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Article Type: Research article

Keywords: Boesenbergia pandurata, age-related periodontitis, gingival inflammation, bone resorption
Inhibitory Effects of *Boesenbergia pandurata* on Age-Related Periodontal Inflammation and Alveolar Bone Loss in Fischer 344 Rats

Haebom Kim¹,#, Changhee Kim¹,#, Do Un Kim², Hee Chul Chung², and Jae-Kwan Hwang¹,*

¹Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul 03722, Republic of Korea

²NewTree CO. Ltd, 11F Tech center, SK n Techno Park, Sungnam 462-120, Republic of Korea

# Co-first authors

*Corresponding author

Phone: +82 2 2123 5881;

Fax: +82 2 362 7265;

E-mail: jkhwang@yonsei.ac.kr

Running title: Anti-periodontitis effect of *Boesenbergia pandurata*
Abstract

Periodontitis, an infective disease caused by oral pathogens and the intrinsic aging process, results in the destruction of periodontal tissues and alveolar bone loss. This study investigated whether *Boesenbergia pandurata* extract (BPE) standardized with panduratin A exerted anti-periodontitis effects using an aging model representative of naturally occurring periodontitis. In aged rats, the oral administration of BPE (200 mg/kg/day) for 8 weeks significantly reduced the mRNA and protein expression of interleukin (IL)-1β, nuclear factor-kappa B (NF-κB), matrix metalloproteinase (MMP)-2, and MMP-8 in gingival tissues (*p* < 0.01). In alveolar bone, histological analysis with staining and micro-computed tomography (micro-CT) revealed the attenuation of alveolar bone resorption of rats in the BPE-treated aged group, which led to a significant reduction in the mRNA and protein expression of nuclear factor of activated T-cells c1 (NFATc1), c-Fos, tartrate-resistant acid phosphatase (TRAP), and cathepsin K (*p* < 0.01). BPE not only increased the expression of osteoblast differentiation markers, such as alkaline phosphate (ALP) and collagen type I (COL1A1), but also increased the ratio of osteoprotegerin (OPG) to RANKL. Collectively, the results strongly suggested that BPE is a natural resource for the prevention or treatment of periodontal diseases.

Keywords: *Boesenbergia pandurata*, age-related periodontitis, gingival inflammation, bone resorption
Introduction

Periodontitis is an inflammatory disease of the periodontal tissues surrounding teeth. When periopathogenic bacteria accumulate on the surface of the teeth or during the intrinsic aging process, periodontal inflammation and the alveolar bone loss occurs [1]. As the severity progresses, the ability to masticate and digest food is limited and a person’s quality of the life is dramatically reduced [2]. Previous studies revealed a high prevalence of periodontitis in adults from the United States aged ≥ 30 years old, with almost fifty-percent affected. This figure includes patients with chronic periodontitis triggered by aging [3, 4]. Chronic periodontitis, which results in more severe outcomes, is highly associated with systemic diseases, such as cerebrovascular diseases, diabetes, osteoporosis, and complications of pregnancy [5]. Thus, the prevention and treatment of periodontitis is an important strategy to maintain health and contribute significantly to an individual’s quality of life and systemic health.

Some loss of periodontal attachment and alveolar bone is often observed in elderly people, which indicates that periodontal disease is time-dependent and aging appears to be inherently responsible for gingival destruction [6]. In the gingival tissues of aged rats, the gene expression changes in the transformed immune system lead to the accumulation of inflammatory mediators, such as interleukin (IL)-1β and nuclear factor kappa B (NF-κB), which cause periodontal destruction [4]. The inflammatory responses increase proteases, including matrix metalloproteinases (MMPs), which degrade protein matrix components, such as collagens and gelatins, and ultimately result in the collapse of gingival tissues [7]. The increased inflammatory responses stimulate the secretion of RANKL in osteoblasts, which binds to the RANK receptor in osteoclasts to initiate osteoclastogenesis and the process of bone resorption [8]. Unlike osteoclasts, osteoblasts play a major role in the formation of new bone. In the aged periodontium, osteoclasts are more prevalent than osteoblasts, which lowers
the expression of bone formation-related markers such as alkaline phosphatase (ALP), collagen type I (COL1A1), and osteoprotegerin (OPG) [9-12]. ALP functions through the release of a phosphate group, leading to mineralization for bone formation in the bone matrix [13]. OPG is a decoy receptor for RANKL, which inhibits the binding of RANKL to RANK, and ultimately affects the bone remodeling activity [14].

