NATIONAL POLYTECHNIC INSTITUTE
SUPERIOR SCHOOL OF MEDICINE
DIVISION OF POSTGRADUATE STUDIES

The effect of hyperbaric oxygen therapy on oxidative stress and inflammation in patients with diabetic foot ulcers

Principal Investigator:

Modesto Gómez López

Molecular Biology of the Inflammatory Process, School of Medicine, National Polytechnic Institute.

Research associates:

Nadia Mabel Pérez Vielma

Sección de Estudios de Posgrado e Investigación, Centro Interdisciplinario de Ciencias de la Salud, Unidad Santo-Tomas, Instituto Politécnico Nacional,

María Magdalena Valencia Gutiérrez

Sección de Estudios de Posgrado, Escuela Superior de Medicina, Instituto politécnico Nacional.

Jennifer Viridiana Sánchez Camacho

Sección de Estudios de Posgrado, Escuela Superior de Medicina, Instituto politécnico Nacional.

José Enrique González Hernández

Clínica Especializada en el manejo de diabetes, de la Secretaría de Salud CDMX

, Ángel Miliar García

Laboratorio de Biología Molecular, Escuela Superior de Medicina, Instituto Politécnico Nacional.

César Ochoa

College of Osteopathic Medicine of the Pacific, Pomona, CA, USA and Center for Clinical, Research at WUHS, Pomona, CA.

Jonathan Labovitz

Clinical Education and Graduate Placement, Western University of Health Sciences, College of Podiatric Medicine, 309 East 2nd Street, Pomona, CA 91766, USA

Mexico, CDMX, December 28th, 2018

The effect of hyperbaric oxygen therapy on oxidative stress and inflammation in patients with diabetic foot ulcers

Background

Type 2 diabetes mellitus (T2DM) is one of the most significant public health conditions worldwide (1, 2). Patients present a metabolic disorder originated by the combination of a deficiency in insulin synthesis by pancreatic β-cells and insulin resistance in the specific tissues where they carry out their action, resulting in hyperglycemia (3, 4).

The severity of insulin resistance depends on numerous factors, such as weight, age, heredity, oxidative stress, and endocrine history (4-8). As a net effect, glucose metabolism is modified, resulting in elevated fasting glucose levels or glucose intolerance and will manifest T2DM within 3 to 5 years (9). On the other hand, hyperbaric oxygen therapy (HBOT) is defined by the Undersea and Hyperbaric Medical Society (UHMS) as treatment where a patient intermittently breathes 100% oxygen at a pressure above sea level pressure (a pressure greater than 1 atmosphere absolute, ATA) (10, 11) and its benefits have been demonstrated in stroke, through the promotion of cerebral oxygenation, metabolic improvement, anti-inflammatory barrier, protection of cerebral blood flow, modulation of intracranial pressure, decrease of oxidative stress and apoptosis, increase of vascular and neural regeneration (12).

HBOT induces an increase of 100% of oxygen in blood and tissues during the procedure helping to maintain the integrity and function of tissues and cells (13), stimulates the antioxidant response in plasma and regulates the processes of angiogenesis, vascular tone by increasing the levels of vascular endothelial growth factor (VEGF) and decreasing the levels of endothelin-1 (ET-1). Different factors such as age, oxidative stress, diabetes and chronic inflammation (14, 15), play an important factor in the development of diabetic foot or diabetic neuropathy (16, 17).

It has been described that the hyperbaric chamber increases the levels of circulating free fatty acids, so that chronic inflammation could be associated with an increase in the production of reactive oxygen species (ROS) and a decrease in antioxidant activity (18).

