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Berberine Ameliorates Cold and Mechanical Allodynia in a Rat Model of Diabetic Neuropathy

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ABSTRACT This study evaluated the antiallodynic properties of berberine on cold and mechanical allodynia after streptozotocin (STZ)-induced diabetes using a rat model. Diabetic neuropathy was induced in rats by intraperitoneal injection of STZ. To measure cold and mechanical allodynia, a 4°C plate and von Frey filament were used, respectively. Cold and mechanical allodynia induced by diabetes were significantly decreased by single and repeated intraperitoneal treatment of amitriptyline at 10 mg/kg, and berberine at 10 and 20 mg/kg. The hepatic malondialdehyde, superoxide dismutase, catalase, and glutathione peroxidase activities were significantly increased in diabetic rats as compared with those in intact rats; however, in amitriptyline- and berberine-treated rats, they were significantly decreased as compared to the STZ control. The overall effects of berberine 20 mg/kg on cold and mechanical allodynia were quite similar to those of amitriptyline 10 mg/kg, and berberine exhibited similar antioxidant effects as the same dosage of amitriptyline. In conclusion, berberine (10 and 20 mg/kg) was observed to have antiallodynic effects against diabetes, which are presumed to be associated with antioxidative effects. It can be considered that the anti-inflammatory or antidepressant capacity of berberine could contribute to the antiallonynic effects shown in this study.

KEY WORDS: • *antioxidants* • *berberine* • *diabetic neuropathies* • *hyperalgesia*

INTRODUCTION

PHARMACOLOGICAL AGENTS with proven efficacy for painful diabetic neuropathy include the tricyclic antidepressants, the selective serotonin and noradrenaline reuptake inhibitors, anticonvulsants, opiates, membrane stabilizers, the antioxidant alpha-lipoic acid, and topical agents, including capsaicin.[1] The wide variety of medical therapies used to treat diabetic neuropathy attests to the lack of an ideal treatment. Moreover, their use is often associated with problems such as development of adverse side effects, insufficient efficacy, and cost–effectiveness. Therefore, clinicians look for other drugs to increase the overall analgesic effect without causing unacceptable side effects.

Berberine is a plant alkaloid with a long history of medicinal use in Oriental medicine. The various plant sources of berberine include *Berberis vulgaris* (barberry), *Hydrastis canadensis* (goldenseal), *Berberis aquifolium* (Oregon grape), *Coptis chinensis* (Chinese goldthread), and *Berberis aristata* (tree turmeric).[2] It has been reported to possess multiple pharmacological properties as a drug for diabetes,[3] coronary artery disease,[4] hyperlipidemia,[5] inflammation,[6] and hypertension,[7] and recently, it is reported that berberine has powerful antioxidative effects.[8]

Since a substantial body of evidence suggests that oxidative stress plays a major role among the putative pathogenic mechanisms of diabetic neuropathy,[9,10] the authors hypothesized that berberine might ameliorate diabetic neuropathic pains, including allodynia. However, no reports thus far have described a direct effect of berberine on the experimental diabetic neuropathic pains.

This study investigated whether acute or subchronic berberine treatment has an analgesic efficacy on mechanical (von Frey filaments) and cold (4°C plate) allodynia after streptozotocin (STZ)-induced diabetes.[11] Amitriptyline (10 mg/kg), a typical tricyclic antidepressant prescribed for diabetic neuropathy,[12] was added as a comparison compound. In addition, to observe the possible antioxidative effects, hepatic levels of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were also measured.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats ($n = 48$), body weights 170–190 g, were housed four per polycarbonate cage, in a room with constant temperature (20–25°C), and humidity (40–45%). Food (Samyang, Seoul, Korea) and water were

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supplied *ad libitum*. They were allocated randomly to six groups ($n = 8$ per group). All procedures of this experiment were approved by the Institutional Animal Care and Use Committee and in accordance with the National Institute of Health guidelines on the laboratory animal welfare.

