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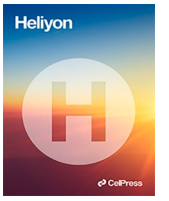

Research article

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

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A R T I C L E I N F O

Keywords:
Diabetic foot
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Inflammation

A B S T R A C T

Introduction: Patients with an uncontrolled glycemic index develop a wide variety of pathologies associated with diabetes, such as diabetic foot ulcers (DFUs). Hyperbaric oxygen therapy (HBOT) is an adjunctive therapy used to heal wounds and prevent lower extremity amputations in this population.

Objective: This preliminary study aimed to evaluate how HBOT impacts inflammation in patients with Wagner stages 2–4 DFUs by analyzing its effect on the gene expression of key oxidative stress regulators SOD1, SOD2, and GPX2, of pro-inflammatory cytokines TNFα, IL-1β, IL-4, and IL-12, and of the NLRP3 inflammasome.

Methods: The effect of HBOT was assessed in 15 patients with Wagner stages 2–4 DFUs that underwent 30 sessions in the hyperbaric chamber. This protocol is registered on Clinical Trials under the title *Hyperbaric Oxygen Therapy in Diabetic Foot* (July 15, 2024) with the number NCT06502808. Blood samples were collected, and relative gene expression was assessed by quantitative real-time polymerase chain reaction (qPCR).

Results: The hyperbaric chamber treatment increased the expression of SOD1 and GPX2 genes (0.4 and 3 times, respectively) after 30 sessions compared to baseline levels. Similarly, the gene expression of pro-inflammatory cytokines IL-1β, IL-12, IL-4, and NLRP3 increased after 30 sessions (2.1, 0.4, 1.5, and 1.2, respectively), while the expression of the TNFα gene decreased (0.5 times). Clinically, the patients' lesions were fully resolved.

Conclusions: HBOT directly influences the gene expression of several potent antioxidants and pro-inflammatory cytokines, thus favoring angiogenesis and blood circulation in the extremities.

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1. Introduction

The World Health Organization (WHO) defines diabetes as a "metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both" [1]. In 2021, the International Diabetes Federation (IDF) reported a prevalence of diabetes of 537 million adults (20–79 years) in the world, of which 90 % is type 2 diabetes mellitus (T2DM) [2]. Diabetes-related foot disease is one of the most serious complications of diabetes, drastically reducing quality of life. According to the International Working Group on the Diabetic Foot (IWGDF), it includes peripheral neuropathy, peripheral artery disease, infection, diabetic foot ulcers (DFUs), neuro-osteoarthropathy, gangrene, and amputation [3]. The prevalence of DFUs in adults varied between 10 % and 30 % worldwide and between 57.3 % and 87.3 % in Mexico (2013–2018) [4].

Hyperbaric oxygen therapy (HBOT) is defined by the Underwater and Hyperbaric Medical Society (UHMS) as a treatment in which a patient intermittently breathes 100 % oxygen at a pressure higher than 1 atm absolute, ATA (the pressure at sea level) [5–9]. Administering HBOT in a pressurized hyperbaric chamber of at least 1.4 ATA induces a 100 % increase of oxygen in blood and tissues during the procedure, helping to maintain the integrity and function of tissues and cells [7,10]. Its benefits have been demonstrated in cerebral vascular accidents, as it promotes cerebral oxygenation and has other effects that include metabolic improvement, anti-inflammatory barrier, protection of brain blood flow, modulation of intracranial pressure, decrease in oxidative stress and apoptosis, and increased vascular and neural regeneration [5,6,8,11]. HBOT has been used in venous leg ulcers and other non-healing wounds, proving to be a valuable aid in healing these wounds [12,13]. In patients with DFU, HBOT stimulates the antioxidant response in plasma and regulates the processes of angiogenesis and vascular tone by increasing the levels of vascular endothelial growth factor and decreasing endothelin-1 levels [8–15]; it also inhibits the growth of anaerobic bacteria and stimulates healing in all tissue planes from skin to bone, leading to a faster recovery.

