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Original Paper

The Natural Rotenoid Deguelin Ameliorates Diabetic Neuropathy by Decreasing Oxidative Stress and Plasma Glucose Levels in Rats via the Nrf2 Signalling Pathway

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Key Words
Deguelin • Diabetic neuropathy • Nrf2 • H_2S • Oxidative stress • Neuroinflammation

Abstract
Background/Aims: Deguelin is a natural rotenoid that shows anti-inflammatory and antimicrobial activities. Rotenoids prevent oxidative damage and potentiate natural antioxidant activity in diabetic conditions, suggesting utility in treating diabetes and its complications. Here, we evaluate the potential efficacy of deguelin against diabetic neuropathy (DN). ***Methods:*** DN was induced by streptozotocin followed by daily treatment with deguelin (4, 6 or 8 mg/kg) for 14 days. Blood glucose was measured, neurobehavioral tests for nociception and motor coordination were performed, and neuron conduction velocities were analysed electrophysiologically. We also assessed (Na^+-K^+) ATPase activity, performed a reactive oxygen species assay, measured the levels of various markers of oxidative stress, and of hydrogen sulphide (H_2S) in dorsal root ganglion (DRG) neurons, conducted immunoblotting studies for proteins and ELISA for inflammatory cytokines. ***Results:*** Deguelin significantly suppressed mechanical and thermal hyperalgesia, as well as cold allodynia, and partially restored the conduction velocities of neurons in DN rats. Significantly decreased expression levels of capspase-3 in DRG neurons, and increased (Na^+-K^+) ATPase activity in sciatic nerves, were observed. In addition, deguelin decreased glucose levels, attenuated oxidative stress and neuroinflammation, and elevated levels of H_2S, nuclear respiratory factor 2 (Nrf2) and heme oxygenase-1, suggesting a disease-attenuating effect of deguelin in DN rats. To shed light on the underlying mechanism of action of deguelin, insulin- and dimethyl fumarate (BG-12)-treated groups were also included. Insulin suppressed glucose levels and BG-12 produced effects on Nrf2 levels similar to 8 mg/kg deguelin, confirming involvement of the Nrf2 pathway in the beneficial effects of deguelin against DN. ***Conclusions:*** Deguelin attenuated DN by decreasing oxidative stress and plasma glucose levels via the Nrf2 signalling pathway.

Chen Ji and Liu Wenjie contributed equally to this work.

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Introduction

Diabetes has emerged as a serious health problem worldwide [1]. Diabetic neuropathy (DN) has been reported to affect at least 50% of all diabetics and is an important factor contributing to disease-related disability. Pain, allodynia and hyperalgesia are major features of DN [2]. To date, no specific therapeutic regimen has been recommended for DN. Therefore, it is of utmost importance to identify an efficacious drug therapy for managing this painful disorder.

Chronic hyperglycaemia activates inflammatory pathways and oxidative stress mechanisms, which together damage nerve tissues, in turn leading to neuropathic pain [3]. Hyperglycaemia generates reactive oxygen species (ROS) via activation of the polyol, hexosamine and protein kinase C (PKC) pathways, leading to the formation of advanced glycation end products (AGEs) [3]. Oxidative stress causes damage to peripheral and dorsal root ganglion (DRG) neurons, constituting an important mechanism underlying DN pain [3]. In addition, the presence of continuous hyperglycaemia leads to neuroinflammation, in turn producing neuropathic pain in diabetes mellitus (DM). Activation of the PKC, polyol and AGE pathways results in inflammation in nerve tissues [4]. These pathways, together with oxidative stress, activate transcription factors such as nuclear factor kappa B (NF-κB), which cause nerve damage in DN via neuroinflammation [3].

NF-κB proteins play an important role in inflammation and immunity, among other processes including cell development, growth, survival and proliferation. ROS are generated via various cellular processes as part of a cellular signalling cascade. Certain NF-κB-regulated genes play a major role in regulating ROS levels in cells, and ROS have various inhibitory and stimulatory roles in NF-κB signalling [5]. To date, studies have shown that both oxidative stress and neuroinflammation are important in the progression of DN. Oxidative stress initiates inflammation by activating NF-κB [6], which in turn undermines antioxidant defences by suppressing the expression of antioxidant genes [7]. We aimed to identify a link between oxidative stress and neuroinflammation and postulated that targeting both of these factors simultaneously could lead to better therapeutic outcomes than addressing either alone.

Deguelin is a natural rotenoid obtained from *Mundulea sericea* (Leguminosae) and certain other plants [8]. Deguelin has shown various pharmacological activities, including apoptotic, anti-angiogenic [9, 10], wound healing, anti-inflammatory [11] and antimicrobial effects [12]. In addition, deguelin targets NF-κB, cyclooxygenase-2 and nucleoporin 98 kDa (Nup98) [13]. Meanwhile, rotenoids have shown free radical scavenging activity, oxidative damage-preventing action, and potentiation of enzymatic and non-enzymatic antioxidants in diabetic conditions, suggesting the antioxidant potential of these substances in the treatment of diabetes [14]. The rotenoid rutin can inhibit oxaliplatin-mediated peripheral neuropathy by attenuating oxidative stress [15]. To date, there have been no reports on the role of deguelin in hyperglycaemia-mediated neuropathic pain. However, on searching the literature, we found reports suggesting the utility of deguelin as an anti-inflammatory agent that acts through inhibiting NF-κB and cyclooxygenase-2; thus, we postulate that deguelin could serve as an important compound in the treatment of DN pain. In the present investigation, we evaluated the action of deguelin in a streptozotocin (STZ)-induced DN rat model and its underlying mechanism of action.

Materials and Methods

Ethics approval and consent to participate

All the animal protocols received prior approval from institutional ethical committee of The First Affiliated Hospital of University of South China with approval number 6780004A. All the animal studies strictly adhered to the draft of Animal protection law of the People's Republic of China-2009 for experimental animals.

Availability of data and materials

The supporting data for present findings is under ethics restrictions and is hence not presented here.