Test drugs

The berberine chloride (berberine) and amitriptyline hydrochloride (amitriptyline) were purchased from Sigma (St. Louis, MO, USA). Berberine was intraperitoneally administered at 5, 10, and 20 mg/kg, in a volume of 5 mL/kg dissolved in saline, according to the previous study.[13] Amitriptyline was also intraperitoneally administered at 10 mg/kg, in a volume of 5 mL/kg dissolved in saline, according to Berrocoso *et al*.[14]

STZ-induced diabetes

The methods used in this model were described previously.[11,15–17] Rats were injected intraperitoneally with STZ (160 mg/kg total dose) freshly dissolved in 50 mM citrate buffer. The 160-mg dose was given in two 80 mg/kg injections on consecutive days. Eight rats were injected with the citrate buffer and served as the intact control group. Diabetes was confirmed 1 week after injection of STZ by measurement of nonfasting blood glucose levels.[18] Blood samples were obtained from the orbital sinus by the heparinized hematocrit capillary tubes.[18] The plasma glucose levels were determined by the glucose oxidase method using the OneTouch Ultra Blood Glucose Meter (LifeScan, Inc., Milpitas, CA, USA). Only animals with final blood glucose levels >400 mg/dL (401–480 mg/dL) were deemed to be diabetic. The rats were retested for hyperglycemia once per week to confirm continued high blood-glucose readings for 4 weeks. Diabetic rats were divided in five groups of eight animals each, including the STZ control group.

Acute study

Four weeks after STZ injection, the antiallodynic effects of test drugs were studied through the cold plate and von Frey tests.

Subacute study

After the acute study, the same animals were treated twice daily (9:00 a.m. and 7:00 p.m.) with vehicle, berberine, or amitriptyline for 14 days. Then, the antiallodynic effects of test compounds were again assessed after the last injection.

Behavioral tests

Behavioral tests were performed by a tester blinded to the experimental conditions.

Cold plate test. Cold-induced allodynia was determined as described previously.[19] The rat was placed on a metal plate (Ugo Basile Hot/Cold Plate; Ugo Basile Srl, Comerio, Italy) kept at a cold temperature (4 ± 1°C). The number of times the animal briskly lifted its left hind paw over a period of 2 min was counted as a nociceptive response. The number of pawlifts was determined 30, 60, and 120 min after injection of the test compound or vehicle.

von Frey hair stimulation test. Rats were placed on an elevated wire mesh floor and confined underneath individual overturned Perspex boxes ($185 \times 210 \times 135$ mm^3) as previously reported.[14] A dynamic plantar aesthesiometer (DPA; Ugo Basile, Comerio, Italy) was used to measure mechanical allodynia.[20] A von Frey filament was placed on the plantar surface of the left hind paw, and the force was increased gradually until a withdrawal response was evoked, and the amount of force needed to cause the withdrawal response was recorded. The threshold, expressed in grams, was determined as the force that induced a withdrawal response, with a 50-g cut-off limit. Paw withdrawal thresholds were determined 30, 60, and 120 min after injection of the test compound or vehicle.

Biochemical assays

After the subacute study, rats were fasted overnight and sacrificed by decapitation under ether anesthesia. The livers were removed and weighed immediately, and homogenized in four volumes of ice-cold Tris–HCL buffer (10 mM, pH 7.4) for 3 min at 16,000 rpm using a homogenizer (IKA Ultra-Turrax T25, Staufen, Germany). The homogenates were then centrifuged for 15 min at 4°C at 12,000 *g*.

MDA assay. The MDA levels were estimated by the double-heating method.[21] Principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 mL of 100 g/L trichloroacetic acid solution was added to 0.5 mL supernatant in each centrifuge tube, and the tubes were placed in a boiling water bath for 15 min. After cooling in tap water, the tubes were centrifuged at 1000 *g* for 10 min, and 2 mL of the supernatant was added to 1 mL of 6.7 g/L TBA solution in a test tube and heated again in a boiling water bath for 15 min. The solution was then cooled in tap water, and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA-TBA complex (absorbance coefficient $\varepsilon = 1.56 \times 10^5$ M/cm) and is expressed as nanomoles (nM) per gram wet tissue.

SOD assay. Total SOD activity was determined according to a previously reported method.[22] The principle of the method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generator. The activity was assessed in the ethanol phase of the supernatant after 1.0 mL ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per gram protein.

CAT assay. CAT activity was measured according to the method of Aebi.[23] The principle of the assay is based on the determination of the rate constant κ (dimension: s^{-1}, κ) of hydrogen peroxide decomposition. By measuring the absorbance change per minute, the rate constant of the enzyme was determined. Activities were expressed as κ per gram protein.

