Rolipram and Pentoxifylline Combination Ameliorates Experimental Diabetic Neuropathy Through Inhibition of Oxidative Stress and Inflammatory Pathways in the Dorsal Root Ganglion Neurons

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Research Article

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Abstract

Diabetic neuropathy (DN) is the most challenging microvascular complication of diabetes and there is no suitable treatment for it, so the development of new agents to relieve DN is urgently needed. Since oxidative stress and inflammation play an essential role in the development of DN, clearance of these factors are good strategies for the treatment of this disease. According to key role of cyclic adenosine monophosphate (cAMP) in the regulation of oxidative stress and inflammatory pathways, it seems that phosphodiesterase inhibitors (PDEIs) can be as novel drug targets for improving DN through enhancement of cAMP level. The aim of this study was to evaluate the effects of rolipram, a selective PDE4 inhibitor, and pentoxifylline, a general PDE inhibitor on experimental model of DN and also to determine the possible mechanisms involved in the effectiveness of these agents. We investigated the effects of rolipram (1mg/kg) and pentoxifylline (100 mg/kg) and also combination of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg), orally for five weeks in rats that became diabetic by STZ (55 mg/kg, i.p.). After treatments, motor function was evaluated by open-field test, then rats were anesthetized and dorsal root ganglion (DRG) neurons isolated. Next, oxidative stress biomarkers and inflammatory factors were assessed by biochemical and ELISA methods, and RT-PCR analysis in DRG neurons. Rolipram and/or pentoxifylline treatment significantly attenuated DN – induced motor function deficiency by modulating distance moved and velocity. Rolipram and/or pentoxifylline treatment dramatically increased the cAMP level, as well as suppressed DN – induced oxidative stress which was associated with decrease in LPO and ROS and increase in TAC, total thiol, CAT and SOD in DRG neurons. On the other hand, the level of inflammatory factors (TNF- α , NF-kB and COX2) significantly decreased following rolipram and/or pentoxifylline administration. The maximum effectiveness was with rolipram and/or pentoxifylline combination on mentioned factors. These findings provide novel experimental evidence for further clinical investigations on rolipram and pentoxifylline combination for the treatment of DN.

Introduction

Diabetic neuropathy (DN) is one of the most and severe challenging microvascular complication of diabetes, which severely limits the life quality in patients (Bönhof et al. 2019). Most patients with DN suffer from the distal symmetrical polyneuropathy, a slowly progressive sensory predominant neuropathy, and experience numbness, weakness, tingling, and pain that usually starts from the toes and progresses to the feet, which is caused by damage to peripheral nerves (Russell et al. 2014).

An estimated about 451 million people worldwide were diagnosed with diabetes in 2017, and this number will raise to 693 million by 2045 (Cho et al. 2018). The prevalence of DN in diabetic patients varies widely from 9.6 to 88.7% worldwide. This variation might be due to several factors such as different types of diabetes, disease duration, age, glucose control, available health facilities, and sample selection. (Amour et al. 2019). Peripheral nerve complications observed in diabetic patients are irreversible, and available treatments at best may only delay the DN progression. Current DN management is based on three tenets: intense blood sugar control; pathogenetic treatments; and symptomatic therapies, which do not have sufficient efficiency (Javed et al. 2015). Accordingly, there is urgent need to clarify the underlying mechanism of DN in order to develop better therapies to treat this refractory disease.

Several molecular pathways are accounted for the pathogenesis and progression of DN, but in recent years, this complication has become increasingly associated with oxidative stress. Indeed, chronic hyperglycemia accelerates the generation of intracellular reactive oxygen species (ROS). Excess production of ROS cause to mitochondrial dysfunction, and cell apoptosis in dorsal root ganglia (DRG) neurons which are the target tissue in diabetic peripheral neuropathy (Mallet et al. 2020). On the other hands oxidative stress leads to NF- κ B activation, which activates inflammatory signaling pathways. Chronic NF- κ B activation is through to be at the center of all the inflammatory elements operating in DN. In addition, all the classical pathways involved in DN can directly or indirectly initiate and expand the production of inflammatory mediators. In fact, oxidative stress and inflammation have been proven to interact with each other and to be inextricably linked to DN (Hosseini and Abdollahi, 2013). Therefore, in this study, we focused on the two pathways of oxidative stress and inflammation in DN and tried to investigate compounds by which one can reduce factors related to these pathways.

DRG neurons contain the highest proportion of sensory neurons, the cells that are primarily liable for transmitting of sensory information from the periphery and for transduction this information to the central nervous system (Pope et al. 2013). DRG neurons are very sensitive to oxidative damage and are known as the target tissue in diabetic peripheral neuropathy due to: their large mitochondria, high oxidative metabolism, disrupted neurotrophic support and leaky blood-ganglia barrier. In addition, DRG neurons absorb higher levels of local blood flow which representing more metabolic and oxygen demand (Vincent et al. 2010).

