Human Umbilical Cord Mesenchymal Stem Cells and their Extracellular Vesicles Modulate Pro- and Anti-inflammatory Cytokines in Ligature-induced Periodontitis

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ABSTRACT

Background: Periodontitis is a chronic inflammatory condition that affects the tissues supporting the teeth, ultimately leading to tooth loss. Mesenchymal stem cells (MSCs) and their extracellular vesicles (EVs) play a crucial role in periodontitis by modulating the activities of gum cells and the immune system.

Objective: To investigate the therapeutic potential of human umbilical cord mesenchymal stem cells (hUCSCs) and EVs in regulating the inflammatory response associated with periodontitis.

Methods: hUCSCs were isolated, subjected to flow cytometry analysis of surface markers, and differentiated into adipocyte and osteocyte. hUCSC-EVs were isolated and characterized using flow cytometry and electron microscopy. A periodontitis animal model was established in 30 female C57Bl/6 mice. Experimental groups received hUCSCs or hUCSCs-EVs, or vehicles intravenously. Animals were monitored for 4 weeks, and the periodontal tissues were used to assess the effects of hUCSCs and hUCSCs-EVs on the expression of pro- (TNF-α, IFN-γ, and IL-17a) and anti-inflammatory cytokines (TGF-β, IL-10, and IL-4). The secretion of these cytokines by splenocytes was also evaluated using ELISA.

Results: The levels of IL-17a, IFN-γ, and TNFα significantly reduced, while TGF-β and IL-10 significantly increased in the periodontal tissues of the hUCSC and hUCSCEVs-treated mice. The expression of TNF-α, IFN-γ, and IL-17a significantly decreased, while the production of IL-10 and TGF-β significantly increased in splenocytes from the hUCSC and EVs-treated mice.

Conclusion: hUCSCs and their EVs have the potential to attenuate the inflammatory response associated with periodontitis, possibly by downregulating pro-inflammatory cytokines and upregulating anti-inflammatory ones.

Keywords: Anti-Inflammatory Cytokine, Extracellular Vesicles, Mesenchymal Stem Cells, Periodontitis, Pro-Inflammatory Cytokine

INTRODUCTION

Periodontitis is a persistent inflammatory condition that impacts the tissues supporting teeth, ultimately causing tooth loss (1). It is driven by an excessive inflammatory response to dental plaque biofilm accumulation. Conventional non-surgical treatment aims to reduce inflammation through the removal of plaque and calculus, but periodontitis often recurs due to an abnormal immune response (2, 3). New regenerative treatments are required to control inflammation and promote tissue regeneration (4).

In recent years, there has been extensive research focusing on the potential utility of stem cells in addressing inflammatory conditions. Mesenchymal stem cells (MSCs) specifically have attracted considerable interest due to their immunomodulatory and reparative capacities (5). These cells can rapidly multiply and are sourced from diverse tissues like umbilical cord, adipose tissue, and bone marrow. However, there are ongoing concerns associated with stem cell therapy, including the risks of immune rejection, genetic abnormalities, and the potential development of cancer (6). New evidence has demonstrated that conditioned medium containing factors secreted by MSCs can provide similar benefits as the parent stem cells (7). As membrane-bound structures, extracellular vesicles (EVs) are secreted by different cells like MSCs. They vary in size from 30-1000 nanometers in diameter and have been suggested to play important roles in mediating MSCs effects (6).

