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Minocycline and Ifenprodil Prevent Development of Painful Diabetic Neuropathy in Streptozotocin-induced Diabetic Rat Model

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ABSTRACT

Painful diabetic neuropathy (PDN) is a common complication of diabetes mellitus which hugely affect life routines and quality. We aimed to investigate the effects of minocycline and ifenprodil on nociceptive behavior response, oxidant-antioxidant status and pro-inflammatory markers level in the PDN rat's spinal cord. Forty-eight male Sprague-Dawley rats were divided into six groups (n=8): non-diabetic control, diabetic PDN control, diabetic PDN rats received minocycline (80µg/day or 160µg/day) and diabetic PDN rats received ifenprodil (0.5µg/day or 1.0µg/day). Diabetes was induced by single injection of streptozotocin at 60mg/kg. The rats were allowed for two weeks period to develop into PDN condition. The intrathecal treatments were given for seven days period. The rat's hind paw was then injected with 5% formalin to induce chronic inflammatory pain and sacrificed three days post-formalin injection. Spinal cord tissue was removed and homogenized (10% homogenate). ELISA for oxidant-antioxidant markers (MDA, catalase and SOD) and pro-inflammatory markers (TNF-α and IL-1β) were carried out. The results showed that untreated PDN rats exhibited increased nociceptive behavior responses indicating hyperalgesia apart from increased MDA activity, reduced catalase with insignificant change in SOD enzymes activities, increased TNF-α and insignificant change in IL-1β level. Minocycline and ifenprodil suppressed MDA activity and improved catalase and SOD activities. Minocycline attenuated both pro-inflammatory cytokines whilst ifenprodil reduced TNF-α level but increased IL-1β level. In conclusion, minocycline and ifenprodil showed potent anti-nociceptive, anti-oxidant and anti-inflammatory effects but possibly via different pathway and mechanism.

Keywords: Painful diabetic neuropathy, minocycline, ifenprodil, formalin test, catalase, interleukin-1β, malondialdehyde, superoxide dismutase, tumour necrosis factor-α

INTRODUCTION

Painful diabetic neuropathy (PDN) is a chronic pain that is developed due to abnormalities in the peripheral somatosensory system in the diabetic patient (Wang *et al*., 2014). The occurrence of PDN arises as a result of hyperglycemia-related with local metabolic and microvascular alterations in both type I and type II diabetes mellitus (DM) (Tesfaye *et al*., 2010, Wang *et al*., 2014). According to the recent reports, approximately 30% of DM patients develop PDN which can be manifested as spontaneous pain, allodynia (pain to normally innocuous stimuli) and hyperalgesia (increased perception of pain to noxious stimuli) (Tavakoli and Malik, 2008). Long-term hyperglycemia in PDN decreases nerve functionality and nerve blood flow which result in malnourished nerve and leads to permanent nerve damage (Vinik and Mehrabyan, 2004). Besides that, diabetic neuropathy may also progressively result in the gradual decreasing in peripheral sensation and eventually complete loss of all sensations to heat, cold, pressure or pain, leading to amputation (Kline *et al*., 2003).

Hyperglycemia is considered a major pathophysiological factor in the development of PDN, however, the mechanisms are still not fully understood. Hyperglycemia has been reported to activate some major pathways like polyol pathway, advanced glycation end products (AGE), hexosamine flux, mitogen-activated protein kinase (MAPK), poly-ADP ribose polymerase (PARP) and cyclooxygenase-2 (COX-2) activation. Cumulative effects of these cascade produce intense oxidative stress, cytokine release and neuroinflammation (Sandireddy *et al*., 2014). Ongoing studies reported the important role of microglial activation and NR2B subunit of NMDA receptors activation in the pathogenesis of PDN and other types of neuropathic pain (Tsuda, 2016, Wang *et al*., 2011, Wang *et al*., 2014). However, the effects of minocycline and ifenprodil treatment on formalin-induced chronic inflammatory pain, oxidant-antioxidant status and pro-inflammatory markers levels in the PDN rat model are still lacking and unclear.

Minocycline is a semi-synthetic second-generation tetracycline (Kraus *et al*., 2005, Mora *et al*., 2014) and has been reported to possess analgesic property via its capability on inhibiting microglial expression (Kobayashi *et al*., 2013, Tikka *et al*., 2001, Tikka and Koistinaho, 2001, Yrjänheikki *et al*., 1998). Kraus *et al.* (2005) have demonstrated that minocycline possesses anti-inflammatory effects via its action in reducing the inducible nitric oxide synthase, caspase-1 activity, metalloprotease activity and formation of cyclooxygenase and prostaglandins. Moreover, other evidence showed the free-radical scavenging activities of minocycline (Miyachi *et al*., 1986) on animal models of stroke and Parkinson's disease (Gilgun-Sherki *et al*., 2002). Minocycline has also shown to suppress lipid peroxidation in rat's brain homogenate (Kraus *et al*., 2005), increase superoxide dismutase activity and reduced the levels of nitric oxide, hydrogen peroxide and malondialdehyde in the mitochondrial cell of *Drosophila* flies treated with manganese (Mora *et al*., 2014).

