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Original Article

Dampened inflammatory response in oral ulcer after topical therapy of adipose mesenchymal stem cell secretome

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الملخص

أهداف البحث: أثبتت الأبحاث أن تعديل الالتهاب يمكن أن يسرع بشكل كبير من شفاء قرح الفم. تركز دراستنا على إفراز الخلايا الجذعية الوسيطة الدهنية، وهو غني بجزيئات التنظيم المناعي القادرة على تثبيط الاستجابة المناعية والتدخل في المسارات الالتهابية. ونحن نهدف إلى تقييم كل من تعبيرات المسار الالتهابي والأنماط الظاهرية للبلاعم في مواقع تقرحات الفم.

طريقة البحث: تم تحفيز حدوث تقرحات الفم في الغشاء المخاطي للقبو السفلي لعشرين من فئران ويستار الذكور الأصحاء. تم علاج هؤلاء الأشخاص موضعيا باستخدام جل إفراز الخلايا الجذعية الوسيطة الدهنية عن طريق الفم ثلاث مرات يوميا لمدة تتراوح بين ثلاثة وسبعة أيام. أجرينا تحليلات كيميائية مناعية لتقييم التعبير عن "تي إل آر-4" و "إن إف كابا بي 65" في مواقع القرحة. بالإضافة إلى ذلك، قمنا بتقييم استقطاب البلاعم من خلال فحص نسبة البلاعم إم1/إم2 ، التي تم تحديدها من خلال خلايا سي دي 68 (إم1) وخلايا سي دي 163 (إم2). تم تحليل البيانات باستخدام أنوفا أحادي الاتجاه، متبوعا باختبار توكي للفرق الهام بصدق.

النتائج: تطبيق جل إفراز الخلايا الجذعية الوسيطة الدهنية عن طريق الفم قلل بشكل كبير من التعبير عن "تي إل آر-4" و "إن إف كابا بي 65". عزز هذا العلاج أيضا استقطاب البلاعم نحو النمط الظاهري "إم2" المضاد للالتهابات في مواقع القرحة.

الاستنتاجات: التطبيق الموضعي لجل إفراز الخلايا الجذعية الوسيطة الدهنية عن طريق الفم ينظم بشكل فعال الاستجابة الالتهابية، ويعزز عمليات الشفاء في نموذج الفئران لقرحة الفم. وهذا يشير إلى فائدته المحتملة كعامل علاجي في إدارة تقرحات الفم.

الكلمات المفتاحية: قرحة الفم؛ تعديل المناعة؛ الخلايا الجذعية الوسيطة؛ طب الأسنان؛ التئام الجروح؛ الطب التجديدي

Abstract

Objectives: Research has demonstrated that modulating inflammation can significantly accelerate the healing of oral ulcers. Our study focused on the adipose mesenchymal stem cell secretome (AdMSCS), which is rich in immunoregulatory molecules capable of dampening the immune response and interfering with inflammatory pathways. We assessed both inflammatory pathway expression and macrophage phenotypes at the sites of oral ulcers.

Methods: We induced oral ulcers in the inferior fornix mucosa of 20 healthy male Wistar rats (*Rattus norvegicus*). These subjects were treated topically with adipose MSC metabolite (AdMSCM) oral gel three times daily, for durations of 3 and 7 days. We performed immunohistochemical analyses to evaluate the expression of Toll-like receptor 4 (TLR4) and nuclear factor kappa B (NF-κB) p65 at the ulcer sites. Additionally, we assessed macrophage polarization by examining the ratio of M2/M1 macrophages, identified through $CD68^{+}\Phi$ (M1) and $CD163^{+}\Phi$ (M2) cells. Data were analyzed using one-way

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analysis of variance, followed by post-hoc Tukey's Honestly Significantly Difference test.

Results: Application of AdMSCM oral gel significantly reduced the expression of TLR4 and NF-κB p65. This treatment also enhanced macrophage polarization towards the anti-inflammatory M2 phenotype at the ulcer sites ($p < 0.05$).

Conclusion: The topical application of AdMSCM oral gel effectively modulates the inflammatory response, enhancing healing processes in the oral ulcer rat model. This suggests its potential utility as a therapeutic agent in managing oral ulcers.

