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Epidermal Growth Factor Reverses the Inhibitory Effects of the Bisphosphonate, Zoledronic Acid, on Human Oral Keratinocytes and Human Vascular Endothelial Cells *In Vitro* via the Epidermal Growth Factor Receptor (EGFR)/Akt/Phosphoinositide 3-Kinase (PI3K) Signaling Pathway

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background:

Medication-related osteonecrosis of the jaw (MRONJ) is due to the direct effects of drug toxicity and the effects on angiogenesis. The aims of this study were to evaluate the effects of treatment with the bisphosphonate, zoledronic acid, on human oral keratinocytes (HOKs) and human umbilical vein endothelial cells (HUVECs) *in vitro*, and whether epidermal growth factor (EGF) could alter these effects.

Material/Methods:

HOKs and HUVECs were incubated with zoledronic acid or EGF. Cell viability was assessed by the cell counting kit-8 (CCK-8), cell apoptosis was studied using Annexin-V conjugated to fluorescein isothiocyanate (FITC). Angiogenesis was studied by observing HUVEC tube formation and cell migrations using a transwell assay. A scratch wound assay investigated cell migration of HOKs. Western blot measured expression levels of phosphorylated epidermal growth factor receptor (EGFR), Akt, phosphoinositide 3-kinase (PI3K), the mechanistic target of rapamycin (mTOR), and endothelial nitric oxide synthase (eNOS).

Results:

Zoledronic acid treatment (5 $\mu\text{mol/L}$) significantly inhibited cell viability and cell migration of HOKs and HUVECs and angiogenesis of HUVECS ($P < 0.05$); EGF partially reversed these effects ($P < 0.05$). Zoledronic acid treatment of HOKs and HUVECs had no significant effects on apoptosis ($P > 0.05$), but significantly reduced expression levels of p-EGFR, p-Akt, p-PI3K, p-mTOR, and p-eNOS ($P < 0.05$); EGF partially reversed these effects and increased the expression levels ($P < 0.05$).

Conclusions:


EGF partially reversed the effects of the bisphosphonate, zoledronic acid, on HOKs and HUVECs *in vitro* via the EGFR/Akt/PI3K signaling pathway. Further studies are required to determine the effects of EGF on MRONJ including bisphosphonate-related osteonecrosis of the jaw.

MeSH Keywords:

Bisphosphonate-Associated Osteonecrosis of the Jaw • Endothelial Cells • Epidermal Growth Factor • Keratinocytes • Proto-Oncogene Proteins c-akt • Receptor, Epidermal Growth Factor

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Background

Bisphosphonate is used in the treatment of osteoporosis and has also been used for the treatment of malignancy, including prostate cancer and breast cancer [1,2]. Since 2003, bisphosphonate-related osteonecrosis of the jaw (BRONJ) has been increasingly reported [3]. In 2014, the American Association of Oral and Maxillofacial Surgeons (AAOMS) renamed BRONJ as medication-related osteonecrosis of the jaws (MRONJ) and is defined as the presence of a necrotic response or fistula formation in the bone of the maxillofacial region for at least eight weeks, in response to therapy with antiresorptive or anti-angiogenic agents, excluding radiotherapy [4]. The mechanism underlying the effects of MRONJ remains unclear [5].

Previously published studies have investigated the influence of bisphosphonate on osteoblasts and osteoclasts and the inhibition of bone remodeling [6,7]. Other mechanisms that have been studied for the effects of bisphosphonate have included an anti-angiogenetic effect by inhibiting endothelial progenitor cells and mature endothelial cells *in vitro* [8]. Zoledronic acid, a third-generation N-bisphosphonate, is also able to suppress pre-osteoclasts that release platelet-derived growth factor-BB (PDGF-BB), resulting in suppression of angiogenesis [9]. Lu et al. [10] demonstrated that zoledronic acid (Zoledronate) promoted Beclin-1-mediated autophagy to induce endothelial cell apoptosis. Other *in vitro* studies have examined the toxicity of bisphosphonate oral tissues. Pabst et al. [11] confirmed the negative influences of highly potent N-bisphosphonates on human oral keratinocytes (HOKs), and this effect could be significantly reversed by geranylgeraniol. Kalyan et al. [12] showed that the expression of genes regulating immune and barrier functions was downregulated in patients with MRONJ.

The EGFR/Akt/PI3K signaling pathway is highly correlated with cell proliferation, apoptosis, cell migration, and endothelial cell angiogenesis. Epidermal growth factor receptor (EGFR) is one of the receptor tyrosine kinases (TKs) and is an important driver of growth and differentiation of epithelial cells [13,14]. Extracellular ligands, such as epidermal growth factor (EGF) and transforming growth factor- α (TGF- α), can interact with the EGFR [13], resulting in the stimulation of Akt/PI3K and downstream molecules, including mTOR, eNOS, and the Bcl2-associated antagonist of cell death (BAD). The mammalian target of rapamycin (mTOR) is associated with cell proliferation, survival, migration, and vascular angiogenesis [15]. Also, endothelial nitric oxide synthase (eNOS) acts as a positive regulator of endothelial NOS, and NO can dilate blood vessels and activate the migration and proliferation of vascular cells [16]. BAD is a member of the pro-apoptosis bcl-2 family of proteins. Non-phosphorylated BAD can interact with Bcl-xl, an anti-apoptotic protein belonging to the Bcl-2 family, inducing cell apoptosis, whereas the phosphorylation of BAD results in the loss of pro-apoptotic activity [17].

Previously published studies have shown that the PI3K/Akt signaling pathway was correlated with the adverse impact of bisphosphonates [18,19]. Tang et al. [19] showed that the inhibitory effects of bisphosphonates on the HIF-1 α /VEGF axis were associated with the PI3K/Akt/mTOR signaling pathways. Inoue et al. [20] showed that alendronate inhibited the PI3K/Akt/NF κ B signaling pathway, which was correlated with the survival of an osteosarcoma cell line.

In view of these previous studies, it is possible to hypothesize that the EGFR/Akt/PI3K signaling pathway might have a role in the anti-angiogenetic effects of bisphosphonate and also in toxicity in the oral mucosa, because EGFR is expressed on the surface of a variety of cells, including epithelial cells and endothelial cells [21,22] (Table 1).

Therefore, the aims of this study were to evaluate the effects of treatment with the bisphosphonate, zoledronic acid, on human oral keratinocytes (HOKs) and human umbilical vein endothelial cells (HUVECs) *in vitro*, and whether epidermal growth factor (EGF) could alter these effects through the EGFR/Akt/PI3K signaling pathway.