HBOT consists of breathing 100% pure oxygen in a pressurized environment (hyperbaric chamber) to at least 1.4 atmospheres absolute (ATAs). The scientific basis for the use of oxygen at elevated pressures is based on the application of the Henry, Dalton and Boyle gas laws. There are other effects, the volumetric ones with an increase of 10 to 15 times the amount of oxygen in the plasma fraction, therefore, the diffusion distance from the vascular space to the tissues increases, and this is directly proportional to the pressure used (19, 20). The aim of this treatment is to evaluate the effect of HBOT on gene expression of superoxide dismutase 1 and 2 (SOD1 and SOD2), pro-inflammatory cytokines (TNFα, IL-1β, IL-12, IL-4, as well as oxidative (NLRP3) and antioxidant (GPX2) enzymes in patients with diabetic foot.

Intermittent exposure to hyperbaric oxygen increases oxygen saturation by 100% in the blood (21, 22) and stimulates various biological processes that promote healing in numerous clinical conditions in which hypoxia is the dominant element (23-25). Hyperbaric oxygen therapy stimulates plasma antioxidant responses and regulates angiogenesis and vascular tone processes by increasing vascular endothelial growth factor levels and decreasing endothelin-1 levels. Different factors including age, oxidative stress (OS), diabetes and chronic inflammation play an important factor in the development of diabetic foot (26-27). It is assumed that the hyperbaric chamber increases the levels of circulating free fatty acids, and chronic inflammation may be associated with an increase in the production of reactive oxygen species (ROS) and a decrease in antioxidant activity (28).

Justification:

Mexico is a country where there is a high population of patients with diabetic foot and little research in response to non-invasive treatments such as hyperbaric chamber. Therefore, studies are needed to determine the mechanisms by which complications are triggered in these patients and to determine possible molecular markers for their timely treatment.

Research question:

In Mexican patients diagnosed with diabetic foot, what are the gene expression changes in inflammation and oxidative stress in diabetic foot patients treated with hyperbaric oxygen?

Hypothesis:

Patients diagnosed with diabetic foot will have significant changes after hyperbaric treatment such as increased gene expression in the antioxidant response and decreased gene expression in the inflammatory response.

General Objective:

To evaluate gene expression changes in inflammation and oxidative stress in diabetic foot patients treated with hyperbaric oxygen.

Specific objectives:

 To evaluate the clinical and biochemical characteristics of the included patients according to sex.

 To evaluate the gene expression of SOD, CAT, GPX, GRd, NLRP3, IL-1b, Casp-1, IL-4, IL-6, IL-10 and IL-13 in the basal state, at 12 and 30 sessions of treatment in the hyperbaric chamber.

Methodology:

Type of study: quasi-experimental, longitudinal, prolective, analytical study.

Characteristics of the population:

Women and men older than 18 years, with Diabetes type 2, residents of the CDMX, with diabetic foot with hyperbaric treatment, attended at the hospital of specialties in diabetes, secretary of health, accepted and signed the informed consent form.

Selection criteria:

Men and women with diabetic foot and hyperbaric treatment.
Ages 30-60 years.
Signed informed consent form.

Exclusion:

Patients with diabetic foot without presence of dermatologic lesions.

Elimination:

Patients who decided to abandon the protocol.
Incomplete data for any variable.

Sample size:

15 patients with diabetic foot.

Operationalization of variables:

Variable	Type	Measuring unit
Age	Quantitative	Years
Sex	Qualitative	Male/ female
Systemic blood pressure	Quantitative	Milimeters of mercury
Waist	Quantitative	Centimeters
Dislipidemias	Qualitative	Positive or negative
Anaemia	Qualitative	Positive or negative
Tabaquism	Qualitative	Positive or negative
Charcot's foot	Qualitative	Positive or negative
Chronic kidney disease by CKD-EPI I-III	Qualitative	Positive or negative
Insuficiencia venosa periférica	Qualitative	Positive or negative
Glucosa en sangre	Quantitative	milligrams/ deciliters
Colesterol	Quantitative	milligrams/ deciliters
Triglicéridos	Quantitative	milligrams/ deciliters
Basal glycosylated hemoglobin basal	Quantitative	Percentage
Creatinina	Quantitative	milligrams/ deciliters
Tasa de filtrado glomerular	Quantitative	milliliters/ square meter of body surface area
glycosylated	Quantitative	Percentage

