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Effect of type I collagen on TLR-3 induced MMP-13 expression in human periodontal ligament fibroblasts

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ABSTRACT

Background: This study examined the effects of type I collagen, alone and in combination with poly I:C—a toll-like receptor 3 (TLR3) agonist—on matrix metalloproteinase-13 (MMP-13) expression and wound healing in human periodontal ligament (hPDL) fibroblasts.
Methods: hPDL fibroblasts were cultured and divided into four treatment groups: control, type I collagen (50 μg/mL), poly I:C (10 μg/mL), and a combination of type I collagen with poly I:C. Cytotoxicity was evaluated using the MTT assay. Cell migration was assessed via scratch assay at 0, 24, and 48 h. MMP-13 expression was quantified at both the mRNA and protein levels by real-time PCR and ELISA, respectively.
Results: After 24 h, the MTT assay indicated that none of the four treatment groups exhibited cytotoxicity toward hPDL fibroblasts. In the scratch assay at 24 and 48 h, type I collagen group demonstrated the fastest wound closure, whereas the poly I:C group showed the slowest migration. Regarding MMP-13 expression, the combination group displayed the highest mRNA levels, while ELISA revealed that both the combination and poly I:C groups had the greatest protein expression relative to the control.
Conclusion: This study provides evidence for an interaction between extracellular matrix signals and innate immune activation. Type I collagen promoted hPDL fibroblast migration, whereas poly I:C—a TLR3 agonist—upregulated MMP-13 expression, with the greatest effect observed in combination with collagen.

1. Introduction

The periodontal ligament (PDL) is a specialized connective tissue that plays a central role in tooth support, homeostasis, and tissue remodeling. The PDL space consists of collagen fiber bundles, fibroblasts, and interstitial fluid, and dynamically adapts to physiological and pathological stimuli, including infection, injury, and orthodontic tooth movement.[1–5] Its extracellular matrix (ECM), predominantly composed of type I collagen[6] is organized in multidirectional bundles that enable effective resistance to mechanical forces.[2] PDL fibroblasts are key regulators of periodontal remodeling and respond to mechanical and biochemical cues through multiple receptor systems, including integrins, focal adhesion complexes,[4,7] receptor tyrosine kinases,[7] phospholipid receptors, G-protein-coupled receptors,[4] and toll-like receptors (TLRs).[8] Through these pathways, PDL fibroblasts modulate the expression and the activity of matrix metalloproteinases (MMPs), which are essential for ECM degradation and turnover during tissue remodeling and wound healing.[9,10] Tight regulation of MMP activity is therefore critical for maintaining the balance between matrix resorption and regeneration required for PDL homeostasis.[1–3,6,10].

MMPs are a family of 23 zinc-dependent enzymes involved in ECM remodeling during embryonic development, tissue repair, and wound healing.[11–13] However, unregulated MMP activity is associated with pathological conditions such as cancer, osteoarthritis, inflammatory diseases, vascular disorders, and neurodegeneration.[13–15] Among these enzymes, MMP-13 or collagenase-3, exhibits strong proteolytic activity against type I and II collagen, which are major structural components of the PDL and alveolar bone.[16] MMP-13 has been shown to contribute to collagen turnover during orthodontic tooth movement[16–18] and to play an essential role in bone formation processes such as endochondral and intramembranous ossification during fetal development.[19]

Recent studies have demonstrated that toll-like receptor 3 (TLR3), a

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pattern recognition receptor expressed on immune and non-immune cells, can regulate MMP-13 expression.[20,21] TLR3 recognizes double-stranded RNA (dsRNA), typically of viral origin, and signals through a MyD88-independent, TRIF-dependent pathway, leading to interferon-β (IFN-β) production[22] and downstream inflammatory responses. In addition to viral dsRNA, TLR3 can be activated by endogenous dsRNA released from necrotic or damaged tissues, implicating this receptor in sterile inflammation and tissue repair.[23] Consistent with this concept, synthetic TLR3 agonists, such as polyinosinic:polycytidylic acid (poly I:C), have been reported to induce MMP-13 expression in various cell types.[24,25].

Wound healing in periodontal tissues is a tightly regulated, multistep process involving inflammation, cell migration, proliferation and matrix remodeling.[26–28] These events are coordinated through reciprocal interaction between cells and ECM, which drive the release and activation of cytokines, growth factors, and MMPs.[1,9,10,28] Although the regulatory effects of type I collagen on MMP-13 expression have been reported in mouse chondrocyte cell lines[29] and human skin fibroblasts,[19] comparable evidence in human PDL (hPDL) fibroblasts remains limited.