Material and Methods

Reagents and antibodies

Zoledronic acid (Zoledronate) was purchased from Sigma-Aldrich (St Louis, MO, USA). Recombinant human epidermal growth factor (EGF) was obtained from Peprotech (Rocky Hill, NJ, USA). Antibodies for p-EGFR (Y845), p-Akt (ser473), p-PI3K (Tyr458), p-mTOR (ser-2448), p-BAD (ser136), and p-eNOS (ser1177) were obtained from Cell Signaling Technology (Boston, MA, USA). Primary antibodies for GAPDH, EGFR, Akt, PI3K, mTOR, BAD, and eNOS were obtained from Hunan Biotechnology (Hangzhou, China).

Cell culture and treatment of human umbilical vein endothelial cells (HUVECs) and human oral keratinocytes (HOKs) and the four treatment groups

Human umbilical vein endothelial cells (HUVECs) and human oral keratinocytes (HOKs) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (GE Healthcare, Hyclone, South Logan, UT, USA) with 10% fetal bovine serum (FBS) (GE Healthcare, Hyclone, South Logan, UT, USA) and 1% penicillin and streptomycin (Sigma-Aldrich, St Louis, MO, USA) in a 37°C humidified atmosphere containing 95% air and 5% CO₂.

After cell starvation for 24 h, four groups were studied, as follows: a control group of untreated HUVECs and HOKs; HUVECs

Table 1. A summary of previously published studies related to the present study.

Study	Title	Conclusions
Pabst et al. (2015) [11]	The influence of geranyl geraniol on human oral keratinocytes after bisphosphonate treatment: An <i>in vitro</i> study	Bisphosphonate treatment had negative effects on human oral keratinocytes (HOKs)
Ziebart et al. (2011) [8]	Bisphosphonates: restriction for vasculogenesis and angiogenesis: inhibition of cell function of endothelial progenitor cells and mature endothelial cells <i>in vitro</i>	The nitrogen-containing bisphosphonates, pamidronate, and zoledronate, had the greatest impact on human umbilical vein endothelial cells (HUVECs)
Kim et al. (2016) [18]	Zoledronic acid is an effective radiosensitizer in the treatment of osteosarcoma	Zoledronic acid decreased the phosphorylation of PI3K-Akt and MAPK pathway proteins
Tang et al. (2010) [19]	Bisphosphonates suppress insulin-like growth factor 1-induced angiogenesis via the HIF-1 α /VEGF signaling pathways in human breast cancer cells	Inhibitory effects of bisphosphonates on the HIF-1 α /VEGF axis are associated with PI3K/Akt/mTOR signaling pathways
Treda et al. (2016) [13]	EGFR activation resulted in cell death, independent of PI3K/Akt/mTOR in the AD293 cell human embryonic kidney cell line	PI3K, Akt, and mTOR were shown to be downstream proteins of the epidermal growth factor receptor (EGFR)
Bhattacharya et al. (2015) [21]	T11TS inhibits angiopoietin-1/Tie-2 signaling, EGFR activation, and the Raf/MEK/ERK pathway in brain endothelial cells, inhibiting angiogenesis in a glioma model	EGFR was expressed by endothelial cells, not only in epithelial cells

MAPK – mitogen-activated protein kinase; HIF-1 – hypoxia inducible factor-1; VEGF – vascular endothelial growth factor; T11TS – T11-target structure.

and HOKs treated with 5, 50, or 100 $\mu\text{mol/L}$ of zoledronic acid; HUVECs and HOKs treated with 10 ng/L of epidermal growth factor (EGF), and; HUVECs and HOKs treated with zoledronic acid combined with EGF (ZA + EGF). The range of concentrations of zoledronic acid was selected based on previous *in vivo* findings of the plasma levels shortly after zoledronic acid infusion, measured at nearly 5 $\mu\text{mol/L}$ [23]. The concentration of EGF was chosen according to previously published recommendations [24]. Also, according to the findings of Shen et al. [25], 10 ng/ml EGF was the maximum effective concentration for stimulating the proliferation of HUVECs.

Cell viability using the cell counting kit-8 (CCK-8) assay *in vitro*

Cell viability and cell proliferation analysis were performed using the cell counting kit-8 (CCK-8) assay (Dojindo, Gaithersburg, MD, USA), as previously described [26]. HUVECs or HOKs were plated in 96-well plates (5,000 cells/well) and incubated overnight. After starvation for 24 h, HUVECs were cultured as described above for 24–48 h [8,10]. HOKs were cultured for 48–72 h with the same stimulation conditions [11]. Then, the CCK-8 solution (10 μl) was added to each well, followed by incubation for 1–4 h at 37°C. The absorbance at 450 nm was measured to analyze the cell viability.

Cell apoptosis using immunofluorescence and flow cytometry

Annexin-V conjugated to fluorescein isothiocyanate (FITC) was used in a detection kit (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, China), as previously described [27]. Briefly, HUVECs or HOKs were seeded at a density of 4×10^5 cells/ml and incubated overnight. After starvation for 24 h, cells were stimulated with 0, 5, 50, or 100 $\mu\text{mol/L}$ of zoledronic acid. HUVECs were collected after 48 h and HOKs were collected after 72 h. The percentage of apoptotic cells was determined using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Wound scratch assay

HOKs were seeded into 6-well plates at a density of 5×10^5 cells/well. After 24 h, a scratch wound was performed in each well using a sterile pipette tip. The size of the scratch wound was measured microscopically and taken as the 100% value. After starvation for 24 h, the cells were treated, as described above. After 72 h, the size of the scratch wounds was measured, as previously described [28].

Angiogenesis assay

HUVECs were seeded and incubated overnight. After starvation for 24 h, the cells were treated, as described above. Matrigel