Oxidative stress causes damaged tissues to release histamine, which stimulates neutrophil chemotaxis; this triggers the inflammatory response and promotes angiogenesis, increasing osteoblast and chondrocyte proliferation [16–21]. NLRP3 inflammasome is one of the most important markers in the inflammatory process. It is a multi-enzyme set that matures both IL-1β and IL-18 and participates in the generation of reactive oxygen species (ROS) [22,23]. Its activation responds to cell damage and ROS, guaranteeing cellular integrity [24,25]. Other important molecules involved in this process are Superoxide Dismutase 1 and 2 (SOD1 and 2), and Glutathione Peroxidase (GPX2) which are potent antioxidants that regulate the generation of ROS [9,26]. Cytokines are involved as follows; IL-1β, IL-4 and TNFα are inflammatory cytokines. On the other hand IL-4 generates IgE-producing plasma cells, and TNFα induces other pro-inflammatory cytokines such as IL-1β and IL-6, further stimulating the local temperature to rise, as well as the inflammatory process.

This preliminary study aimed to evaluate how HBOT impacts inflammation in patients with Wagner stages 2–4 DFUs by analyzing its effect on the gene expression of key oxidative stress regulators SOD1, SOD2, and GPX2, of pro-inflammatory cytokines TNFα, IL-1β, IL-4, and IL-12, and of the NLRP3 inflammasome.

2. Materials and methods

This protocol was approved by the Ethics in Research Committee of the Centro Interdisciplinario de Ciencias de la Salud Unidad Santo Tomás (CONBIOÉTICA-09-CEI-019-20170731. The trial was conducted following the ethical principles of the *Declaration of Helsinki* and is consistent with the *Good Clinical Practice Guidelines*.

This protocol is registered on Clinical Trials under the title *Hyperbaric Oxygen Therapy in Diabetic Foot* (July 15, 2024) with the number NCT06502808.

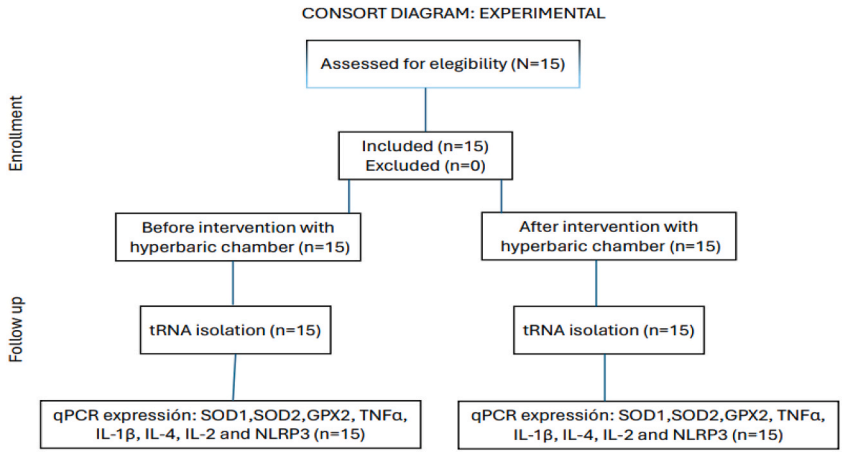

Fig. 1. Consort diagram showing the sequence of the experiment: patient selection, treatment, sample processing and gene expression.

2.1. Subjects

The participants in this study were patients of the Clinic Specialized in the Management of Diabetes "Manuel González Rivera." After signing an informed consent, 15 T2DM patients with Wagner stages 2–4 DFUs were selected by a non-probabilistic method for convenience. All candidates who did not meet this criterion were excluded. Similarly, those patients who were on pharmacological treatments not allowed by the clinic during the use of the hyperbaric chamber were excluded.

The patients underwent a series of 30 HBOT sessions of 60 min at 2 ATA in the hyperbaric chamber for 5 consecutive days per week. At the same time, they received a triple treatment regimen with ceftriaxone, metronidazole, and clindamycin. No statins or antiplatelet agents were administered. Their treating physician managed their glucose levels. Blood samples were drawn before the first HBOT session (baseline) and after sessions 12 and 30 (Fig. 1).

