Protective effect of oryzanol isolated from crude rice bran oil in experimental model of diabetic neuropathy

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Abstract: Several studies have implicated the involvement of poor glycemic control and oxidative/nitrosative stress in the development of diabetic neuropathic pain, an important microvascular complication affecting more than 50% of diabetic patients. However, lack of understanding of the underlying etiology, development of tolerance, inadequate relief and possible toxicity associated with classical analgesics warrant the investigation of the novel agents. Therefore, the present study was carried out to investigate the effect of oryzanol (OZ), a commercially-important potent antioxidant component isolated from crude rice bran oil (cRBO), in streptozotocin (STZ)-induced diabetic neuropathy in rats. After eight weeks, diabetic rats developed neuropathy which was evident from decreased tail-flick latency (thermal hyperalgesia) and increased nociceptive behavior during the formalin test. This was accompanied by decreased motor coordination based on the evaluation of neuromuscular strength. Na⁺ K⁺ ATPase, a biochemical marker associated with the development of diabetic neuropathy, was significantly inhibited in the sciatic nerve of diabetic animals. The activities of antioxidant enzymes and lipid peroxidation levels were significantly elevated in diabetic rats, indicating the involvement of oxidative stress in diabetic neuropathy. Chronic treatment with oryzanol (OZ) (50 and 100 mg/kg) per oral (p.o.) and standard drug glibenclamide (Gl) (10 mg/kg, p.o.) significantly attenuated the behavioral as well as biochemical changes associated with diabetic neuropathy. The findings provide experimental evidence to the protective effects of OZ on hyperglycemia-induced thermal hyperalgesia and oxidative stress which might be responsible for diabetes induced nerve damage.

Keywords: diabetic neuropathy hyperalgesia oryzanol oxidative stress

Introduction

Peripheral diabetic neuropathy (DN), the most common and debilitating clinical complication of long-standing diabetes mellitus, affects more than 50-60% of diabetic patients and is the leading cause of clinically significant morbidities such as pain, decreased motility, foot ulcers, non-traumatic limb amputation and anatomic failure (Sharma et al., 2008; Edwards et al., 2008). Although the pathogenesis is believed to be multifactorial with differing underlying neuroanatomical, neurophysiologic and neurochemical mechanisms (Edwards et al., 2008; Gidal & Billington, 2006), the duration and severity of hyperglycemia is considered as a universal trigger for the development and progression of DN as well as the other microvascular complications of diabetes such as diabetic nephropathy, neuropathy, and retinopathy (Dobretsov et al., 2007). Several evidences implicate that hyperglycemia triggers various pathogenetic pathways leading to increased glucose flux through the polyol pathway resulting in accumulation of sorbitol and fructose; increased hexosamine pathway flux; excess/inappropriate activation of protein kinase C (PKC) isoforms thereby initiating a cascade of stress responses; non-enzymatic glycation of proteins yielding “advanced glycation end-products” (AGE) that disrupt neuronal integrity and repair mechanisms and activation of downstream pathways such as mitogen activated protein kinases (MAPK) and poly ADPribose polymerase (PARP) (Sharma et al., 2008; Vincent et al., 2004). Each of these pathways are intertwined and are collectively responsible for causing an imbalance in the mitochondrial redox state of the cell and an increased production as well as decreased scavenging of reactive oxygen species (ROS) resulting in cellular oxidative stress, which if not controlled, leads to a confluence of metabolic and vascular imbalances, including impaired neural function, reduced nerve conduction velocity, loss of neurotrophic support, and apoptosis of neurons and Schwann cells, leading to nerve damage (Kuhad & Chopra, 2009; Feldman, 2003). Hence, oxidative stress
may be one of the major pathways involved in the development of DN and an antioxidant can potentially prevent or reverse hyperglycemia-induced nerve dysfunctions. Several antioxidants such as α-lipoic acid, taurine, acetyl-L-carnitine, M40403, β-carotene and tocotrienols have been reported to ameliorate nerve function in experimental DN, thus confirming that attenuating oxidative stress may inhibit the development of the complication (Kuhad & Chopra, 2009; Sayyed et al., 2006).

