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Dietary inorganic nitrate attenuates hyperoxia-induced oxidative stress in obese type 2 diabetic male rats

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ABSTRACT

Aims: Hyperoxia has beneficial metabolic effects in type 2 diabetes. However, hyperoxia exacerbates already existing oxidative stress in type 2 diabetes. Nitrate, a nitric oxide donor, is an effective new treatment in type 2 diabetes and also has antioxidant properties. The aim of this study was to determine whether nitrate administration can attenuate hyperoxia-induced oxidative stress in obese type 2 diabetic rats.

Main methods: Fifty-six male Wistar rats (190–210 g) were divided into 8 groups: Controls (non-treated, nitrate-treated, O_2-treated, and nitrate + O_2-treated) and diabetes (non-treated, nitrate-treated, O_2-treated, and nitrate + O_2-treated). Diabetes was induced using high-fat diet and low-dose of streptozotocin (30 mg/kg). Rats in intervention groups, were exposed to 95% oxygen and consumed sodium nitrate (100 mg/L) in drinking water. Serum fasting glucose, oxidized (GSSG) and reduced (GSH) glutathiones, total oxidant status (TOS), catalase and superoxide dismutase (SOD) activities, and total antioxidant capacity (TAC) were measured after intervention. Oxidative stress index (OSI) was calculated as TOS/TAC ratio.

Key findings: Diabetic rats had increased oxidative stress and hyperoxia exacerbated it. In O_2-diabetic rats, nitrate decreased GSSG (102.7 ± 2.1 vs. 236.0 ± 20.1 μM, $P < 0.001$), TOS (67.7 ± 7.3 vs. 104 ± 3.8 μM, $P < 0.001$), and OSI (0.44 ± 0.04 vs. 0.91 ± 0.07, $P < 0.001$) and increased catalase (2.8 ± 0.13 vs. 1.8 ± 0.21 KU/L, $P = 0.014$), SOD (53.4 ± 1.5 vs. 38.4 ± 1.2 U/mL, $P < 0.001$), GSH (43.7 ± 1.4 vs. 17.8 ± 0.5 mM, $P = 0.003$), TAC (152.5 ± 1.9 vs. 116.7 ± 5.0 mM, $P < 0.001$), and GSH/GSSG ratio (0.43 ± 0.01 vs. 0.08 ± 0.01, $P = 0.005$). Nitrate also potentiated effects of hyperoxia on decreasing fasting glucose.

Significance: Our results showed that dietary nitrate attenuates hyperoxia-induced oxidative stress in type 2 diabetic rats.

1. Introduction

Diabetes, the most prevalent endocrine disorder, is one of the leading causes of mortality worldwide [1]. Currently, there is no cure for type 2 diabetes and despite available treatments, there is a need to find new treatments [2]. Tissue hypoxia has been reported in animals [3,4] and humans with type 2 diabetes and obesity [5] and hyperoxia has been suggested as a new treatment for type 2 diabetes [6]. We recently reported the beneficial metabolic effects of hyperoxia in obese type 2 diabetic rats; the effects that are mostly due to browning of white adipose tissue and lowering body weight [7]. Despite safety and cost-effectiveness of using hyperoxia as a treatment for diabetes, it however induces oxidative stress by increasing oxidants and decreasing antioxidants [8–11], a side effect that exacerbates already existing

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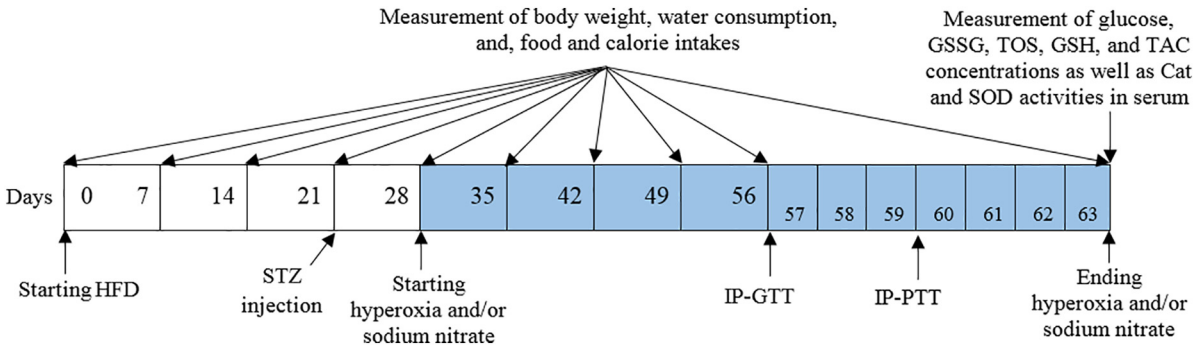

Fig. 1. Study protocol. HFD, high-fat diet; STZ, streptozotocin; IP-GTT, intraperitoneal glucose tolerance test; IP-PTT, intraperitoneal pyruvate tolerance test; GSSG, oxidized glutathione; TOS, total oxidant status; Cat, catalase; SOD, superoxide dismutase; GSH, reduced glutathione; TAC, total antioxidant capacity.

oxidative stress in type 2 diabetes [12].

Type 2 diabetes is also associated with decreased nitric oxide (NO) bioavailability due to decreased activity of NO-producing enzymes and also increased NO quenching [13,14]. Nitrate, as a NO releasing agent, has been shown to be an effective new treatment in type 2 diabetes [13]. Nitrate increases glucose tolerance, insulin sensitivity and secretion, browning of white adipose tissue, and have weight-lowering effects in type 2 diabetes [15,16]. In addition, nitrate has antioxidant properties in animals [17,18] and humans [19]. The traditional view of the harmfulness of nitrate has been changed to the view that it has beneficial metabolic effects [20]. There is no convincing evidence for association between nitrate and incidence of stomach cancer, which thought to be the most relevant site of endogenous nitrosation [21]. In addition, according to a recent meta-analysis, high or moderate intake of dietary nitrate is associated with a decreased risk of gastric cancer [22]. Furthermore, the differences between recommended daily allowance and upper limit of nitrate intake are sufficiently high [23].

