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Effect of long-term sodium nitrate administration on diabetes-induced anemia and glucose homeostasis in obese type 2 diabetic male rats

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ABSTRACT

Purpose: Anemia is common in patients with type 2 diabetes. This aims at determining long-term effects of nitrate administration on diabetes-induced anemia in obese type 2 diabetic rats.
Methods: Male Wistar rats were divided into 4 groups: Control, control + nitrate, diabetes, and diabetes + nitrate. Type 2 diabetes was induced using high-fat diet followed by injection of streptozotocin (30 mg/kg). Sodium nitrate (100 mg/L in drinking water) was administered for six months. After overnight fasting, levels of glucose and erythropoietin (EPO) and complete blood cell count (CBC) were measured at month 0, month 3, and month 6. At month 6, serum iron, and testosterone as well as EPO protein levels and hypoxia-inducible factor-1 (HIF-1) mRNA levels in kidney and liver were measured.
Results: Nitrate administration decreased serum glucose in diabetic rats by 10% and 15% at months 3 and 6, respectively. Nitrate restored decreased red blood cells count, hemoglobin concentration, and hematocrit to control levels in diabetic rats; in addition, nitrate restored decreased serum, kidney, and liver EPO levels to near normal values. Nitrate also increased HIF-1 mRNA levels in both kidney and liver of diabetic rats. Diabetic rats had lower serum testosterone (37%) and iron (20%) and nitrate restored these parameters to near normal values.
Conclusion: Long-term and low dose of nitrate had beneficial effects against anemia in obese type 2 diabetic rats; these effects were associated with increased EPO and HIF-1 levels in kidney and liver as well as increased circulating EPO, testosterone, and iron.

1. Introduction

Anemia is common in patients with type 2 diabetes [1] and intensifies the risk of type 2 diabetes complications [2]. Worldwide prevalence of anemia in diabetic patients ranges between 14 and 45% [2]. Erythropoietin (EPO) and iron deficiencies, hyporesponsiveness to EPO, low testosterone levels, some medications as well as chronic hyperglycemia are among the causes of anemia in diabetic patients [2].

Diabetes and obesity are associated with decreased nitric oxide (NO) bioavailability mostly due to decreased expression and activity of NO synthase (NOS) enzymes and quenching of NO by reactive oxygen species [3]. In addition to classic pathway of conversion of L-arginine to NO by NOS isoforms [viz. endothelial (eNOS), neural (nNOS) and inducible (iNOS)], NO is also produced from nitrate and nitrite in particular in hypoxic conditions [4]. Direct measurement of oxygen pressure indicates obesity-associated adipose tissue hypoxia in human [5] and mice [6–8]. Oxygen pressure in adipose tissue of obese and diabetic mice (15–20 mm Hg) is lower than lean mice (35–55 mm Hg mm Hg) [6–8]; findings indicating a 70% reduction in adipose tissue oxygen pressure that indicate tissue hypoxia in diabetes and obesity [9].

Effects of NO on erythropoiesis and EPO production is conflicting and both inhibitory [10] and stimulatory [11] effects of endogenous NO on EPO production in mice have been reported. In addition, NO could decrease hemoglobin (Hb) synthesis [12] and inhibits the growth of colony-forming unit-erythroid (CFU-E) in mononuclear cells derived from human bone marrow [13].

Nitrate/nitrite has been introduced as a new treatment for diabetes with promising effects mostly in animal studies [14–16]. Few studies have assessed effects of dietary nitrate/nitrite on erythropoiesis [17,18]. Globus et al. have reported that nitrite stimulates embryonic

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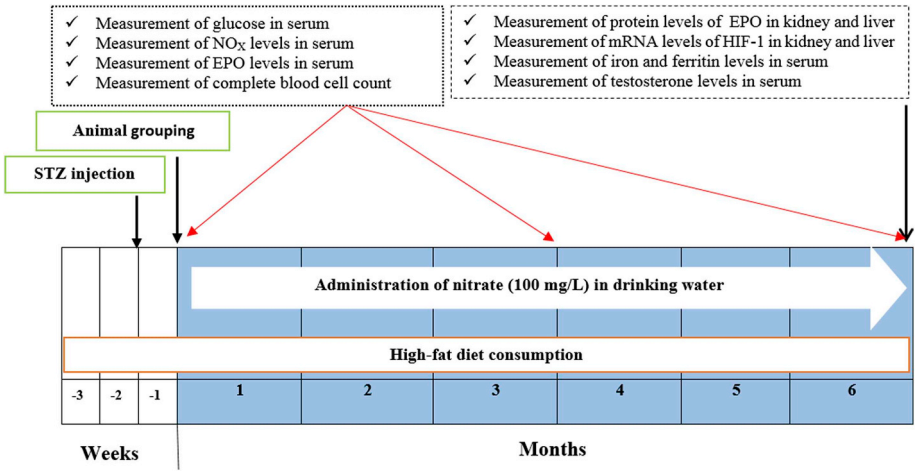

Fig. 1. Experimental procedures over the course of the study. STZ, streptozotocin; NO_X, nitrite + nitrate; EPO, erythropoietin; HIF-1, hypoxia-inducible factors-1.

Table 1
Effects of sodium nitrate (100 mg/L in drinking water) on body weight, water consumption, food intake, and calorie intake.

