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Original Article

Phillyrin attenuates high glucose-induced ferroptosis in gingival cells through the activation of 5′ adenosine monophosphate-activated protein kinase signaling

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KEYWORDS
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Abstract *Background/purpose:* Delayed wound healing in diabetes involves multiple factors, with ferroptosis being one of the key mechanisms. Ferroptosis, marked by iron accumulation and lipid peroxidation, has been implicated in the pathogenesis of various diseases; however, its role in oral wound healing remains unclear. Here, we evaluated its involvement in impaired wound healing, as well as the therapeutic effects of phillyrin, a natural lignan with the potential to ameliorate oxidative stress and diabetic complications.
Materials and methods: Smulow–Glickman (S-G) gingival epithelial cells were exposed to high-glucose conditions, followed by treatment with phillyrin. PrestBlue assay and 2 well culture-insert were utilized to assess cell viability and wound healing capacity, respectively. Lipid peroxidation and oxidative stress were determined using commercial assay kits to measure malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (8-OHdG), respectively. Intracellular Fe^{2+} was measured using FerroOrange fluorescent probe.
Results: High glucose, like the ferroptosis inducer erastin, reduced cell viability and wound-healing capacity, whereas ferroptosis inhibitor Ferrostatin-1 or phillyrin treatment conferred comparable protection. Also, we showed that the characteristics of ferroptosis were elevated

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in high glucose-treated cells, while phillyrin administration reduced these features in a dose-dependent manner. More importantly, we found that this effect may result from phillyrin restoring high-glucose-suppressed 5′ adenosine monophosphate-activated protein kinase (AMPK) phosphorylation, thereby regulating anti-ferroptotic factors and enhancing cellular resilience against ferroptosis.
Conclusion: These findings suggest that phillyrin may improve impaired oral wound healing in diabetes through the activation of AMPK and upregulation of anti-ferroptotic mechanisms, underscoring its promise in the treatment of complications associated with diabetes.

Introduction

Diabetes constitutes a major global health burden, being a leading cause of mortality and disability, with its rising prevalence largely attributed to increasing obesity driven by multifactorial causes.[1] It has been shown that the impaired glucose metabolism in diabetic patients leads to hyperglycaemia and delayed wound healing, with these wounds presenting considerable healing challenges owing to impaired angiogenesis, persistent inflammation, elevated reactive oxygen species (ROS), and susceptibility to bacterial infections.[2,3] The stalled chronic wounds, as one of the most debilitating sequelae of diabetes, have prompted substantial efforts to address the multifaceted challenges of wound healing over the past few years. Among these concerns, diabetic wound healing in soft oral tissues has also received increasing attention.[4,5] It has been reported that injury-related infections in the oral cavity have been associated with an increased risk of bacteremia, as previously demonstrated in studies of dental interventions such as periodontal surgery and tooth extraction.[6] Accordingly, it is of particular importance to investigate strategies for reducing or preventing delayed wound healing and oral infections, as these conditions may contribute to systemic infections and undermine the body's intrinsic capacity for repair.

Ferroptosis represents a distinct form of regulated cell death, driven by the accumulation of iron-dependent lipid peroxides.[7] The peroxidation of polyunsaturated phospholipids (PL-PUFAs), which constitute the lipid bilayers of cellular membranes, is necessary for the execution of ferroptosis. To date, several pathways have been identified to eliminate peroxidized PL-PUFAs, including the glutathione peroxidase 4 (GPX4)-glutathione (GSH) axis and the ferroptosis suppressor protein 1 (FSP1)-coenzyme Q_{10} (CoQ_{10}) axis. It has been shown that GPX4 inhibits ferroptosis by utilizing GSH to reduce phospholipid hydroperoxides to lipid alcohols. System xc^- (composed of xCT/SLC7A11 and SLC3A2/CD98hc), a cystine-glutamate antiporter, regulates intracellular cysteine and GSH levels, thereby indirectly modulating GPX4 activity.[8] On the other hand, FSP1 functions as an oxidoreductase that reduces coenzyme Q10 (CoQ), a lipophilic antioxidant that prevents lipid peroxide propagation.[9] Additionally, nuclear factor erythroid 2-related factor 2 (Nrf2) has been suggested to regulate GPX4 and FSP1,[10,11] thereby rendering it essential for mitigating lipid peroxidation and ferroptosis. Emerging evidence has suggested that ferroptosis may be implicated in diabetic wound healing. One of the recent studies has shown that high glucose exposure increases ROS, lipid peroxidation, and ferroptosis markers in fibroblasts and endothelial cells, thereby reducing their survival and migration.[12] Hyperglycemia further exacerbates intracellular ROS production, leading to persistent oxidative stress and lipid peroxidation. This redox imbalance disrupts cellular homeostasis and impairs the wound-healing trajectory.[13] As such, whether ferroptosis influences impaired oral wound healing in diabetes and how it can be targeted for improvement remain important topics for investigation.

