

# Alterations in glutathione, nitric oxide and 3-nitrotyrosine levels following exercise and/or hyperbaric oxygen treatment in mice with diet-induced diabetes

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**Abstract.** Oxidative stress is involved in the development of diabetes. Nitric oxide (NO) contributes to oxidative stress, affects the synthesis of glutathione (GSH) in tissues and also regulates important physiological processes. The levels of nitrosative stress, assessed by measuring the levels of 3-nitrotyrosine (3NT) as well as the bioavailability of NO are modulated by exercise and hyperbaric oxygenation (HBO). The aim of the present study was to evaluate the effects of exercise and HBO on the levels of NO, 3NT and GSH in tissues of various organs obtained from diabetic mice. Female mice were fed a high-fat/high-fructose diet to induce diabetes. Mice with diabetes were subjected to exercise and/or HBO. Initial and final concentrations of NO, 3NT and GSH were assessed in the muscle, liver, kidney, heart, spleen, lung, brain, visceral adipose, thoracic aorta and small intestine. Diabetes did not affect initial values of NO, although it significantly increased the levels of 3NT. The basal level of GSH in the diabetic group was lower than or comparable to that of the control group in the majority of the organs assessed. A negative correlation was observed between 3NT and GSH levels in the initial values of all tissues of the control group only, whereas all pathological tissues showed a positive correlation between NO and GSH. There was an increase or a stabilization of GSH levels in the majority of the organs in all treated mice despite the increase in nitrosative stress.

## Introduction

Nitric oxide (NO) acts as an intracellular messenger in physiological and pathological conditions (1). The bioavailability

of NO, and therefore its ability to perform physiological functions, can be affected by the excessive production of reactive oxygen species (ROS) involved in the development of diabetes. ROS have the capacity to reduce the enzymatic (2) and non-enzymatic (3) production of NO, or simply eliminate it from tissues. NO reacts with superoxide radicals resulting in peroxynitrite, a very potent oxidant that reacts with tyrosine residues of proteins to form 3-nitrotyrosine (3NT). 3NT serves as an indicator of nitrosative stress, the elimination of NO or modulation of signaling pathways. According to studies using animal models, NO decreases the harmful effects of ROS on biomolecules, whereas ROS limits the effects of NO by eliminating it (4) or affecting its intracellular receptors (5). Diabetes and its complications increase dysfunction of the endothelium (6), the possible mechanisms of which are discussed in a recent review (7).

In general, the bioavailability of NO is diminished during the pathogenesis of diabetes (7-9), despite an increase in the plasma levels in patients, as was shown by a meta-analysis (10). This limited bioavailability may be due to the elimination of NO by the superoxide radical (11), or by modulation of its production through enzymatic mechanisms (12) and/or nonenzymatic conditions such as ischemia or stomach acidity (13,14).

Glutathione (GSH) is an abundant endogenous antioxidant synthesized in large quantities in the liver. It is transported via blood flow to tissues when there is an imbalance between the production of ROS and the endogenous antioxidant mechanisms. Extracellular GSH is not able to pass through cell membranes; however, following enzymatic breakdown into its constituent amino acids, these amino acids are able to traverse the membrane. Once inside the cell, the amino acids are available for the resynthesis of GSH. GSH is irreversibly utilized when its product of oxidation (GSSG) is not recycled into GSH by glutathione reductase (15). The erythrocyte GSH levels are lower in patients with type 2 diabetes (16,17) and there are reports that have shown that GSH production gradually decreases in patients with diabetes with complications (18,19), and that this decrease is dependent on the degree of hyperglycemia (20). In a recent study it was shown that a high-fat diet resulted in GSH deficiency in the kidney of mice (21). GSH has a high affinity for NO, and S-nitrosoglutathione is the primary

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intermediary metabolite in the production of other nitrosothiols which serve as metabolic regulators (22,23). Based on an *in vivo* study using endotoxemic mice, an increase in NO resulted in decreased GSH synthesis, whereas a decrease in NO levels had the opposite effect (24).

Exercise prevents the harmful effects of ROS, and NO may be a central signaling molecule involved in the adaptation of muscles to exercise (25). A recent review analyzed the evidence showing the protective effects of aerobic exercise against the development of complications in patients with diabetes (26). Hyperbaric oxygenation (HBO) shows a beneficial therapeutic effect in patients with ulcers caused by diabetes; however, the underlying mechanisms have not been fully resolved. Pre-treatment with HBO exhibits a protective effect against oxidative stress in different types of tissues in animal models of various pathological processes or stress (27-31). Several studies have explored the hypothesis that oxidative stress serves an important role in HBO-mediated effects at the cellular level (32,33). However, studies examining the effects of HBO on NO metabolism are limited: An increase in pulmonary (34) or cardiac levels of NO (35) results in a vasodilatory effect, and thus an increase of oxygen being delivered to tissues (36). The aim of the present study was to evaluate the levels of NO, 3NT and GSH in mice with type 2 diabetes induced by a high-fat/high-fructose (HFHF) diet, compared with control animals, and the effects of exercise and/or HBO on NO, 3NT and GSH levels in the diabetic mice.

## Materials and methods

**Animals and study design.** A total of 70 3-month-old female CD1 mice (average weight, 35.8 g) were housed in acrylic cages (20x20x30 cm) with a 12:12 h light/dark cycle. Mice pups were used as adult female rodents and humans have a similar pattern of growth, in which weight is stabilized at the adult stage (37). The synchronization of the estrous cycle in the mice was not taken into consideration. In the first 3 months (phase 1), animals were fed a normal (control group) or a HFHF diet in (diabetes group) and parameters were measured in samples taken at the end of the 3 months and were used as the initial values. The use of drinking liquids that contained fructose results in a high fasting levels of glucose in the animals (38). Therefore, these mice were included in the high-fat diet. At the end of the second 3-month period (phase 2), samples were taken again from the untreated groups (final values) and from the experimental groups: After exercise training, HBO or a combination of these two treatments. The treatments were compared to the untreated diabetic mice. The final values of the control mice were used to examine the effects of ageing between the two phases. The time frame of the protocol and the animal groups are illustrated in Table I.