Parameters	Groups			
	Control	Control + Nitrate	Diabetes	Diabetes + Nitrate
Initial body weight (g)	191.1 ± 3.2	186.9 ± 2.9	189.0 ± 4.9	185.3 ± 3.4
Final body weight (g)	363.7 ± 5.9	348.2 ± 10.7	382.1 ± 9.3	351.0 ± 18.4†
Body weight gain (g)	172.3 ± 5.2	161.3 ± 9.9	193.1 ± 9.9*	165.7 ± 19.9
Body weight gain (g/day)	0.86 ± 0.03	0.80 ± 0.05	0.96 ± 0.05	0.82 ± 0.10
Food intakes (g/day/rat)	18.4 ± 0.4	17.8 ± 0.34	14.2 ± 0.2*	14.6 ± 0.2*
Food efficiency ratio[a] (%)	4.7 ± 0.17	4.5 ± 0.21	6.8 ± 0.39*	5.6 ± 0.65*
Calorie intake (kcal/day/rat)	57.0 ± 1.2	55.2 ± 1.0	69.8 ± 0.9*	71.7 ± 0.8*
Energy efficiency ratio[b] (%)	1.51 ± 0.10	1.44 ± 0.10	1.38 ± 0.10	1.14 ± 0.10
Water consumption (mL/day/rat)	34.0 ± 0.7	35.6 ± 0.8	36.1 ± 0.4*	33.4 ± 0.2†

*, † Statistically significant difference compared to the control and diabetes groups, respectively. Values are mean ± SEM (n = 10/each group).
[a] Food efficiency ratio = body weight gain (g/day)/food intake (g/day/rat) × 100 [22].
[b] Energy efficiency ratio (EER) = body weight gain (g/day)/energy intake (kcal/day) × 100 [23].

hepatic production of erythroid cells in mice [17] and Ashmore et al. have reported that dietary nitrate (9 mg/kg/day) suppress erythropoiesis in male Wistar rats by suppressing hepatic EPO expression [18]. To the best of our knowledge, there is no study to address effects of nitrate on EPO levels and red blood cell (RBC) indices in diabetes, the aim of this study was therefore to assess the long-term effect of low dose nitrate administration on diabetes-induced anemia in obese type 2 diabetic rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (weight range: 180–200 g) were kept on a 12:12 h light-dark cycle under controlled temperature (23 ± 2 °C) and humidity (50 ± 6%). All procedures were performed in accordance with the guidelines for the care and use of laboratory animals approved by the ethics committee of the Research Institute for Endocrine Sciences, affiliated to the Shahid Beheshti University of Medical Sciences (Ethic Code: IR.SBMU.Endocrine.Rec.1396.198).

2.2. Induction of diabetes

For induction of type 2 diabetes, rats were fed with a high-fat diet (HFD) for two weeks and then a single low dose of streptozotocin (STZ, 30 mg/kg dissolved in 0.1 mM citrate buffer, pH 4.5; Sigma Aldrich, Hamburg, Germany) was injected intraperitoneally (IP). One week after STZ injection, rats with fasting glucose levels ≥150 mg/dL were considered to be diabetic [19].

2.3. Experimental design

Rats were divided into 4 groups (n = 10/group): Control, control + nitrate, diabetes, and diabetes + nitrate. The nitrate-treated control and diabetic rats received sodium nitrate (100 mg/L in drinking water) and the control and diabetic groups received tap water for 6 months. The 21st day of study was designated as the first day for sodium nitrate administration in nitrate-treated control and diabetic rats. Control rats consumed standard rat chow (~3160 kcal/kg) and diabetic rats consumed HFD from start of study (week −3) until the end of the study (month 6). Details of preparation of HFD (~4900 kcal/kg) as well as components of HFD and standard rat chow has previously been reported [19]. In standard rat chow, 5.7%, 72.2%, and 22.1% of calories were from fat, carbohydrate, and protein, respectively and in HFD, 58%, 27.5%, and 14.5% of calories were from fat, carbohydrates, and proteins, respectively [19].

Timeline of the study is shown in Fig. 1. Body weight (g), water consumption (mL/day/rat), food intakes (g/day/rat), calorie intake (kcal/day/rat), energy efficiency ratio, and food efficiency ratio were measured weekly. Fasting serum glucose and NOx (nitrite + nitrate) were measured at the start of the study (week −3), start of the sodium nitrate administration (month 0), month 3, and the end of the study (month 6). Serum levels of EPO were measured at the month 0, month 3, and month 6. At the end of study (month 6), protein levels of EPO and mRNA expression of hypoxia-inducible factors-1 (HIF-1) in kidney and liver as well as iron, ferritin, and testosterone levels in serum were

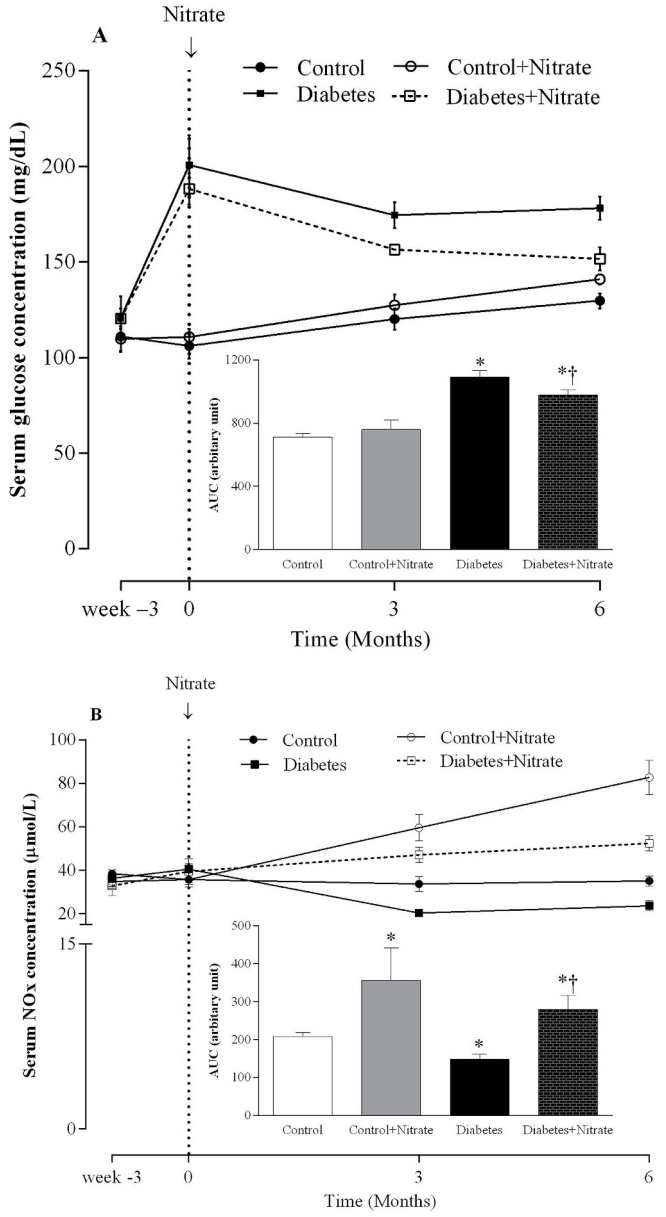

Fig. 2. Effects of sodium nitrate administration (100 mg/L in drinking water) on fasting serum glucose (A) and fasting serum nitric oxide metabolite (NOx) (B). Insets indicate the area under the curves (AUC) after nitrate administration. *, † Statistically significant difference compared to the control and diabetic groups, respectively. Values are mean ± SEM (n = 10/each group).