In the present study, we explored the possible involvement of ferroptosis in mediating the deleterious consequences of high glucose on oral wound healing. Moreover, we assessed the effect of phillyrin, a major lignan extracted from *Forsythia suspensa* (Thunb.) Vahl, on the regulation of ferroptosis and the improvement of delayed wound regeneration, given its reported anti-diabetic properties[14,15] and regulatory effects on ferroptosis.[16]

Materials and methods

Cell culture

The Smulow-Glickman (S-G) gingival epithelial cell line utilized in this study was initially established from human attached gingival tissue.[17] Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; 11965092, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37 °C in a humidified atmosphere containing 5 % CO_2. The culture medium was replaced every 2–3 days. For high glucose treatment, cells were exposed to 70 mmol/L D-glucose or 1 μM erastin[18] (T1765, TargetMol, Wellesley Hills, MA, USA) for 24 h, followed by treatment with 2 μM phillyrin[15] (PHN; HY-N0482, MedChemExpress, Monmouth Junction, NJ, USA) or 5 μM Ferrostatin-1(Fer-1)[19] (T6500, TargetMol) for an additional 24 h. Compound C[20] (Com C; 3 μM), obtained from MedChemExpress (HY-13418A), was used as an 5′ adenosine monophosphate-activated protein kinase (AMPK) inhibitor.

PrestoBlue assay

Cells were seeded in each well and exposed to high-glucose or erastin conditions for 24 h. The high-glucose group was then divided into three subgroups, no treatment, Fer-1, or phillyrin, for an additional 24 or 48 h. Cell viability was assessed after 24 or 48 h of treatment using the PrestoBlue™ Cell Viability Reagent (A13262, Invitrogen, Carlsbad, CA, USA) in a 96-well plate format according to the manufacturer's protocol. Following the addition of 100 μL of 10X PrestoBlue™ reagent, absorbance was measured at 570/600 nm using an iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA).

Wound healing assay

A cell suspension was seeded into both chambers of a silicone Culture-Insert 2 Well in a μ-Dish 35 mm (ibidi GmbH, Martinsried, Germany), allowing the cells to adhere and spread on the substrate, with a 500 μm gap separating the two chambers. After removal of the culture insert, the first wound healing image was captured (designated as 0 h). Cells were then incubated under high-glucose or erastin conditions for 24 h, and the second wound healing image was recorded (24 h). The high-glucose group was subsequently left untreated or treated with Fer-1 or phillyrin for an additional 24 h, followed by the third wound healing image (48 h). The wound area was analyzed by tracing the cell free are in the captured images using ImageJ software (NIH, Bethesda, MD, USA).

Lipid peroxidation, intracellular iron and 8-hydroxy-2-deoxyguanosine (8-OHdG) detection

The malondialdehyde (MDA) concentration was measured using the TBARS Assay Kit (10009055, Cayman, Ann Arbor, MI, USA) in accordance with the manufacturer's instructions to quantify lipid peroxidation. The samples and standards were prepared, and the OD value was measured at 530 nm. To detect oxidative DNA damage, the 8-OHdG ELISA Kit (ab201734, Abcam, Cambridge, UK) was used according to the manufacturer's protocol, and OD value was measured at 450 nm. For intracellular Fe^{2+} detection, cells were incubated with the fluorescent probe BioTracker™ FerroOrange Live Cell Dye (SCT210, MilliporeSigma, Rockville, MD, USA) for 30 min and then observed under a fluorescence microscope.

