Hyaluronic Acid-Astaxanthin Gel: Anti-Inflammatory Effects in Oral Ulcer Diabetes Mellitus Rats

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Abstract

This study was designed to determine the anti-inflammatory effect of a combination of high-molecular-weight hyaluronic acid (HMW-HA) and astaxanthin gel on oral ulcers in diabetes mellitus rats through NF-κB, IL1β, and IL-10 expression. Forty oral ulcer diabetic rats were used as experimental animals and divided randomly into 4 large groups (basic gel treatment (BT); HMW-HA 0.2 % gel treatment (HAT), astaxanthin 1 % gel treatment (AXT), and HMW hyaluronic acid 0.2 % and astaxanthin 1 % gel treatment (HAXT)). They were treated for 3 and 7 days (5 rats per group). The anti-inflammatory effect was measured by immunohistochemical examination of NF-κB, IL1β, and IL-10 expression. The Kruskal-Wallis test was used to analyze the data, followed by a post hoc Mann-Whitney test ($p < 0.05$). The NF-κB expression on day 3 showed a significant difference between BT (5 ± 0.50). and HAXT (3 ± 0.50) ($p < 0.05$); IL1β expression showed no significant differences between BT (4 ± 1.00) with all groups ($p > 0.05$); IL-10 expression showed a significant difference between BT (2 ± 1.00) with AXT ($3 \pm 0,50$) and HAXT ($5 \pm 1,00$) ($p < 0.05$). The NF-κB expression on day 7 showed a significant difference between BT ($5 \pm 0,50$) with HAXT ($2 \pm 1,00$) ($p < 0.05$); IL1β expression showed a significant difference between BT (3 ± 1.00) with HAXT (1 ± 0.50); HAXT (1 ± 0.50) with HAT (2 ± 0.50) and AXT (2 ± 0.50) ($p < 0.05$); IL-10 expression was significantly different between BT (3 ± 1.00) with AXT (4 ± 0.50) and HAXT (7 ± 0.50); AXT (4 ± 0.50) and HAXT (7 ± 0.50) ($p < 0.05$). A combination of HMW hyaluronic acid and astaxanthin gel on oral ulcers has an anti-inflammatory effect seen from reduced NF-κB, IL1β expression, and elevation of IL-10.

Keywords: Hyaluronic acid, Astaxanthin, Anti-inflammation, Oral ulcer, Diabetes mellitus

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by inadequate insulin synthesis and hyperglycemia [1]. This condition leads to several complications such as poor circulation, tissue oxygenation, infection susceptibility, impaired white blood cell function, and nerve damage. Consequently, patients with diabetes may experience delayed wound healing [2]. Diabetes-related wound healing impairments occur owing to decreased neutrophil and macrophage activities. A misalignment between M1

(classically activated macrophages) and M2 (alternatively activated macrophages) polarization results in elevated production of pro-inflammatory cytokines like IL-1β and TNF-α by M1 cells that contribute to activation of nuclear factor kappa B (NF-κB) for inflammation. Moreover, M2 macrophages reduce the levels of anti-inflammatory cytokines, including IL-10 [3,4].

An *in vivo* study using a diabetic rat model showed markedly delayed wound healing in the oral mucosa [5]. One study documented the presence of chronic oral ulcers in patients diagnosed with diabetes mellitus [6]. Oral ulceration is a prevalent lesion that is frequently observed and left untreated. It is characterized by disruption of the oral mucosa resulting from injury to both the epithelium and lamina propria. They also occur in patients with diabetes mellitus. One study revealed that ulcerative lesions were predominant in the oral mucosa, with a prevalence of 24.6 % in traumatic and aphthous ulcers in patients with diabetes mellitus type 1 and 2 [7]. Another study at one hospital in India reported that traumatic ulcers in patients with type 2 diabetes accounted for 2.7 % of all incidents in the oral cavity [8].