Ifenprodil is a non-competitive NMDA receptor of NR2B subunit antagonist. This drug is known for its stimulation of brain circulation and selectively inhibits NR2B-containing NMDA receptors (Suzuki *et al*., 1999). It has been reported to have minimal side effect profiles compared to another similar NMDA antagonists (Chizh *et al*., 2001). The previous study showed NR2B subunit protein has been found in the spinal cord (Boyce *et al*., 1999), thus it may become one of the potential targets to combat PDN. Numerous studies have shown the analgesic effect of ifenprodil on neuropathic pain models (Chizh *et al*., 2001, Boyce *et al*., 1999, Zhang *et al*., 2009), but too limited studies have found the effect of ifenprodil to oxidative stress markers on neuropathic pain, specifically diabetic neuropathy. The present study aimed to determine the effects of microglial activation inhibitor (minocycline) and NMDA receptor NR2B subunit antagonist (ifenprodil) on nociceptive behavior responses induced by formalin, oxidant-antioxidant and pro-inflammatory markers levels in streptozotocin-induced painful diabetic neuropathy rat model. We also aimed to find association of nociceptive behavior produced with oxidant-antioxidant status and levels of pro-inflammatory markers in the spinal cord of the rats. We hypothesized that both minocycline and ifenprodil may reduce the nociceptive behavior responses, oxidative stress and pro-inflammatory markers level in the spinal cord of streptozotocin-induced PDN rats.

MATERIALS AND METHODS

2.1 Animals

All the procedures conducted on the rats have obtained the approval from Ethics Committee of Universiti Sains Malaysia Health Campus, Malaysia [USM/Animal Ethics Approval/2014 (91) (560)]. Forty-eight Sprague-Dawley male rats (200-230 g, 8-10 weeks old) were

randomly assigned into six groups comprising of: (1) non-diabetic control (S+CB), (2) diabetic control rats that developed PDN (S+STZ), diabetic rats with PDN that received (3) minocycline hydrochloride (International Laboratory, USA) at a lower dose (M 80) or (4) minocycline hydrochloride at a higher dose (M 160), and diabetic rats with PDN that received (5) ifenprodil tartrate (International Laboratory, USA) at a lower dose (I 0.5) or (6) ifenprodil tartrate at a higher dose (I 1.0). All the rats were housed individually in an air-conditioned room (20˚C) under 12 h light/dark cycles beginning at 7:00. They were fed with standard food pellets and free access to water and allowed for acclimatization in Animal Research and Service Centre (ARASC) for four days prior to experimentation. The rats were fasted for 14 hours prior to single dose injection of streptozotocin (60 mg/kg) to induce Type-I DM. Meanwhile, non-diabetic rats were injected with single dose injection of vehicle (citrate buffer pH 4.5). Ten percent sucrose solution was given to the rats for one-day post-STZ injection to avoid hypoglycemia. The rats were allowed for two weeks period to develop into painful diabetic neuropathy condition. The selection of PDN rats was determined at two weeks post-STZ injection via tactile allodynia assessment (unpublished data). Only rats with 15% hyperalgesic from the baseline value were included in this study based on a method by Daulhac *et al.* (2006).

2.2 Direct drug delivery

Two weeks after diabetic induction, 20 µL of the treatment by either saline (vehicle), minocycline (80 µg/day or 160 µg/day) or ifenprodil (0.5 µg/day or 1.0 µg/day) was administered via direct intrathecal drug delivery method following Lu and Schmidtko (2013) for seven constitutive days. The rats were initially anaesthetized with the mixture of isoflurane and oxygen in 1:4 ratio using an anesthesia machine. The back of the rat's body was shaved and the area between L5 and L6 spinous processes were chosen as the site of skin puncture. The vertical puncture of the needle to the dura mater was confirmed by either a reflexive flick or the "S" formation of the rat's tail. The treatment was slowly released and the rats were observed for two minutes after the injection to ensure no motor impairment in the rats.

2.3 Formalin test

The chronic inflammatory pain was induced by injecting 5% formalin solution to the dorsal surface of the right hind paw. The rat was immediately transferred to a Plexiglas chamber (26 cm x 20 cm x 20 cm) and pain behavior responses were recorded for one hour and scored by two persons blinded to the treatment groups. A mirror was placed beneath the floor at a 45º angle to allow a clearer view of the injected paw. The pain behavior score assessed was based on the pain score from 0 to 3 referred to Dubuisson and Dennis (1978) as follows:

0 = the foot flat on the floor with all toes splayed indicating rats having no pain
1 = the injected paw has little or no weight on it with no toes splaying, indicating mild pain
2 = the injected paw is lifted up and the paw is not in contact with any surface, indicating moderate pain
3 = the injected paw is shaken, licked or bitten indicating severe pain

The nociceptive behavior responses were tabulated for each minute and averaged at a 5-minute interval (Hayati et al., 2008). The nociceptive responses were divided into phase 1 (mean score from minute-0 to minute-5) and phase 2 (mean score from minute-15 to minute-60).

2.4 Preparation of tissue samples

The rats were sacrificed three days post-formalin injection by an overdose of sodium pentobarbitone and decapitated using a guillotine after the pinch reflex was not detected. In our unpublished work, activated microglia was demonstrated to be clearly expressed and localized to the portion of lamina I-IV in the spinal cord at three days after formalin injection, which is also supported by Fu *et al.* (1999). The lumbar enlargement region of the spinal cord tissue was then removed and homogenized using a tissue homogenizer. The 10% homogenate of the spinal cord tissue was centrifuged at 10,000 x g for 10 minutes at 4˚C to discard nuclei and cell debris. The supernatant was collected and stored at -80˚C until further assay.

