Life Sciences 220 (2019) 58–68

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Hyperoxia improves carbohydrate metabolism by browning of white adipocytes in obese type 2 diabetic rats

Reza Norouzirad[a,b], Mahboubeh Ghanbari[a], Zahra Bahadoran[c], Mohammad Amin Abdollahifar[d], Neda Rasouli[e], Asghar Ghasemi[a,*]

[a] Endocrine Physiology Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Dezful University of Medical Sciences, Dezful, Iran
[c] Nutrition and Endocrine Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[d] Department of Anatomical Sciences and Biology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[e] University of Colorado, School of Medicine, Aurora, CO, USA

ARTICLE INFO

Keywords:
Adipose tissue
Browning
Hyperoxia
Weight gain

ABSTRACT

Aims: Type 2 diabetes and obesity are associated with chronic hypoxia, which contributes to adipose tissue dysfunction and development of insulin resistance and metabolic disorders. We assessed long-term effects of hyperoxia on browning of adipocytes and carbohydrate metabolism in a murine model of type 2 diabetes.

Main methods: Male Wistar rats (190–210 g) were divided into 4 groups: Control, O_2-treated control, untreated diabetes, and O_2-treated diabetes. Diabetes was induced using high-fat diet followed by a low-dose of streptozotocin (30 mg/kg). Hyperoxia sessions were included 2-h exposure to 95% oxygen, repeated 6 days/week for 5 weeks. Serum fasting glucose, insulin, lactate, and lipid profile were measured before, during, and after hyperoxia. Glucose and pyruvate tolerance tests, and histological evaluations of interscapular and epididymal fats were done at the end of study.

Key findings: O_2-treated diabetic rats compared to untreated ones, displayed lower weight gain, improved glucose-tolerance, insulin sensitivity, and more favorable lipid profile. In diabetic rats, hyperoxia increased surface area (6.36 ± 0.93 *vs.* $0.86 \pm 0.16\,mm^2$, $P < 0.001$), and volume density (1.53 ± 0.22 *vs.* $0.21 \pm 0.04\,mm^3$, $P < 0.001$) of interscapular adipose tissue; hyperoxia also increased protein levels of uncoupling protein 1 (UCP1), peroxisome proliferator activated receptor gamma (PPAR-γ), and PPAR-γ coactivator 1 alpha (PGC1-α) in interscapular adipose tissue. The numerical density (541.7 ± 7.3 *vs.* $298.1 \pm 11.7\,mm^3$, $P < 0.001$) of epididymal fat were also higher.

Significance: This study showed that beneficial metabolic effects of hyperoxia in obese type 2 diabetic rats including improved insulin sensitivity and glucose tolerance are at least in part due to browning of adipose tissue.

1. Introduction

Type 2 diabetes, one of the major health challenges in the 21st century [1], is characterized by insulin resistance and β-cell dysfunction [2]. Both type 2 diabetes and obesity are associated with adipose tissue hypoxia [3–5]. Adipose tissue hypoxia in obesity is due to hypertrophy of adipocytes [4] and decreased microvascular density that decreases blood flow [6–8]. In addition, hypoxia uncouples oxidative phosphorylation in mitochondrion and intensifies tissue hypoxia [9].

Adipose tissue hypoxia causes adipocyte dysfunction [5,10,11], by inducing lipolysis and increasing circulating free fatty acids [10] leading to ectopic fat deposition in liver, skeletal muscle, and pancreas [10,11]. Adipose tissue hypoxia impairs glucose and lipid homeostasis [12,13] and causes insulin resistance [4,14]. In addition, intracellular hypoxia may be involved in β-cell dysfunction in glucotoxicity [15].

Despite controversies around the topic, hyperoxia has been proposed as a potential treatment for type 2 diabetes [11]. Hyperoxia restores reduced gene expression of insulin caused by glucotoxicity in INS β-cells [15] and improves insulin sensitivity in patients with type 2 diabetes [16]. In addition, hyperbaric oxygen therapy (HBOT) in patients with type 2 diabetes improves glycemic control by decreasing fasting glucose [17,18], HbA1c [18], and increasing insulin sensitivity [18,19].

The mechanisms underlying beneficial metabolic effects of

* Corresponding author at: Endocrine Physiology Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, No. 24, Parvaneh Street, Velenjak, Tehran, Iran.
E-mail address: Ghasemi@endocrine.ac.ir (A. Ghasemi).

https://doi.org/10.1016/j.lfs.2019.01.045
Received 21 November 2018; Received in revised form 20 January 2019; Accepted 27 January 2019
Available online 28 January 2019

hyperoxia in type 2 diabetes have not been fully addressed. Brown adipose tissue (BAT) has been proposed as a main target for treating type 2 diabetes and obesity in human [20]. BAT has anti-type 2 diabetic effects by decreasing insulin resistance and increasing insulin secretion [21]. The aim of this study was to determine whether hyperoxia improves carbohydrate metabolism in murine model of type 2 diabetes by browning of white adipocytes. The levels of proteins involved in adipocyte browning including uncoupling protein 1 (UCP1), peroxisome proliferator activated receptor gamma (PPAR-γ), and PPAR-γ coactivator 1 alpha (PGC1-α) [22] were also determined.

2. Materials and methods

2.1. Animals

Male Wistar rats (n = 28), weight range 190–210 g, were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature (22 ± 2 °C) and humidity (50 ± 5%) with a 12:12 h light/dark cycle (lights on: 07:00–19:00). All rats were fed with commercially available normal pellet diet (Pars Animal Feed Co.) and water *ad libitum*, prior to the dietary manipulation. Food intake (g/day/rat), water consumption (mL/day/rat), and body weight (using Tefal Scale, sensitivity 1 g) were recorded every week.

Sex is a confounder in animal studies and restriction (studying males and females separately) is an appropriate approach for controlling confounding effect of sex [23]. Therefore, in this study we selected male rats instead of female ones. In addition, circulating levels of some metabolic parameters including insulin, lipid, and glucose are changed during estrous cycle in female rats [24–26].

All performed procedures in this study were in accordance to the animal care protocols of our Institute; in addition, 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.1396.398).

2.1.1. Groups

Rats were randomly divided into four groups (n = 7/group): Control, O_2-treated control, untreated diabetes, and O_2-treated diabetes. Rats had free access to water and food even when received hyperoxia. The control rats (control and O_2-treated control rats) were given standard regular chow (total caloric value of ~3160 kcal/kg), and the rats in diabetic groups (untreated and O_2-treated diabetic groups) were given high fat diet (HFD) (total caloric value of ~4900 kcal/kg) [27]. Details of HFD ingredients were described elsewhere [27].

2.1.2. Induction of type 2 diabetes

As shown in Fig. 1, after 21 days of dietary manipulation, rats were injected intraperitoneally with a single low-dose of STZ (30 mg/kg, dissolved in 0.1 mM citrate buffer, pH 4.5) (Sigma Aldrich, Hamburg, Germany). HFD continued until the end of the study. We used HFD-STZ model of type 2 diabetes in which two weeks after HFD consumption, insulin resistance is developed and the injection of low-dose of STZ causes partial β-cell dysfunction; this model has similar characteristics of human type 2 diabetes [27] and is appropriate for evaluating effects of interventions on carbohydrate metabolism [28].

One week after STZ injection, fasting serum glucose level was measured and the rats with fasting serum glucose levels > 150 mg/dL were considered to have diabetes.

2.2. Exposure to hyperoxia

Rats in hyperoxia groups were exposed to 95% oxygen (near to normobaric atmosphere) in a hypoxia/hyperoxia chamber (SD-EOS) for 2 h/day (10.00–12.00 AM), all days except Fridays, for 5 weeks (Fig. 1). In this study, 95% oxygen was administrated as it has been reported that doses of oxygen of 50% and 60% have no effect on body weight and enzymes related to glucose metabolism [29,30]. Rats had free access to food and water in the chamber; they were at own cages in the chamber to avoid any stressful condition; Rats in control groups were kept in the animal room under normoxia conditions. Conditions in animal room and the chamber was similar in case of temperature, light/dark cycle, and humidity.

2.2.1. Hypoxia and hyperoxia chamber, SD-EOS

To create hyperoxia condition, SD-EOS equipment (Iran Patent No. 89679 dated 22.08.2016; Inventors, Ghasemi Asghar, Norouzirad Reza, and Fallahi Farshad) was used. Briefly, the SD-EOS controls the oxygen, and CO_2 levels in the chamber to create hypoxia/hyperoxia condition (0.1 to 99.9% oxygen). The device monitors temperature, humidity, pressure, CO_2, and oxygen levels of the chamber, every 30 s. Furthermore, it automatically simulates light/dark cycle. The chamber has space for two large-size rat cages and provides *ad libitum* access to food and water. The chamber uses oxygen or nitrogen gases to create hyperoxia or hypoxia conditions, respectively. An internal fan provides uniform gas distribution required for rapid sensors' responses and to avoid gas trapping.