The percentage composition of the normal diet (Chow), consumed by the control mice was 9% fat, 29% protein and 62% carbohydrates. For the high-fat diet, the normal solid food was enriched with lard baked at 100°C, resulting in percentage composition of micronutrients of 38% fat, 19% protein and 43% carbohydrates. The high-fructose diet consisted of 40% carbohydrates (30% fructose and 10% dextrose) in a solution of water. Food and water were provided *ad libitum* for the control group and water with fructose and a HF diet for the

Table I. Time frame of the protocol used and the different groups used.

Animal groups	Phase 1 <sup>a</sup> , diet	Phase 2 <sup>b</sup> , treatment
Control <sup>c</sup>	Normal	-
Diabetes <sup>c</sup>	HFHF	-
Control	Normal	Untreated
Diabetes	HFHF	Untreated
Diabetes	HFHF	Exercise
Diabetes	HFHF	HBO
Diabetes	HFHF	Exercise + HBO

<sup>a</sup>Phase 1, months 1-3; <sup>b</sup>Phase 2, months 4-6. <sup>c</sup>Sacrificed at the end of phase 1. HFHF, high fructose, high fat; HBO, hyperbaric oxygenation.

experimental group. Weight and body composition (Lee index:  $\sqrt[3]{g/l}$ ; where g is the weight in grams and l is the length of mice in mm) were evaluated at the beginning and end of phase 2. Glucose tolerance was quantified at the end of phase 1 by comparing the diabetic group to the control group. A solution of 60% dextrose was injected intraperitoneally at a dose of 3 g/kg. The glucose concentration in the blood was measured after 0, 15, 30, 60, 90 and 120 min by taking blood samples from the tail.

At the start of phase 2, 7 mice from each group (control and diabetes) were sacrificed by the intraperitoneal injection of pentobarbital (100 mg/kg) to determine the initial values of parameters in different tissues. During phase 2, the animals in the control and diabetes groups were fed with their respective diets. The total duration of the experiment was 6 months.

**Treatments.** The exercise protocol started with a 2-week adaptation period of swimming (flotation) sessions 3 times per week. The time of each session was gradually increased from 10 to 60 min. For the following 12 weeks, the mice were trained 3 times per week, 60 min per session. The present protocol was designated as moderate exercise training, based on a comparison to other rodent studies using swimming training (39,40). The water temperature was 32±2°C. For the swimming sessions, animals were placed in transparent tanks divided into cells (25x25 cm), one animal per cell. Following each exercise session, mice were dried and returned to their cages. All sessions were conducted between 11 am and 1 pm. The HBO procedure was carried out from 9 am to 10:30 am in a hyperbaric chamber for small animals, using an oxygen pressure of 2 ATA (15 min pressurization, 60 min exposure and 15 min depressurization). A total of 10 HBO sessions were performed, once every other day, in the second month of phase 2, as the therapeutic use of HBO in humans includes 10-20 sessions (32). Upon completion of HBO and/or exercise, animals were returned to their cages and fed with their corresponding diet.

**Tissue processing.** The values of the measured parameters were assessed in tissues extracted from the liver, skeletal muscle (the *vastus lateralis* from the hind leg), lung, heart, thoracic aorta, brain, spleen, small intestine (lamina propria and mucosa),

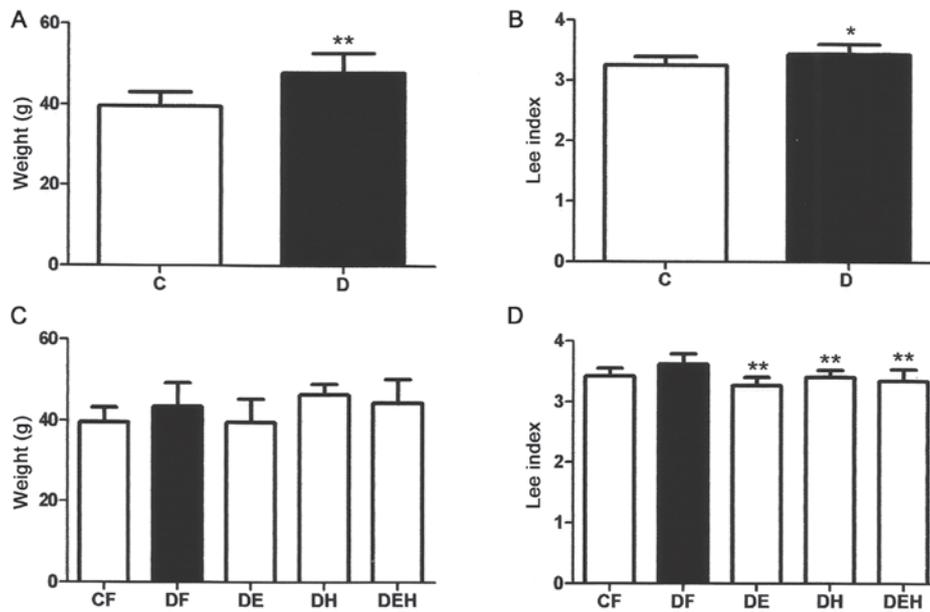


Figure 1. Weight and body composition of the animals in the control and experimental groups. (A) Initial body weight and (B) body composition. (C) Final body weight and (D) body composition. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  vs. C or DF. C, initial values in the control group; D, initial values in the diabetic group; CF, final values in the control group; DF, final values in the diabetic group; DE, values in the diabetic mice treated with exercise; DH, values in diabetic mice treated with hyperbaric oxygenation; DEH diabetic mice treated with both exercise and hyperbaric oxygenation.