2.5 Oxidant-antioxidant Assays

2.5.1 Malondialdehyde (MDA)

The concentration of MDA in the spinal cord sample was measured by the thiobarbituric acid (TBA) test by commercially available MDA assay kit (Northwest Life Science Specialties, Canada). The reaction of MDA with TBA, forming an MDA-TBA_2 adduct that absorbs strongly at 532 nm wavelength. Butylated hydroxytoluene (BHT) was initially added into an Eppendorf tube, followed by the addition of the sample.

Acid Reagent was then added to the tube followed by TBA Reagent. The mixture was then vigorously mixed and incubated at 60˚C for 60 minutes. The mixture was then centrifuged at 10,000 x g for 3 minutes at 4 ˚C. The reaction mixture was then transferred to a cuvette and the absorbance of the sample was measured by a spectrophotometer (PG Instruments, UK) from 400 to 700 nm. The highest peak of the sample was detected at 532 nm wavelength. The sample was then transferred into a 96-wells flat-bottom microplate and the MDA reaction was measured at 532 nm using an automated ELISA reader (Thermofisher Scientific, USA) to perform a third derivative analysis. The results were expressed as mmol thiobarbituric acid reactive substances per mg protein.

2.5.2 Catalase

Catalase catalyzes the breakdown of hydrogen peroxide (H_2O_2) to produce water and molecular oxygen. Catalase activity of the spinal cord sample was determined by using a commercial ELISA kit (Bioassays, USA). H_2O_2 substrate was added to the samples in 96-wells plate and incubated for 30 minutes at room temperature. Detection Reagent was then added to the mixture and re-incubated for 10 minutes at room temperature before the absorbance of an H_20_2 reaction was read at 570 nm wavelength by an ELISA reader. The specific activity of catalase was calculated and expressed in terms of unit U per mg of protein (U/mg protein).

2.5.3 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. This enzyme is an important antioxidant defense in all cells exposed to O_2. The reaction of the SOD in the spinal cord sample was determined using a commercially available kit (Abnova, Taiwan). Working Reagent (Xanthine, WST-1) was firstly added into 96-wells flat-bottom microplate containing samples. XO enzyme was then added immediately and the reaction was measured at 440 nm using an ELISA reader (OD_0). The mixture was then incubated at room temperature for 60 minutes in the dark and again measured at the similar wavelength (OD_{60}). The reaction of SOD (OD_{60}-OD_0) was calculated and expressed as U per mg protein.

2.5.4 Protein measurement

Protein concentration in the spinal cord sample was determined by the method of Bicinchoninic Acid using bovine serum albumin as a standard.

2.6 Assays of pro-inflammatory markers

2.6.1 Interleukin-1β (IL-1β)

Interleukin-1β level of the spinal cord homogenate was determined by using a commercially available ELISA kit (Cusabio, China). The sample was added to the IL-1β readily coated -96 wells flat-bottom microplate and incubated for two hours at 37˚C. The sample was then completely removed and Biotin-antibody (1x) was added to the wells and re-incubated at 37˚C for one hour. The microplate was again re-incubated for 15 minutes at 37˚C in the dark. The reaction was stopped by adding Stop solution and measured with an automated ELISA reader at 450 nm wavelength.

2.6.2 Tumor Necrosis Factor-α (TNF-α)

TNF-α concentration in spinal cord tissue was determined by using a commercially available ELISA assay kit (Raybiotech, USA). Samples were initially added to the TNF-α-readily coated-96 wells flat-bottom microplate and incubated for 2.5 hours at room temperature. The sample was then discarded and the wells were washed with 1X Wash Solution. 1X prepared biotinylated antibody was then added into each well and re-incubated for 1 hour at room temperature. The solution was then discarded and the plate was re-washed. Streptavidin solution was then added to each well and incubated for 45 minutes at room temperature. The plate was again washed before TMB One-Step Substrate reagent was added to each well and incubated for 30 minutes at room temperature in the dark. The reaction was stopped with the addition of Stop Solution and immediately read at 450 nm wavelength using an automated ELISA reader.

2.7 Statistical analysis

The data for each parameter were initially checked for the data distribution. Phase 1 and 2 of formalin test, MDA level, SOD and catalase enzymes activities, IL-1β and TNF-α levels were analyzed with one-way ANOVA with either post-hoc LSD (sphericity assumed) or Dunnett's T3 (sphericity not assumed), according to the value of Levene's test. The correlations between phases of formalin test with oxidant-antioxidant (MDA, catalase and SOD activities) as well as pro-inflammatory cytokines (IL-1β and TNF-α) levels in this study were assessed by Pearson's correlation analysis. The values were expressed as the mean ± standard error of the mean (S.E.M). The significance level was taken as less than 0.05 ($p < 0.05$).