We recorded baseline patient demographics, comorbidities, and biochemical variables: glucose, cholesterol, triglycerides, high-density lipoprotein (HDL), creatinine, glomerular filtration rate (GFR), and glycosylated hemoglobin. To determine the presence of significant differences ($p < 0.05$) in the variables, the Mann-Whitney *U* test and Student's t-test were used to compare patients by gender. A Wilcoxon test was performed to compare changes between the glycosylated hemoglobin at baseline and after 3 months.

2.2. Determination of gene expression

The gene expression of the following genes was determined:

SOD1: F-3′-ATG CAG GCC TTC AGT CAG TC-5′, R-3′-GCA TCA TCA ATT TCG AGC AG-5′, **SOD2:** F-3′-CTG GAC AAA CCT CAG CCC TA-5′, R-3′-TGA TGG CTT CCA GCA ACT C-5′, **GPX2:** F-3′-TTC CTC TCC CAA CCC TCT GG-5′, R-3′-ATT CTG TGA AGG CCC AGA GC-5′, **TNFα:** F-3′-GCC AGA GGG CTG ATT ATT AGA GA-5′, R-3′-CAG CCT CTT CTC CTT CCT GAT-5′, **IL-1β:** F-3′-TTG GGT AAT TTT TGG GAT CTT AC-5′, R-3′-CTG TCC TGC GTG TTG AAA GA-5′, **IL-4:** F-3′-ACG TTT GGC ACA TCC ATC TC-5′, R-3′-CAT CGG CAT TTT GAA CGA G-5′, **IL-12:** F-3′-TCC AGG TGT CAG GGT ACT CC-5′, R-3′-AGA ACT TGC AGC TGA AGC CA-5′, **NLRP3:** F-3′-GCA AGA CTT TGA CAA CAT GC-5′, R-3′-CAC CTG TTG TGC AAT CTG AAG-5, and the **constitutive gene 18s:** F-3′-CGA ACG TCT GCC CTA TCA AC-5′, R-3′-TTG GAT GTG GTA GCC GTT TC-5`.

The expression of each gene for each of the participants was done in triplicate.

The extraction of total RNA from the blood samples was performed using the TRIzol™ method, following the instructions of the provider's protocol. Its concentration (OD-260) and integrity (OD-260/OD-280) were determined using the NanoDrop™ Spectrophotometer equipment.

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 primer hexamers, nitrogenous bases (A, T, G, and C), a reaction buffer, RNase inhibitor, and the enzyme reverse transcriptase. The retro-transcription reaction was carried out in a thermocycler (Eppendorf Mastercycler) programmed for an initial temperature of 25 °C for 15 min, followed by 45 °C for 60 min, and 72 °C for 15 min.

To determine the relative expression with the qRT-PCR technique, a master mix (10 μL) was prepared in microtubes for the LightCycler® Nano (Roche Diagnostics). The mixture was composed of 100 nM of oligonucleotides forward, 100 nM oligonucleotides reverse, 100 nM hydrolysis probe (Human Universal Probe Library, Roche Diagnostics), 2 μL of TaqMan master mix (Taq-polymerase), 0.5 U LightCycler® Uracil-DNA Glycosylase, and 2 μL of cDNA (5100 ng). The mixture underwent 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 min, followed by 45 cycles of denaturation at 95 °C for 10 s, oligos alignment to cDNA at 60 °C for 30 s, and extension (copy) of the cDNA strand at 72 °C for 1 min. The specific sequences used to design the oligonucleotides for the qRT-PCR assays were produced with the online software ProbeFinder (https://lifescience.roche.com/en_mx/articles/Universal-ProbeLibrary-System-Assay-Design.html).

