mechanical stretch to PDLSCs

PDLSCs at passages 3-6 were seeded into six-well 35mm silicone membrane culture plates coated with type I collagen. After the cells reached approximately 80% confluence, cyclic mechanical stretch was performed at 10% deformation and 0.5Hz for 3, 12 and 24 h using Flexercell FX-5000 Strain Unit (Flexcell International Corporation, Hillsborough, NC, USA) [14, 15]. Non-loaded PDLSCs (0 h) were cultivated on similar plate in the same incubator for the maximum stretching period. The cyclic mechanical stretch experiment was performed for three independent times, and the final data were collected from the mean data of each time for statistical analysis.

Reagent Preparation and Application

For NAC treatment, PDLSCs were treated in 10% FBS with different doses of NAC (Beyotime, Nantong, China) (2.5, 5 mM) 12 h prior to cyclic mechanical stretch experiments [11, 16].

Quantitative real-time RT-PCR

Total RNA from PDLSCs was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, USA) according to the manufacturer's instructions. 1000 ng of total RNA of each sample was reverse transcribed to generate cDNA by a PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Tokyo, Japan), and the qRT-PCR reactions were carried out in a 10 μl reaction volume with a TB Green PCR Core Kit (Takara) and the LightCycler ® 96 System following the manufacturer's instructions. The relative mRNA expression was calculated and normalized to GAPDH. The primer sequences used in this research are listed in Table 1.

Western blotting

To extract the total proteins from PDLSCs, cells were washed with PBS and then collected and lysed in RIPA buffer (Solarbio) with 1% PMSF (Solarbio) on ice, and subsequently lysed by ultrasound. Centrifugation was carried out at 12000 rpm at 4 °C for 15 min, and then the supernatant containing the total protein was collected. The concentration of each sample was determined by the BCA Protein Assay Kit (Solarbio). Then, equal amounts of protein from each sample were separated by SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Burlington, MA, United States). The PVDF membranes were incubated with primary antibodies overnight at 4 °C after blocked with 5%

skim milk in tris-buffered saline tween-20 (TBST) and subsequently incubated with secondary antibodies for 1 h at room temperature. The protein bands were visualized with the enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) by the ECL chemiluminescence detection system (Amersham Imager 600, USA). ImageJ software was used to quantitate the protein band intensities, and the target protein expression was normalized to GADPH. The following antibodies were used in this study: COL1A2 (14695-1-AP, ProteinTech), RUNX2 (ab23981, Abcam, Cambridge, MA, USA), OPN (22952-1-AP, ProteinTech), GAPDH (sc-25778, Santa Cruz, CA, USA). GAPDH was used as internal control.

Alkaline phosphatase (ALP) activity

The ALP activity of each sample was measured using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. For ALP staining, PDLSCs were washed with PBS and fixed with 4% paraformaldehyde and then stained using an NBT/BCIP staining kit (Beyotime).

Detection of intracellular ROS

The intracellular ROS levels of each sample were measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Beyotime). The cells were incubated with 10 μM DCFH-DA at 37℃ for 40 minutes in the darkness, and then washed with PBS. And the cells incubated with DCFH-DA were collected and analyzed by BD Accuri™ C6 flow cytometer.

Cellular glutathione

The levels of glutathione in PDLSCs were detected using GSH and GSSG Assay Kit (Beyotime) according to the manufacturer's instructions.

Experimental Animals

The Animal experiments were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. The animal experiments were approved by the Medical Ethical Committee of School of Stomatology, Shandong University (Protocol Number: 20201205). Eight-week-old male Wistar rats were used in this study. The rats were raised at a room temperature of 22-24°C under a 12 h light/dark cycle with a relative humidity of 40-60%. The rats in this study were fed with standard food and water ad libitum. The rats were allowed a week of acclimatization.

Establishing the tooth movement model

Under isoflurane (RWD Life Science Co., Ltd., Shenzhen, China) inhalation anesthesia, a low-speed dental hand piece was applied to make a 0.5 mm groove on the dental cervix of the maxillary incisor. A nickel-titanium coil spring (0.012", GRIMED Medical Co., Ltd., Beijing, China) was ligated between the first upper molar and upper central incisor with a 0.25 mm ligature wire (Changsha Tiantian Dental Equipment Co., Ltd., Changsha, China). The orthodontic force, 25g [17, 18], measured by a dynamometer, was performed

to move the maxillary molar mesially using the two maxillary incisors as anchorage. The light-cure adhesive resin (Shinye Odontology Materials Corp. Co., Hangzhou, China) was used to prevent the loss of orthodontic appliance and protect the lip from injury. The orthodontic devices that were detected to be damaged or detached were reinstalled immediately. All rats kept healthy during the experimental period.

For NAC treatment, the rats were randomly divided in the control and NAC groups. NAC (Beyotime) was dissolved in 0.9% saline for intraperitoneal (i.p.) injections, with NAC doses of 225 mg/kg/d in Wistar rats [19]. The rats in control groups were treated with saline injection.

Six rats from each group were sacrificed by excessive pentobarbital anaesthesia at day 7,14 or 28, respectively and then fixed with 4% paraformaldehyde cardiac perfusion. The maxilla from each rat was separated and collected and then fixed with 4% paraformaldehyde for 24 hours.

Micro-CT scanning

To measure and analyze the micro-structure changes of alveolar bone during orthodontic tooth movement at the tension side, a micro-CT (Perkin Elmer, Waltham, USA) was applied to scan the specimens with scan settings of voltage 90 kV, current 88 μA and voxel resolution 36 μm at 360°.