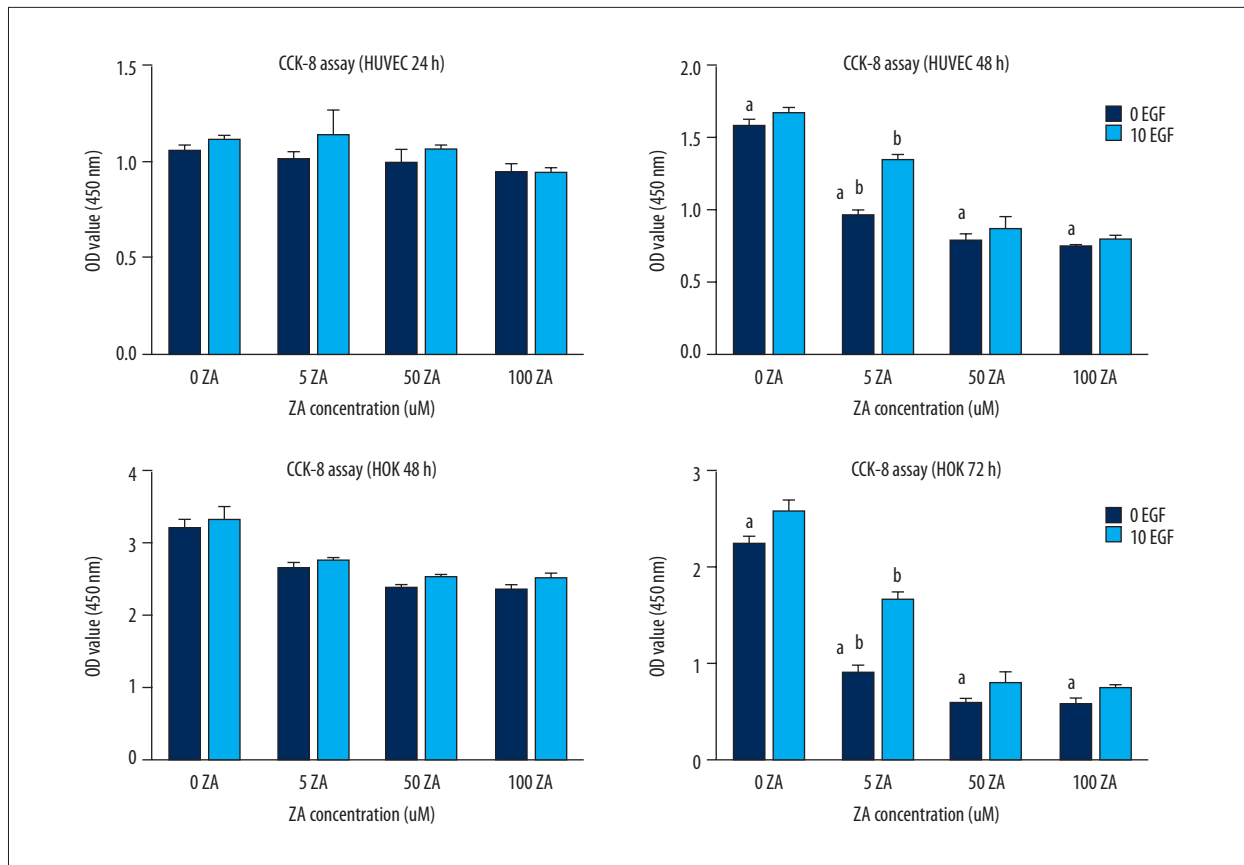


Figure 1. The effects of zoledronic acid (ZA) and epidermal growth factor (EGF) on the cell viability of human umbilical vein endothelial cells (HUVECs) and human oral keratinocytes (HOKs) measured by the cell counting kit-8 (CCK-8) assay. The results are presented as the average \pm standard deviation (SD) of three independent experiments. a: Zoledronic acid treatment showed significant effects on human umbilical vein endothelial cells (HUVECs) after 48 h at the concentrations of 5, 50, and 100 $\mu\text{mol/L}$. Zoledronic acid significantly affected HOKs after 72 h at these concentrations ($P < 0.05$). b: Treatment with epidermal growth factor (EGF) had a significant positive effect when cells were stimulated with 5 $\mu\text{mol/L}$ of zoledronic acid ($P < 0.05$).

(BD Biosciences, San Jose, USA) was thawed at 4°C overnight and used to coat a 96-well plate, which was then incubated at room temperature for 30 minutes on the gel. After 48 h, HUVECs were plated on the Matrigel (3×10^4 cells/well). After incubation for 6 h, the newly-formed vascular tube networks were photographed. The number of branching points was counted in a minimum of three fields per image [29].

Cell migration assay

HUVECs were seeded and incubated overnight. After starvation for 24 h, the cells were treated, as described above. After 48 h, HUVECs (10^5 cells/ml) were cultured in the upper chambers of 24-well transwell chambers with an 8-mm pore size (Costar, Lowell, MA, USA). The lower chambers were supplemented with DMEM containing 10% fetal bovine serum (FBS). After 24 h of incubation at 37°C, the upper chambers were washed with PBS, fixed with paraformaldehyde, and then stained with crystal violet. HUVECs remaining on the upper surface of the

transwell membrane were removed. The number of cells that migrated to the lower part of the chamber was evaluated under a microscope at a magnification of $\times 200$, and five fields were randomly chosen [30].

Western blot

HOKs and HUVECs were treated, as described above. Total protein from the cells was extracted using RIPA lysis buffer (Beyotime Biotech, Shanghai, China), containing 1 mM the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), 10 mM PhosStop phosphatase inhibitor (Sigma-Aldrich, St Louis, MO, USA), and 5 mM of protease inhibitors. The total protein concentration was analyzed by the bicinchoninic acid (BCA) protein assay (Beyotime Biotech, Shanghai, China). The proteins were loaded onto 6–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels at 60–90 V and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (0.22 μm) (Millipore, Burlington, MA, USA) at 200 mA.