Keywords: Dentistry; Immunomodulation; Mesenchymal stem cell; Oral ulcer; Regenerative medicine; Wound healing

Introduction

Oral ulceration is characterized by the complete loss of epithelium accompanied by a variable loss of the underlying connective tissue, resulting in a crateriform appearance. Ulcers are the final common manifestation of a spectrum of immune-mediated diseases or traumatic origins.[1,2] The management of these types of ulcers primarily aims to reduce symptoms and accelerate tissue restoration.[3] Among various strategies, controlling inflammation through the use of immunosuppressive drugs is often employed by general dentists and oral medicine clinicians.[1,4] Topical steroid therapy serves as the predominant first-line treatment for various ulcerative oral diseases of inflammatory origin. However, in cases of long-standing ulcers or highly recurrent lesions, prolonged immunosuppressive therapy becomes necessary. The prolonged application of topical corticosteroids is discouraged due to their potential adverse effects including mucosal atrophy, persistent erythema, and local opportunistic infection.[2,5,6] Furthermore, topical immunosuppressive drugs have limited efficacy in inducing the essential processes during the proliferative phase of wound healing. This condition underscores the imperative for an alternative therapeutic approach with minimal side effects and heightened efficacy in promoting wound healing.

The mesenchymal stem cell secretome (MSCS) represents a novel approach in wound healing management.[7] This technology arises from the new paradigm in the regenerative mechanism of mesenchymal stem cells transplantation, and it is suspected that their main mechanism is by releasing bioactive factors instead of directly replacing damaged cells.[8] Various bioactive factors contained in the MSCS have the capacity to target and modulate various pathways in the wound healing process. One of the prominent effects of these factors is their immunomodulatory capacity, particularly MSC cells (MSCs) sourced from adipose tissue.[9] MSCs are able to produce immunomodulatory molecules during culture, such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), interleukin 4 (IL-4), IL-10, IL-12, and hepatocyte growth factor (HGF), that can be harvested by isolating their culture medium.[10] These factors act via a paracrine mechanism to downregulate the pro-inflammatory gene expression and cytokine production of immune cells, potentially accelerating the resolution of the inflammatory phase.[11,12] This is crucial in facilitating the phenotype switching of several cells into a pro-regenerative phenotype, especially macrophages. The polarization of macrophages from M1 to M2 phenotype is crucial due to M2 function in producing anti-inflammatory cytokines and growth factors to induce mucosal healing.[13] Furthermore, our previous study reported that the topical application of adipose MSC metabolite (AdMSCM) oral gel greatly upregulated the expression of several key growth factors in the proliferative phase of oral ulcer healing. Specifically, AdMSCMs reduce the activity of matrix metalloproteinase (MMP) while increasing the expression of growth factors, such as vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF-2), and epidermal growth factor (EGF), and the crucial expression of proliferative transcription factors SRY-box 2 (Sox2) and Ki-67.[14] A previous study reported that the great regenerative potency of MSCS, pro-regenerative factors of MSCS such as VEGFA, FGF-2, EGF, and antimicrobial agents in MSCS could further support wound healing processes.[8]

Despite the promising potential of MSCs in wound healing, the clinical application of MSCs-derived therapeutic products is still limited. To bridge this gap, our research investigated the immunomodulatory potential of MSCs, particularly those derived from adipose tissue, in an animal oral ulcer model. The results of this study may support development of the AdMSCS into clinically relevant drugs for human use in various wound models, including oral ulcerations.

Materials and Methods

This laboratory experiment obtained ethical approval under Certificate Number 635/HRECC.FODM/V/2023 from the Ethical Clearance Commission at the Faculty of Dental Medicine, Universitas Airlangga (Surabaya, Indonesia). Our use of animals in the experimental process adhered to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and our institution's animal ethical protocols. Additionally, our study adhered to the 3Rs (Replace, Reduce, Refine) to ensure high standards of animal welfare, described as follows. Replacement: Although the use of an animal model is necessary for clinically relevant outcomes, we utilized *in vitro* methods where possible to minimize animal use. Reduction: We optimized our experiment to use the minimum number of animals required for statistical validity, thereby reducing the overall number of animals used. Refinement: Procedures were refined to minimize pain and distress, including the use of anesthesia and analgesia. All handling was performed by trained personnel, and humane endpoints were established to prevent unnecessary suffering. All experimental procedures were approved by the Institutional Animal Care and Use Committee and conducted according to the latest guidelines, ensuring a commitment to ethical practices and animal welfare.

AdMSCM oral gel preparation

The AdMSCM oral gel was prepared following the protocol outlined in our previously published work by Wicaksono et al.[3] (Figure 2C). Briefly, after the fourth passage of AdMSC culture, the culture medium was collected. Subsequently, the culture medium was centrifuged at 1500 rpm for 3 min to remove debris and dead cells. Following this, the culture medium was filtered through a 0.45 μm syringe filter to isolate the bioactive substances released by AdMSCs during their growth. Finally, the purified culture medium was combined with 5% hydroxypropyl-methylcellulose (HPMC) at a volume ratio of 1:3.

