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WORD COUNT	3358	TIME SUBMITTED	20-OCT-2022 03:26PM
		PAPER ID	91764231

Name of Journal: *World Journal of Diabetes*

Manuscript NO: 79456

Manuscript Type: MINIREVIEWS

Hyperbaric oxygen therapy and chemokine administration - a combination with potential therapeutic value for treating diabetic wound healing

Venkataseshan J *et al*. Diabetic wound healing

Abstract

Non-healing wounds impart serious medical problems to people with diabetes. Amongst 15% of diabetic patients, the incidence of foot ulcer is the most prevailing, which confers a significant risk of limb amputation, mainly due to hypoxia and impairment in cell signaling. Alteration in the expression of chemokines and the related factors in diabetic conditions delay the recruitment of different cell types, including fibroblast, keratinocytes, and immune cells such as macrophages to the site of injury, further impairing neovasculogenesis, re-epithelialization and extracellular matrix formation. Thus, proper activation of effector cells through an accurate signal pathway is necessary for better therapeutic application. Hyperbaric oxygen therapy (HBOT) is the current treatment prescribed by medical practitioners, shown to have increased the wound healing rate by reducing the need for significant amputation among the diabetic population. However, the risk of morbidity associated with HBOT needs complete attention through rigorous research to avoid adverse outcomes. Altering the level of pro-angiogenic chemokines may regulate the inflammatory response, further promote vascularization and enhance the complete healing of wounds in diabetic patients. Thus, a combination of better therapeutic approaches could pave the way for developing a successful treatment for diabetic foot and wound healing.

Key Words: Diabetic foot; Wound healing; Hyperbaric oxygen therapy; Chemokines; Combinatorial therapy

Venkataseshan J, Viswanathan P. Hyperbaric oxygen therapy and chemokine administration - a combination with potential therapeutic value for treating diabetic wound healing. *World J Diabetes* 2022; In press

Core Tip: Diabetes induces slow or non-healing of wounds, increasing the risk of developing infection and other complications. Proper management of blood sugar levels is essential to improve the overall health. Hyperbaric oxygen therapy (HBOT)

enhances the efficacy of wound healing rate in chronic diabetic foot ulcer patients. However, the systemic and meta-analysis data contradicts in cases associated with ischemic wounds. Also, the uncordial functioning of effector cells due to the interrupted signaling pathway involving chemokines and related growth factors worsens the condition of wound healing to a greater extent. Thus, a combinatorial approach of HBOT and chemokine administration could have a potential therapeutic value for treating diabetic wounds with the existing clinical protocol.

INTRODUCTION

Diabetic patients encounter non-healing or improper healing of wounds, which is a serious medical problem. Amongst many complications imparted by diabetes, the incidence of diabetic foot ulcer (DFU) is the most prevailing, significantly increasing the risk of limb amputation in 25%-90% of diabetic patients if the proper medication is either not provided or followed[1]. It has been estimated that the current incidence of DFU will affect 15% of all patients related to diabetes[2]. DFU is an open sore or wound that generally begins from minor trauma, pressure, or irritation at the bottom of the foot. The morbidity of DFU leads to chronic pain, suffering and poor quality of life for diabetic patients. The changes in the biomechanics of bones and architecture of soft tissues increase the risk of atherosclerotic arterial diseases and peripheral neuropathy, which could lead to nonenzymatic glycation predisposes causing ligament stiffness and decreased nerve sensation. Due to this, the patient would be unaware of pain on the foot or lower limb[3]. Also, prolonged hyperglycaemia impairs the function of immune cells, making the wound prone to infections. Thus, the overall physiological impairment associated with DFU complicates wound healing and detains precise treatment due to the lack of evidence-based protocol[4]. Though trauma and neuropathy are the critical triads for developing DFU, studies have shown that an intricate signaling mechanism is involved at the molecular level[5].

