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Original Article

Fullerene C60 nanoparticle attenuates pain and tumor necrosis factor-α protein expression in the hippocampus following diabetic neuropathy in rats

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ABSTRACT

Introduction: Diabetic neuropathy is a common complication of diabetes mellitus. It is associated with nerve damage due to oxidative stress and high levels of pro-inflammatory mediators. In the present study, we examined the anti-nociceptive effects of Fullerene nanoparticle, as a potent anti-oxidant, during diabetic neuropathy.

Methods: Diabetes mellitus induced through injection of streptozotocin (STZ) (40 mg/kg). Four groups were used in the study as follows: the control, control+fullerene, diabetes, and diabetes +fullerene groups. All four groups received sesame oil. Treatment rats received fullerene C60 (1mg/kg/day) for 9 weeks by intra-gastric gavage. Then, cold allodynia, histology, and tumor necrosis factor-α (TNF- α) protein expression of the hippocampus were measured 9 weeks after injection of STZ.

Results: Our data revealed that STZ induces cold allodynia in both hind paws and increases the TNF- α protein expression in the hippocampus. Furthermore, STZ induces neural degeneration in the hippocampus. Additionally, fullerene C60 significantly attenuated cold allodynia and TNF- α protein expression. Also, fullerene C60 has neuro-protective effects on hippocampal neurons. However, fullerene C60 did not significantly reduce serum glucose levels in diabetic animals.

Conclusion: Our data suggest that fullerene C60 likely suppressed pain, and neural loss by inhibitory effects on TNF- α protein expression in the hippocampus during diabetes.

Keywords:
Fullerene C60
Diabetic Neuropathy
TNF-α
Hippocampus

Introduction

Painful diabetic peripheral neuropathy (DPN) is a type of neuropathic pain, which is a common devastating complication in patients with diabetes mellitus (DM) (Askary-Ashtiani et al., 2016; Ismail et al., 2018; Zhu et al., 2018). The sign and symptoms of DPN are characterized by allodynia (pain in response to normally innocuous stimuli), hyperalgesia (increased duration and

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amplitude of response to noxious stimuli), and spontaneous pain (Bahari et al., 2014; Schreiber et al., 2015). The development of DPN is multifactorial and the exact underlying mechanism is hence fairly well known. However, two putative mechanisms that lead to pathogenesis and development of DPN are oxidative stress and neuro-inflammation (Sandireddy et al., 2014; Sha et al., 2017). Sustained hyperglycemia, a hallmark of diabetes, can orchestrate excess generation of reactive oxygen species (ROS) and oxidative stress in diabetes conditions (Kandhare et al., 2012). It is accepted that oxidative stress can also link with the pro-inflammatory mediators implicated in progressive nerve fiber damage during DM (Sandireddy et al., 2014). For example, oxidative stress may cause neural apoptosis and nerve damage via increased inflammatory mediators such as TNF-α level. (Kandhare et al., 2012). TNFα is one of the critical mediators of neuro-inflammation in DPN (Debnath and Agrawal, 2016). Moreover, it has been reported that administration of TNF-α to diabetic rats markedly decreases motor nerve conduction velocity (Satoh et al., 2003). Therefore, it is proposed that TNF-α contributes to diabetic-induced nerve dysfunction or damage (Satoh et al., 2003). The current pharmacological treatment against DPN includes analgesic agents and antidepressant chemicals (Ling et al., 2014). However, these agents can suppress pain perception only in some patients and excessive use of these agents may cause complications in clinical trials (Kandhare et al., 2012). In recent years, the use of antioxidants has been considered a therapeutic strategy for pain (Oyenihi et al., 2015; Husseini et al., 2016, Hadipour et al., 2021). Fullerene C60 nanoparticle (C60), comprised of 60 carbon atoms organized as a hollow sphere, has been evaluated for a wide spectrum of activities including free radical scavenger, antioxidant activity, and neuroprotection activity (Rasouli Vani et al., 2016; Sarami Foroshani and Mohammadi, 2016). It is reported that this nanoparticle can easily react with electrons and decrease free radicals in biological environments (Rasouli Vani et al., 2016). However, the contribution of fullerene C60 in STZ-induced DPN has not been investigated. Here, we investigated the protective effects of chronic fullerene C60 treatment (9 weeks) on the serum glucose and nociception in STZ-induced DPN. The hippocampus, a central component of the limbic system, is involved in the development of neuropathic pain (Ignatowski and Spengler, 2018). Several studies have shown hippocampal dysfunction is involved in the pain behaviors of animals (del Rey et al., 2011; Liu and Chen, 2009). The hippocampus also undergoes increased levels of cytokine expressions during chronic neuropathic pain (del Rey et al., 2011). Therefore, we finally evaluated the effects of fullerene C60 treatment on histology and TNF-α protein level in the hippocampus in a STZ-induced DPN model.