Experimental animal groups

This research included 20 Wistar rats (*Rattus norvegicus*) that fulfilled specific criteria: aged 1–2 months, weighing 250–300 g, and lacking oral or systemic pathologies. To ensure the animals were disease-free, the veterinarian conducted clinical assessments on the animals during the acclimatization period (2 weeks). All animals underwent an oral ulcer induction procedure, using an 8 g/3 mm punch biopsy instrument (Premier Medical Products Co., Plymouth Meeting, PA, USA) and precision dissection at the base with No. 15 surgical blades. Clinical evaluation at 24 h post-punch biopsy confirmed the presence of oral ulcers, which were identifiable by a white lesion surrounded by an erythematous border. The animal subjects were categorized into the following four groups (Figure 2A, B).

1. Positive control group: Animals underwent the oral ulcer induction procedure using the punch-biopsy technique, followed by topical application of 5% HPMC gel for 3 days (C3) and 7 days (C7). Each group comprised five rats.
2. Treatment group: Animals in this group underwent the oral ulcer induction procedure using the punch-biopsy technique, followed by topical application of AdMSCM gel for 3 days (T3) and 7 days (T7). Each group comprised five rats.

Euthanasia of the animal models was performed through cervical dislocation after a single intraperitoneal injection of 50 mg/kg pentobarbital solution (Cat no: P-010; Sigma–Aldrich, St. Louis, MO, USA) systemically, administered within the range of 20–40 mL, just before euthanasia.[15]

Proteomic analysis

In this investigation, an immunohistochemical approach was utilized to assess the expression of inflammation-related biomarkers. The process of tissue collection involved excising both the ulcer and adjacent healthy tissue through excisional biopsy, followed by processing into formalin-fixed paraffin-embedded specimens. Subsequently, the tissue was sectioned into histological slides using a rotary microtome. The primary focus of the study was to evaluate the expression of nuclear factor kappa B (NF-κB) p65, Toll-like receptor 4 (TLR4), cluster of differentiation 68 (CD68) (M1), and CD163 (M2) in tissue specimens using immunohistochemistry staining. Horseradish peroxidase-labeled secondary antibodies including NF-κB p65 polyclonal antibody (#51-0500; Invitrogen, Carlsbad, CA, USA), TLR4 polyclonal antibody (#48-2300; Invitrogen), CD68 monoclonal antibody (#A22329; ABclonal, Woburn, MA, USA), and CD163 polyclonal antibody (#A8383; ABclonal) were employed. Following this, two observers examined protein expression, identified by a brown precipitate, in oral ulcer sites under a light microscope at 100×, 400×, and 1000× magnifications across five fields of view.