Wound healing is a cellular response which involves numerous processes such as hemostasis, inflammation, keratinocytes proliferation, angiogenesis, vascular

epithelialization, fibroblast differentiation, collagen production, and tissue remodeling. However, oxygen perfusion to the site of injury is crucial for an effective outcome. Hypoxia, a state of low oxygen tension, induces cellular stress through a complex cascade by delaying the recruitment of pro-inflammatory cells, impaired growth factor expression, defects in angiogenesis and extracellular matrix (ECM) formation[6]. Evidence suggests that diabetes induces hypoxia in the tissues of kidneys, retina, adipose and skin-related wounds[7]. Hypoxia-inducible factor (HIF) is the key transcriptional regulator that plays a prime role in the adaptive response to oxygen homeostasis. In the presence of oxygen (optimal concentration), HIF undergoes hydroxylation and subsequent ubiquitination to degrade in a shorter time. However, under hypoxic conditions, HIF undergoes stabilization and translocates to the nucleus to regulate the activation of genes associated with glucose metabolism and angiogenesis[8]. Studies showed that hyperglycemia destabilizes HIF and dysregulates downstream transcriptional activation, resulting in disease progression. However the exact mechanism is still unknown[7].

In hyperbaric oxygen therapy (HBOT). a patient is treated by delivering 100% oxygen under supra-atmospheric pressure. Evidence suggests that providing HBOT to patients suffering from Wagner grade 3 wound or higher DFUs during the post-operative period have greatly reduced the risk of limb amputation and incomplete re-epithelialization[9]. However, the clinical practice guidelines recommend against using HBOT for patients with Wagner grade 2 or lower DFU as the chance of oxygen toxicity is higher[10]. Thus, more research-based evidence is needed for effective treatment to prevent morbidity and mortality. Though HBOT is currently approved and recommended by the Centre for Medicare Studies for treating DFUs, the management remains complex[9].

Chemokines are signal molecules that play a crucial role in coordinating the activation and migration of immune cells to the site of injury[6]. The cytokines and growth factors produced by the immune cells promote wound healing during the inflammation and proliferation stage. Thus, an imbalance in the micro-environment will alter the network of their functionality, which could lead to prolonged healing or excess

scar formation[11]. The expression profile shows that monocyte chemoattractant protein-1 (MCP-1) production by keratinocytes is significantly high in normal wound healing[12]. However, *in vivo* study revealed that the reepithelization is delayed in MCP-1 deficient mice, indicating that the dysfunction of the chemokine-dependent pathway could impair tissue remodeling[13]. Since chemokines are small proteins that do not have major modification regions other than two disulphide bonds, they are highly stable and can be used as adjuvants corresponding to their functional groups for wound therapy. Also, the ability of chemokines to bind G-protein-coupled receptors increases their likelihood as therapeutic targets for regulating biological activity, thereby mitigating disease progression[14]. This review examines chemokines/their specific receptors as potential targets for treating DFU and emphasizes the possible regulation to attain with HBOT for a combinatorial therapeutic approach to hasten the healing process.

Oxygen in wound healing

Oxygen is essential for maintaining basic cellular functions such as ATP production, protein synthesis and reactive oxygen species (ROS) formation. ROS are oxygen molecules in a reduced format that are highly reactive. These radical derivatives are not only involved in the oxidative killing of bacteria but also act as secondary signal molecules[15]. Most well-known ROS molecules such as $\cdot O_2^-$, O_2^{-2}, H_2O_2, $\cdot OH$, and $\cdot OH^-$ are produced during oxidative phosphorylation. Like ROS, the reactive nitrogen species (RNS) is a normal physiological by-product based on nitrogen oxidation that reacts mainly with thiols and transition metals to form nitrosyl-metal complexes. Cells such as macrophages, platelets, keratinocytes and macrophages utilize these radical complexes as a signal response during wound healing[16]. However, their respective role in the cell cycle, homeostasis, cell-mediated defence and activation of pro-apoptotic proteins for cell death significantly differs based on low, basal, high and excessive concentrations. *In vivo* studies have shown that an optimal and sustained level of ROS mediates the secretion of pro-inflammatory cytokines and induces the matrix of metalloproteases. On the contrary, the addition of excessive reactive species (either ROS or RNS) was found

to damage the ECM and diminish the function of dermal fibroblast and keratinocytes. This shows that the lower and higher level of radical species has respective accelerating and decelerating effects on wound repair[17]. Thus, maintaining a balance in the level of oxidative species is critical for bringing effective outcomes.