Based on the number of cases and the possibility of delayed healing in patients with diabetes, therapy is required to alleviate symptoms and accelerate wound healing. The topical application of corticosteroids, anesthetics, and analgesics is an effective ulcer therapy that minimizes the local immune response, thus avoiding subsequent infections. However, prolonged use of these medications may result in oral flora imbalance, secondary yeast infections, and drug resistance [9]. Ulcer management with 0.2 % hyaluronic acid resulted in successful re-epithelialization of superficial wounds and excellent patient recovery and tolerance [10]. The topical application of a high-molecular-weight hyaluronic acid (HMW-HA) gel reduces the pain intensity in SAR without side effects and is easy to apply [11]. HMW-HA promotes tissue hydration, osmotic equilibrium, and extracellular matrix integrity. However, they cannot infiltrate deeper dermal layers; therefore, topical effects are more effective [12]. This suggests the addition of natural substances, such as astaxanthin, to boost the effect of treating oral ulcers, especially in diabetic conditions.

Astaxanthin is a xanthophyll carotenoid with strong antioxidant properties, which can reduce inflammation, oxidative stress, and apoptosis [13]. Astaxanthin exhibits superior biological activity compared to other antioxidants because of its ability to penetrate and bind to cell membranes from the intracellular side [14]. Astaxanthin exerts its anti-inflammatory effects by targeting biomarkers and several signaling pathways, notably by blocking NF-κB to mitigate inflammation [13,15]. The anti-inflammatory and antioxidant abilities of astaxanthin may be mediated by reducing M1 polarization, increasing M2 polarization, and triggering the Nrf-2/HO-1 antioxidant pathway by generating a negligible quantity of reactive oxygen species [16,17]. Previous studies have shown that topical administration of 1 % astaxanthin gel (Haematococcus pluvialis) improved wound healing of traumatic ulcers by reducing neutrophils and increasing macrophages, fibroblasts, and collagen density on days 3 and 7 compared to the 0.5 % dose [18].

Considering the anti-inflammatory advantages of hyaluronic acid, which can accelerate wound healing, along with the anti-inflammatory and antioxidant benefits of astaxanthin, which may enhance its ability to repair wounds, it is crucial to determine when these 2 components should be combined. This combination has never been studied before. Thus, more research is needed to confirm their anti-inflammatory properties. This study was designed to investigate the anti-inflammatory effects of HMW-HA and astaxanthin gels on oral ulcers in rats with diabetes mellitus by examining NF-κB, IL1β, and IL-10 expression.

Materials and methods

This study employed an experimental laboratory design using a post-test-only control group approach to evaluate the effects of different gel treatments on oral ulcers in diabetic rats. The subjects of our study were 40 male Wistar strain *Rattus norvegicus* rats, with) weighing approximately 200 - 250 g. The rats were randomly allocated to 4 groups, each comprising 5 replicates: BT, basic gel treatment group (control); HAT, hyaluronic acid 0.2 % gel treatment; AXT, astaxanthin 1 % gel treatment; and HAXT, a combination of hyaluronic acid 0.2 % and astaxanthin 1 % gel treatment group. All groups were treated for 3 or

7 days. The sample calculation was based on Sharma *et al.* [19] for 2-variable experiments. The feasibility of this study was evaluated by the Commission for Ethical Clearance in Health Research of the Faculty of Dental Medicine, Universitas Airlangga (no. 1211/HRECC. FODM/X/2023).

Gel preparation

Topical astaxanthin and hyaluronic acid gels were prepared by mixing these ingredients with a gel base containing Hydroxy Propyl Methil Cellulose (HPMC) 2 % hydroxypropyl methylcellulose. We chose a gel-based formulation based on the studies by Shahare *et al.* [20] and Tribhuvan *et al.* [21] by replacing carbapol 934 with HPMC. HPMC was selected over Carbopol 934 due to its biocompatibility, biodegradability, pronounced effects on drug release, and excellent mucoadhesive characteristics [22], which may improve the retention time of the gel on the ulcer surface. Hydroxypropyl methylcellulose (HPMC) was added to methylparaben, propylparaben, and propylene glycol to obtain the gel base. Next, Astaxanthin (Evergen, Indonesia) 1 % or high molecular weight Hyaluronic acid (Sigma-Aldrich, USA) 0.2 % is added to the gel base.