Hyperoxia by increasing oxidative stress decreases NO bioavailability in diabetes [24]. Increased oxidative stress following hyperoxia decreases plasma nitrite, NO metabolite, in healthy humans [25]. In addition, administration of inhaled NO decreases hyperoxia-induced oxidative stress [26]. Recent investigations indicate that pathophysiology of type 2 diabetes includes many organs other than pancreatic β-cell, skeletal muscle, and liver [27]. This leads to rethinking the treatment approaches from only glycemic control to a pathophysiological-based view and warrants combination therapy. These findings justify that co-administration of hyperoxia and nitrate be an effective treatment for type 2 diabetes; an idea that has been proposed by our group [6]. This study therefore aims at determining whether nitrate administration can attenuate hyperoxia-induced oxidative stress in obese type 2 diabetic rats.

2. Materials and methods

2.1. Animal housing, grouping and ethics

Fifty-six male Wistar rats were housed in standard polypropylene cages (three rats/cage) under controlled temperature (22 ± 2 °C) and humidity (50 ± 5%) with a 12:12 h light-dark cycle (lights on: 07:00–19:00). Rats were fed with standard normal pellet diet (Pars Animal Feed Co.) and received water ad libitum, prior to the manipulation. Rats (weight range: 190–210 g) were randomly divided into two control and diabetic groups. Each group was divided into four subgroups (n = 7/subgroup): control (C), control + hyperoxia (C−O_2), control + nitrate (CN), control + hyperoxia and nitrate (CN-O_2), diabetes (D), diabetes + hyperoxia (D-O_2), diabetes + nitrate (DN), diabetes + hyperoxia and nitrate (DN-O_2). Rats to be assigned into diabetic groups were fed a high-fat diet (HFD), described elsewhere [17], throughout the study.

In this study all procedures were in accordance to the local protocols for animal care and use; the proposal of this study was approved by the ethical committee of Research Institute for Endocrine Sciences (RIES), Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran (Ethic code: IR.SBMU.Endocrine.Rec.1396.399).

2.2. Study design

As seen in Fig. 1, diabetes was induced using low-dose streptozotocin (STZ) after HFD manipulation (see below); interventions started at day 28 to day 63. Food intake (g/day/rat), calorie intake (Kcal/day/rat), and water consumption (mL/day/rat) were recorded every week. Body weight (using Tefal Scale, sensitivity 1 g) was recorded at the baseline, before intervention and after intervention. During the last week of intervention, intraperitoneal glucose and pyruvate tolerance tests (IP-GTT and IP-PTT) were performed. At the end of the study, rats were fasted for 12–14 h and blood samples were taken from tail; after blood clot formation, samples were centrifuged at 5000g for 10 min to separate sera; fasting serum glucose, catalase, superoxide dismutase (SOD), reduced glutathione (GSH), oxidized glutathione (GSSG), total antioxidant capacity (TAC), and total oxidant status (TOS) were measured.

2.2.1. Induction of type 2 diabetes

Type 2 diabetes was induced using a combination of HFD and low-dose of STZ; after three weeks on HFD, STZ (30 mg/kg, dissolved in 0.1 mM citrate buffer, pH 4.5; Sigma Aldrich, Hamburg, Germany) was injected intraperitoneally. One week after STZ injection, rats with fasting serum glucose levels > 150 mg/dL were considered to have diabetes. In diabetic rats, HFD was continued throughout the study. In this HFD-STZ model of type 2 diabetes, insulin resistance is induced two weeks after HFD, and following low-dose STZ injection, a partial β-cell dysfunction is caused; this model is appropriate to accessing the metabolic effects of interventions [28].

2.2.2. Interventions

One week after STZ injection, hyperoxia (95% oxygen, absolute pressure 0.998 ± 0.002 atm) was administrated by hypoxia/hyperoxia chamber (SD-EOS) [7] for 2 h/day (10.00–12.00 AM), all days except Fridays, from day 28 to day 63. To decrease stress, rats were kept at own cages in the chamber, while had free access to food and water. The same handlings were done for rats in normoxia groups, except that they receive normoxia in the chamber. Sodium nitrate (100 mg/L in drinking water) was administrated from day 28 to day 63 of the study (Fig. 1).

2.3. Biochemical measurements

2.3.1. Serum catalase activity

Serum catalase activity was measured by the method of Hadwan with slight modification [29]. In brief, 20 μL of the sera and 200 μL of hydrogen peroxide (59 mM, in sodium potassium phosphate buffer,

