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Serum Starvation Regulates Autophagy of Human Periodontal Ligament Cells Through Reactive Oxygen Species Mediated Adenosine Monophosphate-Activated Protein Kinase/Mechanistic Target of RAPAMYCIN Axis

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ABSTRACT

Introduction and Aims: Human periodontal ligament cells (hPDLCs) play a pivotal role in periodontal tissue remodelling, a process essential for orthodontic tooth movement (OTM). Autophagy, a survival mechanism under cellular stress, is induced by nutrient deprivation and impacts hPDLC function. This study aimed to explore the role of autophagy in the adaptive response of hPDLCs to nutritional stress, an environment simulating conditions during OTM.

Methods: Nutrient deprivation in hPDLCs was modelled through serum starvation. Autophagy levels and relevant markers were assessed using electron microscopy, protein assays, and gene expression analyses. Emphasis was placed on adenosine monophosphate-activated protein kinase (AMPK) signalling, specifically phosphorylation of AMPKα at Thr172, as a regulatory node in autophagy induction. Loss- and gain-of-function approaches were utilized to investigate the role of Thr172 in AMPK-mediated autophagy under nutrient stress.

Results: Findings indicated a marked increase in reactive oxygen species-mediated autophagy in hPDLCs under nutrient deprivation. This process was significantly regulated by AMPK activation through Thr172 phosphorylation, establishing AMPK as a critical factor in autophagy induction during cellular adaptation to nutritional stress.

Conclusion: Nutritional stress enhances reactive oxygen species-mediated autophagy in hPDLCs via AMPK signalling, underscoring the role of autophagy in cellular adaptation during OTM. Targeting the AMPK pathway could provide novel insights for optimizing orthodontic treatment by leveraging cellular adaptive mechanisms.

Clinical Relevance: Understanding the molecular mechanisms underlying autophagy in hPDLCs opens potential therapeutic pathways to improve OTM outcomes. Modulating autophagy may lead to advances in orthodontic therapies that facilitate periodontal tissue remodelling, enhancing clinical effectiveness.

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Introduction

Orthodontic tooth movement (OTM) is facilitated by the remodelling of periodontal tissues under mechanical forces. This remodelling is characterized by dynamic cellular homeostasis involving bone resorption by osteoclasts and bone formation by osteoblasts.[1] A critical aspect of this process is the compression of periodontal tissues on the pressure side, leading to a nutrient-deficient environment due to reduced blood supply, causing ischemia and hypoxia.[2-4]

To maintain homeostasis under these conditions, cells must efficiently manage cell death and repair, and autophagy plays a crucial role in this regulation. Autophagy is a lysosome-dependent process that degrades damaged organelles and unnecessary cellular components, thereby maintaining cellular homeostasis and recycling cellular materials for tissue repair and regeneration.[5-8] This process is particularly important in stress conditions such as nutrient deficiency, ischemia, and hypoxia, which are conditions relevant to the pressure side during OTM.[9]

The accumulation of reactive oxygen species (ROS) due to nutrient deficiency is a known trigger for autophagy.[10] A previous study by Mei and colleagues has shown that elevated ROS levels can induce autophagy in human periodontal ligament cells (hPDLCs) in vitro.[11] However, the specific mechanisms by which ROS influences autophagy in hPDLCs under nutritional stress conditions that mimic aspects of OTM remain unclear.

One of the key pathways through which ROS induces autophagy is the adenosine monophosphate-activated protein kinase (AMPK) signalling pathway.[12] AMPK is a heterotrimeric protein kinase that plays a significant role in cellular energy homeostasis. The α subunit of AMPK has a catalytic role, while the β and γ subunits are regulatory.[13] The activation of AMPK, particularly through the phosphorylation of the Thr172 site, is crucial for its role in autophagy induction.[14,15] This pathway negatively regulates the mechanistic target of rapamycin (mTOR), which is a major inhibitor of autophagy.[16]

Research has demonstrated that the AMPK/mTOR pathway is involved in maintaining cellular homeostasis during nutrient deprivation, with the phosphorylation of AMPK at Thr172 being a critical step.[17-20] Given this context, we examined whether autophagy in hPDLCs, triggered by nutrient depletion, is mediated by the ROS-dependent activation of the AMPK/mTOR signalling pathway. Understanding this mechanism not only provides insights on the role of autophagy in periodontal tissue remodelling but also highlights how nutrient stress, which may occur during orthodontic procedures, could influence tissue repair and regeneration in related disciplines.

Materials and methods

Cell and reagents

Primary culture of hPDLCs was used in this study following a previously published protocol.[21] In brief, healthy premolars were extracted from healthy adolescents between ages 10 and 18 due to orthodontic reasons. The periodontal ligament tissues on the middle one-third of the root surface were scraped off with a sharp instrument. The tissues were cut into 1 mm^2 pieces and evenly spread on the bottom of a 25 mL aseptic bottle, and cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) containing 10% foetal bovine serum (GIBCO). hPDLCs at passages 4 to 5 were used for the subsequent experiments. The cells in the nutrient-deficient group were starved with serum-free and low glucose DMEM containing 1% penicillin-streptomycin (Beyotime), while the cells in the control group were treated with DMEM containing 1% penicillin-streptomycin and 10% serum with a low level of glucose.

Transmission electron microscopy (TEM)

hPDLCs were harvested, washed three times with phosphate-buffered saline, and fixed in 2.5% glutaraldehyde for 2 days at 4°C. hPDLCs were postfixed in 1% osmium tetroxide for 2 hours. Following dehydration with a series of ethanol solutions, the samples were embedded in Embed-812 resin and then sliced into ultrathin slices measuring 70 nm in thickness. Afterwards, the ultrathin sections were mounted onto a copper wire and treated with dioxouranium acetate and lead citrate for staining. The ultrastructures of hPDLCs in both groups were observed under TEM (Hitachi). Quantification of autophagy abundance using TEM was done as outlined below. Autophagic bodies appear as vesicles with double or multiple membrane structures. By visually inspecting TEM images, one manually counts the number of autophagic bodies in the images. We conducted three measurements to calculate the average value, which is used to estimate the activity or abundance of autophagy.[22]

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total mRNA of hPDLCs was extracted using TRIzol followed by reverse transcription into cDNA using the ReverTra Ace qPCR RT kit (TOYOBO). PCR amplification of the target genes was performed according to the instructions of the SYBR Green PCR kit (QIAGEN). The primer sequences (Sangon Biotech) are listed in Supplementary Table 1. The relative expression of genes was calculated by the $2^{-\Delta\Delta ct}$ method.