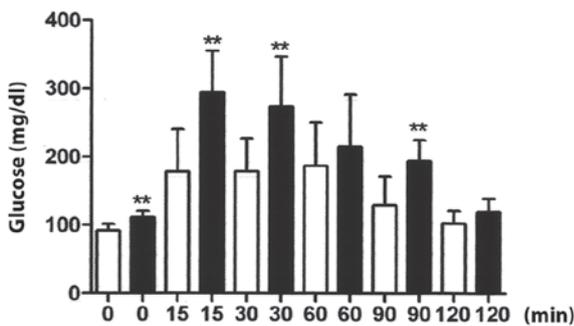


Figure 2. Glucose tolerance curves of mice in the C and D groups at the end of phase 1. \*\* $P < 0.01$ . C, control; D, diabetic.

kidney and visceral adipose tissue. The aforementioned parameters were assessed in these tissues for the following physiological reasons: To evaluate the possible redistribution of GSH from the liver to other tissues during stress, and to examine whether there was a relation between redistribution and the response to NO. Samples were obtained by placing a tissue in 30 mmol cold PBS (pH 7.2) and adding 0.1% of Triton X-100 (1 mg of tissue per 10  $\mu$ l buffer). Tissues were homogenized and centrifuged at 900 x g. for 15 min at 4°C and the supernatants were stored at -80°C for processing within two weeks. Cayman Chemical Company chemical kits were used to measure total proteins (TP; cat. no. 704002), NO (nitrate/nitrite colorimetric assay kit; cat. no. 780001) and total reduced GSH (GSH assay kit; cat. no. 703002) in the tissue homogenates. In the tissues with a significant presence of blood (liver, heart and kidney), the homogenates were treated with an Amicon ultra-0.5 centrifugal filter device (30K) prior to measurement of NO levels. The levels of 3NT (3NT ELISA kit; Abcam; cat. no. ab116691) was measured in the homogenates using ELISA. The values of NO, GSH and 3NT are expressed as nmol/mg of TP.

**Statistical analysis.** GraphPad Prism version 5 (GraphPad Software, Inc.) was used for statistical analysis. The mean values and standard deviations were obtained for each group. The count data were analyzed by ANOVA (7 animals/group), followed by a post-hoc Tukey's test. Bivariate Pearson's correlation analysis was used to assess the relation between the parameters in all tissues.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Lee index.** For all animals, the Lee index and weight was determined at the start (Fig. 1A and B) and end of phase 2 (Fig. 1C and D). During phase 1, a significant gain in weight was observed for the animals fed the HFHF diet ( $P < 0.01$ ; Fig. 1A). The inflammatory response promotes insulin resistance, which is manifested as weight gain and an accumulation of body fat (41). The Lee index, which has a positive correlation with body fat in females with obesity (42), and is an indicator of type 2 diabetes, was significantly higher in the diabetic mice compared with the control mice (Fig. 1B). At the end of phase 2, a significant decrease in the Lee index was observed for all treatments ( $P < 0.01$ ; Fig. 1D).

To analyze the behavior of the tolerance curve for glucose (Fig. 2), it is important to take into account, the measurement at 15 min captures the rapid release of insulin into the system. This measurement provides the first evidence of pancreatic failure. It is also a crucial element in the diagnosis of type 2 diabetes (38,43,44). The results confirm the successful establishment of diet-induced diabetes mellitus type 2 (Figs. 1 and 2). NO, 3NT and GSH levels were evaluated in tissues from various organs at the beginning and end of phase 2 to determine the effect of the treatments with exercise and/or HBO on the diabetic mice. The results of the measured parameters in all tissues are presented in Table II.

Table II. Initial, final and post-treatments levels of NO, 3NT and GSH in all tissues.