Experimental animals and induction of peripheral diabetic neuropathy

All of the animal protocols followed in this study received prior approval from the Institutional Ethical Committee of The First Affiliated Hospital of University of South China, Hunan Sheng, China (approval number 6780004A). All of the animal experiments strictly adhered to the draft experimental animal protection law of the People's Republic of China (2009). We used male adult Sprague-Dawley rats in this study, with an average weight of 210–230 g. The STZ was procured from Sigma-Aldrich Co. (St. Louis, MO, USA) and was injected intraperitoneally (i.p.; 55 mg/kg) to induce painful DN (n = 72). The control group rats were injected with saline (normal control, n = 12). Three weeks after STZ injection, the plasma glucose levels of the rats were evaluated using a plasma glucose estimation kit (Glucose (GO) assay kit, Sigma-Aldrich). Rats with glucose levels above 13.89 mmol/L were regarded as diabetic. Hyperalgesia, allodynia and neuropathy after 3 weeks were diagnosed based on an earlier study [16]. The STZ-mediated DN rats were divided into six groups, as follows: STZ-control (saline, n = 12); deguelin 4 mg/kg (n = 12); deguelin 6 mg/kg (n = 12); deguelin 8 mg/kg (n = 12); insulin (n = 12); and dimethyl fumarate (BG-12; a potent nuclear respiratory factor 2 [Nrf2] activator; n = 12) groups. The doses of deguelin were in accordance with previous studies [17-19]. Dosing with deguelin was started 3 weeks after injecting STZ; the deguelin was dissolved in saline and administered by oral gavage for 14 days [20]. Similarly, after 3 weeks of injecting STZ, the insulin-treated group received a 2 IU insulin injection daily for 14 days, while the BG-12 group received intraperitoneal saline (15 mg/kg daily for 14 days). At the end of the dosing regimen, biochemical (including blood glucose), electrophysiological, protein and behavioural analyses were performed.

Behavioural assessments

Neurobehavioral tests for nociception and motor coordination were performed on all animals, including cold allodynia, mechanical sensitivity and heat nociception tests. The tests were performed prior to STZ treatment, at 3 days after STZ injection, and after completion of the treatment regimen, namely, after 14 days. In addition, the effect of deguelin on mechanical, heat and cold sensation was recorded at 2 h intervals up to 12 h after the final dose of deguelin.

Test for mechanical sensitivity

The mechanical sensation test was performed using the method previously reported by Vogelaar et al [21].. During the test, rats were kept in a cage with a wire-mesh bottom. A Von Frey filament was applied to the foot pads of the hind limbs at least 10 times, followed by recording of the number of positive responses. Care was taken not to apply the filament to the same site on the paw on successive occasions. Licking, shaking and abrupt paw withdrawals were regarded as positive responses. A significant increase in withdrawal frequency was taken to indicate mechanical hyperalgesia.

Test for heat nociception

The heat nociception test was performed in accordance with a previously described procedure [22]. The nociceptive response to heat was measured using a paw thermal stimulation system. A heat source produced stimulation (46–48°C) on the plantar side of the hind paw; the frequency of paw withdrawal was measured, with particular care taken for rats that failed to withdraw the paw within 20 seconds. In these cases, filament heating was stopped to avoid heat injury. The experiments were performed three times within 5 min.

Test for cold allodynia

The cold allodynia test was performed as previously described [23]. Responses to cold stimuli were assessed by applying cold stimulation; the animals were kept on a stainless steel plate with a temperature of 10°C. The frequency of head shaking or lifting of the hind paw was recorded, and particular care was taken with rats that failed to withdraw the paw within 30 s; in these cases, the cold stimuli was stopped to avoid injury. The cold stimulus was delivered twice, separated by an interval of 5 min.

Measurement of the motor nerve conduction velocity (MNCV)

After completing the behavioural screening experiments, the rats were subjected to sodium pentobarbital anaesthesia (40 mg/kg body weight; intraperitoneal injection, 1.0%). After anaesthesia, MNCV was measured electrophysiologically, as previously described [23]. Briefly, the sciatic (proximal to sciatic notch) and tibial (distal to ankle) nerves were stimulated using a bipolar needle (26.5 gauge; 3 V single stimulus). To detect the motor response, surface electrodes were placed on each paw and connected to a bio-potential coupler placed on the rats paws to detect motor response. The motor response was analysed using an oscilloscope (54600B; Hewlett Packard, Palo Alto, CA, USA) and a physiograph instrument (Biodevices, Ambala Cantt, India). The body temperature of the rats was maintained at 37°C and monitored constantly with a rectal probe digital thermometer. The MNCV was calculated using the following formula:

MNCV = (distance between the sciatic and tibial nerve stimulation point) / (sciatic M wave latency – tibial M wave latency)

After MNCV analysis, the DRG neurons of L4–L6 were removed along with the sciatic nerves and frozen at −80°C for further analysis.

Sodium (Na^+) and potassium (K^+) ATPase [(Na^+-K^+) ATPase] activity

Sciatic nerves were isolated from experimental rats under sodium pentobarbitone anaesthesia (60 mg/kg body weight, i.p. injection). Before excision of each nerve, two ligatures (5-0 silk sutures) were applied, one at the sciatic notch and the other 4 cm distal to the first excision. The epineurial and perineurial membranes were excised followed by application of two ligatures at the centre of the endoneurium. Two endoneurial preparations were formed from the sciatic nerves by bisecting the two ligatures, and incubated at 37°C in Krebs-Henseleit bicarbonate Ringer's solution containing bovine serum albumin (4% w/v), glucose (5 mmol/l) and myo-inositol (0.5 mmol/l) with the O_2:CO_2 ratio maintained at 19:1 (v/v). Homogenates of the sciatic nerves were prepared by immersion in 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 10.0 mM Tris-HCl (pH 7.4) pre-chilled solution in a final volume of 200 µL. After 15 min of pre-incubation at 37°C, the homogenate reaction mixture was initiated by the addition of adenosine 5′-triphosphate (ATP) disodium salt hydrate (3.00 mM; Sigma-Aldrich) followed by incubation for 20 min; for controls, 1.0 mM ouabain was added. A previously described coupled enzymatic method [24] was used to measure the (Na^+-K^+) ATPase activity and the results are presented as NADH (mM) oxidised/h.

