Assessment of neuroprotective role of PPAR-gamma agonist in spinal cord injury as possible therapeutic agents

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ABSTRACT

It has been reported that peroxisome proliferator-activated receptor (PPAR)-gamma agonist, pioglitazone, has several beneficial roles in many pathological states of nervous tissues. Then in the present study, we aimed to examine the neuroprotective actions of pioglitazone (PPAR-gamma agonist) on motor function, histopathological changes and oxidative damage during spinal cord injury (SCI) in rats. Twenty-four male Wistar rats were randomly divided into three groups as follows; sham, control injury and pioglitazone-treated injured groups. SCI was performed according to the Ping-Weight Drop (contusion) model in rat. The animals received pioglitazone (3 mg/kg) intraperitoneally at times of 15 min after injury and then each 12 hours for seven days. At day seven after SCI, the malondialdehyde and glutathione levels were assayed using biochemical techniques. Histopathological alterations in injured spinal cord and motor function recovery were also assessed after six weeks. Induction of SCI in control group significantly increased the malondialdehyde levels (56%, P=0.002) and decreased the content of glutathione (39±4 nMol/mL) compared to control group (49±6 nMol/mL). Pioglitazone in treated injured rats significantly decreased the malondialdehyde levels (37%, P=0.018) but not glutathione levels (42±1 nMol/mL) compared to sham group. In addition, pioglitazone noticeably improved the histopathological changes of injured spinal cord but not motor function. Our findings revealed that pioglitazone decreases histopathological changes and oxidative damage of injured spinal cord. However, it is suggested that pioglitazone must be applied at higher doses for improving motor function during SCI.

KEYWORDS: Spinal cord injury, Pioglitazone, Histopathological changes, Motor function, Oxidative damage.
INTRODUCTION

The prevalence of spinal cord injury (SCI) is not well-known in many countries but based on the acknowledged data about 11000 new SCI is happened in the United States every year and about 450000 people live with SCI in this country (Sekhon and Fehlings, 2001; Fatima et al., 2015). Activation of secondary pathophysiological mechanisms after SCI plays a crucial role in development and intensification of primary mechanical injury after trauma to spinal cord (Visavadiya et al., 2016; Sekhon and Fehlings, 2001). These secondary mechanisms, which cause the injury to develop around the epicenter, include the ischemia-reperfusion injury, edema, intracellular calcium accumulation, cellular necrosis and apoptosis, activation of proteases, glutamate-mediated excitotoxicity, and inflammatory processes as well as generation of reactive oxygen species (ROS), (Visavadiya et al., 2016; Hayta and Elden, 2017). An accumulation of intracellular calcium followed a rapid increase in extracellular glutamate levels after SCI results in inhibition of ATP synthesis by mitochondria, a decrease in mitochondrial membrane potential and ultimately an increase in ROS production (Fatima et al., 2015). An increase in activity of plasma membrane oxidases such as NADPH-oxidase, lipoxygenases and xanthine/xanthine oxidase are other significant sources of ROS following traumatic SCI that can contribute to cellular oxidative stress and oxidative damage (Bermudez et al., 2016; Bains and Hall, 2012). Moreover, according to the previous findings the antioxidant defense systems against toxic oxygen free radicals are weakened in the injured areas after damage to spinal cord (Fatima et al., 2015; Bains and Hall, 2012; Vaziri et al., 2004).