The mRNA relative expression of the genes was normalized with the relative expression levels of the housekeeping gene. The relative quantification of the gene expression was determined using the $2-\Delta\Delta CT$ method. A one-way ANOVA was used to compare the change in gene expression from baseline to after sessions 12 and 30. A $p < 0.05$ value was taken as statistical significance.

G*Power was used and is a tool to compute statistical power analyses for many different t tests, F tests, $\chi 2$ tests, z tests and some exact tests. G*Power can also be used to compute effect sizes and to display graphically the results of power analyses.

3. Results

This preliminary study included 15 T2DM patients with Wagner stages 2–4 DFUs who underwent 30 sessions of HBOT in the hyperbaric chamber. 46.7 % were female and 53.3 % were male. 85 % of the patients managed their glucose levels with insulin and metformin, while 15 % were treated with metformin and linagliptin (DPP-4 inhibitor). Their treating physician adjusted the doses according to their weight. All patients recovered regardless of the severity of the initial clinical diagnosis.

Table 1 compares clinical and biochemical characteristics of patients by gender during the 30 sessions in the hyperbaric chamber. Women are significantly older than men. Body Mass Index (BMI) does not show differences among both groups, but most subjects are overweight. Systolic and diastolic blood pressure are similar and within the normal range. Waist-to-hip index (WHI) shows a higher cardiovascular risk in men; however, there is not a significant difference.

The mean glucose level (171.40 mg/dL) was near the upper limit of the normal range (70–180 mg/dL) according to the American Diabetes Association, 2023. The range of glucose levels exceeded the upper limit (121–216) [27]; however, cholesterol, triglycerides, and creatinine levels were within normal ranges.

Patients presented several comorbidities: 73.3 % presented peripheral venous insufficiency, 46.7 % arterial hypertension (SAH), 40 % severe hypertriglyceridemia, 33.3 % anemia, 33.3 % smoking, and 26.7 % Charcot foot. All patients presented chronic kidney disease (CKD) stages I-III, with stage I being the most prevalent (46.7 %). It should be noted that all patients presented a history of diabetic neuropathy.

When comparing the median glycosylated hemoglobin levels at baseline and after 3 months, we observed a statistically significant difference ($p < 0.001$).

Treatment with HBOT increased the gene expression of SOD1 after 12 sessions ($p < 0.05$) and after 30 sessions ($p < 0.05$) compared to baseline. However, gene expression after 30 sessions significantly decreased compared to gene expression after 12 sessions ($p < 0.05$). The gene expression of SOD2 did not change significantly after 12 sessions but after 30 sessions it showed a significant increase ($p = 0.05$). On the other hand, GPX2 gene expression decreased significantly ($p < 0.001$) after 12 sessions and then increased significantly ($p < 0.0001$) after 30 sessions, when compared to the basal data. Fig. 2 shows these results.

We assessed the relative expression of TNFα, IL-1β, IL-4, IL-12, and NLRP3 inflammasome pro-inflammatory cytokines. Although TNFα gene expression decreased after 12 and 30 sessions, only the decrease at 30 sessions was statistically significant compared to baseline ($p < 0.001$). IL-1β and IL-4 gene expression increased significantly after 30 sessions ($p < 0.001$) vs basal data. For IL-12, we observed a significant decrease in gene expression after 12 sessions ($p < 0.001$); however, gene expression increased significantly after 30 sessions ($p < 0.05$) when compared to the basal data. No significant change in gene expression of NLRP3 inflammasome was observed after 12 sessions compared to baseline, but it increased significantly after 30 sessions ($p < 0.001$) vs basal data. Fig. 3 shows these results.

Additionally, effect size and pre-post power were calculated in this preliminary study. Differences were found using G*Power, between the baseline data and the data after 12 sessions; as well as the baseline data and the data obtained after 30 sessions. Table 2 shows medium effect sizes after 30 sessions demonstrating a biological effect in the use of the hyperbaric chamber for the treatment of diabetic foot. Specifically, the differences found in basal vs after 30 sessions were TNFa (−0.58), IL-4 (1.04) and NLRP3 (0.53); respectively, which is considered medium and large, with power of 0.80, 0.95, and 0.77 respectively. While in SOD2, GPX, IL-1 β and IL-12 a small effect and power could be observed, data not included in the table.