Western blotting

Cultured cells and tissues were lysed in modified RIPA lysis buffer (Thermo Fisher Scientific), and then separated by 10% SDS-PAGE (Solarbio). The proteins were then transferred onto polyvinylidene fluoride membranes (Beyotime). The membranes were blocked with 5% skim milk at room temperature for 1 hour and incubated overnight with the following primary antibodies at 4°C: LC3B (1:1000; Abcam), P62 (1:1000; Abcam), Beclin-1 (1:1000; Abcam). Afterwards, the membranes were cultured with the secondary antibody (Bioss). The protein bands were detected using the enhanced chemiluminescence method (Affinity Biosciences) and captured with iBright CL1000 Imaging System (Invitrogen).

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescent probe and flow cytometry for the detection of ROS

hPDLCs in the logarithmic growth phase were collected, digested, and seeded onto 6-well plates. DCFH-DA (Beyotime) was diluted with serum-free DEME at a 1:1000 ratio, resulting in a final concentration of 10 μM. The cells were then mixed with 10 μM DCFH-DA and incubated for 30 minutes to ensure completion of the probe-cell reaction. Subsequently, some cells were photographed using an inverted phase contrast microscope, while others were examined via flow cytometry.

Transfection of mCherry-GFP-LC3 adenovirus and detection of autophagy flow

hPDLCs were transfected with mCherry-GFP-LC3 adenoviral particles (Beyotime) for 48 hours, following supplier's instructions. After this step, the medium was replaced, and the cells were subjected to 24 hours of nutrient deprivation. Subsequently, the medium was removed, and the cells were fixed with 4% paraformaldehyde. The resulting yellow spots (autophagosomes) and red spots (autolysosomes) were counted under a confocal microscope.[23-25] The autophagy flow was represented by the ratio of yellow spot count to red spot count. The transfection efficiency was more than 90%, and the cell death was less than 10%.

siRNA transfection and lentivirus infection

The AMPKα siRNA was purchased from Ribo with sequence GGATGATAGTGCCATGCAT. hPDLCs were seeded onto 6-well plates. Subsequently, transfection compound solution was prepared with 100 nM AMPKα siRNA or its negative control (FAM-siRNA), which was added to the cells. After 6 hours of incubation, the cells were replaced with low glucose DMEM. After 48 hours, the cells were observed and photographed under a fluorescence microscope. The control lentivirus (NC) and the lentiviruses carrying the wild-type AMPKα (WT) and the mutated AMPKα T172A (MT) were designed, synthesized, and packaged by Syngenbio. For the transduction of the hPDLCs with followed a similar protocol to the adenoviral infection.

Statistical analysis

The statistical analysis in this study was performed using the Statistical Package for Social Science version 21.0 (IBM). Initially, the data normality was assessed. For comparing two groups, the *t* test for independent samples was used. For more than two groups, we employed one-way analysis of variance followed by Dunnett's post hoc test. The significance level of $P < .05$ was considered statistically significant.

Results

Nutritional stress induces autophagy in hPDLCs

To investigate the relationship between autophagy and cellular nutrient deficiency, we examined whether nutrient deprivation induces autophagy in hPDLCs in vitro. The hPDLCs were subjected to serum starvation for 6, 12, 24, and 48 hours. TEM revealed a significant accumulation of autophagosomes in hPDLCs from 6 to 24 hours, with a gradual decrease observed after 24 hours of nutritional stress (Figure 1A). Western blot analysis showed elevated levels of Beclin-1 and LC3II/I proteins (Figure 1B, C). Additionally, the autophagy flux, indicated by LC3II/LC3I and p62, was activated under nutritional stress conditions (Figure 1B, C). These results indicated that nutritional stress induces the autophagy of hPDLCs in vitro.

ROS mediates hPDLCs autophagy under nutritional stress by activating the AMPK signalling pathway

Next, to understand the role of ROS in nutritional stress-induced autophagy in hPDLCs, cells were labelled with the DFCH-DA fluorescent probe and analysed using an inverted phase contrast microscope and flow cytometry (Figure 2A, B). The results revealed a significant increase in ROS levels in hPDLCs following nutritional stress treatment. The LC3II/I ratio and Beclin-1 expression were also significantly reduced, while p62 expression increased under nutritional stress conditions. The exogenous application of NAC partially restored autophagy levels towards baseline ($P < .05$) (Figure 2C, D). These findings suggest that ROS mediates autophagy in hPDLCs during nutritional stress.

To further explore autophagy flux in hPDLCs under nutritional stress, cells were transfected with GFP-mcherry-LC3 adenovirus. Autolysosomes (red spots) and autophagosomes (yellow spots) were visualized using confocal microscopy. Notably, cells experiencing nutritional stress showed a substantial increase in autophagy flux (red/yellow spots ratio), while NAC treatment decreased this ratio within 24 hours ($P < .05$) (Figure 2E, F). Moreover, we examined the AMPK signalling pathway, a downstream target of ROS, in this context. In particular, we focused on the phosphorylation at Thr172 because it is crucial for AMPK activation. This residue is essential for AMPK's kinase activity, which is necessary for initiating autophagy under stress conditions. Under nutritional stress, the p-AMPK (Thr172)/AMPK ratio increased, while the p-mTOR/mTOR ratio decreased compared to control cells. Conversely, NAC treatment inhibited ROS content, significantly suppressed p-AMPK (Thr172)/AMPK, and elevated the p-mTOR/mTOR levels ($P < .05$) (Figure 2G). Taken together, these results suggest that the phosphorylation at Thr172 was critical for the AMPK-mediated autophagy observed in hPDLCs under nutritional stress, highlighting its role in the cellular adaptation to nutrient deprivation and the regulation of autophagy through the ROS-AMPK-mTOR axis.