Thiazolidinediones (TZDs) such as pioglitazone and rosiglitazone are powerful synthetic agonists of peroxisome proliferator-activated receptor (PPAR)-gamma. PPAR-gamma is a ligand-activated transcription factor that control many cellular functions including glucose hemostasis and lipid metabolism as well as cell growth and differentiation (Meng et al., 2011; Lee et al., 2011). It has been reported that PPAR-gamma agonists have several beneficial neuroprotective functions and are able to prevent neuronal damage, motor dysfunction, neuropathic pain, myelin loss and inflammation in various pathological states of nervous system (Park et al., 2007; Pei et al., 2016). The findings of McTigue et al. revealed the promising strategy to promote the functional and anatomical repair after SCI (McTigue et al., 2007). Meng et al. demonstrated that rosiglitazone inhibits inflammatory responses and increases the proliferation of neural progenitor cells following SCI (Meng et al., 2011). Also, Zhu et al. reported the neuroprotective roles of PPAR-gamma activation by its agonist, pioglitazone, on retinal ganglion cells in a rat model of optic nerve crush (Zhu et al., 2013). The neuroprotective functions of PPAR-gamma and its agonist, rosiglitazone, have been shown on transient cerebral ischemic damage (Lee et al., 2011). Activation of this receptor delayed the neuronal injuries through interfering with activation of glial and enhanced the anti-inflammatory cytokines in response to ischemic damage (Lee et al., 2011). Moreover, the potent anti-inflammatory and antioxidant effects of PPAR-gamma agonist, pioglitazone, have been demonstrated in renal ischemia-reperfusion injury in rats (Reel et al., 2013).

Controlling of histopathological changes and oxidative damage is an effective policy for prevention of the secondary neuronal damage after SCI. Therefore, we examined the efficacy of PPAR-gamma agonist, pioglitazone, to pre-
vent the histopathological changes and oxidative damage as well as motor dysfunction after SCI in rats.

MATERIALS AND METHODS

Animals

All surgical procedures were performed in accordance with accepted standards of animals use and care of the Animals Committee of Baqiyatallah University Medical Sciences. Male Wistar rats, weighing approximately 300-340 g, were obtained from the animal house facility center of Baqiyatallah University of Medical Sciences. The rats were housed at standard situation with controlled temperature (22-24°C), humidity (40-60%) and light period (07.00-19.00) as well as free access to the rat chow and water.

Experimental protocols and grouping

Twenty-four male Wistar rats were randomly divided into three groups as follows; sham, control injury and pioglitazone-treated injured groups. Spinal cord injury (SCI) was performed according to the Ping-Weight Drop (Contusion) model in rat (Park et al., 2007). In brief, under 2.5% isoflurane (Forane, UK) anesthesia, a T12-L1 laminectomy was performed, and the spinal cord was injured by dropping a 10-g weight from a height of 6 cm. Throughout the procedure, body temperature was maintained at 37°C with a heating pad. Sham-operated rats were laminectomized but not contused. The rats of control injury and treated injured groups were contused. Then, the incisions were sutured, the animals were allowed to recover from anesthesia, and the rats were returned to their cages after recovering from anesthesia. Injured rats underwent manual bladder excretion until reflexive bladder emptying was established. The pioglitazone-treated animals received pioglitazone (3 mg/kg) intraperitoneally at times of 15 min after injury and then each 12 hours for seven days. Pioglitazone was purchased from India (Avinash).

Assessment of motor function

Evaluation of motor function recovery after SCI was performed according to the Basso, Beattie and Bresnahan (BBB) scoring system as described, previously (Basso et al., 1996). In this method a 21-point open-field locomotor scale was used to determine the BBB score. On days 1, 3, 7, 14, 21, 28, 35 and 42 the movements of each rat were scored for five minutes by an evaluator blinded to the study groups.

Histological assessment

At the termination of the study, rats were sacrificed under deep anesthesia. The tissues were removed and fixed in formalin (10%) for two weeks. After fixation and tissue processing, the serial sections (5 μm in thickness) were prepared for conventional histopathological examination. Paraffin embedded sectioning (each 50 μm intervals) was processed routinely for Hematoxylin and Eosin (H&E) staining. The histopathological changes were observed through a light microscope (Nikon, Japan) connected to digital camera (CMEX, Holland) for capturing the photograph.

Tissue preparation to determine oxidative parameters

After deep anesthesia, the tissues were quickly removed, washed in ice-cold phosphate buffer saline (PBS) for glutathione (GSH) and malondialdehyde (MDA) assays. The tissues were quickly weighed and homogenized 1:10 in ice-cold PBS. The homogenates were then centrifuged at 14000×g for 15 minutes at 4°C. The supernatants were separated and used for determination of GSH and MDA levels.
Figure 1: The BBB score after SCI (1-42 days) at control injury (A) and pioglitazone-treated injured group (B). Both groups showed same motor function recovery during the test and there are no significant differences in the BBB score at different days. The values in the graphs (A and B) are means±SEM (n= 6 rats/group).