Hyperglycaemia and oxidative stress

Diabetes is known to be accompanied by an increased cellular oxidative stress. However, recent studies have only shown that hyperglycaemia resulting from unregulated blood glucose levels causes dysfunction in the antioxidant defence system by triggering the overproduction of ROS[18]. The hyperglycaemia-induced cellular damage is mainly associated with: (1) Excessive formation of advanced glycation end products; (2) Protein kinase C activation; and (3) Increased polyol and hexosamine pathway flux, all of which could enhance oxidative stress[19]. The exact mechanism is ambiguous as one influences the other. Still, the prevailing evidence suggests that hyperglycaemia increases the availability of electron transfer donors such as $FADH_2$ and NADH, which increases the electron flux, further creating hyperpolarization of mitochondrial membrane potential due to a change in ATP/ADP ratio. The high electrochemical potential difference leads to the partial inhibition of electron transport between coenzyme Q and complex III, resulting in electron accumulation, further driving the partially reduced O_2 to generate free radical anion superoxide, thus impairing the cell function[20].

Circulatory endothelial progenitor cells (EPC's) produced in the bone marrow play a significant role in the regeneration of the endothelial lining of blood vessels in response to ischemic conditions[21]. The antioxidant enzyme level of EPC cells was found to have enhanced in a low oxygen environment to engraft vasculogenesis. However, their activation and proliferation are significantly impaired in an oxidative stress environment and the baseline pattern is similar to that of diabetic conditions[22]. Other than activation and proliferation, the migration of EPC to the injured sites followed by tissue homing based on chemokine signal is important for wound repair. Nevertheless,

the process is diminished in diabetic condition due to signal deficit, further impairing EPC function[23]. Thus, the elevated level of ROS production is believed to be the prime factor by which hyperglycaemia-mediated diabetes affects the normal wound healing process.

HBOT and oxidative response in diabetic wound healing

The pathological state of delayed wound healing is associated with prolonged oxygen deficit. The increase in the amount of oxygen would generate a favorable gradient for its diffusion into the affected tissues[3]. The management of chronic non-healing wounds by HBOT increases the rate of oxygen perfusion 10-50 folds and shows a correlation by modulating the inflammatory response with an increase in ROS production[24]. Vascular endothelial growth factor (VEGF), a key angiogenic factor responsible for maintaining blood vessel integrity, is stable under hyperoxia and hypoxia conditions. The function of endothelial-1, an endogenous vasoconstrictor responsible for the maintenance of blood pressure and basal vascular tone seems to have significantly decreased under hyperoxic conditions[25]. Thus, it is paradoxical to perceive that the increase and decrease in oxygen concentration have alternative effects on blood vessels together with varying levels of ROS production, thus imparting a setback on HBOT.

A systematic study based on 9000 previous records on the effect of hyperoxygenation shows that HBOT increases the level of oxygen radicals and increases the chance of inducing oxidative stress. On the contrary, the meta-analysis data reveals that HBOT stimulates the release of angiogenesis-promoting cytokines and growth factors, whose function is impaired when oxidative stress is high, as in the case of diabetes[26]. The most remarkable understanding is obtained from the thermal imaging data of an HBOT-treated wound with decreased temperature, indicating a decline in inflammation[27]. Since no significant difference in the profile of anti-inflammatory markers was observed in HBOT, its direct role in anti-inflammation seems less probable. Thus, promoting a wound to an anti-inflammatory state from a prolonged inflammatory condition (where ROS level is high) could be possible by regulating a

nuclear factor that suppresses the pro-inflammatory genes. Nuclear factor kappa beta (NF-κB) is a critical transcriptional factor in inflammation that activates several pro-angiogenic genes together with HIF-1α under hypoxia conditions. However, inhibitor of kappa Balpha (IκBα), another nuclear factor that is stimulated under hyperoxic conditions, inhibits NF-κB, resulting in the downregulation of pro-inflammatory transcription factors and pushing the cellular environment towards an anti-inflammatory state[28]. HBOT could establish the same condition in the wound micro-environment despite oxidative stress and aids healing. *In vivo* study validates that hyperoxia induced during HBOT session is associated with decreased NF-κB expression and stimulated the activation of IκBα, which is generally degraded under hypoxia[29]. Though it seems promising, no significant evidence is available about the cellular damage induced by the preformed oxidative species or its reversal effect by HBOT before the establishment of anti-inflammatory phase, which needs to be addressed thorough research for regulating the interventional procedure.