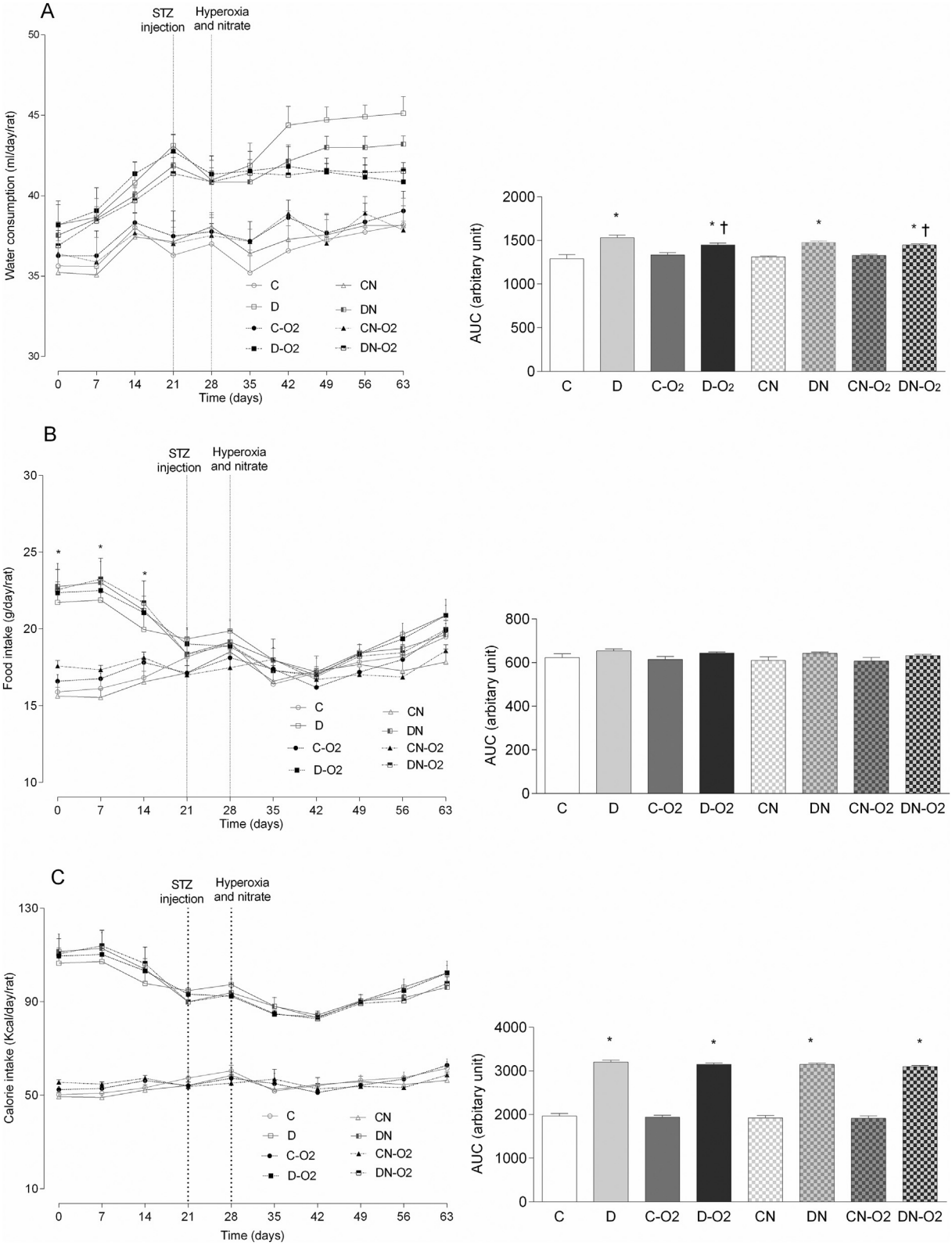

Fig. 2. Effects of hyperoxia and nitrate on water consumption (A), food (B), and calorie intakes (C) in control and obese type 2 diabetic rats (n = 7/group). Area under the curves (AUC), on the right, calculated during the intervention from day 28 to day 63. The comparisons were done by two- and one-way ANOVA for the left and right column, respectively. *, and † statistically significant difference in comparison to control, and diabetes, respectively. C, control; D, diabetes; C-O_2, O_2-treated control; D-O_2, O_2-treated diabetes; CN, nitrate-treated control; DN, nitrate-treated diabetes; CN-O_2, nitrate- and O_2-treated control; DN-O_2, nitrate- and O_2-treated diabetes; STZ, streptozotocin. Values are mean ± standard error of the mean (SEM).

Table 1
Effect of hyperoxia and sodium nitrate on body weight in control and obese type 2 diabetic rats.

Parameters	Groups (n = 7/group, Mean ± *SEM*)							
	C	D	C-O_2	D-O_2	CN	DN	CN-O_2	DN-O_2
Baseline body weight (g)	205.4 ± 2.4	200.3 ± 2.8	201.4 ± 1.9	199.0 ± 1.9	206.6 ± 1.9	199.3 ± 1.9	203.7 ± 1.6	195.9 ± 1.4
Body weight before intervention (g)	254.6 ± 6.1	265.6 ± 2.1	248.6 ± 3.4	260.8 ± 4.5	255.7 ± 4.7	266.0 ± 2.1	250.9 ± 1.9	258.9 ± 2.9
Body weight after intervention (g)	288.6 ± 9.3	315.1 ± 7.9	280.6 ± 3.5	266.2 ± 4.5	288.0 ± 7.4	291.6 ± 2.7	284.3 ± 2.9	265.1 ± 3.4
Body weight gain (g)	34.0 ± 5.4	49.6 ± 6.8*‡	32 ± 4.4	5.4 ± 2.1*†‡	32.3 ± 4.7	25.6 ± 2.9†	33.4 ± 3.4	6.3 ± 1.9*†‡
Body weight gain (%)	13.4	18.7	12.9	1.0	12.6	9.6	13.3	2.4

Intervention was administered with 95% oxygen for C—O_2 and D-O_2 groups, with nitrate (100 mg/L in water consumption) for CN and DN groups, and with both of them for CN-O_2 and DN-O_2 groups. Weight gain (g) was calculated based on the weight difference before and after intervention (s). *, †, and ‡ statistically significant difference in comparison to control, diabetes, and nitrate-treated diabetes, respectively. C, control; D, diabetes; C-O_2, O_2-treated control; D-O_2, O_2-treated diabetes; CN, nitrate-treated control; DN, nitrate-treated diabetes; CN-O_2, nitrate- and O_2-treated control; DN-O_2, nitrate- and O_2-treated diabetes. Values are mean ± standard error of the mean (SEM).