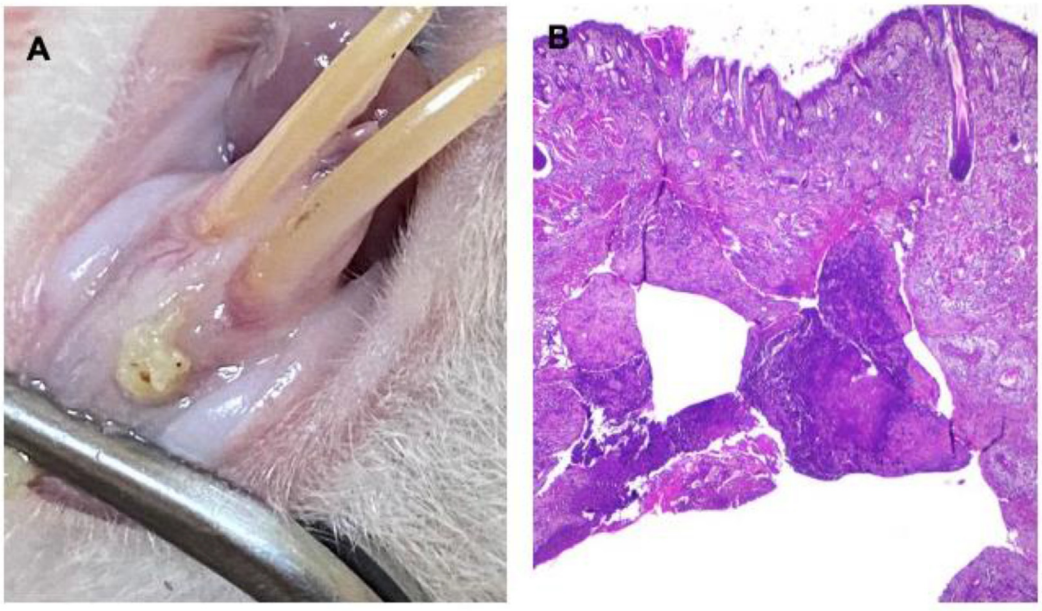

Figure 1: (A) Clinical photograph of an oral ulcer 24 h after induction. (B) Histological assessment confirmed the formation of an ulcer on the oral mucosa following surgical induction.

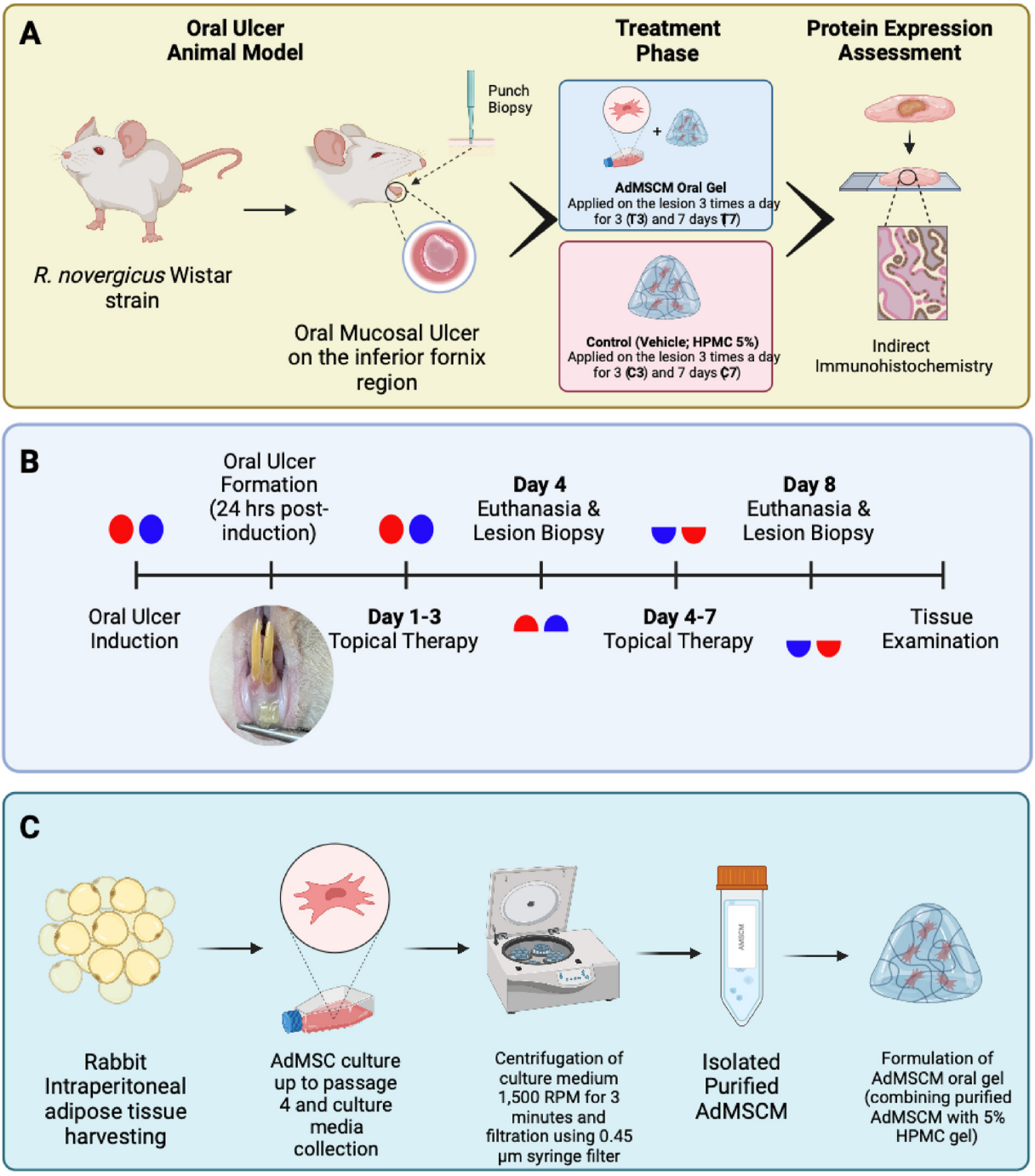

Figure 2: Illustration of the study protocol. (A) *In vivo* experiment depicting oral ulcer modeling and topical treatment with AdMSCM oral gel, followed by immunohistochemical assessment of the tissue. (B) Study timeline outlining key experimental milestones. (C) Formulation process of the AdMSCM oral gel.

