RESEARCH ARTICLE

Sodium nitrate preconditioning prevents progression of the neuropathic pain in streptozotocin-induced diabetes Wistar rats

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Abstract

Purpose The purpose of the study was to evaluate the possible protective effects of low dose sodium nitrate preconditioning on the peripheral neuropathy in streptozotocin (STZ)-induced diabetic model.

Methods Male Wistar rats were randomly divided into five groups: control (no intervention), control treated sodium nitrate (100 mg/L in drinking water), diabetic (no intervention), diabetic treated NPH insulin (2-4 U), and diabetic treated sodium nitrate (100 mg/L in drinking water). Diabetes was induced by intraperitoneal injection of STZ (60 mg/kg). All interventions were done for 60 days immediately following diabetes confirmation. Thermal and mechanical algesia thresholds were measured by means of hot-plate test, von Frey test, and tail-withdrawal test before the diabetic induction and after diabetes confirmation. At the end of the experiment, serum NOx level and serum insulin level were assessed. Blood glucose concentration and body weight have recorded at the base and duration of the experiment.

Results Both hypoalgesia, hyperalgesia along with allodynia developed in diabetic rats. Significant alterations including, decrease in tail withdrawal latency (30th day), decreased mechanical threshold (60th day), and an increase in hot plate latency (61st day) were displayed in diabetic rats compared to control rats. Nitrate and insulin preconditioning produced protective effects against diabetes-induced peripheral neuropathy. Data analysis also showed a significant increase in glucose level as well as a considerable reduction in serum insulin and body weight of diabetic rats, which restored by both insulin and nitrate preconditioning.

Conclusion Sodium nitrate preconditioning produces a protective effect in diabetic neuropathy, which may be mediated by its antihyperglycemic effects and increased serum insulin level.

Keywords Nitrate preconditioning · Diabetes · Peripheral nerve · Thermal algesia · Mechanical algesia

Highlights
- Diabetes decreased mechanical threshold (mechanical allodynia)
- Diabetes caused the first reduction and then a secondary increase in tail withdrawal and hot plate latency
- Sodium nitrate preconditioning decreased the blood glucose and increased the serum insulin level in diabetic rats.
- Sodium nitrate preconditioning reduced allodynia and thermal algesia in diabetic rats

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Introduction

Diabetes mellitus is considered as a chronic progressive metabolic problem with increasing global prevalence, and predict that approximately 640 million will be influenced by 2040 [1]. Diabetic neuropathy is the frequently silent and complex diabetes-associated microvascular complication, described by the progressive loss of autonomic and peripheral nerve fibers [2]. Diabetes-induced peripheral neuropathy is the most prevalent form of diabetic neuropathy observed in 7–50% of diabetic patients, within 1 year to more than 25 years of diagnosis, respectively [3]. Patients with diabetic peripheral neuropathy (DPN) experience functional and structural alterations in peripheral nerves, characterized by lowered nerve conductive velocity, axonal degeneration, and paranodal demyelination [4]. These alterations are a major cause of sensory loss, reduced touch and vibration sensation, limb numbness, pain, tingling, prickling sensations, numbness, and muscular imbalance, fall-related injury, and foot ulceration [5]. The destruction of peripheral nerve fibers in the epidermal layer of skin is reported to be clinical diagnostic criteria for DPN detection [6].

It has been shown that vascular factors have an important role in the incidence and development of DPN [7]. In diabetic models, the thickened basement membrane of endoneurial microvessel is suggesting a close relationship between DPN and microvessel integrity [8]. Microcirculatory disturbances reported in the early stage of diabetes are associated with hyperglycemia [9]. Prolonged hyperglycemia play a vital role in the development of diabetic neuropathy by activation of the hexosamine and polyol pathways, production of advanced glycation end products (AGE), activation of PKC isoforms, and increasing the intracellular ratio of NADH/NAD$^+$ [10–14]. Clinical studies from the past decades indicate that strict control of glucose level reduces the occurrence of DPN, suggesting a key role for hyperglycemia [15]. Therefore, diabetic-induced neuropathy is thought to occur as a result of two important mechanisms including hyperglycemia-induced injury to nerve cells and neuronal ischemia arising from reduction of neurovascular flow owing to hyperglycemia [16].