AMPK signalling pathway is activated during autophagy in hPDLCs under nutritional stress

Western blot analysis indicated that autophagy levels in the blank group remained unchanged after treatment with either compound C or ALCAR ($p > 0.05$, Figure 3A-D). Compound C (dorsomorphin) is a potent AMPK inhibitor, utilized in our study to examine the role of AMPK in autophagy regulation.[26] ALCAR (acetyl-L-carnitine) is an AMPK activator recognized

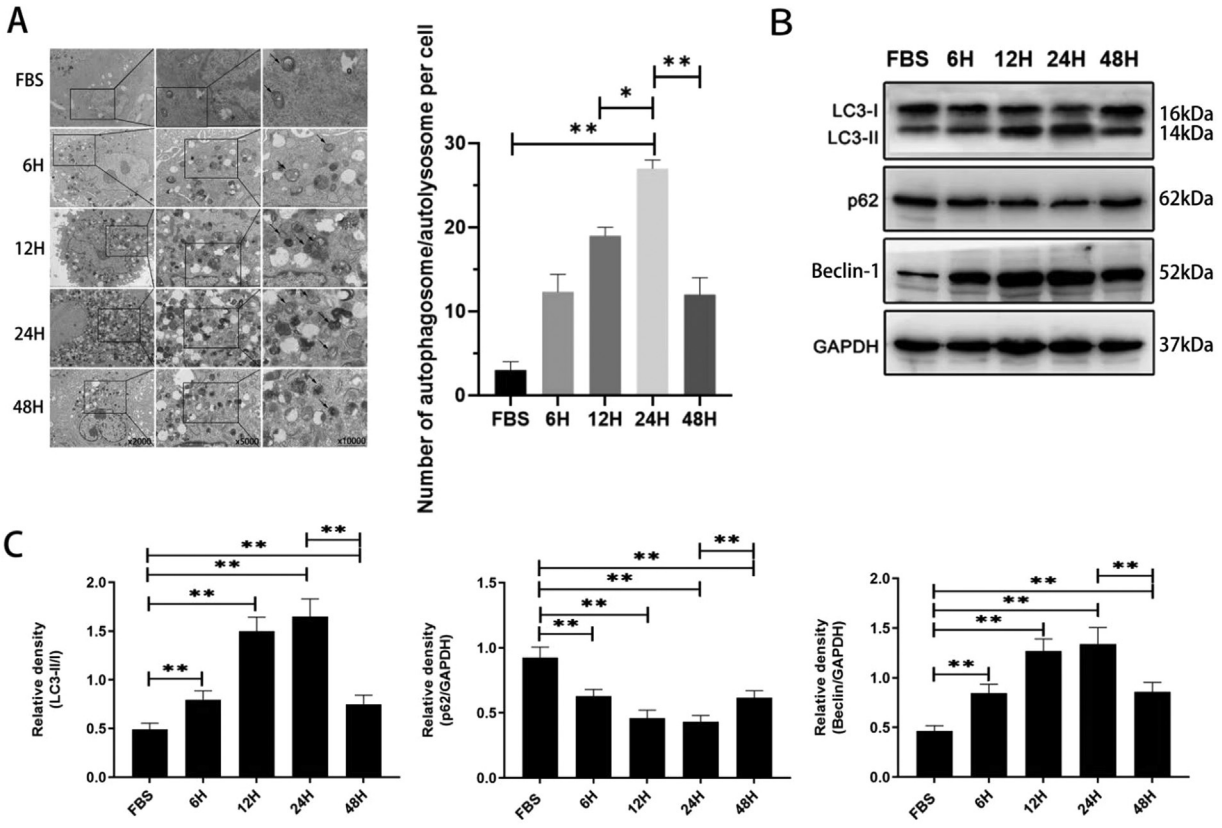

Fig. 1 – Nutrient deprivation induces autophagy in hPDLCs. The figure illustrates the effect of nutrient deprivation on the autophagy levels in hPDLCs over different time points using TEM, and Western blot analysis. (A) TEM images showing the formation of autophagosomes in hPDLCs (left) and relative quantification of autophagosomes per cell (right). Nutrient deprivation under ischemic conditions led to a significant accumulation of autophagosomes at 6, 12, and 24 hours, followed by a gradual decrease at 48 hours. Arrows indicate autophagosomes. (B and C) Western blot results demonstrated a significant increase in the LC3-II/I ratio and Beclin-1 expression upon nutrient deprivation. This increase was accompanied by a decrease in p62, indicating active autophagy. Autophagy peaked at 24 hours and decreased thereafter. 'FBS' denotes normal serum. Data are presented as mean ± standard deviation. Statistical significance is indicated by *P < .05, **P < .01.

for its role in enhancing mitochondrial function, and autophagy.[27] Compared to the nutritional stress group, compound C treatment significantly inhibited the expression of phosphorylated AMPK (Thr172), increased phosphorylated mTOR levels, and suppressed both autophagy activation and its flow ($P < .05$). In contrast, ALCAR treatment upregulated the expressions of phosphorylated AMPK (Thr172), LC3, and Beclin-1, resulting in the inhibition of phosphorylated mTOR and p62 expression, thereby enhancing autophagy levels and flow activation ($P < .05$) (Figure 3E, F).

AMPKα silencing inhibits nutritional stress-induced autophagy in hPDLCs

To determine the role of AMPKα in the autophagy of hPDLCs under nutritional stress, we utilized siRNA to knock down AMPKα expression. This intervention significantly reduced the mRNA levels of AMPKα in conditions of nutrient deprivation (Figure 4A). Consistently, Western blot analysis revealed that AMPKα silencing markedly suppressed the activation of AMPK induced by nutritional stress ($P < .05$) (Figure 4B, C). This suppression led to a decrease in the conversion of LC3-I to LC3-II, lowered Beclin-1 expression, and increased p62 expression ($P < .05$) (Figure 4D, E), ultimately inhibiting autophagic flux. These findings suggest that AMPKα is crucial for the autophagy process in hPDLCs under nutritional stress conditions.