measured. Complete blood count (CBC) including RBC count, hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular Hb (MCH), mean corpuscular Hb concentration (MCHC), were done at the week −3, month 0, month 3, and month 6. At the end of the study, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/kg; Sigma Aldrich, Hamburg, Germany) and kidney and liver were removed and immediately transferred to liquid nitrogen for further assessments.

2.4. Complete blood cell count

After 12–14 h fasting, blood samples were collected in anticoagulant tubes containing K_2EDTA (ethylene diamine tetra-acetic acid, 1.8 mg/mL per milliliter of blood) and CBC was performed by the Sysmex automated hematology analyzer (model Kx 21 N, Japan). Intra- and inter-assay coefficients of variation were < 3% for all CBC parameters.

2.5. Measurement of serum glucose, NOx, EPO, testosterone, iron, and ferritin levels

Serum glucose concentration was measured using an enzymatic colorimetric assay by a commercial kit (Pars Azmoon, Tehran, Iran). Fasting serum NOx levels was measured by the Griess method using a commercial kit (Pazhoheshkave Afagh, Iran). Nitrate content in standard rat chow and HFD were measured according to ISO6635:1984; details of the procedure has previously been reported [20]. In brief, 1 g of the samples were treated with tetraborate sodium, Carrez I, and Carrez II (potassium hexacyanoferrate (II) trihydrate and zinc acetate solution, respectively); extraction was followed by filtration through filter paper. To measure sample nitrate concentration, vanadium (III) trichloride and Griess reagents including Griess I and Griess II (sulfonamide in hydrochloric acid and N-(1-naphtyl) ethylene diamine dihydrochloride in water, respectively) were added to filtrate; the volumetric flask was incubated at 37 °C for 180 min and then the absorbance was read at 538 nm using ELISA reader.

Serum, kidney, and liver levels of EPO were measured using a specific rat ELISA kit (ZellBio Gmbh, Germany). Serum testosterone levels were measured by an ELISA kit (Arka fan azma, Tehran, Iran), and serum level of iron and ferritin were measured by commercial standard kits (Pars Azmoon, Tehran, Iran). Intra-assay coefficients of variation were < 4% for all parameters.

2.6. Measurement of mRNA expression of HIF-1 by qRT-PCR

After anesthesia, the kidney and liver were removed and placed in liquid nitrogen, thereafter stored at −80 °C. Details of RNA extraction, cDNA synthase, and qRT-PCR have been previously reported [21]. Total RNA was extracted by the RNX-Plus solution kit (Cinagen Co., Tehran, Iran) and cDNA synthesis was performed using Thermo Scientific RevertAid Reverse Transcriptase in accordance with manufacturers' instructions. The primers sequences used for HIF-1 gene is forward, 5′-CCGAGCGTGAGCACAGTTAC-3′ and reverse, 5′- TTCATCAGTGGTG GCAGTTGC -3′. The primers sequences used for β-actin gene is forward, 5′- CCGTGAAAAGATGACCCAGATC-3′ and reverse, 5′- CACAGCCTGG ATGGCTACGT -3′. Amplifications were performed in a Rotor-Gene 6000 real time PCR machine (Corbett, Life science, Sydney, Australia). Target gene was normalized with β-actin as reference. The relative mRNA level for HIF-1 gene was calculated by the $2^{-\Delta\Delta C_t}$ method.

2.7. Statistical analyses

Analyses were done using GraphPad Prism software (Version 6) and all values are expressed 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, water consumption, food and calorie intakes, serum glucose, serum NOx, serum EPO levels and CBC parameters. A two-way mixed ANOVA include a repeated measure design with addition of a between subject factor. One-way ANOVA was used for comparing the area under the curves (AUC), CBC parameters and serum level of testosterone, iron, and ferritin as well as levels of EPO in serum, kidney and liver at the end of study. The Mann-Whitney *U* test was used for comparing fold change in mRNA expression levels between the groups. Correlation between EPO and NO_X concentration, RBC count, hemoglobin concentration, and hematocrit was determined using Spearman correlation coefficient. Two-sided P-values < 0.05 were considered statistically significant.

3. Results

3.1. Body weight, water consumption, food intake, and calorie intake

As shown in Table 1, all groups had comparable body weights at start of the study. Compared to controls, diabetic rats had significantly