Macrophage polarization in normal vs diabetic wound

Macrophage infiltration on the wound site is mainly derived from the monocyte precursors in response to pathogen-associated-modifying proteins or damage-associated-modifying proteins. Besides the scavenging activity, macrophages play other roles in tissue regeneration and wound repair[30]. However, depending on the phenotype, their functionality is assigned in the tissue micro-environment.

The macrophages are divided into three subgroups based on the marker it expresses on the surface, such as classical macrophages ($CD14^{++}CD16^{-}$), intermediate macrophages ($CD14^{++}CD16^{+}$) and non-classical macrophages ($CD14^{++}CD16^{++}$)[31]. Classical macrophages are known as M1 or pro-inflammatory macrophages that are triggered by tumor-necrosis factor (TNF), lipopolysaccharides and produce pro-inflammatory cytokines such as interleukin (IL)-12 and IL-23, together with ROS. The non-classical macrophages are known as M2 or resolving macrophages that are stimulated by the anti-inflammatory cytokines such as IL-4 and IL-10 to activate the

release of growth factors such as transforming growth factors and insulin-like growth factors[32]. In normal wound healing, M1 predominates for up to three days in clearing up the pathogens, dead and dying cells from the wound site and causes inflammation. The transition to M2 occurs thereafter with a peak in activity on day seven, promoting wound healing. Studies have shown that impairment in the transition to M2 and persistent polarization of M1 macrophages are responsible for prolonged wound healing in chronic disease conditions[31]. An *in vivo* study showed that the ratio of CCR7-CD48, a respective chemokine receptor and marker found on M1 and M2 macrophages, is higher in diabetic mice with impaired wound healing than in control, indicating the dysfunctionality of macrophage switching/transition and its importance in wound repair. Also, it has been shown that the prolonged M1 polarization reduces the expression of matrix metalloproteinases together with increased pro-inflammatory cytokines secretion such as TNF-α, affecting the keratinocyte migration and leading to the concussion of normal wound healing process[33]. Several factors were found to contribute to the persistent polarization of M1 macrophages, which is why the therapeutic development could be focused on either blocking the inflammatory cascade activating the M1 phenotype or promoting M2 transition to resolve the debilitating diabetic chronic wounds.

Chemokines - a potential regulator of macrophage polarization and wound differentiation

Chemokines are a family of secretory proteins with low molecular weight (8-12 kDa) that have a prominent role in chemotaxis and activation of immune cells. The four subfamilies of chemokines C, CC, CXC and CX3C are classified based on the two conserved cysteine residues present at the N-terminal motif[34]. Chemokines are important in regulating angiogenesis during hemostasis and the inflammatory phase of wound healing for clot formation and the influx and efflux of migratory cells. Also, they control the formation and regression of neovessels during the proliferation and remodeling phase to assist the healing wound in meeting the metabolic need and scar

formation. Thus, playing a pivotal role in orchestrating the precise sequence of events, chemokines are crucial in all stages of wound healing (Table 1)[35]. As discussed earlier, for the establishment of the proliferation phase, the pre-formed inflammation in the tissue microenvironment should get declined by the anti-inflammatory signal cascade to establish the transition of the M1 to M2 phenotype to aid tissue repair.

Adipose tissue macrophages constitute 10%-15% of the total cell population in healthy individuals, and they predominantly show M2 phenotype with high insulin sensitivity. However, in obesity, the adipocytes secrete pro-inflammatory markers that trigger the recruitment of monocytes *via* the CCL5-CCR5 pathway. The macrophages derived from those monocyte precursors acquire the M1 phenotype and contribute to prolonged inflammatory environments[36]. On the contrary, the intrahepatic monocytes in the presence of anti-apoptotic protein BCL2 are mediated by the CX3CL1-CX3CR1 pathway and show a less inflammatory phenotype characterized by decreased TNF-α and nitric oxide synthase production[37]. Thus, macrophage polarization in metabolic disorders could be modulated by regulating the chemokines and their specific receptors.