Western blotting

Cells were lysed using a lysis buffer, and the resulting cell lysates were subjected to 10 % SDS-PAGE, and proteins were subsequently transferred to a PVDF membrane using an electro-transfer system. Membranes were incubated with

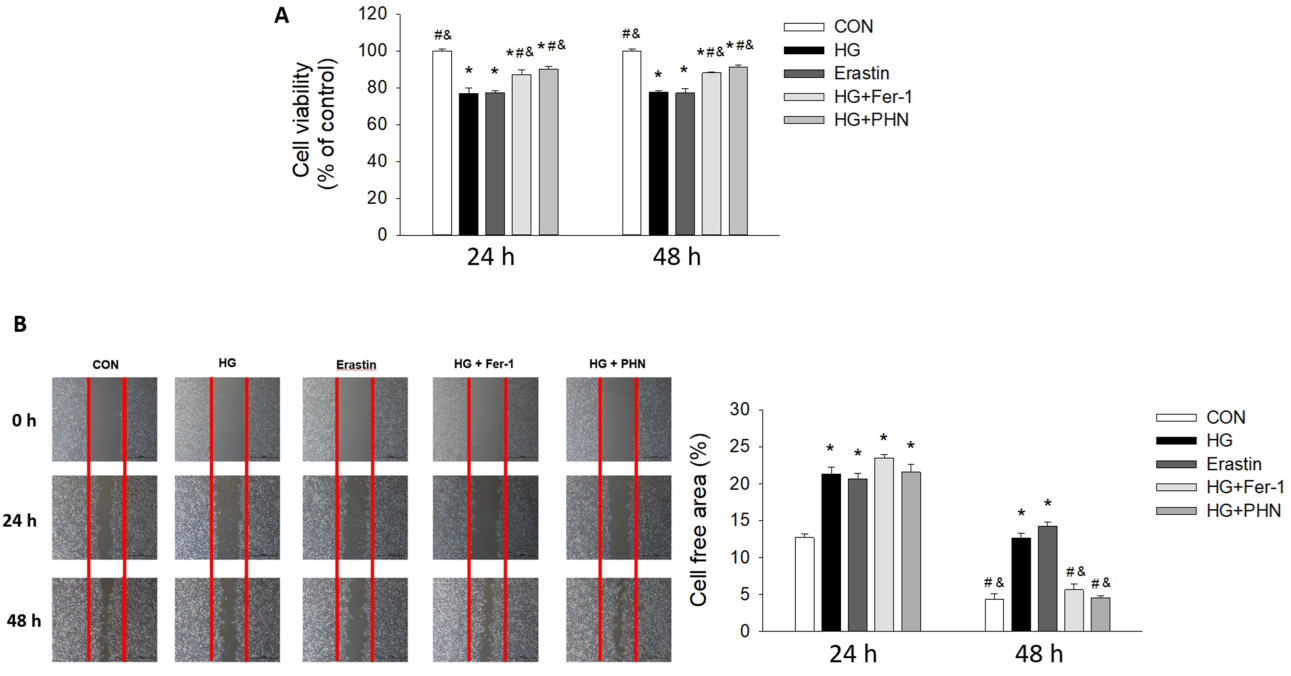

Figure 1 The reduction in cell viability and wound-healing capacity caused by high-glucose-induced ferroptosis is ameliorated by phillyrin (PHN) (A) Cell viability was assessed using the PrestoBlue assay following 24 h of exposure to high-glucose or erastin conditions. Subsequently, the high-glucose group was subdivided into untreated, Fer-1-treated, and PHN-treated subgroups, and cell viability was reassessed after an additional 24 or 48 h. (B) The first wound healing image was taken immediately after removal of the insert (0 h). Cells were then exposed to high-glucose or erastin for 24 h, and a second image was captured (24 h). The high-glucose group was subsequently left untreated or treated with Fer-1 or PHN for an additional 24 h, after which a third image was taken (48 h). Scale bars, 500 μm * $P < 0.05$ compared to control (CON) group at the corresponding time point. #$P < 0.05$ compared to high glucose (HG) group at the corresponding time point. & $P < 0.05$ compared to erastin group at the corresponding time point.

