

## Neuroprotective effect of pioglitazone on acute phase changes induced by partial global cerebral ischemia in mice

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Present study was carried out to investigate the possible neuroprotective effect of pioglitazone, an antidiabetic agent, peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) agonist on acute phase changes in mice model of cerebral ischemia induced by Bilateral Common Carotid artery Occlusion (BCCAO). BCCAO model was used to induce partial global cerebral ischemia. BCCAO induced significant brain infarct size and edema in saline treated control group along with high increase in oxidative stress showed by increase lipid peroxidation and decreased levels of antioxidants like superoxide dismutase, catalase, glutathione peroxidase. Pioglitazone (20 mg/kg, orally) administration showed neuroprotective effects by reducing cerebral infarct size significantly as compared to control group. Postischemic seizure susceptibility was also reduced as number of positive responders decreased to a significant number. Brain edema was subsided to a significant level. Pioglitazone reduced the plasma TNF- $\alpha$  levels as compared to ischemia group significantly. Pioglitazone treatment also improved all the antioxidants levels showing activity against oxidative stress induced by BCCAO. Pioglitazone showed neuroprotection against ischemic insult suggesting the role of PPAR  $\gamma$  agonist in neuroprotective agents.

**Keywords:** Cerebral ischemia, Pioglitazone, PPAR- $\gamma$ , Neuroprotection

Partial global cerebral ischemia (ischemia of forebrain and sub-cortical tissue) is a syndrome characterized by rapid onset of neurological injury due to interruption of blood flow to the brain<sup>1,2</sup> and it leads to various pathophysiological modalities such as ROS, calcium overload, mitochondrial damage, neuronal cell death etc. Global cerebral ischemia results in neuronal death irrespective of postischemic reperfusion<sup>3</sup>. Reperfusion after cerebral ischemia further adds to the complications of stroke by releasing various mediators such as proinflammatory cytokines and free radical generation. This increases the oxidative stress to the brain and ultimately leading to neuronal cell death. Seizures can occur soon after the onset of ischemia or can be delayed<sup>4</sup>. Seizure threshold decreases subsequently with ischemia and reperfusion injury. Repeated seizure-like activity in the setting of cerebral ischemia significantly increases infarct size and can impair functional recovery, an effect that can be ameliorated with the administration of

certain neuroprotective agents<sup>5-7</sup>. Cerebral ischemia is frequently accompanied by inflammation, which can worsen neuronal injury. Activation of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) reduces inflammation and the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. In addition, PPAR  $\gamma$  activators increase levels of Cu-Zn-superoxide dismutase (SOD) in cultured endothelium, suggesting an additional mechanism by which it may exert protective effects within the brain<sup>8</sup>. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that function as ligand-activated transcriptional regulators of genes controlling lipid and glucose metabolism<sup>9</sup>. These receptors have so far not been studied in ischemia reperfusion associated changes in whole body behavior or biochemical parameters in mice model.

Therefore it was thought prudent to investigate the effects of *in vivo* administration pioglitazone on ischemia and reperfusion induced cerebral injury along with roles of PPAR  $\gamma$  activators in ischemia induced seizure susceptibility, behavioral defects and biochemical changes in model of partial global cerebral ischemia in mice.

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## Materials and Methods

Individually caged Swiss Albino mice (n=72) weighing between 20-30 g were maintained under standard laboratory conditions (12 hr light and dark cycle). The mice had free access to food (standard rat chaw) and water. The Institute Animal Ethics Committee (IAEC) approved the experimental design of the study and all the procedures were carried out according to CPCSEA guidelines for animal experimentation in India.

Pioglitazone a gift from Macleod Pharma, Mumbai, India, was administered orally, as a suspension in DMSO (10%).

Animals were divided into following 3 groups of 24 each:

Sham group: (Group 1; 24 mice comprising of 4 subgroups of 6 each)

Mice were subjected to surgical procedure but the arteries were not occluded. After 10 minutes the animals were sutured back and allowed to recover. After 72 hours, each subgroup of mice was subjected to assessment of different parameters as mentioned under methodology.

Surgery group: (Group 2; 24 mice comprising of 4 subgroups of 6 each)

Mice were subjected to the surgical procedure followed by clamping of bilateral common carotid arteries with aneurysm clips for 10 min to produce partial global cerebral ischemia. Subsequently, the carotids were declamped and cerebral reperfusion was allowed for 72 h. After 72 h, each subgroup of mice was subjected to assessment of different parameters as mentioned under methodology.