2.3. Biochemical measurements

After 12–14 h fasting, blood samples were taken from tail vein and after blood clot formation, centrifuged at 5000 ×g for 10 min. Fasting serum levels of glucose, lactate, triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL−C) were measured using commercial kits (Pars Azmoon, Tehran, Iran) on days 0, 21, 28 and 42, and at the end of the study. The intra- and inter-assay coefficients of variation (CVs) for glucose, lactate, TG, TC, LDL-C, and HDL-C measurement were 1.2%, 1.5%, 2.3%, 3.0%, 3.4%, and 1.0% and 2.8%, 2.2%, 3.6%, 2.1%, 3.1%, and 1.1%, respectively.

Intraperitoneal glucose and pyruvate tolerance tests (IP-GTT and IP-PTT), as described in our previous study [27] were performed during the last week. Insulin was measured using rat insulin ELISA kit

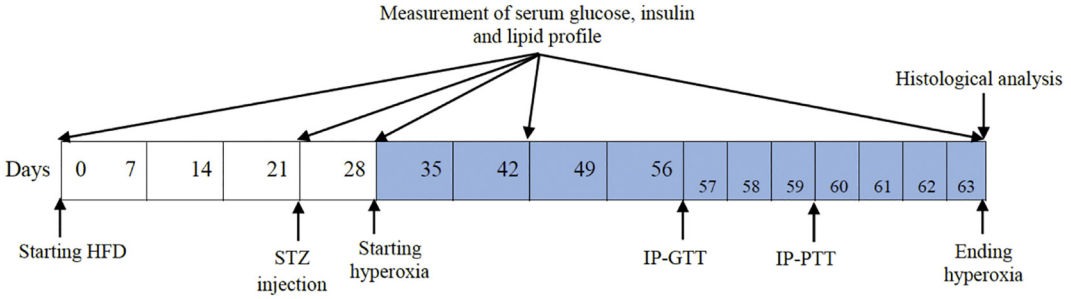


Fig. 1. Study protocol. HFD, high-fat diet; STZ, streptozotocin; IP-GTT, intraperitoneal glucose tolerance test; and IP-PTT, intraperitoneal pyruvate tolerance test.

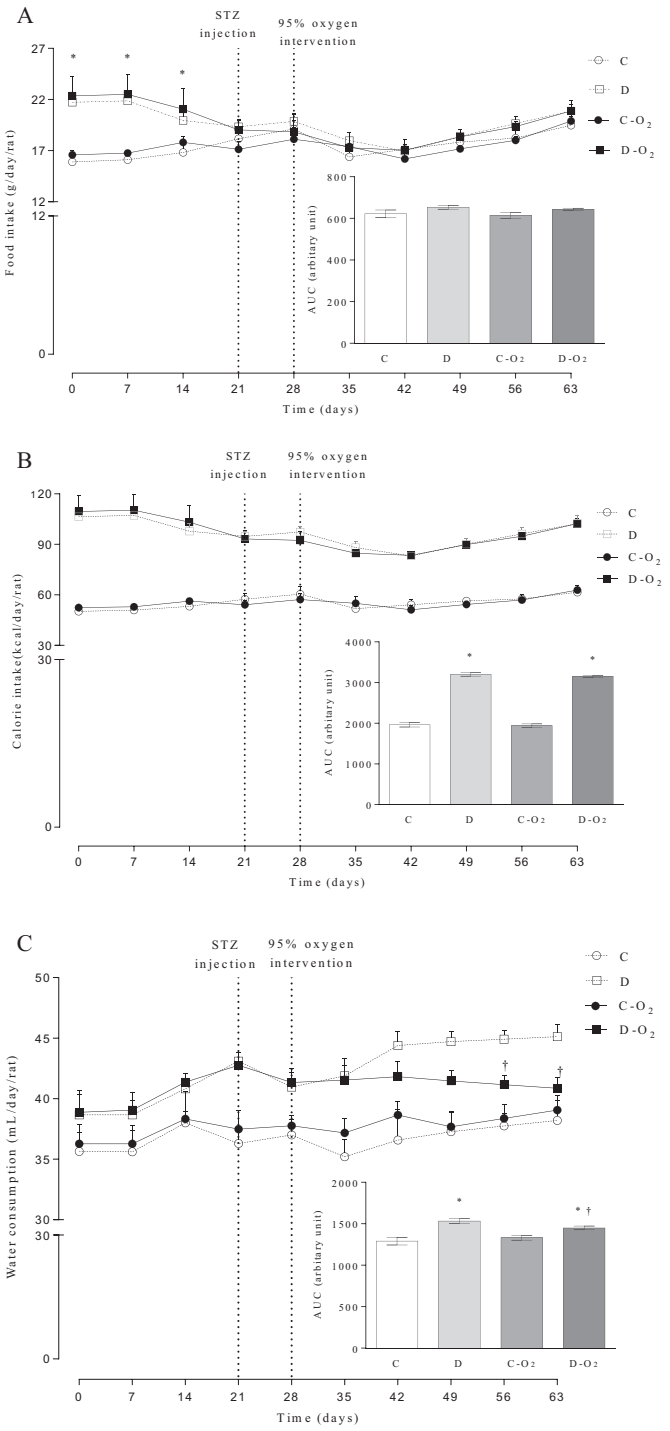


Fig. 2. Effects of hyperoxia on food intake (A), calorie intake (B), and water consumption (C) in control and diabetic rats. Insets show area under the curves (AUC), calculated during the intervention from day 28 to day 63. The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. † Marginally significant ($P = 0.088$) difference compared to untreated diabetic group. C, control; D, untreated diabetes; C—O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

(Mercodia, Uppsala, Sweden), the kit sensitivity was 1 μU/mL (6 pmol/L) and our intra- and inter-assay CVs were 8.0% and 9.7%, respectively.

Concentrations of UCP1, PPAR-γ, and PGC1-α in interscapular adipose tissue were measured at the end of the study using rat ELISA kits (ZellBio Gmbh, Germany); the kits' sensitivity for UCP1, PPAR-γ, and PGC1-α were 0.05, 0.062, and 0.05 ng/mL, respectively and all

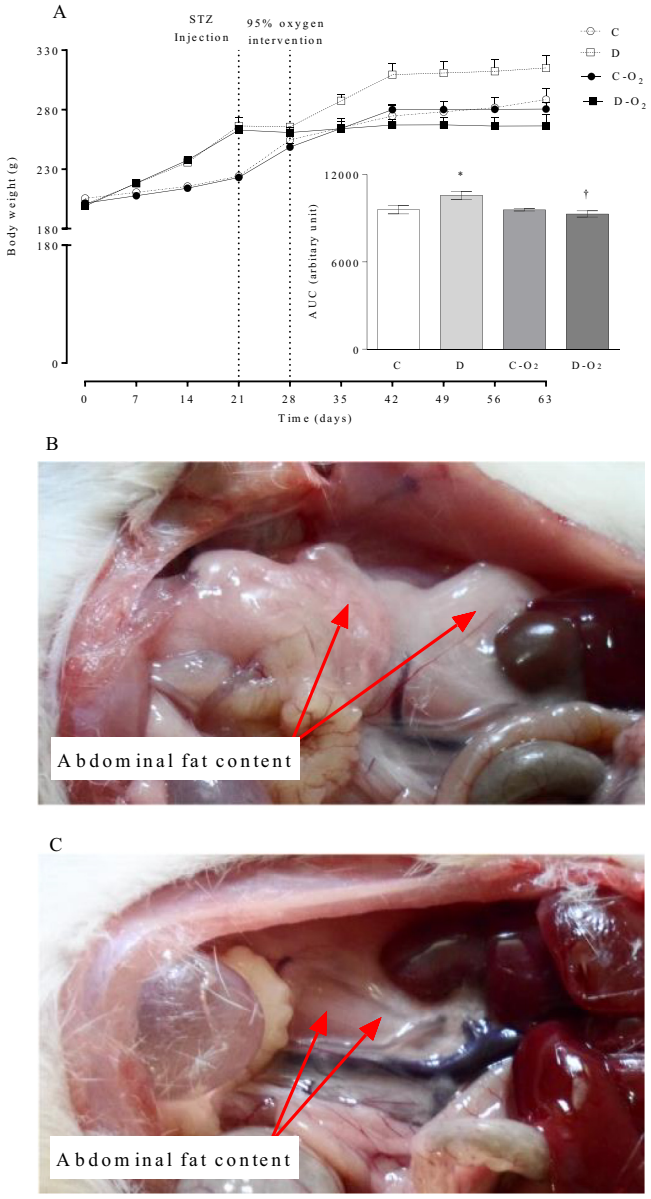


Fig. 3. Effect of hyperoxia on body weight changes of control and diabetic rats (A), and body fat deposition in untreated diabetic rats (B) and O_2-treated diabetic rats (C). Inset shows area under the curves, calculated during the intervention from day 28 to day 63. The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. C, control; D, untreated diabetes; C—O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

intra-assay CVs were < 10%.

Serum levels of total oxidant status (TOS) and total antioxidant capacity (TAC) were measured at the end of the study using commercial assay kits (ZellBio Gmbh, Germany); the kits' sensitivity for TOS and TAC were 0.5 μM and 100 μM, respectively and intra-assay CVs were < 5%.

