The Effects of Hyperbaric Oxygen Treatment on Hypoxia Inducible Factor-1α, Inducible Nitric Oxide Synthase and Vascular Endothelial Growth Factor Levels in Patients with Diabetic Foot Wound

Hiperbarik Oksijen Tedavisinin, Diyabetik Ayak Yarası Olan Hastalarda Hipoksiyle İndüklenen Faktör-1α, İndüklenebilir Nitrik Oksit Sentaz ve Vasküler Endotelyal Büyüme Faktörü Seviyeleri Üzerine Etkisi

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Abstract

A number of studies have proved that hyperbaric oxygen therapy (HBOT) contributes to wound healing by improving hypoxic wound site. However, the mechanisms, by which the enhanced oxygen in the wound site contributes to wound healing, still remain to be elucidated. This study aimed to investigate the effects of the HBOT on hypoxia inducible factor-1α (HIF-1α), inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) levels that we consider influential on diabetic foot healing. 20 patients were included in the study with diabetic foot wounds of Wagner classification grade 2-3 and 4. Tissue samples from the patients' wounds were taken before the first HBOT session and after the 10th session by curettage and stored at -82 °C. Upon collection of all samples, expressions of the stated parameters were examined through polymerase chain reaction (PCR) study. The results revealed that the increase in HIF1-α has a meaningful correlation with the increase in iNOS and VEGF ($p<0.05$). VEGF, iNOS and HIF1-α levels in patients who underwent major or minor amputations went up; while all parameters in patients who recovered indicated decline which is statistically non-significant. According to these data, we concluded that HBOT inhibited prolonged hypoxic stimulus and decreased both the levels of the mentioned parameters and the negative effects of prolonged and intense expressions of them on wound healing.

Keywords: Diabetic Foot, Hyperbaric Oxygen, Hypoxia Inducible Factor, Wound Healing

Öz

Hiperbarik oksijen tedavisinin (HBOT) hipoksik yara ortamını düzelterek yara iyileşmesine katkıda bulunduğu birçok çalışmayla kanıtlanmıştır. Ancak yara bölgesinde arttırılan oksijenin hangi mekanizmalarla yara iyileşmesine katkıda bulunduğu halen aydınlatılmaya çalışılan bir konudur. Bu çalışma diyabetik ayak iyileşmesinde etkisi olduğunu düşündüğümüz hipoksiyle indüklenen faktör-1α (HIF-1α), indüklenebilir nitrik oksit sentaz (iNOS) ve vasküler endotelyal büyüme faktörü (VEGF) seviyeleri üzerine HBOT' nin etkilerini araştırmayı amaçlamıştır. Çalışmaya Wagner sınıflaması evre 2-3 ve 4 diyabetik ayak yarası olan 20 hasta dahil edilmiştir. Hastaların yaralarından küretajla ilk HBOT seansı öncesi ve 10. seans sonrası doku örnekleri alınmış ve -82 °C' de saklanmıştır. Tüm örnekler toplandıktan sonra, bahsedilen parametrelerin ekspresyonları polimeraz zincir reaksiyonu çalışmasıyla değerlendirilmiştir. Sonuçlar HIF1-α' daki artışın, iNOS ve VEGF' deki artışla anlamlı korelasyonu olduğunu göstermiştir ($p<0.05$). Majör ve minor amputasyona giden hastalarda VEGF, iNOS ve HIF1-α seviyeleri artarken iyileşen hastalarda tüm parametreler düşüş göstermiştir, ki bu farklar istatistiksel olarak anlamsız bulunmuştur. Bu verilere göre HBOT' nin uzamış hipoksik stimulusu inhibe ettiği ve bahsedilen parametrelerin seviyeleri ile bunların uzamış ve yoğun ekspresyonlarının yara iyileşmesi üzerindeki olumsuz etkilerini azalttığı sonucuna vardık.