Effective disease modifying therapies for DN are currently limited and unsatisfactory on account of their partial effectiveness and associated side effects (Pabreja et al., 2011). In addition, the medical cost associated with diabetes is unsustainable (IDF, 2011). Therefore, renewed interest has been generated in search for more affordable agents from natural origin that retain the therapeutic efficacy and are devoid of side effects.

In the light of this perspective, rice bran oil (RBO) has been the focus of attention because of its balanced fatty acid profile and due to its rich source of bioactive anti-oxidative polyphenols such as, ferulic acid, its esterified derivatives such as OZ, tocopherols, tocotrienols and other associated phenolic compounds (Sugano et al., 1999). OZ is a mixture of ferulic acid (4-hydroxy-3-methoxycinnamic acid) esters with phytosterols (Lerma-Garcia et al., 2009) and primarily extracted from rice bran. A number of therapeutically useful biological activities have been reported for OZ, such as reduction of cholesterol levels, modulation of the pituitary secretion, inhibition of the gastric acid secretion, antioxidant action and inhibition of the platelet aggregation (Ghatak & Panchal, 2011). Moreover, OZ has been shown to be very safe with no major side effects being reported in either animal or human studies (Ghatak & Panchal, 2011).

Furthermore, although previously conducted research has established the hypoglycemic potential of OZ by virtue of its regulation of adiponectin and insulin secretion and hepatic glucose-regulating enzyme activities (Ohara et al., 2009; Son et al., 2011), the physiological role of OZ in relation to chronic diabetic complications in experimental animal models is not fully established and requires extensive research. Therefore, the present study was designed to investigate the effect of OZ, a potent free radical scavenger, on nociception and oxidative stress in STZ-induced DN model in rats.

Materials and Methods

Plant material

Rice bran was procured from Suryodaya Rice Mills, Ahmedabad, Gujarat, India through their milling process.

Extraction of RBO

The extraction of crude RBO from fresh rice bran and its subsequent analysis for the various physicochemical parameters, such as organoleptic characters, specific gravity, viscosity, moisture content, saponification value, unsaponifiable matter, wax content, iodine value, acetyl value, acid value, hydroxyl value, ester value and peroxide value using various standard official methods have been published previously (Ghatak and Panchal, 2010).

Isolation of OZ from cRBO

The isolation of OZ from cRBO using a modified two-step crystallization process previously described by Zullaikah et al. (2009) has been discussed previously (Ghatak & Panchal, 2012a). The isolated OZ was identified with respect to the standard by melting point determination, thin-layer chromatography (TLC), UV-visible spectrophotometry and high-performance liquid chromatography (HPLC), the results of which have been published recently (Ghatak & Panchal, 2012b).

Experimental animals

Adult wistar rats of either sex weighing 250-300 g were maintained at controlled temperature as well as humidity and fed with standard diet and water provided ad libitum. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Institute of Pharmacy, Nirma University, as per the guidance of committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Dose calculation

Considering the typical daily dosage of OZ as 500 mg/day in humans (Drug Information Online, 2012), test doses for rats were selected as 50 and 100 mg/kg b.w., p. o. based on the dose translation formula on the basis of the body surface area (Reagan-Shaw et al., 2007).

Drugs and reagents

A pure sample of OZ, as reference standard, was purchased from Tokyo Chemical Industry Co.,
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Ltd. Tokyo, Japan. STZ was purchased from Sigma (St. Louis, MO, USA). Glibenclamide was obtained as a gift sample from Torrent Research Centre, Ahmedabad, India. OZ and glibenclamide were freshly prepared by dissolving in double distilled water after triturating with 4% Tween 80. A glucose oxidase peroxidase (GOD-PAP) diagnostic enzyme kit was purchased from Lab Care Diagnostics (India) Pvt. Ltd. All the reagents and chemicals used in the present study were of analytical grade.