*Boesenbergia pandurata* Roxb., commonly known as fingerroot, exerts various bioactivities such as anti-bacterial, anti-oxidant, anti-inflammatory, and anti-obesity effects [15]. Previously, we demonstrated that BPE exerted anti-inflammatory effects in human oral cells and that panduratin A isolated from BPE inhibited periopathogenic microbial growth, which caused periodontitis [16-18]. However, the examination of the anti-periodontitis effect of BPE in animal models has not been conducted. Because the aging model of periodontitis represents a genuinely chronic model, we evaluated the inhibitory effect of BPE on chronic periodontitis in aged rats.
Materials and Methods

Preparation of Standardized Boesenbergia pandurata Extract

Dried rhizomes of *B. pandurata* were obtained from AAT Costech Co., Ltd (Seoul, Korea) and a specimen was deposited in the Department of Biotechnology, Yonsei University (Seoul, Korea). The dried *B. pandurata* rhizomes were ground and extracted with 95% ethanol for 3 days at room temperature. The solvent was filtered and evaporated to obtain BPE at a 12.0% (w/w) yield. The standardized BPE contained 8.0% (w/w) panduratin A as the bioactive compound [19].

Animal Experiment

Eleven-week-old male Fischer 344 (F344) rats were purchased from Central Lab Animal Inc. (Seoul, Korea) and 18–20-month-old male F344 rats were purchased from Laboratory Animal Resource Center (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). All rats were bred in a controlled environment (temperature, 21°C ± 2°C; relative humidity, 50% ± 15%; 12-h light-dark cycle) at the KPC laboratory (Gwangju, Korea). During the entire experiment period, the rats were allowed free access to food and tap water. After a 1-week acclimatization, 24 rats were divided into three groups: (i) young control (Young, n=8); (ii) aged control (Aged, n=8); (iii) BPE-treated aged group (Aged + BPE, n=8). The BPE-treated aged group was orally administered BPE dissolved in saline at a dose of 200 mg/kg/day. After 8 weeks, the animals were euthanized by cardiac puncture under anesthesia. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of KPC Laboratory (P170014).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from gingival tissues and alveolar bone was isolated by the addition of Trizol...
reagent (Takara, Shiga, Japan) and 2 μg of the isolated total RNA was quantified by NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The RNA was mixed with reverse transcriptase premix (Elpis Biotech, Daejeon, Korea) and oligo (dT) to synthesize cDNA (20 μL). Reverse transcription (RT) was conducted by cycles of the following processes: initiation at 70°C for 5 min; incubation at 42°C for 55 min; and termination at 70°C for 15 min. The amplification of cDNA by a polymerase chain reaction (PCR) with specific primer pairs (Bioneer, Daejeon, Korea) (Table 1) was conducted by cycles of the following processes: denaturation at 94°C for 30 s, annealing for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The products were loaded onto a 1.5–2.0% agarose gel, separated by electrophoresis, and stained with Loading STAR dye (Dyne Bio Inc., Seoul, Korea). The dye was visualized using the G:BOX EF imaging system (Syngene, Cambridge, UK) and Genesys software.

Western Blotting

The gingival tissues were lysed using NP40 lysis buffer (Elpis Biotech) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration of the lysate was assessed by the Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) and equal amounts of protein (20 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany), which were incubated with primary antibodies against NF-κB, IL-1β, MMP-2, MMP-8, TRAP, NFATc1, cathepsin K, c-Fos, ALP, COL1A1, OPG, RANKL (1:1000 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and α-tubulin (1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. For the secondary reaction, the membranes were incubated with the appropriate secondary antibody (1:5000 dilution, Bethyl Laboratories Inc., Montgomery, TX,
USA) for 1 h at room temperature. The intensity of the secondary antibody was evaluated through the application of the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK) and visualization using the G:BOX EF imaging system (Syngene) and the Genesys program.