Anahtar Kelimeler: Diyabetik Ayak, Hiperbarik Oksijen, Hipoksiyle İndüklenen Faktör, Yara İyileşmesi

Introduction

Diabetic foot is a serious chronic complication of diabetes mellitus. It is estimated that approximately every 30 seconds one extremity is amputated because of diabetic foot and this information shows us how dramatic a diabetic foot is. The risk for a diabetic patient to have a diabetic foot wound is %15 in his/her lifetime (1). Diabetic foot treatment needs intensive effort and gives economical burden to social security systems.

HBOT is a treatment modality which is effectively used in several diseases including diabetic foot wounds. It is applied in a specially constructed pressure chamber and patient breathes oxygen periodically from a mask, hood, endotracheal tube or chamber environment (if the chamber is pressurized by oxygen) while chamber pressure is higher than 1 atmosphere.

Several mediators which affect wound healing and produced in the hypoxic environment of wound are the scope of many studies. HIF-1α, VEGF and iNOS are attracted attention for this reason.

HBOT corrects tissue oxygenation thus it activates or deactivates many mediators at the cellular level. So it is very important to clarify physiologic basis of HBOT to better understand its action.

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HIF-1 is the main transcription factor which regulates more than one hundred genes under hypoxic conditions (2). The genes which HIF-1 regulates are responsible for oxygen homeostasis and glucose-energy metabolism (3). Its deficient or excessive amount impairs the wound healing.

Nitric oxide (NO) takes part of vasodilatation, angiogenesis, inflammation and different kinds of immune responses. iNOS causes high amounts of NO synthesis in some pathologic conditions. iNOS deficiency is related to delayed wound healing and also its over production causes cytotoxicity. High VEGF levels result in leaky vessels which also impairs wound healing.

Material and Method

Patient Selection: The study group was selected among the patients who applied to our Hyperbaric Medicine Department between October 2008 – July 2009 with diabetic foot wound. All patients were classified according to Wagner Ulcer Classification (Table 1). Wagner grade 2, 3 and 4 patients were included in the study while the other grades were excluded.

Table 1. Wagner ulcer classification

Grade	Lesion
0	No ulcer; but may have deformity, hyperkeratosis or cellulitis
1	Partial or full thickness superficial ulcer
2	Deep ulcer without abscess or osteomyelitis (extending to ligament, tendon, joint capsule, or deep fascia)
3	Deep ulcer with abscess or osteomyelitis
4	Localized gangrene of forefoot, toe or heel
5	Gangrene of the entire foot

All Wagner grade 2, 3 and 4 patients were also examined for systemic hypoxic conditions such as chronic obstructive lung disease, severe congestive heart failure, end-stage renal failure and severe anemia with Hb<9 gr/dl and excluded from the study if they had one or more of these conditions.

Patients were examined for the HBOT suitability beforehand. Exact and relative contraindicative conditions (except mild upper respiratory tract infections and fever below 38.5°C) were also excluded from the study.

Finally, total of 20 patients with Wagner grade 2, 3 and 4 diabetic foot were included in the study.

A written consent, which was approved by Ethics Committee (11.11.2008/123), was signed by each patient.

Sampling: After purifying the wound from debris with physiologic saline solution, tissue sample was taken from the wound-dermis border by curettage. Samples were stored in sterile eppendorf tubes at -82°C until the laboratory studies were performed. These procedures were done before the first and after the tenth HBOT session.

HBOT Procedure: A multiplace chamber was used (Hipertech-Zyron12/Turkey) and 120 minute-treatment sessions were applied to the patients at 2.5 ATA (atmosphere absolute) daily (5 days of week). This 120 minute-treatment session consisted of 20 minute-compression phase with air, three 25 minute-oxygen periods via mask and two 5 minute-air breaks between these oxygen periods and 15 minute-decompression phase. So the patients breathed O2 total of 75 minutes during a session.

PCR Analysis: 30 mg tissue sample for each patient and RNA isolation kit (eZNA, Total RNA Mini Kit, Omega Bio-tek, USA) were used for RNA isolation. During RNA isolation the standart procedure provided by the manufacturer was followed. The primers used for the study were Eurofins MWG Operon, Germany (Table 2).

