Kaempferol Attenuates the Development of Diabetic Neuropathic Pain in Mice: Possible Anti-Inflammatory and Anti-Oxidant Mechanisms

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Abbreviations: Diabetic neuropathic pain (DNP), diabetic neuropathy (DN), diabetic mice (DM), oxidative stress (OS), inflammatory cytokines (IC), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), proinflammatory cytokines (pro-IC), neuropathic pain (NP), nuclear factor-κB (NF-κB), Reactive oxygen species (ROS), nitric oxide (NO), N-methyl-D-aspartate (NMDA), mitogen-activated protein kinase (MAPK), prostaglandins (PGs), dimethyl sulfoxide (DMSO), paw withdrawal latency (PWL), Glutathione (GSH), Malondialdehyde (MDA), streptozotocin (STZ), inducible nitric oxide synthase (iNOS), cycloxygenase-2 (COX-2).

Abstract

BACKGROUND: Diabetic neuropathic pain (DNP) is one of the most difficult types of pain to treat. Many studies emphasized on the role of microglial cells, oxidative stress (OS) and inflammatory cytokines (IC) in the development of diabetic neuropathy (DN).

AIM: Present study was designed to evaluate the effect of kaempferol in attenuation of DN in mice.

METHODS: Diabetes was induced in mice by i.p. injection of a single dose of streptozotocin (STZ) (200 mg/kg). Cold allodynia, thermal hyperalgesia and chemical hyperalgesia were assessed, as well as markers of inflammation and OS.

RESULTS: Diabetic mice (DM) showed an increased pain sensation, IC and OS accompanied with reduced body weigh gain. Treatment of DM with kaempferol (25, 50 and 100 mg/kg/day/orally) attenuated the development of DN and reduced pain sensation. Moreover, it reduced interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), lipid peroxidation and nitrite, concomitant with the improvement of antioxidant defense and body weight gain. In contrast, kaempferol (100 mg/kg) had no effects on the behavioral and biochemical parameters. Our results strongly suggest that activated microglia, IC and OS are involved in the development of DN.

CONCLUSIONS: Kaempferol attenuates the development of DNP in mice probably by inhibition of neuroimmune activation of microglia and, partly mediated by reducing IC and OS.

Introduction

Diabetic peripheral neuropathy is the most common complication of diabetes mellitus and characterized by pain, allodynia, hyperalgesia, dysthesias, parasthesias, foot ulcers and amputations and it was estimated that about 50 % of peoples with diabetes have some degree of neuropathic pain (NP) [1]. Several data indicate that involvement of oxidative-nitrosative stress due to generation of advanced glycation end products [1], mitochondrial dysfunction [2] and activation of nuclear factor-κB

(NF-κB) in the development of DN [3].

Reactive oxygen species (ROS) sensitize nociceptors, which respond more vigorously to the noxious stimuli and start to respond to normally sub-threshold stimuli. This peripheral sensitization not only induces pain directly, but also induces central sensitization in the spinal cord, which indirectly contributes to pain as well [4]. Superoxide and peroxynitrite mediates pain that accompanies inflammation and implicated in diabetes-induced motor and, sensory nerve conduction deficits and peripheral nerve energy deficiency [5]. Moreover, studies implicate infiltration and activation of inflammatory cells as well as pro-IC (proinflammatory cytokines) such as IL-1β and TNF-α [6, 7] which causes neuronal hypersensitization. Pain has been thought to arise primarily from the dysfunction of neurons and activation of non-neuronal cells (microglia and astrocytes); that plays an important role in central sensitization [8]. Previous studies have demonstrated that activation of glial cells, particularly microglia in spinal cord [9], retina [10] and hypothalamus [11] in hyperglycemia. Activated microglia release variety of neuromodulators and neuroactive substances such as; ROS (3), nitric oxide (NO), peroxynitrite [12], prostaglandins (PGs) [13], pro-IC (IL-1β and TNF-α) (14) which have been implicated directly in the induction of NP. Glutamate has been reported to be one of the excitatory amino acid released from neurons and activate microglia (15). A study reported that the development of diabetes-induced hyperalgesia involves spinal mitogen-activated protein kinase (MAPK) activation in neurons and microglia via N-methyl-D-aspartate (NMDA) dependent mechanisms [9].