Assay for reactive oxygen species

The ROS were measured as previously described [25]. Briefly, The sciatic nerve homogenates were subjected to incubation along with 2, 7-dichlorofluorescein diacetate (100 µM) in the dark for 30 min at temperature. The volume was adjusted with phosphate-buffered saline (PBS; pH 7.4), followed by measurement of fluorescence at emission and excitation wavelengths of 525 and 488 nm, respectively.

Markers of oxidative stress

Oxidative stress markers in sciatic nerve homogenates were measured by assays for antioxidant enzymes, such as superoxide-dismutase (SOD), malondialdehyde (MDA), catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (GPx). All assay kits were from Sigma-Aldrich.

Measurement of hydrogen sulphide in neurons

The concentration of hydrogen sulphide (H_2S) in DRG neurons was evaluated as previously described [25]. Briefly, 100 µl of DRG homogenate supernatant was added to 125 µl of 1% zinc acetate solution and 150 µl of distilled water, followed by the addition of 67 µl of 20 mM N,N-dimethyl-phenylene diamine dihydrochloride solution, prepared in HCl (7.2 M) and 67 µl of $FeCl_3$ solution (30 mM). The absorbance of the resulting reaction mixture was measured at 670 nm. The concentration of H_2S was measured in µM/mg protein.

Immunoblotting studies

The DRG neurons harvested from experimental animals were washed with PBS and subjected to lysis using buffer (Sigma-Aldrich) containing protease inhibitor. The I^{ry} antibodies used were rabbit anti-rat phosphorylated-IκBα (p-IκBα), rabbit anti-rat NF-κB, rabbit anti-rat IκBα, mouse anti-rat heme oxygenase-1

(HO-1), rabbit anti-rat caspase-3 and rabbit anti-rat Nrf2 antibodies. All antibodies were obtained from Sigma-Aldrich. Actin was used as a loading standard.

Evaluation of inflammatory cytokines

Homogenates of DRG neurons were used to measure tumour necrosis factor-alpha (TNF-α) levels. The homogenates were subjected to ELISA using kits from Sigma-Aldrich. The developed plates were read at 450 nm using an ELISA reader (BioTek, Winooski, VT, USA).

Statistical analysis

The results in the present study are represented as mean ± percent relative standard deviation (%RSD). One-way ANOVA was performed to compare groups using GraphPad Prism software (GraphPad Software, San Diego, CA, USA), with Tukey's post hoc test applied. P-values <0.05 were regarded as significant.

Results

Deguelin attenuates elevated plasma glucose levels

Deguelin attenuated elevated blood glucose levels in diabetes-induced rats. The mean blood glucose level in the STZ-control group was 24.12 ± 1.45 mmol/l, which was significantly elevated compared with that of the normal control group (3.45 ± 0.45 mmol/l). The plasma glucose levels decreased significantly in the deguelin-treated groups, to 17.45 ± 1.10, 14.35 ± 0.85 and 13.25 ±0.75 mmol/l with deguelin doses of 4, 6 and 8 mg/kg, respectively (P<0.05). The rats injected with insulin showed a significantly lower plasma glucose level, of 5.98 ± 0.24 mmol/l, than the control and deguelin-treated rats (P<0.01). The BG-12-treated group showed a non-significant decrease in mean plasma glucose level (23.1 ± 1.85 mmol/l). The results are presented in Fig. 1.

Deguelin reduces hyperalgesia/allodynia and diabetic neuropathy in rats

Cold allodynia was induced in rats injected with STZ, with mechanical hyperalgesia and heat hyperalgesia seen after 10 days of STZ injection (Fig. 2A–C). After 14 days, the STZ-control group showed a significant elevation in the number of paw withdrawal responses compared with normal control rats following mechanical stimulation with a bending force of 13.2 mN, thus confirming mechanical hyperalgesia (P<0.05; Fig. 2A). The paw withdrawal frequency against cold and heat stimuli in the STZ-control group was significantly reduced compared with that of the normal control group, confirming the induction of cold allodynia and heat hyperalgesia in DN rats (P<0.05; Fig. 2B, C).

We found that deguelin treatment in DN rats attenuated mechanical hyperalgesia, cold allodynia and heat hyperalgesia. A dose-dependent reduction in the number of paw withdrawal responses against mechanical stimuli was observed in rats treated with deguelin at 4, 6 and 8 mg/kg; the reduction persisted for up to 8 h, suggesting that deguelin can attenuate mechanical hyperalgesia in STZ-induced DN rats (P<0.05; Fig. 2A). In the experiment involving heat and cold stimuli, deguelin partially reversed heat hyperalgesia and cold allodynia in a dose-dependent manner (P<0.05; Fig. 2B, C). The effects of deguelin persisted for about 8 h in the 4 mg/kg- and 6 mg/kg-treated groups, compared with 10 h in rats receiving a deguelin dose of 8 mg/kg. In addition, the decreased paw withdrawal frequency in response to cold, heat and mechanical stimuli was partially suppressed

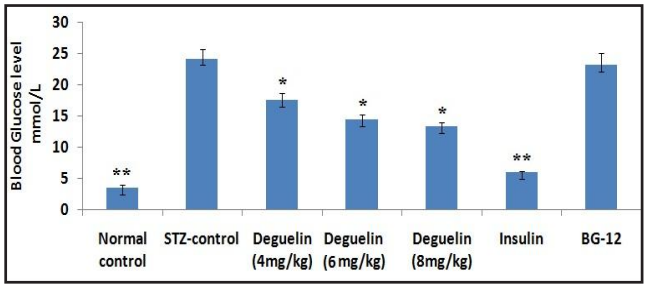

Fig. 1. Deguelin reduced elevated blood glucose levels in diabetes induced rats. (*P<0.05, **P<0.01 compared to STZ-control rats).

in rats after 4 h of treatment with insulin and BG-12 (P<0.05). Deguelin at 8 mg/kg showed better results in terms of the response to cold, hot and mechanical stimuli than both insulin and BG-12, confirming the superior ability of deguelin to ameliorate DN versus insulin and BG-12.