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RESULTS AND DISCUSSION

3.1 Effect of minocycline and ifenprodil on nociceptive behavior score

All the rats challenged with an intraplantar injection of formalin demonstrated biphasic response characteristics with phase 1 and phase 2 separated by a quiescent period (Figure 1A). The result showed a significant increase in the burst activity of nociceptive behavior during phase 1 (Figure 1B) but no significantly differed during phase 2 in (S+STZ) group compared to (S+CB) group (Figure 1C). Minocycline administration of both doses was shown to significantly reduced formalin-induced nociceptive behavior in both phase 1 (Figure 1B) and phase 2 (Figure 1C) compared to (S+STZ) and (S+CB) groups. At the same time, the reduction of nociceptive behavior responses was obvious compared to ifenprodil-treated groups. Meanwhile, ifenprodil treatment at both doses did not alter nociceptive behavior score during phase 1 (Figure 1B) but significantly reduced nociceptive behavior score during phase 2 (Figure 1C).

3.2 Effect of minocycline and ifenprodil on malondialdehyde (MDA) activity

MDA activity was significantly increased in diabetic PDN control (S+STZ) compared to (S+CB) group. This suggests that painful diabetic neuropathy causes lipid peroxidation in the spinal cord. Interestingly, the increased lipid peroxidation level in PDN rat's spinal cord was shown to be corrected by minocycline and ifenprodil at both lower and higher doses (Figure 2).

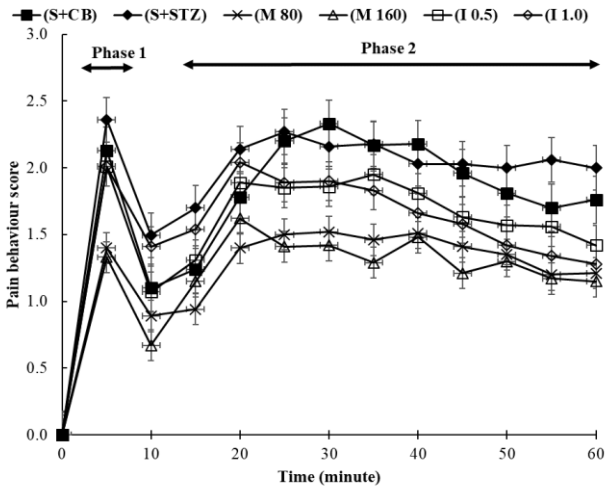

Figure 1 (A)

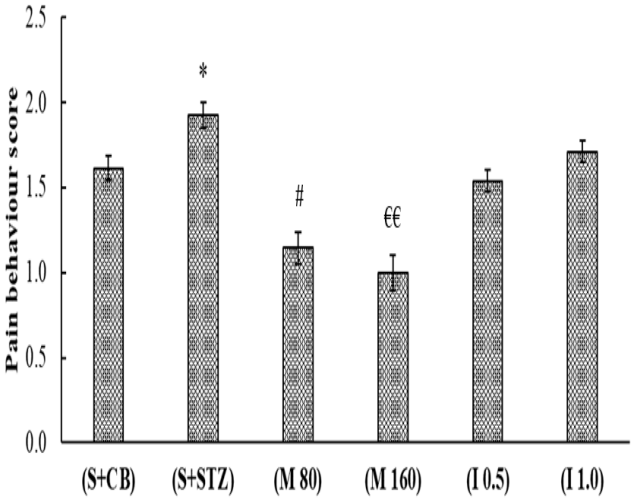

Figure 1 (B)

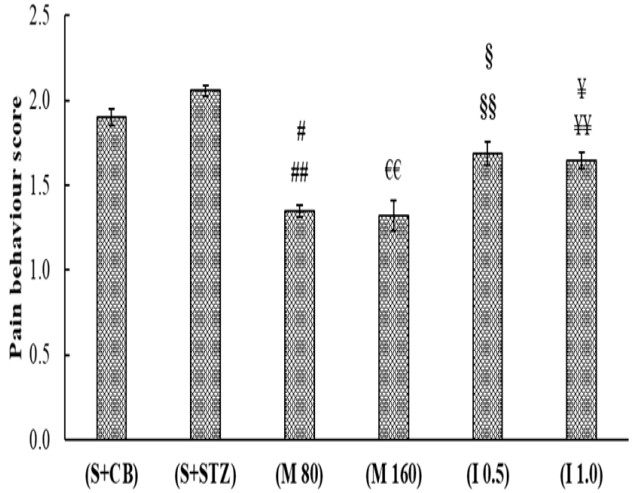

Figure 1 (C)

Figure 1 (A): Nociceptive behavior responses for all groups in one- **Figure 1 (A):** hour period. The values are expressed as mean ± S.E.M (n=8).

Figure 1 (B): Nociceptive behavior response during phase 1. The values are expressed as mean ± S.E.M (n=8). *, p<0.05 Significant compared to (S+CB) groups, #, p<0.001 significant compared to (S+STZ), (S+CB), (I 0.5) and (I 1.0) groups, and €€, p<0.001 significant compared to (S+STZ), (S+CB), (I 0.5) and (I 1.0) groups.