Apart from glycemic control, a corresponding wide range of treatments have been employed to treat patients with NP, such as: opioid, antiepileptics, tricyclic antidepressants, serotonin reuptake inhibitors, NMDA receptor antagonists, lipoic acid, cannabinoids and capsaicin [16]. The most commonly used drug to treat DNP is gabapentin, duloxetine, pregabalin and lidocaine patches (5%) [16]. Drug therapy of DN is often limited and unsatisfactory due to partial effectiveness and associated side effects and further their development is hindered due to incomplete knowledge about induction and maintenance of DN. Epidemiological studies have revealed that a diet rich in plant-derived foods has a protective effect on human health. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavonoid found in many edible plants (e.g. tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries, grapes, Ginkgo biloba, Tilia spp, Equisetum spp, Moringa oleifera, Sophora japonica, cruciferous vegetables, some edible berries and propolis) [17]. Kaempferol has a wide range of pharmacological activities, including anti-oxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-

osteoporotic, anxiolytic, analgesic, anti-hypertensive, antiallergic activities and lipolytic effect [17].

Therefore, the present work was aimed to estimate the effect of kaempferol on experimental DN in mice.

Materials and Methods

Chemicals

Kaempferol, STZ, dimethyl sulfoxide (DMSO) and formalin (37% formaldehyde) were obtained from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany. Rat IL-1β and rat TNF-α ELISA kit were from BioSource International Inc., Camarillo, CA, USA. Unless stated, all other chemicals and biochemical reagents of highest analytical grade quality were used.

Animals

Male Swiss mice (25–30 g) obtained from our animal house facility was used in the present study. The animals were housed under optimal laboratory conditions, maintained on 12 h light and dark cycle with food and water *ad libitum*. Animals were acclimatized to laboratory conditions before the tests. All experiments were carried out between 9:00 and 17:00 o'clock and using age-matched animals in an attempt to avoid variability between experimental groups. Animal body weight was measured at the beginning and at the end of the experiment. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Taif University, Taif, Saudi Arabia (protocol No. IACE/RES 1-434-2205, Dated 15 December 2012). Animals were acclimatized to laboratory conditions for 15 days before starting the experiment. Animals were observed for general health and suitability for testing during this period.

Induction and assessment of diabetes in mice

Mice were treated with a single dose of STZ (200 mg/kg) in citrate buffer (pH 4.4, 0.1 M) intraperitoneally (18). Mice were considered as diabetic if their blood glucose levels were above 200 mg/dl after 48 h of STZ-injection.

Study protocol

Fifteen days after induction of diabetes, mice were randomly divided into 6 groups (8/each). Normal and DM were treated orally (once daily) with DMSO or Kaempferol (25, 50 and 100 mg/kg bwt, freshly prepared in DMSO), in a constant volume of 1 ml/100 g, started on day 16th after induction of diabetes and continued for the next 21 days [19, 20].

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First group; normal mice were treated with DMSO (V).

Second group; DM were treated with DMSO (D).

Third group; normal mice were treated with kaempferol (100 mg/kg) (V+K100).

Fourth, fifth and sixth groups; DM were treated with kaempferol (25, 50 and 100 mg/kg, respectively) (D+K25, D+K50 & D+K100) [19, 21].

The behavioral nociceptive reaction was assessed at 60 min after kaempferol or DMSO treatment [22]. All of the behavioral parameters were evaluated on days 1 (24 h after induction of diabetes) and 15 (the day before DMSO or kaempferol treatment) using 2 h as a time interval, then continued on a different days for each parameter as an explained in the methods section and results to avoid results overlapping and tissue damage.

Behavioral test paradigm

Cold allodynia: Right hind paw of the mice was dipped in water bath maintained at 10 ± 0.5°C (non-noxious stimulus) [23]. The response latency of the right hind paw withdrawal to cold stimulation was estimated thrice with a 5 min interval between (the paw was dried with soft tissue in between) and averaged on days 1, 15, 20, 25, 30 and 35 of the experiment. A fifteen second was imposed as a cut-off time to avoid tissue damage. A marked reduction in paw withdrawal latency (PWL) indicated allodynia.