Statistical analyses were performed using GraphPad Prism 8.0 (for MacBook, version 9.4.1; San Diego, CA, USA). The normality test was performed using the Shapiro–Wilk test, and homogeneity was assessed using Levene's tests. In our study, we employed a comparative analysis using analysis of variance (ANOVA) with a significance level set at $p < 0.05$.

Results

Topical application of AdMSCM oral gel downregulates the expression of NF-κB and TLR4

A histopathological view of NF-κB and TLR4 expression patterns at the oral ulcer site can be observed in Figure 1A to

B. Positive protein expression was indicated by brown precipitates in the macrophages at the ulcer site. According to the results of one-way ANOVA, statistically significant differences were found between all groups ($p = 0.0001$). Subsequent post-hoc Tukey's Honestly Significant Difference analysis demonstrated that topical therapy with AdMSCM oral gel reduced NF-κB expression on the 7th day of the study but not on the 3rd day. Meanwhile, TLR4 expression was significantly reduced in the AdMSCM group on both observation days ($p < 0.05$). In summary, the topical application of AdMSCM oral gel greatly inhibited activation of the pro-inflammatory cytokine production pathway, particularly those involving the TLR4 and NF-kB pathways. These findings suggested a reduced clinical inflammatory state in the rat oral ulcer model (see Figure 3).

Topical therapy with AdMSCM oral gel enhances the M2/M1 MΦ ratio at the ulcer site

In this study, we analyzed macrophage polarization by calculating the M2/M1 MΦ ratio. The numbers of CD68+ macrophages (M1MΦ) and CD163+ macrophages (M2MΦ) were determined in four power fields (at 100× magnification) within the ulcer site. The M2/M1 MΦ ratio was approximately 0.26 ± 0.08 and 0.88 ± 0.3271 in the control group on the 3rd and 7th days of the study, respectively. Meanwhile,

