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Chapter 5

Cell Culture Effects of Altered Oxygen Levels and Hyperbaric Treatment *In Vitro*

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Abstract

Hyperbaric oxygen therapy (HBOT) is a state-of-the-art medical treatment, which is proved to be beneficial in a number of diseases and promising in new fields as well. HBOT is evidence-based treatment for, among others, severe CO intoxication, decompression disease and chronic wound healing. Recent studies promise beneficial effects of HBOT in multiple sclerosis. *In vitro*, cellular models of these complex pathological conditions are limited. In this chapter, we aim to mirror *in vitro* effects of HBOT and other altered oxygen levels on endothelial cells, fibroblast, mesenchymal and pluripotent stem cells. Through these *in vitro* models, the role of HBOT in angiogenesis, blot clotting, wound healing, cell therapy and tissue engineering will be discussed. To summarize *in vitro* effects of HBOT, it has beneficial role on proliferation and viability of most cell types. Furthermore, functional characteristics of the investigated cell types, for example, angiogenesis by endothelial cells, are improved in response to HBOT. Standardized preclinical protocols with HBOT help to translate the benefits to clinical trials and clinical use.

Keywords: hyperbaric oxygen, normoxia, hypoxia, *in vitro*, endothelial cells, fibroblasts, mesenchymal stem cells, endothelial differentiation, wound healing, angiogenesis

1. Introduction

Hyperbaric oxygen therapy (HBOT) is a state-of-the-art medical treatment, which has advantageous therapeutic effects in wide range of pathologies. Despite its high therapeutic potential, its availability is still restricted, and the use of hyperbaric oxygen requires significant organizing steps in most health care systems. Thus, emergent or urgent utilization is very limited.

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HBOT may be used by pulmonologists, internal medicine specialists, surgeons and obstetrics as well. Evidence-based medicine recommends its use in decompression sickness to protect severe lung injury and to enhance recompression [1]. Carbon monoxide intoxication is another severe, life-threatening emergency scenario, where HBOT enhances CO discard and saves lives [2]. HBOT is recommended in severe carbon monoxide intoxication when conservative ventilation techniques are not efficient to eliminate CO, linked with hemoglobin. These time-sensitive conditions shout for widely available HBOT; however, in low-income countries, its use is still optional.

Interestingly, HBOT proved to be effective in wound healing applications, for example, ulcers, scar formation after burn injury or plastic surgery operations [3]. Cardiovascular diseases are the leading cause of death in industrialized countries. Peripheral atherosclerotic diseases and diabetes often go side-by-side. Additionally, venous circulation may also be impaired in these patients. Considering the high burden of cardiovascular diseases, number of patients suffering from not-healing ulcers is constantly increasing. Furthermore, retinal arterial stenosis severely impairs vision, in which condition HBOT is on the palette of treatment applications. Wound healing and scar formation in plastic surgery have a huge esthetic impact and because of this, HBOT draws significant attention from cosmetic companies as well [5, 6].

Next argument for HBOT is that recent publications suggest its beneficial role in neurodegenerative diseases, such as multiple sclerosis [6]. Latest treatment options, for instance mesenchymal stem cell (MSC) implantation, also comprise hyperbaric treatment or preconditioning. Therapeutic potency of MSC improves after hyperbaric modification [8–10].

Other clinical applications of HBOT are severe anemia, crush injury and gas embolism, necrotizing fasciitis, osteomyelitis, brain abscesses and delayed radiation injury. Evidence is lacking in application for Parkinson's disease and autism.

In vitro models of HBOT utilize wide range of cell lines and tissue cultures [9]. HBOT can be combined with modification of cell culture circumstances, for example, adding active drugs, small molecules, growth factors or signaling drives, according to the focus of interest of the study protocol. Mostly, hyperbaric treatments are applied in parallel with normoxic and hypoxic conditions to implicate useful comparative data. Importantly, *in vitro* models have severe limitations as they are not capable to model the whole pathology and tissue characteristics treated with HBOT. *In vitro* models usually follow the clinical protocols of HBOT, regarding timing and incubation periods [10]. In this chapter, altered oxygen levels of human endothelial cell cultures, fibroblasts cultures, human MSC and pluripotent stem cell (PSC) cultures will be discussed, mirroring the effects of HBOT on angiogenesis, blood clotting, wound healing and future cell therapy/tissue engineering issues.