Thr172 site on AMPKα is critical for autophagy elevation in hPDLCs under nutritional stress

AMPKα activation requires phosphorylation at Thr172, and as previously demonstrated (Figure 2G), dietary stress causes an increase in the p-AMPK (Thr172)/AMPK ratio in hPDLCs. To further assess the precise function of Thr172 in this process, we transduced hPDLCs with lentiviral particles expressing the wild-type (WT) or mutated AMPKα (MT), where Thr172 was altered to alanine (T172A) to prevent phosphorylation. qRT-PCR results revealed elevated mRNA levels of AMPKα in both WT and MT groups after nutritional stress, compared to cells infected with control lentivirus (NC) (Figure 5A). Western blot analysis confirmed a notable increase in p-mTOR/mTOR ratio in the MT group under nutritional stress, correlating with suppressed p-AMPK (Thr172)/AMPK levels ($P < .05$)

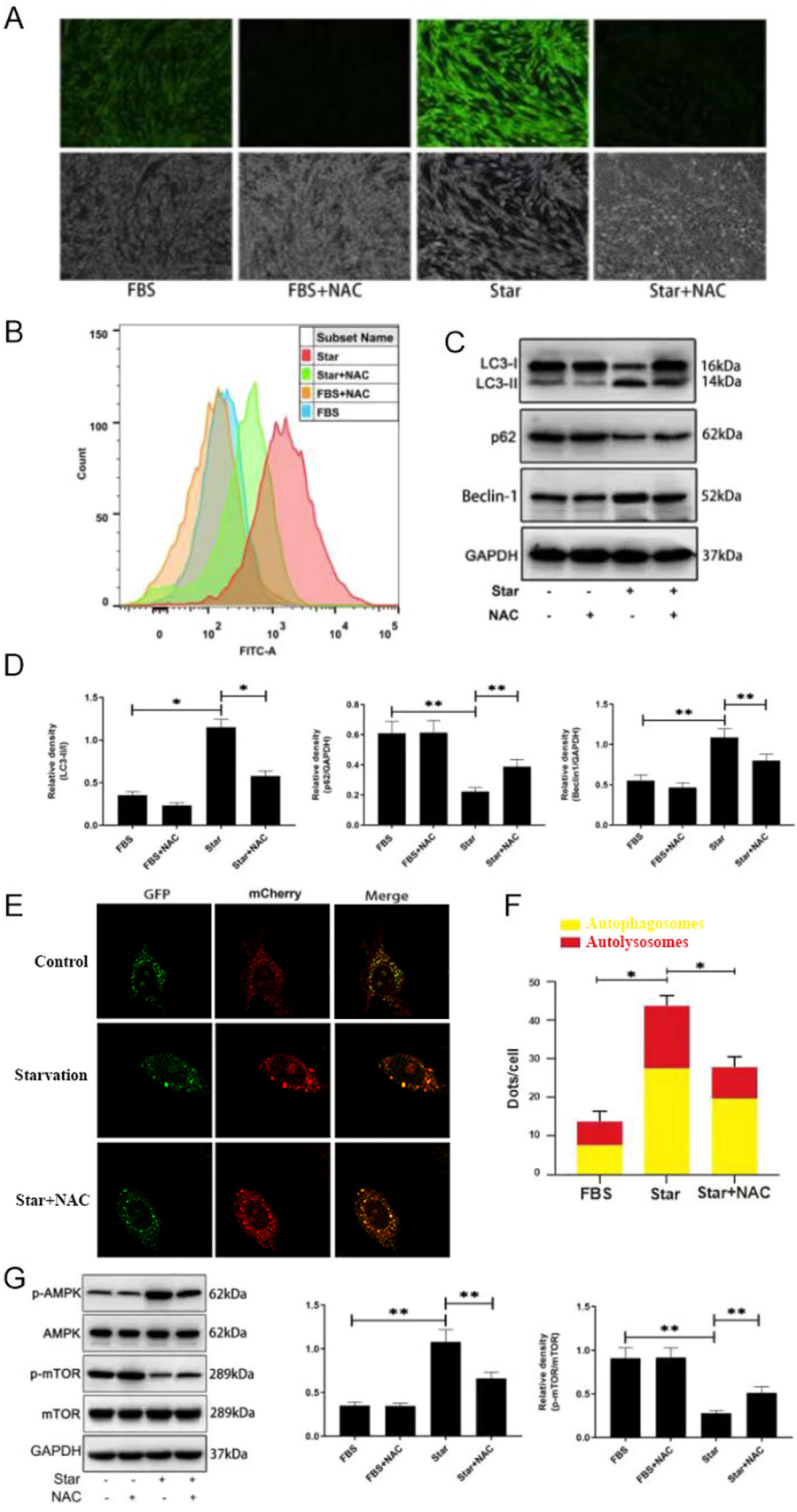

Fig. 2 – ROS-dependent autophagy induced by nutritional stress in hPDLCs. hPDLCs were subjected to different treatments for 24 hours: complete medium, complete medium with NAC (5 mM), serum-free and low glucose DMEM, and serum-free and low glucose DMEM with NAC (5 mM). The results indicate that ROS content in hPDLCs increased under ischemic and

(Figure 5B, C). Additionally, Beclin-1 expression significantly decreased while p62 expression increased in the MT group compared to the WT group under nutritional stress ($P < .05$) (Figure 5D, E). These findings highlight the critical role of the Thr172 site on AMPKα in regulating autophagy in hPDLCs under nutritional stress.

Discussion

Autophagy, an essential catabolic process, facilitates the degradation and recycling of cellular components via lysosomes, thereby maintaining cellular homeostasis and enabling tissue repair and regeneration.[28,29] In conditions relevant to OTM, such as nutrient deficiency and oxidative stress, the hPDLCs experience nutritional and oxidative stress due to mechanical forces. This study investigates how serum starvation, simulating nutritional stress, affects autophagy in hPDLCs,[30,31] particularly through the ROS-mediated AMPK/mTOR signalling pathway.