primary antibodies overnight at 4 °C. Primary antibodies and dilutions are as follows: AMPKα (1:1000; #2532, Cell Signaling, Danvers, MA, USA), Phospho-AMPKα (Thr172) (1:500; #2535, Cell Signaling), Nrf2 (1: 1000; #33649, Cell Signaling), GPX4 (1: 1000; #52455, Cell Signaling), xCT(1: 1000; DF12509, Affinity, Cincinnati, OH, USA), FSP1 (1: 1000; sc-377120, Santa Cruz, Dallas, TX, USA), and β-actin (1:5000; AC-15, Novus, Centennial, CO, USA). After incubation with primary antibodies, the membranes were washed three times and then incubated with the appropriate secondary antibodies. Immunoreactive bands were visualized using the Western-Ready™ ECL Substrate Plus Kit (426316, BioLegend, San Diego, CA, USA) and detected with the ChemiDoc MP Imaging System.

Statistical analysis

All statistical evaluations were conducted with the IBM SPSS Statistics 25.0 (Chicago, IL, USA). Quantitative results were presented as the average value ± standard deviation. Comparisons among various groups were analyzed by one-way ANOVA followed by a LSD post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

It has been known that diabetes fuels periodontal lesions[21] and defective wound healing.[22] In light of the potential benefit of enhanced epithelial proliferation and migration in gingival and mucosal wound healing, we assessed cell viability/proliferation, and wound healing capacity to determine whether phillyrin could improve delayed oral wound healing. We found that cells exposed to high-glucose conditions for 24 or 48 h exhibit reduced viability, similar to cells treated with the ferroptosis inducer erastin when compared at the corresponding time points. However, the inhibition of ferroptosis by Fer-1 attenuated the detrimental effects caused by high glucose, suggesting that the reduction of cell viability may be attributable to high-glucose-induced ferroptosis. In addition, we found that phillyrin treatment under high-glucose conditions exerted protective effects comparable to that of Fer-1 (Fig. 1A). In the wound-healing assay, exposure to high glucose or erastin for 24 h resulted in impaired healing compared with the control group, and this deficit persisted at 48 h. Notably, treatment with Fer-1 or phillyrin for 24 h during high-glucose exposure markedly improved wound-healing capacity, indicating that both interventions can mitigate the high-glucose-induced impairment in wound repair (Fig. 1B).

Given that phillyrin shows similar effects to Fer-1 and has been documented to possess anti-diabetic properties[14,15] and ferroptosis-regulating[16] properties, we examined its anti-ferroptotic potential by assessing various ferroptosis-associated features. MDA is one of the byproducts of lipid peroxidation in cells, and a significant release of 8-OhdG (an indicator of oxidative DNA damage) from ferroptotic cells has also been reported.[23] As shown in Fig. 2A and B, MDA concentration and 8-OHdG levels were elevated in high glucose-stimulated cells compared to the