Figure 1 (C): Nociceptive behavior response during phase 2. The values are expressed as mean ± S.E.M (n=8). #, p<0.05 significant compared to (I 1.0) group, ##, p<0.001 significant compared to (S+STZ), (S+CB) and (I 0.5) groups, €€, p<0.001 significant compared to (S+STZ), (S+CB), (I 0.5) and (I 1.0) groups, §,p<0.05 significant compared to (S+STZ) group, §§, p<0.001 significant compared to (S+CB) group, ¥, p<0.05 significant compared to (S+STZ) and ¥¥, p<0.001 significant effect compared to (S+CB) group.

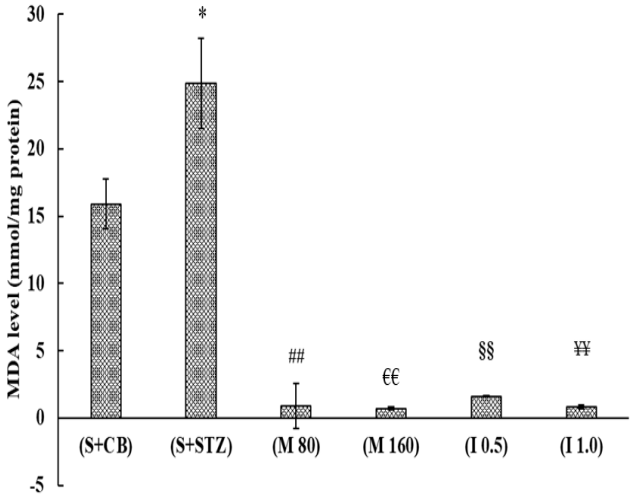

Figure 2

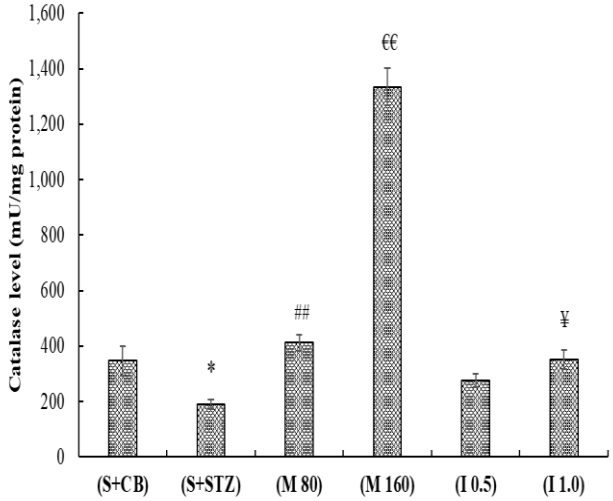

Figure 3

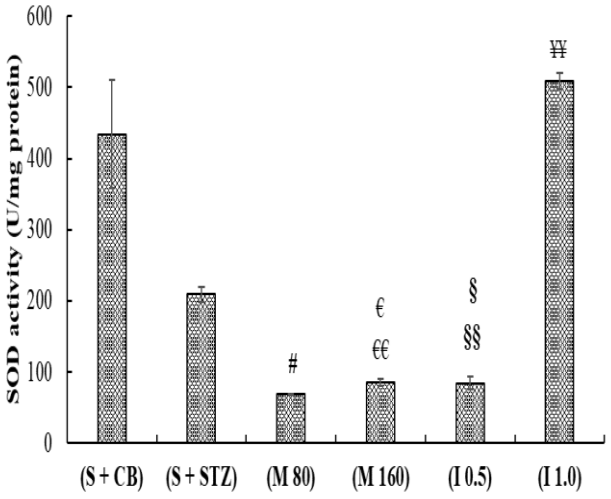

Figure 4

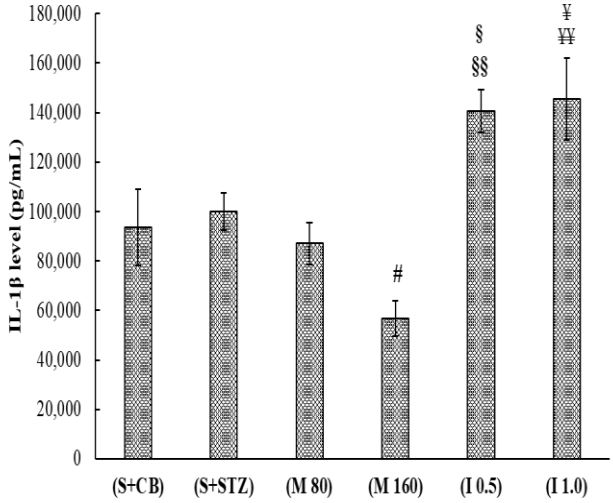

Figure 5

Figure 2: Malonylaldehyde (MDA) activity between the groups. The values are expressed as mean ± S.E.M (n=8). *, p<0.001 significant compared to (S+CB) group, ##, p<0.001 significant compared to (S+STZ) and (S+CB) groups, €€, p<0.001 significant compared to (S+STZ) and (S+CB) groups, §§, p<0.001 significant compared to (S+STZ) and (S+CB) groups and ¥¥, p<0.001 significant compared to (S+STZ) and (S+CB) groups.

Figure 3: Catalase level between the groups. The data was expressed as mean ± SEM (n=8). *, p<0.05 significant compared to (S+CB) group, ##, p<0.001 significant compared to (S+STZ) and (I 0.5) groups, €€, p<0.001 significant compared to (S+STZ), (S+CB), (M 80), (I 0.5) and (I 1.0) groups, and ¥, p<0.05 significant compared to (S+STZ) group.