Table 2. Primers used in the study

	Forward (5′ - 3′)	Reverse (5′ - 3′)
HIF-1α	CTAGCCGGAGGAAGAACTATGAAC	CCCACACTGAGGTTGGTTACTGT
VEGF	ACTGCCATCCAATCGAGACC	GATGGCTGAAGATGTACTCGATCT
iNOS	TCAAATCTCGGCAGAATCTACAAA	CAGGAGAGTTCCACCAGGATG

Following RNA isolation, dNTP, primer, Reverse Trascriptase (RT) and RT buffer solution were added to 10 µL isolated RNA for each sample. This mixture was processed at 37ºC for 1 hour and at 95ºC for 5 minutes to synthesize cDNA.

cDNA samples were processed by using Syber green (Brillant II Syber Green, Agilent Technologies, USA) at Mx3005P (Stratagene, USA) Real Time PCR equipment.

Results were evaluated by measuring the fluorescence values which the samples took and by calculating percentage ratios.

Statistical Analysis: Statistical analysis was done by using SPSS 16.0. Results were given as mean±standart deviation and percentage. For statistical comparison among independent groups, non-parametric Wilcoxon test was used. $P<0.05$ was accepted as statistically significant.

Results

16 patients (80%) were male and 4 (20%) were female. Average age 63.1 (±7.1) years; diabetes duration 15.5 (±6.6) years; fasting glucose level 197.7 (±49.18) mg/dl; HbA1c 9.06 (±2.0) %; BMI 27.89 (±3.46); sedimentation 57.9 (±37.24) mm/h; CRP 38.8 (±50.5) mg/l; Hb 12.3 (±1.95) gr/dl; Hct 37.34 (±6.53) %; WBC 9880 (±4409) /mm^3. 8 patients experienced (40%) minor or major amputation and 12 patients recovered (60%).

Mean PCR values are given for before the first and after the tenth HBOT session, respectively: HIF1-α 5502.85 (±2314.11), 5023.35 (±1337.00);

iNOS 5479.95 (±1580.30), 5448.40 (±1467.29); VEGF 6026.30 (±1838.08), 5949.30 (±2313.13) (Fig.1).

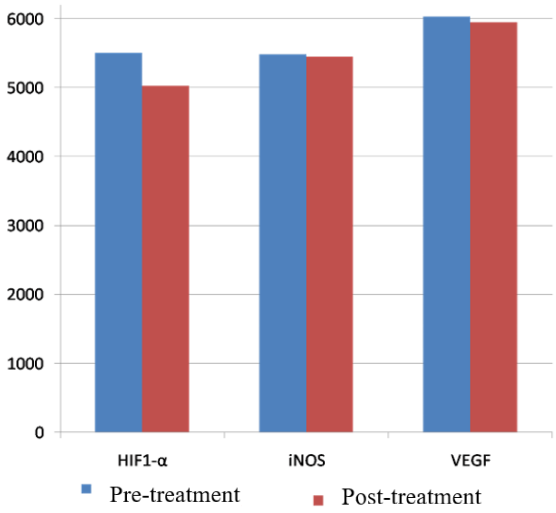

Figure 1. Average values of the parameters (Pre and Post-treatment)

The increase in HIF1-α has a meaningful correlation with the increase in iNOS and VEGF ($p<0.05$). VEGF, iNOS and HIF1-α levels in patients who underwent major or minor amputations went up; while all parameters in patients who recovered indicated a decline which is statistically non-significant. PCR analysis of the mentioned parameters are shown at Fig. 2 (HIF1-α), 3 (iNOS) and 4 (VEGF).

Discussion

Zhang Q et al. (4) studied the effects of HBOT on an ischemic wound model on rats and reported that HBOT showed its beneficial effects by means of HIF-1α downregulation. This study also reported that this downregulation caused decrement of cell apoptosis and wound inflammation.