hemoglobin at 3 months		
Wagner's classification for diabetic feet	Qualitative	Wagner Grade 0: Absence of ulcers in a high-risk foot. Grade 1: Superficial ulcer involving the full thickness of the skin but no underlying tissues. Grade 2: Deep ulcer, penetrating to ligaments and muscles but not involving bone or abscess formation. Grade 3: Deep ulcer with cellulitis or abscess formation, almost always with osteomyelitis. Grade 4: Localized gangrene. Grade 5: Extensive gangrene involving the entire foot.
Gene expression SOD, CAT, GPX, GRd, NLRP3, IL-1b, Casp-1, IL-4, IL-6, IL-10 e IL-13.	Qualitative	International units

Procedure:

1. Patients attended at the diabetic foot clinic in the CDMX were invited to participate. 2.
2. An informed consent process was carried out with the patient's signature to agree to participate in the study.
3. Questions were asked about their personal pathological and non-pathological history.
4. Three tubes of blood samples were taken for determination of blood biometry, urea, creatinine, cholesterol, triglycerides, glycosylated hemoglobin. A 4 ml tube

with EDTA was taken to measure the gene expression of SOD, CAT, GPX, GRd, NLRP3, NLRP3, IL-1b, Casp-1, IL-4, IL-6, IL-10 and IL-13.

5. Complementary DNA (cDNA) synthesis was carried out following the instructions of the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics), using a mixture of random hexamer primers, nitrogenous bases (A, T, G and C), reaction buffer, RNase inhibitor and Reveso Transcriptase enzyme. The retrotranscription reaction was carried out in a thermocycler (Eppendorf Mastercycler) programmed at an initial temperature of 25°C for 15 minutes, 45°C for 60 minutes and 72°C for 15 minutes.
6. The qRT-PCR technique to determine relative expression, a "master mix" (10 µL) was prepared in microtubes for the LightCycler nano (Roche Diagnostics), the mix was composed of 100 nM forward oligonucleotides, 100 nM reverse oligonucleotides, 100 nM hydrolysis probe (Human Universal Probe Library, Roche Diagnostics), 2 µL TaqMan master (Taq-polymerase), 0. 5 U LightCycler Uracil-DNA glycosylase, and 2 µL of DNAc (500 ng). The mixture was subjected to different temperature changes in the LightCycler Nano Real-Time PCR System (Roche Diagnostics) under the following conditions: initial incubation (denaturation) at 95 °C for 10 minutes, followed by 45 cycles of: denaturation (95 °C for 10 seconds), allylation of oligos to cDNA (60 °C for 30 seconds), and extension (copying) of the cDNA strand (72 °C for 1 minute). The specific sequences used to design the oligonucleotides used in qRT-PCR were designed using the online software ProbeFinder: https://lifescience.roche.com/shop/en/us/overviews/brand/universal-probe-library.
7. Relative RNAm expression of genes was normalized to the relative expression levels of the housekeeping gene (18s). Relative quantification (normalization) of gene expression was determined using the 2-ΔΔΔCT method.

Statistical analysis

In the analysis, qualitative variables are presented in frequencies and percentages, compared with the Chi-square test; quantitative variables according to the Shapiro-Wilk normality test, normally distributed variables are presented in means and standard deviation, compared with Student's t-test, those related with ANOVA test; quantitative variables of free distribution are presented in medians and interquartile ranges, compared with Mann Whitney U-test, those related with Kruskall-Wallis test. Data analysis was carried out with SPSS version 23, Graph Pad Prisma. A value $p<0.05$ was taken as statistical significance, 95% confidence interval (95%CI).

