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**REVIEW**

# Hyperbaric Oxygen Influences Chronic Wound Healing – a Cellular Level Review

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**Summary**

Chronic wound is a serious medical issue due to its high prevalence and complications; hyperbaric oxygen therapy (HBOT) is also considered in comprehensive treatment. Clinical trials, including large meta-analyses bring inconsistent results about HBOT efficacy. This review is summarizing the possible effect of HBOT on the healing of chronic wound models at the cellular level. HBOT undoubtedly escalates the production of reactive oxygen and nitrogen radicals (ROS and RNS), which underlie both the therapeutic and toxic effects of HBOT on certain tissues. HBOT paradoxically elevates the concentration of Hypoxia inducible factor (HIF) 1 by diverting the HIF-1 degradation to pathways that are independent of the oxygen concentration. Elevated HIF-1 stimulates the production of different growth factors, boosting the healing process. HBOT supports synthesis of Heat shock proteins (HSP), which are serving as chaperones of HIF-1. HBOT has antimicrobial effect, increases the effectiveness of some antibiotics, stimulates fibroblasts growth, collagen synthesis and suppresses the activity of proteolytic enzymes like matrix metalloproteinases. All effects of HBOT were investigated on cell cultures and animal models, the limitation of their translation is discussed at the end of this review.

**Key words**

Hyperbaric oxygen • Wound healing • Reactive oxygen and nitrogen species • Hypoxia inducible factor • Heat shock protein

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## Introduction

Chronic wound is physiologically affected healing due to a disruption of the healing cycle as a result of impaired angiogenesis, innervation, or cellular migration. Chronic wounds of mixed etiologies showed a pooled prevalence of 2.21 per 1,000 population; for chronic leg ulcers the prevalence was estimated at 1.51 per 1,000 population, so it is a serious medical issue (Martinegro *et al*. 2019). Treatment of chronic wound must be complex. Local treatment consists of debridement and proper dressings along with so-called adjunctive local therapies (Liu *et al*. 2013), including hyperbaric oxygen therapy (HBOT).

HBOT is a therapeutic inhalation of pure oxygen in a chamber pressurized up to 0.2-0.3 MPa, which is equal to submersion up to 10-20 m into water. In the protocols for chronic wound treatment, daily sessions limited to working days lasting 90 min for 4-8 weeks are used. Protocols display high inter-workplace variability in the pressure applied and number of sessions used. Moreover, some facilities employ air breaks, i.e. breathing of air instead of oxygen for approximately 5 min in the middle of treatment, to support vessel contractions (Jain 2009). All these differences can contribute to problematic evaluation of the outcome of HBOT. HBOT has been used in the world (first in the USA) for over 100 years; in the Czech Republic since

1965 (Emmerová *et al.* 2007, Kindwal 1994). The spectrum of indications is broad and often criticized for insufficient scientific justification. Apart from divers' accidents, there is not a single diagnosis that has not been criticized for the possible ineffectiveness of the use of HBOT (Gabb *et al.* 1987). Less controversial diagnoses include non-healing chronic defects that occur with predilection for the lower limbs in patients with diabetes, ischemic disease, or venous insufficiency. The impact of HBOT on so-called diabetic foot syndrome, manifested in non-healing lower limb defect (DFU), has been extensively studied.

Most meta-analyses reported a positive effect of HBOT on DFU, concluding that HBOT reduces the risk of above-knee amputations and improves defect healing, even in one-year follow up (Stoekenbroek *et al.* 2014, Kranke *et al*. 2015, Elraiyah *et al.* 2016). In contrast, another meta-analysis of 6,259 patients did not show any beneficial effect of HBOT (Margolis *et al*. 2013). In addition, recent randomized clinical trials (Santema *et al*. 2018, Fedorko *et al*. 2016) have not shown any positive impact of HBOT on reducing the risk of lower limb amputations or on accelerating DFU healing either.