Deguelin increases motor and sensory nerve conduction velocities in diabetic neuropathy rats

The conduction velocities of sensory and sciatic motor nerves in diabetic rats were significantly lower than in normal rats following deguelin administration, confirming the presence of impaired nerve conduction in DN rats (both P<0.05; Fig. 3A, B). Deguelin administration for 14 days at all three doses (4, 6 and 8 mg/kg) significantly increased the conduction velocities of sensory and motor nerves in DN rats compared with those in the control animals (both P<0.05; Fig. 3A, B). Deguelin at 8 mg/kg showed superior results to both the 4 and 6 mg/kg regimens (P<0.05), confirming a dose-dependent treatment effect. Furthermore, treatment with both insulin and BG-12 was associated with a significant increase in the conduction of sciatic sensory and motor nerves in DN rats (both P<0.05; Fig. 3A, B).

Deguelin increases activity of (Na^+-K^+) ATPase and suppresses the expression of caspase-3 in diabetic neuropathy rats

In the present investigation, deguelin increased (Na^+-K^+) ATPase activity in sciatic nerves and suppressed caspsase-2 in the DRG neurons of diabetes-induced rats (Fig. 4A, B; P<0.05).

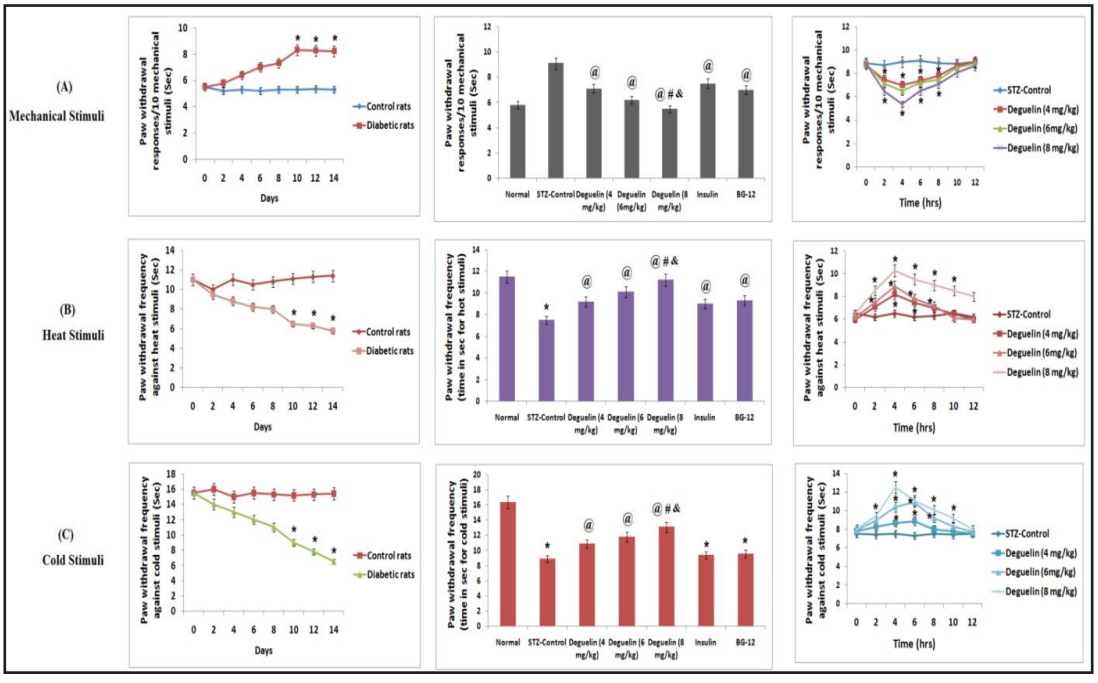

Fig. 2. Deguelin alleviated diabetes mediated mechanical hyperalgesia, cold allodynia and heat hyperalgesia in diabetes induced rats. A: Paw withdrawal frequency increased after induction of diabetes, The treatment of Deguelin (8mg/kg) resulted in significant suppression of number of paw withdrawals compared to STZ-control ($^{@}$P<0.05), Deguelin at the dose 4 mg/kg ($^{\#}$P<0.05), Insulin and BG-12 treatment ($^{\&}$P<0.05) (one way ANOVA followed by Turkeys test) suggesting a dose dependent effect of Deguelin. The treatment of Insulin and BG-12 showed significant effects compared to STZ-control ($^{@}$P<0.05 compared to STZ control). B and C: The induction of diabetes decreased paw withdrawal frequency against heat and cold stimuli. Significant improvement in number of paw withdrawal responses to heat and cold stimuli was seen in rats receiving treatment of 8 mg/kg suggesting a dose dependent effect of Deguelin in diabetic neuropathic rats.

Rats treated with deguelin at 4 mg/kg showed a non-significant increase in (Na^+-K^+) ATPase activity and lower expression of capsase-3; however, the results were significant with doses of 6 and 8 mg/kg (both P<0.05; Fig. 4B). These results suggested that deguelin attenuated nerve damage in diabetes-induced rats in a dose-dependent manner. In the rats receiving insulin and BG-12, (Na+-K+) ATPase activity was enhanced and casapase-3 was suppressed (both P<0.05; Fig. 4A, B).

Deguelin reduced oxidative stress and formation of reactive oxygen species in diabetic rats

The diabetic rats showed elevated ROS and MDA levels, but this was dose-dependently attenuated by deguelin (Fig. 5A). Important markers of oxidative stress were evaluated and inhibition of antioxidant enzymes, such as SOD, GST, CAT and GPx, was seen in the sciatic nerves of the diabetes-induced rats (all P<0.05; Fig. 5C–F).