2. Altered levels of oxygen in cell cultures

2.1. Methods of altered oxygen levels in cell culture

Hyperbaric oxygen treatment of cell cultures can be performed in hyperbaric cell culture chamber *in vitro*. Hyperbaric chambers are available commercially and offer sterile

cell culture conditions for short- or long-term maintenance. Cells are usually exposed to 100% oxygen in these chambers; however, some studies comprise 98% oxygen and 2% CO_2 [11]. The level of hyperbaric pressure varies between 1.5 and 3 atmospheres absolute. Compression and decompression times may be applied according to focus of interest and study protocol. Standardization of basic research protocols is key to move the latest investigations with HBOT to clinical translation. Besides HBOT, oxygen levels may be modified for normal or low oxygen (hypoxic) conditions. For normoxic treatment (21% O_2), general cell culture conditions are suitable (5% CO_2, 95% normal air). Hypoxia can be induced by replacing oxygen with nitrogen in cell culture incubators. Mostly, 5 or 10% oxygen levels are investigated in cell culture studies. The same cell culture media and culturing surface can be used in altered oxygen levels, HBOT and in normal conditions [12, 13].

More detailed studies comprise direct quantification of oxygen consumption levels in cellular cultures. These data provide information also on metabolomics status, indirectly on cellular energy homeostasis and metabolic activity of the investigated cultures [14]. Planning studies with direct measurement of oxygen consumption levels enable investigation of cellular function keep with oxygen consumption.

2.2. Endothelial cells, angiogenesis

It is widely accepted that endothelial cells play a key role in a number of important physiological conditions and in pathological steps as well. Endothelial functions comprise regulation of blood flow via regulating vascular tone, vasodilation or vasocontraction. Furthermore, endothelial cells and their expressed factors are cornerstones in initiating or inhibiting platelet activation and blood clotting. Next role is inflammatory mechanisms, white blood cell rolling and diapedesis. Furthermore, special sites of endothelial barriers are the blood–brain barrier, the renal glomeruli and the portal endothelial cells. All these sites have complex barrier and gating functions. All endothelial functions can be modeled *in vitro* and may be investigated and modified via changing oxygen levels or by application of HBOT for cultures.

Additionally, endothelial cells regulate and are involved in embryonic vasculogenesis and somatic angiogenesis as well. Neo-angiogenesis is a key pathological step in tumorous proliferation and metastases development as well. To fulfill these tasks, endothelial cells produce and secrete wide range of angiogenesis-related proteins and small molecules. These may be investigated on gene expression or on the translational (protein) level.

Endothelial cells are keen to proliferate *in vitro*, wide range of cell lines and primary cultures are also available commercially. Widely used endothelial lines *in vitro* are the human umbilical vein endothelial cells (HUVEC), the human coronary arterial endothelial cell (HCAEC), capillary endothelial cells and others from human and animal sources as well. Arterial and venous endothelial cells can be divided via cell surface markers and genotype properties. Arterial and venous endothelial phenotypes differ also *in vitro* because the arterial and venous vessels have largely different functional tasks *in vivo*. As an example, arterial endothelial cells are the major regulators of peripheral vascular resistance, while venous capillary endothelium is the localization for white blood cells' rolling and diapedesis [15]. Furthermore, venous endothelial junctions are thinner, and vessels have greater compliance. Interestingly, arterial and venous plasticity exists *in vitro*, for example, HUVEC surprisingly express arterial markers *in vitro* [16].

Endothelial cells may be cultured in universal cell culturing dishes, on various surfaces, for example, gelatin, fibronectin, collagen and laminin. Common endothelial cell culture media are DMEM and endothelial growth media. To enhance proliferation of mature cells or differentiation from stem cells, a range of growth factors and cytokines can be applied to culture. Important characteristic of mature endothelial cells *in vitro* is the contact inhibition of proliferation [17]. This means that endothelial cells are only capable to proliferate in monolayer trend and grow onto free surfaces. Once the monolayer surface is full-grown, endothelial cell refuses to proliferate *in vitro*.

When investigating endothelial culture, most important *in vitro* characteristics of endothelial cells are the following: phenotype appearance (cobblestone pattern), expression of endothelial specific cell surface markers (CD31, CD144, vascular-endothelial cadherin), acetylated low-density-lipoprotein uptake, tube formation on Matrigel surface and wound healing assay [18]. During passage mechanisms, usually trypsin-based enzymatic digestion is utilized.

Interestingly, these endothelial characteristics were studied in HBOT circumstances as well (**Figure 1**). The morphology of adult somatic endothelial cells in response to HBOT did not change. They retained their cobblestone pattern after HBOT [19]. Importantly, viability of endothelial cells improved after 24 h of HBOT. Increase in viability was related to increase in proliferative capacity as well. Nitric oxide synthase (NOS) has pivotal role in endothelium-dependent vasoactive actions. Role of HBOT treatment was investigated on gene expression levels and on protein levels of primary microvascular capillary endothelial cell cultures. The mechanisms of actions needed further investigations, briefly NOS levels were increased in genomic and protein levels as well [20]. In-depth micro-array analyses of microvascular endothelial cells' genome proved huge impact of HBOT on angiogenesis-related gene expressions [21]. In these studies, HBOT dramatically increased tube formation capacity of endothelial cells on Matrigel [22]. Other studies also proved that HBOT had significant effects on endothelial cells tube formation and migration capacity. Short-term (6–8 h) HBOT treatment resulted in increased migration capacity and enhanced tube formation also by length and density of the network [20]. Ingenuity pathway analyses of the microarray expression data proved top responder's genes for HBOT. These top responder genes were all related to cell-matrix adhesion and matrix degradation processes. The analyses further provided quantitative data on the absolute percentage of endothelial cells that have a specific modulation, such as cellular growth and proliferation 41%, cell death 39%, gene expression 34%, cell morphology 16% and cell cycle 13% [23].