Rolipram is a selective phosphodiesterase (PDE)-4 inhibitor (Kim et al. 2015). The PDEs are a superfamily of enzymes that promote the hydrolysis of cyclic nucleotides to inactive counterparts and PDEs inhibitors (PDEIs) prolong cyclic nucleotides' function by prevention from their hydrolysis (Milani et al. 2005). PDE4, which is often found in nerve cells and immune cells, hydrolyzes only cyclic adenosine monophosphate (cAMP). Therefore, rolipram by inhibiting PDE4 increases the level of cAMP in nerve cells and immune cells. cAMP is an intracellular second messenger that regulates cellular effectors in many physiological processes. Documents has shown that cAMP levels play an important role in neuroprotection and neuroinflammatory response (Volakakis et al. 2010). cAMP also promotes differentiation, survival, regeneration and outgrowth of neural cells (Hannila and Filbin, 2008; Troadec et al. 2002). Thus, rolipram exert antioxidant, anti-inflammatory and neuroprotective effects in nerve cells through increasing cAMP levels (Kilanowska and Ziolkowska, 2020). In animal studies, rolipram alleviated symptoms of DN (Kim et al. 2015).

Pentoxifylline is a methylxanthine derivative and a non-selective PDEIs; it increases the intracellular cAMP level. It is well known for its immunomodulatory and anti-inflammatory effects (de Oliveira Garcia et al. 2015; Kim et al. 2016; Kreth et al. 2010; Liu et al. 2007). Despite many clinical findings suggested that pentoxifylline may improve DN (Cohen and Harris 1987; Cohen and Mathews 1991; Hosseini et al. 2019; Kalmansohn et al. 1988; Lee et al. 1997; Radfar et al. 2005), but mechanism of action of it is not fully understood yet. It is thought that pentoxifylline useful effects is due to reducing pro-inflammatory cytokines and free radicals through inducing accumulation of cAMP (Neves et al. 2015).

Considering this scenario, the present study was designed to evaluate the effects of rolipram and/or pentoxifylline on experimental model of DN and also to determine the potential mechanisms involved in effectiveness of these compounds. Accordingly, we hypothesized that rolipram and pentoxifylline produce their protective effects with inhibition of oxidative stress and inflammatory pathways through inducing

accumulation of cAMP in DRG neurons. To examine this hypothesis, we investigated the effectiveness of rolipram and pentoxifylline alone and also simultaneous application of them on biomarkers of oxidative stress, antioxidant enzymes, inflammatory factors and motor function in experimental diabetic neuropathy. These findings may provide novel evidence for improving DN by using combination of rolipram and pentoxifylline.

Material And Methods

Animals

The experimental animals were male Wistar rats (200-300 g body weight) purchased from the animal house of Iran University of Medical Sciences. They were kept under standard conditions (Temperature $22\pm 2^{\circ}\text{C}$, the relative humidity of $60\pm 5\%$) and 12 h light/dark cycle. They had free access to a standard pellet diet and water ad libitum. The experiments were approved by the ethical committee of Iran University of Medical Sciences. (96-01-118-29912).

Diabetic neuropathy in type 1 diabetic streptozotocin model

Diabetes mellitus (DM) type 1 was induced through a single intra-peritoneal injection of dissolved streptozotocin (Sigma, USA) (55 mg/kg) in normal saline following 12 h fasting. After 72h of injection, the fasting blood sugar (FBS) levels were measured from animals' tails using a glucometer. The FBS over 250 mg/dl was considered as DM and 5 weeks following confirmed hyperglycemia, DN occurred (Davidson et al. 2011).

Study design

After confirmation of hyperglycemia, the rats were divided randomly into 5 groups, each group consists of 7 animals (n=7): (1) Control group which received DMSO (drugs solvent), orally, 5 days/week for 5 weeks; (2) DN group: diabetic rats which received 0.5% DMSO, orally, 5 days/week for 5 weeks; (3) DN + rolipram group: diabetic rats which received rolipram (1mg/kg), orally, 5 days/week for 5 weeks; (4) DN+ pentoxifylline group: diabetic rats which received pentoxifylline (100 mg/kg), orally, 5 days/week for 5 weeks ; (5) DN + rolipram +pentoxifylline group: diabetic rats which received a combination of rolipram (0.5mg/kg) and pentoxifylline (50 mg/kg), orally, 5 days/week for 5 weeks. All oral treatments were performed by gavage. We selected doses of rolipram and pentoxifylline according to the literature. Among the effective doses of rolipram in previous studies (1-3 mg/kg), due to the side effects of this drug, lower dose (1 mg / kg) was selected in this study. (Mokry et al.2013; Han et al. 2012; Kajana et al. 2008). According to previous studies, the appropriate dose of pentoxifylline 100 mg / kg was chosen. (Neves et al. 2015; Halis et al. 2019). In previous studies, the dose of rolipram in combination with another phosphodiesterase inhibitor was halved. Therefore, the first we chose rolipram dose (0.5 mg/kg) and then adjusted pentoxifylline similar dose (50 mg/kg) (Mokry et al, 2013).