GSH-Px assay. GSH-Px activity was measured by the method of Paglia and Valentine.[24] The enzymatic reaction in the tube that contained reduced nicotinamide adenine dinucleotide phosphate, reduced glutathione, sodium azide, and glutathione reductase was initiated by the addition of hydrogen peroxide, and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units per gram protein.

Statistical analyses

All data were presented as mean ± standard deviation (SD) of eight rats. Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtained data were analyzed by one-way analysis of variance (ANOVA) followed by the least-significant differences (LSD) multicomparison test to determine which pairs of group comparison were significantly different. If significant deviations from variance homogeneity were observed by the Levene test, Kruskal–Wallis H test was conducted. When a significant difference was observed in the Kruskal–Wallis H test, the Mann–Whitney *U* (MW) test was conducted. Statistical analyses were conducted using SPSS for Windows (Release 14.0K; SPSS, Inc., MO, USA). In the present study, $P < .01$ or $P < .05$ was regarded as significant differences.

RESULTS

Acute study

Cold plate test. Diabetic animals showed significantly more paw lifts than sham control rats at all three different measure points (30, 60, and 120 min). However, the paw lifts in amitriptyline 10 mg/kg and berberine 10 and 20 mg/kg treated rats were significantly fewer as compared with the STZ control at all different measured points after single injections. In berberine 5 mg/kg treated rats, slight, but nonsignificantly fewer, paw lifts were observed after treatment, as compared with the STZ controls (Fig. 1a).

von Frey hair stimulation test. The paw withdrawal threshold (g) of diabetic animals was significantly decreased as compared with an intact animal at all different measurement points (30, 60, and 120 min). However, the paw withdrawal threshold in amitriptyline 10 mg/kg and in berberine 10 and 20 mg/kg treated rats were significantly increased as compared with the STZ control at all different measured points after single injections. In berberine 5 mg/kg treated rats, slight, but nonsignificant, increases of paw withdrawal threshold were observed after treatment, as compared with the STZ control (Fig. 1b).

Subacute study

Cold plate test. Similar significant decreases in the numbers of paw lifts were also demonstrated in amitriptyline 10 mg/kg and in berberine 10 and 20 mg/kg treated rats as compared with the STZ control after 14 days of continuous injections. In berberine 5 mg/kg treated rats, slight, but nonsignificant, decreases in paw lifts were observed after treatment, as compared with the STZ control (Fig. 1c).

von Frey hair stimulation test. Similar significant increases in the paw withdrawal thresholds were also demonstrated in amitriptyline 10 mg/kg and in berberine 10 and 20 mg/kg treated rats as compared with STZ control after 14 days of continuous injections. In berberine 5 mg/kg treated rats, slight, but nonsignificant, increases in the paw withdrawal threshold were observed after treatment, as compared with the STZ control (Fig. 1d).

Biochemical assays (Table 1)

Hepatic MDA levels. Forty-two days after of STZ administration, hepatic MDA levels (nM/g wet tissue) of diabetic animals were significantly increased as compared with intact rats. However, the hepatic MDA levels in amitriptyline 10 mg/kg and in berberine 10 and 20 mg/kg treated rats were significantly lower as compared with the STZ control, respectively.

Hepatic SOD levels. Hepatic SOD levels (U/g protein) of diabetic animals were significantly higher as compared with intact rats. However, the hepatic SOD levels in amitriptyline 10 mg/kg and in berberine 10 and 20 mg/kg treated rats were significantly decreased as compared with STZ controls.

Hepatic CAT levels. Hepatic CAT levels (κ/g protein) of diabetic animals were significantly higher as compared with intact rats. However, the hepatic CAT levels in amitriptyline 10 mg/kg and in berberine 10 and 20 mg/kg treated rats were significantly lower as compared with the STZ control, respectively.

Hepatic GSH-Px levels. Hepatic GSH-Px levels (U/g protein) of diabetic animals were significantly higher as compared with the intact rats. However, the hepatic GSH-Px levels in amitriptyline 10 mg/kg and in berberine 10 and 20 mg/kg treated rats were significantly lower as compared with the STZ controls.

