The Effect of the Flavonoid Quercetin on Pain Sensation in Diabetic Rats

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A B S T R A C T

Introduction: Hyperalgesia is considered as one of the marked signs of subchronic diabetes mellitus in patients that could affect their lifestyle. This study was designed to investigate the anti-nociceptive effect of chronic administration of quercetin in streptozotocin (STZ)-diabetic rats using formalin and hot tail immersion tests.

Methods: Rats were divided into control, control or diabetic groups receiving sodium salicylate, untreated diabetic, and quercetin-treated control and diabetic groups. The treatment groups received i.p. administration of quercetin at a dose of 10 mg/kg for 6 weeks. Finally, hyperalgesia were assessed using standard formalin and hot tail immersion tests. Meanwhile, some markers of oxidative stress were also measured in brain tissue.

Results: Quercetin or SS treatment of diabetic rats significantly reduced pain score in chronic phase of formalin test (p<0.05). Regarding hot tail immersion test, diabetic rats showed a significant reduction in tail flick latency as compared to control ones (p<0.05) and quercetin treatment of diabetic rats did significantly increase this latency relative to untreated diabetics (p<0.05). Quercetin treatment of diabetic rats also significantly decreased brain level of malondialdehyde (MDA) (p<0.05) and nitrite (p<0.05) and slightly increased activity of superoxide dismutase (SOD) relative to diabetics.

Discussion: Taken together, chronic administration of quercetin could attenuate nociceptive score in chronic phase of formalin test in streptozotocin-diabetic rats and could also increase threshold of thermal nociception.

1. Introduction

Diabetes mellitus is one of the serious problems worldwide and number of diabetic people is estimated to increase markedly by the year 2030 (Wild, 2004). Uncontrolled chronic hyperglycemia in diabetes leads to severe complications including neuropathy, retinopathy, and autonomic dysfunctions. Diabetic neuropathy as observed with some deranged conditions of nociception (i.e. hyperalgesia) is the most common complication with an incidence of more than 50% (Sima & Sugimoto, 1999). Diabetes-induced deficits in motor and sensory nerve conduction velocities and other manifestations of peripheral diabetic neuropathy have been well correlated with chronic hyperglycemia (Vincent, 2004, van Dam, 2002). Hyperglycemia could also lead to increased oxidative stress (enhanced free radical formation and/or a defect in antioxidant defenses), advanced glycation end product formation, nerve

hypoxia/ischemia, and impaired nerve growth factor support (Vincent , Russell, 2004, van Dam, 2002). Several studies suggest that oxidative stress may be one of the major pathways in the development of diabetic neuropathy and antioxidant therapy could prevent or even reverse hyperglycemia-induced nerve dysfunctions (Nickander, 1996). Recent interests are focusing on the use of non-vitamin antioxidants such as flavonoids in reducing the devastating complications of diabetes in experimental models and in patients (Laight , Carrier & Anggard, 2000). Plant-based pharmaceuticals including flavonoids have been employed in the management of various mankind diseases (Laight , Carrier, 2000). They are as essential part of human diet and are present in plants that have been used for centuries in medicine. Antioxidant properties, reactive oxygen species (ROS) scavenging, and cell function modulation of flavonoids could account for the large part of their pharmacological activity (Laight , Carrier, 2000, Machha & Mustafa, 2005). Since diabetes mellitus is considered as a free radical-mediated disease, there has been renewed interest in the use of flavonoids in diabetes research. In the search for new therapeutic approaches, quercetin, a natural flavonoid, is a strong antioxidant and radical scavenger and a polyphenol component which is abundant in fruits and vegetables (Kleemann, 2011). It has been reported that quercetin has anti-inflammatory (Kleemann , Verschuren, 2011), neuroprotective (Haleagrahara, 2011), antidiabetic (Vessal , Hemmati & Vasei, 2003) effects, and can prevent memory dysfunction in streptozotocin-diabetic rats (Bhutada, 2010). It also exhibits anti-apoptotic property (Choi, 2009), acts as an inhibitor of alpha-glucosidase (Li, 2009), being capable of attenuation of thermal

hyperalgesia and cold allodynia in STZ-induced diabetic rats (Anjaneyulu & Chopra, 2004) and could alleviate oxidative stress in STZ-diabetic rats (Mahesh & Menon, 2004). Therefore, we designed this study to investigate the effect of chronic quercetin treatment, a free radical scavenger on hyperalgesia in streptozotocin (STZ)-diabetic neuropathic rat using standard formalin and hot tail immersion tests and to evaluate some markers of oxidative stress in the brain tissue.