Dorsal root ganglion (DRG) isolation

After the end of treatment period, animals were anesthetized using ketamine hydrochloride 10% (60 mg/kg) and xylazine hydrochloride 2% (8 mg/kg), then the spines were broken and the whole vertebrates were separated, DRG neurons isolated from the second cervical (C2) to second lumbar (L2) spine region on both

sides. DRG neurons was fixed by 10% formalin for histological study and the others immediately transferred to liquid nitrogen for further evaluation (Hosseini et al. 2011).

Processing of DRG neurons for further analysis

DRG neurons were placed in lysis buffer (10mM Tris-base, 150mM NaCl, 1mM EDTA, 1% NP40 in double distill H₂O) including suitable enzyme inhibitors on ice. Then the tissues were homogenized using a mechanical grinding pestle for 30 s on ice after that were centrifuged for

15 min at 16,000×g at 4 °C and finally the supernatant was collected as sample for further analysis (Galeshkalami et al. 2019).

cAMP assay

cAMP level was measured using cyclic AMP ELISA kit (Cayman chemical) according the manufactures' instruction. The level of cAMP in samples were assessed at 405 nm by a plate reader (Synergy HT, BIOTEK, USA).

Measurement of reactive oxygen species (ROS)

For assessment of ROS generation, samples were incubated with a fluorescent dye 2', 7-dichlorofluorescein diacetate (DCFH-DA) at 37°C for 30 min. The intensity of fluorescence was measured at 485 and 528 nm as excitation and emission wavelengths respectively, by a Microplate Reader (BIOTEK instruments, USA) (LeBel et al. 1992).

Measurement of lipid peroxidation (LPO)

The Lipid peroxidation was measured using the thiobarbituric acid reactive substances (TBARS) method. The samples were mixed with thiobarbituric acid (TBA) and then were heated in boiling water for 30 min. The reaction of (TBA) and MDA produced red color which its absorbance was measured at 532 nm using a Microplate Reader (BIOTEK instruments, USA) (Ohkawa et al. 1979).

Measurement of total antioxidant capacity (TAC)

TAC was determined using the FRAP method. The basis of FRAP method is a reduction of Fe³⁺ to Fe²⁺ by the samples. The complex of Fe²⁺ and 2,4,6-tris (2-pyridyl)-1,3,5-triazine (TPTZ) generates a blue color which its absorbance was taken at 593 nm through spectrophotometer (Benzi et al. 1999).

Measurement of superoxide dismutase (SOD) activity

The activity of CAT was measured through assay kits (Cayman Chemical, Ann Arbor, USA). The O₂⁻ which is generated from xanthinoxidase activity reacts with 2-(4-iodophenyl)-3-(4-nitrophenol) 5-phenyl tetrazolium chloride (INT) and produced a red formazan dye. The SOD activity was determined through reaction inhibition from one unit of SOD activity was defined as the amount that leads to 50% inhibition of the rate of reduction of INT. The color reaction was measured at 505 nm by ELISA microplate reader. The results are presented as unit per milligram of protein.

Measurement of catalase (CAT) activity

The CAT enzyme activity by observing the disappearance of hydrogen peroxide in the spectrophotometer at 240 nm was determined. Briefly, supernatant (100 µL) and alcohol ethanol (10 µL) were vortexed and placed in ice water bath for 10min. Then, 10 µL of Triton X-100 was added to mixture and was vortexed in room temperature. Thereupon, 100 µL of phosphate buffer (H₂O₂ 0.66 M) was added to the mixture, and decrease of absorbance was measured at 240 nm by spectrophotometer (Cecil CE7250-7000 series, Milton Technical Centre, Cambridge, UK). The results were presented as µ/milligram protein (Sinha et al. 1972).

Measurement of total thiol groups

The procedure of determination of total sulfhydryl was described previously (Hu et al. 1994). 0.2 ml from sample was mixed with 0.6 ml of Tris-EDTA buffer [Tris base (0.25 M), EDTA (20 Mm), PH 8.2] in a 10 ml test tube, after that mixed with 40 ml of DTNB (10 mM) in methanol. 16 ml of methanol added until the final volume reaches 4 ml. After capping, the test tube was centrifuged at 3000 g for 10 min and after 15-20 min the color appeared and the supernatant absorbance was measured at 412 nm.

Measurement of cyclooxygenase 2 (COX-2)

COX-2 was detected using rat cyclooxygenase-2, ELISA Kit, Cusabio (Houston, TX, USA) according the manufactures' instruction.

Measurement of Tumor necrosis factor-α (TNF-α)

For assessment of TNF-α level, a Tumor necrosis factor-α kit (Zell bio Gmbh, Germany) was used. TNF-α level in samples were assessed at 450 nm using a plate reader (Synergy HT, BIOTEK, USA).