Evaluating the microstructural parameters

We selected the alveolar bone distal to the cervial third of mesiobuccal root of the maxillary first molar at the tension side as the region of interest (ROI) for analysis (Additional file 1: Figure S1) [18, 20, 21]. We measured the microstructure of alveolar bone including bone volume/total volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm), and microstructure model index (SMI) by the CT Analyser software (version 1.17.7.2, Bruker MicroCT).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way ANOVA was used to analyze the variances between more than two experimental groups and control group. Student's t test was used to analyze the differences between two groups with GraphPad Prism software (MacKiev Software, Boston, MA, USA).

Results

PDLSCs culture and identification

PDLSCs were successfully derived and cultured from human periodontal ligament tissues, exhibiting a typical spindle-shaped morphology (Fig. 1a). In flow cytometric analyses, PDLSCs were positively expressed the human mesenchymal stem cell (MSC)-specific surface markers (STRO-1, CD146) and negatively expressed hematopoietic and endothelial cell-specific markers (CD34, CD45) (Fig. 1b). And PDLSCs possessed multipotentiality properties as evidenced by the positive staining of the formation of

Alizarin Red-positive nodules (Fig. 1c), Oil Red O-positive lipid droplets (Fig. 1d) and cartilage (Fig. 1e) after osteogenic, adipogenic, chondrogenic induction, respectively.

The effect of cyclic mechanical stretch on osteogenic differentiation in PDLSCs

To study the influence of cyclic mechanical stretch on osteogenic differentiation in PDLSCs, we examined the expression of osteogenic relative markers, such as COL1, RUNX2, OPN and ALP, under cyclic mechanical stretch of 10% and 0.5Hz deformation for 3 h, 12 h, and 24h, induced by the Flexcell tension system. The results showed an osteogenic differentiation tendency of PDLSC was induced by cyclic mechanical stretch. As shown in Fig. 2a, we found that the mRNA expression of COL1, RUNX2 and OPN in PDLSCs was gradually increased. The western blot experiments confirmed the elevated amount of COL1, RUNX2, OPN protein after cyclic mechanical stretch (Fig. 2b-c). Compared to the control, we observed the increase of cellular ALP enzyme activity, according to the results of ALP staining and ALP activity tests (Fig. 2d-e). The above results indicated that cyclic mechanical stretch with 10% and 0.5 Hz deformation promoted the osteogenic differentiation tendency in PDLSCs.

NAC inhibited ROS generation in PDLSCs in response to cyclic mechanical stretch

Numerous studies have confirmed that cyclic mechanical stretch enhanced ROS generation level in various cellular phenotypes. Therefore, we next examined whether cyclic mechanical stretch could trigger the ROS generation in PDLSCs and the effect of NAC, a well-known ROS scavenger, on ROS production in PDLSCs. According to the results of the CCK8 assay in our previous experiments (unpublished observations) and previous researches [11], we treated PDLSCs with 2.5 mM and 5 mM NAC in the following studies.

Using DCFH-DA probe, we measured temporal ROS production in PDLSCs in response to cyclic mechanical stretch by flow cytometric analyses. Compared to the control, a significant higher fluorescence intensity level of ROS was detected at 3 h stretch, and the increase was maintained throughout the duration of cyclic stretch (Fig. 3). NAC treatment obviously restrained the cyclic mechanical stretch-induced ROS generation. These results demonstrated that cyclic mechanical stretch resulted in the elevation of intracellular ROS production, while NAC treatment inhibited it.

The effect of antioxidant NAC on osteogenic differentiation in PDLSCs under cyclic mechanical stretch

To determine whether NAC might affect osteogenic differentiation in PDLSCs in response to cyclic mechanical stretch. The characteristic markers of osteogenic differentiation, including COL1, RUNX2, OPN, and ALP activity, were examined with NAC treatment.

The mRNA expression levels of osteogenesis markers COL1, RUNX2 and OPN were observed and found to be promoted following NAC treatment, compared with the controls (Fig. 4a). NAC treatment also enhanced the expression of osteoblast differentiation-associated proteins COL1, RUNX2, OPN undergoing cyclic mechanical stretch (Fig. 4b). ALP staining and ALP activity tests showed that, compared to the controls, the increase of cellular ALP enzyme activity was exhibited in response to NAC in PDLSCs

exposed to cyclic mechanical stretch (Fig. 4c-d). These data indicated that NAC treatment could promote the osteogenic differentiation in PDLSCs under cyclic mechanical stretch. And the antioxidant defense might be vital during osteogenic differentiation in PDLSCs in response to cyclic mechanical stretch.

NAC increased GSH concentration in PDLSCs under cyclic mechanical stretch

NAC could facilitate the GSH synthesis, the most abundant antioxidant thiol in cells. To examinate intracellular antioxidant factors potentially related to the beneficial effects of NAC against cyclic mechanical stretch-induced increased ROS level, we next measured the levels of reduced glutathione (GSH), oxidized GSH (GSSG) and the GSH/GSSG ratio with or without NAC treatment. With the increase in ROS production induced by cyclic mechanical stretch, the level of GSH and the ratio of GSH/GSSG were enhanced, accompanied by a reduction in the GSSG expression. The increased level of GSH and the ratio of GSH/GSSG were further induced with NAC treatment in response to cyclic mechanical stretch (Fig. 5).

The effect of NAC on microarchitectural parameters of the trabecular bone at the tension side during orthodontic tooth movement in rats

We next evaluated whether NAC would have an effect on microarchitectural parameters of the trabecular bone at the tension side during orthodontic tooth movement in rats. Our micro-CT analysis showed an increase in BV/TV in the NAC group compared with the control group at day 14. Similarly, Tb.Th was increased in the NAC group at day 14 and day 28. Additionally, Tb.Sp and SMI were significantly lower in the NAC group than that in the control group at day 14 and day 28 (Fig. 6 and Additional file 2: Table S1).