Figure 4: Superoxide dismutase (SOD) activity between the groups. The values are expressed as mean ± S.E.M (n=8). #, p<0.05 significant compared to (S+STZ) group, €, p<0.05 significant compared to (S+STZ) group, €€, p<0.001 significant compared to (S+CB) group, §, p<0.05 significant compared to (S+STZ) group, §§, p<0.001 significant compared to (S+CB) group, ¥¥, p<0.001 significant compared to (S+STZ), (S+CB), (M 80), (M 160) and (I 0.5) groups.

Figure 5 : Interleukin-1β (IL-1β) level between the groups. The values were express as mean ± S.E.M (n=8). #, p<0.05 significant compared to (S+STZ) and (S+CB) groups, §, p<0.05 significant compared to (S+STZ) and (S+CB) groups, §§, p<0.001 significant compared to (M 80) and (M 160) groups, ¥, p<0.05 significant compared to (S+STZ) and (S+CB) groups, ¥¥, p<0.001 significant compared to (M 80) and (M 160) groups.

3.3 Effect of Minocycline and Ifenprodil on Catalase activity

Catalase enzyme activity was found to be significantly reduced in (S+STZ) group compared to (S+CB) group. The reduced catalase activity in PDN rats was improved by minocycline treatment at both doses with the higher administration of minocycline (160 µg/day) was highly boosted catalase enzyme activity to scavenge lipid peroxidation. Meanwhile, the administration of ifenprodil at a higher dose (1.0 µg/day) was demonstrated to increase catalase enzyme activity in the spinal cord of PDN rats whilst the use of ifenprodil at a lower dose (0.5 µg/day) did not give any significant effect on catalase enzyme activity (Figure 3).

3.4 Effect of Minocycline and Ifenprodil on Superoxide dismutase activity

The reduction of SOD enzyme activity in diabetic PDN control (S+STZ) group was demonstrated to be not significantly differed with (S+CB) group. The treatment of minocycline at both doses has been shown to boost SOD enzyme activity in the spinal cord of PDN rats. Meanwhile, the administration of ifenprodil at both doses also significantly boost SOD enzyme activity with the higher dose of ifenprodil (1.0 µg/day) highly boosted SOD enzyme activity in PDN rat's spinal cord. The increase in SOD enzyme activity was also seen to be better in the ifenprodil-treated group at higher dose compared to minocycline-treated groups (Figure 4).

3.5 PRO-INFLAMMATORY MARKERS

3.5.1Effects of Minocycline and Ifenprodil on interleukin-1β level

The increase in pro-inflammatory IL-1β level in diabetic PDN control (S+STZ) group was not significantly differed from (S+CB) group. The treatment with minocycline at a higher dose (160 µg/day) was significantly reduced the level of IL-1β whilst the treatment of minocycline at a lower dose did not give effect to IL-1β level. On the other hand, the treatment of ifenprodil at both doses has been exhibited to significantly increase the release of IL-1β in the spinal cord of PDN rats (Figure 5).

3.5.2 Minocycline and Ifenprodil reduce TNF-α level

The pro-inflammatory TNF-α level was significantly increased in diabetic PDN control (S+STZ) group compared to (S+CB) group. Minocycline and ifenprodil treatments at both doses have been shown to significantly reduced the level of TNF-α in the spinal cord of PDN rats in which not statistically differed from (S+CB) group (Figure 6).

