

Original Article

Pyrroloquinoline quinone plays an important role in rescuing Bmi-1^{-/-} mice induced developmental disorders of teeth and mandible—anti-oxidant effect of pyrroloquinoline quinone

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Abstract: To investigate whether pyrroloquinoline quinone (PQQ) plays an important role in rescuing Bmi-1^{-/-} mice induced developmental disorders of teeth and mandible by regulating oxidative stress. We fed Bmi-1^{-/-} mice a diet supplemented with PQQ (BKO+PQQ), BKO mice with normal diet (BKO) and wild type mice with normal diet (WT) as controls. We compared the differences of dental, mandibular phenotype by means of X-ray photography, micro CT scanning and three-dimensional reconstruction, HE staining, histochemistry, immunohistochemistry, TUNEL staining, Western blot and Flow cytometry in three groups of animals. Results showed that BKO+PQQ mice increased morphology of teeth and mandible, decreased X-ray transmittance, and increased bone density compared with BKO mice. Results also showed that the teeth volume and the dentin sialoprotein (DSP) immunopositive areas, the cortical thickness, alveolar bone volume, osteoblast number and activity, and alkaline phosphatase (ALP), Osteocalcin (OCN) and type I collagen (Col 1) were all reduced significantly in BKO mice compared with their wild-type littermates, whereas these parameters were increased significantly in BKO+PQQ mice compared with BKO mice. Our study indicated that, compared BKO mice, PCNA positive cells percentage of mandibular first molar epithelial root sheath area significantly increased in BKO+PQQ mice, and TUNEL positive cells percentage was significantly decreased. Further studies showed that supplemental PQQ played a role in anti-osteoporosis of teeth and mandible by up-regulating anti-oxidant capacity, inhibiting oxidative stress and reducing DNA damage, down-regulating CDKI proteins levels, and decreasing cell apoptosis. This study demonstrated that PQQ played an important role in rescuing mandible osteoporosis and disorder of teeth development in BKO mice by promoting osteoblastic bone formation of mandibular alveolar bone, inhibiting osteoclastic bone resorption, promoting odontoblast cell proliferation of epithelial root sheath area, inhibiting cell apoptosis, scavenging ROS.

Keywords: Pyrroloquinoline quinone, Bmi-1, teeth, mandible, oxidative stress

Introduction

The development of teeth is characterized by a series of reiterative molecular interactions accompany with the growth of jawbones. The mice have been used very successfully to identify many of the molecular signaling interactions during the early stage of development of mandible. Numerous studies have been performed to investigate canonical signaling pathways of bone morphogenetic proteins (BMPs), Wnt signaling, Notch signaling, fibroblast growth factors (FGFs) and Sonic hedgehog (Shh), which can function synergistically or antagonis-

tically during teeth development and intramembranous ossification in embryonic period [1-4]. However, the molecular basis of teeth and mandible growth is poorly understood in post-natal mice.

B cell-specific Moloney MLV insertion site-1 (Bmi-1) belongs to the polycomb group (PcG) genes, which are transcriptional repressors that are essential for the maintenance of appropriate gene expression patterns during development [5]. It is reported that Bmi-1 is expressed by incisor stem cells and that deletion of Bmi-1 resulted in fewer stem cells, perturbed

gene expression and defective enamel production [6]. It is well established that not only deletion of Bmi-1 prematurely produces a typical osteoporotic phenotype, but also Bmi-1^{-/-} mice exhibit a total body premature aging phenotype including osteoporosis [7]. Protection against oxidative stress and apoptosis emerges as an important Bmi-1-downstream pathway as well, either by reducing P53 levels via Bmi-1-mediated repression of the INK4a/Arf locus or via modulation of the oxidative stress response in an INK4a/Arf-independent manner. The thymocyte and kidney maturation defect characteristic of Bmi-1-deficient mice is largely rescued by treatment with antioxidants [8, 9]. However, in view of the difference between teeth formation and enamel production, intramembranous ossification and cartilaginous ossification, it is unclear whether Bmi-1 deficiency could lead to dentin and alveolar bone defects in development by disturbing redox homeostasis and inducing DNA damage in mandible.

Pyrroloquinoline quinone (PQQ) was first identified as a novel cofactor of ethanol and glucose dehydrogenase in the methylotrophic bacteria, now is considered an important nutritional growth factor [10, 11]. There is mounting evidence that, PQQ is a 4,5-dihydro-4,5-dioxo-1H-Pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid, is thought to be bacterial glucose dehydrogenase redox cofactor, widely distributed in plants, bacteria, animal, food and many biological fluid, it is soluble in water and thermal stability, divided into the oxidized and reduced forms [12, 13]. In recent years, PQQ has become a hot research, because PQQ has been shown to act as an antioxidant by scavenging superoxide radicals and to protect mitochondria from oxidative stress-induced DNA damage [14]. However, the mechanism underlying effect of PQQ on Bmi1^{-/-} mice has not been elucidated.

To investigate the potential of PQQ, in the present study, Bmi-1 knockout mice were treated with PQQ in the drinking water. We hypothesize whether PQQ could rescue Bmi-1^{-/-} mice induced development defective of teeth and mandible through anti-oxidative stress pathway.

Materials and methods

Mice and genotyping

The Bmi-1 heterozygote (Bmi-1^{+/-}) mice (1290la/FVB/N hybrid back-ground) were backcrossed

10-12 times to the C57BL/6J background and mated to generate Bmi-1 homozygote (Bmi-1^{-/-}) and their wild-type (WT) littermates genotyped by PCR, as described previously [7, 15]. Mice were maintained in the Experimental Animal Center of Nanjing Medical University. This study was carried out in strict accordance with the guidelines of the Institute for Laboratory Animal Research of Nanjing Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

Animals treatment and supplementary of dietary PQQ

Purified PQQ was given free by Professor Chuanjun Wen in Academy of Life Science, Nanjing Normal University. PQQ-supplemented diet was made in Beijing cooperation Feed Co. Ltd., China. In vivo, mice were divided into 3 groups of 6 mice each and treated as following:

(1) Normal diet (WT) group: 3-week weaning littermate wild type mice were fed normal diet for 4 weeks.

(2) Normal diet (BKO) group: 3-week weaning littermate Bmi-1 knockout mice were fed normal diet for 4 weeks.

(3) PQQ-supplemented diet (BKO+PQQ) group: 3-week weaning littermate Bmi-1 knockout mice were fed PQQ-supplemented diet (4 mg PQQ/kg in normal diet) for 4 weeks [16]. Seven weeks later, 3 groups of 6 mice each were sacrificed for further analysis.

Radiography

Mandible were removed and dissected free of soft tissue. Contact radiographs were taken using a Faxitron model 805 radiographic inspection system (Faxitron, München, Germany), at 22 kV voltage and with a 4-minute exposure time. X-Omat TL film (Eastman Kodak, Rochester, NY, USA) was used and processed routinely.