Tail immersion (Thermal allodynia) test: Mice tail was immersed in a water bath maintained at 52.5 ± 0.5 °C until tail withdrawal (flicking response) or signs of struggle were observed, on days 1, 15, 21, 27, 32 and 36 of the experiment. The baseline latency of tail withdrawal from thermal source was established three times, using 5 min as a time interval (the tail was dried in between) and averaged. A cut-off time of 12 s was used to avoid tissue damage. The decreased response latency (tail withdrawal time) referred to hyperalgesia and is attributed to central mechanisms. In the tail-flick test, the delay in removing the tail from noxious stimuli is evaluated reflecting the activity of simple spinal reflex arc (24). Moreover, the paw withdrawal responses to noxious thermal stimuli (hyperalgesia) reveal supraspinal sensory processing (24).

Paw immersion test: The hyperalgesic response on the paw immersion test is referred to result from central and peripheral mechanisms. Thermal hypersensitivity associated with NP was measured using mean paw withdrawal latencies of the right hind-paw when immersed in water bath maintained at 52.5 ± 0.5 °C, on days 1, 15, 22, 28, 31 and 34 of the experiment. The response latency time of right hind-paw was estimated three times using 5 min as a time interval and averaged. Fifteen seconds was used as a cut-off time to prevent paw injury.

Reduction in the response latency indicates the development of thermal hyperalgesia [25].

Hot plate test: The hyperalgesic response on the hot plate is considered to result from a combination of central and peripheral mechanisms [26, 27]. Mice were placed individually on a hot plate adjusted to 52 ± 1 °C. The latency of hind-paw licking or jumping was determined using a timer as an index of the pain threshold, on days 1, 15, 19, 24, 29 and 33 of the experiment. A cut-off time of 60 s was used to prevent tissue damage.

Formalin-induced pain and edema test (moderate, tonic, inflammatory pain): Mice were left in the observation cage for 30 min to acclimate before starting the experiment (day 37th). Animals were injected with 20 µl of formalin (5% in saline), under the skin of the dorsal surface of the right hind-paw and returned into the observation (10 x 20 x 24 cm). Every fifth seconds the observation of pain-related behaviors (lifting, licking or no pain of the injected paw) was recorded in the early phase (0-5 min) and in the late phase (30-35 min) after the formalin injection. Results were expressed as the sum of flinching response (lifting & licking) in the two phases. Edema formation was evaluated by measuring the thickness of the right hind-paw (before and after formalin injection) using a dial caliper. Edema size was expressed as the difference in paw thickness (in millimeter) ((26, 27). In the formalin test, phase 1 responses reflect acute nociceptive pain similar to the chemical nociceptive tests whereas phase 2 responses are attributed to the combination of on-going inflammatory-related afferent input from peripheral tissue and functional changes in the dorsal horn of the spinal cord.

Biochemical parameters

Twenty four hours after the last treatment (day 38th), blood was collected and used for serum preparation.

Blood glucose: Serum glucose was estimated using enzymatic assay method [28].

Pro-inflammatory cytokines: IL-1β and TNF-α concentration were determined in the serum using rat ELISA kit according to manufacturer's instructions [29] at 450 nm. Values were obtained from a standard curve and expressed as pg/ml.

Markers of oxidative stress: Nitrite was assayed spectrophotometrically using Griess reagent [30]. Glutathione (GSH) and malondialdehyde (MDA) levels were estimated in the mice serum according to the methods described previously by Draper and Hadley [31] and Ellman [32] respectively. Serum GSH and MDA concentration were measured spectrophotometrically [at 412 nm (GSH) and 532 nm (MDA)] from a standard curve that was obtained from freshly prepared standard solution of GSH and 1,1,3,3-tetramethoxypropane respectively.

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Statistical analysis of data

Data are expressed as mean ± SEM and statistically analyzed using one-way ANOVA followed by Tukey's test. $P \leq 0.05$ was considered as statistically significant.

Results

Treatment of normal mice with kaempferol (100 mg/kg) had no significant effect on all behavior and biochemical parameters.

Streptozotocin injection and induction of diabetes

More than 90 % of the animals were diabetic 48 h after STZ administration as indicated by serum glucose levels more than 200 mg/dl and remained high at the end of the experiment (Table 1).

Table 1: Effect of kaempferol on body weight and blood glucose in normal and diabetic mice.