2.4. Calculation of insulin sensitivity/resistance indices and atherogenic index

Indices of insulin sensitivity/resistance including homeostasis model assessment-insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) were calculated using the

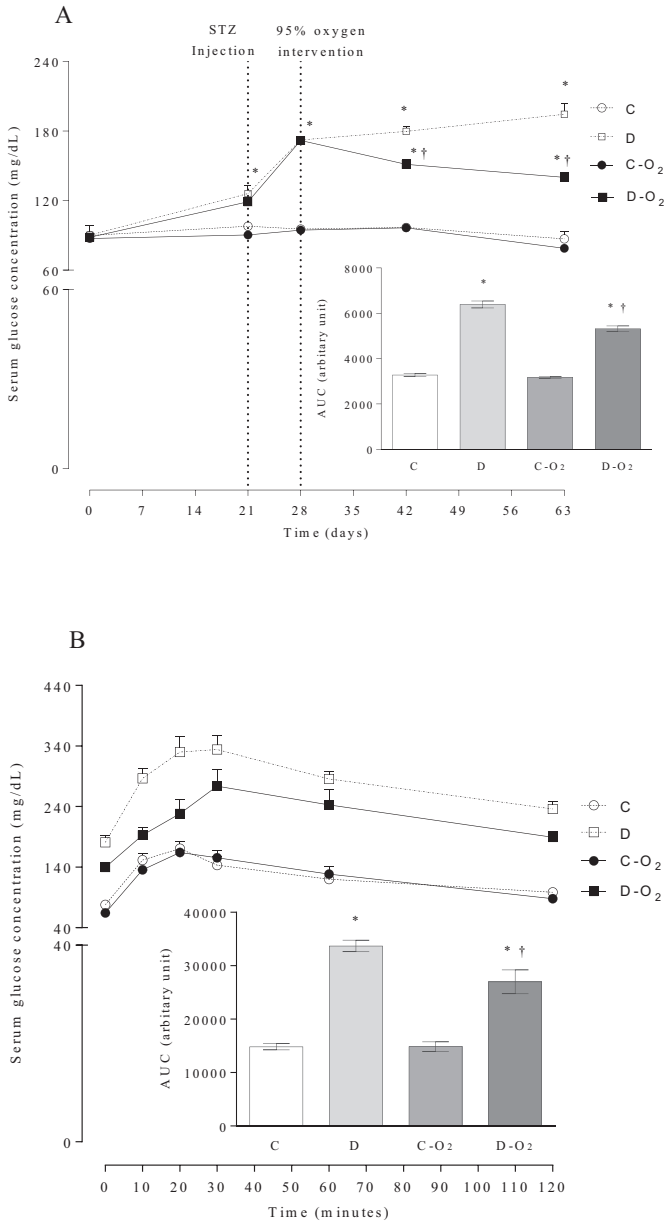


Fig. 4. Effect of hyperoxia on fasting serum glucose (A) and glucose tolerance (B) in control and diabetic rats. Insets show area under the curves (AUC) calculated during the intervention from day 28 to day 63 for fasting glucose and from 0 to 120 min for glucose tolerance). The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. C, control; D, untreated diabetes; C—O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

following formulae: HOMA1-IR (original HOMA model) = [fasting insulin (μU/mL) × fasting glucose (mmol/L)]/22.5, and QUICKI = 1/[log fasting insulin (μU/mL) + log fasting glucose (mg/dL)]. The HOMA2-IR was calculated by the online HOMA2 calculator, which is available from http://www.dtu.ox.ac.uk/homacalculator [31]. Atherogenic index was calculated as the logarithm of the ratio of serum TG to serum HDL-C [32].

2.5. Histological analyses

Histological analyses were done at the end of the study; interscapular and epididymal adipose tissues were removed, under deep anesthetic state (intraperitoneal injection of 60 mg/kg sodium pentobarbital), and fixed in 10% formalin for at least 48 h.

2.5.1. Tissue preparation

The formalin-fixed tissues were decalcified in 10% nitric acid, embedded in paraffin blocks, and cut longitudinally into 10 μm thick sections by a microtome (Leica, Rotary Microtome, Germany); then, they were prepared for microscopic descriptive analysis using hematoxylin and eosin (H&E) staining. The magnifying objective "×4 and ×40" was used to analyze the stained sections.

2.5.2. Stereological study

2.5.2.1. Surface area of interscapular adipose tissue. The surface area (ΣA) of brown adipocytes in interscapular adipose tissue (brown-iAT) was measured by the method of Cavalieri, using a light microscope (Olympus-VANOX, model-AHBS) connected to a camera, as described previously [33,34]. The ΣA was calculated by the formula: $\sum A = \sum P \times \left(\frac{a}{p}\right)$, where "ΣP" is total points hitting the adipocytes and was calculated with the point-counting method, and "$\frac{a}{p}$" is the area of each point projected on brown adipose tissue.

2.5.2.2. Volume density of interscapular adipose tissue. The volume density of brown-iAT and white adipocytes in interscapular adipose tissue (white-iAT) were calculated using the result of ΣA and measured tissue thickness between the saved sections using the formula: $V_{total} = \Sigma A \times t$, where "Σ*A*" is the surface area of brown-iAT, and "t" is distance between sections [33].

2.5.2.3. Numerical density of adipocytes. The number of the adipocytes in epididymal white adipose tissue (eWAT) and white-iAT were measured by optical dissector method [35,36]. Numerical density of adipocytes (Nv) was calculated by following formula [34]:

$$Nv = \left[\frac{\sum Q^{-}}{\sum P \times \frac{a}{f} \times h} \times \frac{t}{BA}\right]$$

where "ΣQ^{-}" is the number of the nuclei, "ΣP" is the total number of unbiased counting frame in all fields, "a/f" is the area of frame, "h" is dissector height, "t" is the real section thickness, and "BA" is the block advance of the microtome (set at 10 μm).

2.6. Statistical analysis

GraphPad Prism software, version 6, was used to perform analyses. The values are reported as mean ± SEM. Two-way mixed (between-within) analysis of variance (ANOVA) followed by Fisher post-hoc test was used for analyzing data of body weight, food and calorie intake, water consumption, glucose, lactate, TG, TC, LDL-C, HDL—C, IP-GTT, IP-PTT, insulin, HOMA-IR, and QUICKI. One-way ANOVA was used for analyzing tissue levels of UCP1, PPAR-γ and PGC1-α proteins, serum TOS and TAC concentrations, histological data, and area under the curves (AUC) of all parameters. AUC was calculated during the intervention from day 28 to day 63. Two-sided *P*-values < 0.05 were considered statistically significant.

3. Results

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

In the first three weeks of the study, food intake was higher in rats on HFD compared to rats who received normal diet; thereafter, there was no significant difference in food intake among groups (Fig. 2A). Total calorie intake and water consumption were significantly higher in diabetic rats compared to controls (Fig. 2B and C). Water consumption was lower in O_2-treated diabetic rats compared to untreated ones; a