DISCUSSION

There are several acceptable pathogenetic factors that explain the various deficits observed in diabetic neuropathy, including advanced glycation end-product formation, aldose

reductase pathway, oxidative/nitrosative/carbonyl stress, and increased protein kinase C activity.[25–28] These pathways together produce a state of disparity between reactive species production and the body's redox homeostasis, resulting in oxidative stress. A role for oxidative stress in the pathogenesis of diabetic neuropathy is further supported by experimental and clinical studies where various antioxidants, including alpha-lipoic acid,[29] glutathione,[30] and metal chelators,[30] have shown to ameliorate biochemical and functional nerve disorders. Despite widespread acceptance of oxidative stress having a role in the pathogenesis of diabetic neuropathy, the precise mechanisms involved remain incompletely resolved, and further research is in progress.

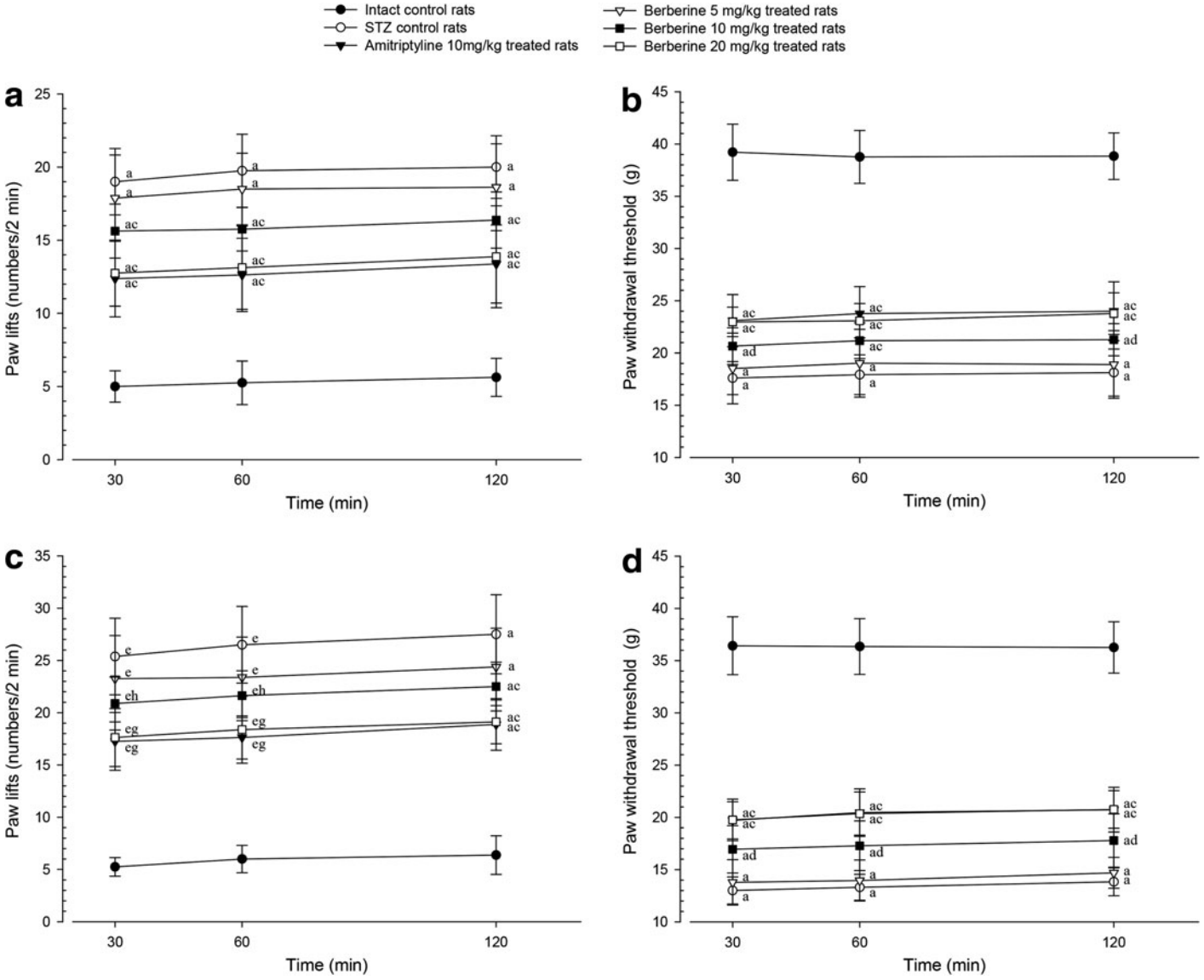

FIG. 1. Acute (28 days after streptozotocin [STZ] administration) **(a, b)** and subacute study (42 days after STZ administration) **(c, d)**); cold plate test **(a, c)** and von Frey hair stimulation test **(b, d). (a)** Diabetic rats showed significantly more paw lifts than the sham control rats. However, the paw lifts in amitriptyline 10 mg/kg and berberine 10 and 20 mg/kg treated rats were significantly decreased as compared with the STZ control after single intraperitoneal injections. **(b)** The paw withdrawal threshold of diabetic rats was significantly decreased as compared with intact rats at all three different measurement points. However, the paw withdrawal threshold in amitriptyline 10 mg/kg and berberine 10 and 20 mg/kg treated rats was significantly increased as compared with STZ control after single intraperitoneal injections. **(c)** Significant decreases in the number of paw lifts were demonstrated in amitriptyline 10 mg/kg and berberine 10 and 20 mg/kg treated rats as compared with the STZ control after 14 days of continuous intraperitoneal injections. **(d)** Significant increases in the paw withdrawal threshold were demonstrated in amitriptyline 10 mg/kg and berberine 10 and 20 mg/kg treated rats as compared with STZ control after 14 days of continuous intraperitoneal injections. Values were expressed as mean ± SD of eight rats. $^{a}P < .01$ and $^{b}P < .05$ as compared with intact control by LSD test. $^{c}P < .01$ and $^{d}P < .05$ as compared with STZ control by LSD test. $^{e}P < .01$ and $^{f}P < .05$ as compared with intact control by MW test. $^{g}P < .01$ and $^{h}P < .05$ as compared with STZ control by MW test. LSD, least-significant differences; MW, Mann–Whitney *U*.