Induction and assessment of diabetes

A single dose of 45 mg/kg STZ prepared in citrate buffer (pH 4.5, 0.1 M) was injected intravenously to induce diabetes. The age-matched control rats received an equal volume of citrate buffer. Diabetes was confirmed after 48 h of STZ injection, the blood samples were collected through retro-orbital plexus and plasma glucose levels were estimated by enzymatic glucose oxidase- peroxidase (GOD/POD) diagnostic kit method. The rats having plasma glucose levels >250 mg/dL (Kuhad et al., 2008) were used for the present study. STZ, a β-cytotoxin, irreversibly enhances the glycosylation of the pancreatic islet O-linked protein in a dose dependent manner and also inhibits the action of N-acetylglucosaminase (O-GlcNAcase) enzyme that removes O-linked N-acetylgalosamine (O-GlcNAc) from proteins thereby contributing to its diabetogenic toxicity (Konrad et al., 2001). General parameters such as body weight, food and water intake and plasma glucose levels were measured before and at the end of the experiment in order to assess the effect of OZ on these parameters.

Treatment protocol

Control and diabetic rats were randomly selected and divided in eight groups of eight animals each. Group 1 animals served as the non-diabetic controls (NC) and received a single vehicle injection of citrate buffer (pH 4.5, 0.1 M) and 4% Tween 80. Group 2, 3 and 4 comprised of control treated animals and received OZ 50 mg/kg (CT-O50), OZ 100 mg/kg (CT-O100) and standard Glibencamide 10 mg/kg (CT-G10) respectively p.o. Group 5 consisted of the diabetic control (DC) and the animals received Tween 80 vehicle p.o. Group 6, 7 and 8 consisted of diabetic animals treated with OZ (50 mg/kg/day, p.o.) (DT-O50), OZ (100 mg/kg/day, p.o.) (DT-O100) and standard Glibencamide (10 mg/kg/day, p.o.) (DT-G10) respectively. All administrations were carried out between 9 am and 11 am daily. Eight weeks following STZ injection, the animals were sacrificed under deep anesthesia, blood was collected from the retro-orbital plexus and plasma was separated. Sciatic nerves were rapidly removed and weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). Homogenates were centrifuged at 200 g for 10 min at 4 °C and supernatant was used for the estimation of Na+-K+ ATPase, lipid peroxidation, reduced glutathione, superoxide dismutase catalase and total nitric oxide assay. The samples were stored at -20 °C until processed for biochemical estimations.

Behavioral assessment

Evaluation of nociceptive threshold

Tail-immersion (hot water) test: tail of rat was immersed in a hot water bath (52.5±0.5 °C) until tail withdrawal (flicking response) or signs of struggle were observed (cut-off time 12 s). Shortening of the tail withdrawal time indicates hyperalgesia and is attributed to central mechanisms (Kannan et al., 1996; Ramabadran et al., 1989).

Tail-immersion (cold water) test: tail of rat was immersed in cold water (10±0.5 °C) until tail withdrawal (flicking response) or signs of struggle were observed (cut-off time 15 s). Shortening of the tail withdrawal time indicates allodynia (Attal et al., 1990).

Hot-plate test: the hyperalgesic response on the hot-plate is considered to result from a combination of central and peripheral mechanisms (Kannan et al., 1996). In this test, animals were individually placed on a hot-plate (Eddy’s Hot-Plate) with the temperature adjusted to 55±1 °C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold; the cut-off time was 10 s in order to avoid damage to the paw.