**Histological Analysis**

The decalcified periodontal tissues were fixed in 10% formalin solution, embedded in paraffin, and cut into 5-μm sections, which were mounted on slides. The paraffin slides were stained with hematoxylin and eosin (H&E) and observed using an Eclipse TE2000U inverted microscope with twin CCD cameras (×200 magnification, Nikon, Tokyo, Japan) to detect cell infiltration and alveolar bone resorption. To determine the expression levels of key biomarkers in the tissues, immunohistochemical staining was conducted in accordance with the following protocol. The paraffin slides were incubated with MMP-2, MMP-8, TRAP, NFATc1, ALP, and COL1A1 (1:200 dilution, Santa Cruz Biotechnology Inc.) at 4°C overnight. The slides were then incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugated IgG secondary antibody (1:5000 dilution, Bethyl Laboratories Inc.) for 1 h at 4°C. The stained slides were observed using an Eclipse TE2000U Inverted Microscope with twin CCD cameras (×400 magnification, Nikon). To quantify protein expression, the stained areas of the images were measured using ImageJ software version 1.47 (National Institutes of Health, Bethesda, MD, USA).

**Micro-Computed Tomography (CT) Imaging**

To evaluate the effect of BPE on alveolar bone loss, micro-computed tomography (CT) imaging was performed using a Skyscan 1076 (Skyscan, Kontich, Belgium) with the following conditions: total rotation, 360°; rotation step, 0.5°; pixel size, 18 μm; voltage, 100
kV; current, 100 μA; and exposure time, 1475 ms. The distance between the cementoenamel
junction (CEJ) and alveolar bone crest (ABC), which represented the level of alveolar bone
loss, was quantified using ImageJ software version 1.47 (National Institutes of Health). The
scanned images were reconstructed to obtain the 3D trabecular structure by NRecon (Skyscan)
and CTAn (Skyscan). The parameters of bone volume per tissue volume (BV/TV), trabecular
thickness (Tb. Th), trabecular separation (Tb. Sp), and bone mineral density (BMD) were
measured and quantified within the region of interest (ROI). The alveolar bone of the first
molar was selected as the ROI.

Statistical Analysis
All experiments were repeated at least three times in triplicate. Each value was reported as
the mean ± standard deviation (SD). Statistical analyses were computed using Statistical
Package for the Social Sciences (SPSS) version 23.0 (SPSS Inc., Chicago, IL, USA).
Differences between groups were evaluated using one-way analysis of variance (ANOVA)
followed by Duncan’s test, with values of \( #p < 0.05, ##p < 0.01, ^{*}p < 0.05, \) and \( ^{**}p < 0.01 \)
considered statistically significant.
Results

BPE Inhibits Gingival Inflammation and Osteoclastogenesis in Aged Rats

In the aged control group, the mRNA and protein levels of IL-1β and NF-κB were significantly enhanced, whereas the BPE-treated aged group showed a significant decrease in these mRNA and protein levels (Figs. 1A and 1B). Similarly, the levels of MMP-2 and MMP-8 were diminished by BPE compared with those of the aged control group. In particular, BPE treatment effectively inhibited MMP-2 expression, with 65.7% and 70.5% reduction in the mRNA and protein expression, respectively. When compared with the aged control group, BPE significantly decreased the mRNA and protein expression of osteoclastic transcription factors including NFATc1, c-Fos, TRAP, and cathepsin K (Figs. 1C and 1D). These results suggested that BPE inhibited the destruction of the periodontium through the control of inflammatory factors and differentiation-related transcription factors and enzymes.

BPE Improves Histological Changes Induced by Periodontitis

The H&E staining analysis revealed high levels of histological changes in the aged control group, such as cell infiltration and bone resorption (Fig. 2A). BPE treatment decreased cell infiltration and improved the irregular surface caused by bone resorption. In addition, the levels of the key proteins expressed in the periodontal tissues were visualized using immunohistochemistry (Fig. 2B). In comparison with the aged control group, BPE downregulated the protein expression of MMP-2, MMP-8, NFATc1, and TRAP, but increased the protein expression of ALP and COL1A1 in periodontal tissues (Fig. 2C). Hence, BPE treatment was shown to be effective against periodontitis at the histological level, which provided evidence to support the anti-periodontitis activity of BPE.