Treatment (mg/kg)	Body weight (g)		Blood glucose (mg/dl)	
	Initial	Final	Initial	Final
V	27.00 ± 0.65	34.88 ± 0.74	100.38 ± 3.31	98.88 ± 2.93
D	27.88 ± 0.48	23.38 ± 0.42[a]	236.25 ± 6.16[a]	320.63 ± 5.72[a]
V+K100	27.13 ± 0.55	36.38 ± 0.68[b]	100.25 ± 3.73[b]	99.25 ± 3.56[b]
D+K25	26.25 ± 0.49	31.38 ± 0.82[a,b,c]	238.50 ± 2.56[a,c]	315.50 ± 6.86[a]
D+K50	26.38 ± 0.53	33.88 ± 0.74[b]	237.38 ± 4.65[a,c]	206.63 ± 4.15[a,b]
D+K100	28.13 ± 0.48	36.13 ± 0.72[a,c]	239.25 ± 5.77[a,c]	149.00 ± 3.60[a,b]

Data are the means ± SEM (n=8). V: vehicle control; D: diabetic group; K: kaempferol. [a]P < 0.05 as compared to vehicle control group; [b]P < 0.05 as compared to diabetic group; [c]P < 0.05 as compared to V+K100 group; [d]P < 0.05 as compared to D+K25 group. Kaempferol was given orally at the recommended doses for 21 days (started on day 16th of the experiment).

Effect of kaempferol on body weight and blood glucose levels

At the end of the experiment, kaempferol treated (25, 50 or 100 mg/kg) and untreated DM exhibited significantly increased glucose levels compared with the control mice. While, administration of kaempferol at doses 50 or 100 mg/kg induced a marked decrease in the blood glucose compared to DM. Interestingly, there was a significant difference in blood glucose levels between kaempferol doses. Moreover, diabetic-untreated and kaempferol treated (25 mg/kg) mice exhibited a marked decrease in the body weight gain compared to control animals, while kaempferol at doses 50 and 100 mg/kg normalized body weight gain in DM (Table 1).

Effect of kaempferol on pro-inflammatory cytokines and markers of oxidative stress

Free radicals and pro-IC significantly increased in DM in response to hyperglycemia, causing oxidative and nitrosative stress, which play an important role in central sensitization. Thus we also evaluated the effect of kaempferol on the markers of oxidative and nitrosative stress as well as pro-IC.

Chronic hyperglycemia induced a marked increase in MDA and nitrite levels, accompanied with reduction of GSH content in serum. Systemic administration of kaempferol during the development of DNP significantly attenuated oxidative and nitrosative stresses in a dose dependant manner (Table 2).

Table 2: Effect of kaempferol on serum GSH, MDA, NO, TNF-α, IL-1β in normal and diabetic mice.

Treatment (mg/kg)	GSH (µmol/ml)	MDA (nmol/ml)	NO (ng/ml)	TNF-α (pg/ml)	IL-1β (pg/ml)
V	202.13 ± 9.42	2.54 ± 0.20	155.00 ± 6.14	50.88 ± 2.42	6.63 ± 0.68
D	63.13 ± 6.41[a]	6.36 ± 0.62[a]	338.63 ± 11.98[a]	552.75 ± 22.93[a]	25.50 ± 1.21[a]
V+K100	224.38 ± 10.35[b]	2.44 ± 0.18[b]	148.63 ± 3.19[b]	51.00 ± 2.56[b]	6.13 ± 0.40[b]
D+K25	91.88 ± 9.09[a,c]	6.06 ± 0.56[a,c]	223.25 ± 19.74[a,b,c]	468.88 ± 24.94[a,b,c]	19.75 ± 1.05[a,b,c]
D+K50	202.50 ± 11.58[b,d]	4.66 ± 0.33[a,b,c]	191.38 ± 6.26[b]	202.50 ± 11.58[a,b,c,d]	13.75 ± 0.77[a,b,c,d]
D+K100	222.38 ± 11.63[b,d]	2.81 ± 0.19[b,d,e]	157.00 ± 6.72[b,d]	57.88 ± 2.67[b,d,e]	6.63 ± 0.38[b,d,e]

Data are the means ± SEM (n=8). V: vehicle control; D: diabetic group; K: kaempferol. [a]P < 0.05 as compared to vehicle control group; [b]P < 0.05 as compared to diabetic group; [c]P < 0.05 as compared to V+K100 group; [d]P < 0.05 as compared to D+K25 group; [e]P < 0.05 as compared to D+K50 group;. Kaempferol was given orally at the recommended doses for 21 days (started on day 16th of the experiment).