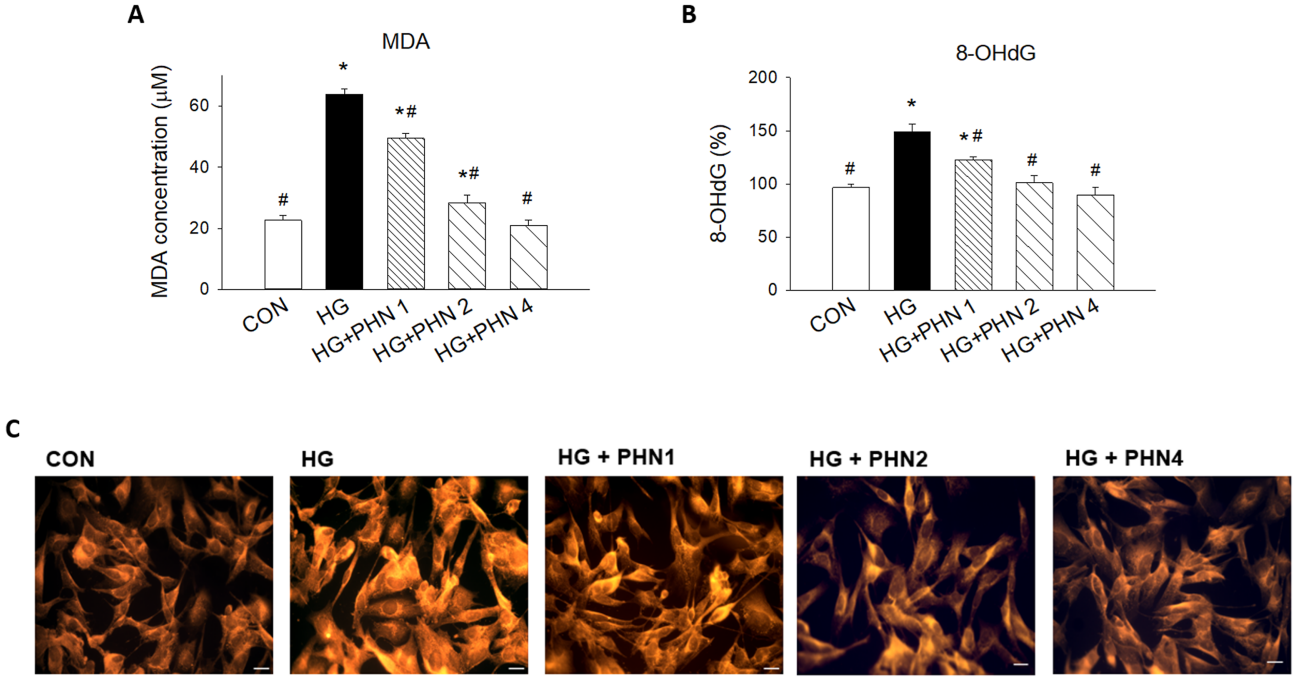

Figure 2 Phillyrin attenuates ferroptosis-related features in a dose-dependent manner. (A) The TBARS Assay Kit and (B) the 8-hydroxy-2-deoxyguanosine (8-OHdG) ELISA Kit were used to measure malondialdehyde (MDA) concentrations and 8-OHdG levels, respectively, as indicators of lipid peroxidation and oxidative DNA damage in gingival epithelial cells treated with various concentrations of phillyrin under high-glucose conditions. (C) Representative live-cell fluorescence images showing intracellular Fe^{2+} detected by the FerroOrange probe. Scale bars, 50 μm. PHN1, PHN2, and PHN4 represent treatment with 1 μM, 2 μM, and 4 μM phillyrin, respectively. * $P < 0.05$ compared to control (CON) group. #$P < 0.05$ compared to high glucose (HG) group.

control group, whereas phillyrin treatment (1, 2, and 4 μM) downregulated these markers in a dose-dependent fashion. Likewise, iron abundance was most pronounced in the high-glucose group, and phillyrin appeared to reduce it proportionally to the administered dose (Fig. 2C). Since a dose of 2 μM was sufficient to restore these measurements close to control levels, this concentration was used for subsequent assays. Our results indicated that phillyrin inhibits the production of lipid metabolites, oxidative stress indicator and iron accumulation under high glucose condition in a concentration-dependent manner.

An intimate link between metabolism and ferroptosis has been increasingly recognized over the past few years. As a critical cellular energy sensor, AMPK has been shown to mediate the regulation of ferroptosis in diabetic kidney disease.[24,25] In gingival epithelial cells exposed to high glucose, AMPK phosphorylation was markedly reduced; however, phillyrin treatment reinstated its activation (Fig. 3A–C). We inferred that high glucose induces ferroptosis based on elevated MDA and 8-OHdG levels, increased iron overload (Fig. 2) and decreased expression of anti-ferroptic factors (Fig. 4). Since Fer-1 mitigated high-glucose-induced suppression of cell viability and wound healing, and phillyrin produced similar effects (Fig. 1), we examined whether phillyrin could also normalize anti-ferroptotic factor expression beyond reducing MDA, 8-OHdG, and iron accumulation.