Real-time PCR

DRG neurons were subjected to quantitative RT-PCR experiments. Total RNA was isolated from DRG tissues by using Trizol reagent in accordance with the manufacturer's protocol. The extracted RNA (1 µg) was reverse-transcribed into cDNA by using a PrimeScript RT reagent kit. RT-PCR was performed. The primer sequences of NF-kB: Forward primer: TTCAACATGGCAGACGACGA, Reverse primer: AGGTATGGGCCATCTGTTGA.

After PCR was completed, the products were analyzed through electrophoresis on 1.2% agarose gel and photographed under UV light in an EC3 Imaging System (UVP, Upland, CA) (Miroliaee et al. 2011).

Measurement of motor function

Motor function was assessed by open-field activity tests. The rats were placed in an open-field box where velocity (cm/sec.) and distance moved (cm) were recorded with a camera on the top of the box for 5 min. The EthoVision tracking system (Noldus Information Technology, Wageningen, the Netherlands) was used to measure motor function by determining speed and distance of animal movement (Hosseini et al. 2011).

Histological preparation

Isolated DRG neurons were fixed in the paraformaldehyde and then embedded in paraffin and after that were cut into 40 µm sections. These sections were stained with hematoxylin and eosin (H&E) and were used for microscopic analysis by Olympus microscope (LX71, Japan).

Statistical analysis

The results were presented as the mean $\pm$ SEM and were analyzed using one-way ANOVA and Tukey's post hoc tests. $p < 0.05$ was considered as statistically significant.

Results

The effect of rolipram and/or pentoxifylline on cAMP level

As shown in figure 1, the level of cAMP in DN group is significantly lower than the control group ($p < 0.01$). Application of rolipram and/or pentoxifylline increased the level of cAMP in comparison to the DN group ($p < 0.0001$). This is while rolipram or pentoxifylline and co-administration of them also enhance cAMP level in comparison to the control group ($p < 0.01$ and 0.001 , respectively).

The effect of rolipram and/or pentoxifylline on oxidative stress biomarkers

The effect of rolipram and/or pentoxifylline on ROS generation

Table 1, illustrates the effect of rolipram, pentoxifylline and co-administration of them on the ROS level. In the DN group, the ROS level is significantly increased compared with the control group ($p < 0.001$). Treatment with rolipram and co-administration of both rolipram and pentoxifylline significantly decreased the DN-induced ROS generation ($p < 0.05$ and $p < 0.01$, respectively).

The effect of rolipram and/or pentoxifylline on LPO level

According to the study results, the LPO level in DN is significantly higher than the control group ($p < 0.01$) while the administration of rolipram or pentoxifylline and also combination of them have reduced the LPO level compared with the DN group ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) (Table 1).

The effect of rolipram and/or pentoxifylline on TAC level

As illustrated in table 1, the level of TAC in the DN group is significantly lowered in comparison with the control group ($p < 0.001$). This is while treatment with rolipram or pentoxifylline ($p < 0.05$) and co-administration of them ($p < 0.01$) considerably increased the TAC level compared with the DN group.

The effect of rolipram and/or pentoxifylline on total thiol level

Total thiol level has decreased in the DN group compared with the control group ($p < 0.01$). Although applying rolipram or pentoxifylline individually had no significant effect on the level of thiol, their co-administration could significantly restore the total thiol level ($p < 0.05$) compared with the DN group (table 1).

The effect of rolipram and/or pentoxifylline on antioxidant enzymes

The effect of rolipram and/or pentoxifylline on CAT activity

As shown in figure 2, the CAT activity in the DN group is significantly lower than the control group ($p < 0.001$). Co-administration of rolipram and pentoxifylline significantly restore the CAT activity in comparison to the DN group ($p < 0.05$)

The effect of rolipram and/or pentoxifylline on SOD activity

In this experiment, a significant reduction of SOD activity was observed in the DN group compared with the control group ($p < 0.001$). This effect was restored in diabetic rats treated with co-administration of rolipram and pentoxifylline ($p < 0.05$) (Fig.3).

The effect of rolipram and/or pentoxifylline on inflammation factors

The effect of rolipram and/or pentoxifylline on COX2 level

The result shows that the COX2 level in the DN group significantly increased compared with the control group ($p < 0.001$) and administration of rolipram + pentoxifylline significantly reduced the COX2 level compared with the DN group ($p < 0.05$) (Fig.4).

The effect of rolipram and/or pentoxifylline on TNF- α level

A prominent increase has been shown in the TNF- α level in the DN group compared with the control group ($p < 0.001$) which was restored by treatment with rolipram and pentoxifylline co-administration ($p < 0.05$) (Fig.5).

The effect of rolipram and/or pentoxifylline on NF- κ B mRNA expression

The result shows that the NF- κ B mRNA expression in the DN group significantly increased compared with the control group ($p < 0.001$) and administration of rolipram ($p < 0.05$) has significantly reduced the NF- κ B mRNA expression compared with the DN group. Co administration of rolipram and pentoxifylline had more improvement on this gene expression ($P < 0.01$) compared with the DN group (Fig.6).