A, NO levels, nmol/mg proteins									
Tissue	Initial values in the control mice	Initial values in the diabetic mice	Final values in the control mice	Final values in the diabetic mice	Values in the diabetic mice treated with exercise	Values in the diabetic mice treated with HBO	Values in the diabetic mice treated with both exercise and HBO		
Muscle	2.7±0.42	1.5±0.37 <sup>b</sup>	1.2±0.17 <sup>d</sup>	1.1±0.29	1.1±0.24	1.6±0.33	1.2±0.13		
Liver	1.02±0.20	0.96±0.15	0.37±0.08 <sup>d</sup>	0.41±0.10 <sup>f</sup>	0.37±0.24	0.36±0.10	0.43±0.10		
Heart	0.28±0.08	0.25±0.06	0.21±0.07 <sup>e</sup>	0.24±0.06	0.18±0.04	0.22±0.05	0.53±0.12 <sup>g</sup>		
Kidney	1.27±0.15	1.48±0.15	0.42±0.08 <sup>d</sup>	0.42±0.11 <sup>f</sup>	0.34±0.05	0.28±0.06 <sup>g</sup>	0.29±0.07 <sup>g</sup>		
Lung	2.79±0.83	3.25±0.69	5.21±1.05 <sup>d</sup>	2.27±0.46 <sup>f</sup>	1.96±0.38	1.09±0.26 <sup>h</sup>	0.37±0.10 <sup>h</sup>		
Brain	0.40±0.10	0.33±0.07	0.30±0.09	0.21±0.07 <sup>e</sup>	0.20±0.07	1.86±0.70	0.18±0.06		
Aorta	4.39±0.98	6.65±1.25 <sup>a</sup>	1.92±0.38 <sup>d</sup>	0.55±0.12 <sup>f</sup>	0.28±0.07 <sup>h</sup>	0.26±0.09 <sup>h</sup>	0.24±0.08 <sup>h</sup>		
Lamina propria	0.35±0.08	0.34±0.07	0.28±0.09	0.26±0.06	0.21±0.06	0.19±0.06	0.19±0.05		
Mucosa	0.32±0.13	0.33±0.10	0.24±0.09	0.23±0.15 <sup>e</sup>	0.18±0.05	0.14±0.04	0.55±0.12 <sup>h</sup>		
Adipose tissue	3.14±0.67	3.56±1.06	2.19±0.44 <sup>d</sup>	5.31±1.46	5.17±0.92	4.99±0.99	3.65±0.92		
Spleen	1.12±0.17	1.24±0.31	0.18±0.06 <sup>d</sup>	0.39±0.09 <sup>f</sup>	0.24±0.08	0.23±0.05 <sup>g</sup>	0.24±0.08 <sup>g</sup>		
B, 3NT levels, nmol/mg proteins									
Tissue	Initial values in the control mice	Initial values in the diabetic mice	Final values in the control mice	Final values in the diabetic mice	Values in the diabetic mice treated with exercise	Values in the diabetic mice treated with HBO	Values in the diabetic mice treated with both exercise and HBO		
Muscle	1.5±0.30	0.58±0.12 <sup>b</sup>	1.9±0.42	0.41±0.11	1.1±0.24 <sup>h</sup>	0.05±0.015 <sup>h</sup>	0.94±0.20 <sup>h</sup>		
Liver	6.5±0.92	8.9±1.6 <sup>b</sup>	8.9±1.7	10.0±1.48	13.6±2.7 <sup>g</sup>	13.5±2.12 <sup>h</sup>	15.8±2.67 <sup>h</sup>		
Heart	4.5±0.89	14.1±1.9 <sup>b</sup>	10.4±2.3 <sup>d</sup>	5.9±0.96 <sup>f</sup>	5.2±0.93	4.7±0.96	4.5±0.85		
Kidney	3.3±0.43	19.7±2.5 <sup>b</sup>	3.6±0.59	12.5±1.85 <sup>f</sup>	18.9±1.9 <sup>h</sup>	19.5±2.73 <sup>h</sup>	18.8±1.96 <sup>g</sup>		
Lung	2.5±0.65	4.1±0.67 <sup>b</sup>	2.4±0.60	3.4±1.06	5.0±0.86 <sup>h</sup>	3.6±0.65	2.9±0.60		
Brain	1.2±0.18	4.9±0.87 <sup>b</sup>	1.3±0.31	3.1±0.59 <sup>f</sup>	2.7±0.61	3.2±0.63	4.1±0.95		
Aorta	4.0±0.78	12.5±2.6 <sup>b</sup>	6.6±1.2 <sup>d</sup>	12.0±2.44	6.1±0.92 <sup>h</sup>	6.5±1.56 <sup>h</sup>	6.2±1.43 <sup>h</sup>		
Lamina propria	2.4±0.58	1.3±0.34 <sup>b</sup>	3.4±0.49 <sup>c</sup>	1.1±0.29 <sup>f</sup>	1.9±0.27 <sup>h</sup>	3.4±0.63	3.6±0.71 <sup>h</sup>		
Mucosa	0.57±0.10	2.0±0.31 <sup>b</sup>	0.75±0.16	2.6±0.45	2.5±0.50	3.1±0.64	3.8±0.90 <sup>g</sup>		
Adipose tissue	2.5±0.67	16.6±2.6 <sup>b</sup>	3.6±0.72 <sup>c</sup>	9.8±2.00 <sup>f</sup>	10.4±2.92	7.4±1.60	6.7±1.92		
Spleen	0.74±0.17	2.6±0.51 <sup>b</sup>	2.2±0.48 <sup>d</sup>	1.8±0.39 <sup>f</sup>	2.0±0.38	2.3±0.40	3.9±0.84 <sup>h</sup>		

Table II. Continued.

Tissue	Initial values in the control mice	Initial values in the diabetic mice	Final values in the control mice	Final values in the diabetic mice	Values in the diabetic mice treated with exercise	Values in the diabetic mice treated with HBO	Values in the diabetic mice treated with both exercise and HBO
Muscle	2.6±0.34	2.3±0.48	0.22±0.03 <sup>d</sup>	0.08±0.02 <sup>f</sup>	0.31±0.06 <sup>h</sup>	0.44±0.07 <sup>h</sup>	0.32±0.06 <sup>h</sup>
Liver	84±18	90±19	75±11	81±16	88±16	84±14	86±15
Heart	3.0±0.63	3.8±0.76	0.40±0.08 <sup>d</sup>	0.20±0.04 <sup>f</sup>	0.36±0.06 <sup>h</sup>	0.34±0.05 <sup>h</sup>	0.47±0.09 <sup>h</sup>
Kidney	0.55±0.22	0.35±0.08	0.04±0.01	0.03±0.01	0.16±0.05 <sup>h</sup>	0.31±0.08 <sup>h</sup>	0.42±0.09 <sup>h</sup>
Lung	3.5±0.71	4.17±0.73	1.2±0.47 <sup>d</sup>	0.16±0.06 <sup>f</sup>	0.17±0.05	0.06±0.02 <sup>h</sup>	0.19±0.05
Brain	2.6±0.52	2.1±0.42	0.82±0.20 <sup>d</sup>	1.4±0.38 <sup>f</sup>	0.98±0.24	2.1±0.50 <sup>h</sup>	0.50±0.11 <sup>h</sup>
Aorta	1.7±0.53	2.7±0.83 <sup>a</sup>	0.18±0.06 <sup>d</sup>	0.50±0.14 <sup>f</sup>	0.18±0.06 <sup>h</sup>	0.27±0.12 <sup>h</sup>	0.37±0.12
Lamina propria	0.96±0.21	1.48±0.33 <sup>b</sup>	0.43±0.11 <sup>d</sup>	0.94±0.24 <sup>f</sup>	0.34±0.08 <sup>h</sup>	0.84±0.26	0.33±0.07 <sup>h</sup>
Mucosa	1.1±0.22	1.1±0.22	0.51±0.16 <sup>d</sup>	0.35±0.09 <sup>f</sup>	0.62±0.14 <sup>h</sup>	0.27±0.07	0.22±0.05 <sup>h</sup>
Adipose tissue	2.7±0.71	1.7±0.39 <sup>b</sup>	2.4±0.62	2.4±0.66	1.8±0.36	1.8±0.37	1.3±0.31 <sup>h</sup>
Spleen	6.7±1.22	4.8±0.68 <sup>b</sup>	0.21±0.04 <sup>d</sup>	0.18±0.02 <sup>f</sup>	0.34±0.08 <sup>h</sup>	0.10±0.04 <sup>h</sup>	0.32±0.09 <sup>h</sup>

Data are presented as the mean ± standard deviation. <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs. Initial values in the control mice; <sup>c</sup>P<0.05, <sup>d</sup>P<0.01 vs. Initial values in the diabetic mice; <sup>e</sup>P<0.05, <sup>f</sup>P<0.01 vs. Initial values in the control mice; <sup>g</sup>P<0.05, <sup>h</sup>P<0.01 vs. Initial values in the diabetic mice. HBO, hyperbaric oxygenation.