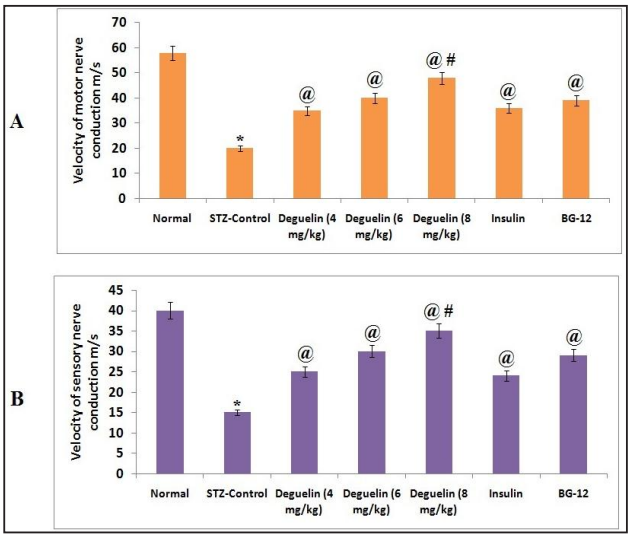

Fig. 3. Deguelin attenuates conduction velocities of both motor and sensory nerves in diabetic rats. Treatment of Deguelin increased the conductance velocities in motor neurons (A) and in sensory neurons (B). Insulin and BG-12 also enhanced the conduction velocities in both motor (A) and sensory nerves (B) in diabetes induced rats. The results showed that the effect of insulin and BG-2 treatment were significantly lower compared to rat's receiving Deguelin 8 mg/kg. *P<0.05 compared to normal rats, @P<0.05 compared to STZ-control rats, #P<0.05 compared to Deguelin 4mg/kg.

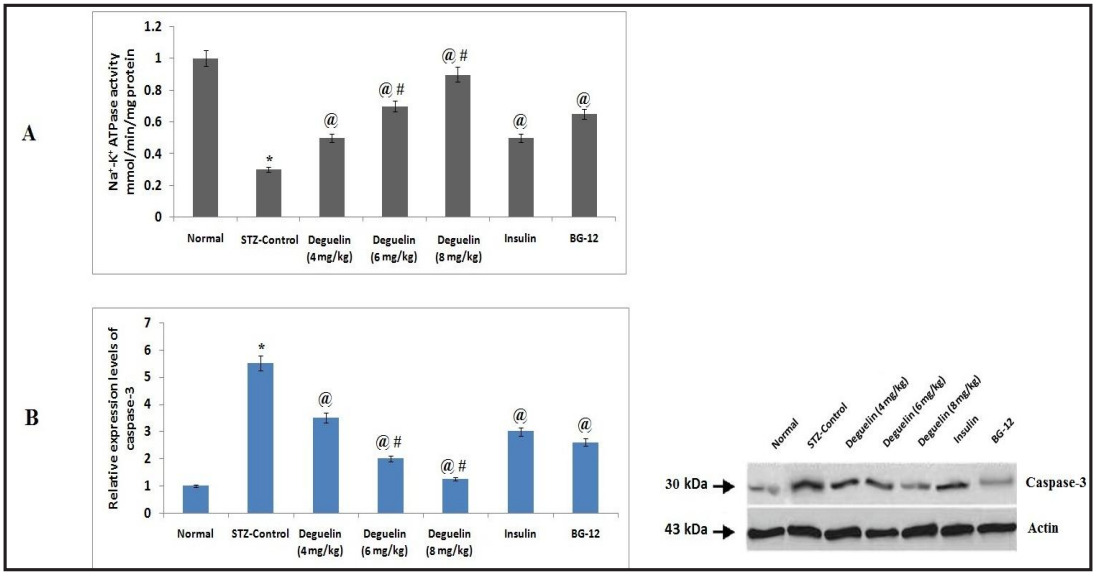

Fig. 4. effect of Deguelin on expression levels of caspase-3 in DRG neurons and Na+, K+-ATPase activity in sciatic nerves. Deguelin resulted in increased Na+, K+-ATPase activity in sciatic nerves (A) and suppressed the levels of caspase-3 in DRG neurons (B). Insulin and BG-12 caused increase in Na+, K+-ATPase activity (A) and suppressed levels of capase-3 in diabetic rats (B) compared to STZ-control (@P<0.05). Deguelin at dose of 6 and 8 mg/kg resulted in significant increase in activity of Na+, K+-ATPase (A) and suppressed levels of caspase-3 (B) compared to STZ control (@P<0.05) and Deguelin 4 mg/kg (#P<0.05).

Partial reversal of the activities of SOD, GST, CAT and GPx was seen after 14 days of treatment with deguelin. The antioxidant enzyme activity increased in all three treatment groups; the significant increases seen with deguelin at doses of 6 and 8 mg/kg suggested a beneficial action of this agent in terms of restoring impaired antioxidant defence systems. Treatment with insulin and BG-12 suppressed the levels of both MDA and ROS, and increased the levels of antioxidant enzymes in diabetic rats.

Deguelin increases H_2S and Nfr2/HO-1 levels in diabetic rats

Deguelin treatment for 14 days elevated H_2S levels in the DRG neurons of diabetes-induced rats ($P<0.05$; Fig. 6A). Since the effect of H_2S on oxidative stress involves the Nrf2-dependent pathway, we evaluated the effect of deguelin administered to diabetes-induced rats on the levels of HO-1 and Nrf2; both showed significant reductions ($P<0.05$; Fig. 6B). With 8 mg/kg deguelin treatment for 14 days, the diabetes-induced rats showed increased levels of HO-1 and Nrf2 (both $P<0.05$; Fig. 6B, C). Furthermore, insulin and BG-12 increased the levels of Nrf2 and HO-1 in the DRG neurons of the rats (both $P<0.05$; Fig. 6B, C); however, the attenuating effect of 8 mg/kg deguelin was superior, suggesting partial involvement of the Nrf2/HO-1 pathway in DN.