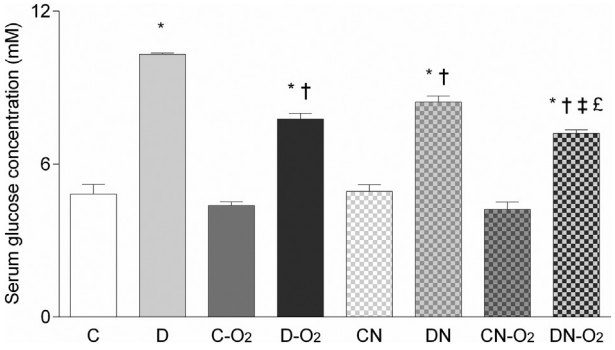

Fig. 3. Effects of hyperoxia and nitrate on fasting serum glucose concentration in control and obese type 2 diabetic rats (n = 7/group). The comparisons were done by one-way ANOVA at the end of the study (day 63). *, †, and ‡ statistically significant difference in comparison to control, diabetic, and nitrated-treated diabetic rats respectively; £ marginally significant (P = 0.098) difference in comparison to O_2-treated diabetic rats. C, control; D, diabetes; C-O_2, O_2-treated control; D-O_2, O_2-treated diabetes; CN, nitrate-treated control; DN, nitrate-treated diabetes; CN-O_2, nitrate- and O_2-treated control; DN-O_2, nitrate- and O_2-treated diabetes. Values are mean ± standard error of the mean (SEM).

pH = 7.4) were added to the test tubes and 20 μL of the sera and 200 μL of distilled water were added to the control test tubes; the standard tube contained 20 μL distilled water and 200 μL hydrogen peroxide, and blank tube contained 220 μL distilled water. The tubes incubated 3 min in 37 °C water bath and immediately 400 μL dichromate potassium 5% (w/v) in glacial acetic acid was added to all the tubes and incubated in boiling water for 10 min; after cooling and centrifugation, the ODs were read at 570 nm. Catalase activity (KU/L) of the sera were calculated using following formula: Log [OD standard/ (OD test-OD control test)] × 23.7977.

2.3.2. Serum superoxide dismutase activity, glutathione (reduced and oxidized) concentration, total antioxidant capacity, and total oxidant status

SOD, GSH, GSSG, TAC, and TOS were measured by colorimetric method using research commercial kits (ZellBio GmbH, Germany) at the end of the study. The ratio of GSH to GSSG, as cellular redox [30], and the ratio of TOS to TAC, as oxidative stress index (OSI) [31], were calculated. The kit's sensitivity for SOD, GSH, GSSG, TAC, and TOS were 1 U/mL, 10.0 μM, 10.0 μM, 100 μM, and 0.5 μM, respectively. All intra- and inter-assay coefficients of variation (CVs) were under 6% and 10%, respectively.

2.3.3. Intraperitoneal glucose and pyruvate tolerance tests

IP-GTT and IP-PTT were performed during the last week. In brief, after 12–14 h fasting, animals were anesthetized using pentobarbital (45 mg/kg); for IP-GTT, glucose solution (1 g/kg) was injected, and tail blood samples were taken at 0, 10, 20, 30, 60, and 120 min; for IP-PTT, sodium pyruvate (2 g/kg) was injected and tail blood samples were taken at 0, 5, 10, 20, 30, and 60 min, then glucose of samples were measured. Serum glucose concentration was measured using glucose oxidase method by commercial kit (Pars Azmoon, Tehran, Iran).

2.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Data are reported as mean ± standard error of the mean (SEM). One-way ANOVA with Fisher's LSD test as post-hoc was used for analyzing data of body weight, and biochemical parameters. Two-way mixed (between-whiten) ANOVA with Fisher's LSD test as post-hoc was used for analyzing data of food and calorie intakes, water consumption, IP-GTT and IP-PTT. Two-sided $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of hyperoxia and nitrate on water consumption, food intake, and calorie intake

Water consumption was higher in diabetic rats than controls. In diabetic rats, hyperoxia decreased water consumption (AUC 1450 ± 23 vs. 1533 ± 28, P = 0.024) as did co-administration of nitrate and hyperoxia (AUC 1448 ± 10 vs. 1533 ± 28, P = 0.022). Food intake was comparable between all groups. Diabetic rats had higher calorie intake that was not affected by hyperoxia and nitrate (Fig. 2A-C).

3.2. Effect of hyperoxia and nitrate on body weight

The baseline body weights of all groups were comparable. At the end of the study, weight gain in non-treated diabetic rats was higher than other groups. Following 5 weeks of intervention, weight gain in O_2-treated (5.4 ± 2.1 g), nitrate-treated (25.6 ± 2.9 g), and O_2 + nitrate-treated (6.3 ± 1.9 g) rats were significantly ($P < 0.001$) lower than that in non-treated diabetic rats (49.6 ± 6.8 g). Hyperoxia and nitrate had no significant effects on body weight in control rats (Table 1).