Discussion

The present study demonstrated that cyclic mechanical stretch of 10% elongation and 0.5 Hz increased the generation of ROS, and promoted the osteogenic differentiation tendency in PDLSCs. The application of NAC obviously restrained the ROS generation stimulated by cyclic mechanical stretch, reinforced cellular antioxidant capabilities by facilitating the GSH synthesis and promoted osteogenic differentiation. The beneficial effect of NAC on osteogenic differentiation of PDLSCs under cyclic mechanical stretch indicated that antioxidant defense might be pivotal during osteogenic differentiation and bone forming.

Osteogenic differentiation in response to mechanical stretch was threshold-driven and relevant to strain rates, amplitude and duration [22]. According to previous studies, mechanical stretch of a physiological magnitude could promote cell differentiation, while high-magnitude mechanical strain could induce a profound damage to cellular activity. Mechanical tensile strain of 30% initiated an enhancement of ROS production and the hallmarks of brain trauma [23]. The unphysiological cyclic mechanical tension would significantly cause DNA damage and the premature senescence in nucleus pulposus cells, compared with the physiological mechanical stretch [24]. Therefore, it is important to choose and determine the amplitude and frequency of cyclic mechanical stretch applied to PDLSCs. Cyclic mechanical stretch of 10% and 0.5 Hz was performed in our study, as it was proved to be physiological and favorable for

osteogenic differentiation [14, 25]. Our results revealed that cyclic mechanical stretch induced the generation of ROS and promoted osteogenic differentiation in PDLSCs.

NAC might have a dual role, antioxidant or a prooxidant, due to the schedule of NAC administration and its concentration. The concentration of NAC used in this study was based on our previous experimental CCK8 results (unpublished observations) and previous researches from others [11, 16]. Xu et al. showed that pretreatment with NAC attenuated LPS-stimulated lipid peroxidation and NAC behaved as an antioxidant, while posttreatment with NAC worsened LPS-induced GSH depletion and NAC behaved as a prooxidant [26]. Based on our results that ROS generation began at 3 h under cyclic mechanical stretch, and remained elevated for the duration of cyclic mechanical stretch. Therefore, we treated PDLSCs with NAC 12 h prior to cyclic mechanical stretch. The data indicated that NAC treatment could promote the osteogenic differentiation of PDLSCs under cyclic mechanical stretch, which was verified in vivo.

The persistent and prolonged overproduction of ROS could alter bone remodeling process leading to an imbalance between osteoclast and osteoblast activities. This could be related to the pathogenesis of osteoporosis [27]. NAC, a powerful exogenous antioxidant, is greatly applied for treatment of a wide range of diseases, such as chronic bronchitis, ulcerative colitis, and Alzheimer [28]. Preincubation of BMSCs with NAC was reported to enhanced cellular resistance against oxidative stress and reinforced the mineralization of newly formed bone tissue after MSC transplantation [29]. Previous studies showed that NAC-pretreated osteoblast-like cells yielded nearly complete bone defect healing with significantly matured mineralized structure after autologous local transplantation in the critical-size defect in a rat femur, compared with the cells without NAC pretreatment [30]. NAC had a preventive and protective effect on orchiectomy-induced osteoporosis by inhibiting osteoclast activity and promoting osteoblast differentiation [12]. Consistent with previous studies, our results showed that application of the antioxidant molecule NAC for PDLSCs promoted the expression of osteogenesis markers COL1, RUNX2, OPN and ALP under cyclic mechanical stretch. And the microarchitectural parameters, such as BV/TV, Tb.Th, Tb.Sp and SMI, of the trabecular bone at the tension side during orthodontic tooth movement in rats in the NAC groups were superior to those in the control groups. These results showed that the antioxidant defense could be vital during osteogenic differentiation under mechanical stretch. NAC might be a promising strategy for promoting osteogenic differentiation at the tension side during orthodontic tooth movement.

As the continuous and sustained overproduction of ROS can oxidize macromolecules, including DNA, proteins and lipids [31]. The endogenous defense system has the ability to scavenge the excessive ROS production, protecting tissues from the excessive ROS-induced damage. GSH, an intracellular major non-enzymatic antioxidant with low-molecular-mass, could serve as a reducing agent and neutralize ROS by binding to electrophiles [32]. Exogenous GSH is unabsorbable outside the cell [30]. NAC, an amino acid derivative with L-cysteine, has a sulfhydryl group acting as a ROS scavenger and exhibiting the antioxidant capability [33]. In addition, as a glutathione precursor, NAC facilitates the generation of GSH to buffer against ROS [34]. NAC might promote osteogenic activity through increasing GSH synthesis [35]. GSH has a vital role in osteoblast and osteoclast differentiation as well as the pathophysiology of

various bone diseases [36]. The reduced intracellular ROS levels and the increased GSH/GSSG ratio could exert protective action in MC3T3-E1 cells [37]. The GSH/GSSG ratio was reported to have a pivotal role in osteogenic differentiation. NAC treatment enhanced ALP activity in the process of osteogenic differentiation, which was related to the increased GSH/GSSG ratio in NAC-treated cells [38]. GSH suppressed RANKL-induced osteoclastogenesis by inhibiting intracellular ROS generation [39]. Our results presented that NAC supplement inhibited the elevation of ROS production and enhanced the level of GSH and the ratio of GSH/GSSG in PDLSCs, suggesting that NAC application improved antioxidant capacity through decreased ROS production and the increased ratio of GSH/GSSG, which might contribute to the promotion of osteogenic differentiation in PDLSCs under cyclic mechanical stretch. The detailed mechanisms remain to be further studied.

During orthodontic tooth movement in vivo, bone formation by osteoblasts at the tension side and bone resorption by osteoclasts at the pressure side are involved in the remodeling of the alveolar bone. And bone resorption caused by osteoclasts at the compression side is related to the rate of orthodontic tooth movement [40, 41]. The effect of NAC on osteoclast differentiation will be determined in the further studies to ensure the delivery time of NAC during orthodontic tooth movement.