Formalin induced flinching

The rats were briefly allowed to acclimatize in open Plexiglas observation chambers for 30 min before injecting the nociceptive stimuli. Age-matched control and diabetic animals were gently restrained and injected with 50 μL of 0.5% formalin solution into the dorsal surface of the right hind paw with a 26-gauge needle. Animals were transferred back to the chambers and nociceptive behavior was observed (cut-off time 12 s). All administrations were carried out between 9 am and 11 am daily. Eight weeks following STZ injection, the animals were sacrificed under deep anesthesia, blood was collected from the retro-orbital plexus and plasma was separated. Sciatic nerves were rapidly removed and weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). Homogenates were centrifuged at 200 g for 10 min at 4 °C and supernatant was used for the estimation of Na+-K+ ATPase, lipid peroxidation, reduced glutathione, superoxide dismutase catalase and total nitric oxide assay. The samples were stored at -20 °C until processed for biochemical estimations.
Na\textsuperscript{+}-K\textsuperscript{+} ATPase activity was measured in the sciatic nerve tissue homogenate by the method described by Katewa & Katyare (2003). The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Ohkawa et al. (1979) and expressed as nanomols of malondialdehyde per milligram of protein. Tissue protein estimation was carried out according to the method of Lowry et al. (1951) using bovine serum albumin as the standard. Reduced glutathione (GSH) levels and the activities of various anti-oxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) were assayed in the tissue homogenate as previously established methods (Moron et al., 1979; Misra & Fridovich, 1972, Aebi, 1983). In vitro quantitative measurement of nitrite level in the sciatic nerve tissue was carried out by the method described by Griess (1879).

Statistical analysis

All the values were expressed as mean±SEM. Statistics was applied using Graph Pad Prism version 5.0 for Windows, Graph Pad software, San Diego, California, USA. One way ANOVA followed by Tukey’s multiple comparison test was used to determine the statistical significance between various groups. Differences were considered to be statistically significant when \( p<0.05 \).

Results

Effect of OZ on body weight, food and water intake, and blood glucose level in rats

At the end of eight weeks of treatment, STZ produced a significant \( (p<0.05) \) decrease in body weight (Figure 1) and a significant \( (p<0.05) \) increase in food and water intake (Table 1) in diabetic rats as compared to the control animals. Treatment with OZ (50 and 100 mg/kg/day, p.o.) and standard GI (10 mg/kg/day, p.o.) for eight weeks showed a significant \( (p<0.05) \) increase in body weight and a significantly \( (p<0.05) \) reduced intake of food and water. Further treatment with OZ and GI did not produce any significant changes in body weight, food and water intake in control rats.

STZ treated rat exhibited a significantly \( (p<0.05) \) increased serum glucose level as compared to control rats. Chronic treatment with OZ (50 and 100 mg/kg/day, p.o.) and standard GI (10 mg/kg/day, p.o.) did not produce any significant change in serum glucose level in control rats.

Effect of OZ treatment on nociceptive threshold

The nociceptive threshold was significantly \( (p<0.05) \) lower in diabetic rats compared to the basal values tested in both the tail-immersion (hot and cold water tests) (Figure 3a and b) and hot-plate assays (Figure 3c). Hyperalgesia was manifested in the tail-immersion and hot-plate tests after one and two weeks, respectively. OZ (50 and 100 mg/kg/day, p.o.) and standard GI (10 mg/kg/day, p.o.) administration to diabetic rats produced a significant \( (p<0.05) \) increase in pain threshold as compared to the untreated diabetic rats following eight weeks of treatment. OZ (100 mg/kg/day, p.o.) exhibited a higher increase in pain threshold than that of OZ (50 mg/kg/day, p.o.) in both tail-immersion (Figure 3a and b) and hot-plate assays (Figure 3c).
Table 1. Effect of oryzanol on food (g) and water intake (mL) of the rats after eight-week treatment schedule.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Food Intake (g)</th>
<th>Water Intake (mL/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>15.84±0.2436</td>
<td>24.77±0.3425</td>
</tr>
<tr>
<td>CT-O50</td>
<td>13.54±0.3027</td>
<td>22.32±0.3620</td>
</tr>
<tr>
<td>CT-O100</td>
<td>11.52±0.3884</td>
<td>19.88±0.3879</td>
</tr>
<tr>
<td>CTG-10</td>
<td>12.18±0.3259</td>
<td>20.96±0.2879</td>
</tr>
<tr>
<td>DC</td>
<td>26.76±2.584*</td>
<td>73.28±8.462*</td>
</tr>
<tr>
<td>DT-O50</td>
<td>22.22±1.533</td>
<td>52.57±4.832*</td>
</tr>
<tr>
<td>DT-O100</td>
<td>19.57±1.400*</td>
<td>48.38±4.699*</td>
</tr>
<tr>
<td>DTG-10</td>
<td>20.52±1.523*</td>
<td>49.92±4.759*</td>
</tr>
</tbody>
</table>