To date, the influence of TLR-3 activation on MMP-13 expression in hPDL fibroblasts has not been systematically investigated, particularly in the context of simultaneous ECM stimulation. Therefore, the present study aimed to investigate the effect of type I collagen alone and in combination with poly I:C on MMP-13 expression in hPDL fibroblasts. Furthermore, the functional implications of these treatments on wound healing related cell migration were examined. We therefore hypothesized that activation of TLR3 by poly I:C, as a model of sterile inflammatory signaling, enhances MMP-13 expression in hPDL fibroblasts and that this response is modulated by type I collagen, reflecting an interaction between innate immune signaling and the ECM.

2. Methods

2.1. Cell culture

Primary hPDL fibroblasts were isolated and cultured following the protocol described by Tiranathanagul et al., 2001.[30] Briefly, periodontal tissues were obtained from three systemically healthy donors (age range: 20-35 years) undergoing premolars extracted for orthodontic purposes with written informed consent. Each tooth was rinsed three times with phosphate-buffered saline (PBS), and the PDL tissue was carefully scraped from the middle third of the root surface. The harvested tissues were minced into small fragments and placed in a cell culture dish with the complete medium containing Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA), 2 mM L-glutamine (GlutaMAX™, Thermo Fisher Scientific, USA), 100 unit/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-antimycotic, Gibco, New York, USA). The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Three independent hPDL fibroblasts cell lines (passage 3 to 6), each derived from a different donor, were used in this study.

The collection and use of human-extracted teeth were conducted in accordance with the Declaration of Helsinki and approved by the Srinakarinwirot University Human Research Ethics committee (approval numbers: SWUEC/E−333/2563 and SWUEC-116/2565X).

2.2. Cell culture treatment

An initial cytotoxic assay by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method was conducted to determine the appropriate dosage of poly I:C (InvivoGen, San Diego, CA USA). hPDL fibroblasts were seeded in 96-well plates (Thermo Fisher Scientific, USA) at a density of 1×10^4 cells/well in complete medium and incubated overnight. The following day, the medium was replaced with serum-free DMEM (SF-DMEM), and the cells were treated with poly I:C at concentrations of 2.5, 5, 10, 20, and 40 μg/mL. After 24 h of incubation, the MTT assay was performed.

In this study, four experimental groups were established. The first was the untreated control group. The second was type I collagen group, in which hPDL fibroblasts were plated on top of 50 μg/mL type I collagen. The third was the poly I:C group, in which hPDL fibroblasts were treated with 10 μg/mL poly I:C. The fourth was the combination group, in which hPDL fibroblasts were plated on top of 50 μg/mL type I collagen and subsequently treated with 10 μg/mL poly I:C.

Type I collagen (Cultrex Rat Collagen I, R&D Systems, Minneapolis, Minnesota, USA) was prepared according to the manufacturer's protocol. A thin-coat collagen layer was generated using a final concentration of 50 μg/mL, as recommended by the manufacturer and commonly used in previous cell culture studies.[31] The collagen solution was aliquoted and pre-warmed to 37 °C for 5 min before dilution. It was then diluted to a final concentration of 50 μg/mL using 0.02 M acetic acid. One milliliter of the diluted solution was added to each well of a 6-well culture plate. After 1 h of incubation at 37 °C in a 5% CO_2 atmosphere, the collagen solution was aspirated, and the wells were rinsed with PBS. The wells were then air-dried for 30 min prior to cell seeding. hPDL fibroblasts were seeded into 6-well plates (onto the collagen-coated or non-collagen –coated wells) at a density of 2.5×10^5 cells/well in complete medium and incubated for 24 h under standard culture conditions (37 °C, 5% CO_2). The next day, the culture medium was replaced with SF-DMEM, and treatments were initiated according to the four previously described groups for a further 24 h.

2.3. Cytotoxic assay

Cellular metabolic activity was evaluated using MTT method. Following 24-h cell culture treatment, MTT solution (0.5 mg/mL; Sigma-Aldrich Co., Saint Louis, Mo, USA) was added to each well and incubated at 37 °C for 30 min. After incubation, the resulting purple formazan crystals were solubilized in dimethyl sulfoxide (DMSO, Sigma Aldrich Co., USA), and absorbance was measured at 550 nm using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, USA). All experimental conditions were tested in triplicate.