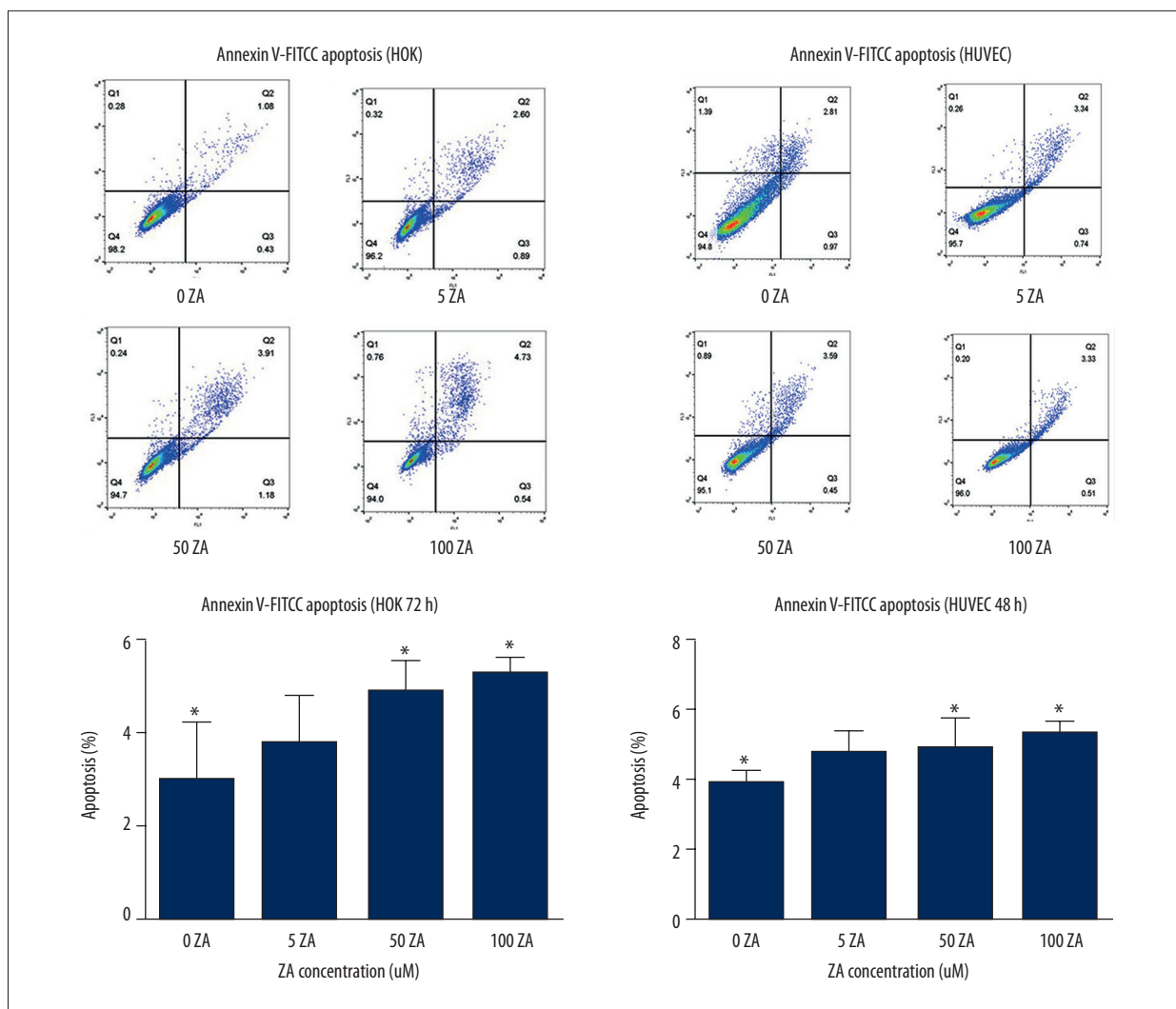


Figure 2. The effects of zoledronic acid on apoptosis in human umbilical vein endothelial cells (HUVECs) and human oral keratinocytes (HOKs) was measured by an Annexin-V conjugated to fluorescein isothiocyanate (FITC) apoptosis assay. The results are presented as the average ± standard deviation (SD) of three independent experiments. There was a significant difference between the control group and the human umbilical vein endothelial cells (HUVECs) treated with 100 μmol/L of zoledronic acid, and a significant difference between the control group and the human oral keratinocytes (HOKs) treated with 50 and 100 μmol/L of zoledronic acid ($P < 0.05$). However, even the highest concentration (100 μmol/L) of zoledronic acid resulted in less than 10% of cell apoptosis. * $P < 0.05$ zoledronic acid-treated vs. control.

After blocking for 1 h with 5% bovine serum albumin (BSA) in TBST, the membranes were incubated with primary antibodies against total and phosphorylated EGFR, Akt, PI3K, mTOR, BAD, and eNOS at 4°C overnight. GAPDH served as the internal control. The membranes were then washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1: 1,000) (Cell Signaling Technology, Danvers, MA, USA) for 1 h. After washing again with TBST, the proteins were finally detected by SuperSignal enhanced chemiluminescence (ECL) substrate, (Pierce, Rockford IL, USA) [5].

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) to explore the relationships between the different samples. Fisher's test of least significant difference (LSD) was used for post hoc analysis. A p-value < 0.05 was considered to represent statistical significance.

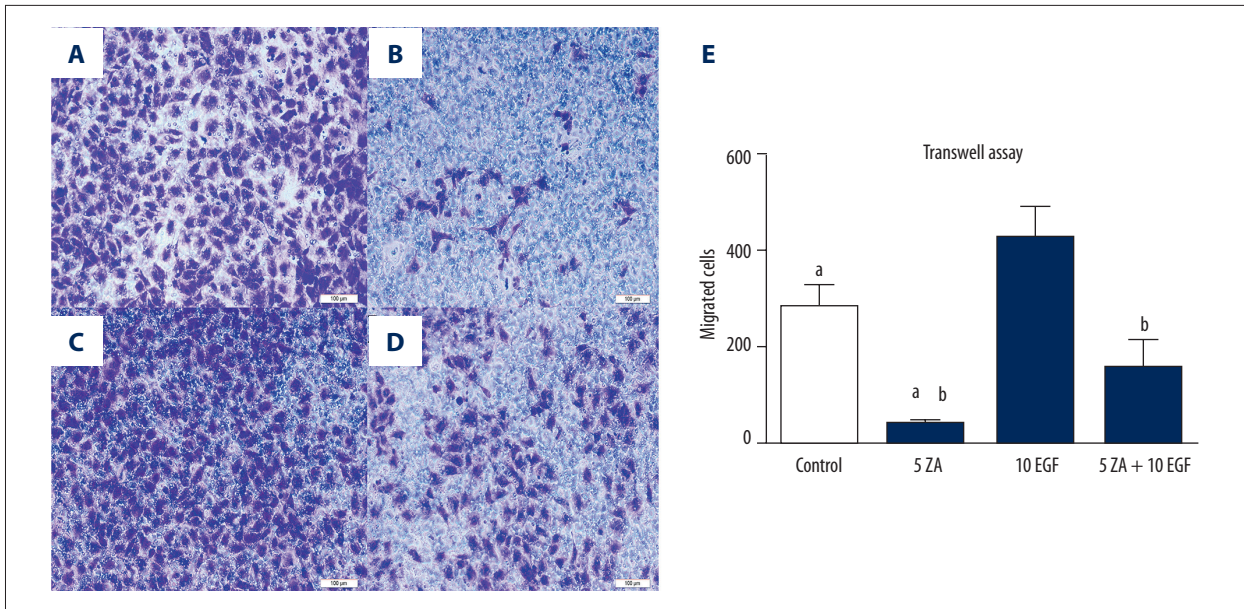


Figure 3. The effects of zoledronic acid and epidermal growth factor (EGF) on the migration ability of human umbilical vein endothelial cells (HUVECs) were measured by a transwell assay. (A) Photomicrograph of the transwell assay of human umbilical vein endothelial cells (HUVECs) treated with Dulbecco's modified Eagle's medium (DMEM) without zoledronic acid or epidermal growth factor (EGF) treatment for 48 h. (B) Photomicrograph of the transwell assay of HUVECs, treated with 5 μmol/L zoledronic acid for 48 h. (C) Photomicrograph of the transwell assay of HUVECs, treated with 10 ng/L EGF for 48 h. (D) Photomicrograph of the transwell assay of HUVECs, treated with 5 μmol/L zoledronic acid and 10 ng/L EGF for 48 h. The results are presented as the average ± standard deviation (SD) of three independent experiments. (E) a: Zoledronic significantly inhibited the migration ability at a concentration of 5 μmol/L ($P < 0.05$). b: EGF could partially reverse the inhibitory effects of zoledronic acid ($P < 0.05$).