3.3. Effect of hyperoxia and nitrate on carbohydrate metabolism

3.3.1. Fasting serum glucose concentration

Diabetic rats had significantly higher serum glucose concentrations compared to controls. Following 5 weeks of intervention, fasting serum glucose in O_2-treated (7.77 ± 0.22 mM), nitrate-treated (8.44 ± 0.63 mM), and O_2 + nitrate-treated (7.21 ± 0.14 mM) diabetic rats were significantly ($P < 0.001$) lower than that in non-treated

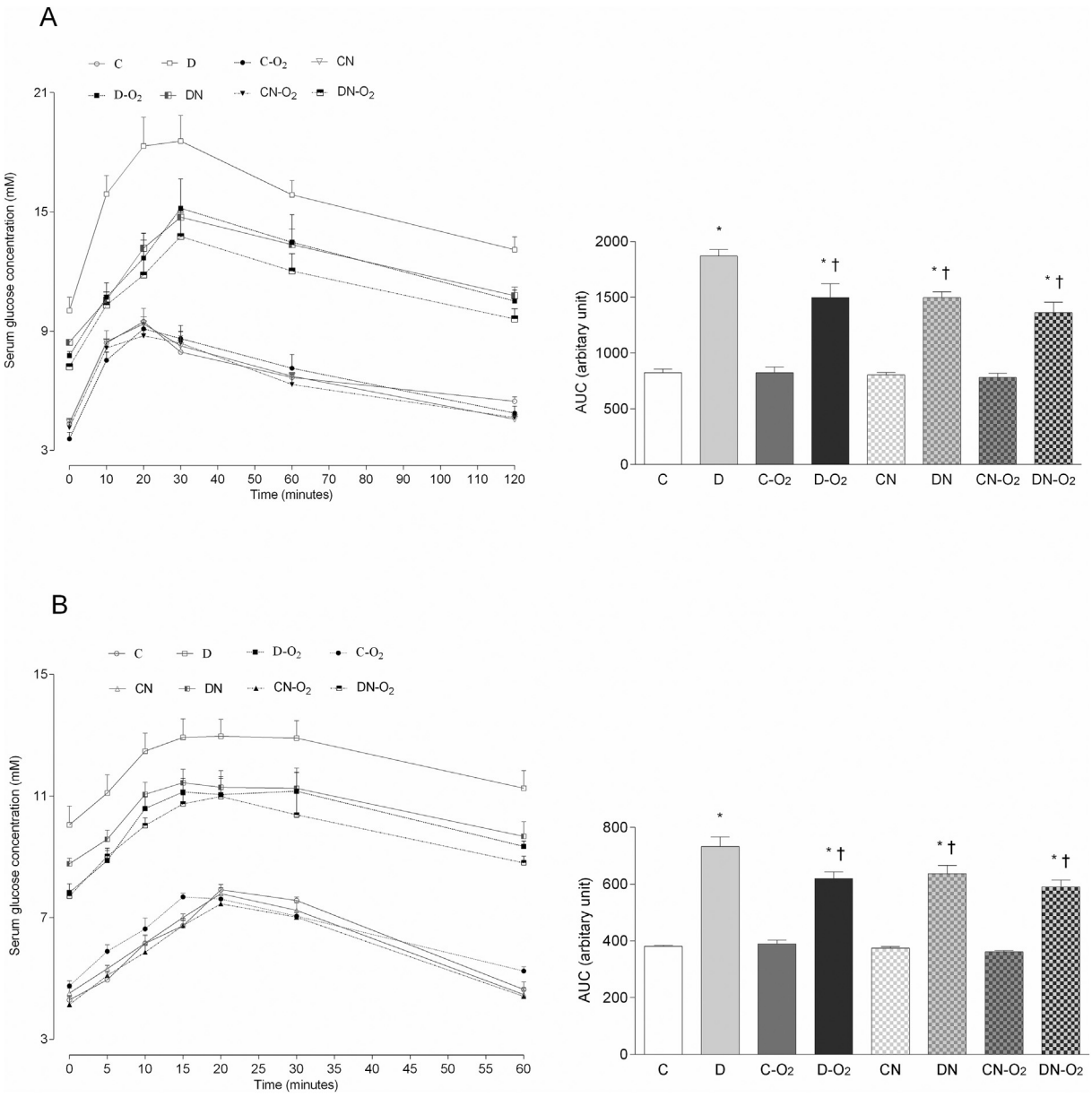

Fig. 4. Effects of hyperoxia and nitrate on glucose tolerance test (A), and pyruvate tolerance test (B) in control and obese type 2 diabetic rats (n = 7/group). Area under the curves are shown in the right columns. The comparisons were done by two-way and one-way ANOVA for the left and right column respectively. *, and † statistically significant difference in comparison to control and diabetic rats respectively. C, control; D, diabetes; C-O_2, O_2-treated control; D-O_2, O_2-treated diabetes; CN, nitrate-treated control; DN, nitrate-treated diabetes; CN-O_2, nitrate- and O_2-treated control; DN-O_2, nitrate- and O_2-treated diabetes. Values are mean ± standard error of the mean (SEM).

ones (10.31 ± 0.10 mM). Administration of nitrate in O_2-treated diabetic rats caused further decrease in fasting serum glucose (7.21 ± 0.14 vs. 7.77 ± 0.22 mM, P = 0.098). Hyperoxia and nitrate had no significant effects on fasting serum glucose in control rats (Fig. 3).

3.3.2. Glucose tolerance test

As shown in Fig. 4A, during the last week of the study, glucose tolerance in O_2-treated (AUC 1498 ± 124), nitrate-treated (AUC 1498 ± 52), and O_2 + nitrate-treated (AUC 1362 ± 94) diabetic rats were significantly (P < 0.001) lower than that in non-treated ones (AUC 1870 ± 60). Nitrate did not increase the effect of hyperoxia on glucose tolerance in diabetic rats. Hyperoxia and nitrate had no significant effects on glucose tolerance in control rats.

3.3.3. Gluconeogenesis

As shown in Fig. 4B, during the last week of the study, gluconeogenesis in O_2-treated (AUC 619 ± 24), nitrate-treated (AUC 637 ± 29), and O_2 + nitrate-treated (AUC 590 ± 24) diabetic rats were significantly (P < 0.001, P = 0.002, and P < 0.001, respectively) lower than that in non-treated ones (AUC 732 ± 35). Nitrate did not increase the effect of hyperoxia on gluconeogenesis in diabetic rats. Hyperoxia and nitrate had no significant effects on gluconeogenesis in control rats.