2.4. RNA extraction and gene quantification by real-time PCR

After 24-h treatment, total RNA was extracted from hPDL fibroblasts using the TRIzol reagent (Thermo Fisher Scientific, USA) following the manufacturer's instruction. The RNA pellet was washed with 70% ethanol, air-dried, and dissolved in RNase-free water. Complementary DNA (cDNA) synthesis was performed using the ImProm-II™ Reverse Transcription System (Promega, Madison, Wiscosin, USA) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was carried out using the LightCycler® 480 SYBR Green Master with the LightCycler® 480 system (Roche Diagnostics, Mannheim, Germany). Expression levels of *matrix metalloproteinase-13* (MMP-13) were quantified, with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) serving as the internal control. The following primer sequences were used in this study: *MMP-13* forward: 5′-AGGCTGAGCTCTTTTTGACA-3′, *MMP-13* reverse: 5′-TCATAACCATTCAGAGCCCA-3′;[32] *GAPDH* forward: 5′-TGAAGGTCGAGTCAACGGAT-3′, *GAPDH* reverse: 5′-TCACACCC ATGACGAACATGG-3′.[33]

2.5. MMP-13 protein quantification by enzyme-linked immunosorbent assay (ELISA)

Following the 24-h experimental treatments, sample collection and processing were performed in accordance with the manufacturer's protocol. The supernatant was carefully harvested and centrifuged at 1,000×*g* for 10 min at 4 °C to remove cell debris. The clarified supernatant was then collected and stored at −80 °C until analysis. Quantification of pro-MMP-13 was conducted using the Human Pro-MMP-13

Quantikine® ELISA Kit (R&D Systems, USA). The optical density (OD) of each well was measured at 450 nm using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, USA). A standard curve was generated from known concentrations of MMP-13 to determine sample concentrations. Final values were normalized to the total protein content and expressed relative to the control group.

2.6. Scratch assay

After hPDL fibroblasts were seeded into 6-well plates (onto the collagen-coated or non-collagen –coated wells) and 4 experimental groups were established as mentioned previously, a linear scratch was then introduced across the confluent monolayer using the SPLScar™ Scratcher (SPL Life Sciences, Korea) to generate a standardized wound area. Following scratch creation, the cells were washed with PBS and the treatment conditions were applied as described in the previous section. Phase-contrast images of the wound area were captured at 4 × magnification using an inverted microscope at three time points: immediately after scratching, 24 h, and 48 h. The same field of view was consistently imaged at each time point. Wound closure was quantified using ImageJ software (version 1.42, NIH, USA). The wound area at each time point was measured and expressed as a percentage of the initial wound area (Day 0). Relative wound closure was calculated using the following formula:

$$\text{Relative wound area } (\%) = \frac{(\text{Wound area at Day 0} - \text{Wound area at specific time}) \text{ x } 100}{\text{Wound area at Day 0}}$$

2.7. Data analysis

All experiments were repeated three times, and data are presented as mean ± standard deviation. Statistical analysis was performed using SPSS version 28.0.1.1 (IBM, New York, NY, USA) with a significance level of 0.05. The Shapiro-Wilk test was used to confirm the normality of the data. For the cytotoxic assay and the expression of MMP-13 mRNA and pro-MMP-13 protein, a one-way ANOVA was used to evaluate differences between groups, followed by Tukey's post-hoc test. For the scratch assay, a two-way repeated-measures ANOVA was used to assess differences between treatment groups and time points, followed by Games-Howell post-hoc analysis.

3. Results

3.1. Cytotoxic assay of poly I:C on hPDL fibroblasts

As shown in Fig. 1, treatment with poly I:C at concentrations of 2.5, 5, 10, 20, and 40 μg/mL for 24 h did not significantly affect hPDL viability, indicating no cytotoxic effects under the tested conditions. A concentration of 10 μg/mL was selected for further experiments, as it represented a non-cytotoxic dose that has been shown in previous studies to effectively activate TLR3-mediated signaling without causing overstimulation or loss of cell viability.[24,34,35]