The serum levels of IL-1β and TNF-α (Table 2) were significantly elevated in DM when compared to that of vehicle-treated control mice. However, the concentrations of these cytokines were significantly lower in DM that had been treated with kaempferol (25, 50 or 100 mg/kg) in a dose dependant manner. There was a significant difference in kaempferol doses in pro-IC and, markers for oxidative and nitrosactive stress (Table 2).

Effect of kaempferol on formalin-induced flinching and edema size

Table 3 showed that all mice challenged with formalin subcutaneously into the dorsal surface of the right hind-paw had characteristic biphasic response with an early phase 1 and a late phase 2 separated by a quiescent phase as well as increased paw thickness. There was a significant difference in the sum of flinches counted in phase 1, phase 2 and edema size between age-matched control and diabetic animals on day 38th. Whereas systemic administration of kaempferol (50 or 100 mg/kg) to DM significantly decreased formalin-induced nociceptive behavior in phase 1, phase 2 and edema size as compared to vehicle treated DM. DM treated with Kaempferol (25 mg/kg) had no significant changes in the phase 1, phase2 and edema size compared to the diabetic untreated mice.

Effect of kaempferol on cold allodynia

The threshold for cold stimuli was markedly reduced at day 15 in DM and maintained to the end of the experiment compared to non DM. Diabetic

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Table 3: Effect of kaempferol on nociceptive responses and edema size in the formalin test indicative of hyperalgesia in development of diabetic neuropathy.

Treatment (mg/kg)	Number of flinches		Edema size (mm)
	Early phase	Late phase	
V	39.75 ± 1.56	24.25 ± 0.49	1.74 ± 0.06
D	54.00 ± 1.44[a]	35.38 ± 0.50[a]	2.93 ± 0.03[a]
V+K100	37.38 ± 1.46[b]	24.00 ± 0.38[b]	1.61 ± 0.13[b]
D+K25	50.88 ± 1.88[a,c]	34.88 ± 1.29[a,c]	2.46 ± 0.22[a,c]
D+K50	45.13 ± 0.90[b,c]	27.75 ± 1.80[b,d]	1.99 ± 0.10[b,c]
D+K100	41.00 ± 1.99[b,d]	23.75 ± 1.36[b,d]	1.76 ± 0.06[b,c,d]

Data are the means ± SEM (n=8). V: vehicle control group; D: diabetic group; K: kaempferol. [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to diabetic group; [c]$P < 0.05$ as compared to V+K100 group; [d]$P < 0.05$ as compared to D+K25 group. Kaempferol was given orally at the recommended doses for 21 days (started on day 16th of the experiment).
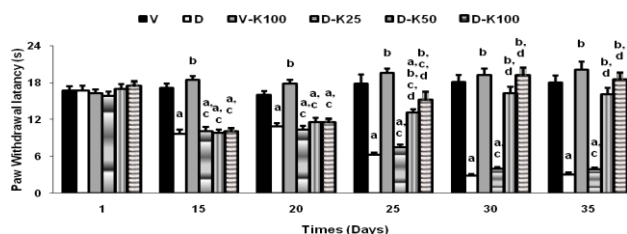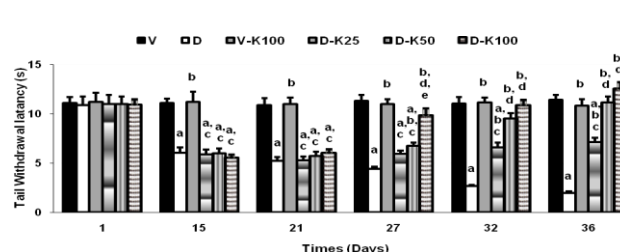
animals exhibited a significant decrease in pain threshold from non-noxious stimuli compared to age-matched, vehicle-treated group. Systemic administration of kaempferol (50 or 100 mg/kg) significantly attenuated the development of hypersensitivity to cold stimulus in DM as compared to vehicle-treated diabetic group. The effect of kaempferol on diabetes induced-hypersensitivity in the cold non-noxious stimuli was dose dependent (Fig 1A).