Micro-computed tomography (micro-CT)

Mandible were fixed overnight in 70% ethanol and analyzed by micro-CT with a SkyScan 1072 scanner and associated analysis software (SkyScan, Antwerp, Belgium) as described [17]. Briefly, image acquisition was performed at 100 kV and 98 mA with a 0.98 degree rotation between frames. During scanning, the samples

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were enclosed in tightly fitting plastic wrap to prevent movement and dehydration. Thresholding was applied to the images to segment the bone from the background. 2D images were used to generate 3D renderings using the 3D Creator software supplied with the instrument. The resolution of the micro-CT images is 18.2 μm .

Histology

Mandible were removed, fixed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate) overnight at 4°C and processed histologically as described [17]. Mandible was decalcified in EDTA-glycerol solution for 5-7 days at 4°C. Decalcified right mandible were dehydrated and embedded in paraffin, and 5 μm sections cut on a rotary microtome. The sections were stained with hematoxylin and eosin (H&E) or histochemically for total collagen (TCOL) or alkaline phosphatase activity (ALP), or tartrate-resistant acid phosphatase (TRAP) activity, or immunohistochemically as described below. Alternatively, undecalcified tibiae were embedded in LR White acrylic resin (London Resin Company, Ltd., London, UK), and 1 μm sections were cut on an ultramicrotome. These sections were observed under fluorescence microscopy.

Immunohistochemical staining

Immunohistochemical staining was carried out for biglycan, dentin sialoprotein (DSP), PCNA, COL 1, OCN using the avidin-biotin-peroxidase complex technique with rabbit anti-mouse biglycan antibody (Abcam, Cambridge, UK), dentin sialoprotein (Santa Cruz, CA, USA), affinity-purified goat anti-rabbit PCNA antibody (Abcam, UK), affinity-purified goat anti-mouse COL 1 antibody (Santa Cruz, CA, USA), affinity-purified goat anti-mouse OCN (Cell Signal, China). Briefly, dewaxed and rehydrated paraffin-embedded sections were incubated with methanol-hydrogen peroxide (1:10) to block endogenous peroxidase activity and then washed in Tris-buffered saline (pH 7.6). The slides were then incubated with the primary antibodies overnight at room temperature. After rinsing with Tris-buffered saline for 15 min, sections were incubated with biotinylated secondary antibody (Sigma, St. Louis, MO, USA). Sections were then washed and incubated with the Vectastain Elite ABC reagent (Vector Laboratories, Burlington,

Canada) for 45 min. After washing, brown pigmentation was likewise produced using 3,3-diaminobenzidine (DAB). Finally, the stained sections were counterstained with H&E staining. Images were acquired with a Leica microscope (Leica DM4000B, Solms, German) equipped with Leica software.

Evaluation of apoptotic cells by TUNEL staining

The apoptotic cell of mandibular first molar epithelial root sheath area was evaluated using the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique (20 mg/mL, Cat. no. 21627, Chemicon International, Temecula, CA, USA) [18]. To count the TUNEL-positive cells in mandibular first molar epithelial root sheath area, the ocular micrometer compatible with an Olympus BX51 microscope was used.

Western blot analysis

Proteins were extracted from mandible and quantitated by a kit (Bio-Rad, Mississauga, Ontario, Canada). Protein samples were fractionated by SDS-PAGE and transferred to nitro-cellulose membranes. Western blot was carried out as described previously [7] using antibodies against P16 (goat anti-mouse, M-156, Santa Cruz Biotechnology, USA), P19 (goat anti-mouse, Santa Cruz Biotechnology, USA), P21 (goat anti-mouse, M19, Santa Cruz Biotechnology, USA), P27 (goat anti-mouse, Zymed Laboratories, Santa Cruz, CA, USA), P53 (goat anti-mouse, Cell Signal, China), SOD1 and SOD2 (goat anti-rabbit, Abcam, UK), prdx I (goat anti-rabbit, Abcam, UK), prdx IV (goat anti-rabbit, Abcam, UK), Caspase-3 (goat anti-rabbit, Cell Signal, China), γH2AX (goat anti-mouse, Santa Cruz, CA, USA), and β -actin (goat anti-rabbit, Santa Cruz Biotechnology, USA). Bands were visualized using enhanced chemiluminescence (ECL, Amersham) and quantitated by Scion Image Beta 4.02 (Scion Corporation, Bethesda, MD, USA).

Detection of ROS levels

The mandible tissues were converted into single-cell suspensions containing 5×10^5 cells/mL. We used 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma, USA) for detection of intracellular ROS. Fluorescence intensity is proportional to oxidant production [19]. DCFH-DA

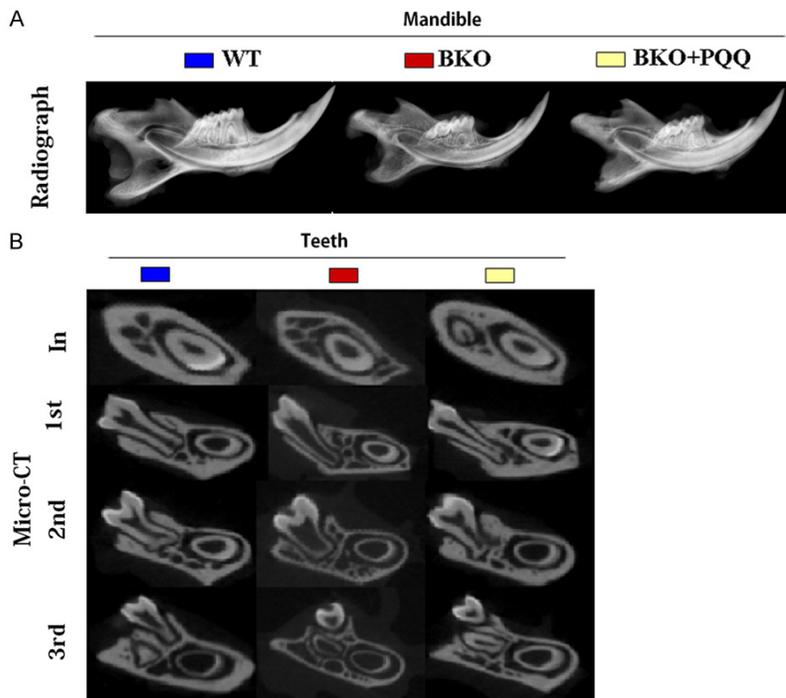


Figure 1. Effect of PQQ on mineralization defects of teeth and mandible in *Bmi-1^{-/-}* mice. A. Radiographs of the mandible from 7-week-old vehicle-treated wild-type (WT) and *Bmi-1^{-/-}* mice and PQQ-treated *Bmi-1^{-/-}* mice. B. Micro-CT scanned sections through the incisor before the first molar (In), and through the first (1st), second (2nd) and third (3rd) molars from vehicle-treated wild-type (WT) and *Bmi-1^{-/-}* mice and PQQ-treated *Bmi-1^{-/-}* mice.

was added to bone cell suspensions to yield final concentrations of 20 $\mu\text{mol/L}$. Then, the cells were incubated at 37°C for 30 min in the dark, washed twice with 0.01 mol/L phosphate-buffered saline (PBS), and centrifuged at $\times 300$ g for 5 min. ROS levels were measured by mean fluorescence intensity (MFI) of 10,000 cells using a flow cytometer (Becton, Dickinson and Co, USA).