The effect of rolipram and/or pentoxifylline on motor function deficiency

There was a notable deficiency in motor function in DN rats. The velocity (cm/s) (Fig. 7a) and distance moved (cm) (Fig. 7b) had a significant decrease ($p < 0.001$) in DN group than in control rats. Although administration of rolipram and pentoxifylline improved motor function, they were no statistically significant. Interestingly, combination of rolipram and pentoxifylline reversed velocity and distance moved significantly ($p < 0.05$) in compared with DN rats.

The effect of rolipram and/or pentoxifylline on qualitative observations of DRG neurons

A stronger basophil staining attitude with more and larger vacuoles was evident in DRG neurons of DN rats. There also appeared to be a tendency to be smaller in DRG neurons of DN animals, as previously documented. This is while, these histological changes were restored in DN rats treated with rolipram, pentoxifylline, and especially with combination of them (Fig.8).

Discussion

DN is an intense diabetic complication presented worldwide which severely reduced the life quality of diabetic patients. DRG neurons are known as the target tissue in diabetic peripheral neuropathy. There is also no suitable treatment for patients suffering and for retarding the progression of DN. Therefore, the development of new agents to relieve DN is urgently needed (Sandireddy et al. 2014). Since oxidative stress and inflammation have been proven to interact with each other and to be inextricably linked to DN, clearance of oxidative stress and inhibition of inflammation are good strategies for the treatment of this disease.

The present study examines rolipram, a selective PDE4 inhibitor, and pentoxifylline, a general PDE inhibitor on experimental model of DN and also determines the possible mechanisms involved in the effectiveness of these agents. The results of the present study demonstrate that rolipram and pentoxifylline attenuate experimental diabetic neuropathy via preventing oxidative stress and inflammation in DRG neurons. The major significance of these findings is the novel therapeutic agent, rolipram and pentoxifylline combination, which can be used to advance our knowledge in the field of new approach to relieve DN. In the present study rolipram and pentoxifylline increase the level of cAMP in DRG neurons by inhibiting PDE4 and general PDEs, respectively. Documents demonstrated that increase of cAMP levels in nerve cells have an important role in reduce oxidative stress and inflammation that result in neuroprotection and reduction of neuroinflammation (Lonze and Ginty 2002; Volakakis et al. 2010). Despite rolipram has been known as a novel antioxidant and anti-inflammation and also is only selective PDE4 inhibitor which has been approved for use in humans, its application is limited due to a narrow therapeutic window along with significant gastrointestinal side effects. Thus, an approach in the pharmaceutical industry is to base on the assumption that additive or synergistic antioxidant and anti-inflammatory effects can be produced with a PDE4 inhibitor in combination with inhibitors that target either two or more PDE families. Obviously, this approach can reduce the therapeutic dose and the side effects of rolipram (Giembycz and Newton, 2011). Accordingly, we selected pentoxifylline, a general PDE inhibitor, for combination to rolipram. Pentoxifylline could theoretically be a useful treatment for DN and several clinical findings suggested that pentoxifylline may improve DN, but there is not enough evidence to prove its effectiveness and the molecular mechanisms involved (Hosseini et al. 2019).

DN develops in response to prolonged hyperglycemia. Evidence suggests hyperglycemia can cause oxidative stress in the peripheral nervous system that can promote DPN development.

Documents have been shown that superoxide overproduction induced by hyperglycemia, activates the pathways of cell injury. One of these pathways has been shown in the DRG, where superoxide induced-oxidative stress is severe and for this reason, DRG neurons have been identified as major target in DN (Vincent et al. 2004). The hyperglycemia in DN induces autooxidative glycosylation which is the main reason for the increased ROS generation, and also it induces glycation of antioxidant enzymes that lead to decreased availability or activity of antioxidant enzymes. As a result, imbalance between ROS production and the cell's ability to scavenge the reactive species, plays an essential role in the DN pathogenesis (Oyenihi et al. 2015; Sytze van Dam, 2002). The present work, like previous studies (Galeshkalami et al. 2019; Ghaznavi et al. 2015; Khasraghi et al. 2018; Najafi et al. 2015; Saberi Firouzi et al. 2018; Sarvestani et al. 2018) revealed an increased ROS generation in DRG neurons in rats with DN. On the other hand, insufficient antioxidant capacity such as the reduced activity of superoxide dismutase (SOD) and catalase (CAT) in DN leads to attack of free radicals to