(A) Cold Allodynia

(B) Tail Immersion

(C) Paw Immersion

(D) Hot-plate

Figure 1: Effect of kaempferol at different doses on (A) PWL to cold stimuli (cold allodynia); (B) tail withdrawal latency to thermal stimuli (Tail immersion); (C) PWL to thermal stimuli (Paw immersion) and (D) PWL to thermal stimuli (Hot plat) in mice indicative of thermal hyperalgesia in development of DN. Data are the means ± SEM (n=8). V: vehicle control group; D: diabetic group; K: kaempferol. [a]P < 0.05 as compared to vehicle control group; [b]P < 0.05 as compared to diabetic group; [c]P < 0.05 as compared to V+K100 group; [d]P < 0.05 as compared to D+K25 group; [e]P < 0.05 as compared to D+K50 group;. Kaempferol was given orally at the recommended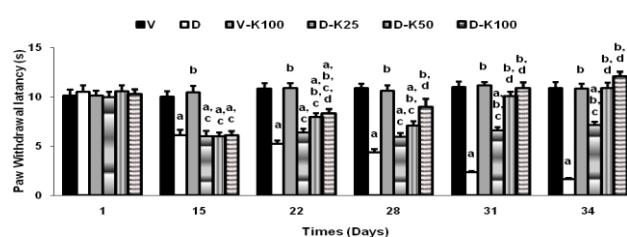 doses for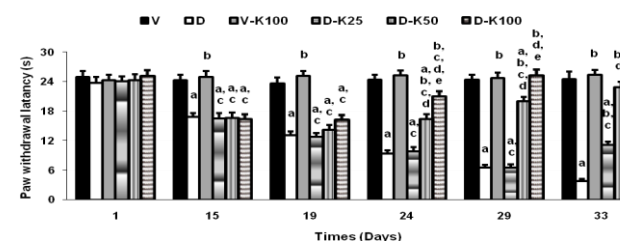 21 days (started on day 16th of the experiment). Fig. 1 showed that diabetic animals exhibited a significant increase in pain sensation in all parameters tested. Administration of kaempferol significantly elevated the pain threshold in all parameters in a dose dependent manner.

Effect of kaempferol on thermal hyperalgesia

The threshold for thermal hyperalgesia was significantly decreased at day 15 and continued to develop down to day 38th after STZ injection as compared to vehicle treated age-matched non-diabetic animals. While, kaempferol (25, 50 or 100 mg/kg) markedly prevented the development of hyperalgesia in DM as compared to vehicle-treated DM. Moreover, the thermal pain threshold was significantly lower in animals treated with 25 mg/kg kaempferol as compared to normal mice. Interestingly, The effect of kaempferol on the thermal pain threshold was dose and time dependent: 25 mg/kg was effective at day 32, 31 and 33 for tail immersion, paw immersion and hot-plate respectively; 50 or 100 mg/kg was effective at day 27, 22 and 24 for tail immersion, paw immersion and hot-plate respectively (Fig. 1B, 1C & 1D).

Discussion

The mechanisms of progressive nerve fiber loss in diabetes, including polyol pathway, glycation, ROS, pro-IC and altered protein kinase C activity, which affect cellular proteins, gene expression and receptor expression and resulting in diabetic complications [33].

In the present study, DM had significantly lower nociceptive threshold and body weight gain, with higher blood glucose, pro-IC and OS. Pre-treatment of DM with kaempferol significantly increased nociceptive threshold and improved body weight gain, with decreased pro-IC and OS. Thus, it is clear from the behavioral studies that kaempferol attenuated the development of DN and suggest the involvement of activated microglia, inflammatory cytokine and OS in nociceptor hypersensitization in diabetes.