Computer-assisted image analysis

After H&E staining or histochemical or immunohistochemical staining of sections from 3 groups of 6 mice each, images of interested fields were photographed with a SONY digital camera. Images of micrographs from single section were digitally recorded using a rectangular template, and recordings were processed and analyzed using Northern Eclipse image analysis software as described previously [7].

Statistic analysis

All data were expressed as mean \pm standard error. Statistical analysis of numeration data

were performed using analysis of χ^2 test, while statistical analyses of measurement data were performed using analysis of student's t-test. The significance level was set at $P < 0.05$.

Results

*Effect of PQQ on mineralization defects of teeth and mandible in *Bmi-1^{-/-}* mice*

To determine whether PQQ-supplemented diet could rescue teeth and mandible growth retardation in *Bmi-1^{-/-}* mice, we comparatively analyzed related parameters of teeth and mandible growth in different groups mice respectively through Radiography and Micro-CT tomography. The results showed that bone mineral density of teeth and mandible was lower in *Bmi-1^{-/-}* mice relative to wild-type mice (**Figure 1A**). Comparison between wild-type and *Bmi-1^{-/-}* littermates of micro-CT scanned sections through the incisor before the first molar, and through the first, second, and third molars showed that the mineralized tooth volume in incisor and molars and the mineralized cortical and alveolar bone volume in mandible were decreased in *Bmi-1^{-/-}* mice (**Figure 1B**). These mineralization defects in teeth and alveolar bone were almost completely rescued by PQQ supplementation.

Comparison between wild-type and *Bmi-1^{-/-}* littermates of micro-CT scanned sections through the incisor before the first molar, and through the first, second, and third molars showed that the mineralized tooth volume in incisor and molars and the mineralized cortical and alveolar bone volume in mandible were decreased in *Bmi-1^{-/-}* mice (**Figure 1B**). These mineralization defects in teeth and alveolar bone were almost completely rescued by PQQ supplementation.

*Effect of PQQ on decreased teeth volume and cortical and alveolar bone volume in *Bmi-1^{-/-}* mandible*

To determine whether phenotype of decreased teeth volume and cortical and alveolar bone volume caused by *Bmi-1* deficiency were improved by PQQ-supplemented diet, Phenotypic differences of mandible between BKO+PQQ mice and littermates WT, BKO mice were analyzed by H&E and TCOL staining. As shown in **Figure 2**, total (mineralized and unmineralized) dentin thickness and the alveolar bone volumes were decreased in *Bmi-1^{-/-}* mice compared with their wild-type littermates (**Figure 2A** and

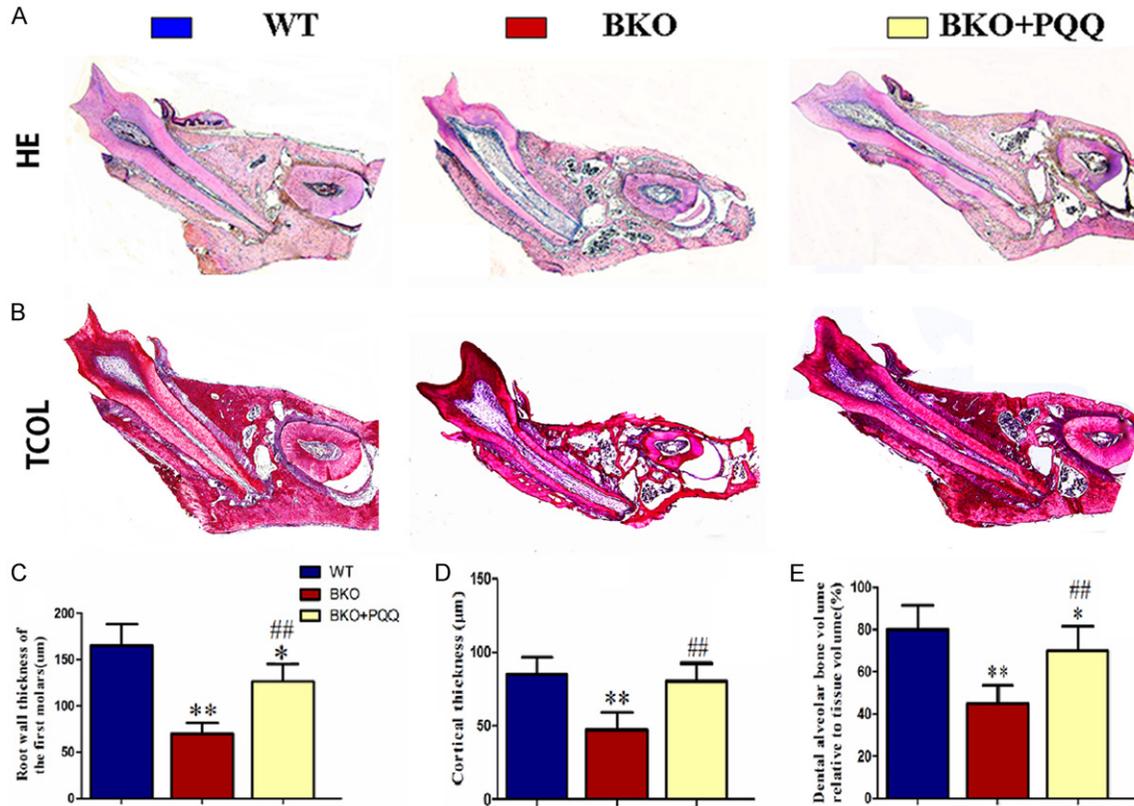


Figure 2. Effect of PQQ on decreased teeth volume and cortical and alveolar bone volume in *Bmi-1^{-/-}* mandible. Representative micrographs of decalcified paraffin-embedded sections through the first molars from 7-week-old vehicle-treated wild-type (WT) and *Bmi-1^{-/-}* mice and PQQ-treated *Bmi-1^{-/-}* mice stained with (A, 50×) HE, and with (B, 50×) serial red for total collagen as described in Materials and Methods. Scale bars represent 400 µm. (C) Dentin thickness of the first molars. (D) The cortical thickness was measured. (E) Alveolar bone volume of the mandible, each value is the mean ± SEM of determinations in six animals of the same groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT mice; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, compared with BKO mice.

2B). Quantitative data showed that the dentin thickness of the first molars (**Figure 2C**), cortical thickness (**Figure 2D**) and the alveolar bone volume (**Figure 2E**) were all reduced significantly in *Bmi1^{-/-}* mice compared with their wild-type littermates. Decreased dentin and cortical thickness and alveolar bone volume in *Bmi-1* deficient mandible were largely rescued by PQQ supplementation.