3.4. Effect of hyperoxia and nitrate on serum oxidants

3.4.1. Oxidized glutathione (GSSG)

GSSG was significantly higher in non-treated diabetic rats compared to controls. Hyperoxia increased the elevated GSSG in diabetic rats (236.0 ± 20.1 vs. 142.0 ± 2.3 μM, P < 0.001); nitrate

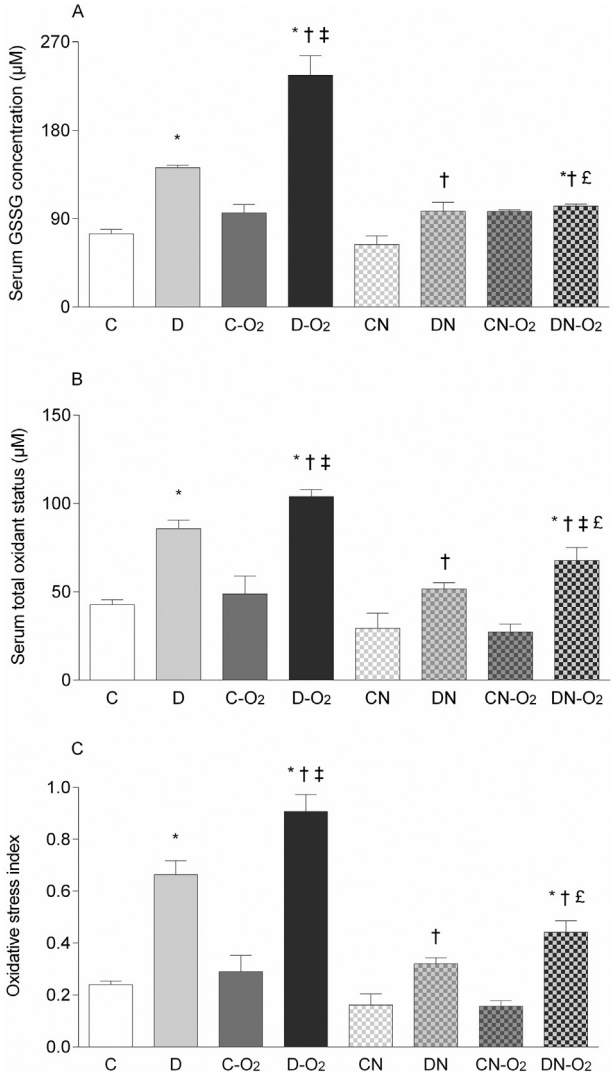

Fig. 5. Effects of hyperoxia and nitrate on serum oxidized glutathione (GSSG) (A), and total oxidant status (TOS) (B) levels and the ratio of TOS to total antioxidant capacity (oxidative stress index, OSI) (C) in control and obese type 2 diabetic rats (n = 7/group). The comparisons of all groups were done by one-way ANOVA at the end of the study (day 63). *, †, ‡, and £ statistically significant difference in comparison to control, diabetes, nitrated-treated diabetes, and O_2-treated diabetes, respectively. C, control; D, diabetes; C-O_2, O_2-treated control; D-O_2, O_2-treated diabetes; CN, nitrate-treated control; DN, nitrate-treated diabetes; CN-O_2, nitrate- and O_2-treated control; DN-O_2, nitrate- and O_2-treated diabetes. Values are mean ± standard error of the mean (SEM).

administration restored elevated GSSG concentration in non-treated, and O_2-treated diabetic rats. Hyperoxia and nitrate had no significant effects on serum GSSG levels in control rats (Fig. 5A).

3.4.2. Total oxidant status (TOS) and oxidative stress index (OSI)

Serum TOS was significantly higher in non-treated diabetic rats compared to controls. Hyperoxia increased elevated TOS in diabetic rats. Nitrate administration restored elevated TOS in non-treated, and O_2-treated diabetic rats. Hyperoxia and nitrate had no significant effects on serum TOS in control rats (Fig. 5B). A similar pattern of changes was observed for OSI, an index of redox state (Fig. 5C).

3.5. Effect of hyperoxia and nitrate on serum antioxidants

3.5.1. Catalase activity

Non-treated diabetic rats had lower catalase activity compared to controls; hyperoxia caused further decrease in catalase activity in diabetic rats (1.8 ± 0.21 vs. 3.0 ± 0.18 KU/L, P = 0.003). Nitrate administration increased serum catalase activity in both non-treated and O_2-treated diabetic rats compared to their corresponding groups. Hyperoxia and nitrate had no significant effects on serum catalase activity in control rats (Fig. 6A).

3.5.2. Superoxide dismutase activity (SOD)

Serum SOD activity was significantly lower in non-treated diabetic rats compared to controls. Hyperoxia caused further decrease in serum SOD activity in diabetic rats (38.4 ± 1.2 vs. 44.6 ± 0.6 U/mL, P = 0.01). Nitrate administration increased serum SOD activity in both non-treated and O_2-treated diabetic rats compared to their corresponding groups. Hyperoxia and nitrate had no significant effects on serum SOD activity in control rats (Fig. 6B).

3.5.3. Reduced glutathione (GSH) and GSH/GSSG ratio

Serum levels of GSH were significantly lower in non-treated diabetic rats compared to controls; although hyperoxia caused further decrease in GSH levels in diabetic rats, it did not reach statistically significant. Nitrate administration increased serum GSH levels in both non-treated and O_2-treated diabetic rats compared to their corresponding groups. Hyperoxia and nitrate had no significant effects on serum GSH levels in control rats (Fig. 6C). A similar pattern of changes was observed for GSH/GSSG ratio, an index of redox state (Fig. 6D).