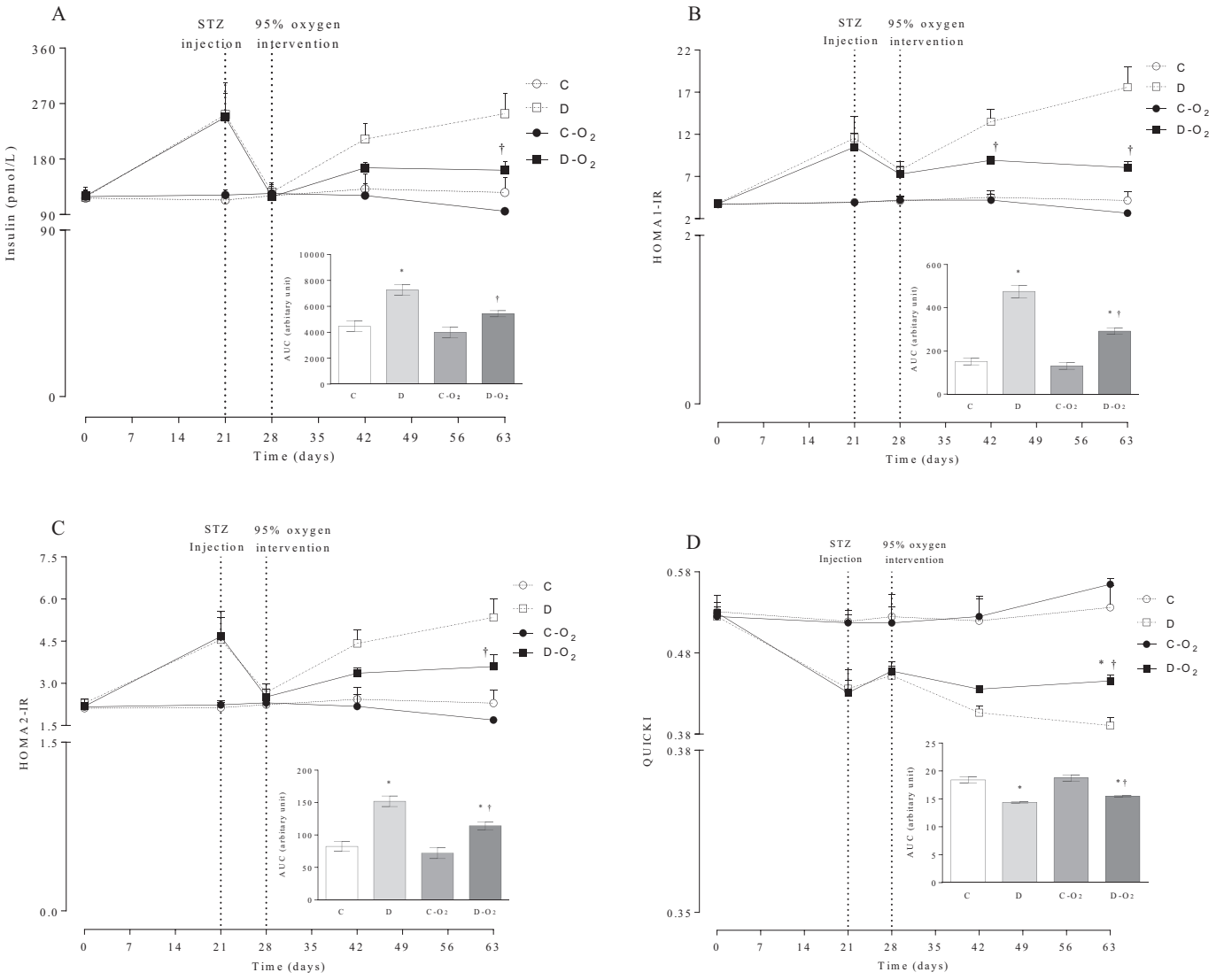


Fig. 5. Effect of hyperoxia on serum levels of insulin (A), homeostasis model assessment-insulin resistance (HOMA1-IR) (B), HOMA2-IR (C), and quantitative insulin sensitivity check index (QUICKI) (D) in control and diabetic rats. Insets show area under the curves (AUC), calculated during the intervention from day 28 to day 63. The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. The AUC changes of QUICKI in O_2-treated compared to untreated diabetic rats were marginally significant, $P = 0.060$. C, control; D, untreated diabetes; C−O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

change that was marginally significant (AUC 1450 ± 23 *vs.* 1533 ± 28, $P = 0.088$) (Fig. 2C). Hyperoxia had no significant effect on food and calorie intakes, and water consumption in control rats.

3.2. Effect of hyperoxia on body weight

Body weight of all groups was comparable at baseline (Fig. 3A). Weight gain of diabetic rats was significantly higher than controls. O_2-treated diabetic rats had lower weight gain (1.3% *vs.* 18.7%) compared to untreated ones (AUC 9297 ± 213 *vs.* 10,573 ± 286, $P < 0.001$). Hyperoxia had no effect on weight gain in control rats. In addition, all O_2-treated diabetic rats had obviously lower abdominal fat content compared to untreated ones (Fig. 3B and C).

3.3. Effect of hyperoxia on carbohydrate metabolism

3.3.1. Serum glucose

Serum glucose levels were comparable between groups at baseline but were significantly higher in HFD-treated rats compared to controls, on day 21 (Fig. 4A). On day 28, one week after STZ injection, serum glucose concentration was significantly ($P < 0.001$) higher in diabetic groups compared to controls. Hyperoxia significantly decreased serum glucose in O_2-treated compared to untreated diabetic rats (AUC 5320 ± 123 *vs.* 6391 ± 152, $P < 0.001$) (Fig. 4A). Serum glucose was not affected by hyperoxia in control rats.

3.3.2. Glucose tolerance

As shown in Fig. 4B, hyperoxia improved glucose tolerance, measured by IP-GTT, in diabetic rats compared to untreated ones (AUC 26982 ± 2226 *vs.* 33,688 ± 1081, $P = 0.002$). Compared to untreated diabetic rats, O_2-treated ones had lower glucose values by 32.6%, 30.7%, and 18.2% at 10, 20, and 30 min during GTT, respectively. After peak of glucose concentration, O_2-treated diabetic rats had lower glucose levels (15.0% and 19.7% at 60 and 120 min), compared to untreated diabetic rats. Hyperoxia had no effect on glucose tolerance in control rats.

3.3.3. Serum insulin and insulin resistance/sensitivity indices

As shown in Fig. 5A, after 5 weeks exposure to hyperoxia, serum insulin levels were significantly lower in O_2-treated diabetic rats

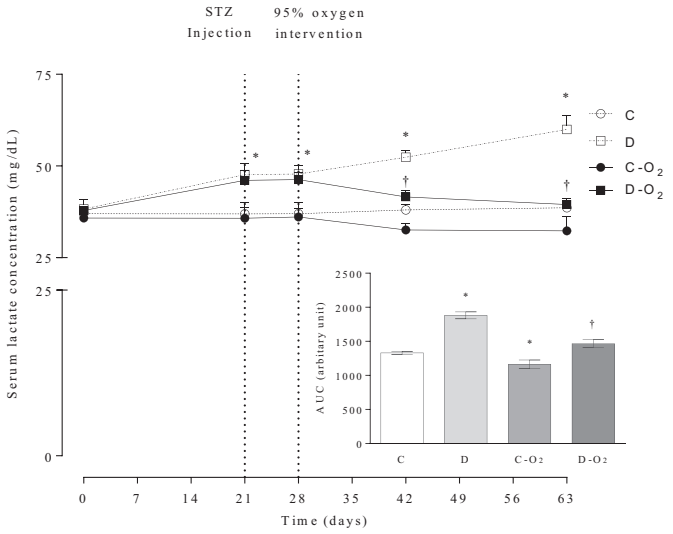


Fig. 6. Effect of hyperoxia on serum lactate levels in control and diabetic rats. Inset shows area under the curves (AUC), calculated during the intervention from day 28 to day 63. The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. C, control; D, untreated diabetes; C−O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

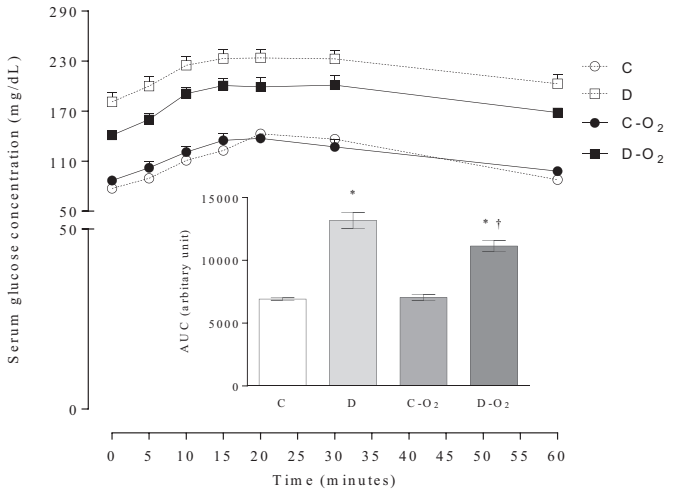


Fig. 7. Effect of hyperoxia on gluconeogenesis as assessed by serum glucose levels during intraperitoneal pyruvate tolerance test (IP-PTT) in control and diabetic rats. Inset shows area under the curves (AUC), calculated during the IP-PTT from 0 to 60 min. The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. C, control; D, untreated diabetes; C−O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

compared to untreated ones (AUC 5436 ± 238 *vs.* 7269 ± 415, $P = 0.002$). Hyperoxia had no effect on serum insulin concentrations in control rats.

At the end of the study, both HOMA1-IR and HOMA2-IR were significantly lower in *O_2-treated diabetic rats compared to untreated ones* (AUC 292 ± 15 *vs.* 476 ± 29, $P < 0.001$ for HOMA1-IR, Fig. 5B; and, AUC 114 ± 6 *vs.* 152 ± 8, $P = 0.002$ for HOMA2-IR; Fig. 5C). At the end of study, QUICKI was also significantly higher in O_2-treated diabetic rats compared to untreated ones (0.446 ± 0.008 *vs.* 0.391 ± 0.010, $P = 0.027$); in addition, hyperoxia increased QUICKI in diabetic rats during intervention; a change that was marginally significant (AUC 16 ± 0.2 *vs.* 14 ± 0.1, $P = 0.060$) (Fig. 5D). Hyperoxia had no effect on insulin resistance/sensitivity indices in control rats.

3.3.4. Serum lactate

As seen in Fig. 6, after 21 days on HFD, serum lactate concentration was significantly higher in diabetic compared to control rats. Hyperoxia significantly decreased elevated serum lactate concentration in diabetic rats compared to untreated ones (AUC 1466 ± 57 *vs.* 1881 ± 51, $P < 0.001$). Hyperoxia also decreased lactate levels in control rats.

3.3.5. Gluconeogenesis

Using intraperitoneal pyruvate tolerance test (IP-PTT), we investigated whether hyperoxia affects gluconeogenesis in diabetic rats. Injection of gluconeogenic substrate precursor pyruvate, was accompanied with lower glucose concentrations in O_2-treated compared to untreated diabetic rats (AUC 11148 ± 439 *vs.* 13186 ± 624, $P = 0.001$). Hyperoxia had no effect on gluconeogenesis in control rats (Fig. 7).

3.4. Effect of hyperoxia on browning of adipose tissue

3.4.1. Histological evaluation of interscapular and epididymal adipose tissues

As shown in Fig. 8A, diabetic rats had lower brown-iAT surface area. Hyperoxia increased the surface area of brown-iAT in O_2-treated diabetic rats compared to untreated ones. The surface area of brown-iAT in O_2-treated diabetic rats were significantly higher (739%) than untreated diabetic rats (6.36 ± 0.93 *vs.* 0.86 ± 0.16 mm^2, $P < 0.001$). The H&E staining images of brown-iAT in the four groups showed lower browning of interscapular adipose tissue (iAT) in untreated rats, while O_2-treated ones showed browning of iAT (Fig. 8A right).