Currently, non-surgical periodontal treatment focuses on mechanical debridement to reduce the microbial load and subgingival administration of antimicrobials (8). However, inflammation often recurs due to the persistence of abnormal immune responses (9). The immunomodulatory properties of human umbilical cord mesenchymal stem cells (hUCSCs)/EVs could be harnessed as an adjunct to conventional treatment to modulate the dysfunctional inflammatory response underlying periodontitis recurrence (10). MSCs show promise as a more effective treatment that could potentially achieve full periodontal tissue regeneration (11). MSC-EVs also affect periodontitis by influencing the behaviors of immune cells, periodontal cells, and the local environment. EVs have a role in controlling the biological activity of immunological and periodontal tissue cells (12). Through these effects on multiple cell types and the microenvironment, EVs work to promote the repair of periodontal injury and the regeneration of periodontal tissues (4). However, the effect of MSCs and MSC-EVs in modulating the pro- and anti-inflammatory cytokines in periodontitis remains unclear. Dental treatments like periodontal work and orthodontics are often done together, especially for certain dental issues (13). Evaluating how MSCs and EVs affect the immune system could potentially lead to a new approach to orthodontic treatment (14). Since periodontal and orthodontic procedures frequently complement each other (15), particularly when managing specific oral health problems, researching the immunomodulatory effects of MSCs and EVs may additionally provide an innovative path for correcting misaligned teeth. The current work aimed to investigate the modulation of anti- and pro-inflammatory cytokines by hUCSCs and their EVs using a ligation-induced periodontitis model. The results provide insights into the immunomodulatory mechanisms of hUCSCs and hUCSC-EVs and their potential utility as a cellular or secretome-based therapy for periodontitis.

MATERIALS AND METHODS

Isolation and Characterization of hUCSCs

Approximately 20 cm sections of umbilical cord were obtained from full-term deliveries of healthy mothers who provided informed consent. The collected umbilical cord sections were chopped into smaller 2 cm pieces after washing with phosphate-buffered saline

(PBS). These pieces were then further cut into smaller 1-3 mm3 sections and digested for one hour at 37ºC using collagenase type II and 0.125% trypsin in PBS. By mixing in 10% fetal bovine serum (FBS), the enzymatic reaction was halted, and then filtered and centrifuged for 5 min at 1500 rpm. After that, the suspension was combined with 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% FBS in low-glucose Dulbecco's Modified Eagle Medium (L-DMEM). For 48 h, the cells were cultured in T75 flasks filled with DMEM and maintained at 37°C with 5% CO_2. The culture medium was refreshed every 3 days. Trypsin/EDTA was used to detach the cells once the cell cultures reached 80% confluence, and the cells were then moved to fresh flasks for growth (16). For characterization, cells from passages 3-5 were surface stained with fluorophore-conjugated markers CD105, CD45, and CD73 and analyzed by flow cytometry. A FACSCalibur flow cytometer was used to collect the data, and FlowJo software (version 10, USA) was used for the analysis. Osteogenic and adipogenic differentiation potential was also assessed by culturing the cells for 21 days in differentiation-inducing media and staining with Alizarin Red and Oil Red O, respectively.

Isolation and Characterization of hUCSC-EVs

For isolating EVs from hUCSCs, previously established methods were followed using cells from passages 3-5. When the hUCSCs reached 85-90% confluency, the cells were cultivated for 48 h in serum-free media after their normal culture medium containing FBS was replaced. The prepared medium was gathered and treated to separate the EVs. Initially, the medium underwent centrifugation at 4°C for 20 min at 500 ×g to eliminate cells. Subsequently, the resulting liquid was subjected to additional centrifugation for 30 min at 18,000 ×g to collect cellular debris. The remaining solution passed through a 0.22 μm filter and was then ultracentrifuged for 90 min at 120,000 ×g. The pelleted EVs were suspended in PBS. For characterization of the isolated hUCSC-EVs, flow cytometry was used to detect surface markers CD9 and CD63, electron microscopy examined size and morphology, and the Bradford quantified total protein concentrations.

Ligature-induced Periodontitis animal Model and Treatment Protocol

Thirty female C57Bl/6 mice, aged 9-10 weeks, were kept in a controlled environment with a temperature of 24°C and regulated light/dark cycles. They were provided with sterile food and water in specific pathogen-free conditions. All animal protocols were approved by the Affiliated Hospital of Tangshan Vocational and Technical College, Tangshan, Hebei Province, China, ensuring minimal animal suffering. The study adhered to the National and International Guidelines for animal care and use. Induction was performed based on the study of Abe et al. (17). Briefly, all animals were first anesthetized by a mixture of xylazine (10 mg/kg) and ketamine (100 mg/kg) intraperitoneally. Using a 5-0 silk suture, their teeth in the mandible were ligated. The suture was threaded through the interdental spaces between the second and third molar teeth using Dumont forceps, then through the spaces between the first and second molars. Subsequently, the suture was looped around the second molar tooth, any slack was removed with suture-tying forceps, and it was securely fastened with a triple knot. The excess suture was trimmed using spring scissors.