Taken together, NAC could improve the antioxidant defense and promote the osteogenic differentiation under cyclic mechanical stretch in vitro and improve the changes of the microstructure of alveolar bone during orthodontic tooth movement in rats, suggesting a potential therapeutic approach for the remodeling of the alveolar bone during orthodontic tooth movement.

Conclusion

NAC could restrain the ROS generation and reinforce antioxidant defense by increasing the level of glutathione, as well as promote the osteogenic differentiation under cyclic mechanical stretch in vitro and improve the changes of the microstructure of alveolar bone during orthodontic tooth movement in rats. These results revealed a promising application of NAC in the therapeutic area of orthodontics.

Abbreviations

ALP
alkaline phosphatase;
BV/TV
bone volume/total volume;
COLI
collagen type I;
GSH
reduced glutathione;
GSSG
oxidized GSH;

NAC
N-acetylcysteine;
OPN
osteopontin;
OTM
orthodontic tooth movement;
PDLSCs
periodontal ligament stem cells;
ROS
reactive oxygen species;
ROI
region of interest;
RUNX2
runt-related transcription factor 2;
SMI
microstructure model index;
Tb.Th
trabecular thickness;
Tb.Sp
trabecular separation

Declarations

Ethics approval and consent to participate

Collection of human periodontal ligament tissues

The experimental protocols and procedures were approved by the Medical Ethical Committee of the School of Stomatology, Shandong University (Protocol Number: 20201206). Human periodontal ligament (PDL) tissues were isolated and collected from premolars, extracted from systemically healthy participants (aged 12–16) for orthodontic reasons at the Stomatological Hospital of Shandong University. The informed consents were obtained from the subjects and subjects' guardians.

Experimental Animals

The Animal experiments were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. The animal experiments were approved by the Medical Ethical Committee of School of Stomatology, Shandong University (Protocol Number: 20201205). Eight-week-old male Wistar rats were used in this study. The rats were raised at a room temperature of 22-24°C under a 12 h light/dark cycle with a relative humidity of 40-60%. The rats in this study were fed with standard food and water ad libitum. The rats were allowed a week of acclimatization.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XX: proposed over all designing ideas and prepared the manuscript, performed the experiments, read and approved the final version of the manuscript. LZX: performed the experiments, read and approved the final version of the manuscript. ZY: performed formal analysis, read and approved the final version of the manuscript. LH: performed the experiments, read and approved the final version of the manuscript. CS: Formal analysis, read and approved the final version of the manuscript. LDX: proposed over all designing ideas and prepared the manuscript, read and approved the final version of the manuscript.

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Table

Due to technical limitations, Table 1 is only available as a download in the supplemental files section