The reasons for these controversial results are difficulties in clinical trials caused by group inhomogeneity and specific problems with the control group: in principle, HBOT cannot be administered in a placebo regimen, control groups are either not treated at all or undergo modified HBOT in a presumed ineffective regimen. One reason for scepticism about HBOT is the lack of explanation for the effect of HBOT at the cellular level. Therefore, the research is also intensively focused on animal models of chronic wounds and cell cultures (e.g. of skin fibroblasts), where standard experimental conditions can be provided. The aim of this review is to summarize the possible effect of HBOT on the healing of chronic wound models at the cellular level of the repair processes. It will be shown that free oxygen radicals in particular play a complex role in regulation and fundamentally affect the positive and negative effects of HBOT.

## Reactive oxygen and reactive nitrogen species

Reactive oxygen (ROS) and reactive nitrogen species (RNS) are naturally occurring metabolites, mostly with a very short reaction time. ROS include free oxygen radicals and non-radical species, e.g. superoxide anion ($O_2^-$), hydroxyl radical ($OH^-$), hydrogen peroxide ($H_2O_2$), singlet oxygen $^1O_2$, and many others. Nitric oxide (NO) and peroxynitrite ($ONOO^-$) are typically referred to as RNS, with some of them being linked to ROS by metabolic pathways. ROS and RNS occur in small amounts during normal metabolism having a signaling role and contributing to pathogen resistance (Pisoschi *et al*. 2015, Yang and Lian 2020). However, high concentrations of ROS and RNS damage nucleic acids, proteins or lipids (Schieber *et al*. 2014). Their increased concentration has been observed in about 100 pathological conditions, typically in various inflammatory and reperfusion syndromes (Gutteridge 1993).

Mitochondria are considered to be the major source of ROS, however a number of enzymes, such as xanthine oxidase and NADPH oxidase can catalyse the formation of ROS in the cell cytoplasm. In the chronic wound that frequently suffers from severe hypoxia, ROS are supposed to be generated preferentially out of mitochondria (Stowe and Camara 2009).

During wound healing process, inflammation associated with a recruitment and activation of immune cells is a typical feature. ROS and RNS play an irreplaceable role in phagocytosis, when pathogens are encapsulated by macrophages, neutrophils or dendritic cells. After pathogen internalization**,** the cells start the process of oxidative burst, when consumption of molecular oxygen increases by activated NADPH oxidases resulting in production of high amounts of ROS that then help in destruction of internalized pathogens. Accumulating evidence suggests that macrophages are dysfunctional in the chronic wounds, not only in the promotion of inflammation, but also in subsequent phases of healing (reviewed in Ganesh and Ramkumar 2020).

Besides direct cytotoxic affects, ROS also interfere with intracellular signaling in a complex way that helps in appropriate timing of individual healing processes (initiation and resolution of inflammation, removal of apoptotic cells and tissue restoration). On the molecular level, ROS are involved in activation MAPK (mitogen-activated protein kinase) pathway, which is a ubiquitous family of serine/threonine kinases playing an essential role in signal transduction from the cell membrane receptors to the nucleus (Brown and Sacks 2009). Specifically in the chronic wounds, MAPK induction makes cells generate signaling proteins like monocyte chemoatracting protein (MCP-1), series of interleukines (IL) IL-1, 8, 6, tumor necrosis factor alpha (TNFα) and macrophage inflammatory protein (MIP-1).

This complex protein system stimulates inflammation and attracts phagocytes, neutrophils, eosinophils, basophils and lymphocytes into the wound (Bryan *et al.* 2012).

ROS also mediate cell division and migration of kerationocytes, endothelial and fibroblast proliferation (Dunnill *et al.* 2017), they are involved in antigen presentation in dendritic cells through expression of CD86, stimulate CD4 differentiation, release Ca ions from endoplasmic reticulum into the cytosol resulting in Ca-dependent MAPK signaling and P selectin expression, which causes adhesion of neutrophils to endothelium. By similar mechanism ROS enable leukocyte extravasation during inflammation. In addition, ROS influence phenotype of fibroblasts stimulating their differentiation into myofibroblasts (Bryan *et al.* 2012).