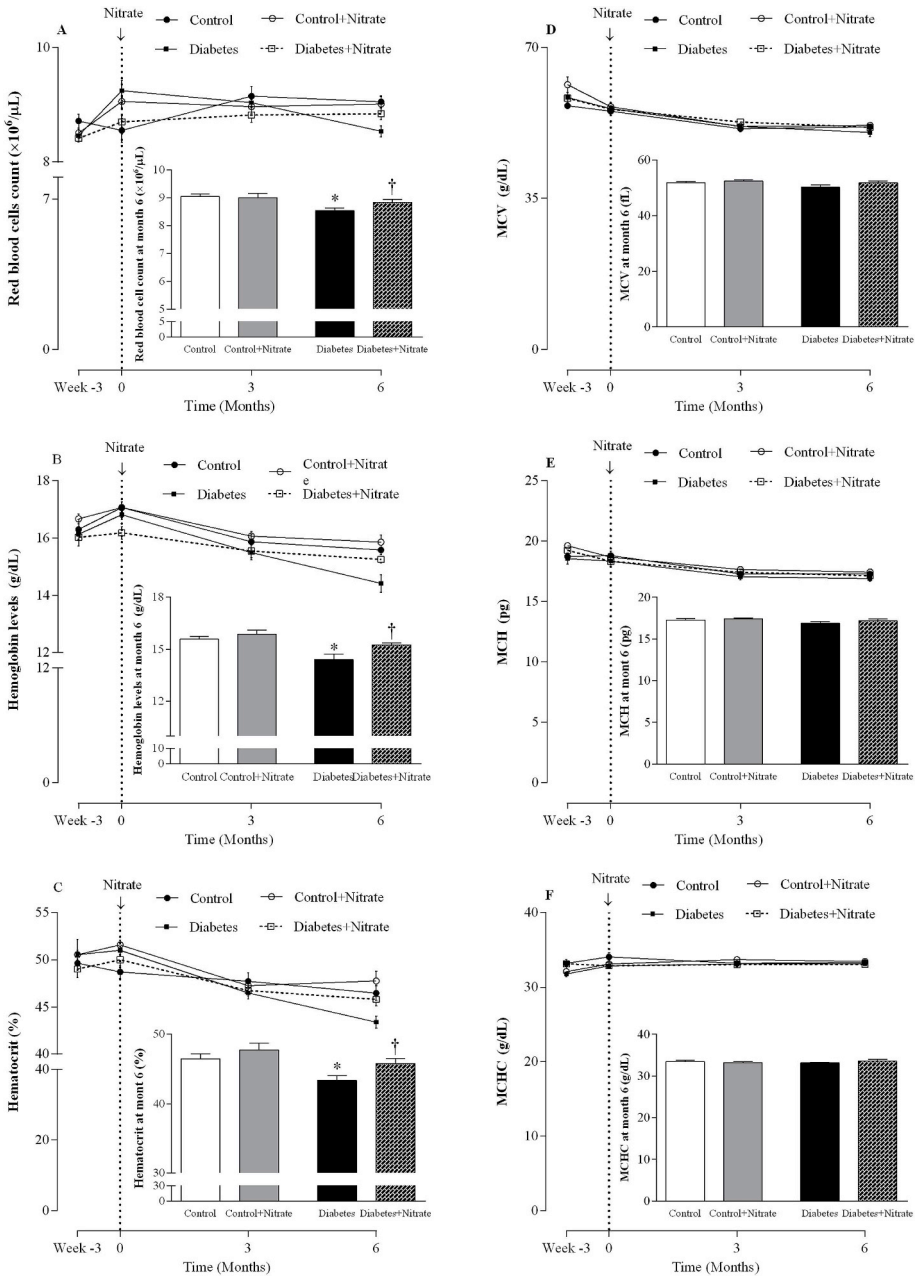

Fig. 3. Effect of sodium nitrate administration (100 mg/L in drinking water) on red blood cell count (A), hemoglobin concentration (B), hematocrit (C), mean corpuscular volume (MCV) (D), mean corpuscular hemoglobin (MCH) (E), and mean corpuscular hemoglobin concentration (MCHC) (F) in control and diabetic rats. Insets indicate the result for CBC parameters at the end of study. *, † Statistically significant difference compared to the control and diabetes groups, respectively. Values are mean ± SEM (n = 10/each group).

higher body weight gain, water consumption, calorie intake, and food efficiency ratio and lower food intake during study. It should be noted that calorie content of HFD was higher than that of normal chow diet (~4900 vs. ~3160 kcal/kg) and therefore diabetic rats had lower food intake but higher calorie intake. Nitrate administration decreased body weight and water consumption in diabetic rats but had no effect on food intake, calorie intake, energy efficiency ratio, and food efficiency ratio (Table 1 and Supplementary Fig. 1).

3.2. Serum glucose concentrations

Serum glucose concentrations were comparable in all groups at the start of the study (week −3). Serum glucose was significantly higher in diabetic rats (200 ± 15 mg/dL) than controls (106 ± 6 mg/dL) at the start of nitrate administration (month 0); an effect that was maintained throughout the rest of the study (Fig. 2A). Nitrate administration significantly ($P < 0.05$) decreased serum glucose concentration in diabetic rats by 10% at month 3 (174 ± 6 to 156 ± 2 mg/dL) and by 15% at month 6 (178 ± 6 to 151 ± 6 mg/dL) (Fig. 2A).

3.3. Serum concentrations of nitric oxide metabolites

As shown in Fig. 2B, serum NOx concentrations did not differ between control and diabetic rats at the start of study and also at month 0 (i.e. before nitrate administration). Compared to controls, diabetic rats had lower NOx concentrations in serum at month 3 (20.3 ± 1.6 vs. 34.1 ± 3.8 μmol/L, $P < 0.05$) and month 6 (23.6 ± 2.4 vs. 34.4 ± 2.4 μmol/L, $P < 0.05$). Nitrate administration increased