Figures

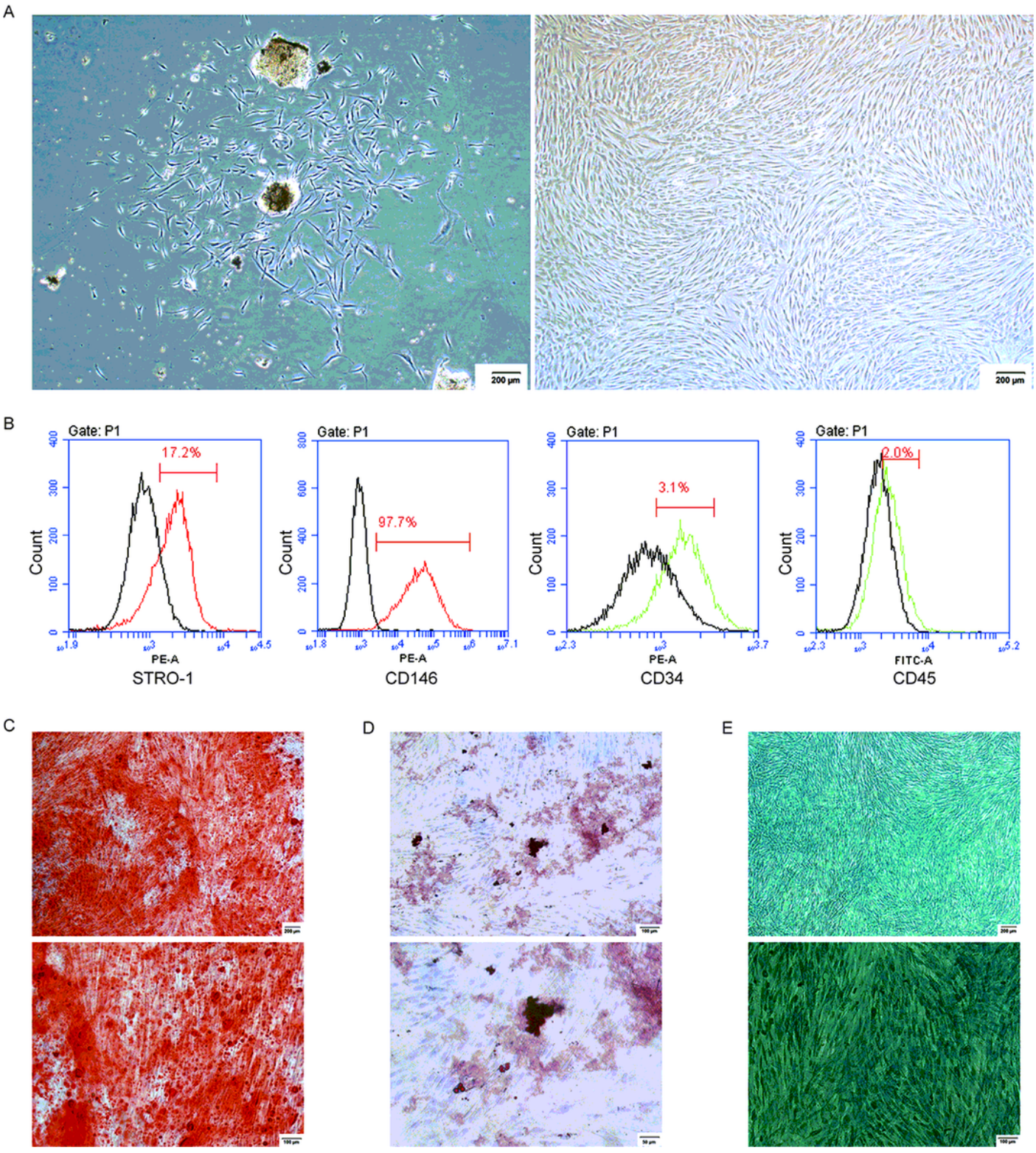

Figure 1

PDLSCs culture and identification. a PDLSCs were derived from human periodontal ligament tissue (scale bar: 200 μm) and cultured from passage 3-6. b the immunophenotypes of PDLSCs were determined by flow cytometry using MSCs-specific positive surface markers (STRO-1, CD146) and MSCs negative markers (CD34, CD45). c Alizarin Red staining after osteogenic induction for 3 weeks (scale bar: 100, 200

μm). d Oil red O staining after adipogenic induction for 3 weeks (scale bar: 50, 100 μm). e Alcian blue staining after chondrogenic induction for 3 weeks (scale bar:100, 200 μm).

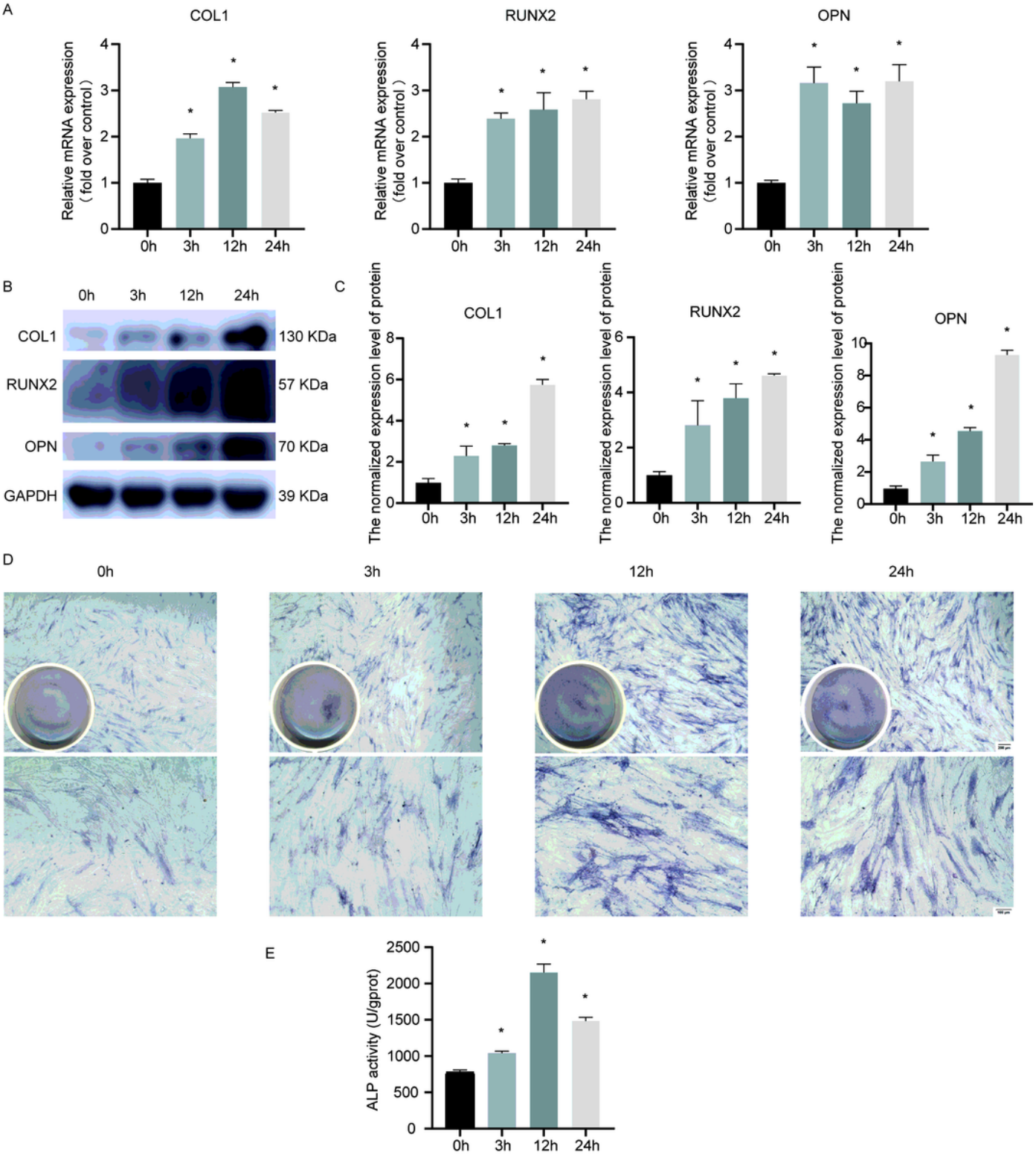

Figure 2

Cyclic mechanical stretch increased the osteogenic differentiation tendency in PDLSCs. a the mRNA expression of osteogenesis markers COL1, RUNX2 and OPN was up-regulated under cyclic mechanical stretch using real-time PCR. b and c the protein expression of COL1, RUNX2 and OPN was elevated by

cyclic mechanical stretch. d ALP staining during cyclic mechanical stretch (scale bar:100, 200 μm). e ALP activity tests during cyclic mechanical stretch. *P < 0.05 vs. 0 h.