Halterman et al. (5) showed that high and prolonged HIF-1α levels changed the adaptive form of HIF-1 (HIF-1α + HIF-1β) into the pathologic form of HIF-1 (HIF-1α + p53) which caused apoptotic cell death on a cerebral ischemia-hypotension model. The severity and the duration of hypoxia states whether the HIF-1 becomes adaptive or pathologic form (6).

In experimental global ischemia and subarachnoid hemorrhage models, HBOT was showed to decrease HIF-1α and most genes controlled by it. Thus the apoptotic cell death decreased. Theoretically, HBOT can decrease the pathologic form of HIF-1α (HIF-1α + p53) by means of its hyperoxic effect in hypoxic tissues (6).

In this study, we found decreased levels of HIF-1α expression in patients whose wounds recovered and increased levels of HIF-1α expression in patients who underwent amputation. It is well known that

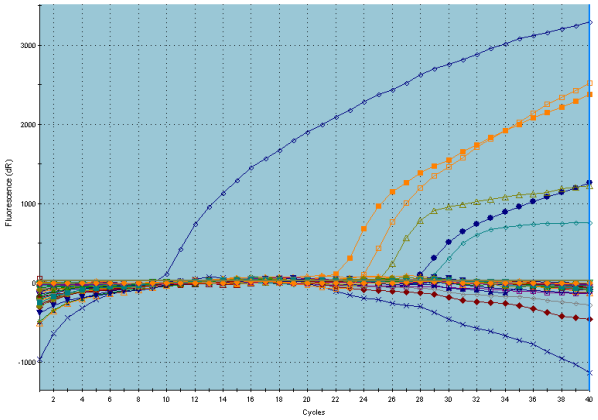

Figure 2. PCR Analysis for HIF1-α

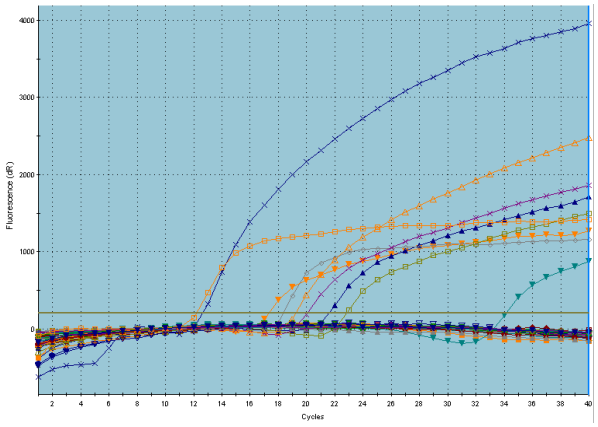

Figure 3. PCR Analysis for iNOS

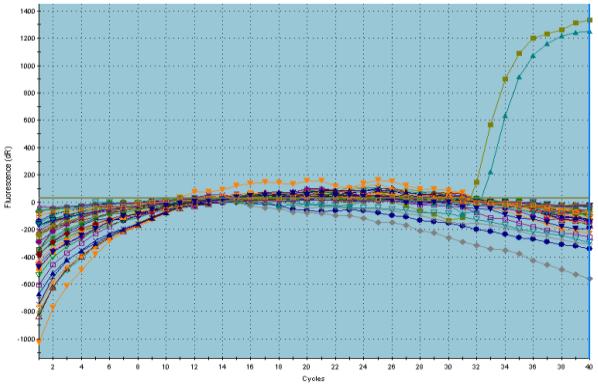

Figure 4. PCR Analysis for VEGF

most diabetic patients have ischemia and hypoxia due to peripheral arterial disease. HBOT seems to decrease HIF-1α levels by its hyperoxic effect and prevents cell apoptosis in diabetic foot wounds as mentioned by Zhang Q et al. (4) in an ischemic wound model. Further studies should include tissue oxygen tension measurements to show the correlation between normalisation of tissue oxygenation and HIF-1α levels.