Our findings demonstrate a significant upregulation of autophagosome formation and autophagy-related proteins, LC3 and Beclin-1, in hPDLCs under serum starvation. This indicates that the nutrient-deficient environment stimulates autophagy in hPDLCs. However, it is important to note that the mere increase in autophagy markers does not fully reflect autophagic activity but rather indicates the induction or inhibition of autophagy clearance. Therefore, a comprehensive evaluation of autophagy must include dynamic observation of autophagy flux.[29] Specifically, autophagy activity was observed to peak at 24 hours of nutritional stress, indicating an optimal adaptive response period that enhances cellular homeostasis and tissue remodelling while maintaining cell viability. Prolonged stress beyond this period was associated with increased apoptosis, diminishing the potential benefits and highlighting the risks associated with dysregulated autophagy under certain conditions. These observations underscore the 'double-edged sword' nature of autophagy, where excessive or insufficient activity can lead to adverse effects, including impaired tissue remodelling and heightened apoptosis.

Interestingly, our study found that autophagy-related proteins and autophagy flux in hPDLCs increased significantly during the early stages of serum starvation but began to decrease at later stages. This decline is likely due to apoptosis or nonapoptotic cell death resulting from prolonged nutritional deficiency. Additionally, under certain stress conditions, autophagy defects may compromise cell viability, leading to cell death and potentially exacerbating periodontal damage during OTM. This emphasizes the need to consider not only the benefits but also the potential negative consequences of autophagy dysregulation during therapeutic interventions.

Intracellular ROS play a crucial role as mediators of autophagy under nutritional stress, contributing to the maintenance of cellular function and homeostasis, a role that could be relevant in contexts like OTM.[32] In addition, ROS are known to induce and participate in autophagy processes.[33,34] In our study, we observed a notable increase in ROS levels in hPDLCs under nutritional deprivation, which corresponded with increased autophagy. The use of NAC, a ROS inhibitor, significantly reduced the expression of autophagy-related factors, suggesting that ROS accumulation mediates autophagy in hPDLCs under nutritional stress.

While the regulatory mechanisms of autophagy in OTM differ from those in cancer cells, they share foundational molecular pathways such as the ROS-mediated AMPK/mTOR axis. In cancer, autophagy functions predominantly as a survival mechanism, aiding tumour cells in withstanding stress conditions like hypoxia and nutrient deprivation to promote growth and therapy resistance. Conversely, in OTM, autophagy facilitates physiological adaptation by enabling tissue remodelling and maintaining cellular homeostasis under mechanical stress. These distinct outcomes are driven by the differing objectives of autophagy within each cellular context. For instance, autophagy in cancer cells is characterized by a dysregulated balance favouring tumour survival, whereas in OTM, it serves a homeostatic role, ensuring controlled tissue remodelling and repair.

Our findings corroborate prior studies demonstrating that the regulatory mechanisms involving ROS and AMPK/mTOR pathways are conserved across different cell types and stress conditions, underscoring their relevance to autophagy modulation.[35-38] However, we acknowledge that in the specific context of OTM, dysregulated autophagy—whether excessive or insufficient—can have negative consequences, including heightened apoptosis, delayed tissue repair, or exacerbated inflammatory responses.

The AMPKα subunit, particularly its Thr172 site, is critical in regulating autophagy under nutrient-deficient conditions.[39-41] This site, along with other essential sites for AMPKα activation, is regulated by upstream kinase phosphorylation.

nutrient-deprived conditions, leading to ROS-mediated autophagy and activation of the AMPK-mTOR signalling pathway. (A) DFCH-DA ROS fluorescent staining (green, top) and inverted phase contrast (grayscale, bottom) images showing an increase in ROS content in hPDLCs due to nutrient deficiency, which was reversed by NAC treatment (magnification × 100). (B) Flow cytometry of hPDLCs stained with DFCH-DA ROS probe demonstrating that nutritional stress-induced ROS accumulation in hPDLCs (Star), which NAC treatment reversed (Star + NAC). (C and D) Western blot analysis showing a significant decrease in LC3-II/I and Beclin-1 expression under nutritional stress, with an increase in p62 expression. NAC addition rescued the autophagic effects. (E and F) Confocal images showing hPDLCs transduced with LC3 adenovirus (GFP-mcherry-LC3) (F). Red spots (autolysosomes) and yellow spots (autophagosomes) reveal autophagy. Nutritional stress significantly increased the red/yellow spot ratio (autophagic flux), while NAC treatment reduced this ratio within 24 hours (magnification × 400). Stacked barplot showing the relative quantification of autophagosomes and autolysosomes per cell (G). (G) Western blots and relative quantifications showing a significant increase in p-AMPK (Thr172)/AMPK and a decrease in p-mTOR/mTOR under nutritional stress. Exogenous NAC decreased p-AMPK (Thr172)/AMPK and increased p-mTOR/mTOR. 'FBS' denotes normal serum, and 'Star' represents serum starvation treatment. Data are presented as mean ± standard deviation. *$P < .05$, **$P < .01$.