n=8; Values are expressed in mean±SEM; NC: Normal control (no treatment); CT-O50: Control treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks; CT-O100: Control treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks; CT-G10: Control treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks; DC: Diabetic control animals induced with a single i.v. injection of streptozotocin, 45 mg/kg in citrate buffer (pH 4.5, 0.1 M); DT-O50: Diabetic treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks following STZ injection; DT-O100: Diabetic treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks following STZ injection; DT-G10: Diabetic treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks following STZ injection; *p<0.05 versus NC, #p<0.05 versus DC; Values are obtained by one way ANOVA followed by Tukey’s multiple comparison test.

Figure 2. Effect of oryzanol on serum glucose level (mg/dL) of the rats after eight-week treatment schedule; n=8; Values are expressed in mean±SEM; NC: Normal control (no treatment); CT-O50: Control treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks; CT-O100: Control treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks; CT-G10: Control treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks; DC: Diabetic control animals induced with a single i.v. injection of streptozotocin, 45 mg/kg in citrate buffer (pH 4.5, 0.1 M); DT-O50: Diabetic treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks following STZ injection; DT-O100: Diabetic treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks following STZ injection; *p<0.05 versus NC, #p<0.05 versus DC; Values are obtained by one way ANOVA followed by Tukey’s multiple comparison test.

Figure 3. Effect of chronic treatment of oryzanol on pain threshold in rats subjected to (a) warm water tail immersion test (b) cold water tail immersion test (c) hot plate test; n=8; Values are expressed in mean±SEM; NC: Normal control (no treatment); CT-O50: Control treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks; CT-O100: Control treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks following STZ injection; DT-G10: Diabetic treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks following STZ injection; *p<0.05 versus NC, #p<0.05 versus DC; Values are obtained by one way ANOVA followed by Tukey’s multiple comparison test.
Control treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks; CT-G10: Control treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks; DC: Diabetic control animals induced with a single i.v. injection of streptozotocin, 45 mg/kg in citrate buffer (pH 4.5, 0.1 M); DT-O50: Diabetic treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks following STZ injection; DT-O100: Diabetic treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks following STZ injection; DT-G10: Diabetic treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks following STZ injection; *p<0.05 versus NC, †p<0.05 versus DC; Values are obtained by one way ANOVA followed by Tukey’s multiple comparison test.

Effect of OZ treatment on formalin-induced flinching

All the rats subcutaneously challenged with 0.5% formalin into hind paw produced characteristic biphasic incidence of flinching with an early phase 1 and a late phase 2 separated by a quiescent period (phase Q). Diabetic rats exposed to 0.5% formalin exhibited a significantly (p<0.05) increased frequency of flinching during both Q phase and phase 2 as compared to the untreated controls. Following injection of 0.5% formalin, OZ (50 and 100 mg/kg/day, p.o.) and standard Gl (10 mg/kg/day, p.o.) significantly (p<0.05) reduced the flinching in diabetic rats during both phase Q and phase 2 but not in phase 1 (Figure 4a and b). The values, however, were not significantly different from normal or treated controls.