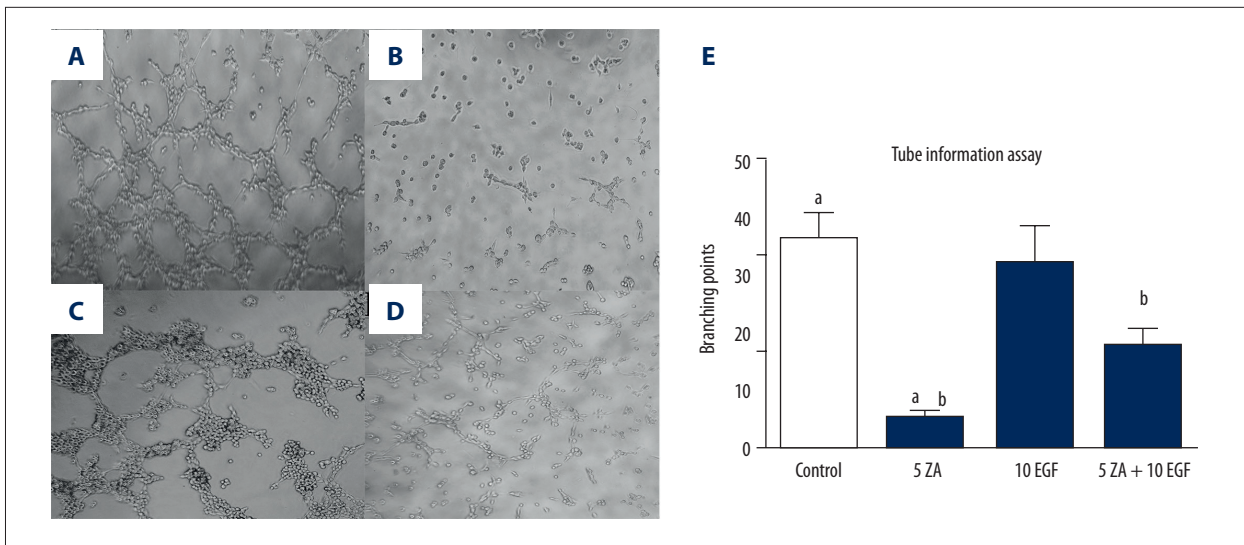


Figure 4. The effects of treatment with zoledronic acid (ZA) and epidermal growth factor (EGF) on angiogenesis of human umbilical vein endothelial cells (HUVECs) were measured using an assay for the formation of vascular tubes. (A) Human umbilical vein endothelial cells (HUVECs) were treated with Dulbecco's modified Eagle's medium (DMEM) without zoledronic acid or epidermal growth factor (EGF) for 48 h. (B) HUVECs were treated with 5 μmol/L of zoledronic acid for 48 h. (C) HUVECs were treated with 10 ng/L of EGF for 48 h. (D) HUVECs were treated with 5 μmol/L of zoledronic acid and 10 ng/L EGF for 48 h. Results are presented as the average ±SD of three independent experiments. (E) a: Zoledronic acid significantly inhibited angiogenesis at a concentration of 5 μmol/L ($P < 0.05$). b: EGF treatment could partially reverse the negative effects of zoledronic acid ($P < 0.05$).

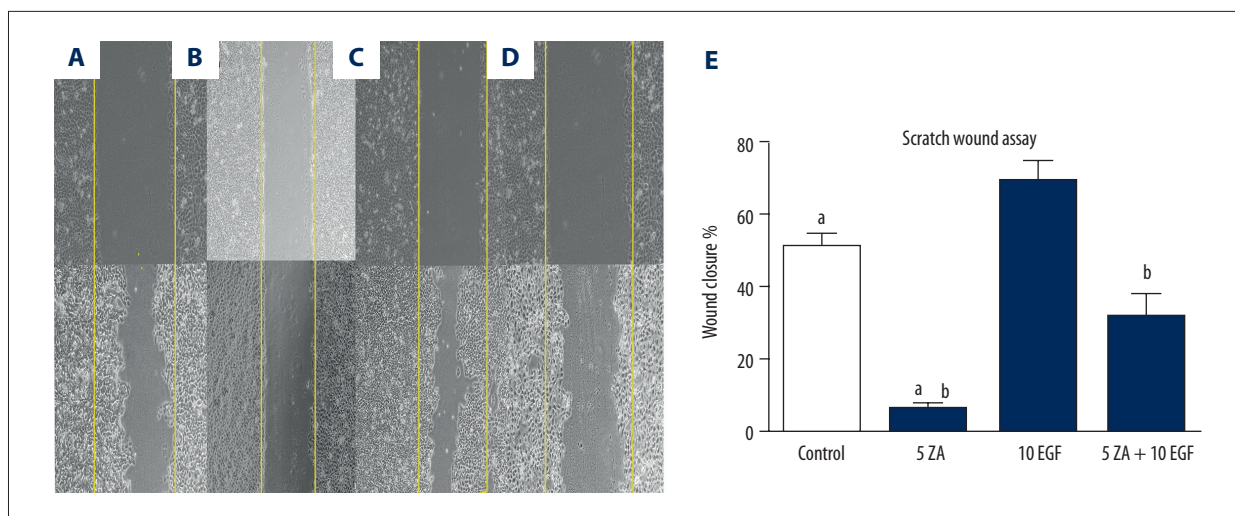


Figure 5. The effects of treatment with zoledronic acid (ZA) and epidermal growth factor (EGF) on the migration ability of human oral keratinocytes (HOKs) were measured by a scratch wound assay. (A) Human oral keratinocytes (HOKs) were treated with Dulbecco's modified Eagle's medium (DMEM) without zoledronic acid or EGF for 72 h. (B) HOKs were treated with 5 $\mu\text{mol/L}$ zoledronic acid for 72 h. (C) HOKs were treated with 10 ng/L EGF for 72 h. (D) HOKs were treated with 5 $\mu\text{mol/L}$ of zoledronic acid and 10 ng/L EGF for 72 h. After 72 h, the sizes of the scratch wounds were measured. The results are presented as the average \pm standard deviation (SD) of three independent experiments. (E) a: Zoledronic acid treatment significantly inhibited the migration ability of HOKs at a concentration of 5 $\mu\text{mol/L}$ ($P < 0.05$). b: EGF could partially reverse the negative effects of zoledronic acid ($P < 0.05$).