In angiogenesis, main initiative steps are orchestrated by VEGF. Both by sprouting and intussusceptive angiogenesis, the main drive brings activation by VEGF isoforms and their receptors. These VEGFs set communication between tip, phalanx, stalk cells and pericytes [24]. Many other endothelial growth factors and small molecules take part in this process, for example, fibroblast growth factor, epidermal growth factor, insulin-like growth factor, Ephrins and Ephrin receptors, angiopoietin-1, angiopoietin-2 and their receptors [25]. Furthermore, the complex regulatory pathway of the renin-angiotensin-aldosteron system also interacts with vascular mechanism. Amount of secreted angiogenesis-related factors can be measured *in vitro* from cell culture supernatants and from cell lysates via proteolysis. Interestingly, angiogenesis-related steps and molecules may also play a role in chronic tinnitus, which tends to be a future disease to be treated with HBOT [26].

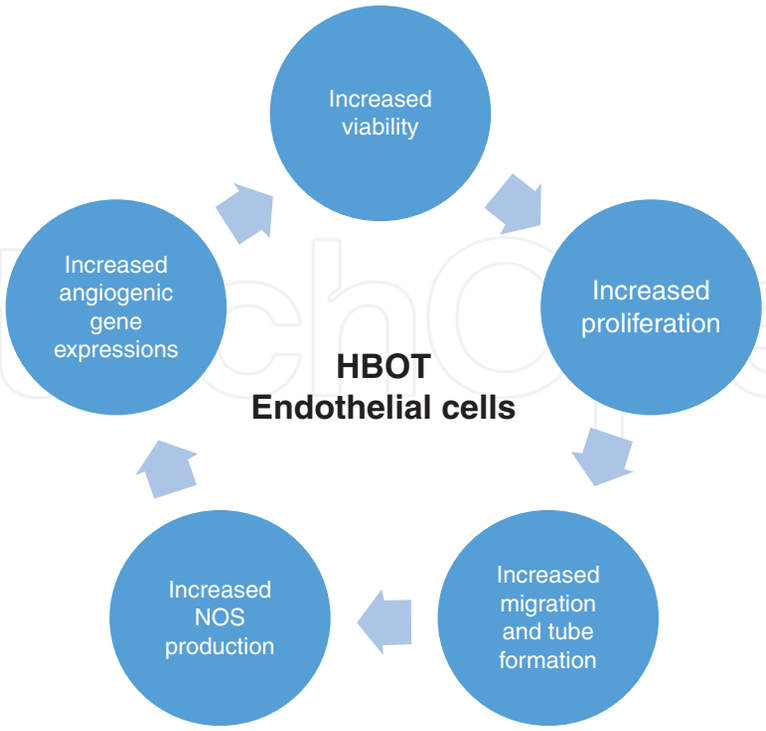


Figure 1. Angiogenesis-related effects of HBOT on endothelial cells.

2.3. Endothelial cells, blood clotting

Besides angiogenesis, orchestrating of blot clotting is a foremost characteristic of endothelial cells. Importantly, altered oxygen circumstances can change endothelial responsiveness, platelet activation and clotting mechanisms. Tissue plasminogen activator is the most powerful enzyme to catalyze thrombin via activating plasminogen to cleave thrombin. Interesting *in vitro* studies proved that HBOT has the potential to modify tissue plasminogen activator secretion from endothelial cells [27]. The changes observed would be clinically significant and beneficial, even more, considering advantageous effects of HBOT on blood-brain barrier function. Others also measured tissue plasminogen activator in combination with plasminogen activator-inhibitor from endothelial supernatants, immediately after HBOT treatment. Surprisingly, both peptides were significantly increased after short-term HBOT. Increased expression was observed immediately after the HBOT and remained also significantly higher at 6 h follow-up of treatment [28].

Additionally, beside regulating endothelial cells-related clot cytokines, HBOT also had notable effects on platelet activity and function as well. Interestingly, platelets responded to HBOT in a manner that their NOS secretion increased significantly [29]. This phenomenon can have significant effects on platelet clotting and thrombus formation as well [9]; however detailed understanding is warranted.