To measure the MDA levels, as an index of lipid peroxidation, 0.5 mL of tissue homogenate was added to 1.5 mL of 10% trichloroacetic acid (TCA), vortexed and incubated for 10 min at room temperature. 1.5 mL of supernatant and 2 mL of thiobarbituric acid (0.67%) were added and placed in a boiling water bath in sealed tubes for 30 min. The samples were allowed to cool at room temperature. 1.25 mL of n-butanol was added, vortexed and centrifuged at 2000 g for 5 min. The resulting supernatant was removed and measured at 532 nm on a spectrophotometer. MDA concentrations were determined by using 1,1,3,3-tetraethoxypropane as standard (Satoh, 1978). Finally, the MDA concentration was expressed as percentage.

To determine the GSH levels, cellular protein was precipitated by addition of 5% sulfosalicylic acid and removed by centrifugation at 2000 g for 10 min. GSH in the supernatant was assayed as follows: 100 µL of the protein-free supernatant of the cell lysate, 800 µL of 0.3
mM Na2HPO4 and 100 µL of 0.04% 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.1% sodium citrate. The absorbance of DTNB was monitored at 412 nm for 5 min. A standard curve of GSH was performed and sensitivity of measurement was determined to be between 1 and 100 µM (Tietz, 1969). The level of GSH was expressed as nMol/mL.

**Statistical analysis**

Statistical analysis was performed using SPSS software (version 21). The comparisons between groups were performed using analysis of variance (ANOVA) followed by Tukey post-hoc test. Also, the method of repeated-measured was used to analyze the data of BBB score. All data was expressed as mean±SEM and all states differences between the groups were reported significant when P<0.05.

**Figure 2:** Photomicrographs show the effects of pioglitazone on histopathological changes in the prepared cross-sections of spinal cord at the injured areas using H&E staining method. A, B and C are the taken photomicrographs for sham, control and treatment groups, respectively, with a magnification 100X. The micrographs D, E and F also show the cross-sections of injured spinal cord for sham, control and treatment groups, respectively, with a magnification 400X (Scale bar 20 µm).
RESULTS

**Motor function test:** As shown in figure 1, the BBB scale or motor function test was improved in the control injured rats (A) during the test. The BBB scale for these rats was near zero at day one after injury but it was 7±1 at termination of the study. The alterations of the BBB scale in the pioglitazone-treated injured rats also were same as control group and there was no significant difference in the BBB scales between control and treated injured groups from the beginning to the end of the study (6 weeks).

![Figure 3](image-url)  
**Figure 3:** The percentage of malondialdehyde (MDA) changes (%) in spinal cord at day 7 after SCI induction. All values are means±SEM.

**Histopathological changes:** As shown in figure 2, photomicrographs show the cross-sections of spinal cord at injured areas stained with H&E at level of T12-L1. Histopathological assessments of the sections by using light microscope didn’t show any histopathological alterations at termination of the study in sham group. Evaluation of injured areas in control rats revealed the different lesions in the obtained photomicrographs such as heterogeneous neuronal changes, pyknotic and shrinkage of nucleus with widened pericellular spaces, eosinophilic neurons characterized by condensed acidophilic cytoplasm and formation of triangular nuclear pyknosis, vacuolization and edema in neuropils (vacuolization of neuropils). Pioglitazone in treated injured group considerably decreased the mentioned histopathological changes.

![Figure 4](image-url)  
**Figure 4:** The glutathione (GSH) levels (nMol/mL) in spinal cord at day 7 after SCI induction. All values are means±SEM. There was no statistically significant difference between groups.