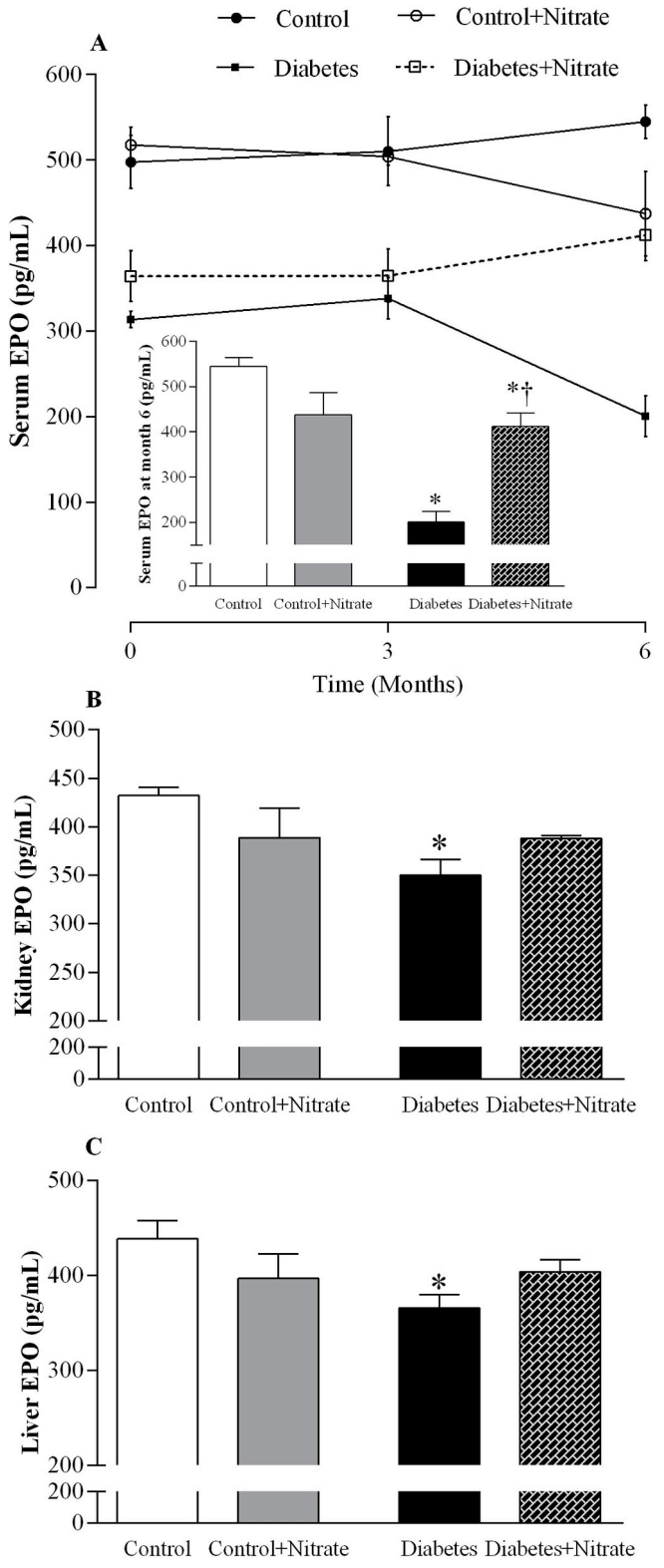

Fig. 4. Effect of sodium nitrate administration (100 mg/L in drinking water) on concentrations of erythropoietin (EPO) in serum (A), kidney (B), and liver (C) in control and diabetic rats. Inset indicates the EPO in serum at the end of study. *, † Statistically significant difference compared to the control and diabetes groups, respectively. Values are mean ± SEM.

serum NOx concentrations of control and diabetic rats by 58% and 54%, respectively.

3.4. CBC

As shown in Fig. 3, RBC count, Hb concentration and Hct did not differ between control and diabetic rats at the start of study, month 0, and month 3. Diabetic rats, compared to controls, had significantly lower RBC count, Hb concentration and Hct at the end of study. Nitrate administration significantly ($P < 0.05$) increased RBC count, Hb concentration, and Hct in diabetic rats by 3.4%, 5.2%, 5.8%, respectively. MCV, MCH, and MCHC did not differ between control and diabetic rats throughout the study. Nitrate administration had no effect on these parameters in both control and diabetic rats.

3.5. Erythropoietin concentrations in serum, kidney and liver

Diabetic rats, compared to controls, had significantly lower concentrations of EPO in serum at the month 0, month 3, and month 6 by 37%, 34%, and 63%, respectively. Diabetic rats also had significantly lower concentrations of EPO in kidney and liver than controls at the end of study (month 6). At the end of study, nitrate-treated diabetic rats compared to non-treated ones had significantly higher concentrations of EPO in serum; in addition, nitrate treatment restored EPO levels to normal values in kidney and liver (Fig. 4).

3.6. Correlation between EPO and NO_X concentration, RBC count, hemoglobin concentration, and hematocrit

As shown in Fig. 5, in diabetic rats, serum EPO concentration was positively correlated with serum NO_X ($r = 0.821$, $P < 0.001$), RBC count ($r = 0.542$, $P = 0.048$), hemoglobin concentration ($r = 0.527$, $P = 0.055$), and hematocrit ($r = 0.488$, $P = 0.078$). In addition, there was an inverse correlation between serum EPO concentration and serum NO_X ($r = -0.462$, $P = 0.096$) in control rats. There was no significant correlation between serum EPO concentrations with CBC parameters in control rats.

3.7. mRNA expression of HIF-1 in the kidney and liver

As shown in Fig. 6, in diabetic rats, compared to the controls, mRNA expression of HIF-1 was significantly lower in the kidney (43% of control) but not in the liver. Six-month nitrate administration increased HIF-1 mRNA expression in kidney and liver in diabetic rats by 2.26 and 2.31 fold, respectively.

3.8. Serum concentrations of testosterone, iron, and ferritin

Diabetic rat compared to controls, had lower serum testosterone (1.70 ± 0.15 vs. 2.77 ± 0.38 ng/mL, $P = 0.03$) and serum iron (135.8 ± 11.2 vs. 170.1 ± 10.3 μg/dL, $P = 0.05$), as well as higher serum ferritin (397.1 ± 29.3 vs. 284.9 ± 41.8 μg/dL, $P = 0.05$). Six-month nitrate administration restored testosterone, iron, and ferritin levels to near normal values (Fig. 7).

4. Discussion

The results of this study showed that low dose and long-term administration of nitrate had beneficial effects against type 2 diabetes-induced anemia in rats; these effects are at least in part due to increased EPO and HIF-1 levels in kidney and liver as well as increased circulating levels of EPO, testosterone, and iron.