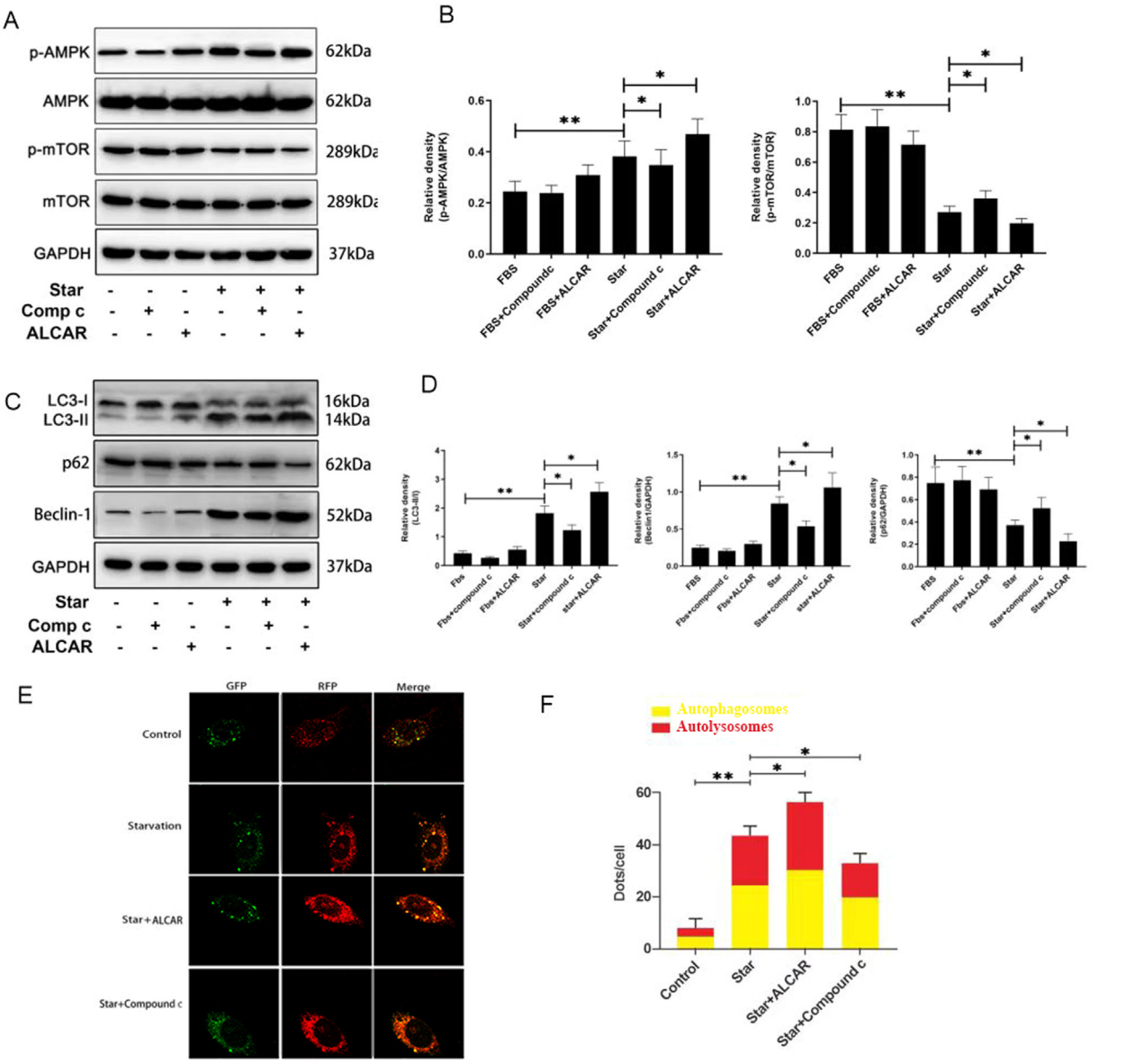

Fig. 3 – ROS-mediated AMPK signalling pathway in autophagy of hPDLCs. To investigate the role of ROS in the AMPK-mTOR signalling pathway and its regulation of autophagy under nutrient-deprived conditions, hPDLCs were cultured in either complete media (FBS) or in serum-free low glucose DMEM (Star) and were treated as follows: (1) control (2) compound C (1.25 μM), (3) AICAR (5 μM). All treatments were applied for a duration of 24 hours. (A and B) Western blot analysis demonstrates that compound C treatment decreased the p-AMPK (Thr172)/AMPK ratio and increased the p-mTOR/mTOR ratio, while AICAR treatment had the opposite effects. (C and D) Western blots showing that compound C significantly inhibited the conversion of LC3-I to LC3-II and the expression of Beclin-1, while enhancing the expression of p62. AICAR treatment produced contrasting results. (E and F) Confocal images showing hPDLCs transduced with LC3 adenovirus (GFP-mcherry-LC3) exhibit a significant increase in the red/yellow spot ratio (autophagy flux) following nutritional stress. Autophagy flux decreased within 24 hours after compound C treatment and increased after AICAR treatment (magnification $\times$ 400). Data are presented as mean ± standard deviation. *P* values calculated with independent samples *t* test on the number of Autolysosomes. *P < .05, **P < .01.

Changes in AMPK activity, particularly at the Thr172 site, have been associated with various pathological conditions, including type I diabetes, papillary thyroid cancer, and mental retardation.[42-44] Our results highlight the significant role of Thr172 in hPDLC autophagy during nutritional deprivation, suggesting it as a potential target for intervention. To mitigate the risks of dysregulated autophagy during OTM, we propose several strategies. Mechanically, adjusting the magnitude, direction, and duration of orthodontic forces could help maintain moderate stress levels, thereby supporting balanced autophagy. Chemically, targeted autophagy modulators such as AMPK activators (e.g., metformin, simvastatin) could be employed to fine-tune cellular responses. The development of real-time monitoring tools for autophagic activity offers a