HBOT leads to an increased accumulation of ROS through elevated oxygen concentration (Kindwall 1994, Jain 2007) by shifting the reaction potentials towards the formation of ROS. It seems logical that an increase in ROS will lead to oxidative tissue damage. These adverse effects on DNA damage have been documented on leukocytes (Dennong *et al.* 1996) and have led to the spread of criticism of HBOT as an ineffective and potentially harmful therapy.

However, HBOT was shown to increase ROS-induced mobilization of stem cells to the wound and to contribute to the activation of transcription factors inducing the secretion of growth factors important for wound repair processes (Dunnill *et al.* 2017). Another ROS-balancing effect of HBOT was documented in the rat model of the chronic wound where NADPH oxidase flavocytochrome b large subunit gp91phox was down-regulated in the HBOT environment (Ma *et al.* 2020, Zhang and Gould 2013).

The cell maintains, or seeks to maintain, a balance between ROS production and their degradation mechanisms that reduce ROS concentration. Increased concentration of ROS induced by HBOT leads to increased activation of antioxidant mechanisms, which might in turn prevent tissue damage. ROS are typically eliminated by the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH) that have been shown to be up-regulated by HBOT (Cozene *et al.* 2020, Thom 2009).

RNS are metabolically linked to ROS at several levels. Three enzymes play a major role in the formation of RNS: inducible-, endothelial- and neuronal nitric oxide synthase (i-, e-, n-NOS). The effect of HBOT on the activity of these enzymes is complex. In a chronic wound model in which a higher initial RNS concentration was determined, HBOT was shown to reduce iNOS activity (Zhang and Gould 2013, Poff *et al*. 2016) and thus to prevent further RNS formation and tissue damage. Conversely, other tissues, such as rat brain, showed higher e-NOS activity under HBOT conditions and increased RNS production (Xu *et al.* 2009, Elayan *et al*. 2000).

A high inter-individual variability was found in the effect of HBOT on iNOS activity. Some authors conclude that this could be a possible cause of HBOT ineffectiveness at least in some cases. Others try to find a genomic differences to explain poor response to HBOT. In human patients subjected to HBOT, approximately 25 % of defects did not respond to the treatment (Zhang and Gould 2013, Löndahl *et al.* 2010). The possibility that this phenomenon is related to the regulation of iNOS activity in the wound should be further explored (Johnston *et al.* 2016).

In conclusion, HBOT undoubtedly stimulates the production of ROS and RNS, as well as it promotes the activity of antioxidant enzymes thus establishing new balance between pro- and antioxidant mechanisms. In addition, HBOT could have both beneficial and detrimental impact in different tissues. E.g. in the lungs, exposure to high oxygen concentrations (even lower than in HBOT) leads to deposition of collagen, inflammation, diffuse alveolar damage, pulmonary edema, chronic pulmonary fibrosis and emphysema (Mach *et al.* 2011, Pereira *et al.* 2014, Thomson and Paton 2014). At the cellular level, lung fibroblasts subjected to HBOT displayed compromised viability and impaired mitochondrial oxygen consumption (Dejmek *et al.* 2018). It is thus not possible to decide on the unequivocal benefit of HBOT, however it seems that the potential benefit of this adjunctive method in the chronic wound treatment should be further explored.

## Hypoxia inducible factor-1

Hypoxia inducible factor-1 (HIF-1) is a complex of proteins that is activated in conditions of hypoxia, i.e. lower oxygen concentration. The proteins are marked with Greek letters; HIF-1α is a protein that is responsible for regulating the concentration of the whole complex, therefore, sometimes only HIF-1α is referred to. HIF-1 is used in many physiological and pathophysiological processes such as embryonic development by promoting formation and differentiation of the vascular and

hematopoietic systems, inhibition of differentiation of preadipocyte fibroblasts into adipocytes and myoblasts into myocytes, proliferation of some tumors, secretion of inflammatory chemokines, attraction of neutrophils and monocytes in inflammation, etc. HIF-1 regulates the division of stem cells, preventing their rapid differentiation (Takubo *et al*. 2010, Semenza *et al.* 1998).