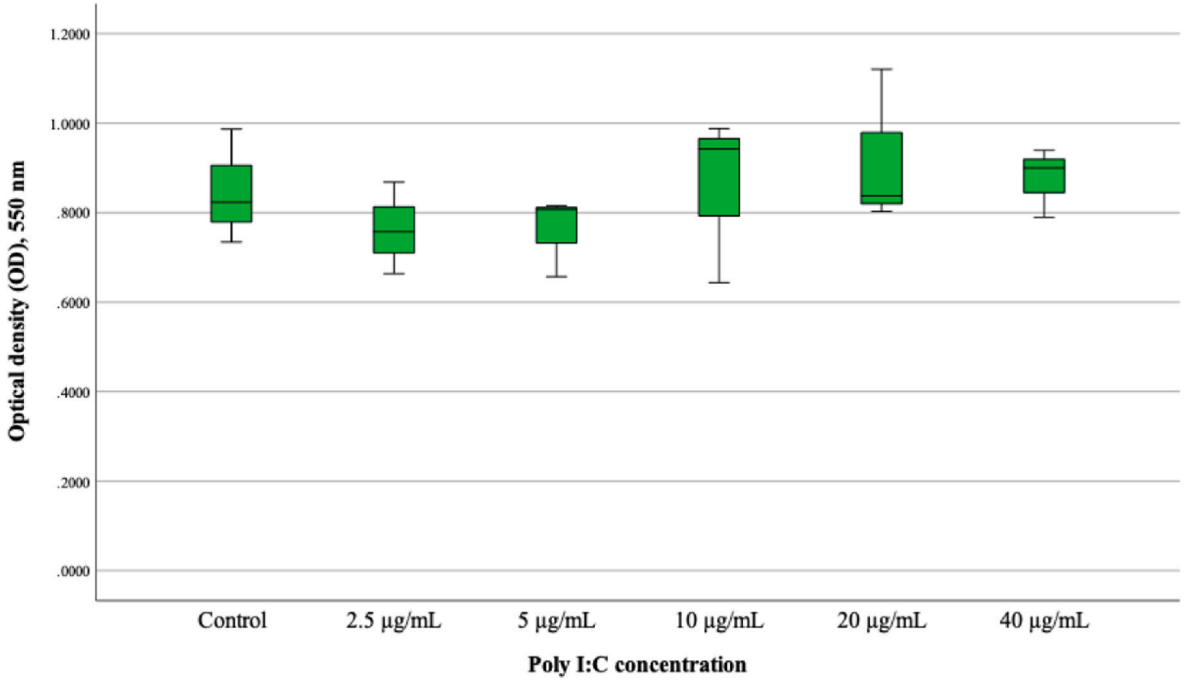

Fig. 1. Cytotoxic assay of poly I:C on hPDL fibroblasts.