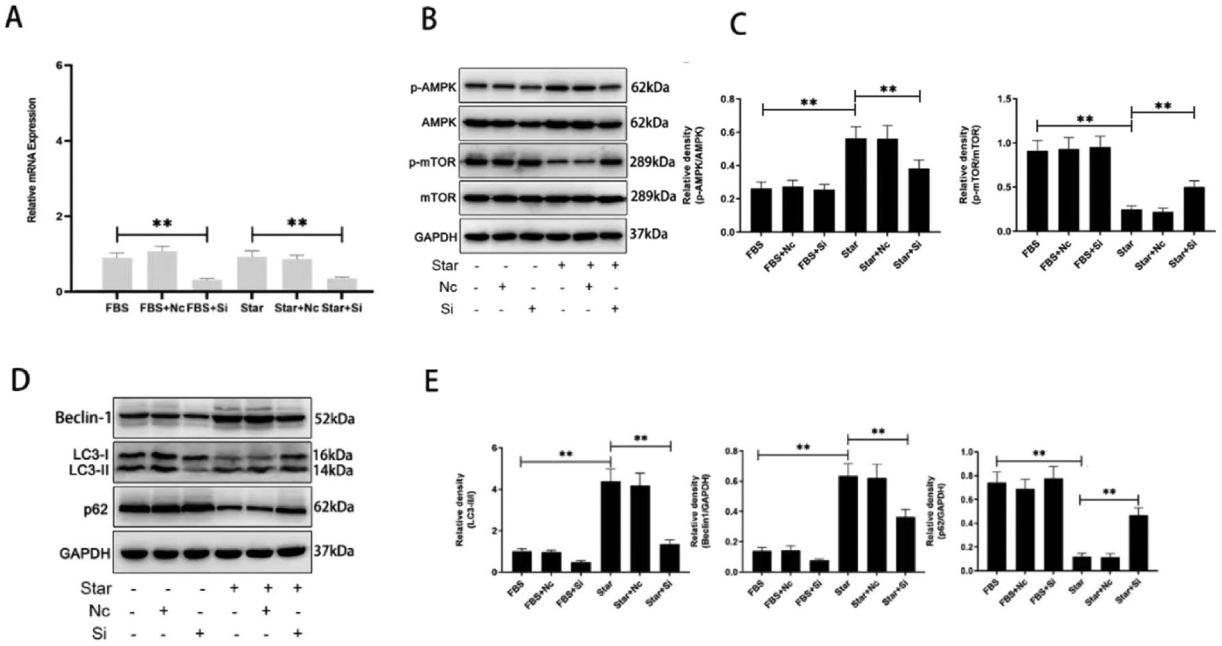

Fig. 4 – Regulatory mechanism of AMPKα in autophagy of hPDLCs. hPDLCs were transfected with AMPKα siRNA (Si) or a negative control (Nc), followed by incubation in serum-free DMEM or complete medium for 24 hours. (A) Quantitative RT-PCR analysis showing significant inhibition of AMPKα mRNA expression compared to the control due to AMPKα silencing. (B and C) Western blot analysis revealing that nutritional stress significantly increased the p-AMPK (Thr172)/AMPK ratio and decreased the p-mTOR/mTOR ratio. AMPKα silencing reversed these effects, decreasing p-AMPK (Thr172)/AMPK and increasing p-mTOR/mTOR. (D and E) Western blot detection of autophagy markers LC3-II/I, p62, and Beclin-1 indicating that AMPKα silencing inhibited autophagy and autophagic flux in hPDLCs under nutrient-deficient conditions. 'FBS' denotes the serum control group, 'Star' represents the starvation treatment group, 'Nc' is the siRNA negative control group, and 'Si' refers to the siRNA transfection group. Data are presented as mean ± standard deviation. *$P < .05$, **$P < .01$.

promising avenue to dynamically optimize therapeutic protocols, further minimizing adverse effects.

From a clinical perspective, these findings highlight that the magnitude and duration of orthodontic forces can be adjusted to optimize autophagic activity and tissue remodelling. For instance, lighter, controlled forces applied intermittently may enhance autophagic processes without inducing excessive cellular stress or apoptosis, leading to more efficient and predictable OTM outcomes. In particular, studies have shown that AMPK activation enhances osteoblast differentiation and bone formation.[45-47] For instance, metformin, an AMPK activator, has been found to promote dental tissue regeneration,[46] while simvastatin, known for its bone anabolic and anti-inflammatory properties, improves tooth anchorage during OTM through AMPK activation.[47] Together, these strategies, combined with the optimization of mechanical forces, offer a pathway to translate our findings into practical orthodontic applications. Nevertheless, while these approaches hold considerable promise, further in vivo studies are essential to validate these mechanisms and refine clinical protocols.

Our results underscore that balancing the activation of AMPK pathways during orthodontic therapy is critical to prevent excessive autophagy or apoptosis, thus preserving PDL integrity. Clinically, the magnitude and duration of orthodontic forces must be optimized to ensure beneficial autophagic activity without exacerbating stress-induced cell death. Moreover, the dual role of autophagy as a cytoprotective and cytotoxic mechanism underlines its 'double-edged sword' nature in OTM. Excessive autophagy can lead to dysregulated cellular functions, while insufficient activation might impair tissue remodelling. Adjunctive strategies, such as localized delivery of autophagy modulators, represent promising avenues to fine-tune autophagic activity, enhancing tissue remodelling and minimizing adverse effects during OTM.

Moreover, our results provide new insights into the potential of modulating autophagy as a therapeutic strategy in orthodontics. By fine-tuning the autophagic response, it may be possible to enhance tissue resilience and remodelling during OTM. For example, the regulation of inflammatory responses through autophagy could mitigate excessive tissue damage while promoting efficient bone formation.[48,49] Additionally, enhancing cellular homeostasis under mechanical stress may prevent pathological outcomes such as periodontal tissue destruction or cell death.[50] From a clinical perspective, real-time monitoring tools for autophagic activity could be integrated to optimize force magnitudes and durations, providing a personalized approach to OTM. These findings pave the way for developing therapies aimed at optimizing autophagic responses to improve clinical outcomes in orthodontic treatments.

However, it is crucial to consider the broader implications of modulating autophagy in clinical settings. While controlled autophagy modulation shows promise in enhancing tissue