There is currently no symptomatic and effective treatment for DPN, it is thereby vital to develop therapeutic ways for this condition. It has been shown that strict glycemic manage and therapies targeting neurovascular function, slow the progression and development of DPN in diabetes [17, 18]. It is recognized that nitric oxide (NO) plays a significant role in the mediation of close communication between neurons and vessels, biological processes of the blood vessels and neurotransmission, and neurovascular coupling [19, 20]. Moreover, nNOS derived NO emerges essentially for neurovascular coupling [21]. In addition to the L-arginine pathway, NO is synthesized from nitrate and nitrite [22]. Previous findings provide evidence showing that both nitrite and nitrate have beneficial effects on various organs in healthy and diabetic cases including the cardiovascular and reproductive system [23, 24]. However, the preconditioning effects of sodium nitrate on diabetes-induced peripheral neuropathy have not been studied yet.

If DPN recognized and treated early, the severe fiber damage and even amputation may be avoided. It is of crucial importance to detect DPN early to prevent its progression and development. Manifestations of diabetic neuropathy such as hyperalgesia (exaggerated pain resulting from a painful stimulus) and allodynia (pain resulting from a non-painful stimulus) have been documented [25]. Therefore, the present study was designed to evaluate the preconditioning effects of sodium nitrate against streptozotocin (STZ)-induced DPN in male Wistar rats.

Materials and methods

Animals

The experiments were carried out according to regulations specified by the National Institutes of Health "Principles of Laboratory Animal Care and protocols of Tabriz University of Medical Sciences for animal studies. Forty male Wistar rats, 85–115 g body weight, were fed standard rat chow and had free access to water. Animals were randomly allocated into 5 groups: C; control group with no intervention, CN; controls treated with 100 mg/L sodium nitrate, D; diabetics with no intervention, DI; diabetics treated with 2-4 U of NPH-insulin and DN; diabetics treated with 100 mg/L sodium nitrate. Animals in nitrate groups were housed individually while animals in other groups were kept two or three rats in standard cages.

Diabetes induction and experimental protocols

Diabetes was induced by 60 mg/kg STZ (Sigma-Aldrich, Germany) as described [26]. Blood samples for glucose measurements were taken from the tail vein 72 h following the STZ injection. The rats with blood glucose more than 250 mg/dl were considered diabetic [27]. All treatments were started immediately after confirmation of diabetes for 60 days. NPH insulin (Pharmaceutical Mfg. Co., Iran) was administered subcutaneously at fixed times, 2-4 U daily adjusted according to the blood glucose level [28]. The dose of 100 mg/L sodium nitrate (Merck KGaA, 64,271 Darmstadt, Germany) administration was started immediately after the confirmation of diabetes in both CN and DN groups [29]. Blood glucose and body weight were assessed at the beginning of the study and during the experiment. After 60 days, blood samples were collected to assess serum insulin and NOx levels. Design of the experimental protocols of the study has depicted in Fig. 1.

Fig. 1 Experimental design
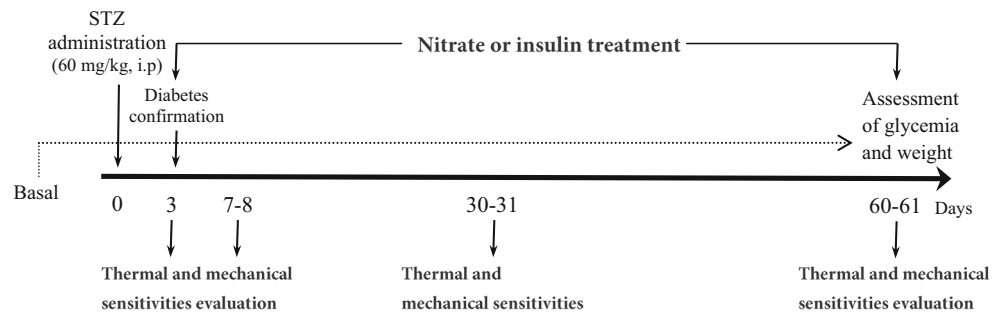
Serum insulin and NOx assessment

Serum insulin concentration was evaluated using ELISA kits (Shanghaicrystal Day Biotech Co., LTD, China) according to the manufacturer's instruction. Nitric oxide metabolites (NOx) level was measured using the Griess method in accordance with a protocol explained by previous studies [29].