The mice were monitored over two weeks to allow the development of periodontitis. After periodontitis development, the mice were divided into three groups, each consisting of ten mice: a control group that received PBS, hUCSC-treated group that received 1×10^6 hUCSCs, and hUCSC-EVs-treated group that received 100μg hUCSC-EVs. Subgingival injection of treatment was performed around the ligated tooth.

Preparation of Splenocytes

After a four-week period following ligation, the mice were anesthetized using xylazine (10 mg/kg) and ketamine (100 mg/kg). The spleens from mice in different groups were extracted. Small splenic tissue fragments were filtered through a 70µm cell strainer with a syringe plunger. After being washed with Roswell Park Memorial Institute (RPMI-1640) media, the cells were centrifuged for five min at 4°C at 400 ×g. To remove red blood cells, a lysis buffer was added and the mixture was incubated at 37°C for 2 min. After centrifuging again at 4°C for 5 min at 400 ×g, the supernatant was removed, and the cell pellet was suspended in complete RPMI media with 1% penicillin/ streptomycin and 10% FBS.

Cytokine Assay

In a 24-well culture plate, splenocytes were collected and cultivated at a concentration of 2 million cells per well. They were stimulated for 48 h using phytohemagglutinin (PHA). After stimulation, the supernatants containing secreted proteins were collected from the wells. The concentrations of various cytokines (including interleukin (IL)-10, IL-4, IL-17a, interferon-gamma (IFN-γ), transforming growth factor-beta (TGF-β), and tumor necrosis factor-alpha (TNF-α)) were assessed using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, California). All samples were tested in triplicate measurements to improve accuracy.

mRNA Levels in the Gingival Tissue

To assess the expression levels of inflammatory and anti-inflammatory cytokines, gingival tissue was extracted at four weeks post-ligation. Total RNA was isolated from the tissue using a BioFACTTM Total RNA Prep Kit. The concentration of RNA was measured using a NanoDrop. Using a cDNA Reverse Transcription Kit, cDNA was generated from 1µg of total RNA, and its purity was confirmed with NanoDrop. Specific primers for cytokines and the reference gene β-actin were designed and validated through Primer-BLAST NCBI online software. Real-time RT-PCR was conducted in triplicate on the cDNA using SYBR® Green PCR Master Mix to assess the expression of five genes: IL-17a, IL-10, TGF-β, IFN-γ, TNF-α, and IL-4. β-actin was utilized as the internal control gene. Gene expression was determined relative to the control using the $2^{-\Delta\Delta CT}$ method. Primer design for the genes was accomplished using AlleleID software, v 7.8, and the sequences of primer are detailed in Table 1.

Statistical Analysis

The Kolmogorov-Smirnov Z test was

Table 1. The sequence of primers used in the current study.

Primer name	Sequence (5'→3')
IL-4	Forward: 5'-AGTTGTCATCCTGCTCTTCTT-3'
	Reverse: 5'- TGTGGTGTTCTTCGTTGCT-3'
IL-10	Forward: 5'- GCTATGCTGCCTGCTCTT-3'
	Reverse: 5'- CAACCCAAGTAACCCTTAAAGT-3'
IL-17a	Forward: 5'- GACTCTCCACCGCAATGA-3'
	Reverse: 5'- ACACCCACCAGCATCTTC-3'
IFN-γ	Forward: 5'- AAAGAGATAATCTGGCTCTGC-3'
	Reverse: 5'- GCTCTGAGACAATGAACGCT-3'
TNF-α	Forward: 5'- GTGGAACTGGCAGAAGAG-3'
	Reverse: 5'- TTGAGAAGATGATCTGAGTGT-3'
TGF-β	Forward: 5'- ATTCCTGGCGTTACCTTG
	Reverse: 5'- GTATTCCGTCTCCTTGGTTC
β -actin	Forward: 5'- GGCTGTATTCCCCTCCATCG-3'
	Reverse: 5'- CCAGTTGGTAACAATGCCATGT-3'