This is in line with previous observation reported that, STZ-injected mice had increased pain

sensation in a model of cold allodynia, thermal hyperalgesia, formalin-evoked flinching and formalin increased edema size [24]. Hyperglycemia induces OS through different pathways such as increased aldose reductase activity, glycation, protein kinase C activity, prostanoids production and superoxide generation [4, 33, 34]. All these pathways converge in the production of OS and these results in NF-κB [35] and TNF-α activation [36] and cycloxygenase-2 (COX-2) gene expression [37]. Indeed, activated microglial cells in the central nervous system are an important source of free radicals [13], express inducible nitric oxide synthase (iNOS) [12] in response to pro-IC. ROS, nitrosative and OS have been implicated in the central sensitization, development and maintenance of DN [2, 5], which are attenuated by antioxidants GSH, α-lipoic acid, taurine [2]. Previous studies reported that kaempferol induce-neuroprotective effect through suppression of iNOS, scavenge superoxide and peroxynitrite in glial cells [38]. Furthermore, it attenuate LPS-induced inflammatory mediators (NO, prostaglandine-2, TNF-α, IL-1β, ROS and phagocytosis) in the microglial cells through down-regulation of Toll-like receptors-4, NF-kB, p38 MAPK, JNK and AKT [39]. Several lines of evidence support the neurotoxic role of NO and prostaglandine-2 in neuronal cell death, which can be attenuated by using NOS inhibitors and NSAIDs [4, 40]. The present study provides evidence that attenuation of hypersensitivity in diabetes by kaempferol is accompanied by reduced OS and further suggesting the role of ROS and reactive nitrogen species in the activation of microglia and vice versa in the development of DN.

Hyperglycemia induce cytokine release (TNF-α and IL-1β), which promotes neuronal injury and demyelinating process [6, 36]. TNF-α is a key downstream mediator in inflammatory responses, and IL-1β is known to be the major microglial signal that promotes the cascade of glial cell reactions. Accumulating data indicate that TNF-α, IL-1β, ROS and peroxynitrite directly activate microglia and induced NP [3, 10, 12]. In addition, pro-IC release lead to accumulation of free radicals and activate enzymes like COX-2 and iNOS, further releasing PGs and NO, well known mediators involved in spinal hypersensitization [41]. These observations combined with our results, that showed significant increase in serum IL-1β and TNF-α level in the development of DN which was markedly attenuated by kaempferol treatment [8, 22]. Moreover, Kaempferol inhibits the gene expression of LPS-induced TNF-α and IL-1β [42] and inhibits pro-IC, COX-2 and iNOS, through accumulation of IκB the inhibitory subunit for NF-κB [39, 43]. Further, it provides significant neuroprotection, anti-inflammatory and antinociceptive effects of kaempferol [22]. Consistent with previous reports, kaempferol had no effect on nociceptive threshold in tail immersion and hot plate which further supports that the microglia are not activated in the normal animals [21]. Moreover, kaempferol had no effect on basal responses indicating that the effect in DM is not due to analgesic or hypoalgesic effect only [20, 21, 22]. These data along with the results of the present study suggest that kaempferol decreases pro-inflammatory cytokine levels that are involved in attenuating DN.

Pain has been thought to arise primarily from the dysfunction of neurons. Growing body of data implicate that the activation of non-neuronal cells (microglia and astrocytes) play an important role in central sensitization [8]. Moreover, It was reported that, increased activation of the glial cells, particularly microglia in the spinal cord [9], retina [10] and hypothalamus [11] in uncontrolled hyperglycemic. Activated microglia release variety of neuromodulators and neuroactive substances like ROS [3], NO and peroxynitrite [12], PGs [13], and pro-IC (IL-1β and TNF-α) [14] which have been implicated directly in the induction of DNP. Spinal administration of activated microglia produced hypersensitivity, but not quiescent microglia and activated astroglia which indicated that activated microglia are involved in spinal sensitization [44]. Glutamate and MAPK activate microglia and induced diabetic hyperalgesia via NMDA dependent mechanisms [9, 15]. Moreover, inhibitors of iNOS and COX-2 expression have been found to exert neuroprotective effects in Parkinson's and Alzheimer disease [37]. These findings suggest that kaempferol may be a promising candidate to inhibit the primary steps in the neuroinflammatory pathway. Because, kaempferol is without any effect on neurons and astrocytes, it seems likely that the antihyperalgesic and antiallodynic effects of kaempferol in attenuating behavioral hypersensitivity could be attributed to its ability to suppress the activation of microglia during the course of disease state [21, 39].

In conclusion, the results demonstrate that kaempferol attenuates the development of hyperalgesia and allodynia in STZ model of DNP probably by inhibiting microglial activation and partly by decreasing the inflammatory mediators and oxidative stress. Moreover, further studies are warranted to explore the exact mechanism of kaempferol's anti-nociceptive effect.

Acknowledgments

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