**Muscle.** The basal level of NO in the muscle tissues of mice with diabetes was ~50% of the levels observed in the control mice; whereas the final level of NO in the muscle tissue of the control group was <50% of the basal value, and a similar decrease was observed in the diabetic mice between the start and end of phase 2. The values for NO in the control and diabetic mice at the end of phase 2 did not differ significantly. Compared with the untreated diabetes group, no significant differences were observed in the levels of NO in the animals treated with exercise, HBO or both. The development of diabetes caused a 2.5-fold decrease in 3NT levels compared with the control group, similar to the decrease in NO levels in muscle tissue between the two groups. In both groups, the final levels of 3NT remained relatively stable at the end of study, but were lower in the diabetic mice. Additionally, 3NT levels increased to a similar degree in the mice subjected to exercise or exercise and HBO compared with the untreated diabetic mice. For the animals treated with HBO alone, there was a significant decrease in 3NT levels to a minimal level, corroborating the protective effect of HBO against nitrosative stress in muscle tissue (4,6). The effect of exercise was more prominent in the mice treated with exercise and HBO, as the response to exercise training alone was similar to the combined treatment. The basal levels of GSH in muscle tissues of mice fed either diet were high and did not differ significantly. The final levels of GSH were >10-fold lower in both groups, with the lowest level observed in the mice with diabetes. All treatments resulted in a significant increase in GSH levels compared with the final values in the diabetic mice. The effect of treatment with HBO was more prominent than with exercise, and the results coincided with the decrease in 3NT levels in the muscle tissues.

**Liver.** The initial levels of NO in liver tissue was not affected by diabetes; whereas the final levels of NO in the liver decreased >50% in both groups. In the animals with diabetes, NO levels were not altered by any of the treatments. The mice fed a HFHF diet had higher initial 3NT levels compared with the mice fed a normal diet, confirming an increased basal level of nitrosative stress in a diabetic state. The final levels of 3NT in the control group increased moderately (P<0.05), but no significant difference was observed in the final values between the groups. In comparison to the final values of the diabetic group, the three treatments increased the levels of 3NT to a similar degree (all P<0.05). Therefore, each treatment increased nitrosative stress in the liver. The initial levels of GSH in the liver was >30-fold higher compared with muscle tissues, but was not significantly different between the diabetic mice compared with the control group. No significant differences were observed in the final levels of GSH between the two groups, and none of the three treatments resulted in significant differences in levels.

**Heart.** The initial levels of NO were similar in the heart tissues of both groups. The final values of NO were moderately (P<0.05) reduced in the control animals and there was no significant change in the diabetic mice. Exercise reduced the NO levels, but HBO alone had no effect. There was large and significant increase in the levels of NO in the mice treated with both exercise and HBO, showing a possible synergistic effect of exercise and HBO on the heart which was absent in either treatment alone. The initial levels of 3NT were low

in the control mice and >3-fold higher for the diabetic mice. Compared with the initial values, the final levels of 3NT were increased in the control mice and decreased in the diabetic mice (both  $P < 0.01$ ). Neither exercise, HBO or both had an effect on 3NT levels in the heart of diabetic mice, suggesting that these treatments did not affect nitrosative stress. The development of diabetes did not significantly affect the initial levels of GSH in the heart, although the GSH levels were elevated in both groups in the majority of other tissues. The elevated initial levels of GSH observed in all mice was followed by a significant and large decrease in the final levels in both groups (all  $P < 0.01$ ). The final levels were lower in the diabetic mice compared with the control mice ( $P < 0.01$ ). All treatments increased GSH levels in diabetic mice to levels similar to those observed in the final values of the control group.

*Kidney.* The initial levels of NO in kidney tissues was comparable between the control and diabetic mice. A ~3-fold decrease in NO levels was observed in the final values in both groups and all treatments moderately reduced NO levels, and treatment with HBO or exercise and HBO combined resulted in a significant reduction in NO levels. In the control group, the initial and final values of 3NT levels were very low. However, the initial value in the diabetes group was ~17-fold higher compared with the control. Despite the decrease in 3NT levels in the final values in both groups, the levels in the diabetic mice were still 4-fold higher than the control group. The three treatments increased the levels of 3NT to a similar degree, reaching levels similar to those of the initial values of the diabetic mice. The initial GSH levels were moderately but not significantly lower in the diabetic group ( $P > 0.05$ ). The final levels of GSH were significantly and drastically reduced compared with the minimal levels in both the untreated groups ( $P < 0.01$ ). All treatments increased this parameter compared with the final values in the diabetic group, with the combined treatment having the most prominent effect, followed by HBO alone and then exercise alone. With both treatments involving HBO, the final levels of GSH were comparable to the initial values of the mice with diabetes. Thus, nitrosative stress was exacerbated in the kidney due to the development of diabetes and with the three treatments, similar to what was observed in the liver. The initial levels of GSH in the kidney in both groups was the lowest compared with all tissues assessed in the present study, consistent with a previous study (45).

*Lung.* In lung tissues, diabetes did not affect the initial levels of NO, and the final levels of NO were increased in the control group ( $P < 0.01$ ) and decreased in the diabetes group ( $P < 0.01$ ), compared with the initial levels. Both treatments involving HBO led to a decrease, and the decrease was more pronounced in the combined treatment group. Lung tissue is the first to be affected by sessions of HBO. The initial levels of 3NT were higher in the diabetic mice, and no significant change was observed in the final values in either the control or diabetic mice. Exercise increased the levels of 3NT, whereas HBO and the combined treatment did not have a significant effect. The changes in GSH levels in lung tissues were similar to those observed in other tissues. The initial values of the control and diabetic mice were comparable, and the final values were significantly decreased in both groups compared to the

respective initial value, but did not differ significantly between groups. HBO reduced GSH levels, whereas exercise and the combined treatment did not have a notable effect.