HIF acts in a specific way in chronic wounds, having a crucial role in the healing process. It supports keratinocyte migration and epithelial regeneration. It stimulates the production of vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and stromal cell derived factor (SDF). The migration of endothelial progenitor cells into the wound is controlled also by the SDF pathway (Benizri *et al*. 2008).

The half-life of HIF degradation is in the order of minutes under normal conditions. The most important pathway of its degradation is hydroxylation (by the enzyme prolyl hydroxylase) and subsequent binding to the von Hippel Lindau tumor suppressor protein (vHL). This complex is then destroyed by the protease system. The degradation, or the binding of HIF-1 to vHL is dependent on the oxygen concentration (Zepeda *et al*. 2013). More precisely, this binding is controlled by redox changes upstream from complex IV of mitochondrial respiratory chain (Stowe and Camara 2009). Therefore a higher HIF level is achieved (by this mechanism) under hypoxic conditions, where the protein is synthesized but not degraded. Under conditions of hypoxia, HIF-1 binds to other hypoxia responsive elements and promotes the synthesis of VEGF, SDF and PDGF.

There are other mechanisms of HIF-1 degradation than by the vHL pathway, for example *via* the activated protein kinase C receptor (RACK1), or through the oxygen-independent E3 ubiquitin ligase pathway (Liu *et al.* 2007, Isaacs *et al*. 2002). None of these pathways is dependent on oxygen concentration; on the contrary, they are affected by the expression of heat shock proteins. Degradation of HIF-1 is summarized in Figure 1.