Results

The effects of zoledronic acid and epidermal growth factor (EGF) on the proliferation of human oral keratinocytes (HOKs) and human umbilical vein endothelial cells (HUVECs) *in vitro*

There was no significant difference when human umbilical vein endothelial cells (HUVECs) were stimulated with zoledronic acid for 24 h compared with the control group ($P > 0.05$). Whereas, the cells viability of HUVECs was reduced in a dose-dependent manner following treatment with zoledronic acid for 48 h ($P < 0.05$), but no difference was found between 50 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$ of zoledronic acid. The cell viability of human oral keratinocytes (HOKs) was significantly inhibited by 50% following treatment with zoledronic acid for 72 hours, at all three dose concentrations ($P < 0.05$). Also, no significant difference was found between 50 $\mu\text{mol/L}$ of zoledronic acid and 100 $\mu\text{mol/L}$ of zoledronic acid.

Treatment with EGF exerted a significant positive effect when cells were stimulated with 5 $\mu\text{mol/L}$ of zoledronic acid ($P < 0.05$) but had little effect on HUVECs and HOKs with at doses of 50 $\mu\text{mol/L}$ or 100 $\mu\text{mol/L}$ for zoledronic acid ($P > 0.05$) (Figure 1).

The effects of zoledronic acid on the apoptosis of both HUVECs and HOKs *in vitro*

In the present study, zoledronic acid had a small negative influence on apoptosis in HUVECs and HOKs, although there was a significant difference between the control group and 100 $\mu\text{mol/L}$ zoledronic acid ($P < 0.05$) in HUVECs. A significant difference was also found between the control and the HOK cell groups treated with 50 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$ of zoledronic acid. However, even the highest concentration (100 $\mu\text{mol/L}$) of zoledronic acid resulted in less than 10% of cell apoptosis in HUVEC and HOK cells (Figure 2).

Effects of zoledronic acid and epidermal growth factor (EGFR) on cell migration and angiogenesis of HUVECs *in vitro*

The migration and angiogenesis of HUVECs were studied when incubated with zoledronic acid and EGF. Zoledronic acid inhibited both cell migration and angiogenesis at a concentration of 5 $\mu\text{mol/L}$. There were significant differences between the control group and the zoledronic acid-treated groups ($P < 0.05$). Compared with the zoledronic acid-treated group, the combination of EGF (10 ng/L) and zoledronic acid (5 $\mu\text{mol/L}$) could partially reverse the properties of cell migration and angiogenesis ($P < 0.05$). However, the migration and angiogenesis in the zoledronic acid and EGF-treated group (ZA + EGF) were

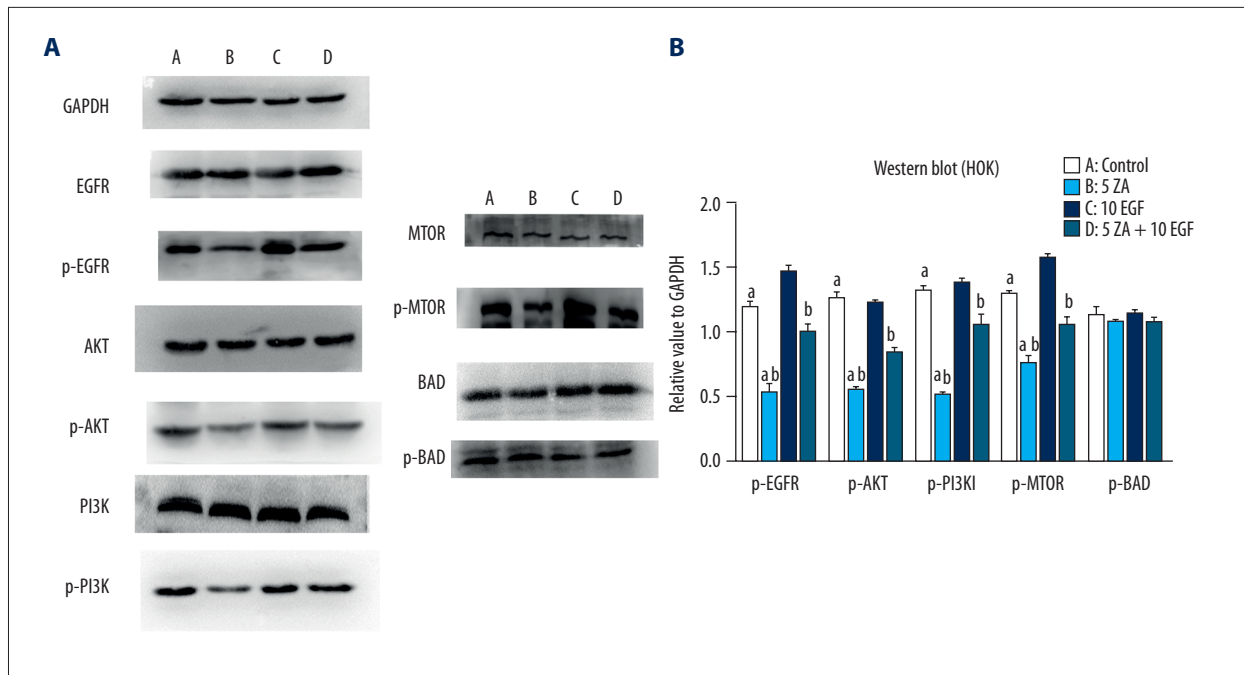


Figure 6. Western blot determined protein levels in human oral keratinocytes (HOKs). (A) Human oral keratinocytes (HOK) cells were treated with Dulbecco's modified Eagle's medium (DMEM) without zoledronic acid or epidermal growth factor (EGF) for 72 h. B) HOK cells were treated with 5 $\mu\text{mol/L}$ of zoledronic acid for 72 h. C) HOK cells were treated with 10 ng/L EGF for 72 h. D) HOK cells were treated with 5 $\mu\text{mol/L}$ of zoledronic acid and 10 ng/L EGF for 72 h. The results are presented as the mean \pm standard deviation (SD) of three independent experiments. (B) a: Zoledronic acid significantly decreased the expression levels of p-EGFR, p-Akt, p-PI3K, and p-mTOR in HOKs at a concentration of 5 $\mu\text{mol/L}$ ($P < 0.05$). b: EGF could partially reverse the negative effects of zoledronic acid ($P < 0.05$).

significantly lower compared with the control and EGF-treated groups ($P < 0.05$) (Figures 3, 4).