*Thoracic aorta.* The initial levels of NO in the thoracic aorta were higher in the diabetic mice compared with the control mice ( $P < 0.05$ ). The final levels were significantly and drastically lower in both groups ( $P < 0.01$ ), and the decrease in the diabetic mice was greater. All treatments resulted in ~50% decrease in the NO levels. The initial levels of 3NT were higher in the diabetic group compared with the control group ( $P < 0.01$ ). The final levels of 3NT levels were increased in the control group only compared with the respective initial values (all  $P < 0.01$ ). All treatments decreased the increased levels of 3NT in the diabetic mice to a similar degree (all  $P < 0.01$ ). The levels of GSH were higher in the diabetic mice compared with the control group ( $P < 0.05$ ). In both groups, the final GSH levels were considerably lower compared with the respective initial values (both  $P < 0.01$ ). Exercise or HBO significantly decreased GSH levels (both  $P < 0.01$ ), and to a lesser extent in the mice treated with the combined treatment.

*Intestinal lamina propria.* The development of diabetes did not significantly affect the initial, final or post-treatments values in the lamina propria. The initial levels of 3NT in the lamina propria were lower in the diabetic mice compared with the control group ( $P < 0.01$ ). For the control mice, there was an increase in the levels of 3NT between the initial and final levels ( $P < 0.05$ ), whereas in the diabetic group there was no significant change. The decreased levels of 3NT in the diabetic mice were increased with exercise ( $P < 0.01$ ), and the increase was greater in the mice treated with HBO alone or the combined treatment (both  $P < 0.01$ ), reaching similar values to the final levels of the control group. This confirmed the predominance of the effects of HBO over exercise in the exacerbation of nitrosative stress. The initial levels of GSH were higher in the mice diabetic mice compared with the control animals ( $P < 0.01$ ). The final levels of GSH decreased in the control group and in the diabetic group ( $P < 0.05$ ), but remained higher in the diabetic mice compared with the control mice ( $P < 0.05$ ). The two treatments involving exercise significantly reduced the levels of GSH (both  $P < 0.01$ ); whereas HBO alone did not notably affect GSH levels, demonstrating the predominance of exercise in regard to this parameter.

*Mucosa.* The initial levels of NO were not affected by diabetes. Only final levels of NO in the diabetic group decreased in intestinal mucosa ( $P < 0.05$ ), and only combined treatment increased this parameter ( $P < 0.01$ ). The initial levels of 3NT were relatively low in the mucosa of control mice and was higher in the diabetic group ( $P < 0.01$ ). There were no significant changes in the final values of 3NT in both groups. 3NT levels were not significantly affected by exercise or HBO treatment alone, the combination treatment resulted in an increase in 3NT levels ( $P < 0.05$ ), suggesting a synergistic effect of these two treatments. The similar initial levels of GSH were followed by a sharp decrease in the final values of both groups in the mucosa. Exercise increased the levels of GSH in the mucosa ( $P < 0.01$ ), whereas the combined treatment decreased this parameter.

Table III. Direction of significant changes of measured parameters in all tissues based on Table II.

A, Animal model									
Tissue	Initial levels, Control vs. diabetic group			Control, final vs. initial levels			Diabetic group, final vs. initial levels		
	NO	3NT	GSH	NO	3NT	GSH	NO	3NT	GSH
Muscle	D	D	0	D	0	D	0	0	D
Liver	0	I	0	D	0	0	D	0	0
Heart	0	I	0	D	I	D	0	D	D
Kidney	0	I	0	D	0	D	D	D	D
Lung	0	I	0	I	0	D	D	0	D
Brain	0	I	0	0	0	D	D	D	D
Aorta	I	I	I	D	I	D	D	0	D
LP	0	D	I	0	I	D	0	0	D
Mucosa	0	I	0	0	0	D	D	0	D
AT	0	I	D	D	I	0	0	D	0
Spleen	0	I	D	D	I	D	D	D	D

B, Treatment

Tissue	NO			3NT			GSH		
	DE	DH	DEH	DE	DH	DEH	DE	DH	DEH
Muscle	0	0	0	I	D	I	I	I	I
Liver	0	0	0	I	I	I	0	0	0
Heart	0	0	I	0	0	0	I	I	I
Kidney	0	D	D	I	I	I	I	I	I
Lung	0	D	D	I	0	0	0	D	0
Brain	0	0	0	0	0	0	0	I	D
Aorta	D	D	D	D	D	D	D	D	0
LP	0	0	0	I	I	I	D	0	D
Mucosa	0	0	I	0	0	I	I	0	D
AT	0	0	0	0	0	0	0	0	D
Spleen	D	D	D	0	0	I	I	D	I

I, significant increase; D, significant decrease; 0, lack of change; LP, lamina propria of the small intestine; AT, adipose tissue; DE, values in the diabetic mice treated with exercise; DH, values in diabetic mice treated with hyperbaric oxygenation; DEH diabetic mice treated with both exercise and hyperbaric oxygenation.

*Adipose tissue.* The initial levels of NO were similar in the adipose tissues of both the control and diabetic mice. The final levels were decreased in the control group ( $P < 0.01$ ), but were not significantly different in the diabetic mice, and none of the treatments altered NO levels significantly. The initial levels of 3NT were low in the control mice and were significantly higher in the diabetic mice ( $P < 0.01$ ). The final levels showed a moderate increase in the control group ( $P < 0.05$ ) and a decrease in the diabetic mice compared with the respective initial values ( $P < 0.01$ ), and none of the treatments significantly altered the 3NT levels. Compared with the adipose tissue of the control group, GSH levels were significantly lower in the diabetic mice ( $P < 0.01$ ), and the final levels were similar in both groups compared with their respective initial values, and

only the combined treatment resulted in a significant decrease in the levels of GSH ( $P < 0.01$ ).