cells membranes and initiation of lipid peroxidation (Dewanjee et al. 2018). In the present work similar to previous studies, MDA level “a main end product from membrane lipid peroxidation” in rats DRG neurons with DN was higher than normal rats (Galeshkalami et al. 2019; Hosseini et al. 2010; Khasraghi et al. 2018; Mallet et al. 2020; Najafi et al. 2017; Sharifzadeh et al. 2017). SOD is an important enzyme for detoxifying reactive O_2^- through catalysis into H_2O_2 and neutralizes the superoxide radicals. CAT is another enzyme that transforms hydrogen peroxide to oxygen and water and abates its toxic effects. Evidence has shown that the activity of SOD and CAT enzymes and also TAC and total thiol levels reduce in DN which promote free radical production (Bertolotto and Massone 2012; Galeshkalami et al. 2019; Mallet et al. 2020; Khasraghi et al. 2018; Hosseini et al. 2010; Najafi et al. 2015; Najafi et al. 2017). Our results in support of mentioned studies revealed that activity of SOD and CAT enzymes and also TAC and total thiol levels decreased in rats DRG neurons with DN. On the other hands, our data showed that treatment of diabetic rats with rolipram or pentoxifylline inhibited the ROS generation and lipid peroxidation as well as, increased SOD and CAT activities, and TAC and total thiol levels in DRG neurons of rats with DN. Previous studies confirm our findings and show that PDE4 inhibitors reduce the main markers of oxidative stress. PDE4 inhibitors reduce the production of free radicals, which is associated with a significant elimination of lipid peroxidation (Bao et al. 2011). In our previous study, we showed rolipram decreased ROS and lipid peroxidation and increased antioxidant enzymes activity in in vitro model of DN (Sarvestani et al. 2018). Rolipram in Alzheimer’s disease reduced ROS and markedly decreased MDA level and also restored antioxidant enzymes (SOD and CAT) activity. Antioxidant effects of rolipram in this neurodegenerative disease may be for its ability to balance the oxidative and antioxidant systems (Zhuo et al. 2016). Several experimental studies also suggest rolipram may be beneficial for peripheral neuropathy (Kim et al. 2015; Sarvestani et al. 2018). On the other hands, documents reveal that pentoxifylline may have attenuated diabetic polyneuropathy. According to pentoxifylline as an inhibitor of xanthine oxidase, an enzyme involved in the ROS generation, inhibits free radical generation and decreases lipid peroxidation and also induces of antioxidant enzymes (Hosseini et al. 2019; Laczy et al. 2009; Satoh et al. 2003). It also is demonstrated that neuroprotective effect of pentoxifylline in treatment of Parkinson’s disease, possibly related to its antioxidant actions (Neves et al. 2015). In support of the hypothesis of additive or synergistic antioxidant effects of rolipram in combination with pentoxifylline, we found that treatment of diabetic rats with this combination further improved ROS and MDA levels, and antioxidant enzymes compared with rolipram or pentoxifylline alone.

It has been proved that the inflammatory pathways are involved in peripheral nerve injury and neuroinflammation plays a main role in the pathogenesis of DN (Sandireddy et al. 2014). Oxidative stress leads to NF- κ B activation, which activates inflammatory signaling pathways. NF- κ B is a fast-acting pleiotropic transcription factor involved in the genesis and exaggeration of inflammatory responses at different tissue sites. The activated NF- κ B via ROS has been shown to increase the genes expression of COX-2, TNF- α , and pro-inflammatory cytokines in DRG neurons following peripheral nerve damage. Proinflammatory factors overproduction in neural tissue also led to neuropathic pain (Goldin et al. 2006; Hayden and Ghosh, 2008). Many documents have shown enhance in expression levels of NF- κ B, COX-2, and TNF- α in DRG neurons, especially in painful neuropathy (Galeshkalami et al. 2019; Hosseini and Abdollahi 2013; Shi et al. 2013; Yang et al. 2016). The present work similar to previous studies revealed an increase in gene expression of NF- κ B, and also COX-2 and TNF- α levels in rats DRG neurons with DN. This work showed that treatment of diabetic rats with rolipram or pentoxifylline decreased DN-induced gene expression of NF- κ B and also COX-2 and TNF- α

levels in DRG neurons. Numerous studies have shown that PDE4 inhibitors especially, PDE4 inhibitors with low molecular-weight, such as rolipram are important pharmaceutical agents with a wide range of anti-inflammatory effects (Chung 2006; Houslay et al. 2005; Perez-Aso et al. 2015; Sarvestani et al. 2018). Rolipram increases cAMP levels in nerve tissue, leading to inhibition of NF- κ B, decreased production of inflammatory cytokines (TNF- α , IL-1 β), and molecules involved in oxidative stress, such as COX-2. Thus, rolipram may ameliorate DN-induced neuropathic pain by decreasing expression of inflammatory cytokines in DRG neurons (Kim et al. 2016; Kim et al. 2011). Pentoxifylline, known as an inhibitor of TNF- α production and its action, reduces TNF- α gene transcription and directly or indirectly affects several stages in the inflammatory pathways, and has beneficial anti-inflammatory effects (Kreth et al. 2010; Satoh et al. 2003). Documents suggested that pentoxifylline can improve DN by reducing inflammatory factors such as TNF- α , NF κ B and COX-2 in DRG neurons and it has also shown antiallodynic effects against various types of neuropathic pain (Kim et al. 2016; Kreth et al. 2010; Liu et al. 2007). Interestingly, treatment of diabetic rats with the combination of rolipram and pentoxifylline in this work provided more improvement of NF- κ B, COX-2 and TNF- α levels compared with rolipram or pentoxifylline alone. In support of the present results, previous studies confirmed the combination of rolipram and pentoxifylline has beneficial inhibitory effect on inflammatory cytokines, especially TNF- α . In this respect, documents have shown co-administration rolipram and pentoxifylline has potential anti-inflammatory activity (Beshay et al. 2001; Luna et al. 2011; Okayama et al. 2004). Our findings support this idea and suggest that this combination is useful for potent inhibition of key inflammatory markers.