Some human clinical studies investigated platelet count and activity after HBOT and surprisingly found no significant difference before and after HBOT [30]. *In vitro* models often use altered levels and timing of HBOT and most *in vitro* effects are not directly translatable to

in vivo human responses. For instance, platelet rich plasma in experimental circumstances improved after HBOT and had more advantageous effects in a pro-inflammatory, pro-thrombotic area *in vivo* [31]. Mostly, these mechanisms act differently in whole bodies *in vivo*.

Some studies concluded that HBOT may also have disadvantageous effects *in vivo*, if the timing and longevity of treatment is not optimal. Interestingly, in experimental setup, HBOT was able to modify renal erythropoietin production. Disadvantageous results came after HBOT was released and rebound effects ameliorated normal erythropoietin levels. Thus, renal tissue failed to cope with sudden and frequent changes in oxygen levels. The observed results were unrelated to circadian rhythm of erythropoietin production [32].

Point-of-care whole blood and platelet clot analyzer systems also brought disappointing data. Some of these ex vivo analyses proved that short-term HBOT may initiate *in vitro* steps which are characteristics of a disseminated intravascular coagulation (DIC) [33]. DIC is a severe, life threating condition, comprising both pro-thrombotic and not-clotting elements, resulting in a severe clinical case, when blood is unable to clot, but small capillaries are impaired by thrombi. In response to HBOT, an increase in the maximum clot firmness and thrombo-elastic component in clot firmness was depicted (**Figure 2**) [33].

2.4. Endothelial cells, barrier and inflammation

Nitric oxide (NO) is one of the most important factors released by endothelial cells. NO plays a pivotal role in setting vascular tone and regulating blood pressure, via arterioles. On the venous circuit site, NO also has vasodilatory effects, thus is a major vasoactive factor at the site of white blood cells diapedesis and extravasation. Beside these, NO also counteracts with angiogenic activities.

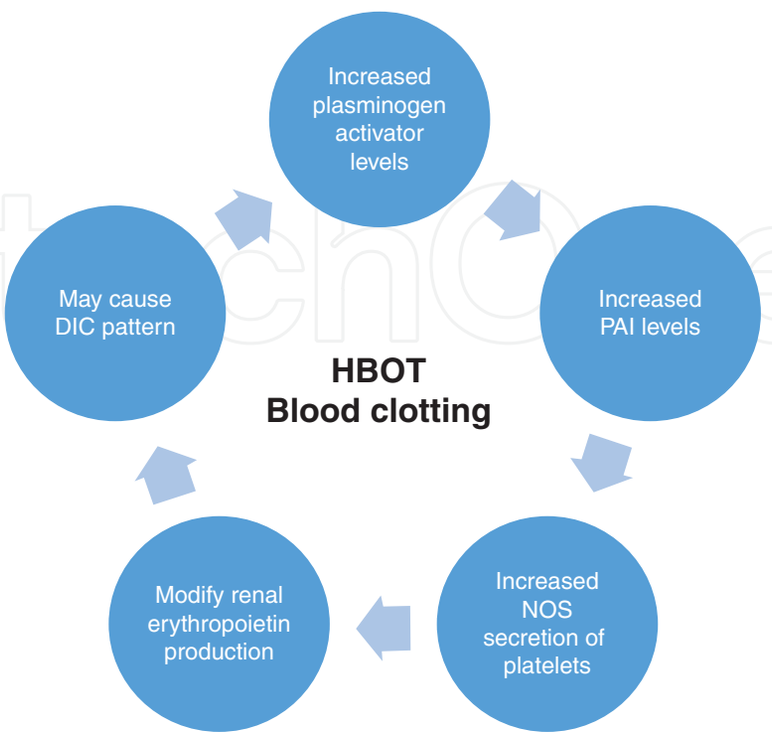


Figure 2. Effects of HBOT on blood clotting. PAI: plasminogen activator inhibitor, DIC: disseminated intravasulcar coagulation.

Interestingly, blood-brain barrier function of endothelial cells can also be modeled and investigated *in vitro*. This very special and crucial endothelial site of the human body is key in pharmacological interventions and critically ill patients. Altered oxygen levels have different effects on blood-brain barrier. It is widely believed that decreased oxygen availability, for example, ischemic attack of the brain, has huge impact on the existence and proper function of blood-brain barrier. In stroke, blood-brain barrier lacks its gating function and medications may have altered neurological side effects as well.

In the in vitro model of blood-brain barrier, brain microvascular endothelial cells can be cultured and trans-endothelial electric potential, as a measure of barrier function, can be evaluated in different oxygen circumstances [34]. Mainly cell interactions, tight junctions and endothelial-pericyte interactions are damaged in blood-brain barrier dysfunction. Cell adhesion molecules are often investigated *in vitro* as well. Endothelial and pericyte co-cultures (e.g., insert plate) offer studying communication between these cellular compartments [35].