*Spleen.* The initial level of NO in the spleen tissue did not differ significantly between the two groups. The final NO levels were significantly lower in both groups compared with the respective initial values (both  $P < 0.01$ ), and the final levels were higher in the diabetic group compared with the control group ( $P < 0.01$ ). All treatments reduced the concentration of NO (all  $P < 0.05$ ). The initial levels of 3NT were lower in the spleen of the control group compared with the diabetic group ( $P < 0.01$ ), and were significantly higher in the final measurement in both groups (both  $P < 0.01$ ). The elevated initial 3NT levels in the diabetes group moderately decreased during phase 2 ( $P < 0.01$ ),

leading to comparable final levels between the two groups. Treatment with exercise or HBO had no significant effect on 3NT, but the combined treatment resulted in a significant increase ( $P<0.01$ ). The initial levels of GSH were higher in the control group compared with diabetic group ( $P<0.01$ ). The final levels decreased considerably in both groups compared with the respective initial values (both  $P<0.01$ ), reaching similar levels. The levels of GSH decreased with HBO alone ( $P<0.01$ ), but increased with exercise and the combined treatment ( $P<0.01$ ), suggesting that exercise predominated in the latter.

**Brain.** The initial levels of NO in brain tissue did not differ significantly between the control and diabetic mice, and there was a moderate significant decrease in the final values of NO only in the diabetic group ( $P<0.05$ ). None of the treatments significantly altered the NO levels. The relatively low levels of 3NT in the control mice remained constant throughout the study. Conversely, the initial values of 3NT in the diabetic mice were significantly higher compared with the control group ( $P<0.01$ ), and the final values in the diabetic group were significantly lower compared with the initial values in the diabetic mice ( $P<0.01$ ). The final value of 3NT was higher in the diabetic mice compared with the control group and the treatments did not significantly affect the 3NT levels. The initial levels of GSH in the brain were not significantly affected by diabetes. The final levels were decreased in both groups (both  $P<0.01$ ), and were higher in the diabetic mice compared with the control mice ( $P<0.05$ ). HBO increased the levels of GSH ( $P<0.01$ ), and the combined treatment resulted in a decrease ( $P<0.01$ ).

**Pearson correlation.** Regarding the initial levels of GSH and 3NT in all the evaluated tissues of the control mice, a significant negative correlation was observed when averaged across all tissues ( $r=-0.734$ ,  $P=0.024$ ) except liver (due to the high levels of GSH in this tissue), and this was not observed in the diabetic group. Under conditions of relative stability of NO levels in the control group, this stability indicates a direct antioxidant affect against peroxynitrite: A greater level of GSH corresponded to a lower level of 3NT. There was a positive correlation observed between the initial levels of NO and the final levels of 3NT in the control group ( $r=0.641$ ,  $P=0.044$ ) suggesting an increase in nitrosative stress: Higher initial levels of NO resulted in higher final levels of 3NT (data not shown).

A positive correlation was observed between the final levels of GSH and NO in the diabetic mice ( $r=0.788$ ,  $P=0.007$ ), suggesting a close association between these parameters in the pathological state as a correlation was not observed in the final values of the control tissues. The statistical analysis of the bivariate correlation of parameters in the tissues evaluated showed a positive correlation between NO and GSH in all tissues from the diabetic mice subjected to exercise (exercise alone:  $r=0.755$ ,  $P=0.012$ ; combination treatment,  $r=0.878$ ,  $P=0.01$ ). Table III shows which parameters were significantly altered in Table II.

The development of diabetes did not affect the level of NO in the tissues assessed except the aorta (Table IIIA). Conversely, induction of diabetes significantly increased the levels of 3NT in the majority of tissues (with a reduction in the muscle and lamina propria), while decreasing the levels of

GSH in adipose tissue and spleen, and increasing its levels in the aorta and lamina propria. The control and diabetes groups showed a drastic and significant decrease in the initial levels of NO and GSH in all tissues between the two phases of the study.

The positive effects of treatments against oxidative stress are reflected by a decrease in 3NT levels or an increase in GSH levels (4). Some treatments resulted in a protective effect against nitrosative stress in the diabetic mice in certain tissues (Table IIIB): Aorta, all treatments; and muscle, HBO. However, a negative effect (an increase in 3NT levels) was observed more frequently: Liver, all treatments; kidney, all treatments; intestinal lamina propria, all treatments; muscle, exercise and combined treatment; lung, exercise; and spleen, combined treatment. An increase in GSH levels were observed following all treatments in the muscle, heart and kidney, after exercise in the mucosa and spleen; after HBO only in the brain; and after combined treatment in the spleen.

## Discussion

The results of the present study confirmed the successful establishment of a diet-induced diabetes mellitus type 2 model in mice. The age of mice is associated with the level of basal metabolism (lower in adults), and increases the likelihood of developing obesity based on diet (46-48). The possible effect of a change of basal metabolic levels in adult diabetic animals during the present study was eliminated by comparison of post-treatment levels of measured parameters to final levels of the diabetic group.

Oxidative stress served an important role in the pathogenesis of diabetes and complications linked to dysfunction of the endothelium. A possible initial mechanism of dysfunction of the endothelium may be associated with a reduction in the bioavailability of NO (6,49,50). In the present study, the development of diabetes did not affect the levels of NO in the majority of tissues. Indirectly, the stability of NO during diabetes emphasizes the importance of its homeostasis in tissues of mice in the experimental model used. The development of diabetes drastically increased the levels of 3NT in the majority of tissues, which is indicative of nitrosative stress (4,51), as it is a product of a reaction of peroxynitrite (derived from the reaction of NO with superoxide radicals) with the tyrosine residues of proteins. Conversely, the role of S-nitrosation in various signaling pathways associated with NO has also been discussed (25) and may be relevant for interpretation of the results of the present study considering the importance of S-nitrosation in the pathogenesis of diabetes. Principally, the effect of NO in the short-term is the vasodilatation of large conduit vessels that can modulate the redox state in tissues.