Material and Methods

Animals

Adult male Wistar rats (about 210-250 g) were used in the experiments. They were housed in plexiglass cages in a group of six. Animals were monitored under standard laboratory conditions of 25 ± 2 ˚C and 12 h dark/light cycle). During the experiment, the animals were allowed free access to the standard pellet diet and tap water. The study was conducted in accordance with the Guidelines of the National Institute of Health (NIH) for the Care and Use of Laboratory Animals (Liu and Chen, 2009), and was approved by the local ethical committee (Ethical code: IR.BMSU.REC.1397.410).

Experimental protocols and groups

After one-week accommodation period, animals randomly were divided into 4 groups (n=6 per group). These groups were as follows: [Group 1: control group (intact)]; [Group 2: control+ fullerene group (intact+ fullerene C60)]; [Group 3: diabetic group (STZ)]; [Group 4: diabetes mellitus+ fullerene group (STZ+ fullerene C60)]. Diabetic rats received a single intravenous injection of STZ (40 mg/kg of body weight) into the tail vein at the start of the experiment for induction of DM. All four groups received sesame oil as vehicle. The animals with a blood glucose concentration of >300 mg/dl were used for the study. Treated rats received fullerene C60 (1mg/kg/day) for 9 weeks (63 days) by intra-gastric gavage. Serum glucose level was evaluated on days 3 and 60 after injection of STZ. Behavioral experiments, histology, and western blot analysis were conducted 9 weeks (63 days) post-treatment.

Chemicals

To perform the current study, STZ and C60 fullerene were purchased from Sigma–Aldrich Inc. (St Louis, MO, USA). STZ and fullerene C60 were dissolved in normal saline and sesame oil, respectively.

Induction of Diabetes Mellitus

Ample proposals reported that STZ injection could successfully imitate DM. In our experiment, DM was induced through a single intravenous (i.v.) injection of STZ (40 mg/kg) into the tail vein of animals (Bayatpoor et al., 2019).

Serum glucose determination

Samples were collected from the tail vein; plasma was separated by centrifuge at 3000 × g for 10 minutes. Serum samples were collected and stored at −20°C until analysis. Serum glucose level was evaluated on days 3 and 60 after injection of STZ. Glucose level was estimated by glucose enzymatic kit using a spectrophotometer and measured using the enzymatic colorimetric method (Schleicher and Friess, 2007).

Cold allodynia

Cold-induced pain is an important feature of neuropathic pain (Mangaiarkkarasi et al., 2015). In cold allodynia, physiologic cool stimuli cause pain perception in animals. In the present study, cold allodynia was assessed using the acetone drop method as described by Choi and colleagues with modification on the 9-weeks post-treatment (Choi et al., 1994). Briefly, animals were placed in a metal mesh cage and allowed to habituate for approximately 20 minutes. Then, an acetone drop was applied gently onto the mid-plantar surface of the hind paw. A sensitive reaction for either paw shaking or withdrawal was recorded as a positive response (pain response). For each measurement, the paw was sampled five times for both paws and the frequency of paw withdrawal (positive responses) was calculated. The interval between each application of acetone was approximately 5 minutes.