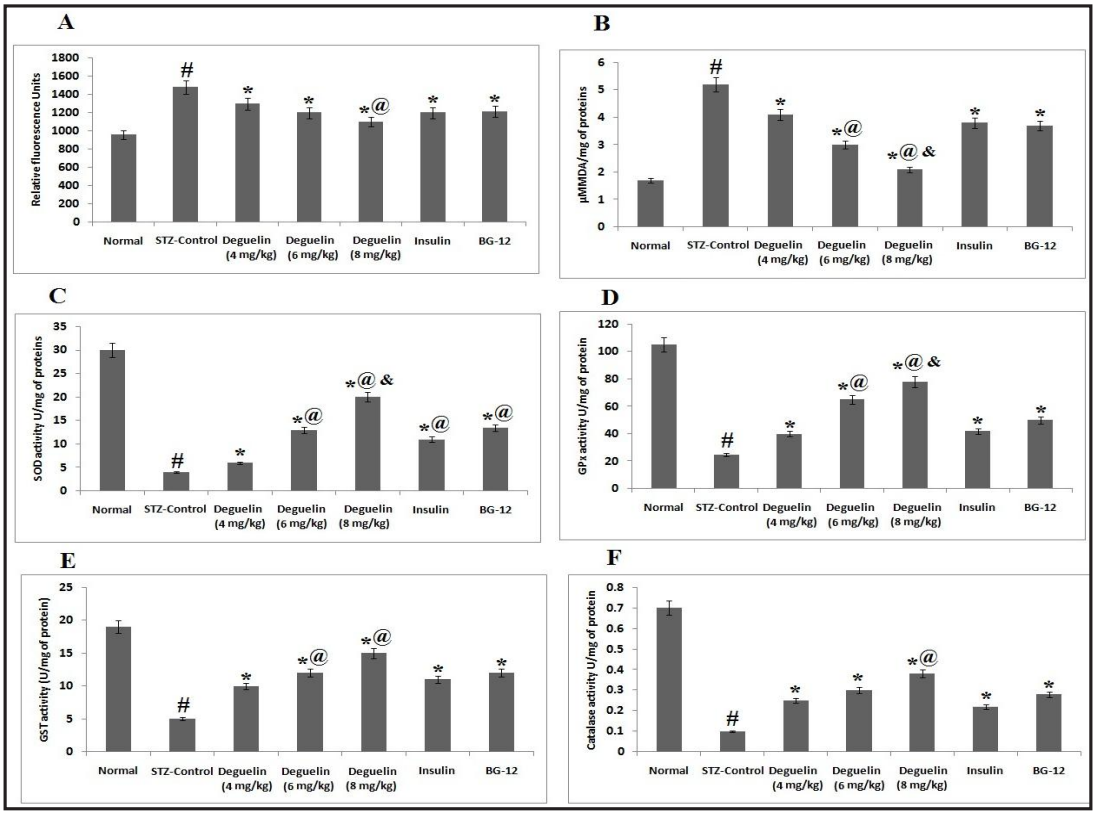

Fig. 5. Effect of Deguelin on oxidative stress in diabetes induced rats. Treatment of Deguelin suppressed levels of ROS (A) and MDA (B) and enhanced activity of antioxidant enzymes SOD (C), GPx (D), GST (E) and Catalase (F) in diabetes induced rats. The treatment of insulin and BG-12 caused significant suppression of ROS and MDA nad caused elevation in activity of antioxidant enzymes SOD (C), GPx (D), GST (E) and catalase (F). Deguelin at dose of 8 mg/kg produced strong suppression of both ROS and MDA (A and B) and resulted in increased antioxidant enzyme activities compared Deguelin at dose of 4 and 6 mg. The results of Deguelin at 8 mg/kg were better than rats receiving Insulin and BG-12. ($^{\#}P<0.05$ compared to control, $^{*}P<0.05$ compared to STZ-control, $^{@}P<0.05$ compared to Deguelin 4 mg/kg, $^{\&}P<0.05$ compared to Insulin and BG-1 treated)