An increased production of superoxide in tissues is well-documented during the pathogenesis of diabetes (7,9,16), suggesting increased elimination of NO by these superoxide radicals in the tissues of the diabetic mice. Therefore, the lack of difference in the levels of NO and elevated levels of nitrosative stress observed in the majority of tissues in the diabetic mice suggests that any increased elimination of NO by superoxides was compensated for by an increase in its production in this animal model. Excessive NO and superoxide production

may disrupt the physiological balance between generation of peroxynitrite, antioxidant defense mechanisms and signaling pathways involving NO in the tissues of diabetic mice (4). GSH is a low-weight multifunctional molecule that also serves as the principal endogenous non-enzymatic antioxidant (15). In the present study, no changes in the levels of GSH were observed in the majority of tissues in the diabetic mice. *In vivo* studies have shown that the concentration of NO is closely associated with the synthesis of GSH in tissues. Increased production of NO inhibits the synthesis of GSH in rodent tissues, and a decreased production results in increased synthesis of GSH (24,52). The stability observed in the levels of NO in the diabetic mice in the present study may therefore be associated with the stability in GSH levels in the majority of tissues in the present study.

In the initial values of the control group only, there was a significant negative correlation between the levels of GSH and 3NT, and a positive correlation between the initial levels of NO and final levels of 3NT in all tissues. In the tissues of the control mice, the lower initial levels of nitrosative stress corresponded with an increase in antioxidant defense, which is manifested as higher levels of GSH. A high level of nitrosative stress resulted in a low level of GSH as the compensatory mechanisms were overwhelmed by excessive production of peroxynitrite and consequently 3NT. Compensatory mechanisms included increased production of GSH and/or its mobilization from the liver, where its concentration was in the mM range, compared with the  $\mu$ M concentrations observed in other tissues.

During phase 2 of the study, both the control and untreated diabetes groups displayed a similar decrease in the levels of NO and GSH in the majority of tissues, suggesting a possible age-related effect and thus may reflect a decrease in the basal levels of metabolism in adult mice. The lower initial levels of 3NT were not significantly altered or significantly increased in the majority of the tissues in the control group between the two phases, whereas the elevated initial levels of 3NT in the diabetic mice were unchanged or decreased between the two phases. The final levels of nitrosative stress (as determined based on 3NT levels) for the control group indicated either stability or an increase in levels, and in the diabetic mice, the levels were either stable or decreased. In the majority of these tissues the final 3NT levels were higher in the diabetic mice compared with the control group. This difference coincided with a positive correlation between the final levels of GSH and NO in all tissues in the diabetic mice only, contradicting the known effect of NO on the synthesis of GSH in rodent tissues without diabetes (24,53) which was also observed in the control group in the present study.

The role of NO-related mechanisms were examined in processes of adaptation to exercise in tissues (25). During a range of pathological processes and in all tissues and organs, NO was determined to be the primary molecule linked to the regulation of vascular endothelium tissue, and exercise may correct alterations in the bioavailability of this radical. Enzymatic production of NO by endothelial nitric oxide synthase has a regulatory role in endothelial dysfunction (54) and NO participates in vascular adaptation to exercise (55,56). Therefore, the association between NO and different nitric oxide synthases in diabetic tissues requires examination.

Studies have described the protective effect of HBO pre-treatment against oxidative stress produced by distinct

factors in different animal models, including experimental damage to the rat spinal cord (27), cerebral ischemia-reperfusion (28), experimental myocardial infarct (53), and the effect of ultraviolet light on mouse skin and liver (29). HBO pretreatment is also reported to reduce the levels of indicators of oxidative stress in rat thoracic aorta, including NO (31) and to decrease oxidative stress in rat lungs (30). In the present study, exercise and HBO did not affect the levels of NO in the majority of diabetic tissues, but the levels of nitrosative stress (3NT) and GSH were increased in the majority of tissues, and contrary to the control group, where a negative correlation was observed between these parameters. An increase in the levels of GSH was interpreted as a positive effect in relation to the redox equilibrium in tissues.

All treatments did not alter or increase the levels of GSH in muscle, liver heart, kidney, lung and brain tissues (except after HBO alone in the lung, or after combined treatment in the brain) suggesting a positive effect on the redox equilibrium of tissues. HBO alone increased the levels of GSH in the brain and decreased the levels in the lung. Conversely, exercise alone resulted in changes in GSH levels in the small intestine, where an increase was observed in the mucosa and a decrease was observed in the lamina propria, and this may be reflective of redistribution of GSH between different intestinal regions. The combination treatment showed a similar effect on GSH levels as exercise in the majority of tissues.

Increased or unchanged levels of GSH by all treatments corresponded to an increase or unchanged levels of nitrosative stress. It is possible that the elevated levels of nitrosative stress facilitated the redistribution of GSH from liver to other tissues. As the final level of GSH in liver of diabetic mice was  $\geq 80$ -fold higher compared with other tissues, alterations in the level of GSH in the liver may have gone unnoticed. In our previous study HBO-induced a notable decrease in the basal levels of GSH in the liver which coincided with an increase in the muscle, brain, small intestine and adipose tissue, without a significant change in the grade of GSH oxidation in the exercised mice (57). That is, the change in GSH without a change in the oxidation of GSH following HBO, suggested that GSH was redistributed from the liver to the other, reflecting an improvement in the antioxidant capability of these tissues.

In conclusion, the development of diabetes in this animal model caused nitrosative stress in the majority of tissues without affecting GSH levels in the majority tissues, and all treatments resulted in an increase or stabilization of GSH levels in the muscle, liver, heart, kidney, lung and brain which coincided with an increase or stabilization in nitrosative stress in the same tissues.

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### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

All authors performed the experiments. AK, GGB and MDCCH performed the analysis and wrote the manuscript. LRGC and ELP performed the analysis and interpreted the data. All authors read and approved the final the manuscript.

### Ethics approval and consent to participate

The study protocol was reviewed and approved by the Institutional Laboratory Use and Care Committee of the High Medical School, National Polytechnic Institute (Mexico City, Mexico; approval no. 02/28-08-2015).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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