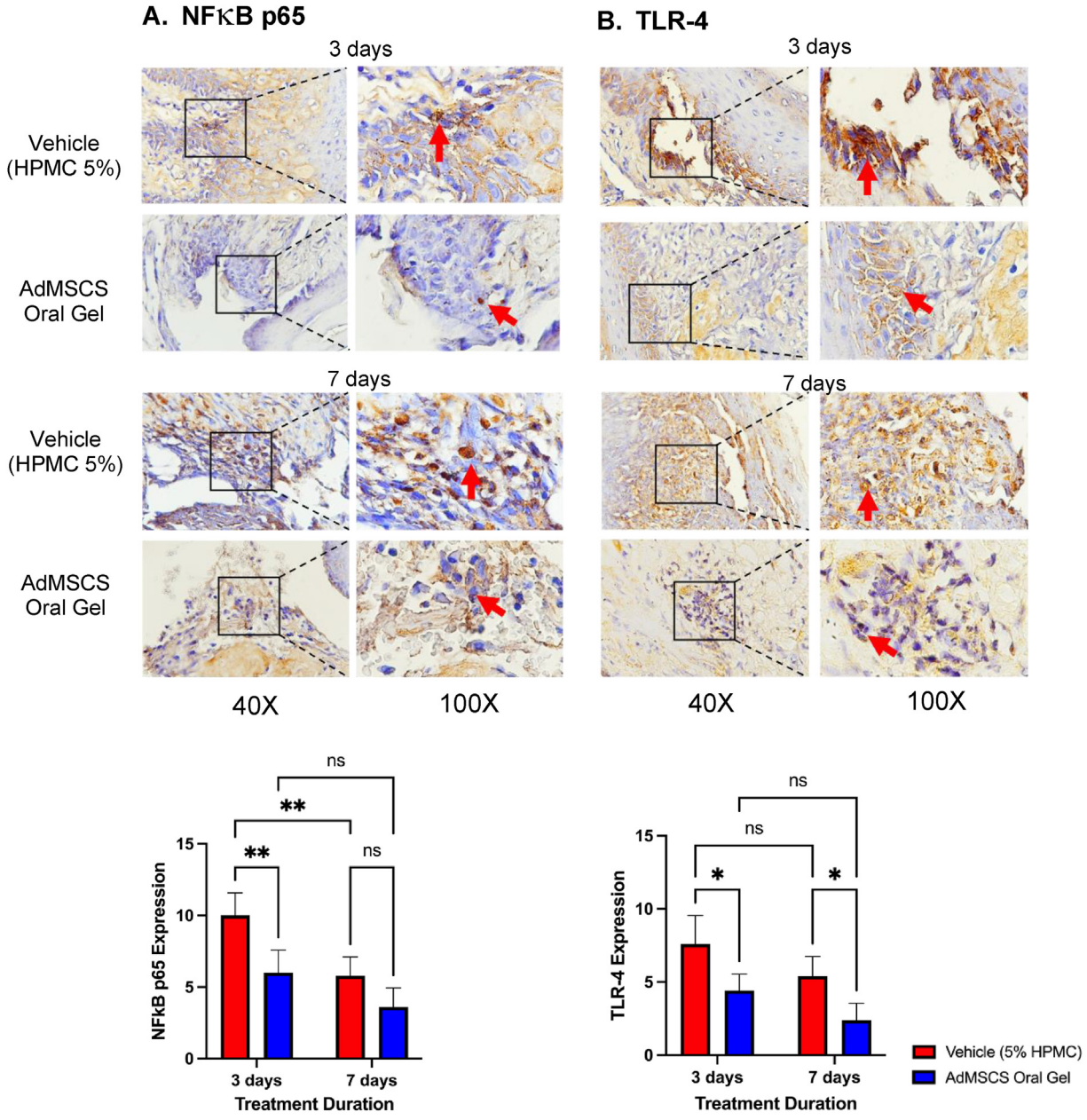

Figure 3: Analysis of inflammatory parameters in the ulcerated area. (A) Topical application of the AdMSM oral gel resulted in the significant reduction in NF-κB p65 expression within the first 3 days of treatment. (B) Topical application of the AdMSCM oral gel consistently reduced TLR4 expression on both days of observation ($p < 0.05$).