3.5.4. Total antioxidant capacity (TAC)

Serum TAC concentration was significantly lower in non-treated diabetic rats compared to controls. Hyperoxia had no additional effect on serum TAC concentrations in diabetic rats. Nitrate administration restored decreased TAC levels to normal values in diabetic rats; in O_2-treated diabetic rats, nitrate significantly increased TAC levels although it did not reach to normal levels. Hyperoxia and nitrate had no significant effects on serum TAC levels in control rats (Fig. 6E).

4. Discussion

This study in obese type 2 diabetic rats showed that long-term administration of nitrate attenuates hyperoxia-induced oxidative stress and that nitrate potentiates favorable effects of hyperoxia on fasting serum glucose. Supports for these findings are decrease in oxidant and increase in antioxidant parameters in serum following nitrate administration in O_2-treated diabetic rats and that co-administration of hyperoxia and nitrate causes further decrease in fasting glucose.

Hyperoxia has favorable metabolic effects in type 2 diabetes as we have seen in the current study by decreasing fasting glucose, improving glucose tolerance, decreasing gluconeogenesis, and weight-lowering effects. In line with these results, favorable metabolic effects of hyperoxia have previously been reported [7,32,33].

In the current study, non-treated diabetic rats had ~20% weight gain/5 weeks, the values for O_2-treated, nitrate-treated, O_2 + nitrate-treated rats were 1%, ~10%, and ~2%, respectively; findings that were independent of food and calorie intakes. Likely explanations for weight-lowering effects of hyperoxia include improved oxygen delivery to tissues, which by itself increases metabolism [34,35]. Weight is determined by the balance between energy intake and expenditure [36]. It has previously been reported that both nitrate administration [16,37] and hyperoxia causes adipocyte browning [7], which increases energy expenditure, so, it is reasonable that both hyperoxia and nitrate administration results in weight loss. The relation between adipocyte browning and weight loss is however not conclusive and it is currently under debate [38]; therefore, the weight loss effect of hyperoxia observed in this study may also have other causes. In our study, neither hyperoxia nor nitrate had effect on body weight in control rats. In line with these results, we previously reported that weight-lowering effect of nitrate, which is mostly due to decrease in adiposity, is started