Western blotting

On the 63rd day (9 weeks post-treatment) of the experiment, the hippocampus of rats was homogenized and centrifuged. Equal amounts of proteins were resolved on a polyacrylamide gel and then transferred to a polyvinylidene fluoride microporous membrane. Then, the membranes were blocked for 1 h at room temperature. The membrane containing the transferred proteins was incubated with primary antibodies: Rabbit polyclonal Anti-TNF alpha (1:2000 ab66579 Abcam), and Rabbit polyclonal GAPDH antibody (1:2000; Abcam Inc.,) overnight at 4°C. After washing, the membranes were incubated with Horseradish peroxidase conjugated secondary antibody (1:5000; Abcam Inc., ab8227) for 1 hour. The density of the protein band was determined using densitometry scanning of blots with Image J software (Liu et al., 2017).

Histological examination (Cresyl Violet Staining)

On the 63rd day of the experiment (9 weeks post-treatment), after the completion of the behavioral experiment, animals were sacrificed with diethyl ether anesthesia and laparotomy was conducted. The hippocampus tissue was removed and fixed in 10% formaldehyde and embedded in paraffin. Sections were floated in a water bath (36 °C) for 5-15 seconds and mounted on poly-L-lysine coated microscope slides. Then, samples were de-paraffinized in a water bath at 56 °C for an hour. Slides were immersed in xylene, 100% ethanol, 95% ethanol, 70% ethanol, and distilled water, respectively. Then, the slides were immersed in cresyl violet staining solution for 15 minutes and washed again in distilled water. Then, the mentioned steps were repeated in reverse. Slides were then cover-slipped with a permanent mounting medium and incubated overnight. Images of the section were examined under light microscopy (Mohd Shafri et al., 2012).

Statistical analysis

All statistical analyses were carried out using the SPSS software (version 21.0). Data were presented as mean±SEM. Differences in measured parameters among 4 groups were analyzed by using one-way analysis of variance (ANOVA), followed by the Tukey HSD post hoc test. The differences were considered to be significant when the probability was less than 0.05.

Results

Effects of fullerene C60 on serum glucose level

As shown in Table 1, fullerene C60 treatment for 9 weeks could not significantly reduce serum glucose level in the diabetes mellitus+ fullerene group as compared with the diabetic group.

Effects of fullerene C60 on cold allodynia

Our data analysis identified that STZ treatment significantly increased paw withdrawal frequency in response to acetone in both hind paws in the diabetic group as

TABLE 1: The effect of fullerene C60 on the serum glucose level.

Groups	Serum Glucose Level (Days after STZ injection)	
	Day 3	Day 60
Control	111.2±3.98	125±4
Control + fullerene	118.7±2.45	122±2
Diabetic	468.2±18.42***	459±14***
Diabetes mellitus + fullerene	424±30***	407±21***

Serum glucose level was evaluated on days 3 and 63 after induction of DM. Data are expressed as mean±SEM. The Control group is included intact animals. The Control+fullerene group included intact animals that received fullerene C60 (1mg/kg/day) for 9 weeks (63 days) by intra-gastric gavage. The diabetic group received a single intravenous injection of STZ (40 mg/kg of body weight) into the tail vein. The diabetes mellitus+fullerene group received a single intravenous injection of STZ and fullerene C60 for 9 weeks. *** denotes a significant difference (P<0.001) with control or control+fullerene groups.