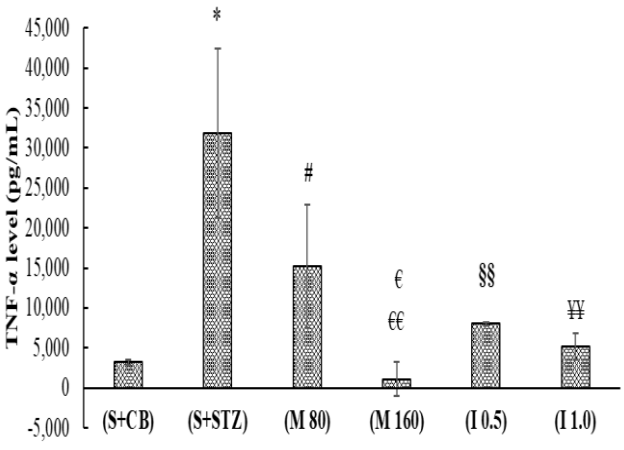

Figure 6

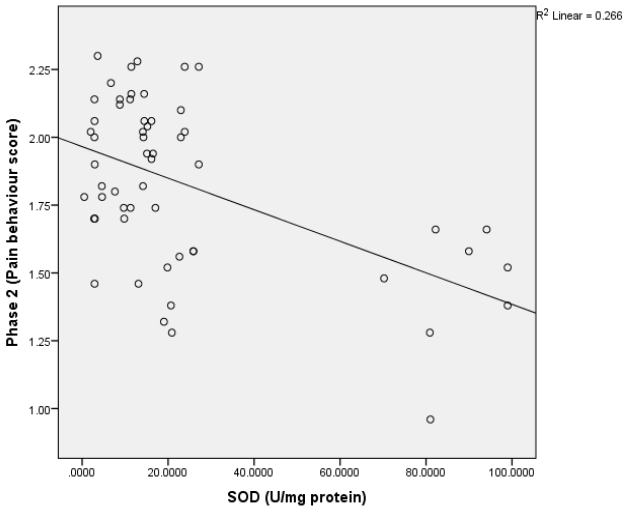

Figure 7

Figure 6: Tumor necrosis factor-α (TNF-α) level between the groups. The values were express as mean ± S.E.M (n=8). *, p<0.05 significant compared to (S+CB) group, #, p<0.05 significant compared to (S+STZ) and (S+CB) groups, €, p<0.05 significant compared to (M 80) group, €€, p<0.001 significant compared to (S+STZ) group, §§, p<0.001 significant compared to (S+STZ) group and ¥¥, p<0.001 significant compared to (S+STZ) group.

Figure 7: Relationship between nociceptive behavior response and SOD enzyme activity (r = -0.516, p<0.001).

3.6 Relationship between nociceptive behavior score and SOD enzyme activity

A Pearson product-moment correlation was run to determine the relationship between phase 2 of formalin test and SOD enzyme activity. There were a strong, negative correlations detected between SOD ($r=-0.516$, $n=48$, $p<0.001$) and phase 2 of pain behavior score (Figure 7). No significant correlation was detected between catalase enzyme activity, MDA, IL-1β and TNF-α level with phases of the formalin test.

DISCUSSION

In the present study, we have explored the effect of minocycline (microglial inhibitor) and ifenprodil (NR2B subunit of NMDA receptor antagonist) on the development of painful diabetic neuropathy. The assessment of hypersensitivity to chemical-induced nociception was performed by formalin test. The results of this study demonstrate that (1) minocycline and ifenprodil treatments attenuated the development of painful diabetic neuropathy (2) that possibly associated with the reduced levels of pro-inflammatory cytokine(s) and oxidative stress as well as increased antioxidant enzymes. This study also supports the fact that (3) NR2B subunit contributes to the increased hypersensitivity and spinal microglia becomes activated in painful diabetic neuropathy which further leads to the increased release of pro-inflammatory mediators and oxidative stress.

In formalin test, phase 1 of nociceptive responses actually represents acute nociceptive pain in which the burst of nociceptive responses is due to the peripheral action as inflammatory mediators released irritate peripheral nerves and tissues after the formalin injection. Whereas, phase 2 of nociceptive responses reflects the combination of on-going inflammatory-associated afferent input from the peripheral nerves and tissues as well as functional modifications in the dorsal horn of the spinal cord (Pabreja et al., 2011). In other words, phase 2 represents the central actions after formalin injection. In the present study, PDN rats demonstrated exacerbated responses in phase 1 but not phase 2, proving the peripheral nerves of PDN rats are still functioning although previous studies reported the damage in peripheral nerves of diabetic rats (Jirmanova, 1993). Minocycline was found to attenuate the nociceptive responses during phase 1 and phase 2 indicating that minocycline may act peripherally and centrally in attenuating nociceptive processes. Meanwhile, ifenprodil has been shown to attenuate nociceptive responses during phase 2 but not phase 1 of the formalin test. It indicates that the action of ifenprodil possibly at centrally in diabetic neuropathy rats model. Although NR2B subunit is also expressed on small and medium sized-primary afferent terminals (Ma and Hargreaves, 2000), however, it is possible that the expression may be reduced due to the effects of diabetic neuropathy on peripheral nerves. The metabolic changes promote demyelination and remyelination processes of peripheral nerves (Pabreja et al., 2011) in which progressively enhance the pathogenesis of diabetic neuropathy. These processes may reduce the expression of NR2B subunit of NMDA receptors on the peripheral nerve of PDN rats. NR2B subunit of NMDA receptors has been found to be denser and more defined distribution in the spinal dorsal horn laminae than other members of NR2 family, therefore the inhibition effect of ifenprodil may be more prominently seen in the spinal level.

As previously mentioned, the imbalance between ROS and antioxidant enzymes and production of oxidative stress has been associated with the occurrence of diabetic related complications. In the present study, we found a marked elevated MDA activity and the prominent reduction in catalase enzyme activity in diabetic PDN rats which are parallel to the previous investigations (Ates et al., 2006, Ates et al., 2007, Delibas et al., 2004, Koneri et al., 2014, Montilla et al., 1998, Pabreja et al., 2011, Qujeq et al., 2004, Zhao et al., 2014). However, the reduction in SOD enzyme activity in diabetic PDN was not significantly differed from non-diabetic rats. In fact, the previous studies revealed that SOD enzyme activity is quite erratic and demonstrated an irregular pattern in the occurrence of diabetes. The amount of SOD enzyme was found to be inconsistently increased, no change and reduced in certain fluids, tissues and organs of STZ-induced diabetic models (El-Khatib et al., 2001, Jang et al., 2000, Kędziora-Kornatowska et al., 1998, Mohan and Das, 1998, Obrosova et al., 2000, van Dam et al., 1998). The altered oxidant-antioxidant balance by the increased oxidant MDA activity and reduced catalase and SOD enzymes activities explains the less protection of antioxidant enzymes the spinal cord of diabetic PDN rats. The higher the free radicals produced, the lower the antioxidant defense protecting the affected cells and tissues from severe oxidative damage (Saini et al., 2007). The treatment with minocycline and ifenprodil has