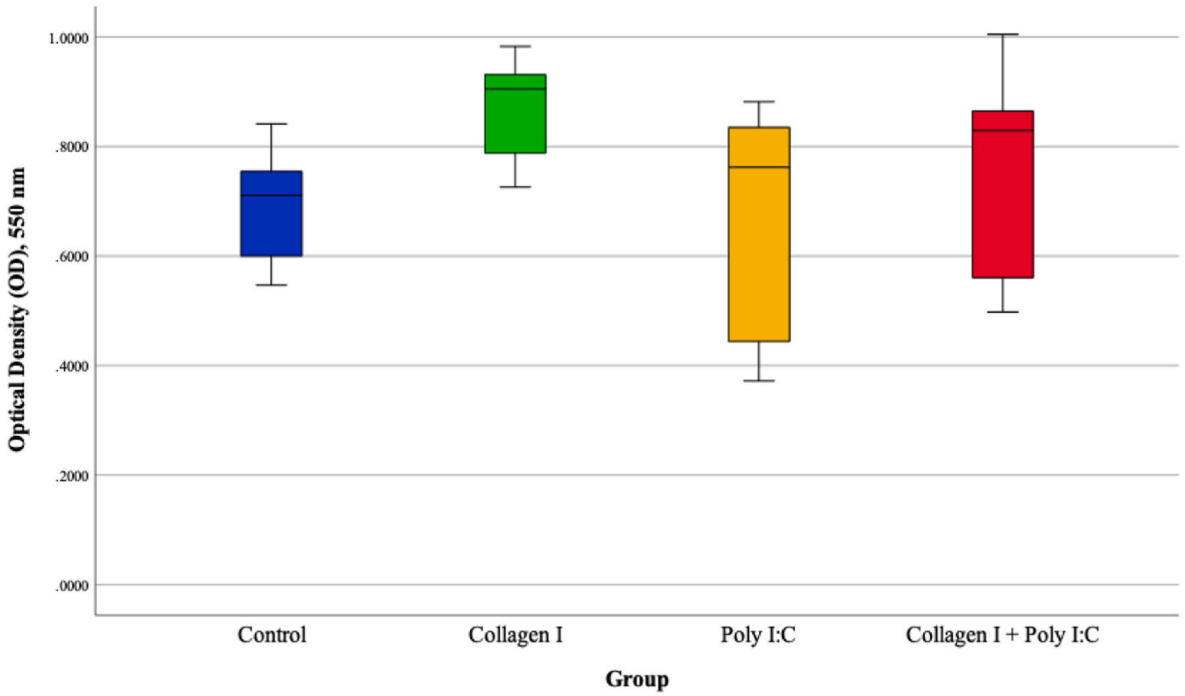

Fig. 2. Cytotoxic assay of type I collagen, poly I:C and their combination on hPDL fibroblasts.

3.2. Cytotoxic assay of type I collagen, poly I:C, and their combination on hPDL fibroblasts

As shown in Fig. 2, there was no significant difference in cell viability as measured by the optical density of 550 nm among any of the treatment groups: type I collagen, poly I:C their sequential combination and the untreated control (**$p > 0.05$**), indicating that none of the treatments exerted cytotoxic effects on hPDL fibroblasts.

3.3. Scratch assay

Cell migration under different treatment conditions was assessed using a scratch wound assay (Fig. 3). At 24 h post-scratch, hPDL fibroblasts cultured on type I collagen showed the greatest reduction in wound area, with 1.3% of the wound area remaining. The combination group exhibited a moderate degree of wound closure, with 21.1% of the wound area remaining. In contrast, larger residual wound areas were observed in the Control (58.5%) and poly I:C (71.4%) groups. Statistical analysis demonstrated significant differences between the type I collagen group and both the control and poly I:C groups at 24 h ($p < 0.05$).

At 48 h post-scratch, complete wound closure was consistently observed in the type I collagen group. In comparison, residual unhealed wound areas persisted in the control (25.2%) and poly I:C (50.0%) groups. The combination treatment further reduced the wound area to 5.7% at 48 h, indicating enhanced wound closure compared with poly I:C treatment alone ($p < 0.05$). However, complete wound closure was not uniformly achieved in the combination group.

Two-way repeated measures ANOVA revealed a significant main effect of time ($p < 0.05$), indicating progressive wound closure from 24 to 48 h across all experimental conditions. A significant main effect of treatment group was also observed ($p < 0.05$), reflecting differences in wound closure among groups. No significant interaction between time and treatment was detected ($p > 0.05$), suggesting that the temporal pattern of wound closure was comparable across treatment conditions.

3.4. The expression of MMP-13 mRNA

Real-time PCR analysis revealed that treatment with type I collagen, poly I:C, and their combination resulted in differential expression levels of MMP-13 mRNA in hPDL fibroblasts. The combination group (collagen I + poly I:C) exhibited a marked upregulation of MMP-13 mRNA expression, with a relative fold-change of 7.94 compared to the control (set at 1.0). In contrast, the poly I:C alone and type I collagen alone groups showed modest expression levels of 1.97 and 1.67, respectively. Statistical analysis confirmed that MMP-13 expression in the combination group was significantly higher than in all other groups ($p < 0.05$). No significant differences were observed among the poly I:C, type I collagen, and control groups (Fig. 4).

3.5. The expression of pro-MMP-13 protein

Pro-MMP-13 protein levels in hPDL fibroblasts were quantified by ELISA to evaluate the translation of MMP-13 mRNA. The highest pro-MMP-13 protein levels were detected in the combination group followed by the poly I:C group, with no significant difference between these two groups. In addition, both the combination and poly I:C exhibited significantly elevated protein expression than the type I collagen and the control groups ($p < 0.05$). No significant difference was observed between the poly I:C and combination groups (Fig. 5).