2. Methods

2.1. Animals

Male albino Wistar rats (Pasteur's institute, Tehran, Iran) weighing 210-270 g (10-12 weeks old) were housed in an air-conditioned colony room on a 12/12 cycle (21-23 □C and 30-40% humidity) and supplied with standard pelleted diet and tap water ad libitum. Procedures involving animals and their care were conducted in conformity with NIH guidelines for the care and use of laboratory animals.

2.2. Experimental Protocol

The rats (n = 48) were randomly allocated and similarly grouped into six groups: vehicle-treated control, control or diabetic groups receiving sodium salicylate (SS), diabetic and quercetin-treated control and diabetic groups. SS (200 mg/kg, i.p.) was administered 1 h before conducting the formalin test as a positive control. The rats were rendered diabetic by a single intraperitoneal injection of 60 mg kg-1 STZ freshly dissolved in
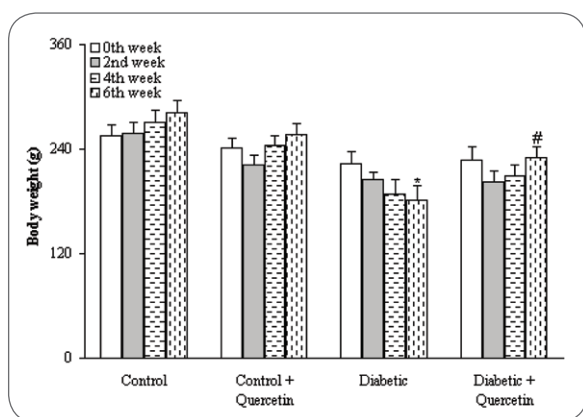
Figure 1. Body weight in different weeks (means ± S.E.M). * p<0.05 (as compared to week 0 in the same group), # p<0.05 (as compared to diabetics in the same week)
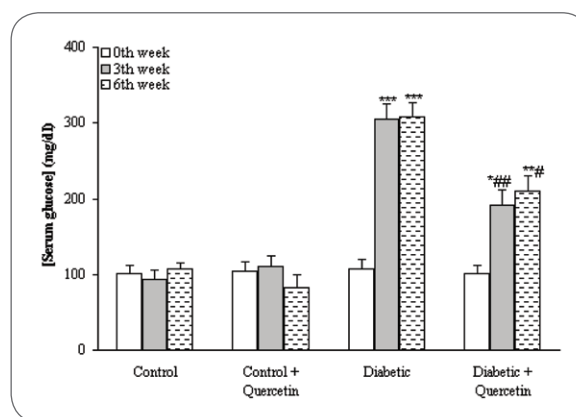
Figure 2. Serum glucose concentration in different weeks (means ± S.E.M).
* p<0.01, ** p<0.005, *** p<0.0001 (as compared to week 0 in the same group),
# p<0.05, ## p<0.01 (as compared to diabetics in the same week)

cold normal saline. Control animals received equivalent volume of normal saline as the solvent of STZ and Cremophor as the solvent for quercetin. Diabetes was confirmed by the presence of hyperglycemia, polyphagia, polydipsia, polyuria, and weight loss. One week after STZ injection, blood samples were collected and serum glucose concentrations were measured using glucose oxidation method (Zistshimi, Tehran). Only those animals with serum glucose level higher than 250 mg dl-1 were selected as diabetic for the following experiments. The day on which hyperglycemia had been confirmed was designated as day 0. Quercetin was daily administered i.p. at a dose of 10 mg/kg for a period of 6 weeks. Quercetin was dissolved in Cremophor. Changes in body weight, food consumption and water intake were regularly observed during the experimental period.