successfully protected the spinal cord of PDN rats by improving the oxidant-antioxidant balance. Both of these treatments attenuated lipid peroxidation and boosted catalase and SOD enzymes activities in the spinal cord of PDN rats. Minocycline has been reported to possess anti-oxidant effect and highly potential to capture free radicals (Dean et al., 2012, Mora et al., 2014). The antioxidant activity of minocycline has also been shown in previous studies on various animal models of neuropathic pain (Aras et al., 2013, Kriz et al., 2003, Pabreja et al., 2011). The inhibition of microglial activation by minocycline proves that activated microglia in CNS is a major source of free radicals in painful diabetic neuropathy (Pabreja et al., 2011, Tsuda et al., 2008). The protection of minocycline by improving the oxidant-antioxidant balance may explain the attenuation of nociceptive behavior responses during phase 1 and 2 of the formalin test. Furthermore, the strong correlation of SOD enzyme activity with formalin-induced nociceptive responses also strengthen this effect. Moreover, the attenuation of lipid peroxidation by ifenprodil may strongly support that activated NR2B subunit of NMDA receptors contributes to the formation of oxidative stress in painful diabetic neuropathy model (Hu et al., 2015, Zhu et al., 2015). Although there is a very limited study that has investigated the effect of NMDA receptor NR2B subunit antagonists like ifenprodil on the oxidative damage, we believe that ifenprodil may have some potential effects on the pathways that boost the anti-oxidation enzymes production and activity, thus causes the significant suppression of lipid peroxidation in spinal cord tissue.

It is also well-known that diabetic neuropathic pain relies on the level of pro-inflammatory release. Hence, in this study we have investigated the markers of pro-inflammatory cytokines to gain the insight into antinociceptive, antioxidant and anti-inflammatory effects of minocycline and ifenprodil. Several clinical investigations have reported that diabetic neuropathy is related with the increased pro-inflammatory mediators (Pabreja et al., 2011, Skundric and Lisak, 2003). Most importantly, the source of pro-inflammatory cytokines in the spinal cord is derived from activated microglia and the release of these cytokines may activate NR2B receptor of NMDA receptors. The TNF-α expression has been demonstrated to induce the phosphorylation of c-Jun N-terminal kinase 1 and stimulates NF-κB leading to the release of chemokine ligand 2 (CCL2). CCL2 may then acts on C-C chemokine receptor type 2 (CCR2) on neurons and communicate positively with neuronal NMDA receptors and α-amino-2-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors to produce central sensitization and synaptic plasticity (Sun et al., 2015). The release of these inflammatory cytokines and free radicals may then directly activate microglia (Pabreja et al., 2011, Tangpong et al., 2008). Marchand *et al.* (2009) suggested that the production of TNF-α seems to be dependent on microglial activation since these cytokines are produced by many types of cells during inflammation including microglial cells (Stirling et al., 2005). Both TNF-α and IL-1β may enhance membrane K^+ ion conductance in a non-voltage-gated fashion which leads to neuronal hyperexcitability (Leung and Cahill, 2010) therefore leading to persistent central sensitization. Moreover, these cytokines may also enhance their productions from glial cells as well as another cell type(s) (Pabreja et al., 2011) after the release. Persistent with the previous studies, we also observed the obvious increase in TNF-α with the increase of IL-1β level although not statistically different from the non-diabetic group. The release of pro-inflammatory mediators may also contribute to the accumulation of free radicals as demonstrated by the increased lipid peroxidation (MDA) in the spinal cord of diabetic PDN rats in this study. The increased of these cytokines was markedly inhibited by minocycline treatment, similarly reported by the previous studies (Krady et al., 2005, Pabreja et al., 2011, Raghavendra et al., 2003). Apart from that, ifenprodil demonstrated a dramatic attenuation of TNF-α level dose-dependently. In fact, there is limited research investigating the effect of ifenprodil on pro-inflammatory markers, but the present study showed ifenprodil enhances the production of IL-1β level in the spinal cord of the PDN rats. Although the IL-1β release is implicated in the phosphorylation of NR2B subunit of NMDA receptor in normal rats, it is unclear how ifenprodil could cause the increased level of IL-1β. It is assumed that ifenprodil may also affect another mechanism or signaling pathway that may affect other cells to be activated, therefore increase the release of IL-1β. There is not a single study that has explored the effect of ifenprodil on astroglia and oligodendrocytes in neuropathic pain model. It is possible although not yet determined that ifenprodil may affect these cells to exaggeratively release pro-inflammatory cytokines (Noguchi, 2010, Vela et al., 2002). However, further investigation should be carried out to confirm the aforementioned mechanism.

CONCLUSION

The results demonstrated that both minocycline and ifenprodil attenuates the development of painful diabetic neuropathy by attenuating formalin-induced nociceptive responses, improve oxidant-antioxidant balance and reduced pro-inflammatory marker(s) level in streptozotocin-model of painful diabetic neuropathy. Further studies are required to identify this specific mechanism that may be involved in the inhibition by both minocycline and ifenprodil on the pathogenesis of painful diabetic neuropathy.

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