Treatment of experimental animals

The experimental animals were first allowed to adapt for 7 days in a cage with sufficient room temperature, air, and lighting. The experimental animals were weighed and their glucose levels were measured. Before induction, the animals were fasted for 8 - 12 h. The streptozotocin (STZ) dose of 50 mg/kg BW intraperitoneally was selected based on Parisihni *et al.* [23] as it effectively induces hyperglycemia while minimizing mortality. To prevent acute hypoglycemia, 10 % dextrose monohydrate was administered orally overnight. dissolved in citrate buffer injected intraperitoneally, and 10 % dextrose monohydrate was administered orally overnight. Four days later, we checked the glucose level and declared diabetes mellitus if the blood sugar was >200 mg/dL, made ulcers on experimental animals by anesthetizing them first using *ketamine* (0.1 cc/100 gr BW) and *xylazine* (0.1 cc/100 gr BW) intramuscularly. The lower lip mucosa of the rats was smeared with 3 - 10 % *povidone-iodine* using a sterile cotton bud. An ulcer was created in the central area of the rat's lower labial mucosa using a *punch* biopsy with a diameter of 2 mm and a depth of 0.5 mm. Observations were made when ulcers formed, and therapy was administered 1 - 24 h after treatment.

Sample collection

Samples were collected on days 3 and 7 with a biopsy performed on the ulcerated lip under the influence of ketamine and xylazine anesthesia, after which the rats were sacrificed according to ethical procedures. The collected samples were then preserved in a 10 % formalin buffer solution for the preparation of immunohistochemical (IHC) assessment, as described by Santi *et al.* [24]. The tissue samples were sectioned to a thickness of 0.3 - 0.5 mm and prepared for paraffin block preparation. The tissue within the paraffin block was subsequently sectioned to a thickness of 5 micrometers with a microtome and positioned on a glass slide. Samples were prepared for IHC staining.

NF-κB, IL1β, and IL-10 inflammasome measurement

NF-κB, IL1β, and IL-10 expression were measured with immunohistological staining (NF-κB p65 Monoclonal Antibody (bsm-33059M, Bioss); IL1β Monoclonal Antibody (IL-1 beta/IL1B Antibody (11E5): sc-52012; Santa Cruz); IL-10 Monoclonal Antibody (IL-10 Antibody (E-10): sc-8438; Santa Cruz)). The measurements were performed using a light microscope (Nikon Eclipse SiRS), supported by Nikon NIS Element software at 400× magnification. Expression was assessed by quantifying the presence of brown precipitates on macrophages in 8 selected fields of view, followed by calculation of the average value for each group.

Results and discussion

This study intends to prove the anti-inflammatory effects of the combination of HMW-HA and astaxanthin in gel formulation as a therapy for diabetic oral ulcers by evaluating NF-κB, IL1β, and IL-10 expression in macrophages. Macrophages play a crucial role in both inflammation and healing. Macrophages play a key role in healing. However, their extensive presence in wounded conditions, along with their dysregulation during repair, leads to poor wound healing and fibrosis. The inability of macrophages to transition from the pro-

inflammatory M1 phenotype to the pro-healing reparative M2 phenotype is associated with insufficient wound healing [25]. Skin wound healing improves once the inhibition of the IL-1β signaling pathway results in a decrease in pro-inflammatory M1 macrophages and an increase in anti-inflammatory M2 macrophages and growth factors [26]. NF-κB regulates
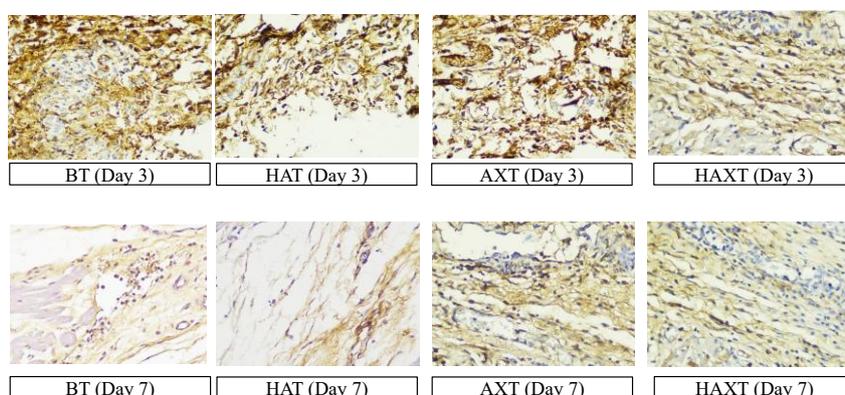
pro-inflammatory cytokines in adaptive immunity, activating immune cell infection or damage to produce cytokines like TNF-α, IL-6, and IL-1β, initiating inflammation and immune responses [27].