Behavioral tests

The behavioral tests have been done in the following order: mechanical algesia was assessed using flexible von Frey filaments and thermal algesia was evaluated by both tail withdrawal test and hot plate test. Von Frey and tail withdrawal tests were done at 0th, 7th, 30th, and 60th days of the experiment. The hot plate test was performed 1 day later, at 0th, 8th, 31st, and 61st days. All three behavioral tests were performed in a quiet room between 8:00 a.m. and 2:00 p.m. The von Frey and tail immersion tests were performed with an interval of 1–2 h [30]. All behavioral tests were performed in a blinded fashion.

Mechanical allodynia

Mechanical allodynia was assessed the mechanical touch sensitivity with flexible von Frey filaments (Touch Test™ Sensory Evaluator Kit, USA) ranged from 0.04 to 300 g on the mid-plantar part of both hind paws. Von Frey monofilaments are nylon or metal hairs of different diameter that exert exact levels of force against the skin. Their use allows assessment of mechanical stimulation. The test was done by placing an animal on an elevated wire mesh platform 30 min before the examination for habituation. The Von Frey hair was inserted perpendicular to the plantar surface of both right and left hind paw. The test was started with the thinnest hair filament; the pressure increased slowly for 2 s. The typical withdrawal responses were considered as a shaking of the hind paw or quickly flicking the paw away from the stimulation. Lifting the hind paw and licking it were also reported as a positive response. When a withdrawal response was observed to the stimulus, a lighter filament was tested, and a heavier filament was applied in the absence of a response. At least two

positive withdrawal responses out of three trials were required to describe the positive response [31].

Thermal algesia

Both hot-plate (supraspinal pain) and tail withdrawal (spinal pain) tests were used to evaluate thermal hyperalgesia. The hot plat machinery was set at a temperature of $53 \pm 0.5^{\circ}C$. Each animal was placed individually on the heated surface, and then the time interval between placement and the licking of any hind paws was recorded. The cut-off time, selected to avoid any probable tissue damage, was 40 s [32].

Rats were gently restrained by placed in a restrainer, and then the distal 5 cm of their tail was dipped into the hot water bath maintained at $48 \pm 0.5^{\circ}C$. The time or latency (second), between exposure to the hot water and the tail sudden withdrawal, was recorded. To minimize the possibility of tissue damage from heat exposure, a cut-off time of 40 s was established, at which time the animal was removed from the test [33].

Blood sampling

At the end of the study, all rats were anesthetized under i.p. injection of 80 mg/kg ketamine and 10 mg/kg xylazine. Blood samples were taken from the inferior vena cava and then centrifuged at 3500 g for 10 min. Then sera were separated and stored at −20 °C until the analysis.

Statistical analysis

Statistical analysis was done using SPSS version 21 statistic software package. Data were represented as mean ± SEM. All data are expressed as mean ± SEM. A One-way ANOVA analysis followed by Tukey's post-hoc tests, were used to determine the significance of differences in behavioral tests measurements. Comparisons between the different times in all groups were performed by Bonferroni post hoc-test in behavioral tests. For all other results, One-way ANOVA analysis followed by Tukey's post-hoc test was done. A *p* value less than 0.05 was considered as statistically significant.

Results

Effect of sodium nitrate preconditioning on blood glucose level and body weight of diabetic rats

As shown in Fig. 2, there were no significant differences in either blood glucose or body weight among the groups before treatment. Three days after diabetic induction blood glucose significantly increased in diabetic rats (D, DI and DN groups) compared to the control group (Fig. 2a, $p < 0.001$). Surprisingly, both the sodium nitrate and insulin administration significantly prevented the increase of blood glucose compared to diabetic rats ($p < 0.001$). In addition, blood glucose levels were elevated in the diabetic group over time ($p < 0.001$). Nitrate and insulin preconditioning in diabetic groups immediately after diabetes confirmation led to a gradual decrease in blood glucose level which persisted until the end of the study ($p < 0.01$). Blood glucose in DN group on 60th day after STZ was higher than that of DI group ($p < 0.05$).