2.3. Formalin Test

The previously described method was applied (Dubuisson & Dennis, 1977). Briefly, each animal was acclimatized to the observation box before any testing began. Then, it was given a subcutaneous injection of 50 □l of 2.5% formalin into the plantar surface of one hind paw. It was then immediately placed in a Plexiglas box. Observations continued for the next 60 min. A nociceptive score was determined for 5 min blocks by measuring the amount of time spent in each of the four behavioral categories: 0, the position and posture of the injected hind paw is indistinguishable from the contralateral paw; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is

licked, bitten, or shaken. Then, a weighted nociceptive score, ranging from 0 to 3 was calculated by multiplying the time spent in each category by the category weight, summing these products and dividing by the total time for each 5 min block of time. The first 10 min post-injection was considered as the early (first) phase and the time interval 15–60 as the late (second) phase.
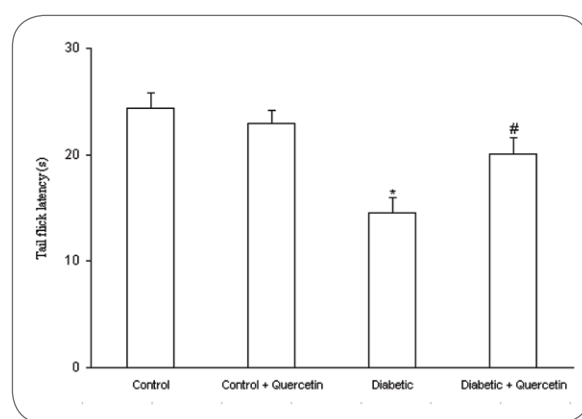
2.4. Hot Tail Immersion Test

Diabetic thermal hyperalgesia was assessed using tail immersion test(Baluchnejadmojarad , Roghani & Khastehkhodaie, 2010). After adaptation, rat tail was immersed in warm water (49 °C) and the tail flick response latency (withdrawal response of tail) was observed as the end point response. Each experiment was repeated 4 times for each animal with an interval of 2 min and its average was reported. Meanwhile, a cut-off time of 30 s was also considered.

2.5. Determination of Brain Malondialdehyde (Mda) Concentration

The rats were anesthetized with ketamine (100 mg/kg), decapitated, brains were removed, blotted dry, weighed, then made into 10% tissue homogenate in ice-cold 0.9% saline solution, centrifuged (1000×g, 4 ℃, 10 min) to remove particulates, obtained supernatant was aliquotted, then stored at -70 °C until assayed. The MDA concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured as described before (Bagheri, 2011). Briefly, trichloroacetic acid and TBARS reagent were added to supernatant, then mixed
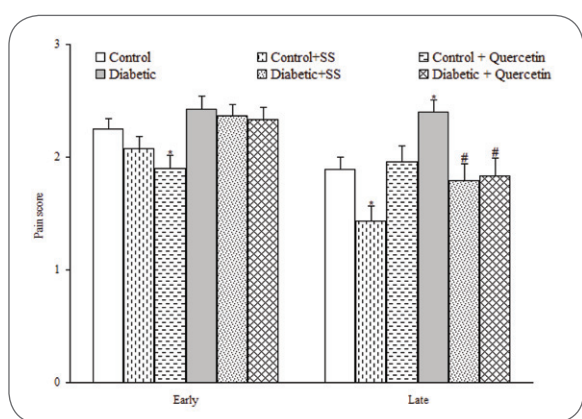
Figure 3. The effect of quercetin and sodium salicylate (SS) on nociceptive scores in the first (early) and second (late) phases of the formalin test. All data represent mean ± S.E.M.
* p<0.05 (as compared to control); # p<0.05 (as compared to diabetic)

Figure 4. The effect of quercetin treatment on nociception threshold in hot tail immersion test. All data represent mean ± S.E.M. * p<0.05 (as compared to control), # p<0.05 (as compared to diabetic)

and incubated at 100 ºC for 80 min. After cooling on ice, samples were centrifuged at 1000×g for 20 min and the absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

2.6. Measurement of Brain SOD Activity

The supernatant of brain homogenate was obtained as described earlier. SOD activity measurement was according to previous works (Bagheri , Joghataei, 2011). Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 ºC) for 40 min and NBT was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity.