Ethical aspects:

The project was carried out in accordance with the General Health Law on Health Research published in the Official Journal of the Federation on February 7, 1984, published in the Official Journal of the Federation the General Health Law, regulating the third paragraph of Article 4 of the Political Constitution of the United Mexican States, which came into force on July 1 of the same year; as well as the guidelines of the Declaration of Helsinki of the World Medical Association. of the Political Constitution of the United Mexican States, which came into force on July 1st of the same year; as well as the guidelines of the Declaration of Helsinki of the World Medical Association on Ethical Principles for Medical Research Involving Human Subjects according to the 64th General Assembly, Fortaleza, Brazil, October 2013. The study is of greater than minimal risk, adhering to Chapter 1, Article 17, Section III of the ethical aspects of research on human beings, the authorization of the participants will be requested through an Informed Consent Letter (ICF) where the objective of the study and its significance, the procedures, the potential benefits and the possible risks will be stated; the name of those responsible for the study will also be mentioned and the autonomy and freedom they have to participate will be clarified.

In addition, according to the Helsinki declaration, specifically paragraph 33 of the last amendment of 2013, we will mention to the patient the possible benefits, risks, costs and efficacy of any new intervention, being evaluated by comparison with the best proven interventions, in this study the patient will be told which intervention could be more effective. As part of the ethical considerations, it will be informed that 14 ml of blood (one tablespoonful) will be drawn and that this amount does not put the health of the participants at risk, producing minimal discomfort with the aim of studying the presence of biochemical alterations.

Biosafety aspects.

- The present study has safety implications when obtaining blood samples, which can be considered infectious-contagious material. In the present study, the authors and co-authors are aware of and adhere to the General Law of Ecological Equilibrium and Environmental Protection and the General Law for the Prevention and Integral Management of Waste, which are of public order and social interest, Official Mexican Standard NOM-CRP-001-ECOL/1993, which establishes the characteristics of hazardous waste, the list of such waste and the limits that make a waste hazardous due to its toxicity to the environment; Official Mexican Standard NOM-002-SCT/2003 List of the most commonly transported Hazardous Substances and Materials; Official Mexican Standard NOM-002-SCT/2003 List of the most commonly transported Hazardous Substances and Materials; Official Mexican Standard NOM-002-SCT/2003 List of the most commonly transported Hazardous Substances and Materials.

As the information will be obtained through consultation with highly trained personnel with more than 10 years of experience, without endangering the health or physical integrity of health personnel or patients, the environment will not be affected since we have the training for the handling of RPBI, with full respect for

the rights of patients avoiding any unnecessary physical or mental suffering or damage as dictated by the Nuremberg Code 1947.

Bibliographic references

1. Loyola-Leyva A., et al., (2019). Altered erythrocyte morphology in Mexican adults with prediabetes and type 2 diabetes mellitus evaluated by scanning electron microscope. Microscopy, pp: 1-10. doi: 10.1093/jmicro/dfz011.
2. International Diabetes Federation (IDF). IDF Diabetes Atlas 8th edition. 2017.
3. Stettler C, Christ E, Diem P (eds): Treatment Goals in Diabetes Andreas Melmer a · Markus Laimer Novelties in Diabetes. Endocr Dev. Basel, Karger 2016, vol 31, pp 1–27 (DOI: 10.1159/000439364).
4. Galicia-García U., et al., (2020). Pathophysiology of Type 2 Diabetes Mellitus. Int. J. Mol. Sci. 21, 6275; doi:10.3390/ijms21176275.
5. Sigal RJ, El-Hashimy M, Martin BC, et al: Acute postchallenge hyperinsulinemia predicts weight gain: a prospective study. Diabetes 1997;46:1025–1029.
6. Odeleye OE, de Courten M, Pettitt DJ, et al: Fasting hyperinsulinemia is a predictor of increased body weight gain and obesity in Pima Indian children. Diabetes 1997;46:1341–1345.
7. Harris MI, Flegal KM, Cowie CC, et al: Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in U.S. adults. The Third National Health and Nutrition Examination Survey, 1988– 1994. Diabetes Care 1998;21:518–524.
8. Klein BE, Klein R, Moss SE, et al: Parental history of diabetes in a population-based study. Diabetes Care 1996;19:827–830.
9. Nathan DM, Davidson MB, DeFronzo RA, et al: Impaired fasting glucose and impaired glucose tolerance: implications for care. Diabetes Care 2007;30: 753–759.
10. Calvert JW, Cahill J, Zhang JH (2007) Hyperbaric oxygen and cerebral physiology. Neurol Res 29:132-141.