Hypoxia increases iNOS expressions via HIF dependent pathway (7). Activation of HIF due to hypoxia leads to expression of some genes which decreases oxygen demand and also provides oxygen supply. One of these mentioned genes is iNOS (8,9). Our findings support the correlation between HIF and iNOS.

Thomas et al. (10) showed that when NO synthesis was below a critical level in diabetic foot wound, the wound failed to heal. In hypoxic and inflammatory conditions NO levels are majorly in control of iNOS. Hypoxic and inflammatory nature of diabetic foot induces iNOS and leads to NO production (11). Jude et al. (12) reported higher iNOS protein levels and enzymatic activity in diabetic patients' skin than non diabetics'. And it was reported that iNOS deficient mice showed delayed wound healing and after adenoviral transfer of iNOS gene, healing rate became normal levels (13). If NOS activity is blocked, collagen synthesis and wound healing are impaired (14,15). Delayed wound healing accompanies to decreased NO (16,17).

Cross et al. (18) showed prolonged high NO levels, produced by iNOS, were proinflammatory and destructive. NO shows its cytotoxic effects via peroxynitrite (19).

When these studies are evaluated, it is clear that both low and high NO levels impair wound healing. Our study showed low iNOS levels in patients who healed and high iNOS levels in patients who underwent amputation. This result shows us when HBOT manages to normalize the oxygenation of diabetic foot wound, iNOS activity and NO levels decrease to optimal levels. So the cytotoxic effects of high NO concentrations are blocked and wound healing can occur.

One of the most important steps of wound healing is angiogenesis which is induced by VEGF. VEGF production is mainly stimulated by hypoxia. Theoretically, it can be said that hyperoxia induced by HBOT suppresses VEGF, but some studies report the opposite (20).

Some in vitro studies showed that hyperoxia led to upregulation of PDGF receptors and some oxidants (like hydrogen peroxide) increased VEGF production in endothelial cells and keratinocytes. In addition to these, it was also reported that macrophages and endothelial cells exposed to hyperoxia upregulated IL-8, TGF-β and VEGF mRNA (20).

Hypoxic periods between HBOT sessions are believed to increase VEGF protein levels in wound fluid and oxidants were reported to increase VEGF levels in macrophages. It is also believed that the high pO_2 levels cause oxidant production from leukocytes and this process leads to VEGF production.

Shenberger et al. (21) reported the decrease of VEGF mRNA levels in lungs due to hyperoxia. Zhang Q et al. (4) showed a peak in VEGF expression in an ischemic wound model on rats at the 7th day but the HBOT group showed significant decrease.

In a recently published article by Zhang M et al. (22) reported decreased HIF-1α and VEGF expressions in patients' keloid tissue who had HBOT compared to non-HBOT keloid group and declared differences between HBOT and non-HBOT groups were statistically significant. They explained this decrease by an increase of oxygen in keloid tissue due to HBOT. Correspondingly, Lu et al. (23) applied HBOT to mice with glioma and showed statistically significant decrement of HIF-1α and VEGF expression in tumor tissues compared to non-HBOT control group. They stated that HBOT enhanced pO_2 in tumor tissues and caused low expression of HIF-1α and VEGF.

Our study showed that the VEGF expression decreased with HBOT in patients who healed and increased in patients who underwent amputation. So it can be said that when the wound bed is oxygenated well enough with HBOT, excessive VEGF expression becomes limited.

Conclusions:

Our research showed that HBOT decreased HIF-1α, iNOS and VEGF levels in diabetic foot wounds which recovered. We concluded that HBOT inhibits prolonged hypoxic stimulus and decreases both the levels of the mentioned parameters and the negative effects of prolonged and intense expressions of these on wound healing even if the differences between pre and post-treatment are not statistically significant. However small size of the study group and early sampling at the tenth session might have affected our results.

Ethics Committee Approval: Gülhane Military Medical Academy Ethics Committee Permission was obtained with the letter dated 11.11.2008 and numbered 123.

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