BPE Promotes Osteoblast Differentiation in Aged Rats
BPE significantly promoted the mRNA and protein expression of ALP and COL1A1, and also reduced the expression of RANKL stimulated by gingival inflammation (Fig. 3). The OPG/RANKL ratio directly affects the degree of bone resorption and bone remodeling. In the BPE-treated aged group, the ratio of OPG to RANKL was increased at both the mRNA and protein level compared with the aged control group, which stimulates bone remodeling. These results indicated that BPE prevented alveolar bone resorption and contributes to bone healing.

**BPE Prevents Alveolar Bone Loss and Repairs Bone Defects**

The level of alveolar bone loss was analyzed using micro-CT. The distance between the CEJ and the ABC, which represents bone loss, was measured on each image. In the aged control group, the distance between the CEJ and the ABC was $1.13 \pm 0.05$ mm, which was 45.2% longer than that of the young control group. However, compared with the aged control group, BPE treatment decreased this distance by 9.7%, which represented an inhibition of alveolar bone loss (Fig. 4B). The 3D image analysis revealed that BPE treatment significantly increased BV/TV, Tb. Th, and BMD by 34.4%, 36.2%, and 21.6%, respectively, whereas Tb. Sp. was decreased by 26.4% compared with the aged control group (Fig. 5). These results suggested that BPE administration restored the alveolar bone loss caused by periodontitis.
In the aging process, gingival inflammation and bone resorption are caused not only by lipopolysaccharide (LPS) infection of oral bacteria, but also by gene expression alterations in the immune system [6]. The high secretion of LPS by oral pathogens activates gingival fibroblasts and periodontal ligament fibroblasts to upregulate inflammatory factors, such as IL-1β and NF-κB [20, 21]. The age-related gene expression changes also caused the accumulation of IL-1β and NF-κB and stimulated chronic periodontal inflammation in the periodontium [4]. These reports implied that gingival tissue, when exposed to bacterial LPS, may be more vulnerable to periodontitis in conjunction with aging.

BPE treatment significantly reduced the expression of MMPs, in addition to inflammatory biomarkers, such as IL-1β and NF-κB, in the aged rats (Figs. 1A and 1B), which provided an indication that BPE could simultaneously inhibit the gingival inflammation caused by bacterial LPS and abnormal gene expression in aged rats. Previously, we reported that BPE suppressed MMP-2 expression in human gingival fibroblasts, which are main cells that constitute gingival tissues and induce the inflammatory responses [17]. Accordingly, BPE can reduce the destruction of gingival tissues through the inhibition of inflammatory responses in human gingival fibroblasts. BPE also reduced the increased secretion of osteoclastic factors, such as NFATc1, c-Fos, TRAP, and cathepsin K (Figs. 1C and 1D). Osteoclastogenesis is activated through the recruitment of c-Fos to the NFATc1 promoter, which induces the expression of NFATc1 in the early stages of osteoclast differentiation [22, 23]. NFATc1, a key transcription factor, plays a major role in the amplification of genes involved in bone resorption, including TRAP and cathepsin K [24]. The results suggested that BPE ameliorates the initiation and amplitude of the age-related osteoclastic processes caused by gingival inflammation.

Histological analysis revealed that BPE attenuated the aggressive immune response-
induced cell infiltration, represented by the destroyed matrix on the surfaces of the alveolar bone (Fig. 2A). Cell infiltrates can induce excessive amounts of reactive oxygen species, proinflammatory cytokines, and osteoclastic enzymes [9]. Immunohistochemical analysis demonstrated that BPE attenuated the expression of MMPs and osteoclastic factors, such as NFATc1 and TRAP, in identical regions (Figs. 2B and 2C). These results indicated that BPE reduced the resorption of alveolar bone through the efficient downregulation of the increased cell infiltration caused by bacterial stimulus (LPS) and hyperactivation of the immune system in aged rats.