4. Discussion

In this preliminary study, we found that HBOT directly influences the gene expression of several potent antioxidants and pro-inflammatory cytokines, thus favoring angiogenesis and blood circulation in the extremities.

HBOT is used as an adjunctive treatment of refractory diabetic lower extremity wounds that seeks to correct the processes of

Table 1
Baseline clinical biochemical variables in patients with Wagner stages 2–4 diabetic foot ulcers treated with HBOT.

Variable	Total N = 15	Men 8 (53.3 %)	Women 7 (46.7 %)	P-value
Clinical variables (m±SD)				
Age (years)	54.20 ± 12.17	48.50 ± 9.710	60.71 ± 11.95	0.048[a]
BMI (kg/m^2)	26.98 ± 6.55	25.39 ± 4.69	28.79 ± 8.19	0.334
PAS (mmHg)	128 ± 21.85	126 ± 21.42	132 ± 23.58	0.614
PAD (mmHg)	76 ± 14.25	79.63 ± 12.17	71.86 ± 16.23	0.310
WHI (cm)	0.96 (0.94–1)	0.97 (0.95–1)	0.95 (0.87–0.99)	0.889
Comorbidities N (%)				
Systemic arterial hypertension	7 (46.7)	4 (57.1)	3 (42.9)	0.782
Dyslipidemias	6 (40.0)	2 (25.0)	4 (57.1)	0.205
Anemia	5 (33.3)	2 (25.0)	3 (42.9)	0.464
Smoking	5 (33.3)	1 (12.5)	4 (57.1)	0.067
Charcot foot	4 (26.7)	1 (12.5)	3 (42.9)	0.185
CKD I	7 (46.7)	1 (12.5)	1 (14.3)	0.388
CKD II	5 (33.3)	2 (25.0)	4 (57.1)	
CKD III	3 (20.0)	5 (62.5)	2 (28.6)	
Peripheral venous insufficiency	11 (73.3)	6 (75.0)	5 (71.4)	0.876
Biochemical variables (m ± p)				
Glucose (mg/dl)	171.40 (121–216)	145.50 (119.50–239.75)	169 (138–183)	0.619
Cholesterol (mg/dl)	157 (126–188)	137 (111.75–184.75)	159 (119–159)	0.619
Triglycerides (mg/dl)	139 (113–191)	136 (99–183.75)	170 (113–222)	0.619
Cholesterol HDL (mg/dl)	40 (35–50)	35 (34.25–42.75)	45 (40–53)	0.132
A1C baseline (%)	9 (7–10)	9.75 (6.55–10.52)	8.50 (7–9.5)	0.315
Creatinine (mg/dl)	0.90 (0.73–1.10)	0.92 (0.65–1.04)	0.90 (0.73–1.20)	0.999
GFR (ml/m^{2}SC)	87 (76–96)	95.5 (83.25–117.50)	85 (52–91)	0.315
A1C 3 months (%)	6.8 (6–7.5)	6.65 (6.12–7.75)	7 (6–7.5)	0.619

Abbreviations: BMI = Body mass index, kg/m^2 = kilograms per square meter, WHI = waist-to-hip index, A1C = glycosylated hemoglobin, HDL = High-density lipoproteins, mg/dl = milligrams per deciliter, GFR = glomerular filtration rate obtained by CKD-EPI, ml/m^{2}SC = milliliters per square meter of body surface area, m ± p = median and percentiles, m±SD = mean and standard deviation.

[a] p-value<0.05.