4. Discussion

Many previous studies have reported the effects of collagen and poly I:C individually in various cell lines, including skin fibroblast,[19,25] mouse chondrocyte cell lines[29] and primary human chondrocyte.[34] To our knowledge, this is the first study to investigate the biological responses of hPDL fibroblasts to type I collagen, poly I:C (the TLR3 agonist) or the combination of both. The cytotoxic assay demonstrated that none of the treatment exerted cytotoxic effects on hPDL fibroblasts after 24 h of exposure. These findings confirm the biocompatibility of all tested conditions and are consistent with previous studies reporting that poly I:C, at various concentrations, does not adversely affect the viability of various cell types, including chondrocytes, dermal fibroblasts and

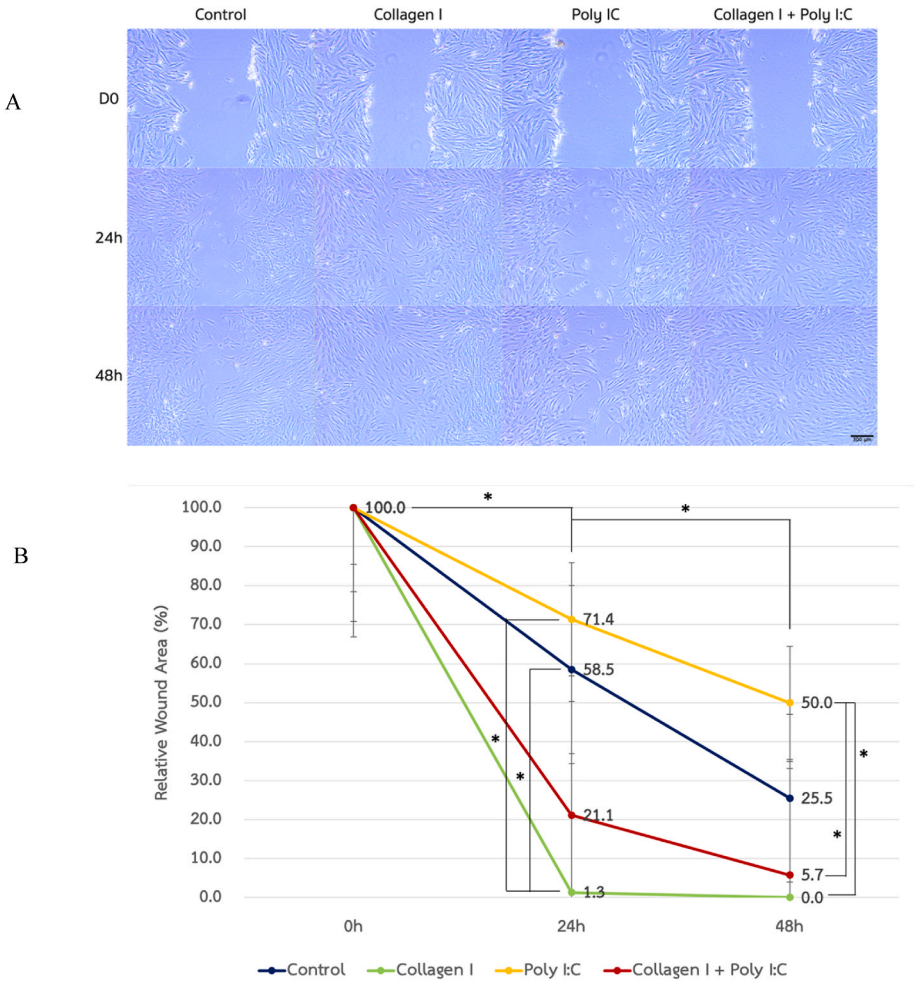

Fig. 3. Scratch assay evaluating hPDL fibroblasts migration under different treatment conditions. (A) Representative phase-contrast images of scratch wounds at 0, 24, and 48 h for each group. Images were taken at the same field of view for each time point. (scale bar 300 μm) (B) Quantitative analysis of relative wound area over time. Wound closure was expressed as the percentage of wound area remaining, normalized to the initial wound width at 0 h.