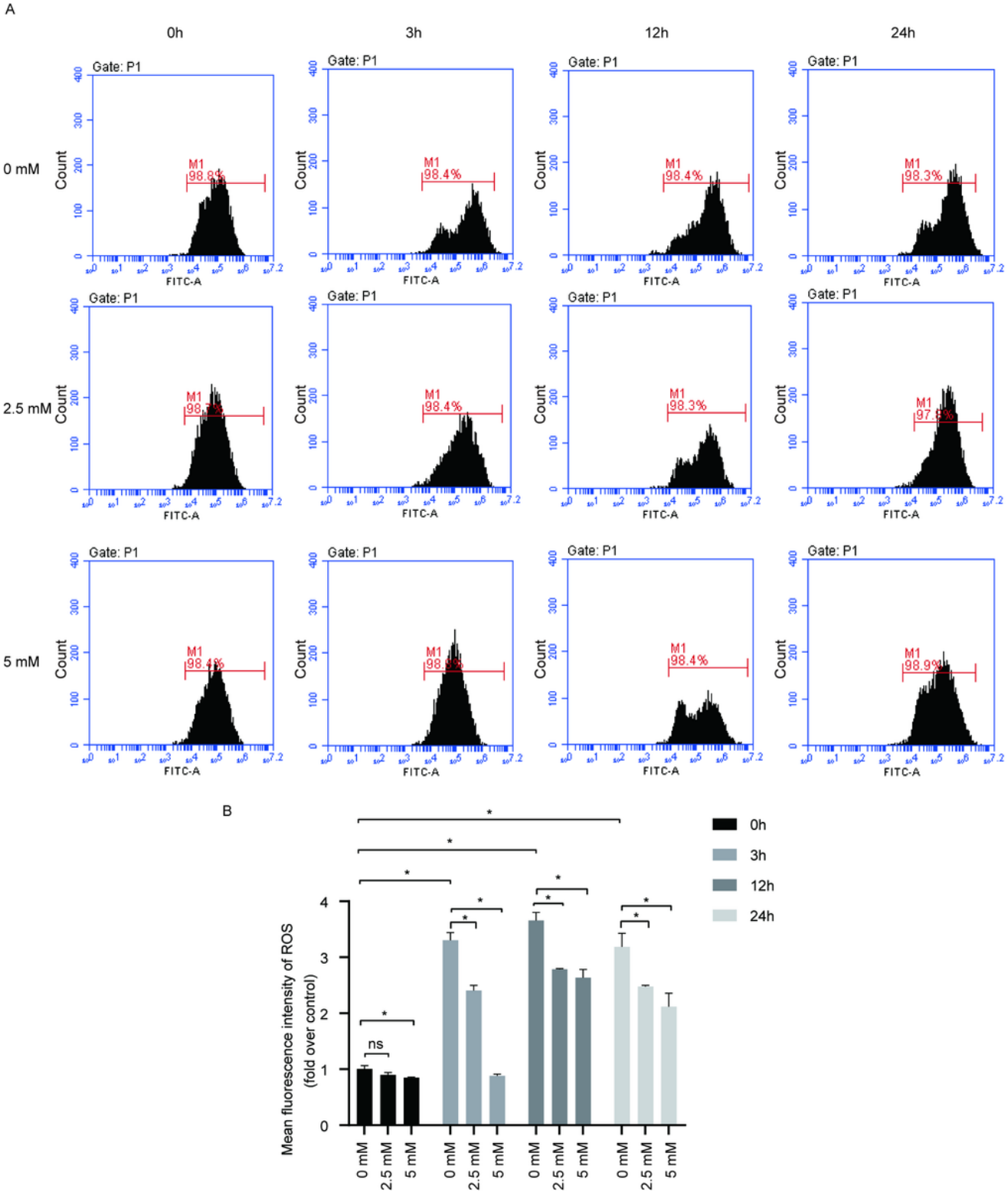

Figure 3

NAC inhibited ROS generation in PDLSCs in response to cyclic mechanical stretch. a ROS levels in PDLSCs were measured via DCFH-DA probe using flow cytometry. b relative quantitative analysis of

intracellular ROS according to flow cytometry results. *P < 0.05 vs. the control group, respectively; ns, not significant.