In bone homeostasis, the balance of osteoblast and osteoclast is of primary importance to maintain bone health. Osteoblasts are the cell type that produce bone formation, whereas the matured osteoclasts resorb bones [9]. It is known that osteoblast differentiation markers such as ALP, COL1A1, and OPG are decreased with aging in periosteal osteoblastic cells [10]. BPE upregulated the expression of the osteoblast differentiation markers (Fig. 3), which indicated that BPE compensated for the collagen matrix surrounding MMP- and osteoclastic enzyme-induced bone defects. As the downregulation of osteoblast activity induces systemic bone loss, such as osteoporosis, and contributes to more rapid alveolar bone loss [25], BPE was determined to efficiently suppress bone resorption through the control of osteoblast-related biomarkers. Histological observations performed using micro-CT also demonstrated the potential action of BPE in the attenuation of periodontitis-induced alveolar bone resorption, through the increased height of the bone crest and protection against the deterioration of trabecular bone structure (Figs. 4 and 5). Previously, it was reported that several natural ingredients, such as green tea extract, curcumin, and myricetin, exerted an anti-periodontitis effect that was only related to bone loss [8, 26, 27]. Conversely, BPE appears to contribute to the recovery of the defected bone matrix, as well as the prevention of bone loss.
Similar to age-related periodontitis, bacterial LPS acutely induces periodontitis in gingival tissues by overexpressing IL-1β and NF-κB [20, 21]. In this study, BPE had an inhibitory effect on these markers in the gingival tissues. In previous study, BPE inhibited Porphyromonas gingivalis supernatant-induced NF-κB activity in human epidermoid cells. In addition, BPE exerted anti-inflammatory effects in P. gingivalis supernatant-treated human gingival fibroblasts [16, 17]. Therefore, BPE could have therapeutic effect in acute periodontitis.

The anti-periodontitis effects of plant extracts and phytochemicals to date have been predominantly examined in an LPS-induced acute periodontitis model, with very limited research documented in age-related periodontitis models. For example, it has been reported that kaempferol reduced inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α) through the inactivation of the NF-κB and MAPK pathways in the gingival tissues of aged rats [28]; however, the amelioration of bone defects triggered by gingival inflammation was not demonstrated. The administration of co-enzyme Q10 or a vitamin-like oil-soluble molecule reduced the number of TRAP-positive osteoclasts in aged gingival tissues [29], but the effect on osteoblast differentiation to protect bone loss remains unclear. In contrast, this study demonstrated that BPE conferred an anti-periodontitis effect through the suppression of alveolar bone loss, as well as the inhibition of age-related inflammation.

Several bioactives, such as cardamonin, pinocembrin, alpinetin, and geraniol in BPE exert anti-inflammatory activities by inhibiting expression of IL-1β and NF-κB [30-33]. Particularly, panduratin A, a major bioactive in BPE, reduces the expression of NF-κB, IL-1β, and the formation of TRAP-positive multinucleated osteoclasts in vitro [34]. Therefore, it is anticipated that BPE standardized with panduratin A can be employed as a therapeutic material for elderly people through complementary clinical trials for anti-periodontitis.
Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest.


**Figure legends**

**Fig. 1.** Inhibitory effect of BPE on inflammation in gingival tissues and osteoclastogenesis in the alveolar bone of aged rats.

(A) The mRNA levels of IL-1β, NF-κB, MMP-2, and MMP-8 in gingival tissues were measured using RT-PCR; β-actin was used as the housekeeping gene. (B) The protein levels in gingival tissues were evaluated by western blotting; α-tubulin was used as the loading control. (C) The mRNA levels of NFATc1, c-Fos, TRAP, and cathepsin K in alveolar bone were measured using RT-PCR; β-actin was used as the housekeeping gene. (D) The protein expression in alveolar bone was evaluated by western blotting. All data are presented as the mean ± SD (% of control) of eight rats per group. ##p < 0.01 (young control vs aged control group) and **p < 0.01 (aged control vs BPE-treated aged group).