IL: interleukin, IFN-γ: interferon-gamma, TNF-α: tumor necrosis factor-alpha, TGF-β: transforming growth factor-beta

performed to determine whether the data distribution was normal. Comparisons between groups were made using the Kruskal-Wallis test. Data were presented as mean±standard deviation, with significance determined for *p*-values below 0.05. IBM SPSS Statistics for Windows, Version 24.0, was employed for statistical analyses, and GraphPad Prism 8 software was utilized for generating graphs.

RESULTS

Characterization of hUCSCs

The characterization of hUCSCs was performed using flow cytometry and differentiation staining. Flow cytometry confirmed hUCSCs were positive for surface markers CD73 and CD105 and negative for CD45 (Figs. 1A, 1B, and 1C). Morphology appeared fibroblast-like and adherent (Fig. 1D). Lipid droplets indicating adipogenic differentiation were detected by Oil Red O staining after 21 days (Fig. 1F). After 21 days, calcium deposits were visible as a result of osteogenic differentiation as visualized by Alizarin Red staining (Fig. 1E).

Characterization of hUCSC-EVs

The characterization of hUCSC-EVs was done using flow cytometry and electron microscopy. Flow cytometry showed expression of CD9 (Fig. 2A) and CD63 (Fig. 2B) on hUCSC-EV surfaces. Electron microscopy provided additional verification of the size and structure of EVs (Fig. 2C).

The Effects of hUCSC and hUCSC-EVs Therapy on the Balance of Anti- and Pro-Inflammatory Cytokines

The research examined the immunomodulatory effects of treating ligation-induced periodontitis mice with hUCSC and hUCSC-EVs. Pro- and anti-inflammatory cytokine expression was analyzed using real-time RT-PCR. Compared with the control mice, a significant downregulation of IFN-γ, IL-17a, and TNF-α mRNA levels in the hUCSC and hUCSC-EVs treated group were observed ($p<0.01$, $p<0.001$,
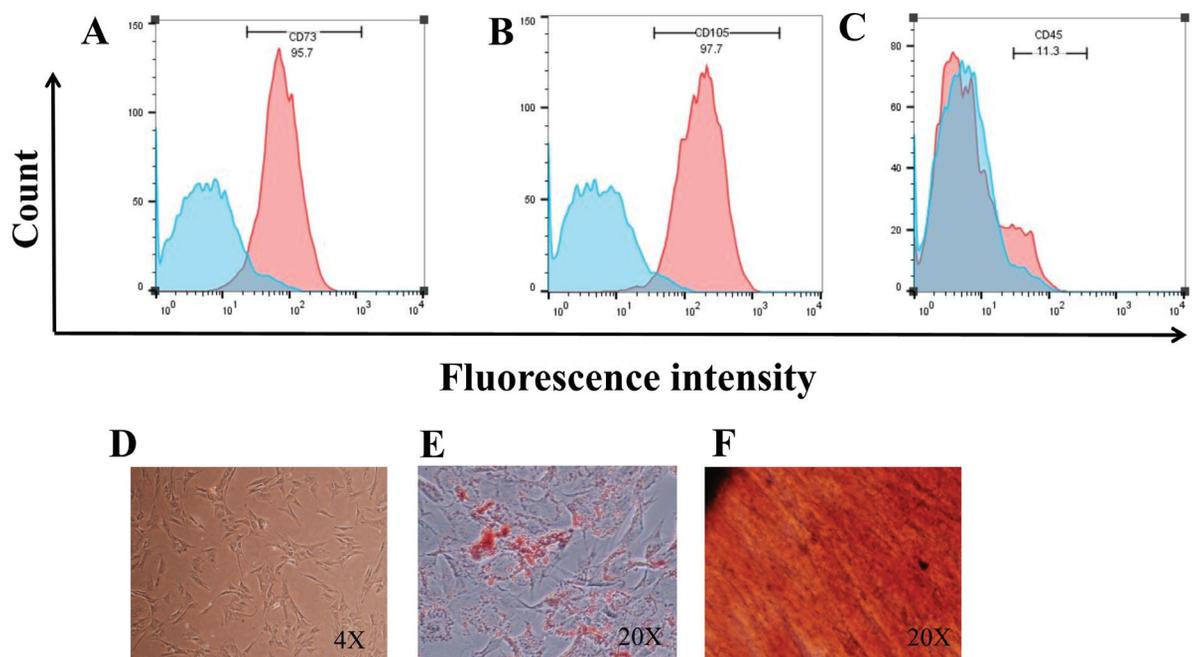