The effect of nitrate preconditioning on body weight was evaluated in male rats. Sixty days after STZ, body weight was significantly lowered in diabetic group compared to control rats ($p < 0.001$). In diabetic animals treated with nitrate or insulin, we observed a statistically significant normalization of body weight (Fig. 2b, $p < 0.001$). Moreover, nitrate preconditioning in CN group caused significant increase in body weight compared to control group ($p < 0.001$ to $p < 0.01$). Weight gain in the control and diabetic received nitrate group, as well as diabetic received insulin group was continued over time ($p < 0.001$).
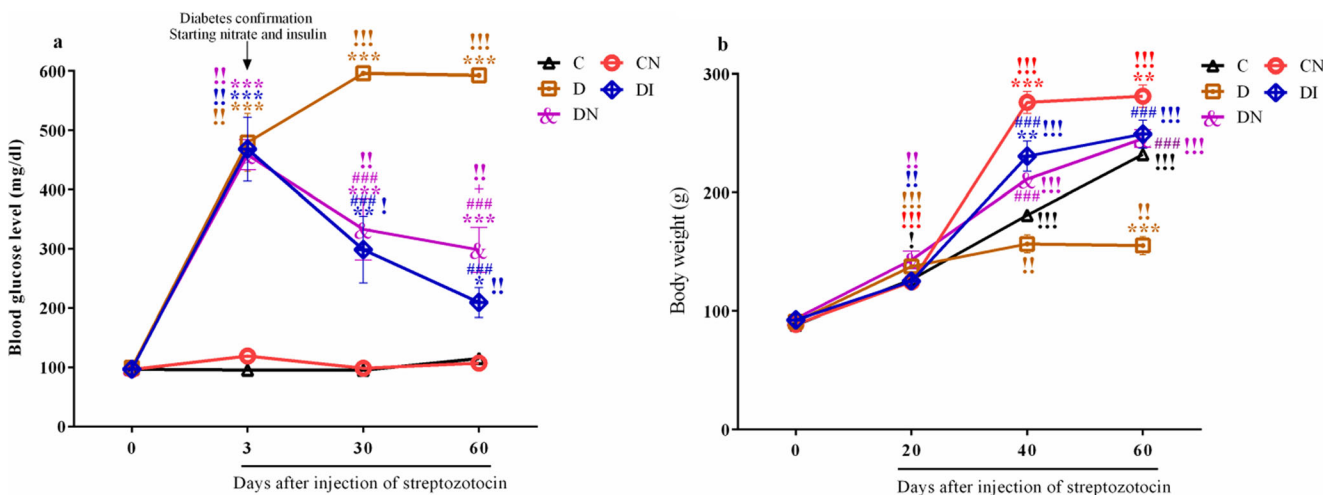
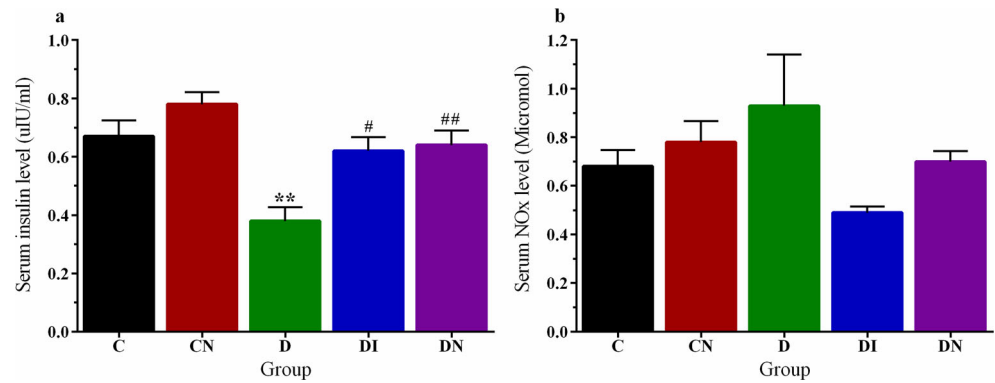### Effect of sodium nitrate preconditioning on serum insulin and NOx level of diabetic rats

In order to elucidate how nitrate preconditioning was therapeutically effective at blood glucose, we measured the serum level of insulin at 60 days after STZ. As shown in Fig. 3 panel a, the serum level of insulin of diabetic rats was reduced compared to controls ($p < 0.01$). Both insulin and nitrate preconditioning were able to prevent insulin level reduction compared to D group ($p < 0.05$ and $p < 0.01$, respectively). There was no significant difference in the levels of serum NOx between the groups (Fig. 3b).