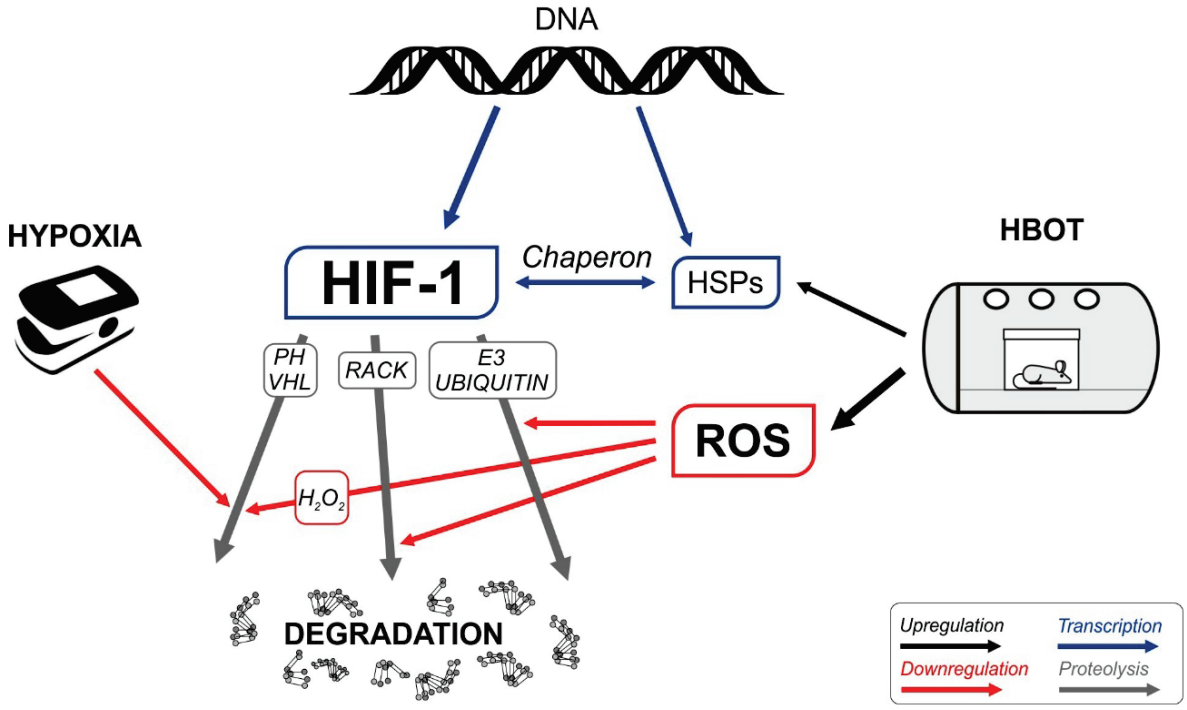


**Fig. 1.** Regulatory chain of HIF-1 signal protein degradation in relation to HBOT, ROS, HSPs and hypoxia. HIF-1 hypoxia inducible factor-1; HSPs heat shock proteins; PH VHL degradation pathway *via* prolyl hydroxylases and von Hippel Lindau tumor suppressor protein; RACK, E3 UBIQUITIN degradation pathways through activated protein kinase C receptor and E3 ubiquitin ligase; up- and down-regulations are color-coded. Hypoxia directly stimulates HIF-1 gene expression, degradation of which is also oxygen dependent *via* PH vHL pathway. Other degradation pathways (RACK, E3 ubiquitin ligase) are oxygen-independent; however, they seem to be inhibited by ROS. HBOT does not interfere with HIF-1 synthesis, but it might contribute to its stabilization, not only through ROS-regulated pathways, but also *via* activation of HSP binding to HIF-1 that further stabilizes HIF-1 thereby increasing its intracellular levels.

It seems paradoxical that the concentration of HIF-1 could be up-regulated by HBOT, logically the opposite would be expected. HBOT has been shown to comprehensively affect HIF-1 metabolism in human skin

fibroblast cultures (Sunkari *et al.* 2015). Immediately after HBOT exposure, HIF destabilization is observed, presumably through activated oxygen-dependent vHL degradation. About 4 h after HBOT initiation, there is an increase in HIF-1, VEGF and SDF levels. This phenomenon probably means that the degradation of HIF is, in addition to the oxygen concentration, also dependent on ROS (Isaacs *et al*. 2002, Sunkari *et al.* 2015). Under conditions of hypoxia, ROS are overproduced and thus the degradation of HIF-1 by the vHL pathway is partially blocked. ROS production is also increased in HBOT, as explained above. Another possible explanation for HIF stabilization under HBOT conditions the degradation block by binding to heat shock proteins.

## Heat Shock Proteins

Heat shock proteins (HSP) represent a group of proteins generally responsible for the cell's response to stress, not just thermal stress. They are massively synthesized in inflammation and intoxication. HSP are denoted by numbers corresponding to their atomic weight in kD (kilodaltons). They serve as chaperones – they bind to older proteins and help their proteolysis, aggregation of other proteins, their secretion outside the cell, etc. (De Maio 1999, Santoro 2000, Borges and Ramos 2005).

HSP 90 is one of the most studied HSPs, reaching almost 1 percent of the concentration of all cellular proteins (Dzialoszynski *et al*. 2016). HSP 90 is involved in metabolism even under normal conditions, not only in stress, when it functions in many signaling pathways as a chaperone, i.e. it allows the participation of other proteins in the reaction. It is involved in the control of the steroid and dioxin receptor. Several protein kinases, including the Src and Raf components of the mitogen-activated protein kinase system, are also bound to HSP 90 (Pratt 1997). Src and Raf kinases belong to a group of so-called oncogenes, where Src is involved in tyrosine residues phosphorylation, while Raf is involved in serine and threonine residues phosphorylation. HSP 90 plays a role in the activation of steroid hormones, has metabolic effects in tumors, CNS injuries, neurodegenerative and other diseases (Tran and Frost 2003, Slimen *et al*. 2014).

HSP 90 is referred to as the HIF-1 chaperone, as shown in Figure 1. The activity of HIF-1 is thus directly conditioned by the presence of HSP 90. When it is blocked, HIF-1 is inactive, which has been repeatedly demonstrated on cell cultures (Minet *et al.* 1999, Sunkari 2015, Pratt 1997).