Effect of OZ treatment Na⁺-K⁺ ATPase activity from sciatic nerve

Sciatic nerve Na⁺-K⁺ ATPase activity was significantly (p<0.05) reduced in diabetic rats as compared to the normal control (Figure 5). This was significantly (p<0.05) corrected by OZ (100 mg/kg/day, p.o.) and standard Gl (10 mg/kg/day, p.o.) treatment after eight weeks.

Effect of OZ on diabetes-induced changes in lipid peroxidation

Thiobarbituric acid-reactive substance levels were increased significantly (p<0.05) in the sciatic nerves of diabetic rats as compared to the normal control group (Figure 6). Chronic treatment with OZ (50 and 100 mg/kg/day, p.o.) produced a significant (p<0.05) and dose dependent reduction in thiobarbituric acid-reactive substance levels in STZ-treated rats, which was more effective than that exhibited by standard Gl (10 mg/kg/day, p.o.).
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Figure 5. Effect of chronic treatment of oryzanol on sciatic nerve Na⁺-K⁺ ATPase activities in rats; n=8; Values are expressed in mean±SEM; NC: Normal control (no treatment); CT-O50: Control treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks; CT-O100: Control treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks; CT-G10: Control treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks; DC: Diabetic control animals induced with a single i.v. injection of streptozotocin, 45 mg/kg in citrate buffer (pH 4.5, 0.1 M); DT-O50: Diabetic treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks following STZ injection; DT-O100: Diabetic treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks following STZ injection; DT-G10: Diabetic treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks following STZ injection; *p<0.05 versus NC, #p<0.05 versus DC; Values are obtained by one way ANOVA followed by Tukey’s multiple comparison test.

Effect of OZ on diabetes-induced changes in the antioxidant profile

In the eighth week, the GSH contents and the enzyme activity of superoxide dismutase significantly (p<0.05) decreased in the sciatic nerves of diabetic rats as compared to the normal control group (Figure 7a and b). Although a marked reduction in the levels of catalase was evident in the sciatic nerves of diabetic rats, the difference was not statistically significant when compared to the normal control animals (Figure 7c). The reduction in the levels of GSH was significantly (p<0.05) improved by treatment with OZ (100 mg/kg/day, p.o.) and standard GI (10 mg/kg/day, p.o.) in the sciatic nerves of STZ-treated rats. However, no significant increase in the levels of superoxide dismutase and catalase was observed in treated vs diabetic rats upon administration of OZ (50 and 100 mg/kg/day, p.o.) and standard GI (10 mg/kg/day, p.o.) during the eight-week treatment period.

Figure 6. Effect of chronic treatment of oryzanol on lipid peroxidation in sciatic nerve of rats; n=8; Values are expressed in mean±SEM; NC: Normal control (no treatment); CT-O50: Control treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks; CT-O100: Control treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks; CT-G10: Control treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks; DC: Diabetic control animals induced with a single i.v. injection of streptozotocin, 45 mg/kg in citrate buffer (pH 4.5, 0.1 M); DT-O50: Diabetic treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks following STZ injection; DT-O100: Diabetic treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks following STZ injection; DT-G10: Diabetic treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks following STZ injection; *p<0.05 versus NC, #p<0.05 versus DC; Values are obtained by one way ANOVA followed by Tukey’s multiple comparison test.

Effect of OZ on diabetes-induced nitrosative stress

Tissue nitrite levels were significantly (p<0.05) elevated in the diabetic rats (Figure 8). OZ (100 mg/kg/day, p.o.) and standard GI (10 mg/kg/day, p.o.) treatment significantly (p<0.05) inhibited this increase in nitrite levels.