Figure 1 shows the expression of NF-κB, IL1β, and IL-10 in macrophages. The expression is shown in brown macrophages.
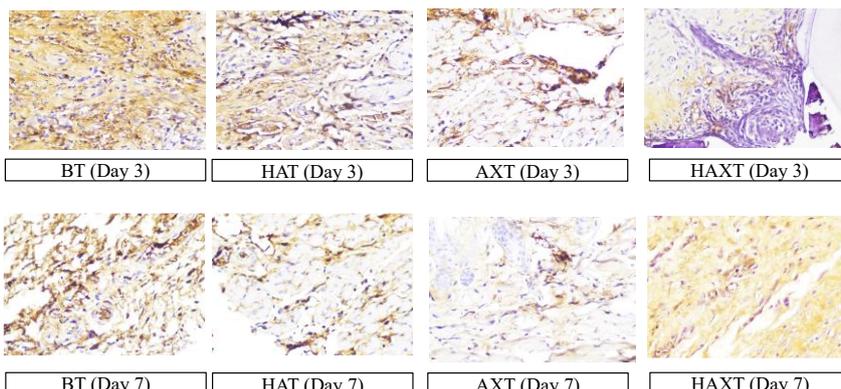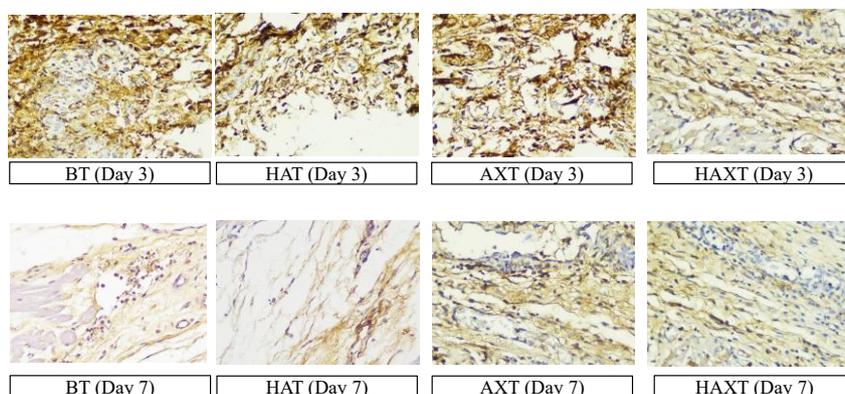

Figure 1 NF-κB, IL1β, and IL-10 in macrophages in oral ulcer shown in brown color (Immunohistochemistry staining, 400× magnification).

The results of the data analysis of NF-κB, IL1β, and IL-10 expression from all groups showed that it was not normally distributed ($p > 0.05$) both on day 3 and day 7, so the test was carried out using the non-parametric Kruskal Wallis followed by the post hoc Mann-Whitney test ($p < 0.05$). The results on day 3 are presented in **Table 1** and **Figure 2**, whereas those on day 7 are presented in **Table 2** and **Figure 3**.

Table 1 The table represents the median ± interquartile deviation of expression of NF-κB, IL1β, and IL-10 in macrophages on day 3.

Groups	n	NF-κB		IL-1b		IL-10		p
		Median ± IQD	Min-Maks	Median ± IQD	Min-Maks	Median ± IQD	Min-Maks	
BT	5	5 ± 0.50[g]	5 - 6	4 ± 1.00[e]	3 - 5	2 ± 1.00[a]	1 - 3	
HAT	5	5 ± 1.50[defg]	3 - 6	4 ± 0.50[e]	3 - 4	3 ± 0.50[ab]	2 - 3	0.000*
AXT	5	4 ± 1.00[defg]	3 - 6	3 ± 0.50[e]	3 - 4	3 ± 0.50[bcd]	3 - 4	
HAXT	5	3 ± 0.50[cd]	3 - 4	3 ± 1.00[cde]	2 - 4	5 ± 1.00[e]	4 - 6	