Pioglitazone group: (Group 3; 24 mice comprising of 4 subgroups of 6 each)

Group 3 comprised of surgically operated mice as mentioned under group 2. The mice were treated with four doses of pioglitazone (20 mg/kg, orally). Dosing time intervals were:

30 min before producing cerebral ischemia (day 1)

24 hrs after the first dose (day 2)

48 hrs after the first dose (day 3)

72 hrs after the first dose (day 4)

After 72 h (i.e. on day 4, 1h after the last dose), each subgroup of mice was subjected to assessment of different parameters as mentioned under methodology.

*Induction of cerebral ischemia*— Mice were anaesthetized with chloral hydrate (400 mg/kg, ip). A midline incision was made in the region between neck and sternum and trachea was exposed. Both the right and left common carotid arteries were located lateral to sternocleidomastoid, freed from the surrounding tissues and vagus nerve was separated. Cerebral ischemia was induced by clamping both the arteries with the help of aneurysm clips<sup>10</sup>. After 10 min of cerebral ischemia, the clips were removed from both the arteries to allow the reflow of the blood through carotid arteries. The incision was sutured back in layers with surgical suture<sup>11</sup>. The sutured area was cleaned with 70% ethanol and was sprayed with antiseptic powder. After completion of surgical procedure, the animals were shifted individually to their home cage and were allowed to recover. While performing the surgical procedure, the body temperature was maintained at 37°C by heated IR lamp. All the surgical instruments used in the surgical procedure were sterilized prior to use.

*Measurement of cerebral infarct size*— Animals were sacrificed under ether anaesthesia and the brain removed. The brain was kept overnight at -4°C. Frozen brain was sliced into uniform sections of 1mm thickness. The slices were immersed in 1% triphenyltetrazolium chloride (TTC) at 37°C in 0.25 M phosphate buffer (pH 8.5) for 20 min; tissue sections were dipped in a 10% formaldehyde solution for 5 min<sup>12</sup>. Triphenyltetrazolium chloride (TTC) is converted to red formazone pigment and therefore stained the viable cells deep red. The infarcted cells have lost the enzyme and cofactor and thus remained unstained dull yellow. The brain slices were placed over glass plate. A transparent plastic grid with 100 squares/1cm<sup>2</sup> was placed over it. Numbers of squares falling over non-stained dull yellow area and total number of squares covered by each brain slice was counted. Infarcted area was expressed as a percentage of total brain volume.

*Measurement of brain edema*— Animals were sacrificed by decapitation under ether anaesthesia; brain removed and weighed immediately to yield wet weight. Brain water content, an indicator of brain edema was measured with the wet dry method<sup>13</sup>.

*Seizure susceptibility*— Seizure susceptibility was assessed by giving PTZ at the subconvulsive dose of

30 mg/kg, ip after 72 h of reperfusion. Seizures were recorded according to the following scale (0-6 score)<sup>14</sup>:

0 = no response; 1 = ear and facial twitching; 2 = one to 20 myoclonic body jerks in 10 min; 3 = more than 20 body jerks in 10 min; 4 = clonic forelimb convulsions; 5 = generalized clonic convulsions with rearing and falling down episodes; and 6 = generalized convulsions with tonic extension episodes.

### Behavioral assessments

Following behavioral assessments were carried out after 72 h of cerebral ischemia.

(i) Short term memory evaluation using elevated plus maze<sup>5</sup>: After 72 h of cerebral ischemia the animal was put to elevated plus maze test. Transfer latency time (TLT) measured on plus maze, on first and second day serve as an index of learning or acquisition, whereas TLT on third day served as an index of retrieval or memory. Utmost care was taken not to change the relative location of plus maze with respect to any object serving as visual clue in laboratory.

(ii) Hole board test: This is an test to evaluate the exploratory behavior, with help of an open field with holes on the bottom into which animals could poke their noses. Each animal was placed on the hole board and tested for 5 min before and after subjecting to cerebral ischemia. The number of counts for nose poking was calculated to evaluate the exploratory behavior of the animal<sup>5</sup>.

(iii) Rota rod test: Rota rod test<sup>5</sup> was employed to evaluate fore and hind limb motor coordination. Animals were prescreened based upon their ability to remain on the revolving rod for 1 min.

### Biochemical parameters

(i) Brain protein assay: Mouse brain protein assay was done according to the method of Lowry *et al*<sup>15</sup>.

(ii) Lipid peroxidation assay: Tissue lipid peroxidation was evaluated by measurement of thiobarbituric acid-reactive substances (TBARS). Mylanoadehyde (MDA) has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give red light absorbency at 535nm<sup>16</sup>.