2.7. Assay of Brain Nitrite Concentration

Supernatant nitrite content was assayed by the Griess method. Because NO is a compound with a short half-life and is rapidly converted to the stable end products nitrate (NO3 –) and nitrite (NO2–), the principle of the assay is the conversion of nitrate into nitrite by cadmium and followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium. The total nitrite was measured by Griess reaction. The absorbance was determined at 540 nm with a spectrophotometer.

2.8. Protein Assay

The protein content of the supernatant was measured with Bradford method using bovine serum albumin (Sigma Chemical, St. Louis, MO) as the standard (Bradford, 1976).

2.9. Chemicals

Quercetin, Cremephor and reagents for oxidative stress assessment were purchased from Sigma Chemical and Fluka (St. Louis, Mo., USA). Sodium salisylate was obtained from Darupakhsh (Tehran, Iran) and streptozotocin from Pharmacia and Upjohn (USA). All other chemicals were purchased from Merck (Germany) and Temad (Tehran).

2.10. Statistical Analysis

All results are expressed as means ± S.E.M. For multiple comparisons, one-way analysis of variance (ANOVA) was used. When ANOVA showed significant difference, Tukey's post hoc test was applied. Statistical significance was regarded as $p<0.05$.

3. Results

After 6 weeks, the weight of the vehicle-treated diabetic rats was found to be significantly decreased as compared to control rats ($p<0.05$) and quercetin treatment caused a less significant decrease in diabetic rats as compared to vehicle-treated diabetics ($p<0.05$) (Fig. 1). In addition, diabetic rats had also an elevated serum
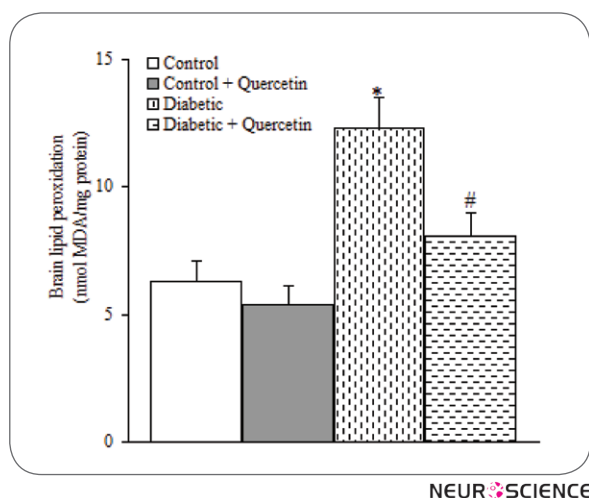
Figure 5. MDA concentration in whole brain homogenate from different groups
* P<0.01 (in comparison with controls)
# P<0.05 (in comparison with diabetics)
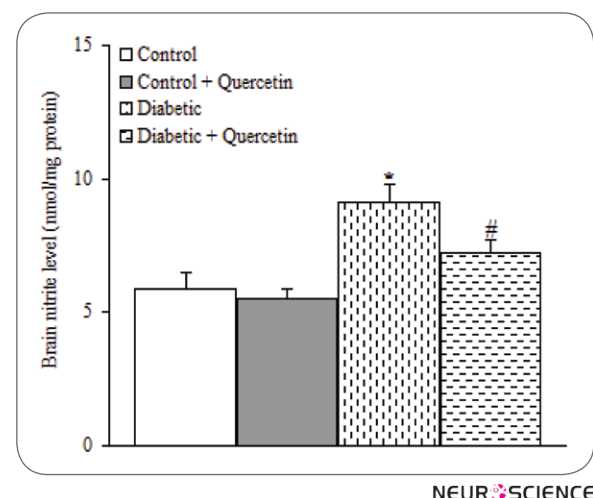
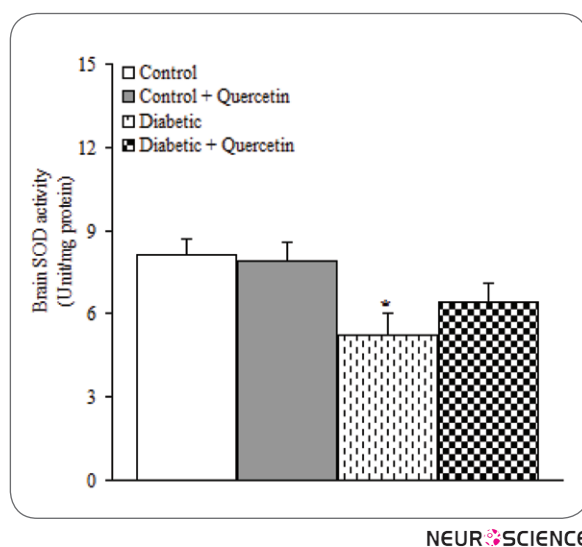**Figure 6.** Nitrite content in whole brain homogenate from different groups.
* p<0.05 (in comparison with controls)
# P<0.05 (in comparison with diabetics)