In co-culture, cellular models of endothelial interactions with white blood cells were also observed. White blood cells' diapedesis, rolling and pooling in microcirculation are the determinants of local inflammatory responses. Attenuating these would have dramatic therapeutic effects, for example, in chronic, not-healing wounds. Neutrophils' adhesion to endothelial cells was reversed and delayed in HBOT circumstances [36]. The underlying molecular mechanism was mainly the reduced expression of neutrophil-endothelial adhesion molecule, ICAM-1. As a result of low neutrophil adhesion, local levels of ROS were also decreased [36].

Further studies proved that HBOT may have direct effects on endothelial gene expression as well. HCAEC modified their angiogenesis-related gene expression, shortly after HBOT. Short-term HBOT (4–6 h) resulted in increased TNF-α secretion from HCAEC. Related to this, HBOT also modified expression of a range of peptides and small molecular, which have strong role in glucose metabolism and inflammatory reactions as well [20]. Additionally, all of these mechanisms were also linked to altered expressions of certain kinases and altered phosphorylation status. These were related to visceral fat accumulation, atherosclerosis, inflammation and increased cardiovascular risk. Remarkable results proved that HBOT also have metabolomics effects on treated endothelial cells. Short-term HBOT altered glucose uptake in HCEAC. These key results showed that metabolomics disturbances may also be modified under HBOT circumstances, which has key message to future therapeutic human applications [20].

Interestingly, HBOT had robust effect on inflammation-related cytokine expression, for example, level of anti-inflammatory angiogenin decreased, while the level of pro-inflammatory cytokines (IL-6 and IL-8) significantly decreased in response to HBOT. This *in vitro* model was established from and *in vivo* septic small animal model. Endothelial cells from septic and control rats were cultures and inflammatory cytokines were measured from endothelial supernatants [37]. Others also showed significant decrease in pro-inflammatory cytokines, such as TNF-α following HBOT [38].

Latest *in vitro* studies demonstrated that hypoxic damage of blood-brain barrier may be reversed via HBOT [34]. Hypoxia induced cellular endothelial fragmentation and impair of cell adhesion molecular. On the contrary, HBOT after hypoxia was able to attenuate the effects and improve cellular junctions [34]. These data have very important message to clinical trials, as HBOT may have undistinguishable role in stroke treatment in the acute clinical phase (**Figure 3**) [39].

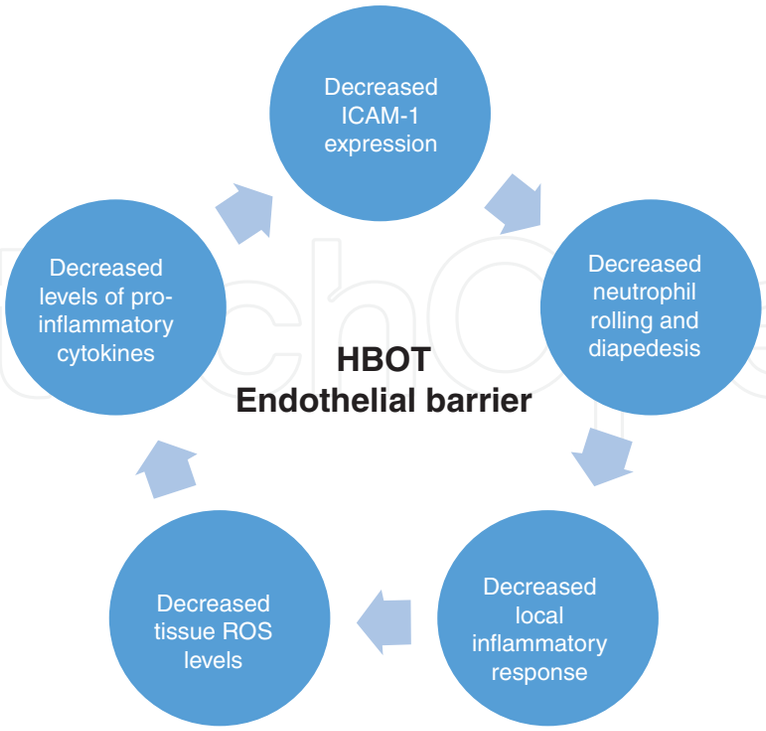


Figure 3. Effects of HBOT on endothleial barrier and local inflammatory reactions.