PQQ supplementation improves abnormal predentin maturation and dentin formation in Bmi-1^{-/-} mice

As seen in sections stained with H&E, the ratio of the areas of predentin to dentin was increased in the first molars (**Figure 3A and 3D**) in *Bmi1^{-/-}* mice compared with their wild-type littermates. Positive immunoreactivity for biglycan was detected in the region of the predentin (**Figure 3B**). The ratio of biglycan positive area

to dentin was increased in the first molars (**Figure 3E**) in *Bmi1^{-/-}* mice compared with their wild-type littermates. Positive immunoreactivity for DSP was detected in the predentin and dentin in the molars (**Figure 3C**). The DSP-positive area was decreased dramatically in the first molars in *Bmi1^{-/-}* mice compared with their wild-type littermates (**Figure 3F**). Decreased dentin maturation and formation in *Bmi-1* deficient teeth were significantly rescued by PQQ supplementation.

Effect of PQQ on osteoblast formation of alveolar bone in Bmi1^{-/-} mice

To detect the effect of PQQ on osteoblast formation of alveolar bone, we analyzed number of osteoblast of alveolar bone with H&E staining, ALP staining, Col I and OCN immunohistochemical staining. As shown in **Figure 4A-I**, the results showed that, compared with BKO mice,

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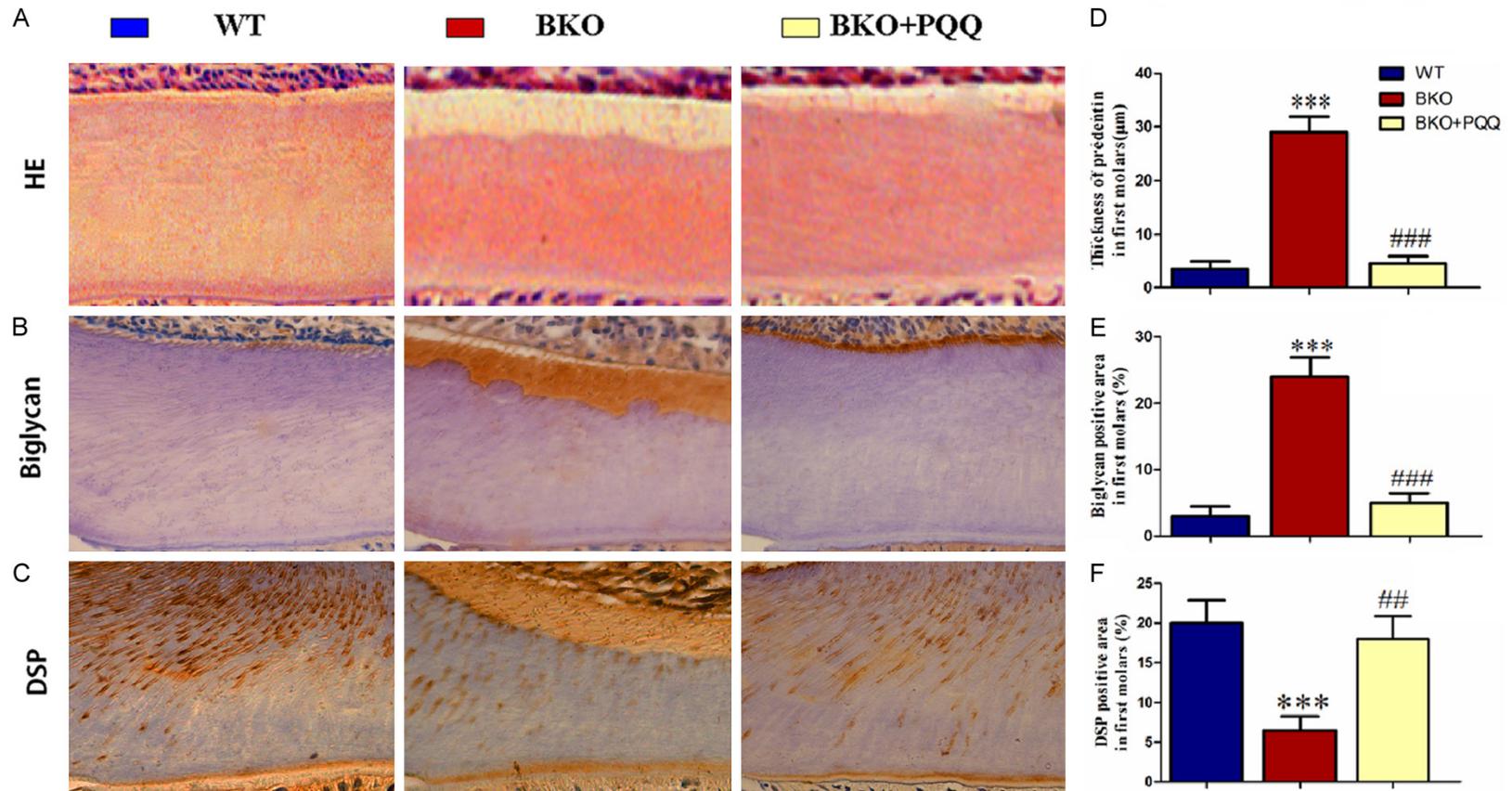


Figure 3. PQQ supplementation improves abnormal predentin maturation and dentin formation in *Bmi-1*^{-/-} mice. Paraffin embedded sections through the first molars from 7-week-old vehicle-treated wild-type (WT) and *Bmi-1*^{-/-} mice and PQQ-treated *Bmi-1*^{-/-} mice stained with (A, 400×) HE, immunohistochemically for (B, 400×) biglycan and (C, 400×) dentin sialoprotein (DSP) and photographed. (A) Representative HE staining of micrographs of the root walls of the first molars. (B) Representative micrographs of the root wall of the first molars stained immunohistochemically for biglycan, and (C) the root wall of the first molars stained immunohistochemically for DSP. (D) Quantitative thickness of predentin in the root walls of the first molars. (E) Quantitative biglycan immunopositive areas in the first molars. (F) Quantitative DSP immunopositive areas in the first molars. Each value is the mean ± SEM of determinations in six animals of the same groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT mice; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, compared with BKO mice.