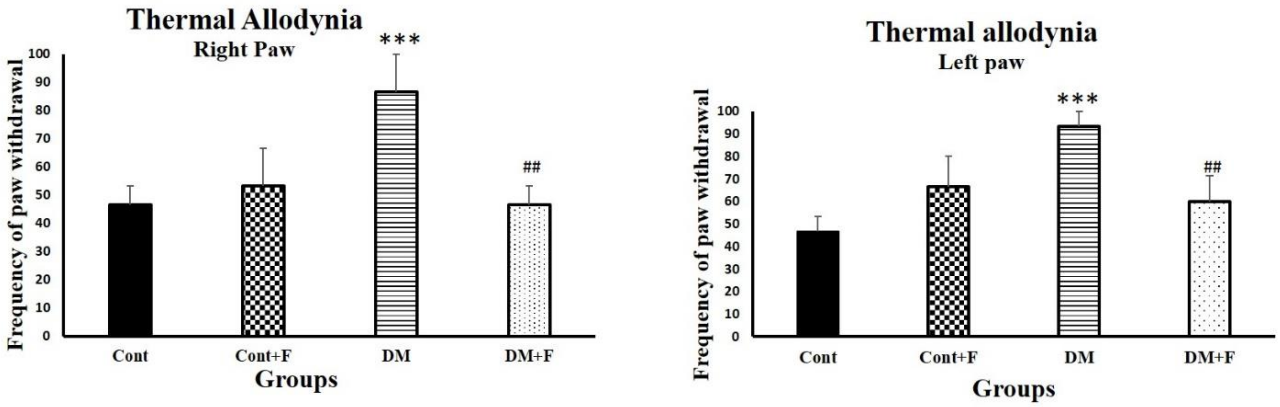

FIGURE 1. Effects of Fullerene C60 were assessed on the frequency of paw withdrawal (cold allodynia). All values are expressed as mean±SEM. ***P<0.001 in comparison with Cont group; ##P<0.01 in comparison with DM group. Cont: control, Cont+F: control + Fullerene, DM: diabetes mellitus, DM+F: diabetes mellitus+ fullerene.

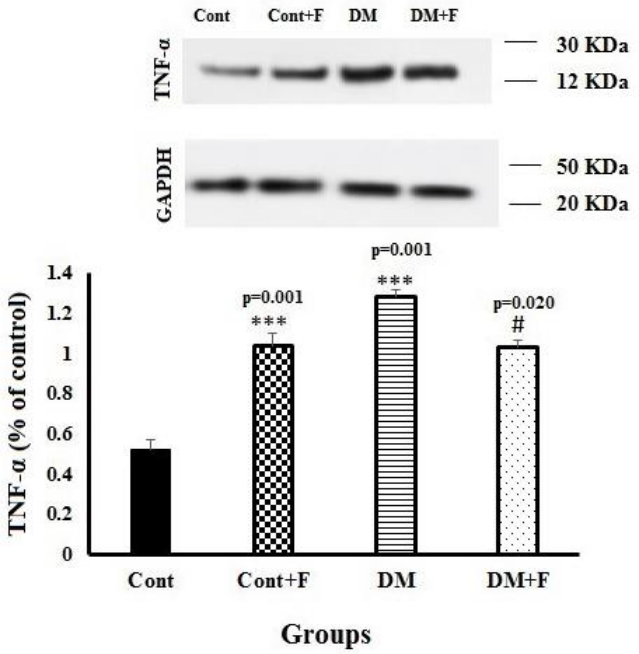

FIGURE 2. Effects of Fullerene were assessed on the TNF-α protein level of the hippocampus. Data are expressed as mean±SEM. ***P<0.001 in comparison with Cont group; #P<0.05 in comparison with DM group. Cont: control, Cont+F: control + fullerene, DM: diabetes mellitus, DM+F: diabetes mellitus+ fullerene.