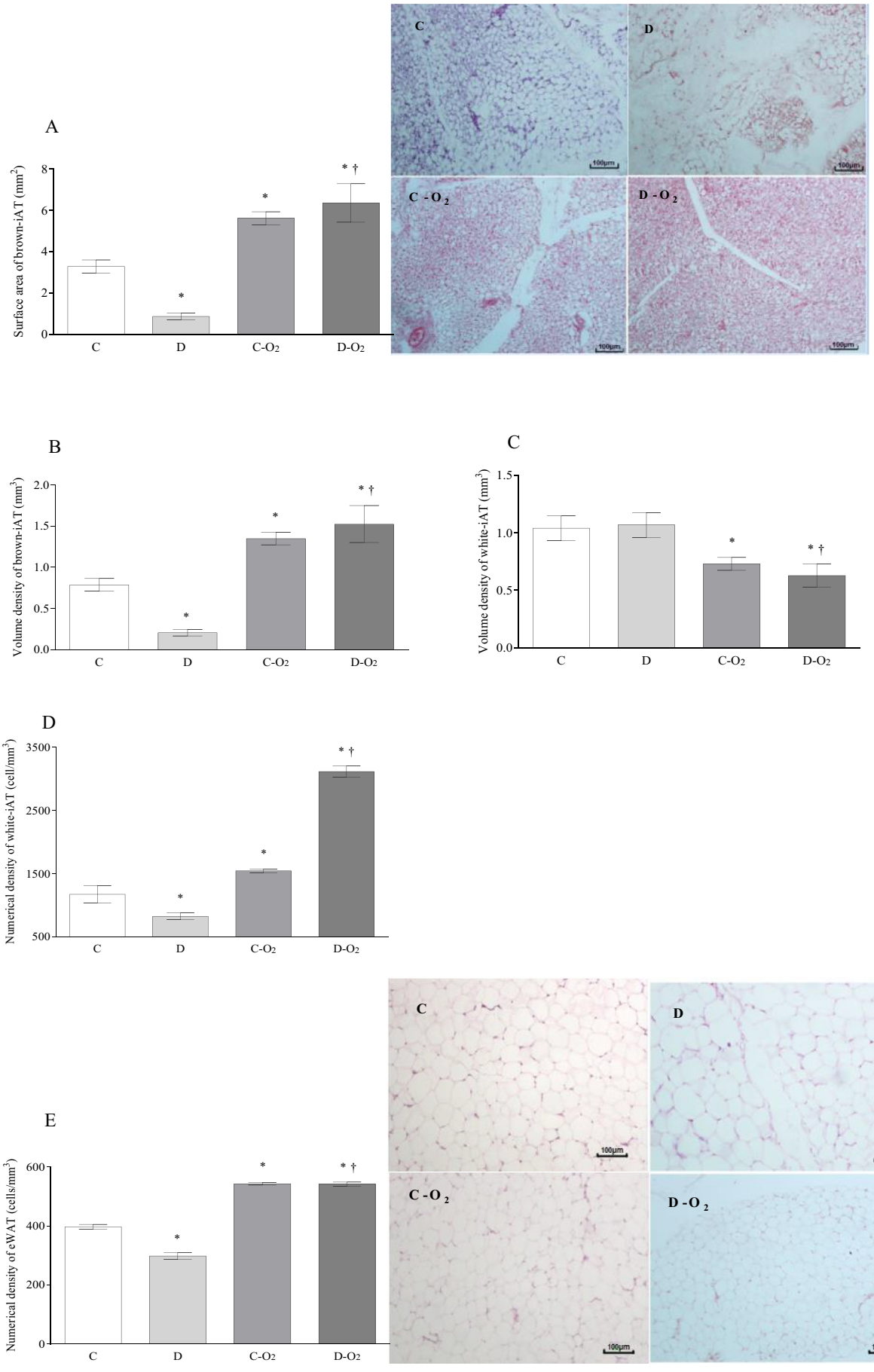
The volume density of brown-iAT in diabetic rats were lower compared to controls. Hyperoxia increased the volume density of brown-iAT in O_2-treated diabetic rats compared to untreated diabetic rats (1.53 ± 0.22 *vs.* 0.21 ± 0.04 mm^3, $P < 0.001$) (Fig. 8B). Hyperoxia intervention increased the number of adipocytes, as measured by numerical density, in O_2-treated diabetic rats compared to untreated ones in both white-iAT (3116 ± 89 *vs.* 827 ± 54 cell/mm^3, $P < 0.001$) and eWAT (542 ± 7 *vs.* 298 ± 12 cell/mm^3, $P < 0.001$) (Fig. 8C, D, and E). The H&E staining images of eWAT in the four groups showed lower adipocyte size in O_2-treated control and diabetic rats compared to untreated ones (Fig. 8E right). Hyperoxia also caused browning of adipose tissue in control rats.

3.4.2. Protein levels of UCP1, PPAR-γ, and PGC1-α in interscapular adipose tissue

Concentrations of UCP1 protein in interscapular adipose tissue were not different between diabetic and control rats. Hyperoxia significantly increased UCP1 levels in both control and diabetic rats (Fig. 9A). Diabetic rats had significantly lower levels of PPAR-γ protein in interscapular adipose tissue compared to controls. Hyperoxia increased the tissue levels of PPAR-γ in diabetic but not control rats (Fig. 9B). Levels of PGC1-α protein in interscapular adipose tissue were comparable between control and diabetic rats. Hyperoxia significantly increased PGC1-α levels in diabetic but not control rats (Fig. 9C).

3.5. Effect of hyperoxia on serum lipid profile

As shown in Fig. 10A–E, serum concentrations of TG, TC, and LDL-C were significantly higher and HDL-C was significantly lower in untreated diabetic rats compared to controls. Compared to untreated diabetic rats, O_2-treated ones had lower serum concentrations of TG (128 ± 3 *vs.* 175 ± 15 mg/dL, $P < 0.001$), TC (162 ± 6 *vs.* 188 ± 11 mg/dL, $P < 0.001$) and LDL-C (60 ± 3 *vs.* 81 ± 3 mg/dL, $P < 0.001$) at the end of study. Hyperoxia also restored decreased serum HDL-C concentrations to near normal values in diabetic rats. Lipid profile in control rats was not affected by hyperoxia. Atherogenic index was higher in untreated diabetic rats compared to control and hyperoxia significantly decreased the index in diabetic rats compared to



(caption on next page)

Fig. 8. Effect of hyperoxia on surface area of brown adipocytes in interscapular adipose tissue (brown-iAT) (selected H&E staining images of brown-iAT in 4 groups are shown on right) (A), volume density of brown-iAT (B), volume density of white adipocytes in interscapular adipose tissue (white-iAT) (C), the numerical density of white-iAT (D), and numerical density of epididymal white adipose tissue (eWAT) (selected H&E staining images of eWAT in 4 groups are shown on right) (E) in control and diabetic rats. The values are mean ± SEM. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. C, control; D, untreated diabetes; C−O_2, O_2-treated control; D-O_2, O_2-treated diabetes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

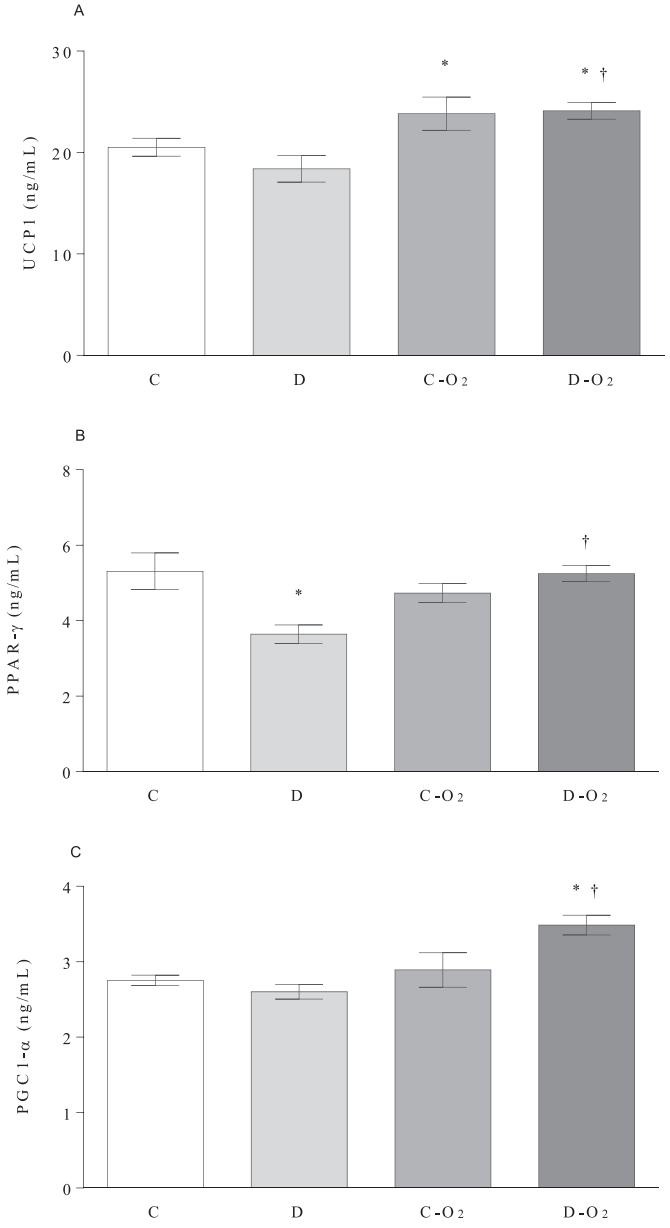


Fig. 9. Effect of hyperoxia on levels of uncoupling protein 1 (UCP1) (A), peroxisome proliferator-activated receptor gamma (PPAR-γ) (B), and PPAR-γ coactivator 1 alpha (PGC1-α) (C) in interscapular adipose tissue in control and diabetic rats. The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. The changes of UCP1 in O_2-treated control rats compared to control were marginally significant, $P = 0.065$. C, control; D, untreated diabetes; C−O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

controls (AUC 20.1 ± 0.5 *vs.* 23.5 ± 1.1, $P = 0.021$).