Effect of sodium nitrate preconditioning on mechanical sensitivity of diabetic rats

Since the sensory alterations in diabetic neuropathy are often different, we decided to test the action of nitrate preconditioning on mechanical hyperalgesia immediately after diabetes confirmation. The mechanical allodynia was evidence in STZ-diabetic animals compared to the control group at 60th day compared to controls ($p < 0.001$). The mechanical threshold in diabetic animals was decreased and maintained significantly lower up to the end of the study ($p < 0.01$). Nitrate preconditioning was able to provide a fast and persisted antiallodynic effect compared to D group (Fig. 4, $p < 0.001$). Insulin therapy also counteracted mechanical allodynia in DI group compared to diabetic group ($p < 0.001$). There was no significant difference in the levels of the mechanical allodynia between diabetic received insulin and diabetic received nitrate groups.

Fig. 2 Sodium nitrate preconditioning rescued the increase of blood glucose and decrease of body weight in diabetic rats. a Changes of blood glucose among groups at 0th, 3th, 30th, and 60th days. **b** Changes of body weight among groups at 0th, 20th, 40th, and 60th days. Data represent mean ± SEM of 8 rats per group. One-way ANOVA followed by Tukey post hoc test was used for multiple comparisons. $*p < 0.05$,

$**p < 0.01$ $***p < 0.001$ versus control group; $###p < 0.001$ versus D group; $+p < 0.05$ between the diabetic received insulin group and the diabetic received nitrate group. Comparisons between the different times in each group were performed by Repeated measures one-way ANOVA and Bonferroni post-test. $!p < 0.05$, $!!p < 0.01$, and $!!!p < 0.001$

Fig. 3 Sodium nitrate preconditioning increased the serum insulin level in diabetic rats. **a** Changes of serum insulin level among groups. **b** Changes of serum NOx level among groups. Data represent mean ± SEM of 8 rats per group. One-way ANOVA followed by Tukey test was used for multiple comparisons. $**p < 0.01$ versus control group; $\#p < 0.05$, $\#\#p < 0.01$ versus D group

Effect of sodium nitrate preconditioning on thermal sensitivity of diabetic rats

Both tail immersion and hot plate tests were performed for spinal and supraspinal pain, respectively. As reported in Fig. 5, panel a, the tail withdrawal thresholds (TWT) of D group at 30 days after STZ were reduced compared to controls ($p < 0.001$). After that, we observed a significant and fast increase in tail withdrawal latency in the 60th day compared to 30th day ($p < 0.05$). Furthermore, insulin treatment was able to significantly correct tail withdrawal thresholds compared to diabetic animals ($p < 0.001$ at 30th day and $p < 0.01$ at 60th day).

As seen in Fig. 5, panel b, a significant reduction of thermal sensitivity was evident at 31th day compared to 0th in STZ-diabetic group ($p < 0.05$). At day 61 after STZ, a significant increase in hot plate latency was detectable in D group compared to controls ($p < 0.001$). Both treatments at that time

were able to significantly reduce thermal sensitivity compared to diabetic rats ($p < 0.001$).

Discussion

This is the first research to investigate the effects of dietary sodium nitrate preconditioning on the diabetic-induced peripheral neuropathy of Wistar rats with type 1 diabetes. The principal findings of this study were that dietary nitrate preconditioning reduced the blood glucose level, increased the body weight and serum insulin level and recovered the thermal and mechanical algesia. We also found that nitrate preconditioning had no effect on the serum NOx level. The pathophysiological factors leading to peripheral neuropathy in diabetes are included chronic hyperglycemia, insulin deficiency, and increased oxidative stress [16, 34, 35]. Studies have shown a close relationship between neurovascular flow and diminished NO bioavailability [36, 37]. The possible explanations for the beneficial effects in the present study related to elevated plasma insulin concentration and subsequent reduced serum blood glucose.