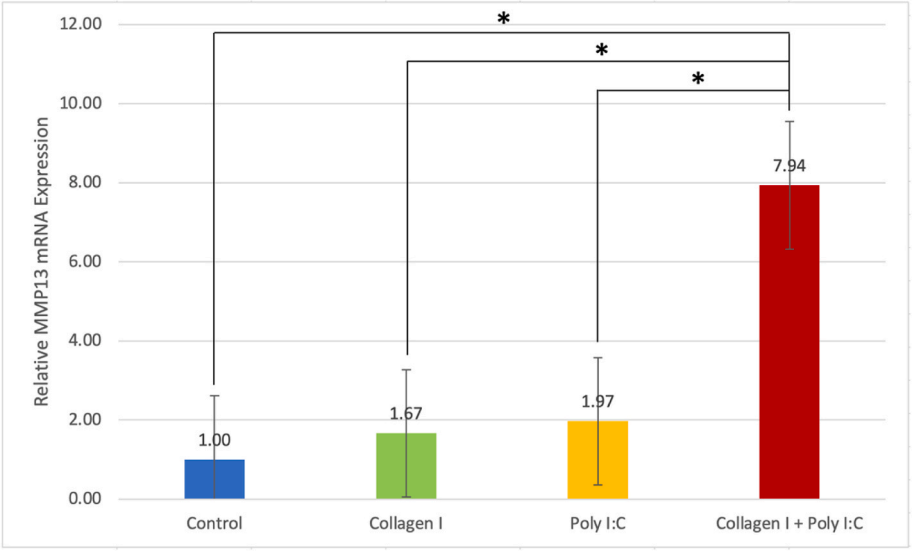

Fig. 4. MMP-13 mRNA expression in hPDL fibroblasts under different treatment conditions.

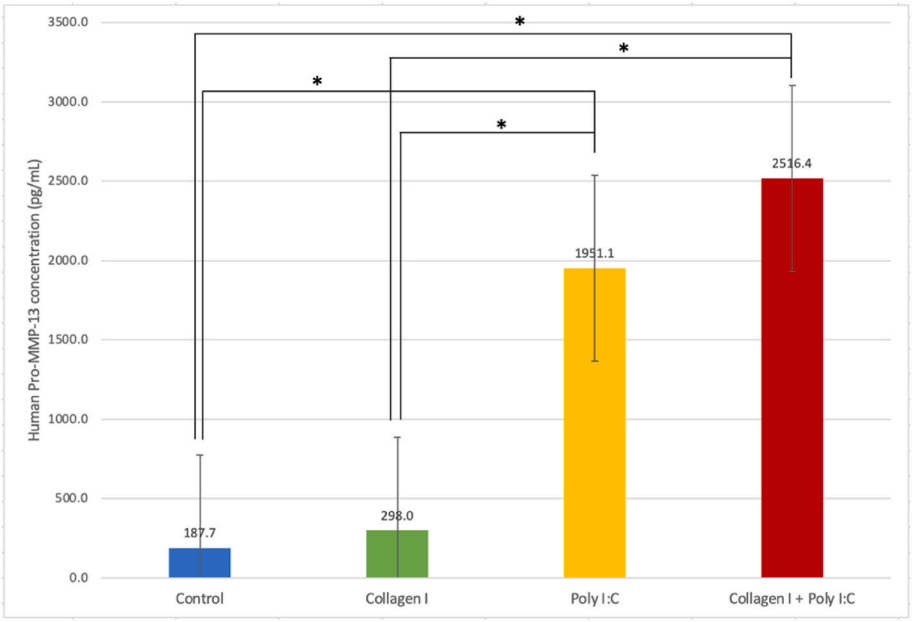

Fig. 5. Quantification of Pro-MMP-13 protein levels in hPDL fibroblasts under different treatment conditions.

hPDL fibroblasts.[24,25,34–36] The non-cytotoxic nature of the treatments provides a reliable foundation for subsequent analyses of gene expression, protein production, and cellular behavior in response to extracellular matrix and immune stimuli.

Based on cytotoxicity screening, a concentration of 10 μg/mL of poly I:C was selected for subsequent experiments as it represents a biologically active, non-toxic dose known to activate TLR3-mediated signaling in various cell types,[24,34,35] including hPDL fibroblasts. Additionally, the combination group (type I collagen + Poly I:C) further supports the suitability of this experimental model for examining interactions between ECM cues and innate immune activation under inflammatory conditions. This approach was designed to investigate molecular and cellular mechanisms relevant to periodontal tissue remodeling, rather than to directly replicate mechanical loading or orthodontic force.

Scratch assay analysis demonstrated that type I collagen significantly promoted hPDL fibroblasts migration, as evidenced by enhanced wound closure at 24 h post-injury compared to the control and poly I:C-treated groups. Given that MTT assays showed no significant difference in cell proliferation among the groups, the observed enhancement in wound closure is likely attributable to increased migratory activity. This finding is consistent with the established role of type I collagen in promoting cytoskeletal organization and directional cell movement.[37–39].