Inhibition of HSP 90 degrades HIF-1 by a pathway different from vHL, e.g. by the RACK1 pathway. Conversely, this pathway is repressed when HSP 90 is expressed, which competes for binding to HIF-1 (Liu *et al*. 2007).

The importance of HSP 90 for chronic wound healing was reviewed elsewhere (Guo *et al.* 2017). Briefly, HSP 90α protein is deposited into the wound bed and increases motility of skin cells needed for successful healing process (keratinocytes, dermal fibroblasts and endothelial cells) even under conditions of hyperglycemia that causes HIF-1 destabilization. The precise source of HSP 90 should be further explored (skin cells, immune cells).

Only scarce data are available on the effect of HBOT on HSP expression or metabolism in general and in relation to chronic wound healing in particular. It has been documented that hyperbaric oxygen improves the tolerance to decompression sickness probably *via* increased expression of HSPs (27, 70, 90) in the rat lungs and spinal cord (Huang *et al*. 2014, Ni *et al*. 2013) or alleviates the symptoms of altitude sickness in a rat model, where overexpression of HSP 70 was documented (Wu *et al*. 2018). Another effect of HBOT on HSP 90 function was described in the rat heart subjected to short-term pre-exposure to hyperbaric oxygen before the onset of myocardial ischemia and reperfusion, where increased association of HSP 90 with NOS contributed to increased cardioprotection (Cabigas *et al*. 2006).

In conclusion, potentially beneficial HSP-related effect of HBOT on chronic wound healing should be further studied in relation to putative stabilization of HIF-1, subsequent secretion of VEGF, SDF and PDGF, i.e. stimulation of the healing process.

## Antimicrobial effects

Chronic wound suffers from hypoxia and circulatory disorders. It has weakened natural immune protection and it is therefore regularly colonized or infected by various bacterial strains (Rahim *et al*. 2017). The possible antimicrobial effects of HBOT have been reviewed recently and comprise both direct and indirect mechanisms (Memar *et al.* 2019).

The increase in oxygen concentration *per se* is believed to be directly toxic to anaerobic microorganisms, typically Clostridia strains (Montrief *et al*. 2019). ROS and RNS, production of which is

stimulated by HBOT, react spontaneously with pathogen organelles, oxidize their proteins and lipids and irreversibly damage them (Cabiscol *et al*. 2000, Ezraty *et al.* 2017). In addition, HBOT promotes protection against bacteria *via* transient suppression of inflammatory signaling pathways and proinflammatory cytokines expression and release, e.g. from macrophages. It has been documented that the release of interleukins (IL) 1, 6, 8 and tumor necrosis factor α (TNFα) by circulating monocytes can be compromised by HBOT (Al-Waili and Butler 2006, Weisz *et al.* 1997, Rinaldi *et al*. 2011) while anti-inflammatory cytokine IL-10 production is elevated (Pan *et al.* 2013). HBOT also promotes phagocytosis by leukocytes (Almzaiel *et al*. 2013), as demonstrated in the neutrophil model – HLA 60 cells. The same study showed an increase in the respiratory burst activity of neutrophil-like cells after HBOT.

HBOT increases the effectiveness of some antibiotics, such as cefazolin, ciprofloxacin, tobramycin, vancomycin and linezolid (Mendel *et al.* 1999, Kolpen *et al.* 2017, Lerche *et al.* 2017, Kurt *et al.* 2015, Koomanachi *et al.* 2011). In the study of Gupta *et al*. (2016), the authors tested fourteen antibiotics against common pathogens *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus* in different oxygen environments, from anoxic to hyperbaric conditions. They concluded that oxygen improved susceptibility to majority of antibiotics evaluated. In addition, this effect significantly correlated with the growth capability of the bacteria studied. One of suggested mechanism of HBOT-induced enhanced sensitivity to antibiotics is revitalization of the dormant bacteria by inducing aerobic metabolism (Jensen *et al.* 2019).