glucose level over those of control rats (p<0.0001) and treatment of diabetic rats with quercetin caused a significant decrease in the serum glucose (p<0.05-0.01) relative to vehicle-treated diabetics. Meanwhile, quercetin treatment of control rats did not cause any significant change regarding serum glucose level (Fig. 2).

In this study, we used formalin test (with its two marked early and late phases) to evaluate the antinociceptive effect of the flavonoid quercetin in diabetic rats and its comparison with sodium salicylate (positive control) as a standard and generally used drug in this field. Hind limb formalin injection produced a marked biphasic response in the rats of all groups (Fig. 3). Hyperalgesia was significantly (p<0.05) greater in untreated diabetics than in control rats only in late phase of the test. Pretreatment of control and diabetic rats with sodium salicylate (200 mg/kg, i.p.) 1 hour before the test caused a significant reduction (p<0.05) in nociceptive score only in the second phase of the formalin test. In addition, chronic treatment of diabetic rats with quercetin (10 mg/kg) significantly caused lower nociceptive scores in late phase of the formalin test as compared to vehicle-treated diabetics (p<0.05). No such effect was observed for quercetin-treated controls.

In this study, hot tail immersion test was used as a thermal test to evaluate the antinociceptive effect of the flavonoid quercetin in diabetic rats. A significant decrease in tail flick latency was observed in diabetic rats after 6 weeks in hot tail immersion test (p<0.01) (Fig. 4). This deficit in tail flick response latency was significantly reversed on treatment with quercetin (p<0.05).

Figure 7. Superoxide dismutase (SOD) activity in whole brain homogenate from different groups.
* p<0.05 (in comparison with controls)

In our study, we also measured some markers of oxidative stress in the brain homogenate to find whether quercetin could affect it. In this respect, chronic STZ-induced diabetes resulted in significant elevation of MDA and nitrite content (p<0.01) and significant reduction of SOD activity (p<0.05) and treatment of diabetic rats with quercetin significantly attenuated the increased MDA and nitrite content (p<0.05) and level of SOD was slightly higher in quercetin-treated diabetics as compared to vehicle-treated diabetic group (Figures 5-7).

4. Discussion

In this study, development of diabetic neuropathy in STZ-induced diabetic rats was confirmed after 6 weeks, which was consistent with previous reports (Cotter , Jack & Cameron, 2002). It has been well established that oxidative stress finally leads to nerve deficits in diabetic rats(Cameron & Cotter, 1999). Some studies indicated that a hypoxic mechanism in involved in peripheral diabetic neuropathy. Decreased nerve blood flow and ensuing endoneurial hypoxia may cause functional and morphological abnormalities of nervous system(Obrosova, 2003). In addition, increased free radicals due to hyperglycemia may affect central and peripheral nervous system (van Dam, 2002, Obrosova, 2003). Furthermore, there is some evidence for involvement of sorbitol pathway related to glucose metabolism in the pathogenesis of diabetic neuropathy (van Dam, 2002).