**Fig. 2.** Effect of BPE on histological changes in age-related periodontitis in rats.

(A) Histological analysis of periodontium using hematoxylin and eosin (H&E) staining (×200 magnification). Black arrow, cell infiltration; yellow arrow, alveolar bone resorption; A, alveolar bone; PDL, periodontal ligament; C, cementum; D, dentin. (B) Histological analysis of periodontium using immunohistochemical staining (×400 magnification). Yellow arrow, expressed proteins; A, alveolar bone; PDL, periodontal ligament. (C) Quantification of relative stained area in immunohistochemical images. The relative stained areas are expressed as the mean ± SD (% of control) of three rats per group. ##p < 0.01 (young control vs aged control group) and **p < 0.01 (aged control vs BPE-treated aged group).

**Fig. 3.** BPE-induced stimulation of BPE on osteoblast differentiation in alveolar bone of aged rats.

(A) The mRNA levels of ALP, COL1A1, OPG, and RANKL in alveolar bone were estimated
using RT-PCR; β-actin was used as the housekeeping gene. (B) The protein expression was examined by western blotting. All data are expressed as mean ± SD (% of control) of eight rats per group. \#\#p < 0.01 (young control vs aged control group) and \*\*p < 0.01 (aged control vs BPE-treated aged group).

**Fig. 4** Reversible effect of BPE on alveolar bone loss in age-related periodontitis in rats.

(A) Micro-computed tomography (CT) images. Yellow arrow, CEJ; green arrow, ABC. (B) Quantification of the CEJ-ABC distance. CEJ, cementoenamel junction; ABC, alveolar bone crest. The alveolar bone loss is expressed as the mean ± SD (mm) of five rats per group. \#\#p < 0.01 (young control vs aged control group) and \*\*p < 0.01 (aged control vs BPE-treated aged group).

**Fig. 5** Inhibitory effect of BPE on alveolar bone destruction in age-related periodontitis in rats.

Using reconstructed 3D images, (A) BV/TV, (B) Tb. Th, (C) Tb. Sp, and (D) BMD of alveolar bone covering the root of first molar were measured. BV, bone volume; TV, tissue volume; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; BMD, bone mineral density. Each parameter is expressed as the mean ± SD (% or μm) of five rats per group. \#p < 0.05, \#\#p < 0.01 (young control vs aged control group), \*p < 0.05, and \*\*p < 0.01 (aged control vs BPE-treated aged group).
### Table 1. Primer sequences used in RT-PCR analysis.

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Fig. 1.

(A) Products of various cell lines were subjected to Western blot analysis to quantify the expression of proteins related to inflammation and bone metabolism. The results indicate a significant increase in IL-1β, NF-κB, MMP-2, and MMP-8 in the aged group compared to the young group, with a further reduction observed in the aged + BPE group. 

(B) Similarly, the expression of these proteins was measured using RT-qPCR. The data showed a marked rise in IL-1β, NF-κB, MMP-2, and MMP-8 in the aged group, which was partially reversed in the aged + BPE group.

(C) The expression levels of NFATc1, c-Fos, TRAP, and Cathepsin K were also assessed by RT-qPCR. The results revealed a substantial rise in NFATc1 and c-Fos in the aged group, with a notable decrease in the aged + BPE group. TRAP and Cathepsin K expression showed a similar trend.

(D) The protein levels of these markers were further examined by Western blotting. The findings were consistent with the RT-qPCR results, highlighting the protective effects of BPE in the aged group.

In summary, these findings support the role of BPE in mitigating the detrimental effects of aging on bone health.
Fig. 2.

A

B

MMP-2

MMP-8

NFATc1

TRAP

ALP

COL1A1

C

Relative stained area (% of control)

MMP-2  MMP-8  NFATc1  TRAP  ALP  COL1A1

Young  Aged  Aged+BPE
Fig. 3.

A

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<td>β-Actin</td>
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B

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<th>Aged+BPE</th>
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<td>α-Tubulin</td>
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</tbody>
</table>
Fig. 4.

A

Young  Aged  Aged+BPE

B

![Bar chart showing CEJ-ABC (mm) for Young, Aged, and Aged+BPE groups.](image)

- Young
- Aged
- Aged+BPE

Legend:
- # #
- *
Fig. 5.