Note: * significant at $\alpha = 0.05$ (Kruskal-Wallis test) the same superscript [abcdefg] indicates no difference between groups (Mann-Whitney test).
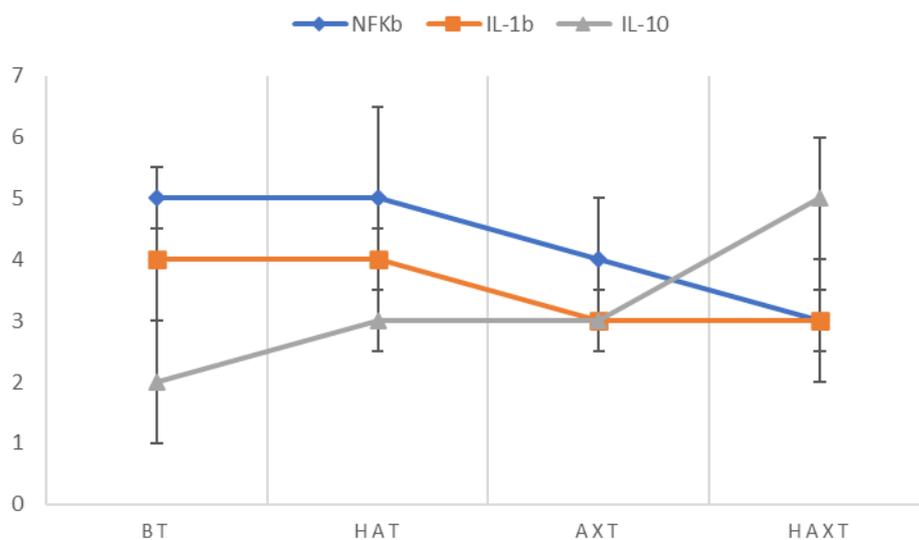

Figure 2 Graphic of the median ± interquartile deviation of expression of NF-κB, IL1β, and IL-10 in macrophages on day 3.

The Kruskal Wallis test results indicated significant differences across groups ($p < 0.05$) in the expression of NF-κB, IL1β, and IL-10 on day 3. On day 3, NF-κB expression exhibited a significant difference between BT and HAXT ($p < 0.05$), but no significant differences were seen between BT with HAT and AXT as well as between HAT and AXT ($p > 0.05$). The IL1β expression exhibited no significant differences between BT and other groups ($p > 0.05$). IL-10 expression was significantly different between BT with AXT and HAXT ($p < 0.05$); however, no significant differences were observed between BT+HAT, HAT+AXT, and HAXT, or AXT+HAXT ($p > 0.05$).

Table 2 The table represents the median ± interquartile deviation of expression of NF-κB, IL1β, and IL-10 in macrophages on day 7.

Groups	n	NF-κB		IL-1b		IL-10		p
		Median ± IQD	Min-Maks	Median ± IQD	Min-Maks	Median ± IQD	Min-Maks	
BT	5	5 ± 0.50^{efg}	4 - 5	3 ± 1.00^{de}	2 - 4	3 ± 1.00^{abcd}	2 - 4	
HAT	5	3 ± 1.00^{cde}	3 - 5	2 ± 0.50^{bcd}	2 - 3	4 ± 1.00^{cde}	3 - 5	
AXT	5	3 ± 0.50^{bc}	2 - 4	2 ± 0.50^{abc}	1 - 3	4 ± 0.50^{e}	4 - 5	0.000*
HAXT	5	2 ± 1.00^{a}	1 - 3	1 ± 0.50^{a}	1 - 2	7 ± 0.50^{f}	6 - 7	