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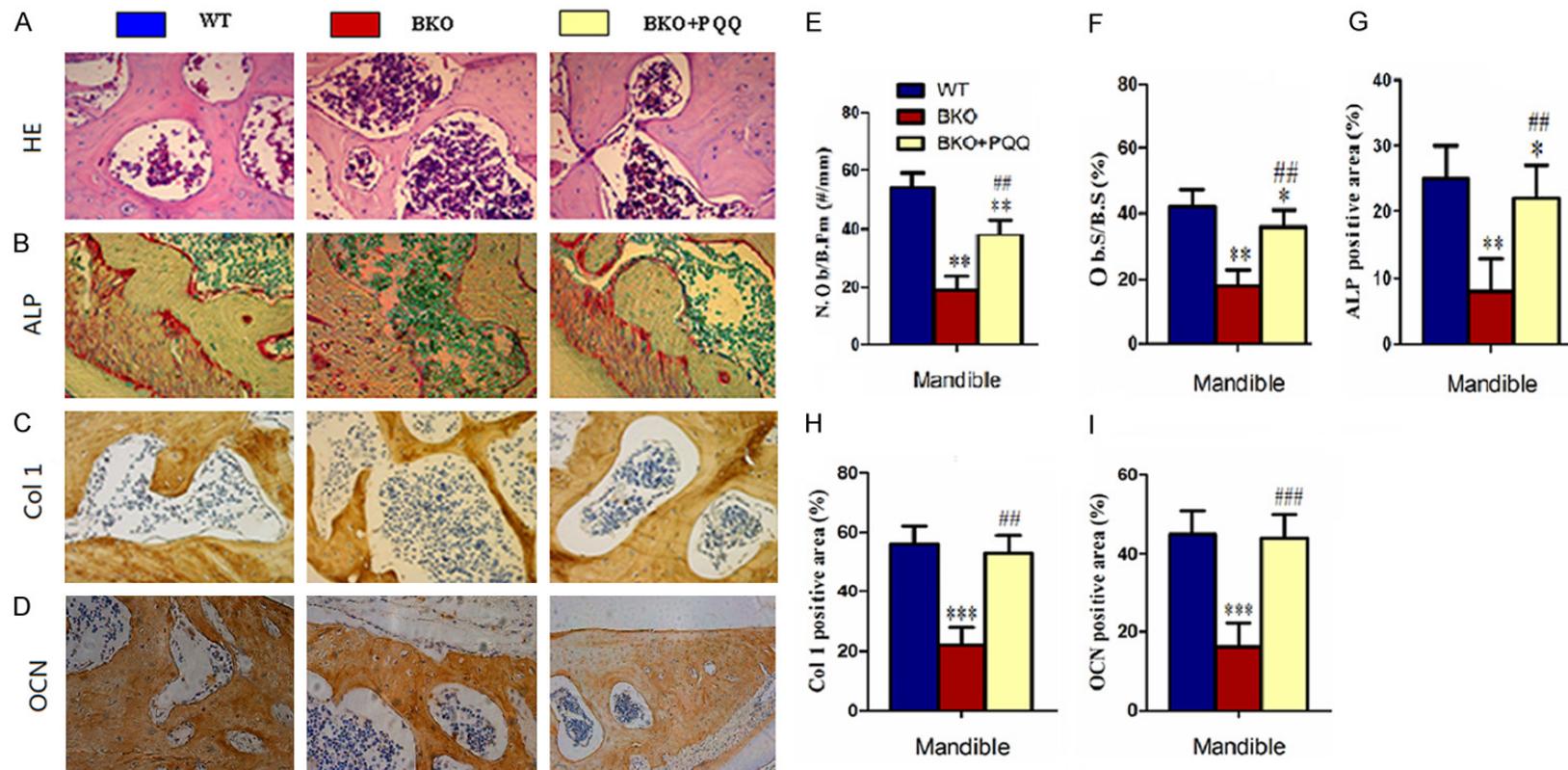


Figure 4. Effect of PQQ on osteoblast formation of alveolar bone in *Bmi1*^{-/-} mice. A. Micrographs of H&E staining of alveolar bone (400×). B. Micrographs of ALP staining of alveolar bone (400×). C. Micrographs of Col I immunohistochemical staining of alveolar bone (400×). D. Micrographs of OCN immunohistochemical staining of alveolar bone (400×). E. Number of positive osteoblasts of alveolar bone. F. Percentage of positive areas osteoblast of alveolar bone. G. ALP positive areas of alveolar bone. H. Col I positive areas percentage of alveolar bone. I. OCN positive areas percentage of alveolar bone. Each value is the mean ± SEM of determinations in six animals of the same groups. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001, compared with WT mice; #, *P*<0.05; ##, *P*<0.01; ###, *P*<0.001, compared with BKO mice. Number of positive osteoblasts (N.Ob/B.Pm (#/mm)). Percent ratio of positive areas osteoblasts (Ob.S/B.S (%)).

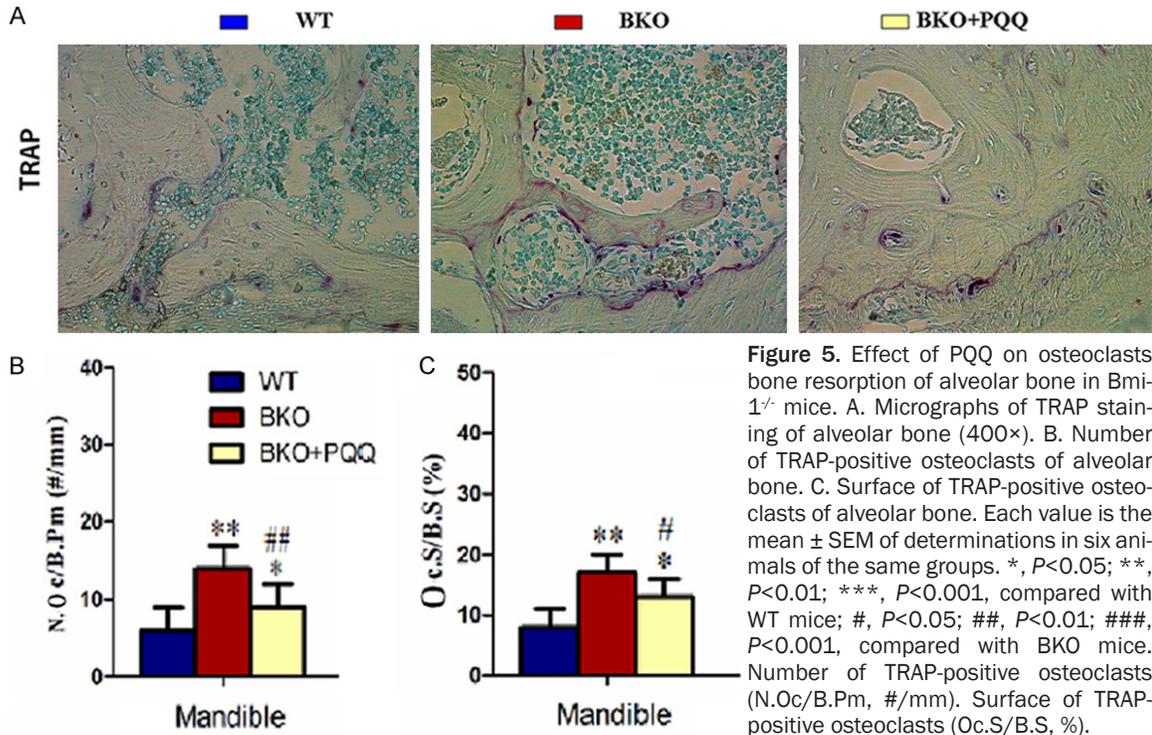


Figure 5. Effect of PQQ on osteoclast bone resorption of alveolar bone in *Bmi-1^{-/-}* mice. A. Micrographs of TRAP staining of alveolar bone (400×). B. Number of TRAP-positive osteoclasts of alveolar bone. C. Surface of TRAP-positive osteoclasts of alveolar bone. Each value is the mean ± SEM of determinations in six animals of the same groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT mice; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, compared with BKO mice. Number of TRAP-positive osteoclasts (N.Oc/B.Pm, #/mm). Surface of TRAP-positive osteoclasts (Oc.S/B.S, %).