Discussion

In the current study, STZ induced rats exhibited a significantly higher blood glucose level, increased food and water intake and a significantly decreased body weight, lower nociceptive threshold and a reduced grip strength as compared to non-diabetic animals thereby confirming allodynia, thermal hyperalgesia and muscle weakness in diabetic animals. Similar models of thermal hyperalgesia and formalin-induced flinching have been previously reported in STZ-induced animals (Calcutt et al., 1996; Kuhad & Chopra, 2008; Tembhurne & Sakarkar, 2010). In the current study, OZ treatment restored body weight,
blood glucose, food and water intake along with pain threshold, pain perception and neuromuscular strength in diabetic rats.

Large proportions of diabetic patients are characterized by an altered perception threshold coupled with an increased sensitivity to noxious and non-noxious stimuli. The tail-flick test depicts the activity of simple spinal reflex arc by assessing a delay in withdrawing the tail from noxious stimuli (Calcutt, 2001; Ilnytska et al., 2006). Hyperalgesia or the paw withdrawal latencies to noxious thermal stimuli, on the other hand, denotes supraspinal sensory processing (Calcutt, 2001; Obrosova et al., 2005). In the current study, marked thermal hyperalgesia observed in diabetic animals was reversed by treatment with OZ.

In the formalin test, phase 1 responses are indicative of acute noxious stimulation, whereas phase 2 responses are ascribed to the combinatorial effect of the inflammation in the peripheral tissue and functional alterations in the dorsal horn of the spinal cord (Calcutt et al., 1994; Kidd & Urban, 2001). In the present study, the marked effects of OZ treatment on flinching responses during phases Q and 2 in diabetic rats, following 0.5% formalin injection, appear to be selective for the diabetes-induced abnormality, as no significant effect was observed in control rats by similar treatments. Thus, it appears that OZ precludes the disruption of normal nociceptive function and can selectively prevent hyperalgesic responses to formalin in diabetic rats.

**Figure 7.** Effect of chronic treatment of oryzanol on (a) reduced glutathione activity (b) superoxide dismutase activity (c) catalase activity in sciatic nerve of rats; n=8; Values are expressed in mean±SEM; NC: Normal control (no treatment); CT-O50: Control treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks; CT-O100: Control treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks; CT-G10: Control treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks; DC: Diabetic control animals induced with a single i.v. injection of streptozotocin, 45 mg/kg in citrate buffer (pH 4.5, 0.1 M); DT-O50: Diabetic treated animals receiving oryzanol 50 mg/kg, orally per day for eight weeks following STZ injection; DT-O100: Diabetic treated animals receiving oryzanol 100 mg/kg, orally per day for eight weeks following STZ injection; DT-G10: Diabetic treated animals receiving standard glibenclamide 10 mg/kg, orally per day for eight weeks following STZ injection; \*p<0.05 versus NC, \#p<0.05 versus DC; Values are obtained by one way ANOVA followed by Tukey’s multiple comparison test.
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Na+-K+ ATPase activities were significantly reduced in the sciatic nerve tissue of diabetic rats as compared to the control animals. OZ supplementation (100 mg/kg/day, p.o.) significantly restored the Na+-K+ ATPase activity, possibly by attenuation of oxidative stress (Son et al., 2011) and amelioration of vascular function (Kihara et al., 1994). OZ treatment (100 mg/kg/day, p.o.) significantly regenerated intracellular GSH content in the sciatic nerve of diabetic animals in the current study. The observations are in accordance to the previous findings implicating lowered GSH levels in diabetes (Kuzumoto et al., 2006; Arora et al., 2008). Furthermore, abnormal GSH metabolism in diabetes may also enhance TNF-α expression (Sagara et al., 1994). OZ treatment (100 mg/kg/day, p.o.) significantly reduced the extent of lipid peroxidation to near control levels.

The levels of GSH, a potent endogenous antioxidant considered as a first line of defense against free radicals were significantly reduced in the sciatic nerve of diabetic animals in the present study. The observations are in accordance to the previous findings implicating lowered GSH levels in diabetes (Kuzumoto et al., 2006; Arora et al., 2008). Furthermore, abnormal GSH metabolism in diabetes may also enhance TNF-α expression (Sagara et al., 1994). OZ treatment (100 mg/kg/day, p.o.) significantly reduced the extent of lipid peroxidation to near control levels.