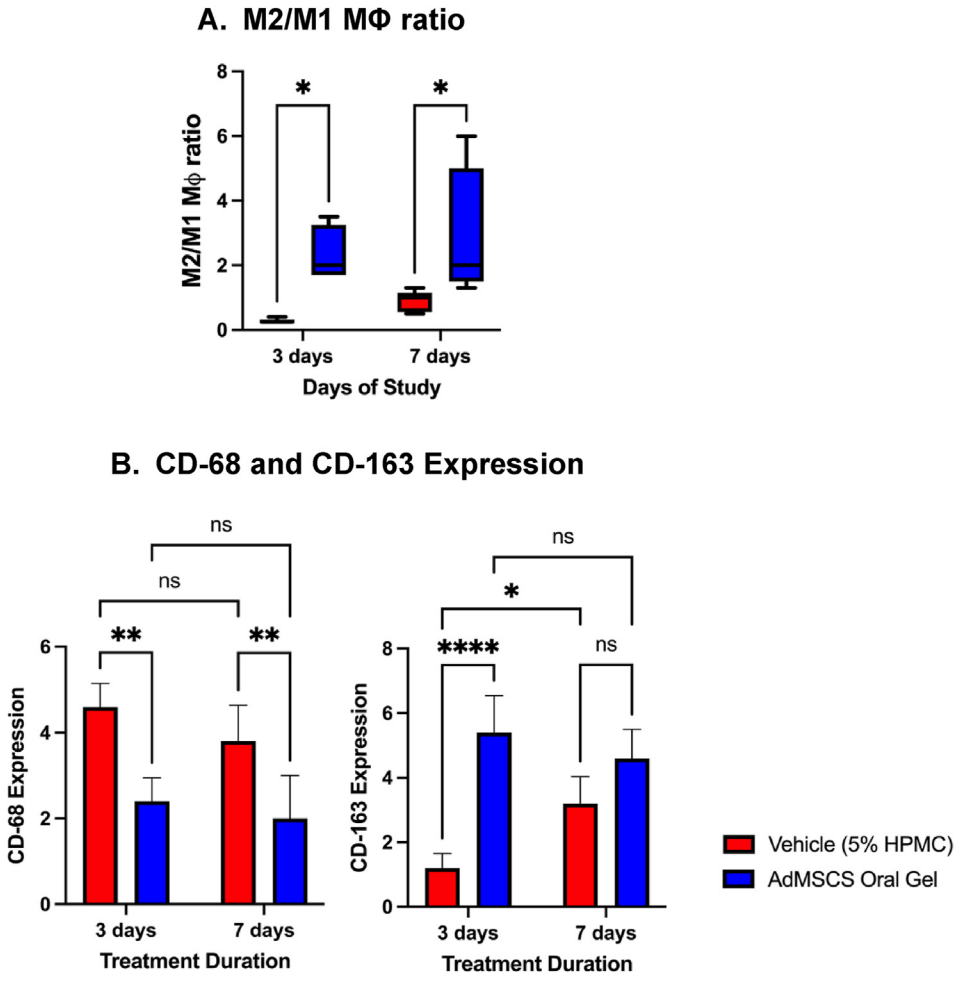

Figure 4: Expression of biomarkers for M1 and M2 macrophage phenotypes, CD68 and CD163. (A) The M2/M1 macrophage (MΦ) ratio was significantly higher in the group treated with AdMSCM oral gel compared to the control group. (B) The graphs show a decrease in CD68+ macrophages and an increase in CD163+ macrophages in the ulcerated area following topical therapy with AdMSCM oral gel, relative to the control group ($p < 0.05$).

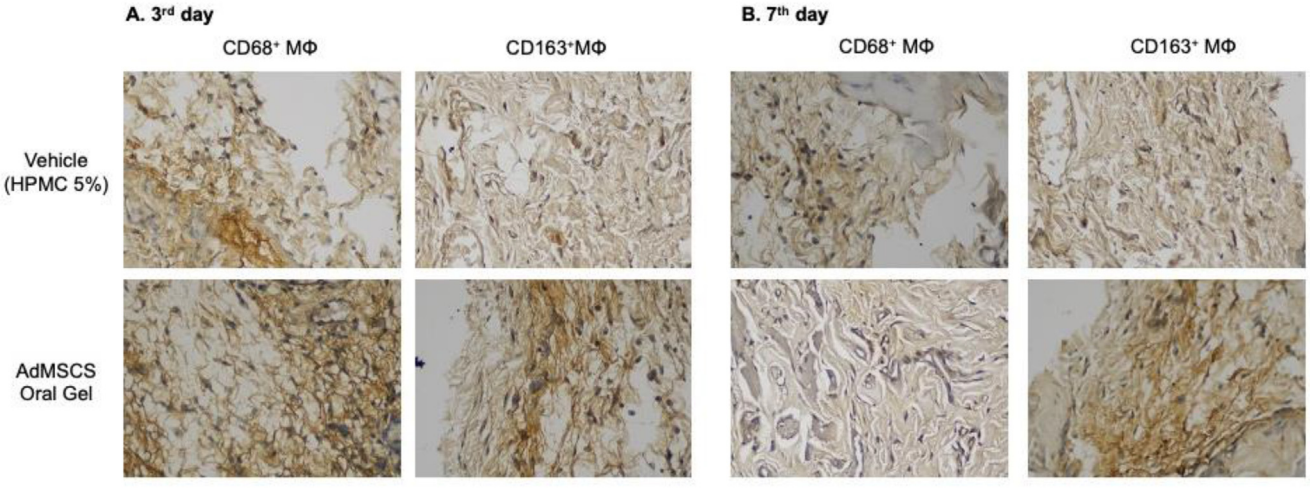

Figure 5: Immunohistochemical slides of $CD68^{+}\Phi$ and $CD163^{+}\Phi$ in the area of oral ulcer (100× magnification).

the M2/M1 MΦ ratio in the AdMSCS group was 2.38 ± 0.8228 and 3.0 ± 1.974 on the 3rd and 7th days of the study, respectively. Further comparative analysis demonstrated a significantly higher M2/M1 MΦ ratio in the AdMSCS oral gel group on both days of the study ($p < 0.05$). These results indicate that topical therapy with AdMSCM oral gel significantly increased wound healing capacity by enhancing the polarization of macrophages into pro-proliferative properties (see Figures 4 and 5).