11.- Michalski D, Hartig W, Schneider D, Hobohm C (2011a) Use of normobaric and hyperbaric oxygen in acute focal cerebral ischemia-a preclinical and clinical review. Acta Neurol Scand 123:85-97

12. Sunkari V. G., Lind F., Botusan I. R., et al. Hyperbaric oxygen therapy activates hypoxia-inducible factor 1 (HIF-1), which contributes to improved wound healing in diabetic mice. Wound Repair and Regeneration. 2015;23(1):98–103. doi: 10.1111/wrr.12253.

13. William J. Ennis, Enoch T. Huang, and Hanna Gordon, Impact of Hyperbaric Oxygen on More Advanced Wagner Grades 3 and 4 Diabetic Foot Ulcers: Matching Therapy to Specific Wound Conditions. Adv Wound Care (New Rochelle). 2018; 7(12): 397–407

14. Zhai Ww, Sun L, Yu Zq, Chen G. Hyperbaric oxygen therapy in experimental and clinical stroke. Med Gas Res 2016;6:111-8

15. Gjedde A (2005) The pathways of oxygen in brain. I. Delivery and metabolism of oxygen. Adv Exp Med Biol 566:269-275

16. Clokie M1, Greenway AL1, Harding K2, Jones NJ3, Vedhara K4, Game F5, Dhatariya KK6. New horizons in the understanding of the causes and management of diabetic foot disease: report from the 2017 Diabetes UK Annual Professional Conference Symposium. Diabet Med. 2017; 00, 00-00

17. Singh N, Armstrong DG, Lipsky BA. Preventing foot ulcers in patients with diabetes. JAMA 2005; 293: 217–228

18. Matchett GA, Martin RD, Zhang JH (2009) Hyperbaric oxygen therapy and cerebral ischemia: neuroprotective mechanisms. Neurol Res 31:114-121

19. Hoogeveen Ruben C. Dorresteijn Johannes AN. Complex interventions for preventing diabetic foot ulceration;Cochrane Database of Systematic Reviews 2015.

20. Stoekenbroek R.M., Santema T.B., Legemate D.A. Hyperbaric oxygen for the treatment of diabetic foot ulcers: A systematic review European Journal of Vascular and Endovascular Surgery, 47 (6) , pp. 647-655. 2014

21. Arenas MD, Gil MT, Gutiérrez MD, et al.: Management of calcific uremic arteriolopathy (calciphylaxis) with a combination of treatments, including hyperbaric oxygen therapy. Clin Nephrol 2008; 70: 261—264.
22. Tuk B., Tong M., Fijneman E. M. G., Van Neck J. W. Hyperbaric oxygen therapy to treat diabetes impaired wound healing in rats. PLoS ONE. 2014;9(10) doi: 10.1371/journal.pone.0108533.e108533
23. Thom SR (1985) Oxidative stress is fundamental to hyperbaric oxygen therapy (2009) J Appl Physiol. 106: 988–995.
24. Milovanova T, Bhopale VM, Sorokina EM, et al. Hyperbaric oxygen stimulates vasculogenic stem cell growth and differentiation in vivo. Journal of Applied Physiology. 2009; 106:711–728.
25. Tepper OM, Capla JM, Galiano RD, et al. Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow-derived cells. Blood. 2005; 105:1068– 1077.
26. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest. 2007; 117:1219–1222
27).- Van Neck JW., et al., (2017). Hyperbaric oxygen therapy for wound healing in diabetic rats: Varying efficacy after a clinically-based protocol. PLoS One. 2017; 12(5): e0177766.
28).-. Godman CA., et al., Hyperbaric oxygen treatment induces antioxidant gene expression. Ann. N.Y. Acad. Sci. 1197 (2010) 178–183.