2.5. Fibroblasts, wound healing

Fibroblasts are easy to culture and maintain. They have high proliferative capacity and low maintenance circumstances. They grow in any cell culture media, mostly in fibroblast growth media or DMEM. They adhere to plastic surfaces or to any additional, for example, gelatin or fibronectin. Interestingly, fibroblasts proliferative from skin biopsy samples *in vitro* as well. Fibroblasts have rod-shaped, elongated phenotype in culture. Usually they proliferate in monolayer; however, contact inhibition of growth is not as prominent as it is by endothelial cells [40].

Chronic, not-healing wounds are major challenge in dermatology, surgery and plastic surgery [4, 7]. These wounds have valuable impact on diabetic and cardiovascular patients' quality of life. Furthermore, these wounds often become infected or colonized with resistant species, for example, MRSA [41]. Mechanisms of action in these chronic wounds include reactive oxygen species, chronic inflammation and chronic ischemia [42]. The connective tissue, extracellular matrices are also affected and hyper-oxidant status seems to be the common clue behind non-healing. Growth and proliferation of fibroblasts are often impaired due to aforementioned pathological mechanisms. Thus, fibroblasts offer platform to monitor cellular events on one important component of these wounds. *In vitro* studies are suitable to monitor effects of altered oxygen levels, especially focusing on cytokine release, apoptosis and leukocyte activation.

In vitro studies proved that HBOT on ischemic wound tissue increased the activity of superoxide-dismutase (SOD) enzyme, which is known to be one of the most potent enzymes acting against ROS species-related harm [43]. Interestingly, *in vivo* studies on small animal models of chronic ulcers also proved significant effects of matrix-metalloproteinases (MMP) in the chronic ongoing

damage. Placing the animals in HBOT and treating them resulted in increased MMP inhibitor activity and in parallel the tissue damage, cellular apoptosis and necrosis decreased [44].

HBOT has a significant effect on the growth of fibroblast cultures. HBOT in increasing pressure and time interval had advantageous effects on the proliferation of fibroblast cultures, suggesting beneficial effects in wound healing steps as well [45]. Parallel with timing and pressure of HBOT, cell numbers increased as well [45]. Additionally, HBOT increased tube formation of endothelial and fibroblast co-cultures [20]. In a wound healing assay, *in vitro*, significant increase was observed after HBOT treatment [22]. Linking to previous clues, HBOT also increases collagen proliferation [22], parallel with beneficial effects on fibroblast growth, and thus all together, HBOT has a beneficial effect on extracellular matrix proliferation, growth and structure [20]. Furthermore, recent experiments proved the increase of fibroblast proliferation and growth also *in vitro* and *in vivo* in epidural fibrous tissue. Mechanisms of action behind these were downregulation of canonical TGF-β and interleukin pathways, which were responsible for maintaining fibroblasts' viability and proliferation (**Figure 4**) [46].

TGF-β is also involved in uncontrolled scar formation, known as keloid scars [47]. Keloid scars contain highly proliferative fibroblasts and connective tissues, which cause significant biomechanical and esthetic problems on affected skins. HBOT were successful to reduce TFG-β levels in these keloids and interestingly proliferation of keloid scar was postponed in response to HBOT [48]. The regulatory steps are not yet characterized in detail, and further investigation is needed to understand the process [49].

In vitro models for burned skins also exist; however, modeling the complex mechanisms of local and systemic response to severe burn and demonstrating and measuring accurately the cytokine storm *in vitro* are almost impossible. Interesting *in vitro* model for burned skin evolved ex vivo available burned skin tissues [50]. Recently, HBOT has been emerged for the treatment of chronic burn-related wounds as inflammatory cytokine release was decreased and bacterial viability also decreased in wound [34, 35]. Burn models proved hyperemia (improved microcirculation) and reduced size of the burned lesions after HBOT of burn wounds [53]. Additionally, fluid homeostasis of burned wounds was also altered beneficially after HBOT [51, 52]. Intercellular edema decreased after HBOT, resulting in better microcirculatory responses and increased debris elimination [54].

2.6. Mesenchymal and pluripotent stem cells, cell therapy and tissue engineering aspects

MSC and other cell types such as the pluripotent stem cells have huge potential for cell therapy and tissue engineering in various diseases. Recently, most clinical trials in cardiovascular field have been performed with MSC or MSC-derivatives [55]. Furthermore, cardiovascular derivatives of pluripotent stem cells are promising tools to differentiate new cardiovascular cells and to build cardiovascular tissue. Latest tissue engineering methods comprise biodegradable matrices combined with cellular building blocks.