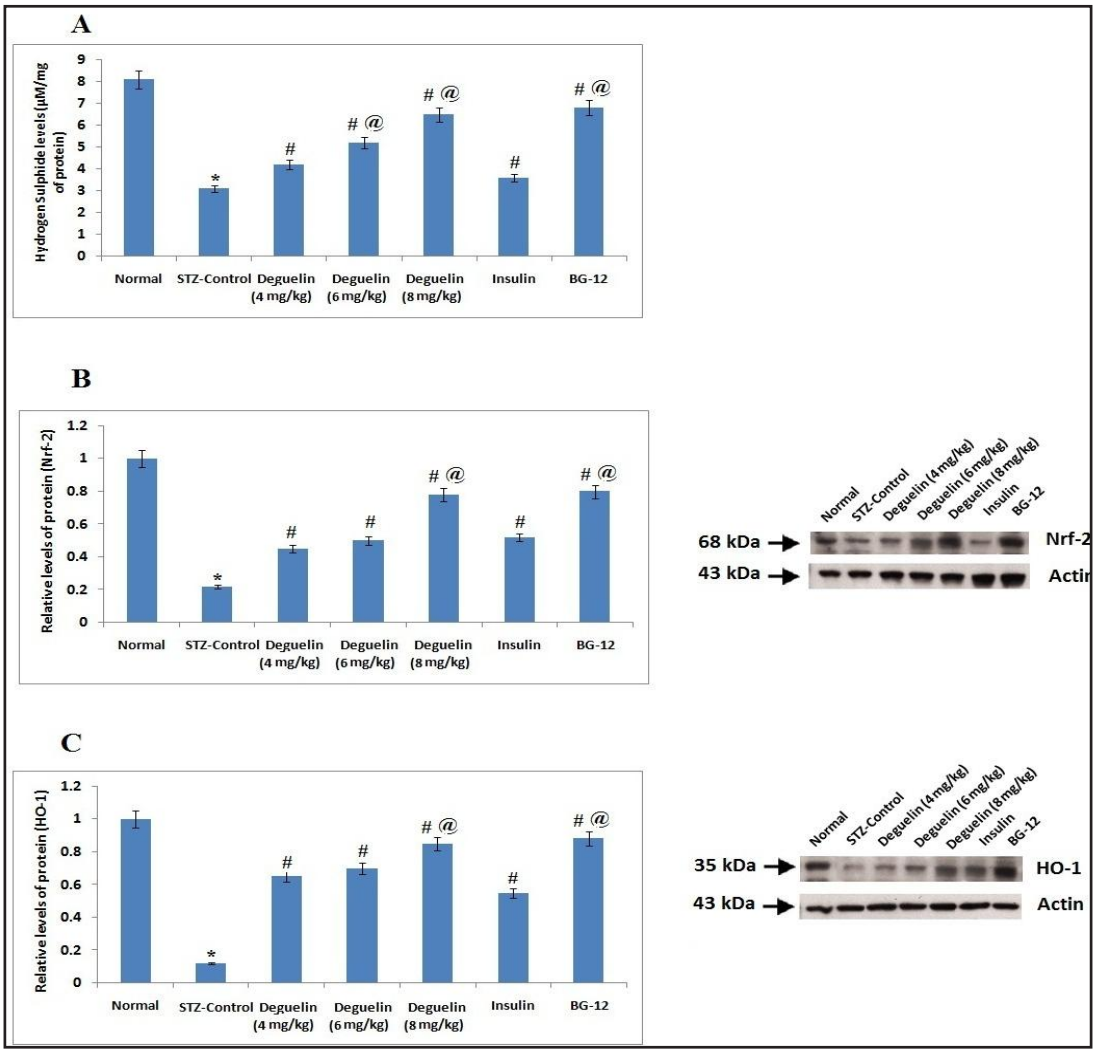

Fig. 6. Effect of Deguelin on levels of H_2S, HO-1 and Nrf-2 in DRG neurons in diabetes induced rats. Deguelin increased levels of H_2S (A), Brf-2 (b) and HO-1(C) in DRG nerves of diabetes induced rats, similarly treatment of Insulin and BG-12 also showed protective effect and unregulated levels of H_2S, Nrf2 and HO-1. The dose of Deguelin 8 mg/kg and BG-12 produced similar effect on levels of Nrf-2 and HO-1 (*P<0.05 compared to normal rats, #P<0.05 compared to STZ-control rats, @P<0.05 versus Deguelin 4 mg/kg.

Deguelin attenuated neuroinflammation in diabetic rats

The diabetic rats showed elevated levels of NF-κB, p-IκBα and IκBα in DRG neurons compared with normal rats (all P<0.05; Fig. 7A–D), suggesting the presence of neuroinflammation in diabetes. Treatment with deguelin suppressed NF-κB, p-IκBα and IκBα levels compared with those in diabetic control rats, indicating attenuation of neuroinflammation, which is important feature of DN. The levels of NF-κB, p-IκBα and IκBα were significantly reduced in diabetic rats treated with 8 mg/kg deguelin versus those in rats receiving 4 mg/kg (all P<0.05; Fig. 7), suggesting a dose-dependent effect. Both insulin and BG-12 suppressed the levels of NF-κB, p-IκBα and IκBα in DRG neurons compared with those in diabetic control rats (all P<0.05; Fig. 7). Deguelin at a dose of 8 mg/kg showed higher potency in suppressing the levels of NF-κB, p-IκBα and IκBα compared with insulin and BG-12 (all P<0.05). The levels of TNF-α and interleukin (IL)-6, which are important markers of diabetic neuroinflammation, were elevated in diabetic rats. Deguelin attenuated the increased levels of TNF-α and IL-6 in a dose-dependent manner (both P<0.05; Fig. 7). Rats treated with insulin and BG-12 also showed significantly lower TNF-α and IL-6 levels in DRG

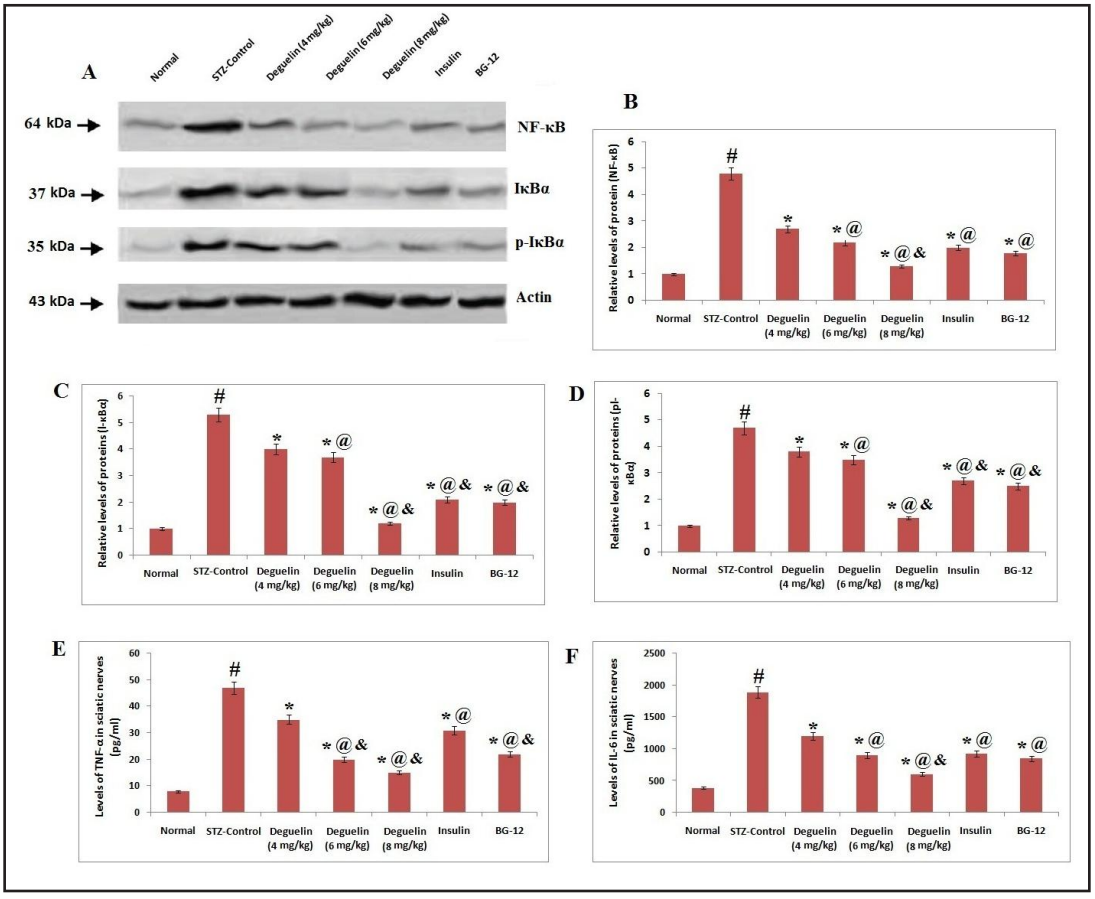

Fig. 7. Deguelin attenuates neuroinflammation in diabetes induced rats. Treatment of Deguelin suppressed the levels of NF-κB, I-κBα, p-I-κBα (A-D) in DRG neurons along with levels of inflammatory cytokines (IL-6 and TNF-α) (E-F). Insulin and BG-12 suppressed levels of NF-κB, I-κBα, p-I-κBα, IL-6 and TNF-α. Deguelin at dose of 8 mg/kg demonstrated significant effect by decreasing the levels of neuroinflammatory markers in dose dependent manner. #P<0.05 compared to normal rats, *P<0.05 compared to STZ-control rats, @P<0.05 compared to Deguelin 4 mg/kg treated, &P<0.05 compared to Deguelin 6 mg/kg treated rats.

neurons (both P<0.05), but deguelin at 8 mg/kg showed the most promising suppressant effects (P<0.05; Fig. 7).