Recent advancement in stem cell-based approaches has garnered significant interest as they have potential therapeutic values. Studies have shown that exosomes derived from mesenchymal stem cells (MSCs) possess immunomodulatory effects that can induce the transition of pro-inflammatory macrophages to anti-inflammatory phenotype in various inflammatory-associated disease conditions[38]. These MSC-derived exosomes exhibit high expression of angiogenic and tissue repair factors such as VEGF, extracellular matrix metalloproteinases, and matrix metallopeptidase 9[39]. The adipocyte-derived MSCs that express Arg-1 and IL-10 based on the activation of STAT3 transferred by the exosomes are shown to alleviate inflammation through M2 polarization[40]. The genes present downstream of macrophage transcriptional factors, such as interferon regulatory factor/STAT, which arranges the polarization. Bruton's tyrosine kinase with STAT1/STAT5 and Kruppel-like factor 4 with STAT3/STAT6 induce the polarization of the M1 and M2 phenotype, respectively[41,42]. However, for

the complete induction of the anti-inflammatory phenotype, the peroxisome proliferator-activated receptor gamma is essential, and its absence diminishes insulin sensitivity, further resulting in hyperglycaemia that impairs cellular function[43].

Other than MSCs, epidermal stem cells aid in tissue repair by modulating the migration and proliferation of EPCs to the injury site. Abnormal EPC migration and a lack of tubularization cause impaired angiogenesis in diabetic patients[21]. Stromal cell-derived factor 1 (SDF-1), a chemokine belonging to the CXC family, recruits EPC to the wound site by interacting with CXCR receptors 4 and 7. A study has shown that the expression of SDF-1 between acute and chronic wounds differs significantly. In the case of chronic wounds, no influence was observed with EPC migration, thus, an exogenous administration of SDF-1 is inevitable to accelerate the wound healing rate[44]. However, the duration of the chemokine gradient and its bioavailability are essential factors to consider in the effectiveness of the wound-healing rate. Instead of a single dose administration, a formulation that enhances a slow release of the element might have a significant positive effect on tissue regeneration. To achieve this, a biomaterial scaffold that retains the bioactivity of chemokine could be developed for tissue engineering purposes. SDF-1 encapsulated in poly (poly ethylene glycol citrate-*co*-N-isopropyl acrylamide) has improved the tissue healing rate in diabetic mice with sustained release of the factor for up to 3 wk without any burst[45]. Modifying the hydrogel systems, such as integrating anti-oxidant properties, could render more advantages for rapid healing, and developing such state-of-the-art techniques could revolutionize the therapeutic aspects of treating chronic diabetic wounds.

CONCLUSION

Diabetes is a chronic disease that brings delirious effects through prolonged inflammation that could lead to other metabolic disorders such as cardiovascular diseases, hypertension and renal diseases. Several interventions have been suggested, including a healthy diet, exercise and proper medication to lessen the adverse outcomes. However, a better therapeutic approach is needed for an effective outcome

despite the standard procedures. The problem with delayed wound healing and persistent infection in diabetic patients is attributed to the deficiency of oxygen perfusion to the injured site. The resulting hypoxic environment alters the sequence of cellular events from the normal wound healing and complicates the process further. HBOT is found to fasten the wound healing rate in DFU cases by inducing angiogenic factors and other critical components of the cellular cascade. Although HBOT is found to be efficient in reverting the ischemic condition of the wound, complete reliance on the interventional procedure is not enough, as wound healing is a multifactorial process. Thus, the efficiency of chemokine-mediated response is essential for activating the effector cells that participate in wound healing.

The combinatorial therapeutic approach could be of interest as it will likely lead to a better outcome. HBOT and simultaneous administration of tissue-specific chemokine/receptor modulating factors could overcome multiple wound healing deficits observed in diabetic conditions (Figure 1). Since not much research was carried out earlier with the proposed combination, this review emphasizes the researchers to conduct various controlled trial studies with FDA-approved biologics to explore the potential and develop novel strategies and better clinical practices for treating diabetic wounds.

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