MSC and PSC behave and differentiate altered in normal hypoxic or in hyperbaric oxygen conditions PSC studies concluded that altered oxygen levels may mimic in utero conditions

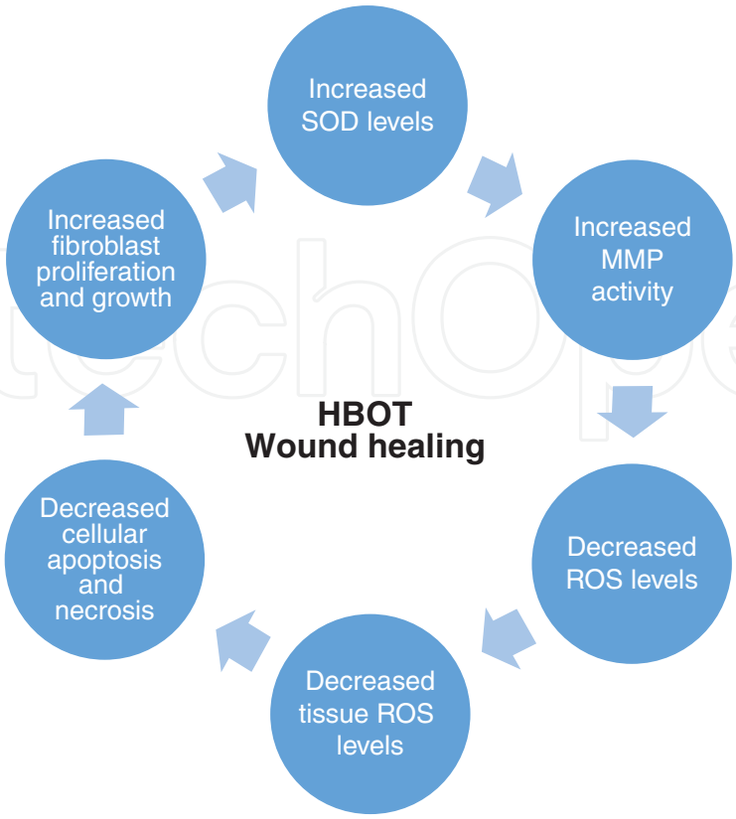


Figure 4. Effects of HBOT on wound healing and fibroblasts SOD: superoxide dismutase, MMP: matrix-metalloproteinase, ROS: reactive oxygen species.

better and thus may initiate differentiation potency [56]. Latest state-of-the-art molecular biology protocols comprise epigenetic or genetic modifications for example reprogramming and CRISPR/Cas9 genome editing technique [57, 58]. These are often utilized parallel with altered oxygen levels. Signaling steps related to these mechanisms also changed including MAP kinases [44].

MSCs are multipotent stem cells which by definition have the potency to differentiate into cartilage bone muscle tendon ligament and fat tissue. MSC can be characterized via cell surface markers: they widely express CD73, CD90 and CD105 but do not express CD11, CD14, CD19, CD34 and CD45 [57]. They are easy to culture adhere to plastic and most cell culture surfaces and can proliferate in MSC media and others as well. By directed differentiation they can differentiate into chondrogenic osteogenic myogenic and adipogenous linage [57]. It is debated if mature cardiomyocytes can derive from MSC.

Hypoxic preconditioning is currently being investigated also in human clinical trials as a protective mechanism of ischemia-reperfusion injury in the ischemic myocardium [59]. Related to this, ischemic preconditioning is being evaluated in the *in vitro* setting and in clinical trials. Recently, MSC were the most robust players in cardiovascular cell therapy trials. For instance, the CHART-1 clinical trial involved hundreds of patients suffering from chronic ischemic heart failure. Cardiopoietic cells, derived from MSC, were implanted endomyocardially.

Despite huge promises, the CHART-1 trial failed to reach primary composite endpoint and cell implantation did not improve functional status of the patients [38–40, 60, 61]. The pre-implanted cells received a growth factor cocktail, but none-of oxygen level modification was performed during pretreatment [62]. Earlier studies proved that hypoxic preconditioning of MSC increases their secretion of pro-angiogenic, anti-fibrotic, anti-apoptotic secretome, which are known as the paracrine mechanism [63]. Some of these trials comprised temporary anoxia as well [64]. Beside hypoxic pretreatment, other studies aimed hyperbaric pretreatment of regenerative studies [65]. Preconditioning in HBOT circumstances had advantageous effects on neuronal cells as well [65]. *In vitro* part of small animals' trials proved that HBOT can induce hypoxia tolerability of spinal neurons. The mechanisms of actions behind these beneficial effects were metabolic coping, especially altered glucose homeostasis [66]. Thus, these experiments underpinned that HBOT has direct effect on metabolomics and energy homeostasis of cellular compartments, as different oxygen levels await altered metabolic actions [66].

HBOT in MSC resulted in increased proliferative capacity of the cells when compared to those MSC treated in normal oxygen circumstances [11, 29]. In this study, secretome of MSC was evaluated via the ELISA method and levels of BDNF were investigated. This peptide has pivotal role in neurodegenerative diseases but also reported to play a role in salvage mechanisms of the central nervous system after a cardiac arrest [67]. BDNF secretion of MSC significantly increased after HBOT treatment, but was also improved in hypoxia [45]. These results widely clue if normal oxygen levels are suitable to culture and maintain MSC and their derivatives. Further cell therapy trials are needed to standardize cell culture protocols, because recent variations disable direct comparative analyses.