Notably, wound closure was most delayed in the poly I:C group, whereas the combination of type I collagen and poly I:C partially restored migratory capacity at both 24 and 48 h. This finding suggests that type I collagen may support cell motility even in the presence of inflammatory stimuli such as poly I:C. Previous studies have demonstrated that poly I:C can impair wound healing in various experimental models, likely through strong activation of pro-inflammatory signaling pathways.[40,41] In addition, excessive MMP-13 expression has been reported to disrupt normal wound remodeling by degrading extracellular matrix components required for tissue repair.[10,40,42] However, based on the present data, it cannot be conclusively determined whether the delayed wound healing observed in the poly I:C group is directly related to MMP-13 induction, and this relationship warrants further investigation. Overall, these findings highlight the complex interplay between extracellular matrix cues and innate immune activation in regulating cell migration and wound healing, where the promigratory effects of type I collagen may be modulated or counterbalanced by inflammation-associated signaling pathways.

At the molecular level, real-time PCR and ELISA analyses demonstrated that the combined treatment with type I collagen and poly I:C significantly increased both MMP-13 mRNA and pro-MMP-13 protein expression in hPDL fibroblasts. In contrast, poly I:C alone increased pro-MMP-13 protein levels without a corresponding increase in mRNA, while type I collagen alone had no significant effect on either mRNA or protein expression. It should be noted that the ELISA employed in this study quantified pro-MMP-13 rather than the enzymatically active form; therefore, the functional matrix-degrading capacity of MMP-13 under these conditions cannot be directly inferred.

Moreover, the finding that poly I:C alone increased MMP-13 protein levels without a parallel rise in mRNA suggests the involvement of post-transcriptional regulation mechanisms in TLR3 signaling. This is consistent with previous reports emphasizing the importance of mechanisms such as mRNA stability, translational efficiency, or protein degradation in modulating protein expression.[43,44] Alternatively, MMP-13 transcription may occurs transiently at an earlier time point, with protein accumulation becoming evident at later stages.[18] However, the present analyses were conducted at a single time point, the temporal dynamics of MMP-13 transcription, secretion, and activation could not be fully characterized. Time-course experiments will therefore be required to clarify these regulatory processes.

These in vitro findings underscore the need for further investigation into the interplay between ECM components and TLR3-mediated signaling pathways in regulating MMP-13 expression in hPDL fibroblasts. Type I Collagen, acting through integrins[4] and receptor tyrosine kinases,[7] and poly I:C, via TLR3,[36] may converge on shared downstream pathways such as MAPK and TRIF signaling, to regulate MMP-13 expression.[7,19,29,34,45,46] Notably, in contrast to studies conducted in other cell types or within 3D collagen matrices,[19,25,29,34,36] neither stimulus alone significantly increased MMP-13 mRNA expression in the present 2D model. This discrepancy may reflect the limitations of simplified 2D model,[47] which may not fully recapitulate the mechanical and structural complexity of the periodontal microenvironment.

Accordingly, further studies incorporating multiple time points, assessments of enzymatically active MMP-13, and more physiologically relevant models such as 3D matrices or mechanically loaded systems will be required to validate and extend the mechanistic insights obtained in this study.

5. Conclusion

In summary, the present study demonstrates an interaction between ECM cues and innate immune activation in regulating MMP-13 expression in hPDL fibroblasts. Type I collagen was found to promotes cell migration, a process relevant to periodontal tissue remodeling, whereas poly I:C, a TLR3 agonist, modulates inflammatory signaling and increased pro-MMP-13 production, particularly in the presence of type I collagen. Importantly, these findings indicate a regulatory role rather than direct evidence of enhanced matrix degradation, highlighting the complexity of PDL remodeling in which ECM and inflammatory signals act in concert.

Patient's/Guardian's consent

The authors declare that this manuscript has no need of Patient's/Guardian's consent. This work is in vitro study.

Ethics clearance

This research was performed in compliance with the Declaration of Helsinki and received approval from the Srinakarinwirot University Human Research Ethics Committee (SWUEC/E−333/2563, SWUEC-116/2565X).

Author contributions

ST and PC contributed to the conception and design of the study. RN, ND, and ST conducted the project. PC, ND, and ST interpreted the results. RN drafted the initial manuscript. PC, ND, and ST critically revised the manuscript. All authors approved the final version for submission.

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Declaration of competing 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 paper.

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None.

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