3.6. Effect of hyperoxia on indices of oxidative stress

At the end of the study, diabetic rats compared to controls had significantly higher levels of TOS and lower levels of TAC that indicates increased oxidative stress. In diabetic rats, hyperoxia aggravated oxidative stress by increasing TOS and decreasing TAC, although the changes of TAC were marginally significant ($P = 0.088$). Hyperoxia has no effect on these indices in control rats (Fig. 11A and B).

4. Discussion

This study for the first time showed that five weeks exposure to hyperoxia resulted in browning of interscapular adipose tissue and decreased adipocyte size in epididymal adipose tissue of diabetic rats. In addition, O_2-treated diabetic rats had lower serum glucose, improved glucose tolerance and insulin sensitivity as well as lower weight gain.

In the current study, untreated diabetic rats had insulin resistance, and glucose intolerance; hyperoxia improved insulin sensitivity and glucose tolerance and decreased gluconeogenesis in diabetic rats. In addition, untreated diabetic rats had ~20% weight gain/5 weeks, while in the same period, O_2-treated ones had only ~1% weight gain, independent of food and calorie intake. The improvement of glucose tolerance and insulin sensitivity related to weight loss has been previously reported [37,38]. In line with our results, HBOT improves insulin sensitivity in obese men with and without type 2 diabetes [39] and decreases weight gain in monosodium glutamate (MSG)-treated mice [40]. Possible explanations for improved insulin sensitivity following weight loss include decreased inflammation [41] and adiposity [42] as well as decreased circulating free fatty acid levels [43], and increased circulating adiponectin concentrations [44].

Improvement of systemic metabolism by transfer sufficient oxygen to tissues is a suggested mechanism by which hyperoxia reduces body weight [19,40]. O_2-treated diabetic rats had obviously lower abdominal fat compared to untreated ones (Fig. 3B and C), which provide a mechanism for body weight lowering effect of hyperoxia. In addition, browning of WAT may be another mechanism as we observed in this study (see later). Visceral fat is inversely correlated with supraclavicular BAT in healthy human. [45]. Body weight lowering effect of hyperoxia could explain the improved glucose tolerance and insulin sensitivity following exposure to oxygen. Even modest weight loss of 5–10%, has protective effects against cardiovascular risk in overweight and obese patients with type 2 diabetes [46]. Hyperoxia-induced weight loss may therefore be promising for management of obesity and diabetes. Wilkinson et al. have reported 40% improvement in peripheral insulin sensitivity (assessed by hyperinsulinaemic clamp) following HBOT (2.0 absolute atmospheres for 2 h, six sessions per week for 5 weeks) in patients with type 2 diabetes [19]. The authors themselves interpret that such improvement is similar in magnitude to that observed following 12% body weight loss after one year lifestyle intervention in type 2 diabetic men [47].

In this study, hyperoxia restored elevated serum lactate levels in diabetic rats. Increased lactate levels in obese subjects with type 2 diabetes [48] contributes to insulin resistance and diabetes development [49]. It has been shown that large adipocytes from obese diabetic rats compared to cells from obese control ones have 60–65% higher lactate production [50]. Lactate production is increased in insulin

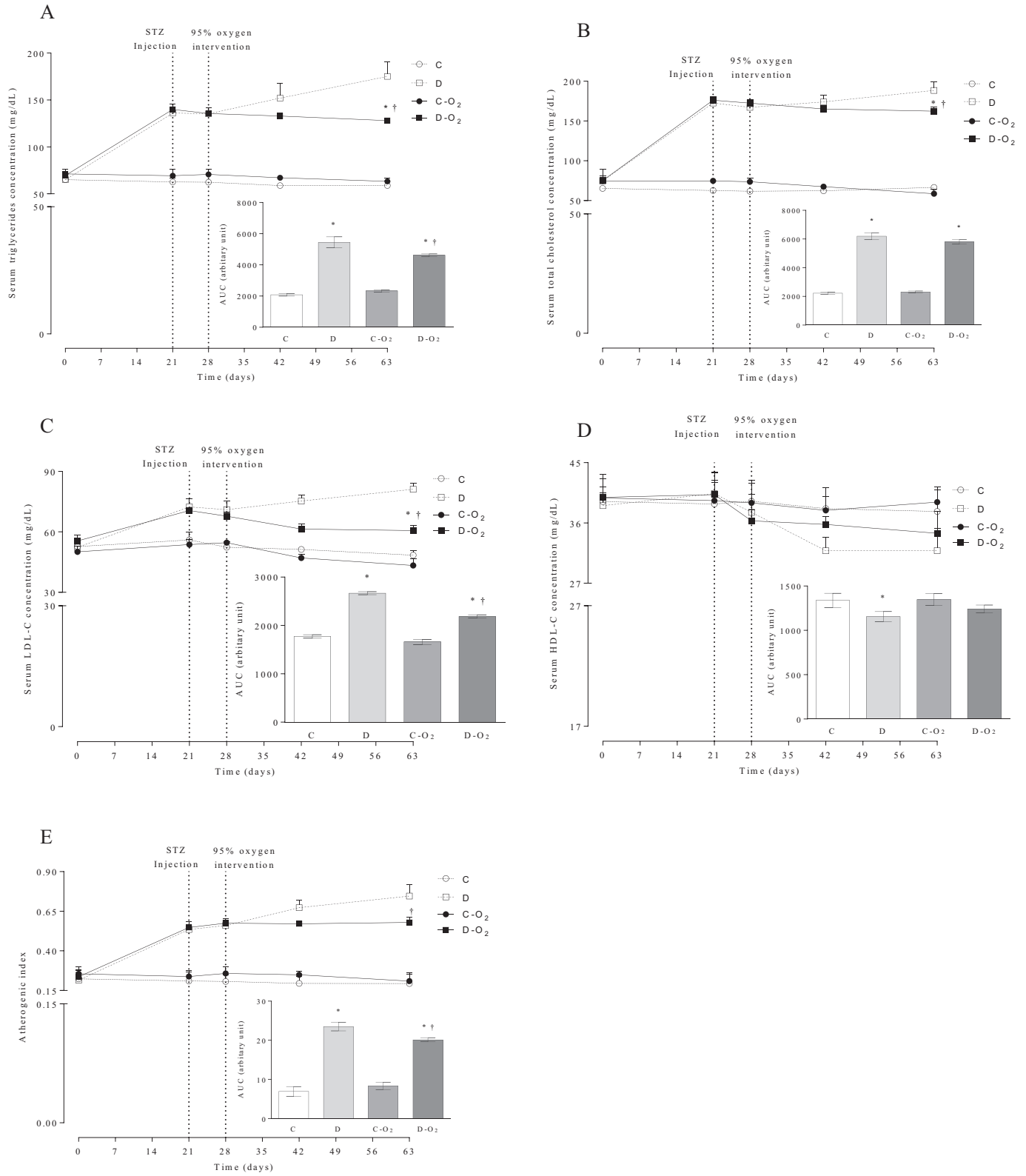


Fig. 10. Effects of hyperoxia on serum triglycerides (A), total cholesterol (B), low-density lipoprotein cholesterol (LDL-C) (C), high-density lipoprotein cholesterol (HDL−C) (D) and atherogenic index value (Log TG/HDL-C) (E) levels in control and diabetic rats. Insets show area under the curves (AUC), calculated during the intervention from day 28 to day 63. The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. C, control; D, untreated diabetes; C−O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