Endothelial progenitor cells [68] are circulating in blood and released from bone marrow. Some studies outline their potential biomarker role for ischemic cardiovascular conditions, as far as their level is increased in acute myocardial infarction and chronic hind-limb ischemia [69]. Endothelial progenitor cells may also have therapeutic effects and phase II/III clinical trials aim boosting them by external infusion of activating factors [70]. An activator drive of these circulating progenitor cells could also be HBOT [71]. Repeated HBOT resulted in significant release of circulating CD34 positive progenitor cells in the peripheral blood. The mechanisms were NOS dependent [71].

If directed differentiation is aimed to be supported, MSC may be cultured in HBOT circumstances. Interestingly, HBOT enhanced osteogenic differentiation of MSC, which *in vitro* description was further proved via *in vivo* proof-of-concept studies as well [11]. Interestingly, metabolic activities, especially calcium influx and exchange, were also modulated by HBOT in MSC. HBOT increased the activity of calcium homeostasis, which is key for osteoblasts via proliferation and bone formation [11].

Pluripotent stem cells are sensitive cell cultures *in vitro*. Their maintenance requires special techniques and expertise in the field. Pluripotent cells form pluripotent cell clusters *in vitro*. They require special maintenance pluripotent stem cell media. Through passaging, spontaneously differentiated cells have to be picked and removed from culture. Recently, enzymatic passage became more advantageous than mechanical breaking of pluripotent colonies.

Pluripotent stem cell maintenance and differentiation are new and difficult cell culture techniques. These involve monolayer or three-dimensional/cell suspension culture as well. Pluripotent stem cell may be cultured on feeder layer of feeder-free surface on biomatrices. The pluripotent stem cells themselves have excellent viability and proliferative capacity in normal oxygen circumstances [72]. Additionally, they are immortal and can continuously proliferate in pluripotent state. Reasonably, most study protocols emphasize the importance of altered oxygen levels, once differentiation steps are in progress. After differentiation, steps are initiated altered oxygen levels usually increase the yield of developed cells and increase functional activity, for example, insulin secretion of beta cells, derived from pluripotent cells [14].

Pluripotent stem cells proliferate in low-oxygen levels in utero. It is also agreed that MSC have low oxygen circumstances *in vivo* in the bone marrow niche. Taking these into consideration, *in vitro* culture of the cells in normal oxygen circumstances is out of their normal niche. Interestingly, all of these cell types improved performance in therapeutic potential when cultured in hypoxic environment [45]. MSC improved angiogenesis-related gene expressions and protein expressions in hypoxia, furthermore implanting them into various *in vivo* models of ischemia resulted in better outcomes [73].

HBOT would have a significant role in tissue engineering and preconditioning the engineered construct *in vitro*, before *in vivo* transplantation. As an example, tissue engineered mucosa were further developed in HBOT. The mucosal cells enhanced expression of angiogenesis-related factor (e.g., VEGF, FGF and HGF) [74]. Enhanced angiogenesis by mucosal tissue may be beneficial for graft homing and retention.

Wide range of differentiation protocols exist, which aim improving the number of cardiovascular derivatives after the differentiation steps. These are increasing in endothelial cell and cardiovascular cells as well. With endothelial cells, recent protocols reached about 50% differentiation yield. Latest studies aim hypoxia as a diver to mesodermal and then to endothelial lineage specification [75].

3. Conclusion

In conclusion, HBOT is an interesting novel medical tool with wide range of therapeutic potential.

In vitro cellular models utilize different HBOT protocols and need to be standardized to bring translatable data to clinicians.

This chapter outlined that HBOT increases endothelial and fibroblast viability and proliferation *in vitro*. Furthermore, tube formation and wound healing assay improved in response to HBOT. HBOT has significant effects on endothelial-related blot-clotting and platelet mechanisms as well. Furthermore, HBOT decreases ROS-related harm in not-healing wound model and improves blood-brain barrier after ischemic event in *in vitro* model. In new therapeutic promises, the stem cells would also benefit from HBOT in maintenance, proliferation and tissue engineering aspects as well.

Conflict of interest

The author declares no conflict of interest.

Abbreviations

CO	carbon monoxide
CO_2	carbon dioxide
HBOT	hyperbaric oxygen treatment
NO	nitric oxide
NOS	nitric oxide synthase
MRSA	methicillin-resistant *Staphylococcus aureus*
MSC	mesenchymal stem cells
PSC	pluripotent stem cells
ROS	reactive oxygen species
SOD	superoxide-dismutase

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