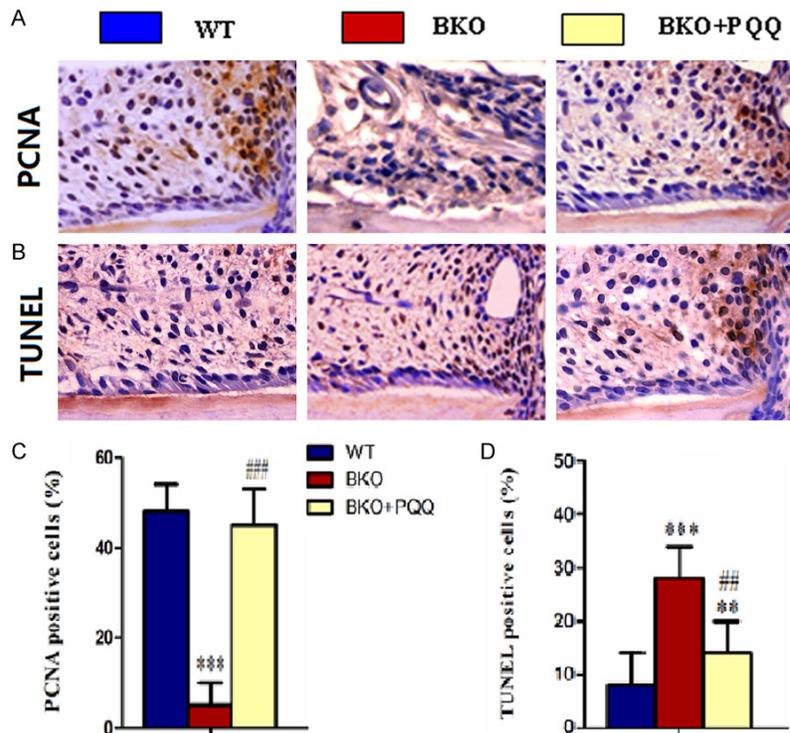


Figure 6. Effect of PQQ on proliferation and apoptosis of mandibular first molar epithelial root sheath area in *Bmi-1^{-/-}* mice. A. PCNA immunohistochemical staining micrograph of mandibular first molar epithelial root sheath area (400×). B. TUNEL staining micrograph of mandibular first molar epithelial root sheath area (400×). C. Statistics of PCNA positive cell percentage. D. Statistics of TUNEL positive cell percentage. Each value is the mean ± SEM of determinations in six animals of the same groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT mice; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, compared with BKO mice.

osteogenic cell, osteoblast number areas, ALP, Col I and OCN positive areas significantly increased in alveolar bone of BKO+PQQ mice. All of the results suggested that PQQ was conducive to osteoblastic bone formation of mice.

*Effect of PQQ on osteoclast bone resorption of alveolar bone in *Bmi-1^{-/-}* mice*

To determine the effect of PQQ on osteoclast bone resorption of alveolar bone, we analyzed number of osteoclast of alveolar bone with TRAP staining. As shown in **Figure 5A-C**, the results showed that, compared with BKO mice, number of osteoclast positive cell, osteoclast positive cell areas significantly decreased in alveolar bone of BKO+PQQ mice. All of the results suggested that PQQ attenuated osteoclast bone resorption ability.

Effect of PQQ on proliferation and apoptosis of mandibular first molar epithelial root sheath area in Bmi-1^{-/-} mice

To determine whether PQQ-supplemented diet could promote proliferation and reduce apoptosis of mandibular first molar epithelial root sheath area in Bmi-1^{-/-} mice, We comparatively analyzed related parameters of mandibular first molar epithelial root sheath area in different groups mice respectively through PCNA immunohistochemistry and TUNEL staining. The results showed that: as shown in **Figure 6A-D**, compared with BKO mice, PCNA positive cell percentage of mandibular first molar epithelial root sheath area were significantly increased in BKO+PQQ mice, however, the percentage of TUNEL positive cells were apparently decreased in BKO+PQQ mice compared to BKO mice. The result was well established that PQQ could improve teeth growth retardation of Bmi-1 deficiency by promoting cell proliferation and inhibiting cell apoptosis.

Effect of PQQ on DNA damage and cell apoptosis of mandible in Bmi-1^{-/-} mice

To determine whether PQQ reduces BKO mice mandible DNA damage and cell apoptosis, we performed one most commonly used markers for double strand DNA breaks γ H2AX and representative markers of apoptosis Caspase-3 (**Figure 7A**). The results found that BKO mice significantly induced cell DNA damage and cell apoptosis, as indicated by the protein level of γ H2AX and Caspase-3. The increased DNA damage and cell apoptosis were prevented by PQQ-supplemented diet (**Figure 7B, 7C**). Therefore, these results suggested PQQ partially played anti-DNA damage and anti-cell apoptosis roles of mandible in Bmi-1^{-/-} mice.

Effect of PQQ on antioxidant proteins of mandible in Bmi-1^{-/-} mice

To further explore whether the effect of PQQ was related to the enhanced expressions of various antioxidant proteins, we performed western blot to examine the expression of BCL-2, prdx I, prdx IV, SOD1 and SOD2 of mandible (**Figure 7D**). Results showed that all the antioxidant proteins were down-regulated in BKO mice compared to WT mice. Strikingly, the expression levels of all the antioxidant proteins were increased by viable levels in BKO+PQQ mice

compared to BKO mice (**Figure 7E-I**). These data suggested that PQQ played antioxidant roles in protecting BKO mice from oxidative stress.

Effect of PQQ on cell cycle proteins of mandible in Bmi-1^{-/-} mice

It is well elucidated that cell proliferation are mediated by the expression and activation of tumor-suppressor genes, we examined the expression of P16, P19, P21, P27, P53 of mandible (**Figure 7J**). Results showed that BKO mice promoted the expression of tumor-suppressor, P16, P19, P21, P27, P53. However, PQQ-supplemented diet on BKO mice decreased expression of P16, P19, P21, P27, P53 (**Figure 7K-O**).

Effect of PQQ on ROS levels of Femur, Thymus, liver and skin in Bmi-1^{-/-} mice

It was well established that BKO mice increased ROS levels and hydroxyl free radicals by oxidative stress, which subsequently resulted in the increase of DNA double strand breaks. Our results showed that PQQ-supplemented diet decreased BKO induced double strand DNA breaks in Femur, Thymus, liver and skin. To determine whether PQQ inhibits the ROS formation, we examined the ROS levels by flow cytometry (**Figure 8A, 8C, 8E, 8G**). The results showed that the increased ROS levels in BKO mice were inhibited by PQQ-supplemented diet (**Figure 8B, 8D, 8F, 8H**).

Discussion

In the study, we investigated that Bmi-1 knockout mice resulted in teeth and mandible development defects with decreased dentin formation and osteoblastic alveolar bone formation. Our results also demonstrated that teeth and alveolar bone development defects caused by Bmi-1 deficiency could be partly rescued by PQQ supplementation. These findings suggest that PQQ plays a critical role in the protection from teeth and alveolar bone development defects by regulating oxidative stress.