Table 1. Hepatic Malondialdehyde, Superoxide Dismutase, Catalase, and Glutathione Peroxidase Levels of Diabetic Rats After 42 Days of Streptozotocin Treatment

	Control			*Berberine treated*		
	Intact	*STZ*	*Amitriptyline*	*5 mg/kg*	*10 mg/kg*	*20 mg/kg*
MDA (nM/g wet tissue)	23.06 ± 3.88	37.31 ± 3.13[a]	30.38 ± 2.42[ac]	35.74 ± 4.84[a]	29.35 ± 3.56[ad]	24.19 ± 5.38[c]
SOD (U/g protein)	232.63 ± 35.37	389.13 ± 57.22[a]	308.63 ± 32.77[ac]	358.25 ± 50.93[a]	304.75 ± 27.76[ad]	284.13 ± 42.82[bc]
CAT (κ/g protein)	1.50 ± 0.32	2.93 ± 0.33[a]	2.28 ± 0.39[ac]	2.72 ± 0.21[a]	2.29 ± 0.33[ad]	1.92 ± 0.14[ac]
GSH-Px (U/g protein)	0.23 ± 0.08	1.76 ± 0.17[e]	1.34 ± 0.32[eh]	1.57 ± 0.37[e]	1.32 ± 0.28[eg]	1.01 ± 0.53[eg]

Values are expressed as mean ± SD of eight rats.

[a]$P < .01$ and [b]$P < .05$ as compared with intact control by the LSD test.

[c]$P < .01$ and [d]$P < .05$ as compared with STZ control by the LSD test.

[e]$P < .01$ and [f]$P < .05$ as compared with intact control by the MW test.

[g]$P < .01$ and [h]$P < .05$ as compared with STZ control by the MW test.

STZ, streptozotocin; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; LSD, least-significant differences; MW, Mann–Whitney *U*.

Assessment of behavioral responses to external stimuli in diabetic rats has led to the identification of a number of mechanisms of abnormal sensation and pain in diabetes. STZ, a glucosamine–nitrosourea compound obtained from *Streptomyces achromogenes*, is commonly used to induce diabetes through pancreatic beta-cell death in rodents to study diabetes and associated complications that include diabetic neuropathy.[11] Although it has been extensively studied, the exact mechanism underlying sensory abnormalities in STZ-administrated rats remains unclear.[31] One study suggested that the spinal dorsal horn is involved in allodynia and hyperalgesia in STZ-induced diabetic rats through a mechanism involving the microglia.[32]

In the present study, marked increases in paw lifts and decreases of paw withdrawal threshold were observed in diabetic rats as compared with the intact control rats after STZ administration, indicating that cold and mechanical allodynia were well induced in these diabetic rats. Cold and mechanical allodynia induced by diabetes were significantly decreased by single and repeated intraperitoneal treatment of berberine 10 and 20 mg/kg. Berberine 20 mg/kg exhibited a similar antiallodynic efficacy to amitriptyline 10 mg/kg in the present study. These findings are considered as direct evidence that berberine has antiallodynic effects against diabetes-related peripheral neuropathic pains. Berberine 5 mg/kg was not sufficient to decrease allodynia significantly.