The antimicrobial effect of HBOT has been documented not only for lower limb defects, but especially for deadly soft tissue infections such as Fournier gangrene (Anheuser *et al.* 2018, Wilkinson and Doolete 2004). Furthermore, HBOT could be considered as a supportive treatment for some antibiotic-resistant pathogens as it has been shown to inhibit the division of methicillin-resistant *Staphylococcus aureus* – MRSA (Kurt *et al.* 2015).

It thus cannot be excluded that in the chronic wounds suffering from severe hypoxia, HBOT through direct and indirect toxic effects of oxygen along with recovery of aerobic metabolism of colonizing bacteria leading to enhanced bactericidal activity of antibiotics might improve the healing process.

## Further effects of HBOT in chronic wounds

Chronic wound is characterized by tissue loss, given by excessive matrix degradation. This process is caused by the up-regulation of proteolytic enzymes, typically matrix metalloproteinases (MMPs). MMPs are a complex group of enzymes, numbered 1 to 19. According to the substrate, they are further divided into groups of collagenases, gelatinases, stromelysins and membrane-type MMPs (Bode and Maskos 2003, Maskos and Bode 2003). MMPs have natural inhibitors of their function, called the tissue inhibitor of MMPs (TIMPs) (Snoek-van Beurden *et al*. 2005). Activity of MMPs is regulated primarily by MAPK pathway that is tightly linked to ROS production. MMPs production might be increased by activation of c-Jun N-terminal kinase (JNK) and extracellular signal–regulated kinases 1/2 (ERK1/2) members of MAPK families (Han *et al.* 2001). MMPs downregulation is mediated by TIMPS, activity of which is stimulated by HBOT.

In a rat chronic wound model, HBOT was shown to reduce MMP1, 2 and 8 expression and activity from day 7 of treatment, while MMP9 was reduced as early as day 3 of HBOT. From day 3 of treatment, TIMP2 expression also increased. Histological analysis of the wound tissue identified MMPs mainly in macrophages and fibroblasts (Zhang and Gould 2013).

In human dermal fibroblast cultures, HBOT has been shown to increase fibroblast proliferation, however, not from the beginning of the treatment but from day 7 of therapy. HBOT further increases the production of the basal growth factor of fibroblast, VEGF and transforming growth factor β1 (Kang *et al.* 2004, Kunnavatanna *et al.* 2005). Similarly, HBOT promotes the growth of human keratinocyte cultures (Hollander 2000) and neovascularization by stimulating release of growth factors, as discussed in the previous chapters.

Last, but not least, HBOT ameliorates collagen production in chronic wound. On the model of chronic wound in diabetic rat with ischemia delayed collagen deposition was found, especially when hyperglycemia was combined with ischemia. HBOT significantly counteracted these negative effects on collagen deposition, most successfully in animals with ischemia and normoglycemia together (André-Lévigne *et al*. 2016). This was the only one study *in vivo*, but more studies *in vitro* documented that skin fibroblast cultures produced more collagen under condition of HBOT (Huang *et al*. 2020, Gould and May 2016, Brismar *et al.*

1997, Conconi *et al.* 2003, Modarressi *et al.* 2010, Kang *et al.* 2004).

All these phenomena in combination might contribute to acceleration in wound healing with HBOT *via* prevention of tissue degradation and promotion of its repair. The question is whether the commonly used clinical set-up in medicine, i.e. a maximum of 20-30 treatment exposures, is sufficient. It is possible the effect would only occur with an even greater number of exposures.

## Conclusions and Limitation

To conclude, HBOT enhances ROS and RNS production, which damage certain tissues and organs. On the contrary, ROS and RNS seem to have a positive effect on pathways accelerating the healing of chronic wound. This claim, based on animal and cell culture experiments, should be commented on with the following limitations.