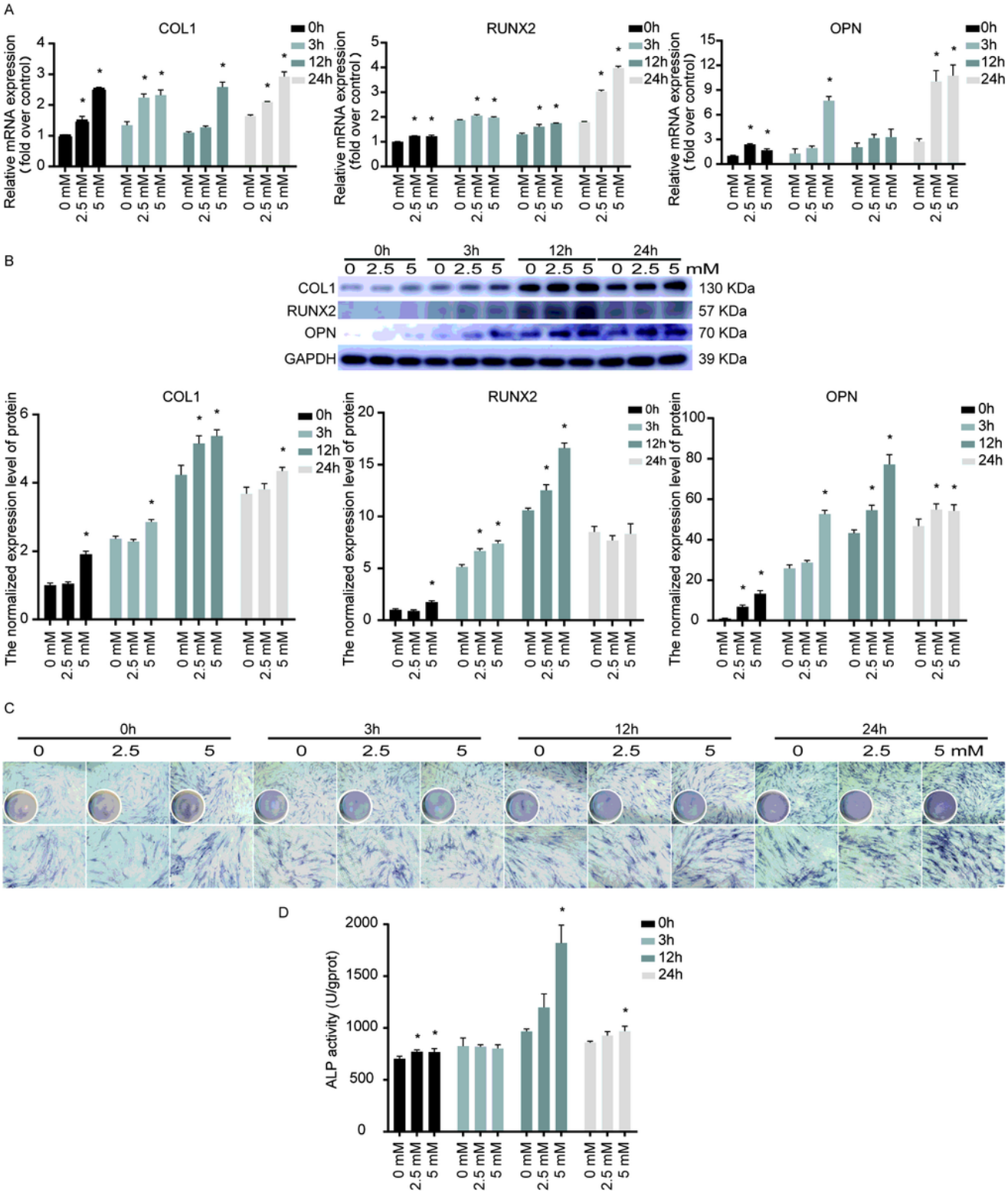

Figure 4

NAC promoted the osteogenic differentiation in PDLSCs under cyclic mechanical stretch. The enhanced expression on COL1, RUNX2 and OPN after NAC treatment according to qRT-PCR (a) and western blot experiments (b). The increase of cellular ALP enzyme activity was noticed in response to NAC by ALP

staining (c) (scale bar:100, 200 μm) and ALP activity tests (d). *P < 0.05 vs. the control group, respectively.