As expected, we showed that phillyrin upregulated the expression of anti-ferroptotic factors, including Nrf2, xCT, GPX4, and FSP1, which were suppressed under high-glucose conditions. Nevertheless, this protective effect was negated by the AMPK inhibitor Compound C, suggesting that the anti-ferroptotic property of phillyrin requires AMPK activation (Fig. 4). Collectively, these results indicated that phillyrin boosts anti-ferroptotic capacity by reactivating AMPK, which may compensate for glucose-induced impairment of oral wound healing.

Discussion

It is well established that oral wound healing is compromised under high-glucose conditions. Previous studies have consistently shown that high glucose levels lead to increased oxidative stress and inflammation, which in turn adversely affects cell proliferation and migration.[26–28] In this study, we demonstrated that high glucose may not only lead to elevated oxidative stress but also induce ferroptosis, as evidenced by increased levels of MDA and 8-OHdG, as well as iron accumulation. It has been known that diabetic

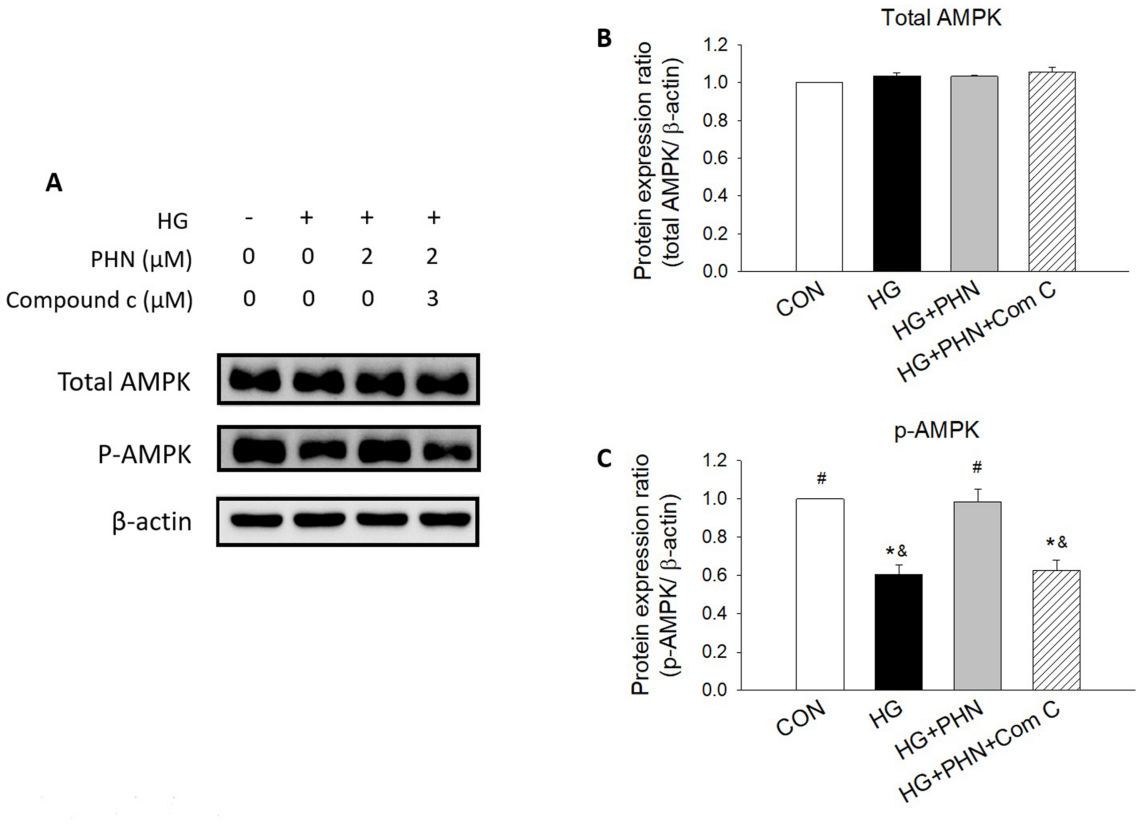

Figure 3 Phillyrin enhances 5′ adenosine monophosphate-activated protein kinase (AMPK) activation suppressed by high glucose and mitigates the effects triggered by erastin. (A) Representative Western blot images and relative densitometric bar graphs of (B) total AMPK and (C) phosphorylated AMPK in cells treated with phillyrin or Compound C under high-glucose condition were shown. * $P < 0.05$ compared to control (CON) group. #$P < 0.05$ compared to high glucose (HG) group. & $P < 0.05$ compared to HG + PHN group.