There are motor and sensory conduction disorders in chronic DN, but defect in motor conduction appear early during DN and progress in a period of time. Therefore, we evaluated motor function to confirm neuropathy. Deficiency of the bioenergy system of DRG neurons alters cellular functions, leading to neuronal atrophy resulting in impaired motor function. Motor abnormality can also be induced by the mitochondrial dysfunction which accompanied by an accumulation of oxygen radicals and supports 'oxidative hypothesis' (Kishi et al. 2002). Similar to previous studies (Galeshkalami et al. 2019; Hosseini et al. 2011; Kishi et al. 2002), our findings showed a significant decrease in motor function in rats with DN compared with control, and treatment with rolipram and pentoxifylline, especially combination of them showed an improvement in velocity and distance moved in this condition. Previous studies in support of our findings showed inflammatory factors inhibitors prevent motor nerve conduction deficits (Obrosova, 2009). Accordingly, it is expected that rolipram and pentoxifylline can improve DN-induced motor function deficiency through anti-inflammatory effect caused by increase of cAMP level. It has also been seen in studies that rolipram and pentoxifylline improved motor nerve conduction velocity in experimental models of DN (Flint et al. 2000; Kim et al. 2011; Laczy et al. 2009; Satoh et al. 2003).

Conclusion

The findings of this study clearly showed that administration of rolipram, a selective PDE4 inhibitor, and pentoxifylline, a general PDE inhibitor, and especially their combination was beneficial at improving oxidative stress and inhibiting inflammation in experimental diabetic neuropathy. These modulatory effects of rolipram and/or pentoxifylline are mediated through increasing TAC, total thiol, CAT and SOD, preventing lipid peroxidation, ROS, COX2 and TNF- α , modulation of NF- κ B mRNA expression, and improving motor function. The better improvement observed following the use of combination of rolipram and pentoxifylline in the present study can be due to additive or synergistic antioxidant and anti-inflammatory effects this two PDEIs through

increase of cAMP level. Thus, these findings provide novel experimental evidence for further clinical investigations on rolipram and pentoxifylline combination for the treatment of DN. Of course continuing this study on apoptosis pathway is necessary to reach a better conclusion.

Declarations

Author contributions

Mona Dastgheib: methodology, validation, investigation, data analysis, writing - original Draft; Seyed Vahid Shetab-Boushehri: investigation, review & editing; Maryam Baeeri: methodology, validation, investigation; Gholami Mahdi: methodology, validation; Mohammad Yahya Karimi: resources, methodology; Mohammad Abdollahi: review & editing; Asieh Hosseini: methodology, writing- review & editing, supervision, project administration, funding acquisition.

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Data availability

All data generated or analyzed during this study are included in this published article

Competing interest The authors declare no conflict of interest.

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Tables

Table.1 Rolipram and/or pentoxifylline modulate DN-induced oxidative stress biomarkers changes in DRG neurons. Diabetic rats were treated with rolipram (1 mg/kg) or pentoxifylline (100 mg/kg) and also with co-

administration of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg) for five weeks, then ROS, LPO, TAC and Total thiol were measured in DRG neurons. Results are mean $\pm$ SEM, n=7. Difference between control and other groups is significant at $p < 0.001$ (aaa), $p < 0.01$ (aa) and $p < 0.05$ (a). Difference between DN and other groups is significant at $p < 0.01$ (bb) and $p < 0.05$ (b). DN: diabetic neuropathy.