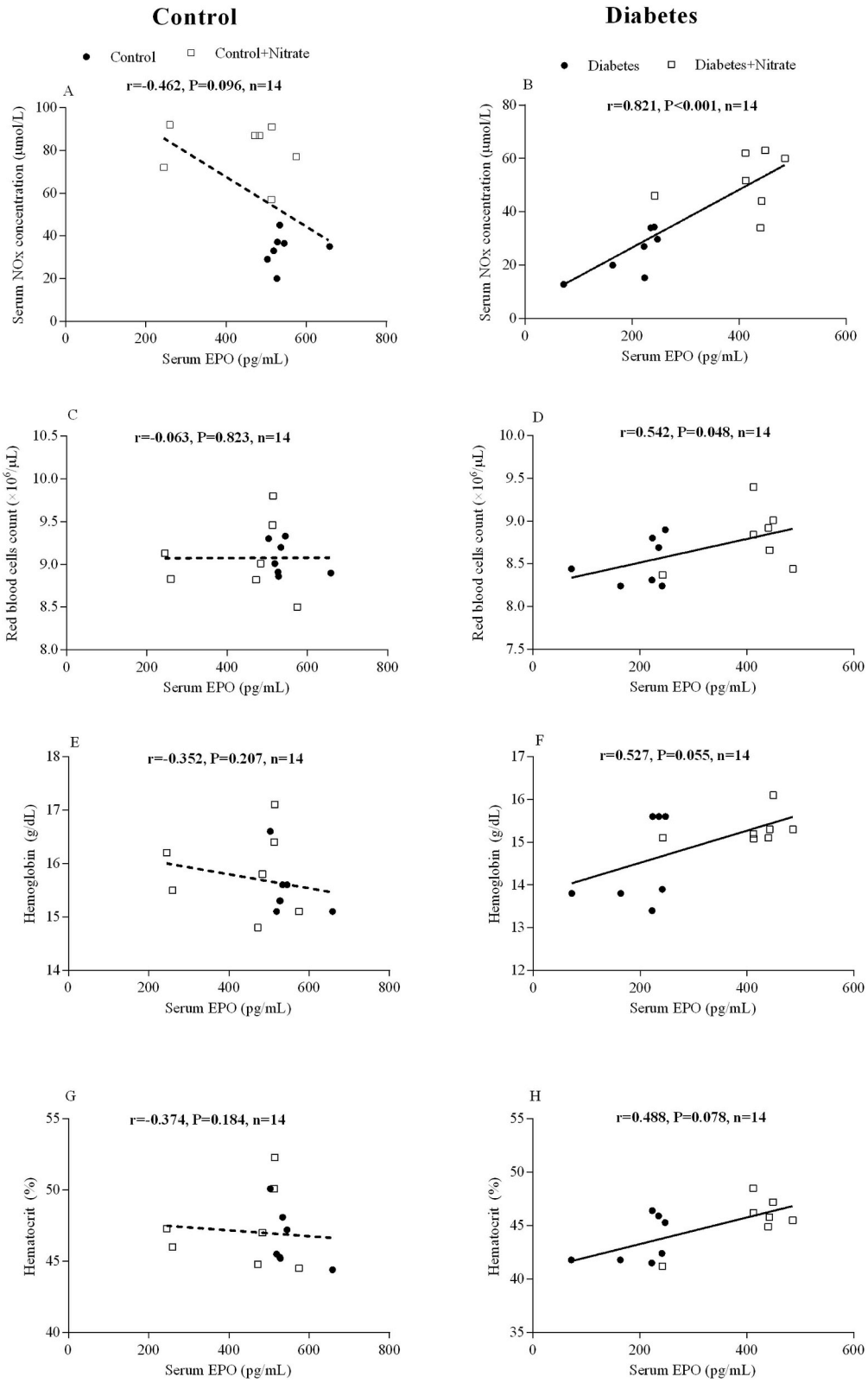

Fig. 5. Correlation between EPO and serum NO_X concentration and CBC parameters in control (left column) and diabetic (right column) rats. EPO, erythropoietin; NO_X, nitric oxide metabolite.

In this study, we showed that in type 2 diabetic rats, six months nitrate administration decreased fasting glucose by 15%; this finding are in support of previous studies on antihyperglycemic effects of nitrate/nitrite during 1 [24], 2 [15], and 3 [25,26] months of intervention. Anti-hyperglycemic effect of nitrate/nitrite is due to decreased insulin resistance, increased insulin secretion [27], increased glucose uptake in skeletal muscle [24], and increased pancreatic islet blood flow [27]. In addition, nitrate administration decreased body weight but had no effect on food intake, calorie intake, energy efficiency ratio and food efficiency ratio in type 2 diabetic rats; we previously reported