Discussion

The inflammatory phase plays a pivotal role in facilitating the formation of an optimal microenvironment for tissue restoration. This biological mechanism is mediated by immune cells such as neutrophils and macrophages for debridement and pathogen clearance at the wound site.[16] In the event of injury, danger molecules released by damaged cells, called damage-associated molecular patterns (DAMPs), are sensed by receptors expressed on cells called pattern recognition receptors (PRRs), which initiate an inflammatory response.[17] Among the PRRs, TLR4 reportedly has significant involvement in the early phase of wound healing. A previous study reported that TLR4 is highly upregulated after incisional skin wounds in wild-type mice.[18] This receptor plays a significant role in immune activation after injury by distinguishing between DAMPs (e.g., extracellular high mobility group box 1 [HMGB1]) and pathogen-associated molecular patterns (e.g., lipopolysaccharides), and then activating the main transcription factor for pro-inflammatory cytokine synthesis, namely NF-κB.[17,19] Activation of this pathway initiates the production of proinflammatory cytokines, such as IL-1 and tumor necrosis factor alpha (TNF-α), resulting in activation of the immune response. The physiological immune response is pivotal in maintaining optimal wound healing. However, an aberrant inflammatory response may lead to delayed ulcer healing process, resulting in highly morbid long-standing oral ulceration.

In wound therapy, immunomodulatory strategies control immune responses after tissue damage by providing an anti-inflammatory microenvironment. Our study highlights the potent immunomodulatory capacity of topical therapy with AdMSCM oral gel, marked by dampening of the inflammatory response at the oral ulcer site. Immunohistochemical analysis has demonstrated the significant downregulation of TLR4 and NF-κB p65 compared to the vehicle group. These findings correspond to a previous study by Wang et al.[20] that described the decreased IL-6 expression in the dermal wound of diabetic mice treated with the AdMSCS. The immunomodulatory action of the AdMSCS may be mediated by anti-inflammatory cytokines (e.g., IL-1Ra, IL-4, IL-13, IL-10, transforming growth factor beta [TGF-β]), microRNAs, and resolution-associated molecular patterns such as IDO and PGE2.[21,22] The anti-inflammatory cytokines can directly inhibit the activation immune response or induce the release of anti-inflammatory molecules. For instance, IDO can induce T-cell anergy by depleting tryptophan concentration and stimulating macrophage polarization to the M2 phenotype, thereby upregulating IL-10 production.[23,24]

In the process of physiological wound healing, macrophages undergo a transition from an initial proinflammatory state, marked by the release of various substances such as reactive oxygen species, nitric oxide, IL-1, IL-6, TNF-α, and heightened phagocytic activity typical of M1-like behavior, to a resolution phase characterized by an M2-like polarization. This phase involves the secretion of anti-inflammatory cytokines such as IL-10, VEGF, FGF-2, HGF, and MMPs responsible for extracellular matrix remodeling.[21,25,26] Our study found that the topical application of AdMSCM oral gel significantly suppressed M1 macrophage activation and promoted M2 macrophage polarization. The event of macrophage polarization is an important biomarker in the resolution of the inflammatory phase of wound healing and transition to the proliferation phase. Our findings correspond to a study of Park et al.,[26] where the topical application of AdMSCS significantly reduced $CD68^{+}$MΦ and increased $CD163^{+}$MΦ at the dermal wound site.[26] Moreover, a previous study by Sicco et al.[27] reported that MSC-extracellular vesicles exert regulatory effects on inducing macrophage polarization to M2 phenotype by downregulating IL-23 and IL-22.[27] This finding provides new insights into the mechanism by which AdMSCM oral gel improves the process of oral ulcer healing reported by our previous study.[3]

One limitation of this study is that it did not directly compare the efficacy of AdMSCM oral gel with currently available therapies, such as topical steroids. Furthermore, the assessment of biomarkers of wound healing relied solely on immunohistochemistry. This semi-quantitative method may have introduced biases. Therefore, investigating the efficacy of AdMSCM oral gel in healing oral ulcers through alternative assessment modalities would be beneficial. Despite these limitations, our findings strongly suggest that this formulation is ready to progress to more advanced *in vivo* studies.

Conclusion

Accelerating the resolution of the inflammatory phase is an effective strategy for efficient wound healing. Our study demonstrated the efficacy of AdMSCM oral gel in modulating inflammation, which was characterized by downregulation of the main inflammatory pathway in the early phase of wound healing, namely, the TLR4/NF-κB pathway, and increasing the rate of macrophage polarization to the anti-inflammatory and pro-reparative M2 phenotype.

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

This study received ethical approval on May 31, 2023 under Certificate Number 635/HRECC.FODM/V/2023

from the Ethical Clearance Commission at the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

Author contributions

SW: Investigation, Methodology, Study Design, Writing – Original Draft, Formal Analysis, Visualization; NN: Investigation, Formal Analysis, Writing – Review & Editing; HS: Investigation, Formal Analysis, Writing – Review & Editing; APN: Study Design, Methodology, Validation, Supervision; DSE: Conceptualization, Funding Acquisition, Project Administration, Supervision. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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