Note: * significant at α = 0.05 (Kruskal-Wallis test) the same superscript [abcdefg] indicates no difference between groups (Mann-Whitney test).
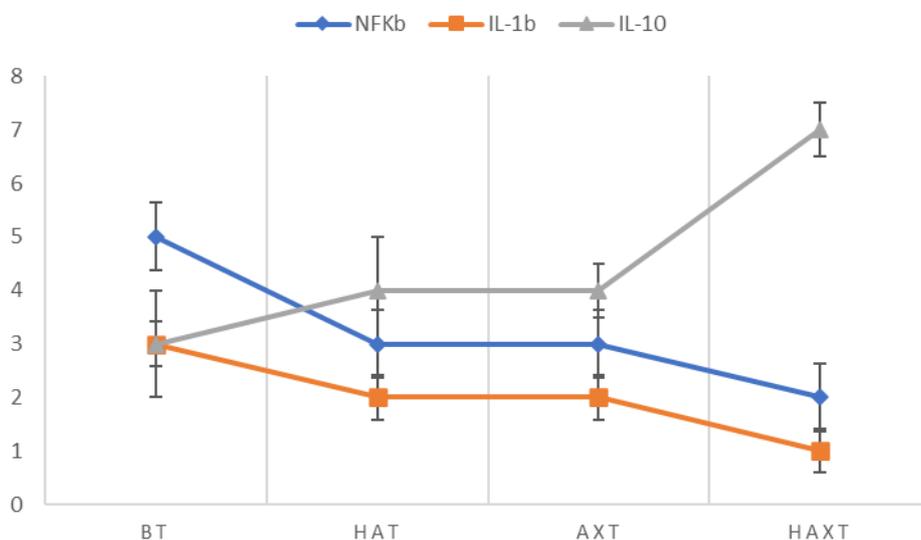

Figure 3 Graphic of the median ± interquartile deviation of expression of NF-κB, IL1β, and IL-10 in macrophages on day 7.

Consistent with day 3, the findings of the Kruskal Wallis difference test indicated significant differences between groups ($p < 0.05$) in NF-κB, IL1β, and IL-10 expression on day 7. On day 7, NF-κB expression exhibited a significant difference between BT with HAXT ($p < 0.05$), whereas no significant differences were seen between BT and HAT, as well as between HAT and AXT ($p > 0.05$). The IL1β expression exhibited a significant difference between BT with HAXT, as well as between HAXT and both HAT and AXT ($p < 0.05$). However, no significant differences were observed between BT and HAT, HAT and AXT, or AXT and HAXT ($p > 0.05$). IL-10 expression was significantly different between BT with AXT and HAXT, and AXT and HAXT ($p < 0.05$), whereas no significant differences were observed between BT and HAT, HAT, and AXT ($p > 0.05$).

The research results revealed that NF-κB expression on days 3 and 7 was significantly lower in the group receiving HMW-HA and astaxanthin combination therapy compared to the group undergoing gel base therapy, HMW-HA gel therapy, and astaxanthin gel. This indicates that the combination of HMW-HA and astaxanthin gel therapy can effectively reduce NF-κB levels. High molecular weight hyaluronic acid (>1,250 kDa) at a dose of 100 µg/mL inhibits immunological responses in both the M1 and M2 phases [28]. HMW-HA (5,000 kD) greatly decreases NF-κB activation [29,30]. Research by Wen *et al.* [30] demonstrated that astaxanthin's effect on microglia cells can inhibit the activation of the NF-κB pathway, a key

transcriptional regulator of the inflammatory response. NF-κB serves as a transcription factor in M1 macrophages, triggering several inflammatory genes, including TNF-α, IL-1β, and IL-6 [30]. The reduction in NF-κB expression due to HMW-HA and astaxanthin has an anti-inflammatory effect by lowering cytokines that lead to inflammation, potentially aiding in wound healing. Growth factors and cytokines are essential in skin healing, as they trigger inflammation followed by epidermal and dermal regeneration. Keratinocytes synthesize IL-1α and IL-1β, facilitating the proliferation, differentiation, and migration of keratinocytes, which leads to reepithelization [26].

A different investigation demonstrated a rise in the quantity of NF-κB, accompanied by pro-inflammatory cytokines in patients with metabolic syndrome disorder [31]. These findings are consistent with the present research, which discovered that IL-1β, a pro-inflammatory cytokine, decreased in parallel with NF-κB levels. This indicates that combining HMW-HA and astaxanthin gel therapy reduces IL-1β as well as NF-κB levels during ulcer healing. This could be attributed to the anti-inflammatory effects of hyaluronic acid and astaxanthin. Astaxanthin stimulates the transcription factor nuclear factor-erythroid-2 related factor 2 (Nrf2), which regulates the human body's defenses and mitigates the inflammatory response induced by NF-κB [32].