Groups	ROS (μ /mg protein)	LPO (μ mol/mg protein)	TAC (mM)	Total Thiol (μ M)
Control	0.6550 $\pm$ 0.03538	119.3 $\pm$ 8.010	213.1 $\pm$ 7.287	143.9 $\pm$ 10.31
DN	1.112 $\pm$ 0.05806 ^{aaa}	177.2 $\pm$ 8.251 ^{aa}	140.5 $\pm$ 6.314 ^{aaa}	85.30 $\pm$ 8.124 ^{aa}
DN+Rolipram	0.8483 $\pm$ 0.05778 ^b	133.4 $\pm$ 3.716 ^b	179.1 $\pm$ 10.35 ^{a,b}	113.0 $\pm$ 1.779
DN+Pentoxifylline	0.9697 $\pm$ 0.02630 ^{aa}	142.3 $\pm$ 9.933 ^b	173.3 $\pm$ 5.125 ^{a,b}	101.0 $\pm$ 7.932 ^a
DN+Rolipram+Pentoxifylline	0.7947 $\pm$ 0.04134 ^{bb}	129.1 $\pm$ 3.467 ^{bb}	195.5 $\pm$ 4.769 ^{bb}	121.1 $\pm$ 7.310 ^b

Figures

Figure 1

Rolipram and/or pentoxifylline enhance cAMP level in DRG neurons. Diabetic rats were treated with rolipram (1 mg/kg) or pentoxifylline (100 mg/kg) and also with co-administration of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg) for five weeks, then cAMP was measured in DRG neurons. Results are mean $\pm$ SEM, $n=7$. Difference between control and other groups is significant at $p < 0.001$ (aaa) and $p < 0.01$ (aa). Difference between DN and other groups is significant at $p < 0.0001$ (bbbb). DN: diabetic neuropathy.

Figure 2

Rolipram and/or pentoxifylline restore DN-reduced CAT activity in DRG neurons. Diabetic rats were treated with rolipram (1 mg/kg) or pentoxifylline (100 mg/kg) and also with co-administration of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg) for five weeks, then CAT activity was measured in DRG neurons. Results are mean $\pm$ SEM, n=7. Difference between control and other groups is significant at $p < 0.001$ (aaa) and $p < 0.05$ (a). Difference between DN and other groups is significant at $p < 0.05$ (b). DN: diabetic neuropathy.

Figure 3

Rolipram and/or pentoxifylline restore DN-reduced SOD activity in DRG neurons. Diabetic rats were treated with rolipram (1 mg/kg) or pentoxifylline (100 mg/kg) and also with co-administration of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg) for five weeks, then SOD activity was measured in DRG neurons. Results are mean $\pm$ SEM, n=7. Difference between control and other groups is significant at $p < 0.001$ (aaa), $p < 0.01$ (aa) and $p < 0.05$ (a). Difference between DN and other groups is significant at $p < 0.05$ (b). DN: diabetic neuropathy.

Figure 4

Rolipram and/or pentoxifylline modulate DN-induced COX2 level in DRG neurons. Diabetic rats were treated with rolipram (1 mg/kg) or pentoxifylline (100 mg/kg) and also with co-administration of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg) for five weeks, then COX2 level was measured in DRG neurons. Results are mean $\pm$ SEM, n=7. Difference between control and other groups is significant at $p < 0.001$ (aaa), $p < 0.01$ (aa) and $p < 0.05$ (a). Difference between DN and other groups is significant at $p < 0.05$ (b). DN: diabetic neuropathy.

Figure 5

Rolipram and/or pentoxifylline modulate DN-induced TNF- α level in DRG neurons. Diabetic rats were treated with rolipram (1 mg/kg) or pentoxifylline (100 mg/kg) and also with co-administration of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg) for five weeks, then TNF- α level was measured in DRG neurons. Results are mean $\pm$ SEM, $n=7$. Difference between control and other groups is significant at $p < 0.001$ (aaa) and $p < 0.05$ (a). Difference between DN and other groups is significant at $p < 0.05$ (b). DN: diabetic neuropathy.

Figure 6

Rolipram and/or pentoxifylline modulate DN effect on NF-κB mRNA expression in DRG neurons. Diabetic rats were treated with rolipram (1 mg/kg) or pentoxifylline (100 mg/kg) and also with co-administration of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg) for five weeks, then NF-κB mRNA expression was measured in DRG neurons. Results are mean ± SEM, n=7. Difference between control and other groups is significant at $p < 0.001$ (aaa) and $p < 0.05$ (a). Difference between DN and other groups is significant at $p < 0.01$ (bb) and $p < 0.05$ (b). DN: diabetic neuropathy.

Figure 7

Rolipram and/or pentoxifylline modulate DN-induced velocity (a) and distance moved (b) changes in rats. Diabetic rats were treated with rolipram (1 mg/kg) or pentoxifylline (100 mg/kg) and also with co-administration of rolipram (0.5 mg/kg) and pentoxifylline (50 mg/kg) for five weeks, then motor function was evaluated by open-field activity tests. Results are mean $\pm$ SEM, n=7. Difference between control and other groups is significant at $p < 0.001$ (aaa) and $p < 0.05$ (a). Difference between DN and other groups is significant at $p < 0.05$ (b). DN: diabetic neuropathy.

Figure 8

HE-stained DRG neurons. DN: diabetic neuropathy. DN neurons tend to be small and expose a stronger basophilic staining attitude and have more and larger vacuoles. Administration of rolipram and/or pentoxifylline abolished these changes. Scale bar: 50 μ m (400 $\times$).