The effects of zoledronic acid and epidermal growth factor on cell migration of HOKs *in vitro*

Cell migration of HOKs was evaluated by a scratch wound assay. Treatment with zoledronic acid significantly suppressed the migration of HOKs compared with the control group. ($P < 0.05$), which was partially reversed following treatment with EGF. There was a significant difference between the zoledronic acid-treated group and the zoledronic acid and EGF-treated group (ZA + EGF) groups ($P < 0.05$). Similar to the above assays, the migration ability of HOKs in the ZA + EGF group was significantly lower compared with the control and EGF groups ($P < 0.05$) (Figure 5).

The effects of zoledronic acid and epidermal growth factor on the EGFR/Akt/PI3K signaling pathway in HOKs and HUVECs *in vitro*

Western blot for the levels of p-EGFR, p-Akt, and p-PI3K in HOKs and HUVECs in the zoledronic acid-treated group were expressed at lower levels compared with the control and EGF

groups ($P < 0.05$). Also, the levels of downstream proteins, including p-mTOR and p-eNOS, were also significantly decreased in the zoledronic acid-treated group compared with the control and EGF groups. However, zoledronic acid did not significantly decrease p-BAD levels, and there were no significant differences among all of the groups, which was consistent with the results of the apoptosis assay. In the zoledronic acid and EGF-treated group (ZA + EGF), the relative values of p-EGFR, p-Akt, p-PI3K, p-mTOR, and p-eNOS were significantly increased compared with those of the zoledronic acid-treated group ($P < 0.05$) (Figures 6, 7).

Discussion

The findings of this *in vitro* study on the effects of treatment with the bisphosphonate, zoledronic acid, on human oral keratinocytes (HOKs) and human umbilical vein endothelial cells (HUVECs), showed a significant negative effect of zoledronic acid on cell viability, cell migration, and angiogenesis. However, these negative effects could be partially reversed by treatment with epidermal growth factor (EGF). with the effects mediated by the EGFR/Akt/PI3K signaling pathway.

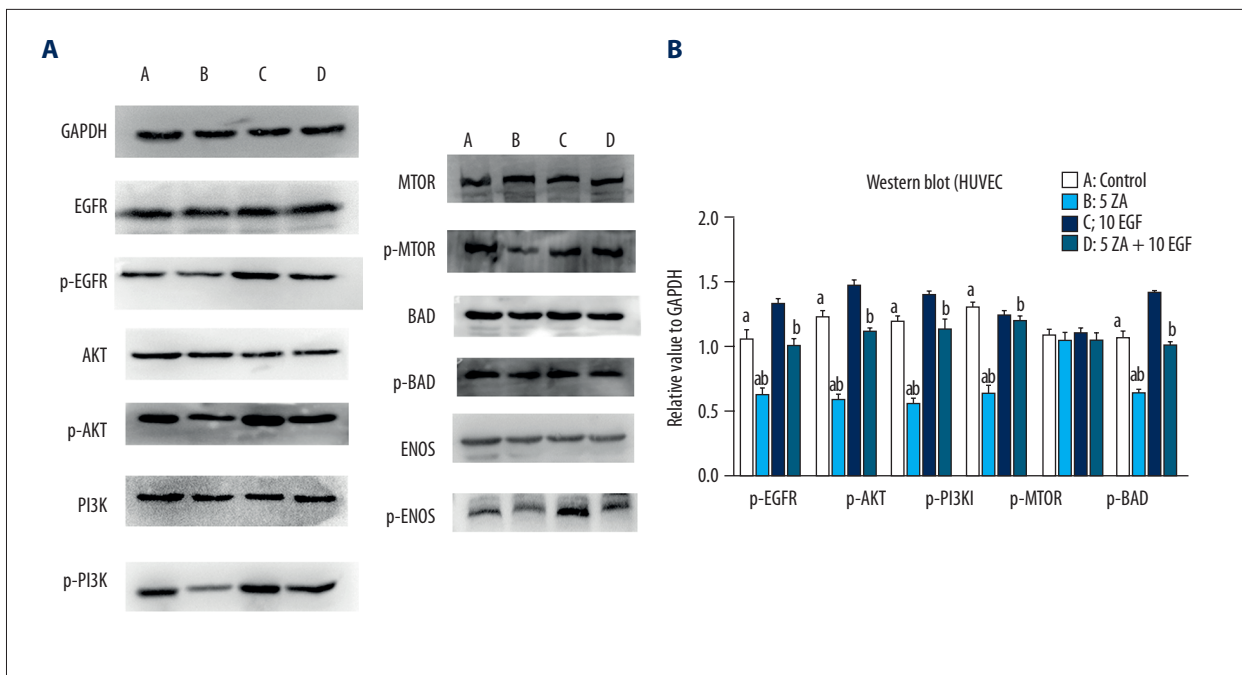


Figure 7. Western blot determined protein levels in human umbilical vein endothelial cells (HUVECs). **(A)** Human umbilical vein endothelial cells (HUVECs) were treated with Dulbecco's modified Eagle's medium (DMEM) without zoledronic acid or epidermal growth factor (EGF) for 48 h. **(B)** HUVECs were treated with 5 $\mu\text{mol/L}$ zoledronic acid for 48 h. **(C)** HUVECs were treated with 10 ng/L EGF for 48 h. **(D)** HUVECs were treated with 5 $\mu\text{mol/L}$ of zoledronic acid and 10 ng/L EGF for 48 h. The results are presented as the mean \pm standard deviation (SD) of three independent experiments. **(B)** a: Zoledronic acid significantly decreased the expression levels of p-EGFR, p-Akt, p-PI3K, p-mTOR, and p-eNOS in HUVECs at a concentration of 5 $\mu\text{mol/L}$ ($P < 0.05$). b: EGF could partially reverse the effects of zoledronic acid ($P < 0.05$).

This study confirmed the potent inhibitory effects of zoledronic acid on the viability of HOKs and HUVECs at concentrations of 5, 50, and 100 $\mu\text{mol/L}$, which is similar to previous reports [8,11]. The HOK proliferation ability was reduced by over 50% with zoledronic acid treatment at 72 h in culture and at a concentration of 5 $\mu\text{mol/L}$ ($P < 0.05$), while 50 and 100 $\mu\text{mol/L}$ concentrations of zoledronic acid could inhibited almost 70% of the proliferation ability. This result is higher than those reported by most previous studies, in which the proliferation abilities were reduced to 60–80% at a concentration of 5 $\mu\text{mol/L}$ of zoledronic acid [31,32]. This difference might be due to the cells in this experiment having been starved for 24 h before stimulation. After starvation, cells were maintained under the same conditions, allowing zoledronic acid to fully exert its effect on cells. In addition, few studies have examined the influence of zoledronic acid on HOKs. Pabst et al. [11] reported that the HOK proliferation rate was reduced to nearly 50% in their study, which supports the findings of the present study.