The research discovered that HMW-HA significantly elevated IL-1β and IL-8 levels in keratinocytes, indicating the potential advantages for wound treatment [33]. An investigation showed that treating LPS-stimulated macrophages with HMW-HA over 1,250 kDa (HMW-1,500) reduced the expression of pro-inflammatory genes like TNF-α, IL-6, and IL-1β [27]. Another study revealed the anti-inflammatory action of astaxanthin through the inhibition of pro-inflammatory cytokines. Astaxanthin decreased M1 polarization by reducing Il-1β mRNA levels [16]. Astaxanthin inhibits the release of pro-inflammatory cytokines (e.g., tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1β (Il-1β), monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1)) by activating reactive oxygen species (SHP-1), suppressing NF-κB expression and IkappaB kinase (IκB) degradation [28].

The anti-inflammatory effect can be observed not only via a reduction in inflammatory markers but also through the elevation of anti-inflammatory cytokines, including IL-10. Our research findings indicated that the administration of a gel combining hyaluronic acid and astaxanthin enhanced the expression of the anti-inflammatory cytokine IL-10 on day 3 compared to the other groups. This was similar to that observed on day 7, although the amount of IL-10 was the same as that in the astaxanthin gel group. Lee *et al.* [28] revealed that HMW-HA increased the expression of genes associated with anti-inflammatory responses (M2 phenotype), including IL-10. The mechanism of IL-10 enhancement by astaxanthin has also been studied. Pan *et al.* [34] showed an increase in IL-10 and M2 macrophages in rats with acute myocardial infarction rats given astaxanthin therapy.

NF-κB plays a vital role in the pro-inflammatory response of macrophages, a critical component of the immune system. Certain conditions induce diverse macrophage states: M1 macrophages release pro-inflammatory cytokines, leading to inflammation, whereas M2 macrophages generate anti-inflammatory substances, assisting in wound healing and inflammation resolution [35]. M1 macrophages persist in diabetic wounds for an extended duration, hindering progression from the inflammatory to the proliferative phase and impairing the overall wound healing process. Dysregulation of the NF-κB pathway may impede diabetic wound healing by triggering aberrant angiogenesis, proliferation, and remodeling [36]. Considering the negative effects of NF-κB activation may be critical for establishing successful therapies to accelerate diabetic wound healing.

NF-κB regulates the pro-inflammatory activity of macrophages. When activated, macrophages can differentiate into 2 types: M1 macrophages, which produce pro-inflammatory cytokines such as IL-1, IL-6, IL-12, TNF-α, and chemokines; and M2 macrophages, which produce anti-inflammatory cytokines such as IL-10 and IL-13, essential for wound healing [35]. Our findings confirm the concept, indicating a decrease in NF-κB expression, followed by a decrease in IL-1β expression and an increase in IL-10 with combination therapy HMW-HA and astaxanthin. Considering that delayed wound healing mostly results from sustained NF-κB activation and insufficient IL-10, the

combination of HMW-HA and astaxanthin may serve as a potential therapeutic approach to enhance ulcer healing. This study has limitations, including the absence of gene analysis related to NF-κB expression, the antioxidant effect of astaxanthin via Nrf-2, lack of clinical examination data, pre-clinical tests, or tests on human subjects. Therefore, further research is needed regarding the effects of this combination therapy at the molecular level with gene expression analysis, pre-clinical trials, and human subject testing.

Conclusions

The results indicate that the combination of HMW hyaluronic acid 0.2 % and astaxanthin 1 % gel demonstrates anti-inflammatory effects. This is evidenced by the reduced expression of NF-κB and IL-1β, accompanied by an elevation in IL-10 levels. The anti-inflammatory effect on day 3 was evidenced by the reduction of NFkb expression and the increase of IL-10 expression compared to the baseline control, HMW-HA, and astaxanthin. On day 7, a decrease in NF-κB and IL-1β expression and an increase in IL-10 expression were observed compared to the baseline control, HMW-HA, and astaxanthin. The combination of HMW hyaluronic acid and astaxanthin gel seems promising as a product that can accelerate wound healing. Further investigation is required to reveal the mechanism of these 2 materials, such as the antioxidant mechanism through the Nrf2 pathway, gene expression analysis, pre-clinical trials, and human subject testing.

Acknowledgments

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