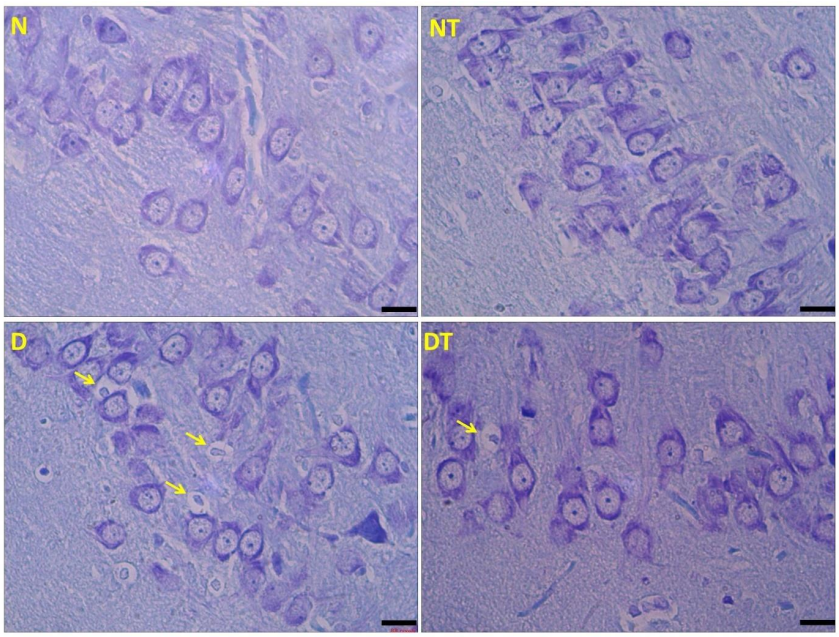

FIGURE 3. Effects of Fullerene were assessed on histology of hippocampus. Photomicrographs of cresyl violet-stained hippocampus sections (dentate gyrus region) are shown in the different experimental groups. Control and control+ fullerene groups show the normal architecture of the hippocampus, respectively (N, normal, and NT, normal+fullerene, groups). STZ-induced DM decreased the hippocampal neurons (D: diabetic group) and application of fullerene C60 prevented STZ-induced neuron loss (DT: diabetes mellitus+fullerene group). (Magnification=400X).

compared with the control group (P<0.05, Figure 1). Application of fullerene C60 significantly reduced paw responses to acetone in both hind paws in the diabetes mellitus+ fullerene group as compared with the diabetic group (P<0.05). Fullerene C60 injection cannot induce significant alteration in paw withdrawal frequency in the control+fullerene group as compared with the control group.

Effects of fullerene C60 on TNF-α protein level of the hippocampus

As shown in Figure 2, The level of TNF-α protein was determined by Western blot analysis in rat hippocampus. The present results identified that injection of STZ significantly increased TNF-α protein level as compared with the control group (Figure 2, P<0.001). Furthermore, application of fullerene C60 significantly decreased TNF-α expression in the diabetes mellitus+fullerene C60 group as compared with the diabetic group (Figure 2, P<0.05). Additionally, application of fullerene C60 significantly increased TNF-α expression in the control+ fullerene group as compared with the control group (Figure 2, P<0.05).

Effects of fullerene C60 on histology of hippocampus

The histological analysis of dentate gyrus region neurons of the hippocampus is shown in Figure 3. We observed normal architecture of the hippocampus in both control and control+ fullerene groups (Figure 3). However, the injection of STZ decreased the hippocampal neurons in the diabetic group. Additionally, application of fullerene C60 prevented STZ-induced neuron loss in the diabetes mellitus+fullerene group as compared with the diabetic group (Figure 3).

Discussion

Allodynia, which is pain perception in response to normally non-noxious stimuli, has been observed in several patients with DM, denoting the painful type of DPN (Ismail et al., 2018). The present study revealed that induction of DM caused cold allodynia, which was shown by a positive response of the hind paw to non-noxious cold (acetone) stimulus 9 weeks after STZ injection. The most common type of nerve damage in DM condition is bilateral distal nerve impairment (Callaghan et al., 2012; Feldman et al., 2017). Similarly, in the present study, cold allodynia in response to acetone drop was observed in both right and left hind paws in diabetic rats. Accumulating evidence highlights that the application of STZ suppresses neuronal survival of the hippocampus,