Our study shows that in the STZ model of type 1 diabetes, serum insulin levels were significantly lower than in controls; sodium nitrate preconditioning increases serum insulin level in diabetic rats. Findings from in vitro and in vivo investigations have supported the hypothesis that the nitrate and nitrite signaling pathway plays a significant role in insulin secretion, insulin signaling, and glucose homeostasis [38, 39]. Intraperitoneal administration of sodium nitrite, increased pancreatic islet blood flow and also enhanced plasma insulin concentration in rats [39]. However, oral supplementation with nitrate or infusion of sodium nitroprusside in healthy men had no effect on plasma insulin or glucose levels [40]. Nitrate and nitrite elevated insulin secretion via various mechanisms including increased pancreatic islet blood flow and activation of guanylyl cyclase and the cGMP pathway [39, 41]. However, in contrast with our results, Gheibi and colleagues have shown that administration of sodium nitrite to rats with type 2 diabetes, induced by the combination of a low-
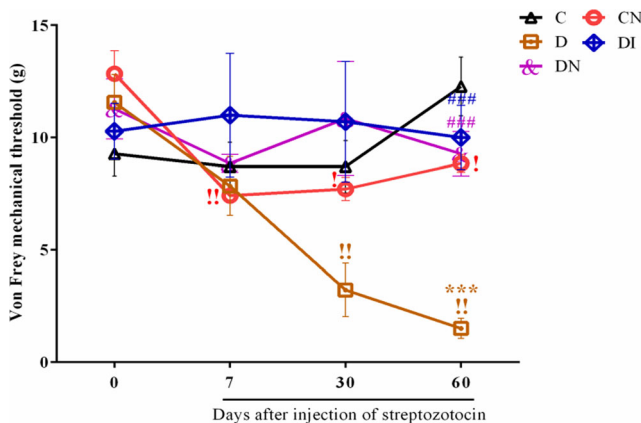

Fig. 4 Sodium nitrate preconditioning reduces allodynia in diabetic rats. Changes of Von Frey mechanical threshold at 0th, 7th, 30th, and 60th days. Data represent mean ± SEM of 8 rats per group. One-way ANOVA followed by Tukey test was used for multiple comparisons. $***p < 0.001$ versus control group; $\#\#\#p < 0.001$ versus D group. Comparisons between the different times in each group were performed by Repeated measures one-way ANOVA and Bonferroni post-test. $!p < 0.05$ and $!!p < 0.01$
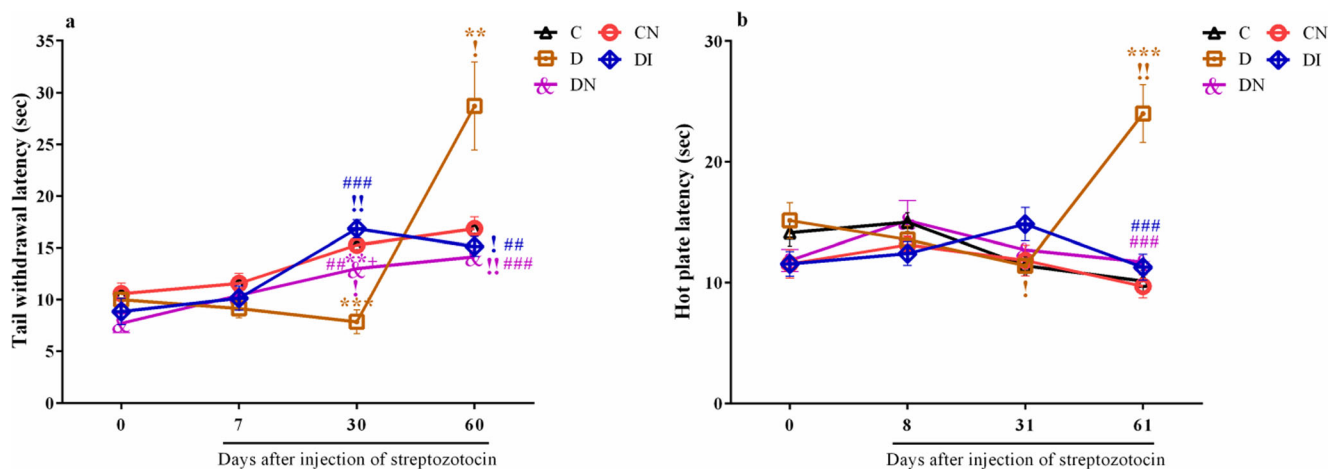
Fig. 5 Sodium nitrate preconditioning reduces thermal algesia alterations in diabetic rats. a Changes of tail withdrawal latency among groups at 0th, 7th, 30th, and 60th days. **b** Changes of hot plate latency among groups at 0th, 8th, 31th, and 61th days. Data represent mean ± SEM of 8 rats per group. One-way ANOVA followed by Tukey test was used for multiple comparisons. $**p < 0.01$, $***p < 0.001$ versus control group; $^{##}p < 0.01$, $^{###}p < 0.001$ versus D group; $^{+}p < 0.05$ versus diabetic received insulin group. Comparisons between the different times in each group were performed by Repeated measures one-way ANOVA and Bonferroni post-test. $!p < 0.05$ and $!!p < 0.01$