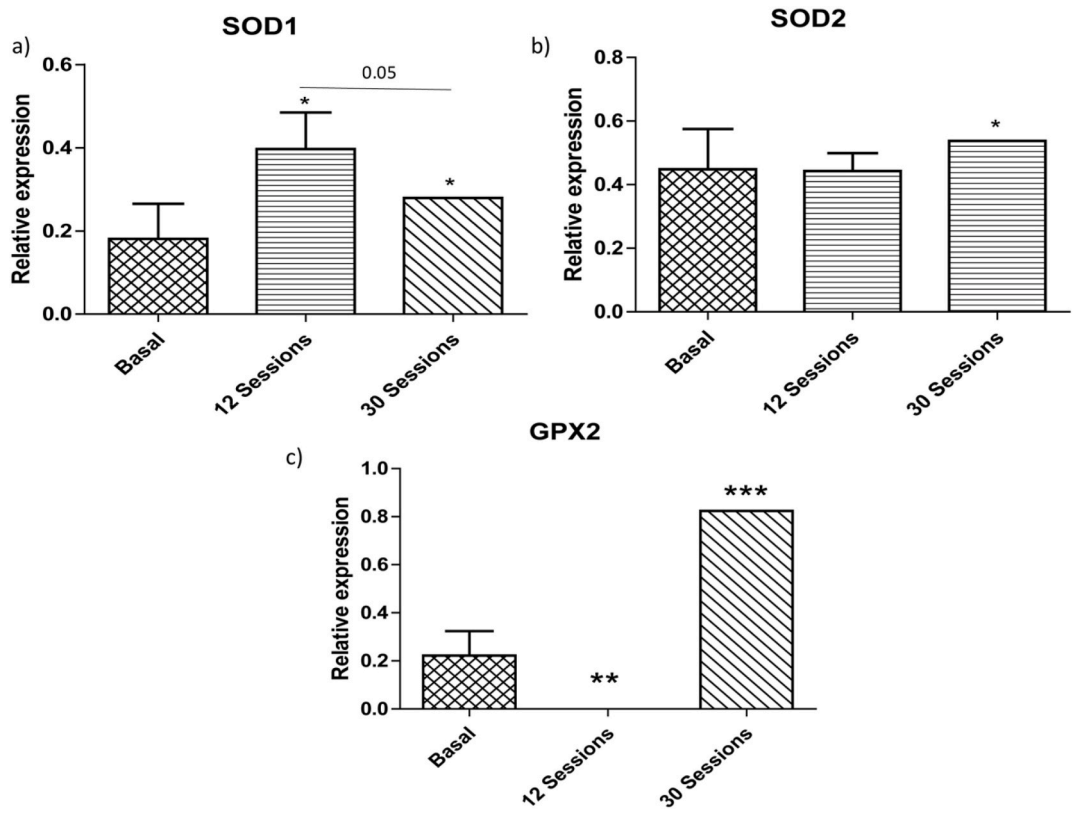

Fig. 2. Relative gene expression of (a) SOD1, (b) SOD2, and (c) GPX2 in diabetic patients with a Wagner stage 2–4 diabetic foot ulcer. Each bar represents the average ± standard deviation of 15 patients treated with HBOT. An ANOVA with a significance of $p < 0.05$ was used. *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$.

depletion of epithelial and stromal cells, chronic inflammation, fibrosis, imbalance, or abnormalities in the components of the extracellular matrix and remodeling processes, and the functions of impaired keratinocytes [28]. The mechanism of action of HBOT in diabetic patients involves increased oxygen in poorly vascularized areas, as well as increased hemoglobin, oxygenation, and perfusion [24]. Thus, it has been suggested that HBOT has a direct effect on oxygen available in tissues and promotes angiogenesis during wound healing through increased availability of growth factors such as VEGF [22] or the hypoxia-inducible factor (HIF) [9]. It has been shown that HBOT promotes angiogenesis through chemotaxis and hematopoietic progenitor cell (HPC) differentiation in the formation of *de novo* blood vessels [26,28–30].

Recent studies in diabetic rats show that HBOT has beneficial effects in the first days of treatment. These include decreased inflammation, improved capillarity of venous oxygen saturation (SO_2), and blood flow in the microvasculature [31,32]. The higher oxygen concentration —as occurs when doing moderate exercise— requires an increase in ATP. This increased metabolism elevates the production of ROS, which in turn induces endogenous defense mechanisms [28].