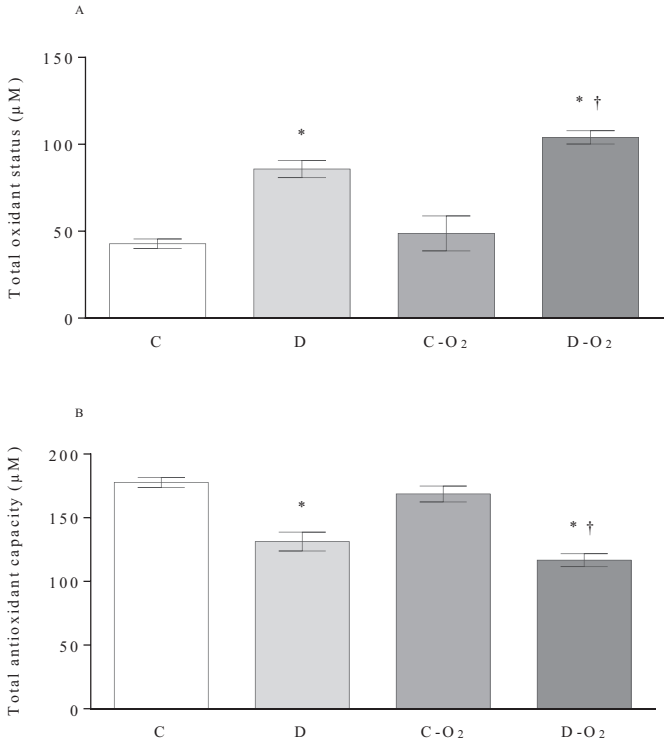


Fig. 11. Effects of hyperoxia on serum total oxidant status (A), and total antioxidant capacity (B) levels in control and diabetic rats at the end of the study. The values are mean ± SEM for 7 rats in each group. *Statistically significant difference compared to control group. †Statistically significant difference compared to untreated diabetic group. The changes of TAC in O_2-treated compared to untreated diabetic rats were marginally significant, $P = 0.088$. C, control; D, untreated diabetes; C−O_2, O_2-treated control; D-O_2, O_2-treated diabetes.

resistance due to stimulation of glycolysis [51]. Hyperoxia improves aerobic metabolism and decreased lactate production [52,53] that could improve insulin sensitivity.

In the current study, untreated diabetic rats had lower surface area and volume density of brown interscapular adipose tissue and higher surface area and volume density of white interscapular adipose tissue. Furthermore, these rats had larger adipocyte size; findings similar to our previous report [34]. In our study, increased browning of interscapular adipose tissue by hyperoxia, corroborated by increased surface area and volume density of brown interscapular adipose tissue, in O_2-treated rats. In addition, in diabetic rats, hyperoxia increased tissue levels of proteins (UCP1, PPAR-γ, and PGC1-α) involved in adipose tissue browning. In line with our results, it has been reported that hyperoxia increases PPAR-γ gene expression in 3T3-L1 murine adipocytes [52]. Furthermore, increased expression of UCP1 and PGC1-α genes and proteins contributes in nitrate-induced browning effect in ob/ob obese mice [22]. PPAR-γ and PGC1-α proteins increase fatty acid oxidation, which decreases lipolysis and ectopic fat deposition that increases oxygen consumption and insulin sensitivity in adipose tissue [10,22,54].

These findings provides a new mechanism for the positive effects of hyperoxia on lowering weight gain as well as improving glucose tolerance and insulin sensitivity. It has been previously reported that adipocyte browning has beneficial metabolic effects [55,56]. In this study, O_2-treated rats had smaller adipocyte size in the eWAT, as measured by numerical density and provide another mechanism for beneficial metabolic effects of hyperoxia. While reduced adipocyte size is associated with improved metabolism [40], adipocyte hypertrophy is an independent predictor of type 2 diabetes [57].

In our study, diabetic rats had dyslipidemia and hyperoxia improved it; in addition, hyperoxia decreased elevated atherogenic index in diabetic rats. These findings are in contrast to some studies that not found lipid profile improvement after HBOT [40,58]. NBOT (100% O_2, 2 h/day, for 6 weeks) decreased serum TG levels in young pigeon but not in older ones, while, had no effect on serum cholesterol [59]. Few studies have reported effects of hyperoxia/hypoxia on lipid profile; in obese mice, sterol regulatory element-binding protein (SREBP)-1, a master regulator of TG synthesis, is upregulated by 300% following chronic intermittent hypoxia [60]; it is therefore speculated that hyperoxia may improve lipid profile by affecting genes involved in lipid biosynthesis; this issue however needs further investigations.

In the current study, diabetic rats had increased oxidative stress and hyperoxia exacerbated it. In line with our study, increased oxidative stress has been reported in diabetes [61] whereas no effect of hyperoxia has been observed on cellular antioxidants in healthy volunteers [62]. In line with our results, increased oxidative stress has been reported following hyperoxia in both *in vitro* [52] and *in vivo* [63] studies. These findings warrant further investigations regarding increased oxidative stress when using hyperoxia.

As a strength, we simultaneously measured changes in metabolic and histologic parameters following a relatively long-term hyperoxia intervention. However, these findings in male rats may not extend to females; an issue warrants further study. In the current study, no mortality was observed in O_2-treated rats (control and diabetes). Given that a living day in rats is equivalent to 26 days in humans, 5 weeks of intermittent hyperoxia intervention in rat could be considered as a long-term intervention in human [64]. In line with our study, it has been reported that intermittent normobaric hyperoxia is safe and has more beneficial effects than continuous hyperoxia [65].

5. Conclusion

This study showed beneficial metabolic effects of hyperoxia in obese type 2 diabetic male rats including improved insulin sensitivity and glucose tolerance; these effects were at least in part due to browning of adipose tissue, decreased adipocyte size, lowering body weight, decreased lactate level and improved dyslipidemia.

Conflict of interest

No competing interests are declared by the authors.

Contribution statement

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. M. Ghanbari contributed in data collection, statistical analysis, and drafting the manuscript. Z. Bahadoran contributed in drafting the manuscript, and final revision of the manuscript. N. Rasouli critically revised the manuscript and approved final manuscript. M.A Abdollahifar contributed in data collection, statistical analysis, and drafting the manuscript.

Acknowledgments

This manuscript has been extracted from a part of the thesis written by Reza Norouzirad, Ph.D. candidate, Endocrine Physiology Research Center, Research Institute of Endocrine Sciences (RIES), Shahid Beheshti University of Medical Sciences. Authors would like to thank from Research Institute of Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran for supporting this work (grant NO. 1005). The authors acknowledge Dr. Sajad Jeddi and Ms. Hanieh Gholami for their assistance in rat's handling and housing.