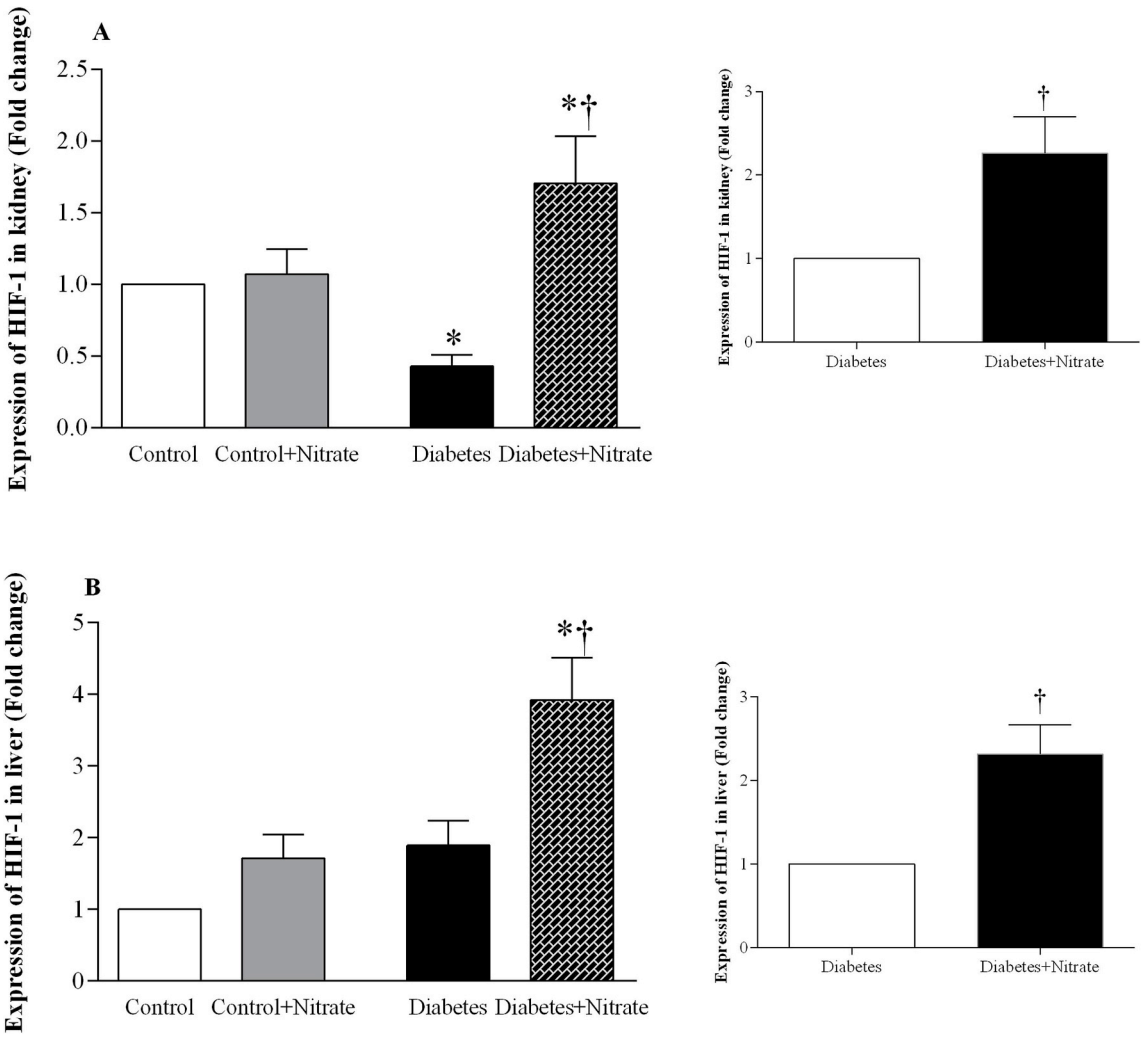

Fig. 6. Effect of sodium nitrate administration (100 mg/L in drinking water) on mRNA expression of hypoxia-inducible factors-1 (HIF-1) in kidney (A) and liver (B). *, † Statistically significant difference compared to the control and diabetes groups, respectively. Values are mean ± SEM (n = 8/each group).

that body weight lowering effect of nitrate or hyperoxia in type 2 diabetic rats is due to browning of white adipose tissue [25,28].

In our study, diabetic rats had significantly lower RBC count, Hb and Hct and normal MCV, MCH and MCHC; these results indicate a normocytic normochromic anemia, characteristic of anemia of chronic disease [29]. In line with our results, normocytic normochromic anemia has been reported in rats [30,31] and humans with type 2 diabetes [32].

Anemia of chronic disease characterize by lower serum iron and higher serum ferritin [33] as observed in our study and is also in line with reports from type 2 diabetic patients [34–37]. Another cause of anemia in diabetic rats is decreased serum testosterone as observed in our study and is in line with previous reports from rats and humans with type 2 diabetes [38,39]; decreased serum testosterone is associated with normocytic normochromic anemia [40]. Testosterone deficiency in diabetes may be associated with decreased stimulatory effect of insulin on Leydig cells [41], which decreases testosterone secretion [38,42].

Diabetic rats in our study had lower mRNA expression of HIF-1 in kidney and also lower levels of EPO in serum, kidney, and liver (63%, 19% and 16%, respectively). In support of our results, chronic hyperglycemia in diabetes decreases stabilization of HIF in mice [43,44], and human with type 2 diabetes [45,46] and also decreases production of EPO by kidney due to increases injury in renal tubular cells [47–49]. Regarding tissue hypoxia reported in diabetes [7], our data on decreased mRNA expression of HIF-1 in diabetic rats seems to be paradoxical. It has however been reported that cellular adaptation to hypoxia is impaired in diabetes [50]. In addition, chronic hypoxia increased HIF-1 degradation by destabilizing its mRNA [51,52]. In diabetes, EPO and iron deficiencies decrease activity of bone marrow and then decrease production of RBC [2,29]. In addition, oxidative stress and inflammation decreases expression of HIF-1 and EPO production by kidney that contribute to development of anemia in diabetes [47,48,53].

Our results showed for first time that administration of low dose of nitrate for 6 months increased RBC count, Hb concentration, and Hct levels in diabetic rats; in addition, serum EPO levels were positively correlated with serum NOx and RBC count. There is no report on the effects of nitrate administration on diabetes-induced anemia; however in control rats, increased erythropoiesis has been reported after nitrate administration at dose 45, 90, and 135 mg/L [54] and NO gas inhalation at dose 8 ppm [55]. Unlike to our results, decrease in erythropoiesis has been reported following administration of nitrate/nitrite in control rats, it has however been observed with high doses of nitrate (1000 and 3000 mg/L) [56] and nitrite (50 and 30 mg/kg/day) [57,58] as well as after short-term administration of nitrate (18 day) [18]. High doses but not low doses of nitrite/nitrate increases lysis and decreases production of RBC [57–59] and induces methemoglobinemia [60]. In addition, it has been suggested that nitrate at low dose increases bone marrow activity and at high dose decreases it [59].