dose STZ and high-fat diet, reduced insulin secretion, a discrepancy may be associated with the type of diabetes [42].

In this study, sodium nitrate preconditioning for 60 days decreased blood glucose levels in diabetic rats, findings that also reported by previous studies. In this context, it has also been shown that nitrite decreased serum glucose concentration in diabetic rats by 27.6% in type 2 diabetic rats [43]. Moreover, infusion of sodium nitroprusside in type 2 diabetic patients resulted in higher glucose uptake independent of plasma insulin level [44]. However, there is some evidence that reported these anions to have no effects on glucose level [45]. A discrepancy may be associated with the duration of nitrate/nitrite administration, study design, and differences in the animal and clinical methods. It has been suggested that nitrate and nitrite decreased glucose level, possibly owing to increased glucose uptake in skeletal muscle, glucose transporter type 4 (GLUT4) translocation to the membrane, insulin secretion, and improving insulin signaling [39, 46]. Insulin-independent stimulatory effect of nitrate and nitrite on GLUT4 translocation suggested that nitrite could improve insulin signaling via restoration of NO-dependent nitrosation of GLUT4 signaling [39].

In our study, STZ-induced diabetic rats had decreased body weight. Nitrate preconditioning increased body weight in both control and diabetic rats. Although a number of animal investigations reported increased body weight following nitrate administration in type 1 diabetic models [28, 29], other researchers observed reduced [38] or no significant changes [47] in body weight at various doses of nitrate and nitrite. There are several probable explanations for these discrepancies, including the type of diabetes, duration, and a dose of nitrate/nitrite administration; a dose-dependent decrease in body weight has been shown following 5 months of nitrate

treatment with various doses of 50, 150, and 500 mg/L in rats [48]. Mechanisms underlying weight-gaining effects of nitrite are not exactly understood and may be at least in part owing to increased food intake [39]. Moreover, in line with our findings, Seethalakshmi and coworkers reported that insulin supplementation recovered body weight [49].

Decreased [24], increased [50], and no change [51] in serum NOx concentrations of diabetes have been documented by previous reports. In our study, neither diabetes nor any of the treatments led to significant alterations in serum NOx concentrations. Although these findings cannot describe from the results of this study, it may be associated with the duration of diabetes. Dissimilar to our findings, increased NOx levels have been reported following both nitrate and nitrite administration in aorta, heart, lung, brain, liver, kidney, and skeletal muscles [52, 53]. A discrepancy may be related to the duration of their administration.

Although the detection of "pain" is challenging because of its mental nature in experimental models [53], there are a number of behavioral tests such as tail withdrawal, von Frey, and hot-plate tests that allow for the reproducible, rapid, and sensitive determination of the mechanical and thermal nociceptive thresholds in rats [54]. Present study displayed that STZ-induced diabetic rats developed considerable thermal and mechanical hyperalgesia and then thermal hypoalgesia. It has been documented that mechanical and thermal hyperalgesia is mediated by A-fibers and C-fibers, respectively [55, 56]. Moreover, downregulation of GABA$_B$ receptors on nociceptive fibers in the spinal cord may contribute to the development of allodynia in diabetic rats [57].