References

[1] P.Z. Zimmet, et al., Diabetes: a 21st century challenge, Lancet Diabetes Endocrinol. 2 (1) (2014) 56–64.
[2] J.J. Meier, R.C. Bonadonna, Role of reduced beta-cell mass versus impaired beta-cell function in the pathogenesis of type 2 diabetes, Diabetes Care 36 (Suppl. 2) (2013) S113–S119.
[3] C.F. Bento, P. Pereira, Regulation of hypoxia-inducible factor 1 and the loss of the cellular response to hypoxia in diabetes, Diabetologia 54 (8) (2011) 1946–1956.
[4] N. Rasouli, Adipose tissue hypoxia and insulin resistance, J. Investig. Med. 64 (4) (2016) 830–832.
[5] L. Hodson, Adipose tissue oxygenation: effects on metabolic function, Adipocytes 3 (1) (2014) 75–80.
[6] M. Pasarica, et al., Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response, Diabetes 58 (3) (2009) 718–725.
[7] S.K. Kota, et al., Aberrant angiogenesis: the gateway to diabetic complications, Indian J. Endocrinol. Metab. 16 (6) (2012) 918–930.
[8] H. Thangarajah, et al., The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues, Proc. Natl. Acad. Sci. U. S. A. 106 (32) (2009) 13505–13510.
[9] Y.S. Lee, et al., Increased adipocyte O_2 consumption triggers HIF-1α, causing inflammation and insulin resistance in obesity, Cell 157 (6) (2014) 1339–1352.
[10] J.O. Ebbert, M.D. Jensen, Fat depots, free fatty acids, and dyslipidemia, Nutrients 5 (2) (2013) 498–508.
[11] R. Norouzirad, et al., Hypoxia in obesity and diabetes: potential therapeutic effects of hyperoxia and nitrate, Oxidative Med. Cell. Longev. 2017 (2017).
[12] C. Regazzetti, et al., Hypoxia decreases insulin signaling pathways in adipocytes, Diabetes 58 (1) (2009) 95–103.
[13] H.C. Lee, S.J. Tsai, Endocrine targets of hypoxia-inducible factors, J. Endocrinol. 234 (1) (2017) R53–R65.
[14] N. Hosogai, et al., Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation, Diabetes 56 (4) (2007) 901–911.
[15] T. Matsunaga, et al., Hyperoxia reverses glucotoxicity-induced inhibition of insulin secretion in rat INS-1 beta cells, Biosci. Biotechnol. Biochem. 78 (5) (2014) 843–850.
[16] Q. Xu, et al., Repetitive hyperbaric oxygen treatment increases insulin sensitivity in diabetes patients with acute intracerebral hemorrhage, Neuropsychiatr. Dis. Treat. 13 (2017) 421–426.
[17] N.S. Al-Waili, et al., Influences of hyperbaric oxygen on blood pressure, heart rate and blood glucose levels in patients with diabetes mellitus and hypertension, Arch. Med. Res. 37 (8) (2006) 991–997.
[18] N. Karadurmus, et al., Potential benefits of hyperbaric oxygen therapy on atherosclerosis and glycaemic control in patients with diabetic foot, Endokrynol. Pol. 61 (3) (2010) 275–279.
[19] D. Wilkinson, et al., Hyperbaric oxygen therapy improves peripheral insulin sensitivity in humans, Diabet. Med. 29 (8) (2012) 986–989.
[20] A.C. Carpentier, et al., Brown adipose tissue energy metabolism in humans, Front. Endocrinol. 9 (2018).
[21] A.L. Poher, et al., Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance, Front. Physiol. 6 (2015).
[22] L.D. Roberts, et al., Inorganic nitrate promotes the browning of white adipose tissue through the nitrate-nitrite-nitric oxide pathway, Diabetes 64 (2) (2015) 471–484.
[23] A.M. O'Connor, J.M. Sargeant, Critical appraisal of studies using laboratory animal models, ILAR J. 55 (3) (2014) 405–417.
[24] S. Morimoto, et al., Insulin gene expression pattern in rat pancreas during the estrous cycle, Life Sci. 68 (26) (2001) 2979–2985.
[25] D. Rebolledo-Solleiro, A. Fernandez-Guasti, Influence of sex and estrous cycle on blood glucose levels, body weight gain, and depressive-like behavior in streptozotocin-induced diabetic rats, Physiol. Behav. 194 (2018) 560–567.
[26] S.L. Mumford, et al., Variations in lipid levels according to menstrual cycle phase: clinical implications, J. Clin. Lipidol. 6 (2) (2011) 225–234.
[27] S. Gheibi, et al., Nitrite increases glucose-stimulated insulin secretion and islet insulin content in obese type 2 diabetic male rats, Nitric Oxide 64 (2017) 39–51.
[28] S. Gheibi, et al., A practical guide for induction of type-2 diabetes in rat: incorporating a high-fat diet and streptozotocin, Biomed. Pharmacother. 95 (2017) 605–613.
[29] Z. Gan, et al., Differential responses of targeted lung redox enzymes to rat exposure to 60 or 85% oxygen, J. Appl. Physiol. (1985) 111 (1) (2011) 95–107.
[30] G.F. Benderro, et al., Decreased VEGF expression and microvascular density, but increased HIF-1 and 2alpha accumulation and EPO expression in chronic moderate hyperoxia in the mouse brain, Brain Res. 1471 (2012) 46–55.
[31] M. Tohidi, et al., Age- and sex-specific reference values for fasting serum insulin levels and insulin resistance/sensitivity indices in healthy Iranian adults: Tehran Lipid and Glucose Study, Clin. Biochem. 47 (6) (2014) 432–438.
[32] P. Mirmiran, et al., Dietary intake, changes in lipid parameters and the risk of hypertriglyceridemia: a prospective approach in the Tehran lipid and glucose study, Int. J. Vitam. Nutr. Res. 84 (5–6) (2014) 269–276.
[33] A. Noorafshan, et al., Curcumin and sertraline prevent the reduction of the number of neurons and glial cells and the volume of rats' medial prefrontal cortex induced by stress, Acta Neurobiol. Exp. (Wars) 74 (1) (2014) 44–53.
[34] T. Varzandi, et al., Effect of long-term nitrite administration on browning of white adipose tissue in type 2 diabetic rats: a stereological study, Life Sci. 207 (2018) 219–226.
[35] H. Gundersen, et al., The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis, APMIS 96 (7–12) (1988) 857–881.
[36] H. Gundersen, et al., Some new, simple and efficient stereological methods and their use in pathological research and diagnosis, APMIS 96 (1–6) (1988) 379–394.
[37] R.G. Vink, et al., Diet-induced weight loss decreases adipose tissue oxygen tension with parallel changes in adipose tissue phenotype and insulin sensitivity in overweight humans, Int. J. Obes. 41 (5) (2017) 722–728.
[38] S.D. Long, et al., Weight loss in severely obese subjects prevents the progression of impaired glucose tolerance to type II diabetes. A longitudinal interventional study, Diabetes Care 17 (5) (1994) 372–375.
[39] D. Wilkinson, et al., Hyperbaric oxygen therapy increases insulin sensitivity in overweight men with and without type 2 diabetes, Diving Hyperb. Med. 45 (1) (2015) 30–36.
[40] K. Tsuneyama, et al., Advantages and disadvantages of hyperbaric oxygen treatment in mice with obesity hyperlipidemia and steatohepatitis, ScientificWorldJournal 11 (2011) 2124–2135.
[41] A.S. Ryan, B.J. Nicklas, Reductions in plasma cytokine levels with weight loss improve insulin sensitivity in overweight and obese postmenopausal women, Diabetes Care 27 (7) (2004) 1699–1705.
[42] B.H. Goodpaster, et al., Effects of weight loss on regional fat distribution and insulin sensitivity in obesity, Diabetes 48 (4) (1999) 839–847.
[43] J. Holte, et al., Restored insulin sensitivity but persistently increased early insulin secretion after weight loss in obese women with polycystic ovary syndrome, J. Clin. Endocrinol. Metab. 80 (9) (1995) 2586–2593.
[44] M.W. Hulver, et al., Adiponectin is not altered with exercise training despite enhanced insulin action, Am. J. Physiol. Endocrinol. Metab. 283 (4) (2002) E861–E865.
[45] M. Saito, et al., High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity, Diabetes 58 (7) (2009) 1526–1531.
[46] R.R. Wing, et al., Benefits of modest weight loss in improving cardiovascular risk factors in overweight and obese individuals with type 2 diabetes, Diabetes Care 34 (7) (2011) 1481–1486.
[47] J.B. Albu, et al., Metabolic changes following a 1-year diet and exercise intervention in patients with type 2 diabetes, Diabetes 59 (3) (2010) 627–633.
[48] Y.D. Chen, et al., Plasma lactate concentration in obesity and type 2 diabetes, Diabetes Metab. 19 (4) (1993) 348–354.
[49] Y. Wu, et al., Lactate, a neglected factor for diabetes and cancer interaction, Mediat. Inflamm. 2016 (2016) 6456018.
[50] F.D. Newby, et al., Effects of streptozocin-induced diabetes on glucose metabolism and lactate release by isolated fat cells from young lean and older, moderately obese rats, Diabetes 38 (2) (1989) 237–243.
[51] J.A. Simoneau, et al., Skeletal muscle glycolytic and oxidative enzyme capacities are determinants of insulin sensitivity and muscle composition in obese women, FASEB J. 9 (2) (1995) 273–278.
[52] P. Quintero, et al., Effects of hyperoxia exposure on metabolic markers and gene expression in 3T3-L1 adipocytes, J. Physiol. Biochem. 68 (4) (2012) 663–669.
[53] M. Menzel, et al., Increased inspired oxygen concentration as a factor in improved brain tissue oxygenation and tissue lactate levels after severe human head injury, J. Neurosurg. 91 (1) (1999) 1–10.
[54] F. Zheng, Y. Cai, Concurrent exercise improves insulin resistance and nonalcoholic fatty liver disease by upregulating PPAR-gamma and genes involved in the beta-oxidation of fatty acids in ApoE-KO mice fed a high-fat diet, Lipids Health Dis. 18 (1) (2019) 6.
[55] M. Scheideler, et al., Endocrine and autocrine/paracrine modulators of brown adipose tissue mass and activity as novel therapeutic strategies against obesity and type 2 diabetes, Horm. Mol. Biol. Clin. Invest. 31 (2) (2017).
[56] D.X. Liu, et al., Activation of mTORC1 is essential for beta-adrenergic stimulation of adipose browning, J. Clin. Investig. 126 (5) (2016) 1704–1716.
[57] C. Weyer, et al., Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance, Diabetologia 43 (12) (2000) 1498–1506.
[58] K. Ghebremeskel, et al., Serum-lipids, cholesterol, alpha-tocopherol, and retinol in multiple-sclerosis patients before and after hyperbaric-oxygen therapy, J. Clin. Biochem. Nutr. 5 (1) (1988) 87–91.
[59] I. Alhazza, A. Haffor, Effects of hyperoxia and aging on cholesterol levels in pigeon, J. Appl. Behav. Sci. 1 (2005) 55–59.
[60] J. Li, et al., Chronic intermittent hypoxia upregulates genes of lipid biosynthesis in obese mice, J. Appl. Physiol. (1985) 99 (5) (2005) 1643–1648.
[61] I. Escribano-Lopez, et al., The mitochondrial antioxidant SS-31 increases SIRT1 levels and ameliorates inflammation, oxidative stress and leukocyte-endothelium interactions in type 2 diabetes, Sci. Rep. 8 (1) (2018) 15862.
[62] C. Dennog, et al., Antioxidant status in humans after exposure to hyperbaric oxygen, Mutat. Res. 428 (1–2) (1999) 83–89.
[63] C.K. Narkowicz, et al., Hyperbaric oxygen therapy increases free radical levels in the blood of humans, Free Radic. Res. Commun. 19 (2) (1993) 71–80.
[64] R. Quinn, Comparing rat's to human's age: how old is my rat in people years? Nutrition 21 (6) (2005) 775.
[65] M.R. Bigdeli, et al., In vivo normobaric hyperoxia preconditioning induces different degrees of antioxidant enzymes activities in rat brain tissue, Eur. J. Pharmacol. 611 (1–3) (2009) 22–29.