In addition, it has been clearly described that the levels of lipid peroxidation and oxidative stress increase in diabetes mellitus.[33] Moreover, the increased oxidative stress due to increased oxygen free radical production is an important mechanism underlying diabetic vascular complications,[34] nephropathy,[35] and neuropathy, including peripheral pains.[36] The level of MDA, an end product of lipid peroxidation, increases significantly in the livers of diabetic rats.[37] However. there is no consensus in the level of antioxidant enzymes, such as SOD and CAT, of many organs in diabetic rats; although some studies measuring activities of SOD and CAT in diabetes mellitus showed the reductions in the levels of these enzymes,[38] some other studies reported increases in the activities of both enzymes with STZ-induced diabetes.[39] The increase in SOD and CAT in diabetic liver tissue in this suggests increased oxidative stress due to chronic exposure to H_2O_2 *in vivo*.[39] H_2O_2 may be an important mediator for any possible tissue damage in STZ-induced diabetes.[40] GSH-Px is an important antioxidant enzyme that plays a role in the elimination of H_2O_2 and lipid hydroperoxides and reduces peroxides by using reduced glutathione as a hydrogen donor.[41] In the current study, the hepatic MDA, SOD, CAT, and GSH-Px activities were significantly higher in diabetic rats as compared with intact rats, but all were dose-dependently lowered by berberine administration, providing strong evidence that berberine exerts potent antioxidative effects. The causal relationship between the antioxidative and antiallodynic effect of berberine was more strongly suggested when observing that a greater antiallodynic efficacy was associated with antioxidative effects, although statistical significance was weak. To understand the precise mechanism of action of these compounds, further studies are required. Berberine 10 mg/kg showed quite similar antioxidative effects in diabetic rats as compared with an equal dosage of amitriptyline, 10 mg/kg. Berberine 5 mg/kg was not sufficient to produce significant antioxidative effects.

Berberine is well known for its anti-inflammatory capacity and can inhibit the expression of proinflammatory cytokines[42,43] through inhibiting the nuclear factor-kappa B (NF-κB) pathway.[44,45] Considerable evidence has accumulated to emphasize the importance of inflammatory processes in the etiology of diabetic complications, including diabetic neuropathy.[46,47] The NF-κB inflammatory cascade occupies an intermediate position in the schema of pathogenesis, downstream of oxidative stress, advanced glycation end-product formation, and mitogen-activated protein kinase activation, but also contributing to them.[48] Therefore, this anti-inflammatory capacity of berberine could contribute to the antiallonynic and antioxidant effects shown in this study, although NF-κB activation was not studied. Furthermore, it is reported that berberine inhibits monoamine oxidase enzyme, particularly the monoamine oxidase-A

isoform.[49] It is well documented that monoamine oxidase inhibitors increase the concentrations of norepinephrine, serotonin, and dopamine in the brain and have antidepressant effects. Further, berberine exerts antidepressant-like effect in various behavioral paradigms of despair, possibly by modulating brain biogenic amines (norepinephrine, serotonin, and dopamine) with the nitric oxide pathway and/or sigma receptors.[13] These results provide clues that this antidepressant efficacy of berberine may be related to the mechanism of the antiallodynic effect in this study, because antidepressants have improved various neuropathic pains. Accordingly, future studies are needed to further address the molecular mechanism of berberine and effects on other types of neuropathic pains excluded from our experiment.

In conclusion, this study demonstrated that berberine (10 and 20 mg/kg) has antiallodynic effects against diabetes, through its antioxidative effects. It can be considered that anti-inflammatory or antidepressant capacity of berberine could contribute to this antiallonynic effects. Overall effects of berberine 20 mg/kg on the cold and mechanical allodynia in diabetes were quite similar to those of amitriptyline 10 mg/kg, and berberine showed similar antioxidative effects as compared with equal dosages of amitriptyline in STZ-induced diabetic rats. The present data suggest that berberine could have utility against diabetes-induced neuropathy.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no competing financial interests.

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