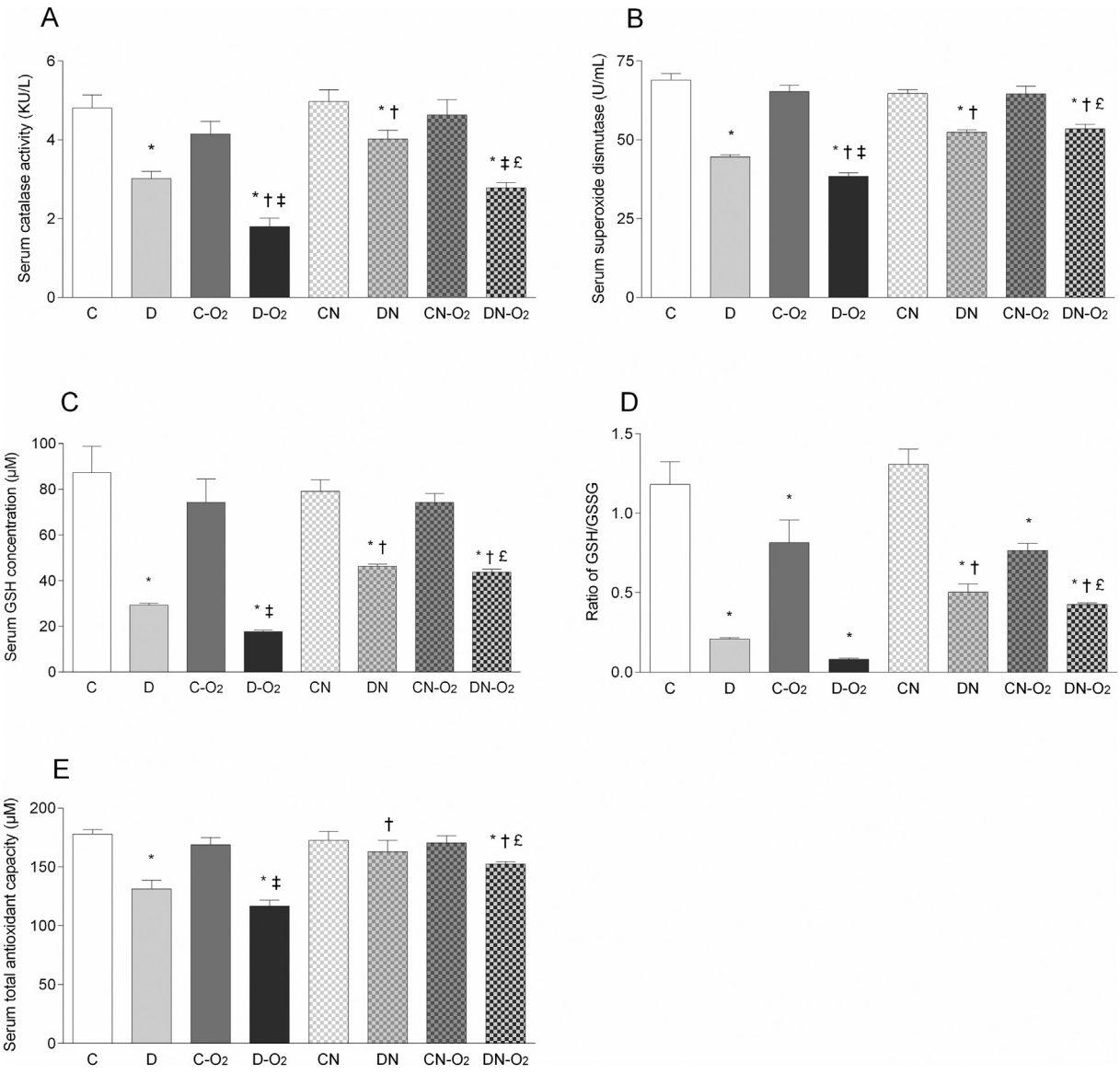

Fig. 6. Effects of hyperoxia and nitrate on serum catalase (A), superoxide dismutase activity (B), reduced glutathione (C), the ratio of GSH/GSSG (D), and total antioxidant capacity (E) levels in control and obese type 2 diabetic rats ($n = 7$/group). The comparisons of all groups were done by one-way ANOVA at the end of the study (day 63). The changes of GSH and ratio of GSH/GSSG in O_2 + nitrate-treated diabetic rats compared to non-treated diabetic rats were marginally significant, $P = 0.092$ and $P = 0.069$, respectively. *, †, ‡, and £ statistically significant difference in comparison to control, diabetes, nitrated-treated diabetes, and O_2-treated diabetes, respectively. C, control; D, diabetes; C-O_2, O_2-treated control; D-O_2, O_2-treated diabetes; CN, nitrate-treated control; DN, nitrate-treated diabetes; CN-O_2, nitrate- and O_2-treated control; DN-O_2, nitrate- and O_2-treated diabetes. Values are mean ± standard error of the mean (SEM).

following 2 months of administration [39].

Despite these beneficial effects, increased oxidative stress limits applicability of hyperoxia for management of type 2 diabetes. In our study, type 2 diabetes in rats increased oxidative stress, a predicted finding that has previously been reported in both animal [40] and human [41] studies. In addition, hyperoxia exacerbated elevated oxidative stress in diabetic rats, again this finding was predictable according to previous reports by ours [7] and others [42]. In addition, NO, produced from NO synthase enzyme [43,44] or nitrate-nitrite-NO pathway [45], can induce mitochondrial biogenesis [46] and has favorable metabolic effects [47]. Our findings provide some evidence that nitrate can decrease hyperoxia-induced oxidative stress in type 2 diabetes.

In the current study, nitrate increased catalase and SOD activities as well as levels of GSH and TAC and also GSH/GSSG ratio in serum of O_2-treated type 2 diabetic rats by 55%, 39%, 146%, 431%, and 31%, respectively. In line with our results, 2 months administration of nitrate (100 mg/L in drinking water) increased serum catalase activity [17,48] and serum TAC level [48] in type 2 diabetic rats. In addition, reports on antioxidant effects of NO-producing agents indicate that 4 weeks administration of L-arginine (300 mg/kg) and sodium nitroprusside (3 mg/kg per day), increase catalase and SOD activities as well as GSH levels in liver and kidney of type 1 diabetic rats [49] and 3 weeks administration of nitrite (50 mg/L drinking water) restores decreased aortic SOD activity in aged mice. These data support our results that indicate nitrate potentiates antioxidant system.

In the current study, nitrate decreased elevated serum TOS and GSSG levels in O_2-treated type 2 diabetic rats by 31% and 40%, respectively. There are few studies on oxidative stress-lowering effects of NO donors. In support of our results, decreased serum malondialdehyde levels has been observed following 2 months administration of nitrate (100 mg/L in drinking water) in type 2 diabetic rats [48] and 4 weeks administration of nitrite (15 mg/kg daily gavage) in hypertensive rats [50]. In addition, nitrate (1.0 mmol/kg/day for 2 weeks) decreases

renal superoxide generation following renal ischemia-reperfusion in mice [18]. These data support that nitrate/nitrite can decrease oxidants. In our study, oxidative stress index (OSI), which was higher in diabetic rats, was exacerbated in O_2-treated ones by 37%. Nitrate administration restored the elevated OSI to normal levels in type 2 diabetic rats and decreased elevated OSI in O_2-treated diabetic rats by 51%. These findings indicate that nitrate attenuates hyperoxia-induced oxidative stress via decreasing oxidants and increasing antioxidants.

Type 2 diabetes is associated with tissue hypoxia [3–5] and decreased NO bioavailability [13,14]; both hyperoxia [7,32,33] and nitrate [20] per se have beneficial metabolic effects in type 2 diabetes. Here we showed that combination therapy with hyperoxia and nitrate has beneficial metabolic effects while precluding increased oxidative stress; these findings may be considered for future management of type 2 diabetes. Oxygen therapy is currently indicated for other diseases including carbon monoxide poisoning, diabetic foot, and myocardial infarction [51].

As a strength, we evaluated in vivo and long-term effects of nitrate against hyperoxia-induced oxidative stress using a HFD-STZ rat model of type 2 diabetes; this model has similar pathophysiology to human type 2 diabetes [28]. As a limitation, our study was only conducted in the male rats and findings cannot be extrapolated to female rats as behavior of both oxidant/antioxidant system [52] and carbohydrate metabolism [53,54] are different between males and females.

5. Conclusion

In conclusion, this study for the first time showed that dietary inorganic nitrate attenuates hyperoxia-induced oxidative stress and that nitrate potentiates serum glucose-lowering effect of hyperoxia.

Author's contribution

R. Norouzirad contributed in design the study, data collection, statistical analysis, and drafting the manuscript. A. Ghasemi contributed in design the study, drafting the manuscript, and final approval for submission. H. Gholami contributed in data collection, statistical analysis, and drafting the manuscript. M. Ghanbari contributed in data collection, statistical analysis, and drafting the manuscript. H. Hedayati contributed in drafting the manuscript, and final revision of the manuscript. P. González-Muniesa critically revised the manuscript and approved final manuscript. S. Jeddi contributed in data collection, statistical analysis, and drafting the manuscript.

Declaration of Competing Interest

The authors declare no competing of interest.

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