(iii) Assay for the estimation of super oxide dismutase (SOD): The method<sup>17</sup> is based on the principle of the inhibitory effect of SOD on reduction

of nitro blue tetrazolium (NBT) dye by super oxide anions which are generated by the photo oxidation of hydroxylamine hydrochloride (NH<sub>2</sub>OH.HCl).

(iv) Estimation of catalase: Activity of catalase was measured<sup>18</sup>. The reaction mixture contained 3 ml of 0.66 M phosphate buffer, pH 7.0 and 1.25×10<sup>-2</sup> M H<sub>2</sub>O<sub>2</sub> in the sample cuvette. Reference cuvette contained only 3 ml of 0.66 M phosphate buffer, pH 7.0. The reaction was started by adding brain tissue homogenates to the sample as well as reference cuvettes. The rate of elimination of H<sub>2</sub>O<sub>2</sub> by catalase was measured by recording the time (in sec) required for 0.05 decline of absorbance at 240 nm. Catalase activity in International Units (IU) was calculated by the formula:

$$\text{IU} = \frac{17}{\text{time in second for 0.050 absorbance change}} \times \frac{1}{\text{weight of homogenate}} \times 13$$

Catalase activity was expressed in terms of nmole of H<sub>2</sub>O<sub>2</sub> consumed/min/mg of protein.

(v) Estimation of glutathione peroxidase (GSH-Px): GSH-Px was estimated as per Paglia and Valentine<sup>19</sup>, using brain tissue homogenate. Samples of brain tissue homogenate were diluted with 50 mM phosphate buffer, pH 7.4 and were added to a reaction mixture containing 5mM EDTA, 0.01 ml of 1.125 M sodium azide, 0.1 ml of 0.15 M glutathione (GSH), 2.4 units of glutathione reductase (10 µl) and 0.1 ml of 8.4 mM NADPH to make a final volume of 2.9 ml. Reaction mixture was incubated at 22°C for 10 min. Reference cuvette contained 100 µl of distilled water instead of brain tissue homogenate. Following addition of 0.1 ml of 2.2 mM H<sub>2</sub>O<sub>2</sub> solution, decrease in absorbance at 340 nm was recorded for 3-4 min. Concentration of the enzyme was finally calculated in terms of NADPH consumed/min/mg homogenate using extinction coefficient of 6.22 mM<sup>-1</sup>cm<sup>-1</sup>.

(vi) Brain TNF-α estimation: Brain TNF-α was estimated by using ELISA kit as per the methodology provided by manufacturer in the kit. A monoclonal antibody, specific for mice TNF-α was coated on to the well of microtitre strip provided. Samples including serially diluted standards of known concentrations of TNF-α and the test sample of TNF-α (obtained from brain homogenate) were pipetted into these wells in 100 µl volume. The blank was created with 100 µl of standard diluents.

The micro well strip was covered with a plate cover and incubated for 2 h at room temperature (25°C). Each well was washed thrice with 0.3 ml of washing solution. To each well 50 µl of diluted biotinylated anti-mTNF-α was added. Strips were incubated for 1 h at room temperature. The wells were washed again. To each well 100 µl of diluted streptavidin-HP solution was added. Strips were incubated for 30 min at room temperature. Wells were again washed. To each well 100 µl of ready-to-use tetramethylbenzidine (TMB) substrate solution was added and incubated in the dark for 30 min at room temperature. The enzyme-substrate reaction was stopped with 100 µl of H<sub>2</sub>SO<sub>4</sub>. Readings were taken immediately thereafter at 450 nm as the primary wavelength using an ELISA reader. The concentration of TNF-α was determined by extrapolating the OD value onto the standard curve obtained from the known concentration of TNF-α provided by the manufacture. Plasma TNF-α level was expressed in terms of pg/ml of brain supernatant.

**Statistical analysis**— All the data are expressed as mean ± SE. Inter-group comparisons were made using ANOVA followed by post-hoc Tukey's test. Number of positive responders and seizure score was evaluated by Fischer's exact probability test. *P* value <0.05 was considered significant.

## Results

Effect of pioglitazone on brain infarct size, brain water content, seizure score, motor coordination, exploratory behavior and plasma TNF-α levels has been given in Table 1.