The reduced threshold of harmful tactile stimuli was detected in the von Frey test for diabetic rats in hind paws after 60 days of STZ injection. Hind paw withdrawal threshold was

also progressively decreased during this study in diabetic rats, indicating this group of rats experienced allodynia throughout the experiment period. In line with our findings, tactile allodynia and spontaneous pain have been reported in a number of patients with diabetes mellitus [58]. Moreover, Hong Gong et al. have documented that STZ-induced diabetic rats developed considerable mechanical hyperalgesia 4 days after STZ injection [59]. A dose of 100 mg/l/day inorganic nitrate or 2–4 U/day NPH insulin preconditioning for 60 days considerably modified the diabetes-induced mechanical allodynia. A number of beneficial effects of nitrate/nitrite have been demonstrated, including improved endothelial dysfunction and pancreatic islet function, reversal of features of metabolic syndrome, increased islet blood flow, and insulin secretion, as well as lowering blood glucose [41]. It is clear that chronic hyperglycemia leads to peripheral nerve damage through activation of several key metabolic pathways including polyol, hexosamine, and AGE/RAGE pathways [60]. Therefore, we suggest that these beneficial effects of sodium nitrate preconditioning in diabetic rats appear to be more related to the increase in serum insulin level and a decrease in blood glucose concentrations. In line with our findings Calcutt and coworkers have shown that diabetes-induced allodynia can be prevented and recovered by insulin replacement in STZ-diabetic rats [61]. Furthermore, a recent review has demonstrated that excess insulin or insulin deficiency has a key role in neuropathic changes in sensory neurons [35].

It is possible that various types of peripheral nerve fibers are affected in rat models of the diabetic neuropathy since paw withdrawal reactions to thermal signals are associated with supraspinal sensory processing [62]. In our study, diabetic rats revealed a significant decrease in hot plate latency at 31st day compared to the basal value as well as a marked increase at 61st day compared to the control group. Results from the tail withdrawal test showed that diabetes causes a significant reduction in tail withdrawal latency (hyperalgesia) at 30th day compared with control group, however, a thermal hypoalgesia was observed on the 60th day in the tail withdrawal latency test. In line with our findings, the alteration pattern of thermal withdrawal threshold in diabetic rats remained inconsistent in previous studies, and both decrease and increase in thermal withdrawal threshold have been observed [59, 63]. Prnova et al. have reported that type 2 diabetes male rats (Zucker Diabetic Fatty) expanded symptoms of thermal hypoalgesia as showed by increased tail-flick latencies [63]. Moreover, Hong Gong et al. demonstrated thermal hyperalgesia occurred following STZ-induced diabetes [59]. Hyperglycemia-induced overproduction of oxidative stress, as well as pro-inflammatory agents, may contribute to the alterations in behavioral responses in peripheral diabetic neuropathy [64, 65]. Our results show that both insulin and nitrate preconditioning prevented thermal hyperalgesia and hypoalgesia in diabetic rats, probably mediated by the modulation of serum insulin and blood glucose level. Insulin may directly supply strong support of

neurons via its effects on brains insulin receptors or may indirectly by its beneficial effects on glycemia [66]. The results are in agreement with our previous findings that showed nitrate supplementation after 1 month of STZ-induced diabetes confirmation improved peripheral neuropathy [30].

Conclusion

In conclusion, this study revealed that long-term nitrate preconditioning in streptozotocin-induced diabetic rats had beneficial and preventive effects against diabetes-induced peripheral nerve damage. Improved blood glucose and serum insulin level may contribute to the favorable effects of sodium nitrate on diabetes-induced peripheral neuropathy.

Compliance with ethical standards

Ethical issues All of protocols were approved by the Ethics Committee of Animal Research of Tabriz University of Medical Sciences (No. IR.TBZMED.REC.1395.960).

Conflict of interests Authors declare no conflict of interests.

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