The results of this preliminary study show that, after 12 sessions, HBOT induces the expression of SOD1, but decreases the expression of GPX2. However, after 30 sessions, it induces the gene expression of SOD1, SOD2, and GPX2, and significantly increases the expression of pro-inflammatory cytokines IL-1β, IL-4, and IL-12, although the expression of TNFα decreases significantly. Therefore, our study demonstrates that HBOT could significantly alter the inflammatory response by modulating the gene expression of antioxidants and inflammatory cytokines. In future studies, we consider it would be very interesting to evaluate the expression of the protein to verify if it has the same behavior as the expression of the genes reported in this study. Our results also indicate that HBOT may induce protection against oxidative stress within endothelial cells and angiogenesis due to the significant increase of inflammatory cytokines after 30 sessions and this association between the inflammatory process and angiogenesis has been observed by Dariusz Szukiewicz in 2024 [33].

In relation to the sample size, a statistical programme was used. This programme allows us to evaluate the effect size and its power. The effect size and power of the hyperbaric chamber were analyzed in patients with diabetic foot with the use of G*power. The obtained effect sizes of −0.53, 0.58 and 1.04 suggest that there are clinically relevant differences between the analyzed groups, which could show a real and significant effect. The power of 0.77, 0.80 and 0.95 suggest that the results are robust and that there is a high probability of observing a real effect in this study, which reinforces the validity of the findings. It is also important to recognize the limitations of the sample size, even though this was a preliminary study, the results obtained can be used as a basis for future studies in larger populations, which would allow validation and scaling up of these results [34,35].