Fig. 1. Characterization of hUCSCs. Flow cytometry analysis for hUCSCs confirmed the expression of the mesenchymal stem cell markers CD73 (A) and CD105 (B) and the lack of expression of the hematopoietic marker CD45 (C). The hUCSCs exhibited an adherent, fibroblast-like shape when viewed under the microscope (D). They demonstrated the ability to undergo osteogenic differentiation as shown by positive staining with Alizarin red (E). The cells also showed adipogenic differentiation potential through positive staining with Oil red O (D). hUCSC: human umbilical cord mesenchymal stem cell
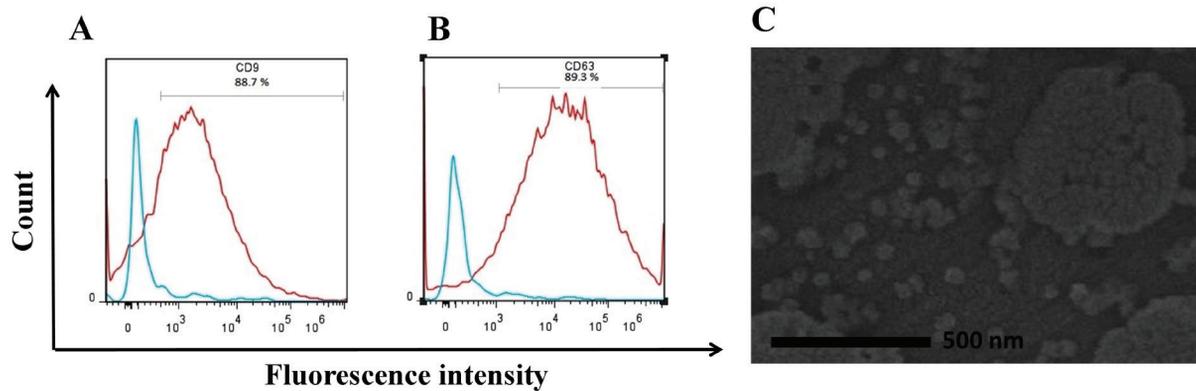
Fig. 2. Characterization of hUCSC-EVs. Flow cytometry demonstrated the expression of the exosomal markers CD9 (A) and CD63 (B). Electron microscopy also confirmed the size and cup-shaped morphology of the EVs (C). hUCSCs: human umbilical cord mesenchymal stem cells; EVs: extracellular vesicles

and $p<0.001$ respectively). Conversely, TGF-β and IL-10 mRNA upregulated significantly in the hUCSC and hUCSC-EVs mice in comparison with the controls ($p<0.01$ and $p<0.001$ respectively). There were no notable alterations found in IL-4 expression among the groups. Additionally, the study observed no significant alterations in cytokine expression among hUCSC-EVs and hUCSC mice. In other words, for all the cytokines measured,

there was no statistically significant effect of treatment with the whole stem cells versus treatment with just the extracellular vesicles released by those stem cells (Fig. 3).