Previous study [1] was well established that NAC played treatment effect on defective teeth and mandible caused by Bmi-1 deficiency through promoting osteoblast bone formation, reducing osteoclast bone resorption, scaveng-

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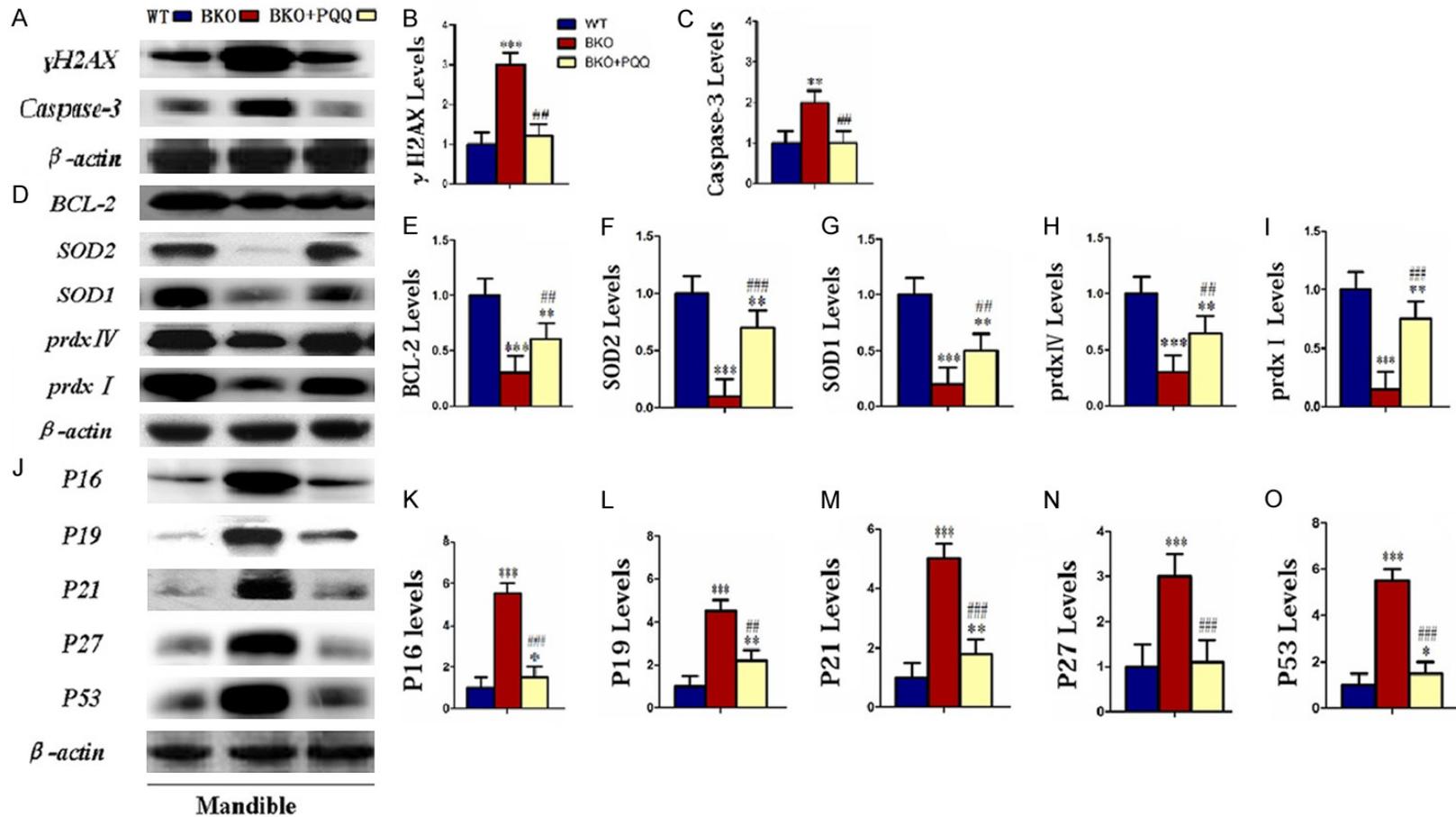


Figure 7. (A) Effect of PQQ on DNA damage and cell apoptosis of mandible in *Bmi1*^{-/-} mice. Representative mandible western blot for expression of γ H2AX and Caspase-3. β -actin was used as loading control for western blot in WT mice, BKO mice and BKO+PQQ mice respectively; γ H2AX and Caspase-3 proteins levels relative to β -actin protein levels were assessed by densitometric analysis and expressed relative to levels of WT mice (B, C). (D) Effect of PQQ on antioxidant proteins of mandible in *Bmi1*^{-/-} mice. BCL-2, SOD1, SOD2, prdx I, prdx IV proteins levels relative to β -actin protein levels were assessed by densitometric analysis and expressed relative to levels of WT mice (E-I). (J) Effect of PQQ on cell cycle proteins of mandible in *Bmi1*^{-/-} mice. P16, P19, P21, P27 and P53 proteins levels relative to β -actin protein levels were assessed by densitometric analysis and expressed relative to levels of WT mice (K-O). Each value is the mean \pm SEM of determinations in six animals of the same groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT mice; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, compared with BKO mice.