In this study, we observed a reduction in tail flick latency in hot tail immersion test in diabetic rats, which indicates thermal hyperalgesia. Several researchers have reported hyperalgesia in diabetic rats (Anjaneyulu & Chopra, 2004, Mahesh & Menon, 2004). Such mechanisms including tissue injury due to ischemia, sensitization of peripheral receptors and ectopic activity in sprouting fibers and alterations in dorsal root ganglia cells have been reported to contribute to changes in nociception (Anjaneyulu & Chopra, 2004, Mahesh & Menon, 2004). It is a well-established fact that diabetic rats display exaggerated hyperalgesic behavior in response to noxious stimuli that may model aspects of painful diabetic neuropathy and for this reason, STZ-diabetic rats have been increasingly used as a model of painful diabetic neuropathy to assess the efficacy of potential analgesic agents (Malcangio & Tomlinson, 1998). Although evaluation of mechanisms causing these symptoms is complicated because of the overlap between the systemic effects of hyperglycemia and its toxic effects within the peripheral nervous system, but direct functional toxicity of hyperglycemia in the peripheral nervous system (Malcangio & Tomlinson, 1998), an increased activity

of primary afferent fibers leading to an increased excitatory tone within the spinal cord, increased release of glutamate and activation of the N-methyl-D-aspartate (NMDA) receptor, reduced activity of both opioidergic and GABAergic inhibitory systems (Malcangio & Tomlinson, 1998), altered sensitivity of the dopaminergic receptors and altered responsiveness of the dopaminergic system, possibly through the enhancement and/or deactivation of the endogenous met-enkephalinergic system (Rutledge, 2002) and alterations in L-type Ca2+ channels (Gullapalli, 2002) are also involved in the modulation of nociception in diabetic rats. As we have previously shown, increased free radical mediated-toxicity has been shown in STZ-diabetic rats (Baluchnejadmojarad , Roghani, 2010, Nourooz-Zadeh, 1997))The elevated level of toxic oxidants in diabetic state may be due to processes such as glucose oxidation and lipid peroxidation (Baluchnejadmojarad , Roghani, 2010). STZ-diabetes is also characterized by several derangements in endogenous antioxidant enzymes (Baluchnejadmojarad , Roghani, 2010) and induction of antioxidant enzymes is a critical approach for protecting cells against a variety of endogenous and exogenous toxic compounds such as reactive oxygen species (ROS) (Baluchnejadmojarad , Roghani, 2010). Increased level of MDA and nitrite and a reduction in the activity of SOD were observed in brain tissue of diabetic rats in our study which is due to increased lipid peroxidation and overproduction of ROS, which is in agreement with previous reports (Vincent , Russell, 2004).

In our study, administration of quercetin for 6 weeks produced a significant analgesic effect at late phase of the formalin test in diabetic rats and sodium salicylate also significantly reduced the nociceptive score in the second phase of the formalin test in control and diabetic rats. It has been known that central acting drugs like narcotics inhibit both phases of the formalin test equally (Shibata, 1989), while peripheral acting drugs like aspirin only inhibit the late phase (Rosland, 1990). Therefore, the effect of quercetin in this study could be mediated possibly through a peripheral mechanism. One of the possible mechanisms that could partially explain the beneficial analgesic property of quercetin may be attributed to its hypoglycemic and antioxidant effects. Since hyperglycemia in diabetic state could induce some functional alterations in the nervous system (Baluchnejadmojarad , Roghani, 2010), quercetin may have attenuated the hyperalgesia in diabetic rats through lowering blood glucose that was observed in this study.

Different mechanisms like enhanced oxidative stress are responsible for hyperalgesia in diabetic rats (Vincent

, Russell, 2004, van Dam, 2002). In this respect, hyperalgesia is partly due to altered functioning of pain receptors and their associated afferent fibers and partly due to changed processing of nociceptive signals (Anjaneyulu & Chopra, 2004). For this reason, oxidative stress was measured in the brain in our study. If we have measured this parameter in the serum, the work was more complete and this is one of the limitations of the work, which should be considered in design of future works.

Overall, chronic quercetin showed anti-hyperglycemic and antinociceptive effect in diabetic rats as it was evident from a reduction in chemical and thermal hyperalgesia and it seems that quercetin may be a good adjuvant in the present armamentarium of antidiabetic drugs to prevent the development of some diabetic complications.

Acknowledgement

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