Interpretation of the cell culture experiments is associated with the difficulty of inducing suitable experimental conditions (Place *et al.* 2017). Merely induction of normoxia for individual cell types is a complicated task. It appears that the oxygen partial pressure ($pO_2$) corresponding to the physioxia ranges between 1 to 13 kPa, whereas current *in vitro* experimentations are usually performed in 18.8 kPa of $pO_2$. It is important to realize that most of the experiments performed in so-called normoxia might be dangerously misleading, especially as control to HBOT (Carreau *et al*. 2011). In HBOT, the situation is worse, complicated by the determination of the real amount of dissolved oxygen. The value of oxygen solubility for clear water is significantly different from inhomogeneous media, such as plasma or culture solutions. Weak interactions between oxygen and water are disrupted by the presence of charged electrolytes and proteins, which form much stronger interactions with water. The Henry constant for these liquids then depends not only on $pO_2$, but also on the concentration of electrolytes, proteins and the temperature of the solution. The value can only be roughly estimated using experimentally derived values of equilibrium dissolved oxygen (Place *et al.* 2017). Oxygen solubility is similar in most commercially available culture media for human cell lines. Their ionic strength (the sum of all electrically charged particles present in the solution) is in the range of 150-200 mmol/l, equivalent to the average ionic strength of human plasma (Wagner *et al*. 2011). At an ambient temperature of 37 °C, an atmospheric air pressure of 101.08 kPa (760 mm Hg) in the incubator (37 °C, 100 % relative humidity, 5 % $CO_2$), the oxygen partial pressure above the medium level is 18.8 kPa. The theoretical extrapolated concentration of dissolved oxygen in the medium under these conditions is 0.194 mmol/l (Koppenol and Butler 1985). The values of dissolved oxygen in the culture medium after HBOT are expected to be higher, unfortunately they have not been determined experimentally yet. The time that is required to fully equilibrate the medium varies with the external oxygen concentration and is mostly longer than 3 h in most experiments, which is much longer than the normal intermittent exposure of the patient. In conclusion, the study of the effect of HBOT on isolated cell lines must be taken with caution, because the values of dissolved oxygen in the culture medium are much higher than the real physiological values, even in the case of control cell culturing in incubator.

Another complication is the interpretation of animal experiments. Lack of optimal preclinical animal models that are capable of properly simulating human chronic wounds still remains a significant translational issue. Animals do not develop chronic wounds in a way that closely resembles those arising in humans. Animal wounds heal faster, with contraction, differ in microbiome, do not create hypertrophic scars or keloids, skin is in the hair etc. Animals do not present the same pathology in metabolism, which is usually the cause of healing problems in human patients. As a most suitable model of Type 2 diabetes serve obese ob/ob mouse (leptin receptor deficient), db/db mouse (a point mutation in the leptin receptor gene), NONcNZO10 mouse, and Zucker fa/fa rat with leptin receptor defect (Grada *et al.* 2018, Fang *et al.* 2010). Unfortunately, none of the cited articles in this review used such model, as the authors are mostly using the common Type 1 diabetes induced by streptozocin. However, patients suffering from the Type 1 diabetes don't exhibit non healing wounds as frequently as the Type 2 diabetics since they are not impaired by insulin resistance (Apelqvist 2008, Rasmussen *et al.* 2017).

And finally, timing of the treatment is a problem. Studies made on animal models and on cell cultures have a clear design and compact HBOT intervention, on a certain number of consecutive days, contrary to clinical use. Every clinical study and meta-analysis cited in this review had HBOT intervention

only on working days. According to the authors' best knowledge, there are no hyperbaric centers in the Czech Republic nor in other European countries that would treat chronic indications on the weekends. The majority of hyperbaric centers are private, some of them don't offer treatment even in acute indications (CO poisoning, anaerobic infections, etc.) (Jain 2009). This is due to the complicated logistics of treatment: patients should be transported somehow to the center, treatment is challenging for staff resources, and patients often do not complete treatment even in the common working day regime of HBOT. For example, in the most recent randomized prospective multi-center trial (Santema *et al*. 2018), only 39 patients finished treatment out of 60 patients enrolled in this study. And according to the authors' experience, the daily reality of non-study enrolled patients is much worse. All of this can substantially reduce the outcome of possible effective therapy.

We believe further research will clarify the aforementioned problems and limitations.

## Conflict of Interest

There is no conflict of interest.

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