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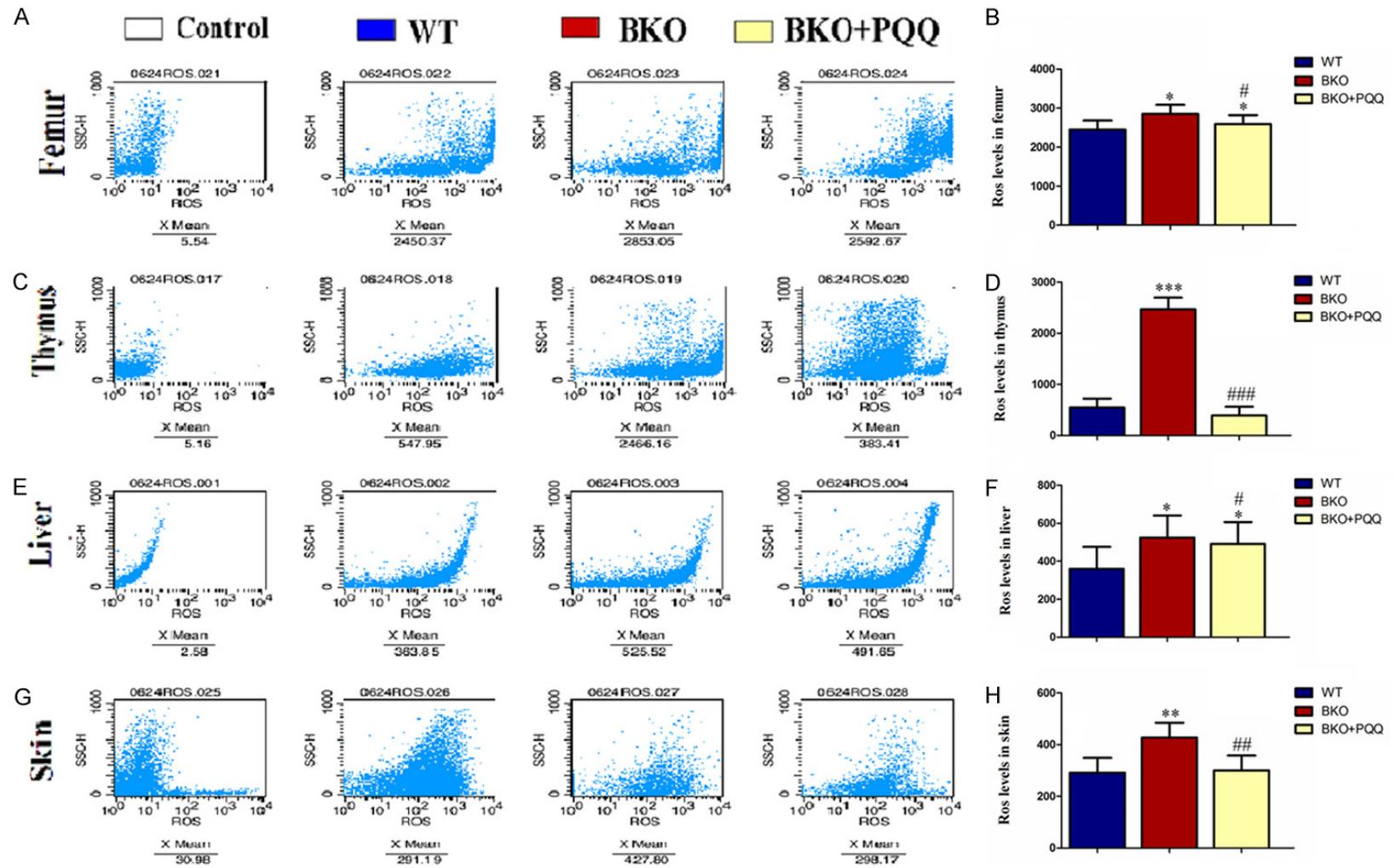


Figure 8. Effect of PQQ on ROS levels of Femur, Thymus, liver and skin in *Bmi1*^{-/-} mice. (A) Representative flow cytometric analysis for ROS levels of Femur, Thymus, liver and skin in WT mice, BKO mice and BKO+PQQ mice respectively (A, C, E, G); ROS levels of Femur, Thymus, liver and skin (B, D, F, H). Each value is the mean ± SEM of determinations in six animals of the same groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ relative to WT mice; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, compared with BKO mice.

ing ROS and preventing DNA damage. As we know, osteogenesis way of teeth and mandible belong to intramembranous ossification, and which of long bones belong to cartilage ossification. In view of this, we put forward to the hypothesis that whether antioxidant PQQ also could play a role in rescuing defects of teeth and mandible induced by Bmi-1 deficiency. Our results from the present study revealed that the ratio of predentin to dentin and that of biglycan positive area to the molar area were increased severely in Bmi-1^{-/-} mice compared with their wild-type littermates. Dentin sialoprophosphoprotein (DSP) distribution in collagen matrix of the forming dentin suggests the protein plays an important role in regulating mineral deposition. Our results also showed a decreased DSP immunopositive area in Bmi-1^{-/-} mice consistent with a reduced dentin thickness. Our findings therefore investigated that Bmi-1 deficiency impaired dentin formation and mineralization. However, PQQ could rescue impaired dentin formation and mineralization.

Bmi-1, derived from the polycomb family, inhibits space-specific and time-specific expression of the Hox gene in growth and development. Bmi-1 gene deficiency leads to shortened life span and growth retardation [9]. Miao et al [7] demonstrated that aging-associated osteoporosis was induced in Bmi-1 knockout mice, as determined by down-regulated self-renewal capacity of bone marrow mesenchymal stem cells. In the study, we analyzed the phenotype of mandible from the Bmi-1 deficient mice and found that the bone mineral density, the mineralized cortical and alveolar bone volume, osteoblast numbers, ALP activity in osteoblasts and expression of the osteoblastic genes in mandible were all decreased. However, the study has evidence suggesting that PQQ significantly reduce the defect of teeth and mandible density in Bmi-1^{-/-} mice. Results from this study indicated that PQQ rescued osteoporosis of teeth and mandible by promoting osteogenesis and inhibiting apoptosis. At the same time, these results suggest that deletion of Bmi-1 disturbs osteoblastic bone formation in both long bones and mandible. The mandibular body develops by intramembranous bone formation. These results therefore suggest that Bmi-1 plays an important role not only in cartilage ossification, but also in intramembranous ossification.

As we know, oxidative stress is a pivotal pathogenic factor for age-related bone loss in mice and rats [20-22], leading to an increase in osteoblast and osteocyte apoptosis, among other changes, and a decrease in osteoblast numbers and the rate of bone formation via Wnt/ β -catenin signaling pathways [21]. Recent studies showed that oxidative stress inhibited osteoblastic differentiation [23, 24] via extracellular signal-regulated kinases (ERKs) and ERK-dependent NF- κ B signaling pathways [25]. Osteoblasts can produce antioxidants, such as glutathione peroxidase, to protect against ROS [26], as well as transforming growth factor β (TGF- β), which is involved in a reduction of bone resorption [27]. ROS are also involved in bone resorption with a direct contribution of osteoclast-generated superoxide to bone degradation, and oxidative stress increases differentiation and function of osteoclasts [28-30]. Our results demonstrated that the increased ROS levels and cell cycle dependent kinase inhibitor (CDKI) in BKO mice were inhibited by PQQ-supplemented diet. The above results showed that, in vivo, PQQ reduced DNA damage and promoted cell proliferation through scavenging ROS of oxygen free radicals and regulating cell cycle proteins.

PQQ, as an antioxidant, is not only involved in redox maintenance and DNA protection from damage, it is also involved in cell cycle regulation by inhibiting p16INK4a/Rb and p19AFR/p53 pathways. Our results investigated that the expression levels of p16, p19, p53, p21 and p27 proteins were down-regulated significantly by PQQ supplementation in Bmi-1 deficient teeth and mandible. We also found that these antioxidant protein expression levels were up-regulated significantly by the antioxidant PQQ supplementation. These results suggest that PQQ regulate p16 and p19 signal pathways by up-antioxidant abilities. However, the exact regulating mechanism of antioxidants on these molecules remains to be investigated.

In conclusion, our study demonstrated that teeth and mandible development defects caused by Bmi-1 deficiency were associated with increased oxidative stress and DNA damage, however, these alterations were partly rescued by PQQ supplementation. Our findings indicate that PQQ plays an important role in stimulating dentin formation and alveolar bone formation

through promoting osteoblast bone formation, reducing osteoclast bone resorption, up-regulating antioxidant abilities, scavenging ROS, decreasing DNA damage and inhibiting cell cycle independent kinase inhibitors.

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Disclosure of conflict of interest

None.

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