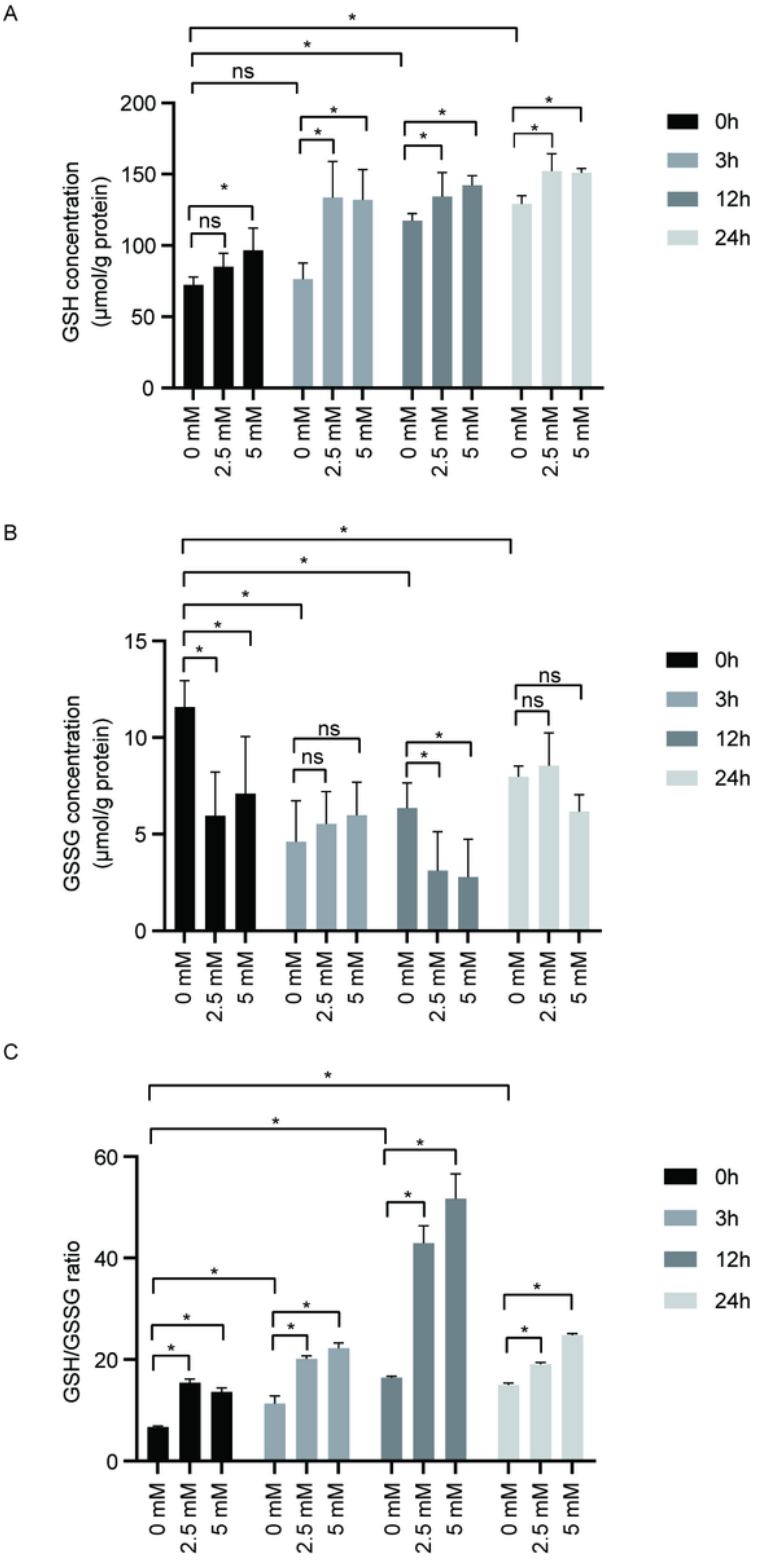

Figure 5

NAC increased GSH concentration in PDLSCs under cyclic mechanical stretch. The level of intracellular GSH (a), GSSG (b) and GSH/GSSG (c) were measured under cyclic mechanical stretch. *P < 0.05 vs. the control group, respectively; ns, not significant.

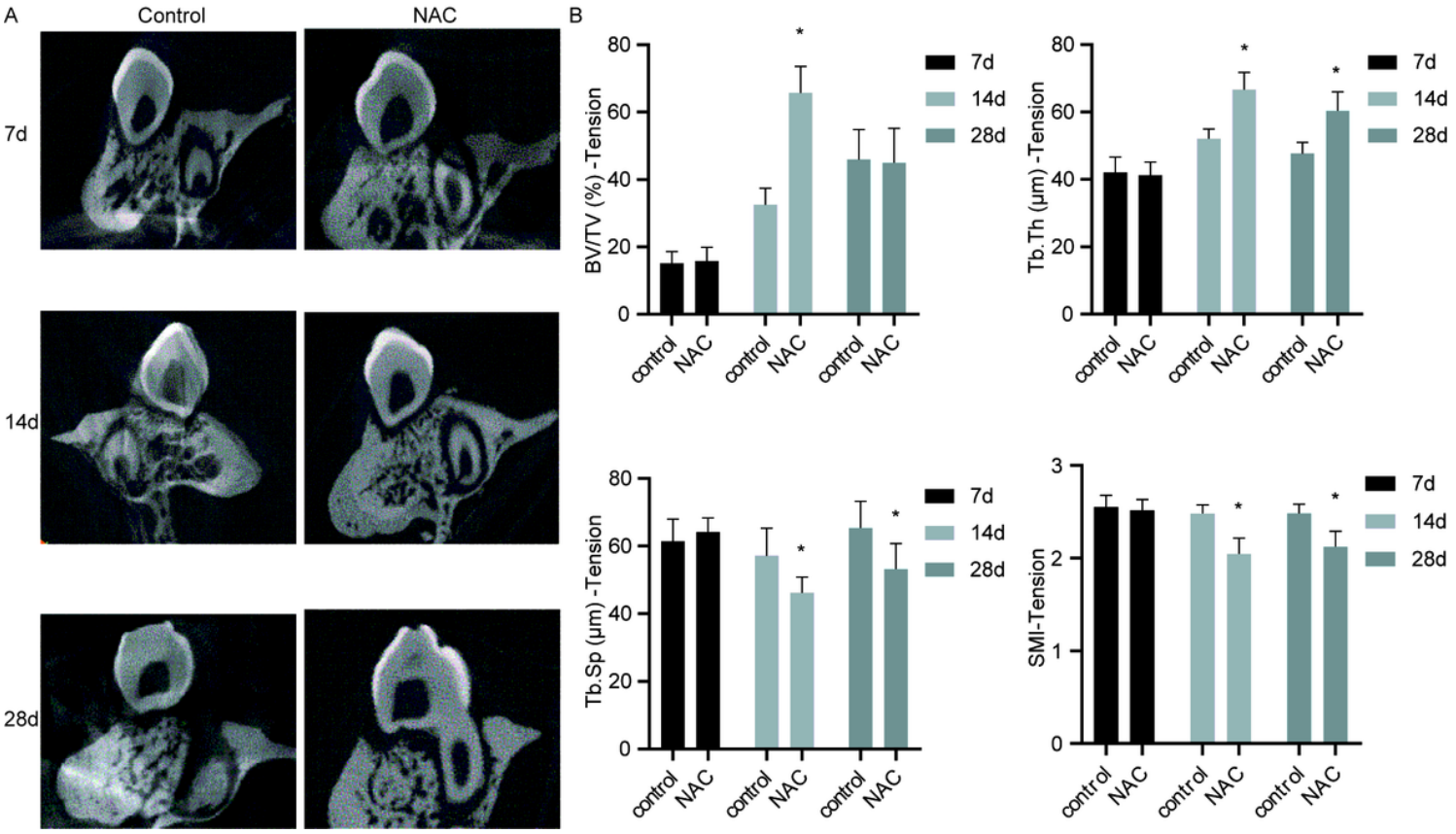

Figure 6

The effect of NAC on microstructure of alveolar bone during orthodontic tooth movement in rats. a micro-CT images of the maxillary first molar at the tension side (coronal view) with or without NAC treatment. b BV/TV, Tb.Th, Tb.Sp and SMI at the tension side with or without NAC treatment (n = 5 specimens/group). *P < 0.05 vs. the control group, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1NAC.xls
- Additionalfile1FigureS1..tif
- Additionalfile2TableS1.xls