The Impact of hUCSC and hUCSC-EVs Therapy on Splenocyte Cytokine Secretion

To demonstrate the beneficial effects of hUCSC and hUCSC-EVs therapy on cytokines, we analyzed anti-inflammatory (IL-4, TGF-β,
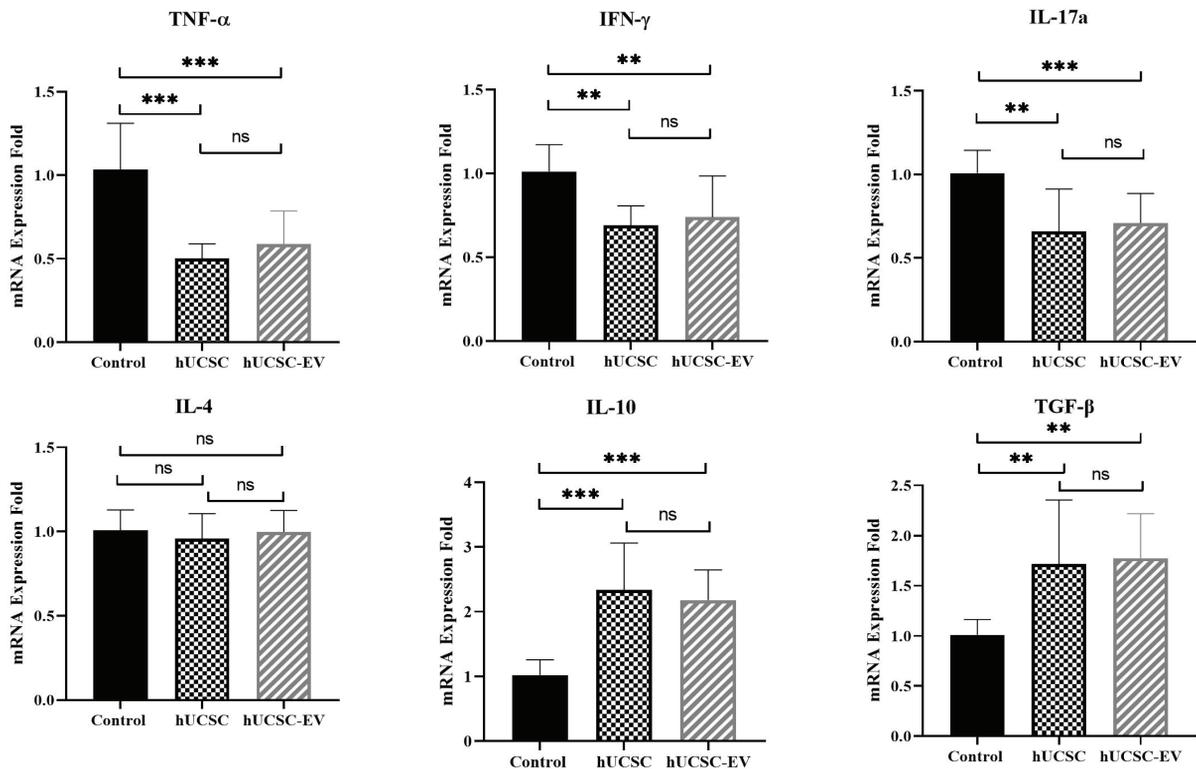

Fig. 3. Differential expression of the pro-/anti-inflammatory cytokine genes in the gingival tissue of experimental groups. Data are shown as mean±SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$. TNF: tumor necrosis factor; SD: standard deviation; IL: interleukin; IFN: interferon; hUCSC-EV: human umbilical cord mesenchymal stem cell-extracellular vesicle