particularly in the dentate gyrus (Duarte, 2015; Yang et al., 2018). In line with this idea, our data similarly revealed that injection of STZ induced neurodegeneration in the diabetic group. It is proposed that the hippocampus, which is received painful sensory information, is associated with cognitive impairment in the DPN condition (Romero-Grimaldi et al., 2015). Extensive evidence supports the involvement of the hippocampus in process of pain information (Covey et al., 2000). In the present study, we also showed that a single injection of STZ can induce up-regulation of TNF-α protein in the hippocampus of animals. It is proposed that excessive production of ROS in DM is considered a critical mediator of neural dysfunction and neurodegeneration in the development of DPN (Farshid and Tamaddonfard, 2015; Negi et al., 2011). Additionally, high production of ROS can trigger neuro-inflammation in many tissues (Fischer R, Maier, 2015; Solleiro-Villavicencio and Rivas-Arancibia, 2018). Neuro-inflammation can further lead to ROS production, which promotes neuro-degeneration (Fischer R, Maier, 2015). Therefore, the cross-talk between oxidative stress and neuro-inflammation can amplify the development of DPN and subsequently pain perception (Debnath and Agrawal, 2016). Among several pro-inflammatory cytokines, up-regulation of TNF-α protein in the central nervous system during DM is considered an important mediator for initiating sensory nerve damage and the development of DPN (Kuhad and Chopra, 2009). Ignatowski and colleagues have reported that TNF-α can synthesize in the brain and promote neuro-plastic changes in neurons that are critical in the process of pain information (Ignatowski et al., 1999). Altogether, oxidative stress and increased TNF-α protein expression are two key factors in pain perception (allodynia) in DM. Therefore, one of the important goals of the current study was to investigate the analgesic effects of a strong antioxidant, fullerene C60 nanoparticle, in DPN. Our data revealed that the application of fullerene C60 improved STZ-induced cold allodynia and neurodegeneration in the hippocampus. Moreover, fullerene C60 treatment increased the percentage of entries or time in open arms in the diabetes mellitus+fullerene group. This means that fullerene C60 increased the curiosity and adventure of treated rats. Additionally, the application of fullerene C60 reduced the expression of TNF-α in the hippocampus. Also, fullerene C60 significantly increased the expression of TNF-α in the control+fullerene group. One possible explanation is that fullerene C60 alone can stimulate macrophage activity, leading to increased generation of TNFα in the control+fullerene group. However, in the diabetes group, many other factors (in addition to fullerene C60) such as oxidative stress and neuropathy can further increase TNFα production. Therefore, the expression of TNFα protein increased more in the diabetic group than in the control+fullerene group. It is reported that the administration of fullerenes C60 can reduce oxidative stress and neuropathy via its antioxidant activity. Therefore, it is suggested that fullerene C60 can decrease TNFα expression via its antioxidant activity in the diabetes mellitus+fullerene group as compared with the diabetic group. However, due to its stimulatory effects on the activity of macrophages, the expression of TNFα is still high in the control+fullerene group. However, in terms of antidiabetic effects, fullerene C60 treatment could not markedly reduce serum glucose levels. A large body of work demonstrates that prolong hyperglycemia is a critical factor in the initiating and maintenance of painful DPN through various pathologic pathways (Xu et al., 2011). However, the injection of fullerene C60 in the present study suppressed the pain sensation (allodynia) only by reducing the expression of the TNF-α protein in the hippocampus, without having any effect on serum glucose levels.

Conclusion

Our results suggest that a single application of STZ can induce cold allodynia in both hind paws, as an index for the development of DPN, neuron loss, and increased TNF-α protein expression in the hippocampus. Furthermore, chronic treatment of fullerene C60 (for 9 weeks) suppressed thermal pain perception and TNF-α protein expression in the hippocampus.

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Conflict of interest

The authors report no conflict of interest.

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