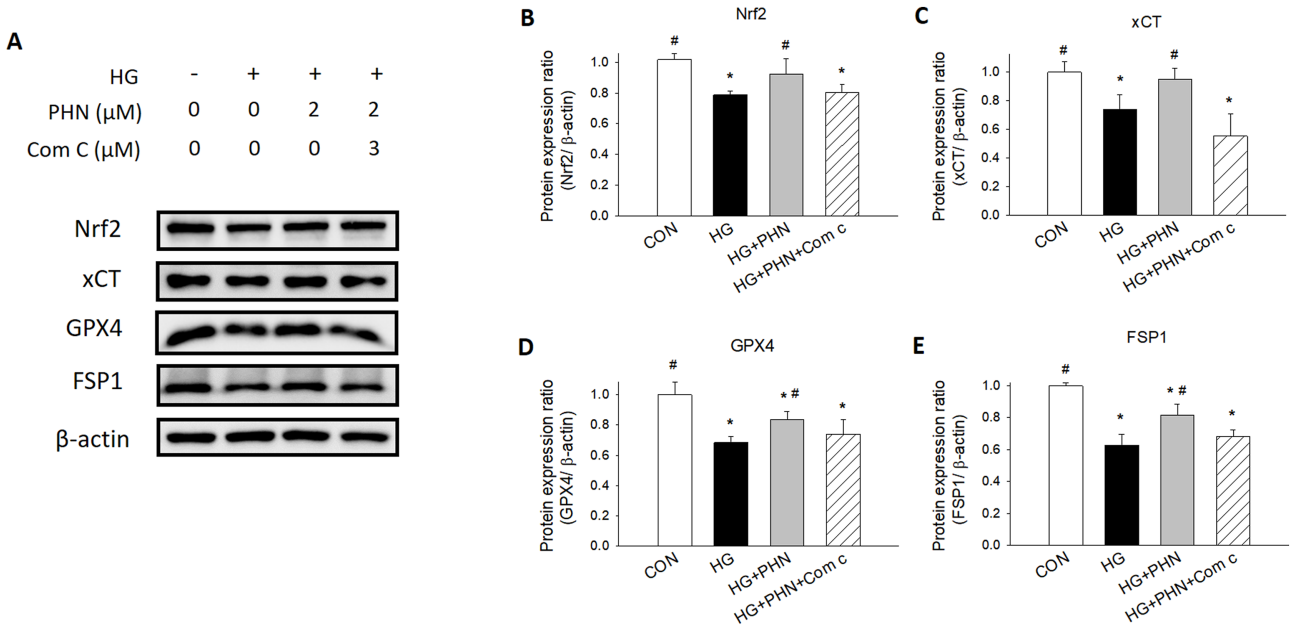

Figure 4 Phillyrin exerts anti-ferroptotic effects, potentially through the upregulation of AMPK signaling. (A) Representative Western blot images and relative densitometric bar graphs of (B) nuclear factor erythroid 2-related factor 2 (Nrf2), (C) xCT/SLC7A11, (D) glutathione peroxidase 4 (GPX4) and (E) ferroptosis suppressor protein 1 (FSP1) in cells treated with phillyrin in the presence or absence of Compound C under high-glucose condition were shown. * $P < 0.05$ compared to control (CON) group. #$P < 0.05$ compared to high glucose (HG) group. & $P < 0.05$ compared to HG + PHN group.

wound models exhibited hallmark features of ferroptosis, and the phosphoinositide 3-kinase (PI3K)/Akt signal pathway seemed to be a key pathway to accelerate wound healing.[12] Likewise, our findings indicated that delayed gingival wound healing is closely associated with the occurrence of ferroptosis. Moreover, we showed that treatment with phillyrin enhanced cell viability and wound healing capacity, which was accompanied by down-regulation of ferroptosis indicators and elevated expression of anti-ferroptotic markers. The data implied that attenuating ferroptosis may facilitate the healing of delayed wounds, which was in agreement with several studies showing that suppression of ferroptosis improves cell proliferation and migration in oral keratinocytes[29] and high glucose-treated endothelial cells.[30] Furthermore, our results demonstrated that the inhibitory effect of phillyrin on ferroptosis is likely mediated via increased AMPK phosphorylation in gingival epithelial cells.