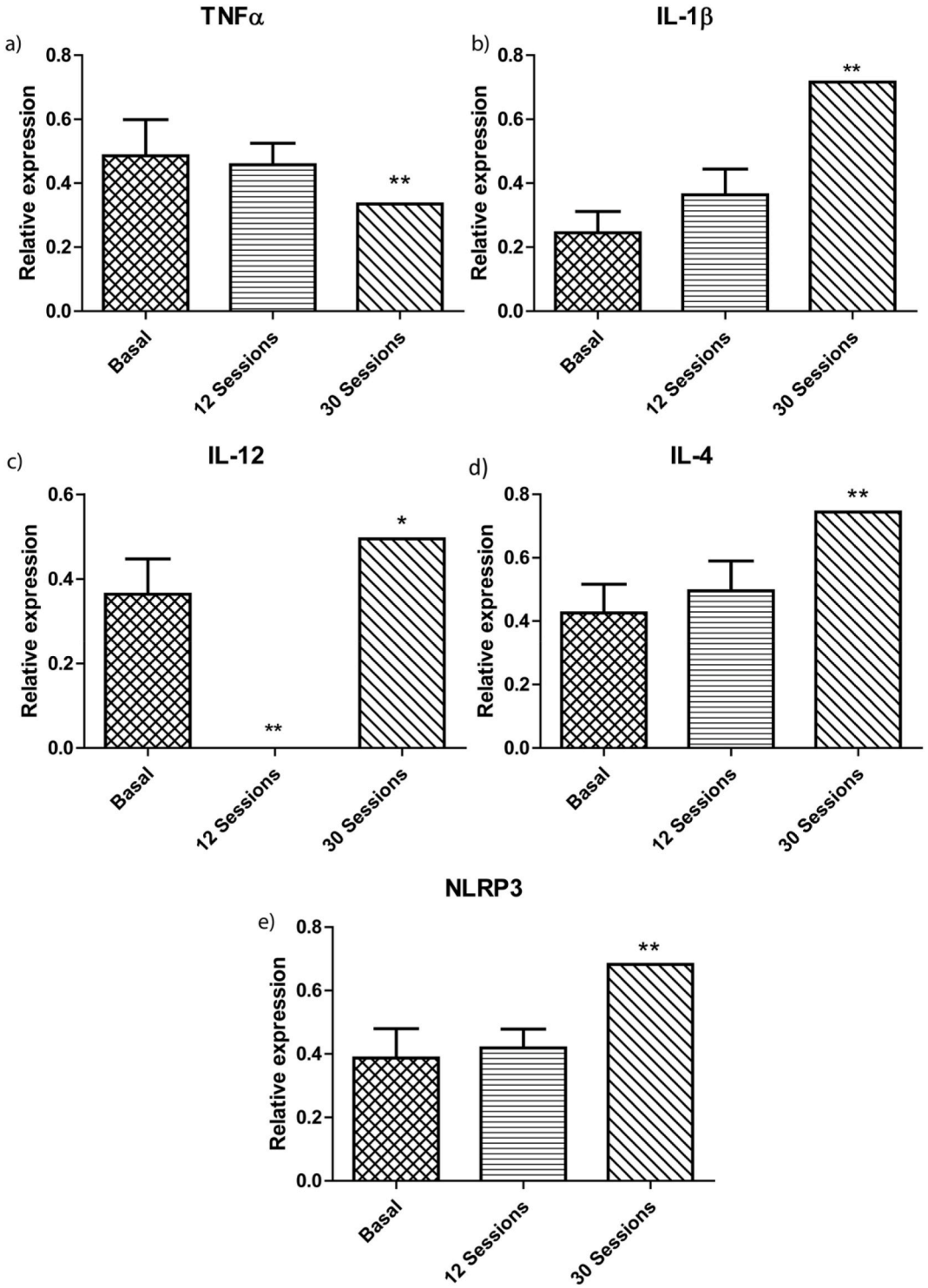

Fig. 3. Gene expression of pro-inflammatory cytokines (a) TNFα, (b) IL-1β, (c) IL-12, (d) IL-4, and (e) NLRP3 inflammasome in patients with a Wagner stage 2–4 diabetic foot ulcer. Each bar represents the average ± standard deviation of the 15 patients treated with hyperbaric oxygen therapy. An ANOVA with a significance of $p < 0.05$ was used. *$p < 0.05$, **$p < 0.001$.

Table 2
The table shows the effect size and potency obtained with G*power, induced by hyperbaric chamber on the expression of SOD2, TNFa, IL-4 and NLRP3 genes.

	Effect size	Power
SOD2_12-SOD2_Basal	0.33909199	0.64
SOD2_30-SOD2_Basal	0.32546244	0.63
TNFa_12-TNFa_Basal	−0.28209986	0.60
TNFa_30-TNFa_Basal	−0.58478037	0.80
IL4_12-IL4_Basal	−0.22698801	0.57
IL4_30-IL4_Basal	1.04218214	0.95
NLRP3_12-NLRP3_Basal	−0.01916606	0.50
NLRP3_30-NLRP3_Basal	0.53715394	0.77

The main limitation found in this study was the population's insufficient access to HBOT due to its cost and the small number of hospitals that provide this treatment. Previous studies of HBOT in patients with DFUs are heterogeneous; therefore, further research is necessary [31].

In conclusion, the use of HBOT in subjects with Wagner stages 2–4 DFU could enhance the improvement of diabetic foot through gene modulation of molecules involved in inflammation and protection against oxidative stress, however, it would be interesting to measure the expression of the protein and to verify that the same behavior is maintained. It would also be important to extend the number of subjects. Therefore, the results obtained from this preliminary study are favorable for healing DFUs and thus help to prevent lower extremity amputations.

CRediT authorship contribution statement

Nadia Mabel Pérez-Vielma: Writing – original draft, Investigation, Conceptualization. **María Magdalena Valencia Gutiérrez:** Investigation, Formal analysis. **Jennifer Viridiana Sánchez Camacho:** Supervision, Formal analysis. **José Enrique González Hernández:** Resources, Methodology. **Ángel Miliar García:** Visualization, Validation. **César Ochoa:** Writing – review & editing, Supervision. **Jonathan Labovitz:** Writing – review & editing, Supervision. **Modesto Gómez López:** Writing – original draft, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

None.

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