Fig. 4. Analysis of the secretion of pro-/anti-inflammatory cytokines by splenocytes in experimental groups. Data are shown as mean±SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$. TNF: tumor necrosis factor; SD: standard deviation; IL: interleukin; IFN: interferon; hUCSC-EV: human umbilical cord mesenchymal stem cell-extracellular vesicle
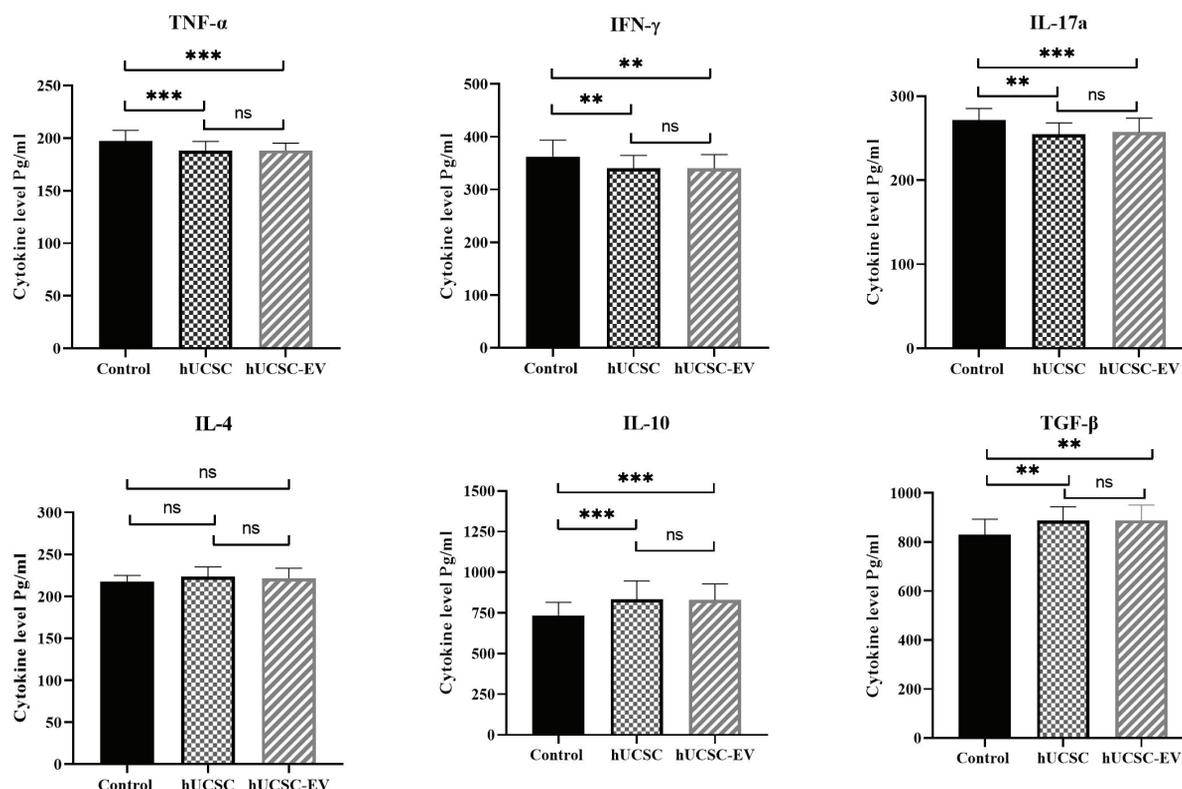
IL-10) and pro-inflammatory (IL-17a, IFN-γ, TNF-α) cytokine levels in splenocyte cultures restimulated with PHA. In the treated mice, the expression of IL-17a, IFN-γ, and TNF-α significantly reduced ($p<0.001$, $p<0.01$, and $p<0.001$ respectively) in comparison with the controls. The mRNA levels of the anti-inflammatory cytokines (TGF-β and IL-10) notably increased in hUCSC-Exo-treated mice versus the controls ($p<0.01$ and $p<0.001$ respectively). The alteration in IL-4 production by splenocytes between the groups was not significant. The research also found that for all of the cytokines examined, there was no statistical difference in the levels of cytokines between the mice that received treatment with hUCSC and mice that received treatment with hUCSC-EVs (Fig. 4).

DISCUSSION

The current study suggests novel insights

into the immunomodulatory mechanisms of hUCSCs and their EVs in periodontitis. The results demonstrated that both hUCSCs and hUCSC-EVs have the ability to upregulate anti-inflammatory cytokines and downregulate pro-inflammatory cytokines in a mouse model of ligature-induced periodontitis. The immunomodulation appears to be mediated through shifting the cytokine balance away from a pro-inflammatory Th1/Th17 response toward an anti-inflammatory Th2 response (18). This modulation of the cytokine milieu indicates a therapeutic potential for attenuating excessive inflammation associated with periodontal disease. These findings are consistent with prior studies emphasizing the immunomodulatory characteristics of MSCs and their EVs (19, 20).