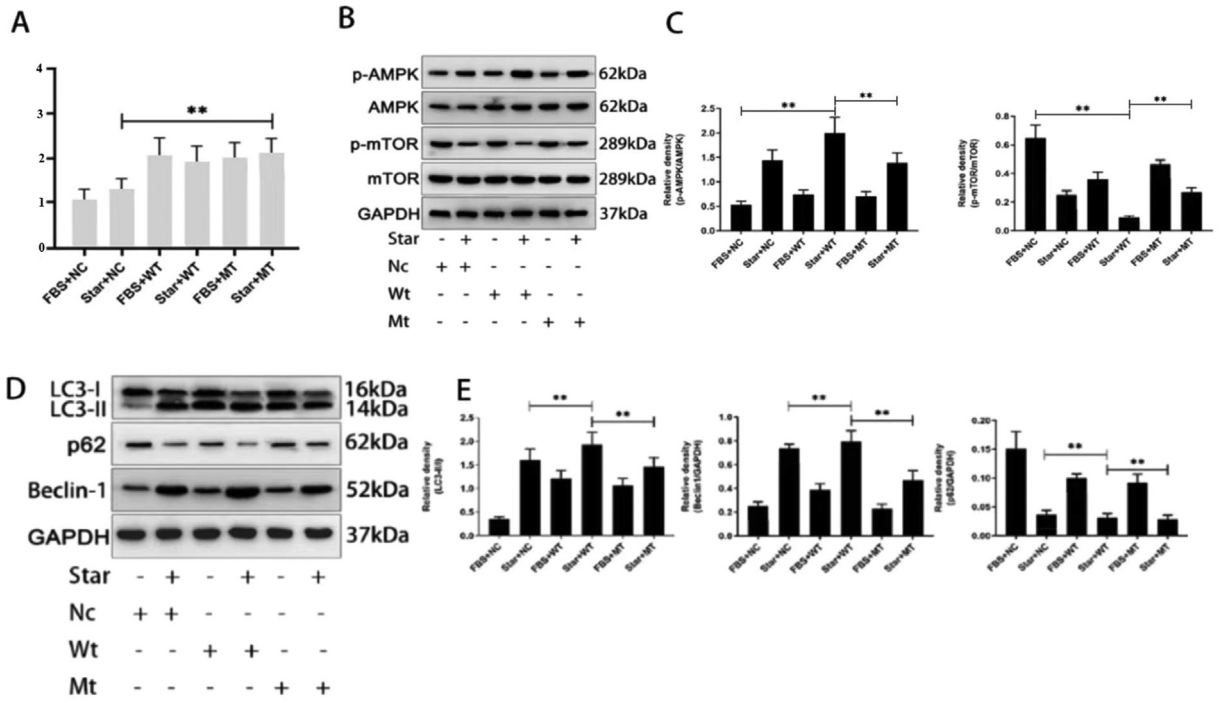

Fig. 5 – Site-specific elevated autophagy in serum-starved hPDLCs at Thr172 of AMPKα. hPDLCs were transduced with lentiviral particles carrying the wild-type (WT) or mutated AMPKα T172A (MT). (A) qRT-PCR analysis showing upregulation of mRNA expression for AMPKα in both the wild-type and mutant groups compared to the control group (NC, empty lentivirus) following 24 hours of treatment. (B and C) Western blot analysis indicating an increase in the p-mTOR/mTOR ratio in the mutant group under nutritional stress, whereas the p-AMPK (Thr172)/AMPK ratio was significantly decreased. (D and E) Comparison with the wild-type group revealed a significant reduction in Beclin-1 expression and an increase in p62 expression in the mutant group under nutritional stress conditions. 'FBS' denotes the serum control group; 'Star' represents the starvation treatment group; 'NC' stands for the lentivirus negative control group; 'Si' refers to the siRNA transfection group; 'WT' indicates the wild-type group; 'MT' represents the mutant group. Data are expressed as mean ± standard deviation. $^{*}P < .05$, $^{**}P < .01$.

remodelling during orthodontic therapy, the potential risks of long-term dysregulation cannot be overlooked. For instance, sustained autophagy activation has been linked to increased apoptosis in ageing PDL cells, potentially compromising tissue integrity.[51] Similarly, dysregulated autophagy has been associated with periodontal tissue destruction and inflammation.[52] These findings underscore the need for further research to establish safe thresholds and to ensure sustained periodontal health and cellular function while mitigating risks associated with prolonged cellular stress. Future studies are warranted to investigate these therapeutic pathways in clinical models, ensuring their safety and efficacy before translation into practice.

In summary, our study provides evidence that autophagy in hPDLCs under conditions of nutritional stress, potentially relevant to orthodontic treatment, is regulated by ROS accumulation in a nutrient-deficient environment (Figure 6). Activation of the Thr172 site on AMPKα and the subsequent AMPK/mTOR signalling pathway induces autophagy. While autophagy serves as a critical process for cellular adaptation and tissue remodelling during OTM, its regulation must be approached with caution to avoid adverse effects. By integrating mechanical adjustments, pharmacological interventions, and real-time monitoring systems, it may be possible to balance autophagy activity effectively, optimizing therapeutic outcomes while minimizing risks.

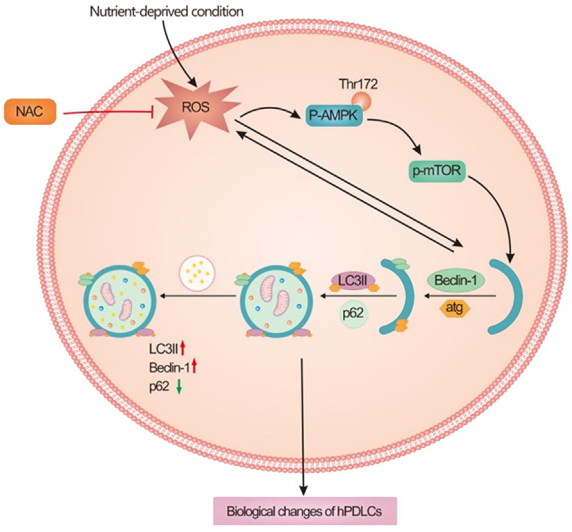

Fig. 6 – Illustration of proposed model.

Ethics statement and consent to participate

This study was approved by the Ethics Committee of the Affiliated Stomatology Hospital of Southwest Medical University (Approval No. 20200916002). Informed consent was obtained from all the participants. All included patients fulfilled the criteria and completed the study.

Author contributions

Mianxing Wei: Contributed to the study's design, experimental execution, data acquisition, and interpretation. Additionally, drafted and critically revised the manuscript. Yujie Wu: Contributed to experimental execution, data acquisition, and interpretation, performed some statistical analyses, and critically revised the manuscript. Qian Yang: Participated in experimental execution, data acquisition, and interpretation, and carried out some statistical analyses. Zheng Zhou: Contributed to the study's conception and design, and critically revised the manuscript. Xiaomei Xu: Conceived and designed the analysis, oversaw the entire experimental design, collected the data, participated in the entire data collection process, and critically revised the manuscript. All authors provided final approval and agreed to be accountable for all aspects of the work.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.identj.2025.02.012.

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