Superoxide anions are also involved in some of the prominent hyperglycemia-induced oxidative changes, including activation of aldose reductase and protein kinase C activity which are further implicated in alterations in pain perception (Kamei et al., 2001). A significantly higher lipid peroxidation levels observed in the sciatic nerve of diabetic animals in the current study have been reported previously (Kamboj et al., 2010; Nickander et al., 1994). OZ, being a potent free-radical-scavenger (Parrado et al., 2003), significantly reduced the extent of lipid peroxidation to near control levels.

The levels of GSH, a potent endogenous antioxidant considered as a first line of defense against free radicals were significantly reduced in the sciatic nerve of diabetic animals in the present study. The observations are in accordance to the previous findings implicating lowered GSH levels in diabetes (Kuzumoto et al., 2006; Arora et al., 2008). Furthermore, abnormal GSH metabolism in diabetes may also enhance TNF-α expression (Sagara et al., 1994). OZ treatment (100 mg/kg/day, p.o.) significantly regenerated intracellular GSH content in the sciatic nerve; this might be attributed to endogenous GSH repletion, high free-radical-scavenging capacity (Parrado et al., 2003) and inhibition of TNF-α activity (Islam et al., 2008).

Implication of oxidative stress in the pathogenesis of diabetes is also suggested by the altered expression of antioxidant enzymes such as SOD and CAT (Moussa, 2008). The current study, in agreement with earlier reports (Cui et al., 2008), manifested a significant reduction in the SOD activity in the sciatic nerve preparations of diabetic animals, which might involve non-enzymatic glycosylation (Arai et al., 1987). In addition to GSH and SOD, a decreased CAT activity in diabetes might further reduce the defense mechanisms against free radicals (Kamboj et al., 2010). Improvement in the SOD and CAT activities after OZ administration in the diabetic animals is in similar lines to previously reported restoration of these antioxidant enzymes.
enzymes by OZ in the liver (Jin Son et al., 2010; Ismail et al., 2010). Thus, it is evident that the simultaneous reduction in the activities of both SOD and CAT might be the underlying factor for the enhanced vulnerability of the sciatic nerve to hyperglycemia induced oxidative stress. The findings, hence obtained, substantiate that OZ protects the sciatic nerve from hyperglycemia induced damage by reinstating the levels of both these enzymes. Nitrosative stress (peroxynitrite-induced injury) plays an important role in functional abnormalities associated with motor (MNCV) and sensory nerve conduction velocity (SNCV) deficits and sensory neuropathy in STZ-induced diabetic rats (Obrosova et al., 2007). A marked increase in the tissue nitrite levels was observed in our study which was indicative of nitrosative stress in diabetic animals. OZ treatment (100 mg/kg/day, p.o.) significantly attenuated the elevated tissue nitrite levels, possibly by virtue of its inducible NO synthase (iNOS) suppression potential (Nagasaka et al., 2007). Moreover, Rao et al. (2010) had recently demonstrated that the rice bran extracts possess potent nitric oxide scavenging activities and inhibit nitrite formation by directly competing with oxygen in the reaction with nitric oxide.

Based on the present preliminary results, it can be concluded that OZ treatment significantly ameliorates hyperglycemia-induced hyperalgesia, reduces formalin-induced nociception and attenuates oxidative stress mediated biochemical changes which might be responsible for diabetes induced nerve damage. Thus, OZ can be considered as a novel antinociceptive agent and can be used as a possible therapeutic option in the treatment of neuropathic pain associated with diabetes mellitus. However, further studies are warranted to elucidate the exact molecular mechanism involved in OZ's antinociceptive effect.

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Protective effect of oryzanol isolated from crude rice bran oil in experimental model of diabetic neuropathy

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