Discussion

In the present research, deguelin showed a protective effect against diabetic neuropathic pain in diabetic rats. A previous rat study confirmed the suppressive effects of deguelin on pro-inflammatory markers, such as CXCR4 and ICAM-1 [26]. In the present work, deguelin attenuated heat hyperalgesia, mechanical hyperalgesia and cold allodynia in diabetes-induced rats, suggesting an antinociceptive effect in painful DN. Previous studies showed that DN is accompanied by decreased (Na^+-K^+) ATPase activity in sciatic nerves, and is also associated with reduced capsase-3 levels in DRG neurons [27]. We found that deguelin significantly increased (Na^+-K^+) ATPase activity and suppressed the expression of caspase-3 in the DRG neurons of diabetic rats, corresponding to minimisation of nerve damage and neuronal apoptosis. Furthermore, deguelin improved the conduction velocity of sensory and motor nerves in DN rats, thus confirming its potential utility for treating damaged nerves in diabetes-induced rats.

A previous study showed that chronic hyperglycaemia was the major cause of reduced sensory and motor nerve conduction velocities, and of the other symptoms associated with peripheral DN [28]. In the present research, deguelin was administered to rats after the successful induction of nociceptive DN, that is, after 21 days of STZ injection. We found that deguelin decreased plasma glucose levels and the nociceptive response in DN. In the experiment on plasma glucose levels, insulin had a superior glucose-lowering effect than deguelin (at both 4 and 8 mg/kg) in diabetic rats; however, the glucose-lowering effect of insulin was not associated with the amelioration of DN pain. The outcome with deguelin at 8 mg/kg was superior to that of insulin in terms of ameliorating DN. Therefore, we suggest that other mechanisms aside from elevated glucose levels may be responsible for the attenuation of DN pain by deguelin.

Oxidative stress has been identified as a major factor in the nerve damage seen in diabetes, which in turn causes the abnormal pain impulses that characterise DN [3]. Diminished antioxidant capacity and high oxygen consumption render nerve tissues susceptible to oxidative attack [29]. Another report showed that minimising oxidative injury to nerve tissues attenuated DN pain in animal models [29]. In the present research, deguelin effectively suppressed oxidative stress in diabetes-induced rats, as confirmed by decreases in the levels of both MDA and ROS. We believe that the reduction of oxidative stress in the sciatic nerves may have been responsible for the antinociceptive effect of deguelin in DN rats.

The literature suggests that H_2S possesses ROS scavenging activity and can activate the endogenous antioxidant system [30]. We estimated the levels of H_2S in diabetic and deguelin-treated rats; deguelin significantly increased H_2S levels in diabetes-induced rats, suggesting a possible role for H_2S in activating the antioxidant defence system. A previous study reported involvement of the Nrf2-dependent pathway in the effects of H_2S against oxidative stress [31]. Therefore, we measured the levels of both HO-1 and Nrf2 in DRG neurons; both were markedly increased in diabetes-induced rats after treatment with deguelin. To confirm involvement of the Nrf2 pathway in the effects of deguelin on DN, we included a BG-12-treated (15 mg/kg) group in this study. BG-12 showed minimal effects on the blood glucose levels of rats. Deguelin at 8 mg/kg showed a similar ability to activate the Nrf2 pathway to that of BG-12, suggesting partial involvement of the Nrf2 pathway in the favourable effect of deguelin against DN.

Previous research suggested the involvement of hyperglycaemia-mediated classical pathways, such as the AGE, polyol and PKC pathways, along with oxidative stress, in activating NF-κB [3]. NF-κB activity is considered important in nerve damage and upregulation of the expression of proinflammatory cytokines [3]. Furthermore, prolonged hyperglycaemia-mediated inflammation causes alterations in the structure of neurons [4]. Neuroinflammation is the main cause of painful DN, resulting in both functional and structural damage to the peripheral neurons [32]. In the present investigation, increased levels of inflammatory cytokines (TNF-α and IL-6), as well as NF-κB, were seen in the DRG neurons of diabetes-induced rats. Deguelin inhibited the activation of NF-κB and suppressed the levels of both TNF-α and IL-6 in DRG neurons, thus suggesting that deguelin could supress neuroinflammation and attenuate DN.

It has also been established that inflammatory pathways and mechanisms of oxidative stress interact at different stages in the DN disease process, exacerbating diabetes-induced neuronal damage [3]. ROS has been shown to activate kinases, leading to phosphorylation of IκB. Poli et al [33]. found that NF-κB generates TNF-α and IL-6 expression. Activation of NF-κB suppresses antioxidant genes by inhibiting the Nrf2 pathway, thereby disrupting the antioxidant defence system [34, 35]. In the present study, DN was induced in diabetic rats, along with activation of both the Nrf2 and NF-κB pathways. Treatment with deguelin inhibited the Nrf2 and NF-κB pathways in the DRG neurons of diabetic rats, which is important in the development of DN [8]. Our findings establish a link between neuroinflammation and oxidative stress, and shed light on the possible mechanism underlying the effect of deguelin on pain-associated DN.

Conclusion

In the present study, deguelin ameliorated DN by improving nociception and the conduction velocities of motor and sensory nerves. The effects of deguelin against DN may be attributable to its ability to lower plasma glucose levels, suppress oxidative stress, improve the antioxidant defence system and inhibit neuroinflammation. Our results confirm the potential utility of deguelin in the management of painful DN, but more detailed studies including clinical observations are necessary.

Abbreviations

DN (Diabetic neuropathy); Nrf2: Nuclear (factor-E2-related factor-2); HO-1: Heme (oxygenase-1); NF-κB: Necrosis (factor kappa B); STZ: Streptozotocin.

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Disclosure Statement

The authors declare no competing interests.

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