**Effect of pioglitazone on transfer latency time (TLT)**— The data on the transfer latency time are summarized in Table 2. Acquisition phase denotes the transfer latency time after 3 days of training session to animals. Upon training TLT was reduced from cut off TLT i.e. 90 sec in all the groups. Retention phase denotes the TLT at different time intervals with subsequent dosing of responsive drugs. At 24 hours i.e. 24 hrs after ischemia and 1 hour after second dose of drug, there is no significant difference in TLT in sham group. Even after 48 and 72 hrs TLT was not different from acquisition phase in sham group. Making comparisons in different groups in acquisition also shows not a significant difference in TLT. Upon surgery and BCAA, TLT was significantly increased to 90 secs at all the time positions (*P* < 0.05). Comparing the groups, TLT was increased in surgery group as compared to sham group showing, the memory impairment as an effect of BCAA. But treatment with pioglitazone at 24 hrs had significantly reduced TLT as compared to surgery group (*P* < 0.001) and treatment with pioglitazone at 72 hours shown reduction in TLT even more significantly (*P* < 0.0001).

Table 1— Effect of pioglitazone on brain infarct size, brain water content, seizure score, motor coordination, exploration behavior and plasma TNF-α levels in stroke model of mice  
[Values are mean ± SE from 6 animals in each group]

Groups	Water contents (%)	Infarct size (mm)	Seizure score	Motor coordination (sec)	Exploratory behavior (no. of dips)	TNF- α level (pg/ml)
Sham	74.23 ± 0.36	26.86 ± 2.91	0.5 ± 0.34	112.17 ± 6.1	34.00 ± 2.93	40.67 ± 6.38
Surgery	76.25 ± 0.51*	87.65 ± 1.95*	3.5 ± 0.87*	23.17 ± 5.1*	24.83 ± 2.14*	241.67 ± 22.46*
Pioglitazone	69.94 ± 0.37 <sup>#</sup>	48.79 ± 2.41 <sup>#</sup>	0.67 ± 0.49 <sup>#</sup>	97.17 ± 16.9 <sup>#</sup>	37.9 ± 2.25 <sup>#</sup>	146.50 ± 17.89 <sup>#</sup>

\**P* < 0.01; sham vs. surgery. <sup>#</sup>*P* < 0.001; surgery vs pioglitazone

Table 2—Effect of Pioglitazone on the transfer latency time (TLT) in mice  
[Values are mean ± SE from 6 animals in each group]

Groups	Acquisition phase	Retention phase 24h	Retention phase 48h	Retention phase 72h
Sham	21.25 ± 2.17	26.50 ± 1.32	28.50 ± 2.96	26.00 ± 3.24
Surgery	20.75 ± 5.02	90.00 ± 0.00* <sup>#</sup>	90.00 ± 0.00* <sup>#</sup>	90.00 ± 0.00* <sup>#</sup>
Pioglitazone	28.25 ± 10.58	36.50 ± 12.02 <sup>S</sup>	17.75 ± 2.81 <sup>S</sup>	10.50 ± 0.29* <sup>@</sup>

\**P* < 0.05 Acquisition phase vs. retention phase

<sup>#</sup>*P* < 0.01 sham vs. surgery

<sup>S</sup>*P* < 0.001 surgery vs. sioglitazone

<sup>@</sup>*P* < 0.0001 surgery vs. sioglitazone at 72 hrs

Retention phases followed 24, 48 and 72 h after acquisition phase. Ischemic surgery was done immediately after acquisition phase. After 1 hour of dosing, retention phase was noted at each level.

*Effect of pioglitazone on cerebral ischemia induced antioxidant activity*— All the values of antioxidant parameters are outlined in Table 3. Upon the ischemic insult LPO activity increased significantly as compared to sham group ( $P < 0.001$ ) which was attenuated significantly with pioglitazone treatment  $P < 0.001$ . Further, antioxidant activity was decreased with ischemia shown by low levels of SOD, GPx and CAT as compared to sham group ( $P < 0.001$ ). Treatment with pioglitazone raised all the antioxidant enzyme levels to a significant value indicating improved antioxidant enzyme activity against cerebral ischemia and reperfusion induced oxidative stress.

### Discussion

Peroxisome-proliferator-activated receptors (PPARs) are ligand-activated transcriptional factor receptor belonging to the so-called nuclear receptor family. The three isoforms of PPAR ( $\alpha$ ,  $\beta/\Delta$  and  $\gamma$ ) are involved in regulation of lipid or glucose metabolism<sup>20</sup>. Beyond metabolic effects, PPAR- $\alpha$  and PPAR- $\gamma$  activation also induces anti-inflammatory and antioxidant effects in different organs. Thiazolidinediones (TZDs) are potent synthetic agonists of the ligand-activated transcription factor PPAR- $\gamma$ . TZDs were shown to induce neuroprotection after cerebral ischemia by blocking inflammation<sup>21</sup>. Role of thiazolidinediones naming pioglitazone and rosiglitazone in neurodegenerative diseases with inflammation is well reported. Studies were undertaken using rosiglitazone, showing it as neuroprotective agent<sup>22</sup>. But there are very few studies relating pioglitazone with cerebral ischemia and reperfusion induced neurological disorder, known as stroke. Tureyen *et al.*<sup>23</sup> reported beneficial effect of pioglitazone in cerebral ischemia in rats with middle carotid artery occlusion (MCAO). Then, showed pre-treatment and post-treatment with TZDs rosiglitazone and pioglitazone significantly decreased the infarct volume and neurological deficits in normotensive,