Phillyrin is a lignan extracted from *Forsythia suspensa* (Thunb.) Vahl (Oleaceae) with various pharmacological properties, such as anti-emetic,[31] anti-oxidative,[32] and anti-inflammatory[33] effects. Several studies have demonstrated its protective potential on diabetes, including reduction of insulin resistance[34] and anti-obesity activities.[15,35] Besides, phillyrin has been shown to confer protective effects against diabetic nephropathy by attenuating the proliferation and inflammation in glomerular mesangial cells[14] and reducing renal dysfunction *in vivo*[36] through regulation of the PI3K/Akt signaling. Moreover, a few studies have indicated that phillyrin may exert modulatory effects on ferroptosis in various cancer cells.[16,37] In line with these results, we demonstrated that phillyrin may promote oral wound healing under high glucose conditions through its inhibitory effects on oxidative stress and ferroptosis. Aside from regulating PI3K/Akt[14,36] or peroxisome proliferator-activated receptors (PPARs)[35,38] pathways, emerging evidence suggests that phillyrin may function as an AMPK activator.[15,39] Our findings are consistent with previous studies and demonstrate that phillyrin restores AMPK expression suppressed by high glucose, thereby attenuating oxidative stress accumulation and subsequent ferroptosis.

AMPK has been acknowledged as a central regulator of cellular energy homeostasis, functioning to suppresses energy-consuming processes while promoting energy-generating pathways. It has been reported that activation of AMPK by metformin mitigates senescence of the junctional epithelium in periodontitis driven by hyperglycemia[40] and alleviates the inflammatory response in diabetic periodontitis.[41] Additionally, oxidative stress, metabolic imbalance, and apoptosis induced by high glucose in nerve cells are reduced by taurine via AMPK activation.[42] Similarly, the AMPK/mTOR signaling pathway also mediates the effects of quercetin on the suppression of high-glucose-induced oxidative stress in cardiomyocytes.[43] These findings suggest that activation of AMPK may be beneficial for the repair of periodontal tissues and the downregulation of oxidative stress under high glucose conditions. In the current study, we showed that the expression of phospho-AMPK was inhibited in response to high glucose, which was aligned with a previous study showing that phospho-AMPK expression was lower in high-glucose-treated gingival fibroblasts.[44] Moreover, we demonstrated that it was re-elevated by phillyrin treatment, accompanied by reductions in MDA, 8-OHdG levels, and intracellular iron, as well as improvements in cell viability and wound healing

capacity. Several studies have demonstrated that AMPK pathway mediates the regulation of ferroptosis in diabetic complications. For instance, activating AMPK signaling has been shown to prevent ferroptosis in renal podocyte,[24] renal tubular cells[25] and glomerular endothelial cells[45] in diabetic kidney disease. Our results were consistent with these findings and showed that activation of AMPK resulted in upregulation of anti-ferroptotic markers, which may have contributed to the improvement of high-glucose-induced deficits in cell viability and wound healing.

In summary, we demonstrated that the impairment of cell proliferation and wound healing in gingival epithelial cells exposed to high-glucose conditions may be ascribed to ferroptosis. Moreover, we showed that phillyrin treatment ameliorated these issues, potentially by counteracting the effects of high glucose on MDA, 8-OHdG, iron accumulation, and anti-ferroptotic factors. More importantly, our results suggest that the regulation of anti-ferroptotic factors may be mediated through the modulation of AMPK phosphorylation. These findings indicated that supplementation with phillyrin may contribute to oral tissue regeneration in diabetes.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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