However, there appears to be no direct interaction between the application of zoledronic acid and apoptosis in either HOKs or HUVECs. Even the highest concentration (100 $\mu\text{mol/L}$) of zoledronic acid could induce less than 10% apoptosis, although there was a statistical difference between the control

group and the group treated with 100 $\mu\text{mol/L}$ of zoledronic acid. This result is in contrast to the findings of several reports. Lu et al. [10] found that zoledronic acid could induce apoptosis and autophagy in HUVECs and that the inhibition of autophagy attenuated zoledronic acid-induced apoptosis. Huang et al. [31] showed that zoledronic acid treatment induced apoptosis in osteoblasts in a dose- and time-dependent manner. One reason for this discrepancy might be that methods for assaying apoptosis were different between studies. Other kits were selected in previous experiments, rather than the Annexin-V conjugated to fluorescein isothiocyanate (FITC) apoptosis detection kit [31]. Tai et al. [33] reported that zoledronic acid could activate the p38 MAPK signaling pathway, which would increase the expression of anti-apoptotic Bcl-XL and increase cell survival, which could also explain why zoledronic acid induced less than 10% apoptosis in the present study. Considering that zoledronic acid had little impact on the apoptosis of HOKs and HUVECs, this study did not assay the effects of adding EGF on apoptosis.

The migration ability of HOKs and HUVECs and the angiogenesis ability of HUVECs were also suppressed by zoledronic acid treatment. According to the wound scratch assay, there was little wound healing in HOKs treated with 5 $\mu\text{mol/L}$ of zoledronic

acid for 72 h, and a significant difference was found between the zoledronic acid and control groups. In the tube formation and transwell assays, 5 $\mu\text{mol/L}$ of zoledronic acid strongly affected the migration and angiogenesis abilities of HUVECs, in contrast with the control group. These results are in accordance with those reported by published studies [11,34]. The concentration of 5 $\mu\text{mol/L}$ of zoledronic acid was selected because it is the *in vivo* concentration found in plasma shortly after zoledronic acid infusion [23], and 5 $\mu\text{mol/L}$ zoledronic acid had a visible impact on HOKs and HUVECs in the viability assay described above.

To examine whether the EGFR/PI3K/Akt signaling pathway was associated with the negative effect of bisphosphonate and whether EGF can reduce the effects of bisphosphonate via the EGFR/PI3K/Akt signaling pathway, Western blot analysis was used to examine the expression of proteins of interest. Based on the results, zoledronic acid treatment downregulated p-Akt and p-PI3K in HOKs and HUVECs. Also, zoledronic acid decreased the expression levels of downstream phosphorylated proteins. The reduced expression level of p-mTOR observed in this study is in accordance with the aforementioned findings that zoledronic acid inhibited proliferation and the migration ability. p-EGFR was also obviously decreased in HOKs. These results suggest that zoledronic acid might inhibit HOKs through the Akt/PI3K signaling pathway after zoledronic acid interacts with the EGFR. In HUVECs, the expression level of p-EGFR was also decreased. This result could be because there are also epidermal growth factor receptors on the surface of endothelial cells, and the activation of EGFRs are required for vascular remodeling and angiogenesis [21,22]. Zoledronic acid also downregulated p-eNOS, which is associated with the angiogenesis ability of HUVECs [35]. This finding supports those from the tube formation assay. The level of p-BAD was not affected by zoledronic acid in both HOKs and HUVECs, which could explain why the apoptosis assay did not show much difference between the zoledronic acid and control groups.

The present study analyzed protein expression levels in the presence of EGF and zoledronic acid simultaneously. Consequently, the expression levels of p-EGFR, p-Akt, p-PI3K/p-mTOR, and p-eNOS were partially elevated by EGF. Although the expression levels were still lower than those in the control and EGF groups, they were significantly higher than those in the zoledronic acid only group. This result is in accordance with the results of the proliferation, migration, and angiogenesis assays described above. When HOKs and HUVECs were treated with both EGF and zoledronic acid, the negative effects of zoledronic acid could be partially reversed by EGF. The outcome of the Western blot analysis illustrates the direct toxicity of bisphosphonate to the oral mucosa, and the anti-angiogenic effects of bisphosphonate on the endothelium can be

partially neutralized by EGF through the EGFR/Akt/PI3K signaling pathway.

This study aimed to investigate neutralization of the *in vitro* cytotoxic effects of bisphosphonates on oral keratinocytes, as well as the endothelium, based on the theory of an anti-angiogenesis effect. After a series of assays it was found that EGF could attenuate the negative effects of bisphosphonate, and this might be due to the activation of the EGFR/Akt/PI3K signaling pathway. After the systemic or topical application of bisphosphonates, direct contact with the mucosa can lead to cytotoxic effects for the oral mucosa, and high systemic concentrations in the circulating blood can cause angiogenesis inhibition, which can eventually develop into medication-related osteonecrosis of the jaw (MRONJ). Based on these experiments, EGF might represent an option for the prevention and treatment of MRONJ. EGF could be administered to patients by topical delivery systems, such as collagen membranes or mouth rinses [11,36]. Further research should investigate the application of EGF to MRONJ lesions in animal models.

However, several practical considerations remain to be clarified and require further study. In the present study, EGF could only partially neutralize the adverse effects of low-level bisphosphonates. Therefore, other agents should be evaluated to amplify the positive effect of EGF, such as transforming growth factor- α (TGF- α). In addition, the optimal therapeutic EGF concentrations should be confirmed, as this study only analyzed the effects of EGF at a concentration of 10 ng/L. Furthermore, this *in vitro* study used zoledronic acid, the bisphosphonate most frequently associated with the development of MRONJ. Other bisphosphonates, such as pamidronate and clodronate, and other agents related to MRONJ, including bevacizumab [37], denosumab [38], and sunitinib [39], should be evaluated in further studies.

Conclusions

The present study explored whether the effects of the bisphosphonate, zoledronic acid, on human oral keratinocytes (HOKs) and human umbilical vein endothelial cells (HUVECs) could be partially neutralized with the treatment of epidermal growth factor (EGF) *in vitro*. EGF reversed the effects of bisphosphonates via the EGFR/Akt/PI3K signaling pathway, which supports that further *in vivo* studies in animal models are required to investigate the effects of EGF treatment on medication-related osteonecrosis of the jaw (MRONJ).

Conflict of interest

None.

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