normoglycemic, hypertensive and hyperglycemic rats. Rosiglitazone also increased the expression of the anti-inflammatory gene suppressor of cytokine signaling-3 and prevented the phosphorylation of the transcription factor signal transducer and activator of transcription-3 after focal ischemia.

Therefore, based on these assumptions, pioglitazone was investigated as a neuroprotective agent in BCCAO model of cerebral ischemia in mice. Bilateral common carotid artery occlusion increased brain infarct size as compared to sham group. Administration of pioglitazone at the dose of 20 mg/kg, orally, decreased the infarct size significantly. In the present study, there was an increase in susceptibility to seizures and the number of positive responders increased significantly after cerebral ischemia. Administration of pioglitazone decreased the seizure score almost near to the baseline showing increased threshold for seizures after PTZ administration (subconvulsive dose 30 mg/kg, ip). Pioglitazone also decreased the brain water content to a significant level as compared to increased brain edema after cerebral ischemia and reperfusion injury. Hence, pioglitazone showed beneficial effect in reducing brain edema following cerebral ischemia. Short term memory impairment was seen in surgery group with cut off TLT i.e. 90 sec. Administration of pioglitazone improved short term memory with decreased TLT as compared to surgery group at all time intervals. At 72 h TLT was reduced to much significant level proposing memory enhancement with pioglitazone. Pioglitazone (20 mg/kg, orally) attenuated the motor incoordination quite significantly and improved the grip strength. Though pioglitazone increased the number of dips as compared to surgery group, it cannot be concluded as an anxiolytic effect of pioglitazone. Zhao *et al.*<sup>24</sup> showed that activation of neuronal PPAR  $\gamma$  considerably contributes to neuroprotection by prevention of COX-2 up-regulation *in vitro* and in peri-infarct brain areas.

Table 3—Mean values of lipid peroxidation, SOD, GPx and CAT levels  
[Values are mean  $\pm$  SE ]

Groups	LPO levels (nmoles/ml)	SOD levels (units/mg protein)	GPx levels (units/mg protein)	CAT levels (units/mg protein)
Sham	1.53 $\pm$ 0.18	3.76 $\pm$ 0.39	8.99 $\pm$ 0.64	3.13 $\pm$ 0.30
Surgery	9.55 $\pm$ 0.67*	1.59 $\pm$ 0.17*	3.29 $\pm$ 0.22*	0.79 $\pm$ 0.12*
Pioglitazone	3.41 $\pm$ 0.34 <sup>#</sup>	5.16 $\pm$ 0.23 <sup>#</sup>	9.56 $\pm$ 0.81 <sup>#</sup>	1.79 $\pm$ 0.29 <sup>#</sup>

\* $P < 0.001$  sham vs. surgery in all parameters

<sup>#</sup> $P < 0.001$  surgery vs. pioglitazone

During ischemia, and especially during reperfusion, these radicals may be produced to such an extent that endogenous antioxidant systems are overwhelmed. Free radicals are demonstrated to promote lipid peroxidation. In the present study, there was a significant increase in lipid peroxidation levels and significant decrease in all the other antioxidant enzymes. Treatment with pioglitazone decreased the lipid peroxidation levels and increased all the antioxidant enzymes like SOD, GPx and CAT. Improvement in antioxidant enzyme levels to significant values suggests antioxidant and free radical scavenger activity of pioglitazone against cerebral ischemia and reperfusion induced oxidative stress. During ischemia, a lot of mediators are released in progressive manner like O<sub>2</sub> radical, ET1, and TNF- $\alpha$  etc. which leads to various vascular complications<sup>25</sup> but on treatment with pioglitazone has reduced the levels of TNF- $\alpha$  combating the vascular complications during acute phase of ischemic stroke.

To conclude, the neuroprotective effect of pioglitazone, a PPAR- $\gamma$  agonist, in cerebral ischemia and reperfusion induced by BCCAO in mice can be confirmed. Nevertheless, further studies are needed to elucidate these findings in clinical settings.

### Conflict of interest

The authors have no financial or proprietary interest in any of the products mentioned in this manuscript.

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