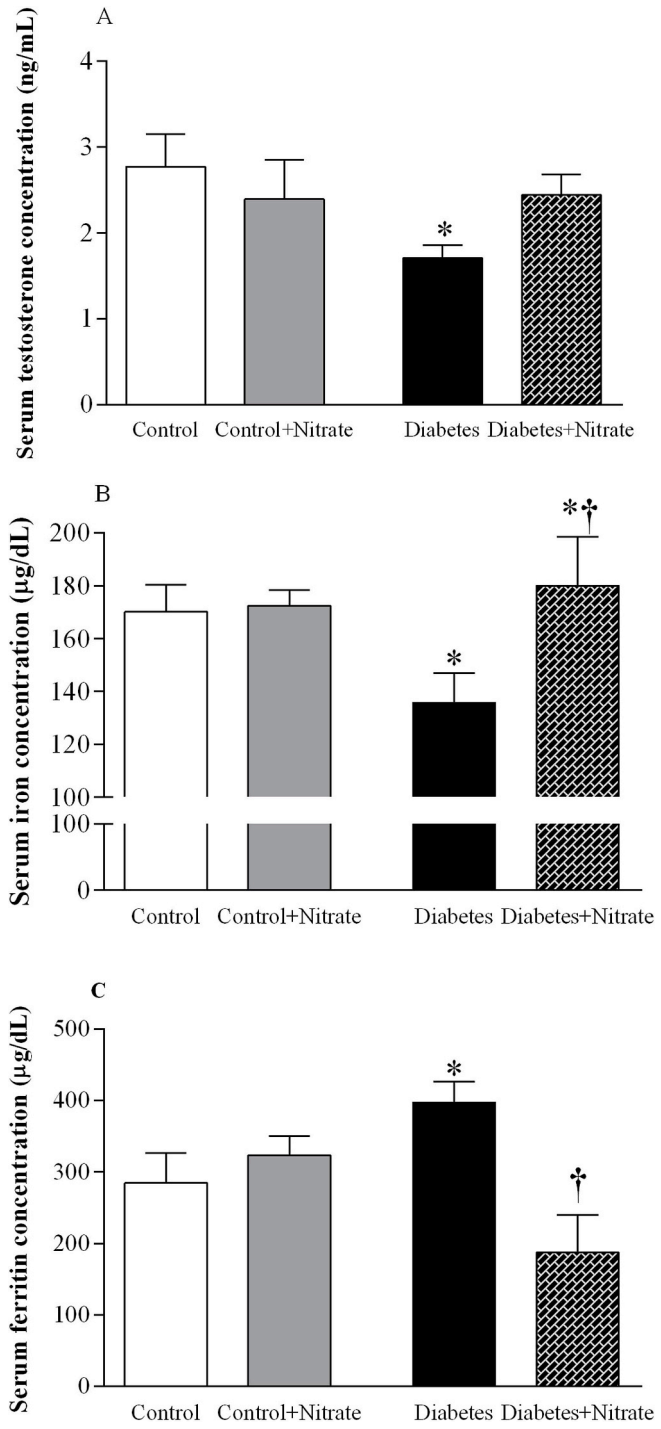

Fig. 7. Effect of sodium nitrate administration (100 mg/L in drinking water) on serum concentrations of testosterone (A), iron (B) and ferritin (C) in control and diabetic rats at the end of the study. *, † Statistically significant difference compared to the control and diabetes groups, respectively. Values are mean ± SEM (n = 8/each group).

In this study, nitrate administration restored serum testosterone, iron, and ferritin levels to near normal values in diabetic rats. Nitrate and NO-donors increase serum testosterone levels in rats wit type 1 diabetes [61] and testosterone secretion in cultured Leydig cells of rats [42,62]. Diabetic patients have higher levels of hepcidin, which decreases iron absorption [63,64]. Testosterone increases EPO production [65,66], iron absorption and therefore serum iron [63], iron incorporation into RBC [63,64] and therefore erythropoietic activity. These effects may partly explain anti-anemia effects of nitrate in diabetes.

In this study, long-term nitrate administration increased concentrations of EPO in serum, kidney, and liver as well as mRNA expression of HIF-1 in kidney and liver in diabetic rats. In line with these results, administration of sodium nitroprusside (a NO donor) increases serum levels of EPO [11], increases gene and protein expression of EPO [11] and L-NAME (a nonspecific NOS inhibitor), decreases EPO production [67]. NO also stabilizes HIF-1α and increases transcription of its target genes including EPO [68]. In this regard, Ashmore et al. reported that administration of nitrate (9 mg/kg/day) increases mRNA levels of HIF-1 and EPO in kidney of control rats [18]. In addition, Ikuta et al. reported that NO increases the differentiation of hematopoietic progenitors toward erythroid-lineage cells by increases cGMP levels [55]. The HIF increases erythropoiesis by increasing EPO production, promoting the uptake and utilization of iron, and altering the bone marrow microenvironment to facilitate erythroid progenitor maturation and proliferation [69]. Nitrate/nitrite converts to NO and increases cGMP levels in male type 2 diabetic rats [25,70], which could stimulates erythropoiesis by increasing testosterone levels, HIF-1 and EPO expression. These data highlight the favorable effect of nitrate/nitrite-nitric oxide-cGMP pathway against diabetes-induced anemia in obese type 2 diabetic rats.

This study has some strengths; first, low dose of nitrate was administrated to diabetic rats for a long-time (i.e. 100 mg/L in drinking water for 6 months). The same dose of nitrate used in this study is derived from eNOS under normal conditions in mice [71] and also achievable through vegetable and fruit consumption in human and is sufficient for induction of NO-like activity in humans and animals [72,73]. In our study, nitrate content of standard rat chow, HFD, and water were 112 ± 15 mg/kg, 88 ± 13 mg/kg, and 0.29 ± 0.22 mg/L, respectively; according to these values, nitrate intake in control, control + nitrate, diabetes, and diabetes + nitrate groups were 2.07 ± 0.14, 4.60 ± 0.31, 1.26 ± 0.05 and 3.73 ± 0.07 mg/day/rat respectively. According to Bryan et al. large range of nitrate from very low dose (85 mg/L equal to 6.2 mg/kg) to high dose (1000 mg/L equal to 160 mg/kg) has been studied in both humans and mice that provide therapeutic effects without any signs of toxicity [74]. Therapeutic potential effects of nitrate/nitrite against type 2 diabetes have been reviewed recently and seems to be promising for prevention and treatment of type 2 diabetes [72]. Regarding high levels of nitrate/nitrite in green leafy vegetables, this can provide a cost-effective and nutritional-based approach for managing type 2 diabetes [72].

Second, we used the HFD-STZ model of type 2 diabetes that causes long-lasting and stable hyperglycemia and mimics the pathogenesis of human type 2 diabetes [19]; in this model, HFD induces insulin resistance and then STZ partially destructs β-cells [19]. Finally, given that a living day in rats is equivalent to 26 days in humans [75], 6 months of nitrate administration in rat could be considered as a long-term intervention in human. As a limitation, we measured serum NO_X but not nitrate and nitrite separately. This is because the Griess method used in our study is useful for the NO_X measurement but provides less reliable results for nitrite [76,77].

In conclusion, long-term and low dose administration of nitrate had beneficial effects against anemia in obese type 2 diabetic rats; these effects were associated with increased EPO and HIF-1 levels in kidney and liver as well as increased circulating EPO, testosterone, and iron. Nitrate therapy could be achieved through nutrition-based interventions and may be therefore considered for managing diabetes-induced anemia.

Declaration of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.02.003.

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