**MDA levels:** Figure 3 shows the percentage of MDA changes of injured spinal cord for experimental groups. Induction of the SCI in control rats significantly increased the MDA levels by 56% compared to sham group (P=0.002). However, treatment in pioglitazone-treated injured rats noticeably decreased the MDA levels by 37% in comparison to control group (P=0.018).

**GSH levels:** As shown in figure 3 the GSH level of spinal cord in sham group was 49±6 nMol/mL. Induction of SCI decreased the GSH levels of control rats (39±4 nMol/mL) but not significantly. This value was 42±1 nMol/mL in pioglitazone-treated injured rats.

DISCUSSION

Previously, several beneficial roles of PPAR-gamma agonists have been reported in various pathological states of nervous system (Lee et al., 2011; Kapadia et al., 2008). The
findings of the present study indicated the neuroprotective functions of pioglitazone (PPAR-gamma agonist) in the contusion model of SCI in rats. According to the results, treatment with pioglitazone improved the histopathological changes and oxidative damage of injured spinal cord. However, pioglitazone did not affect the BBB score or glutathione levels in the injured areas of spinal cord.

Activation of secondary pathophysiological mechanisms of injury after occurrence of SCI plays a key role in intensification and development of damage (Hayta and Elden, 2017; Fatima et al., 2015). Accordingly, the findings of the present revealed the free radicals overproduction and oxidative damage in the injured areas of spinal cord (increment of MDA levels). Several mechanisms are involved in development of tissue oxidative damage. Based on previous studies, weakening of the antioxidant defense system and activation of the prooxidant enzyme such as plasma membrane oxidases (NADPH-oxidase and xanthine oxidase) are the main sources of free radicals and oxidative damage (Fatima et al., 2015; Bermudez et al., 2016). According to our results, the glutathione levels of the injured areas considerably decreased but not significantly. Glutathione is necessary for action of glutathione peroxidase, which neutralizes hydrogen peroxide by converting it to H₂O (Valko et al., 2007). It has been reported that tissue oxidative damage is one of the main mechanisms of neuronal injury after SCI (Vaziri et al., 2004). Based on histopathological assessments of the present study, several neuronal and tissue damages were well observed at injured areas. However, it is noticed that other neurodegenerative signals such as apoptosis and necrosis signals, inflammatory factors and proteases play a crucial role in development of neural and tissue damage after SCI.

It has been demonstrated that pioglitazone (PPAR-gamma) is beneficial for the injury of nervous tissue (Zhu et al., 2013; Lee et al., 2011). Our findings indicated that treatment with pioglitazone has decreased the histopathological changes of neuronal and tissue damage in injured spinal cord. Previously, the neuroprotective functions of pioglitazone have been reported in several pathological states of central nervous system. Neuroprotective role of pioglitazone via the reduction of Muller glial activation has been reported in rat retina following optic nerve injury (Zhu et al., 2013). The results of another study indicate that enhanced expression levels of PPAR-gamma in microglia following ischemia delays neuronal damage by interfering with glial activation and increases the anti-inflammatory cytokines in response to ischemic damage (Lee et al., 2011). Likewise, the anti-apoptotic action of PPAR-gamma agonists has been demonstrated (Li et al., 2013). Moreover, according to our results, pioglitazone has the antioxidant property because it decreased the MDA levels (an index of oxidative damage) at injured areas. Other studies also have confirmed the antioxidative effects of this agonist in other pathological states (Reel et al., 2013; Al Rouq and El Eter, 2014). For example pioglitazone might be helpful to reduce renal ischemia-reperfusion injury because of its antioxidant properties (Reel et al., 2013). However, in the present study pioglitazone did not change motor function (BBB score) and also glutathione levels. It is possible that the level of injury was intensive or the utilized dose of pioglitazone was low.

The findings of present study revealed that pioglitazone improves the histopathological changes and oxidative damage of the injured spinal cord. Then, it is suggested that PPAR-gamma agonist, pioglitazone, can be a candidate for treatment of SCI but high doses
of this agonist (>3 mg/kg) is proposed for better response.

**CONFLICT OF INTERESTS**

The authors declare that they have no conflict of interests.

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