A few key findings support this conclusion. First, gene expression analysis revealed that hUCSC and hUCSC-EV treatments suppressed mRNA expression of primarily pro-inflammatory cytokines IL-

17a, IFN-γ, and TNF-α in gingival tissue. Meanwhile, an increase was observed in anti-inflammatory cytokines IL-10 and TGF-β. Similar trends were observed when measuring cytokine secretion directly from splenocyte cultures. These gene and protein level changes corroborate each other and demonstrate hUCSCs and their EVs can robustly modulate both local periodontal and systemic inflammatory responses.

The downregulation of IL-17a, IFN-γ, and TNF-α is especially noteworthy given their prominent roles in driving periodontitis pathology. Overproduction of these cytokines leads to the excessive recruitment and activation of immune cells including neutrophils, leading to tissue destruction (21). Therefore, decreasing their levels through hUCSC-EV therapy could help resolve inflammation and promote healing (22).

Besides suppressing pro-inflammatory cytokines, enhancing anti-inflammatory TGF-β and IL-10 is therapeutically beneficial. IL-10 prevents excessive or prolonged inflammatory activity to prevent collateral tissue damage (23). TGF-β promotes wound healing, angiogenesis, and tissue regeneration (24). Thus, hUCSC-EV modulation appears to achieve a balanced cytokine profile that is simultaneously anti-inflammatory and pro-resolving/reparative. The unchanged expression of IL-4 across treatments indicates that this cytokine might not be the mediator for the therapeutic impacts of hUCSCs and EVs.

The results also carry significance in comprehending the mechanisms involved in MSC immunomodulation. While other studies have investigated paracrine factors secreted by MSCs in periodontitis (25), this work establishes EVs derived from hUCSCs are equally effective in influencing inflammation as the parent cells. EVs are emerging as important vehicles for transferring proteins, mRNAs, and microRNAs between cells (26). They likely mediate hUCSC effects by modulating recipient cell transcription, protein expression, and signaling pathways involved in controlling cytokine production (27).

Overall, this study provides a strong preclinical rationale for using hUCSCs or their EVs as a cellular/secretome-based therapeutic to regulate periodontal inflammation. This could benefit not only periodontitis but also inflammatory complications during orthodontics (28). In orthodontics, periodontal inflammation is a risk factor for accelerated tooth movement and increased root resorption during orthodontic tooth movement (29). Using hUCSCs or their EVs could help reduce unwanted side effects by attenuating the inflammatory response to orthodontic forces. This may allow for safer orthodontic treatments by preventing excess root resorption in patients prone to periodontal inflammation. The ease of administering EVs over direct stem cell therapy also makes this a clinically translatable approach (30).

A notable strength was utilizing both gene expression and direct cytokine measurement assays. However, future studies could interrogate specific intracellular signaling pathways modulated in immune cells. One of the limitations of the current research is that the animal model used in this study does not fully represent the complexity of human periodontitis. In addition, the exact mechanism behind the observed effects remains unclear. Future studies could focus on understanding these mechanisms and exploring the long-term effects and safety of hUCSC and EV treatment.

CONCLUSION

In conclusion, this work provides an exciting initial validation of hUCSCs and their EVs as a potential new regenerative therapeutic approach for periodontitis. Their impressive ability to restore balance between pro- and anti-inflammatory cytokines through paracrine mechanisms warrants further investigation as an adjunct or alternative to current antimicrobial therapies. With additional preclinical development and optimization, hUCSC/EV immunotherapy

holds promise to revolutionize periodontal disease management and outcomes.

AUTHOR'S CONTRIBUTION

XW designed the study and performed the experimental protocols, data analysis, interpretation, and writing and editing the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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