Aquaporin-4 Inhibition Mediates Piroxicam-Induced Neuroprotection against Focal Cerebral Ischemia/ Reperfusion Injury in Rodents

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Abstract

Background and Purpose: Aquaporin-4(AQP4) is an abundant water channel protein in brain that regulates water transport to maintain homeostasis. Cerebral edema resulting from AQP4 over expression is considered to be one of the major determinants for progressive neuronal insult during cerebral ischemia. Although, both upregulation and downregulation of AQP4 expression is associated with brain pathology, over expression of AQP4 is one of the chief contributors of water imbalance in brain during ischemic pathology. We have found that Piroxicam binds to AQP4 with optimal binding energy value. Thus, we hypothesized that Piroxicam is neuroprotective in the rodent cerebral ischemic model by mitigating cerebral edema via AQP4 regulation.

Methods: Rats were treated with Piroxicam OR placebo at 30 min prior, 2 h post and 4 h post 60 minutes of MCAO followed by 24 hour reperfusion. Rats were evaluated for neurological deficits and motor function just before sacrifice. Brains were harvested for infarct size estimation, water content measurement, biochemical analysis, RT-PCR and western blot experiments.

Results: Piroxicam pretreatment thirty minutes prior to ischemia and four hour post reperfusion afforded neuroprotection as evident through significant reduction in cerebral infarct volume, improvement in motor behavior, neurological deficit and reduction in brain edema. Furthermore, ischemia induced surge in levels of nitrite and malondialdehyde were also found to be significantly reduced in ischemic brain regions in treated animals. This neuroprotection was found to be associated with inhibition of acid mediated rise in intracellular calcium levels and also downregulated AQP4 expression.

Conclusions: Findings of the present study provide significant evidence that Piroxicam acts as a potent AQP4 regulator and renders neuroprotection in focal cerebral ischemia. Piroxicam could be clinically exploited for the treatment of brain stroke along with other anti-stroke therapeutics in future.

Introduction

Cerebral edema is a potentially devastating complication of various acute neurologic disorders which accounts for much of the morbidity and mortality [1,2]. Various secondary mechanisms contribute to further progressive deterioration with limited treatment options available which include osmotherapy and surgical decompression. None of these are successful to obliterate the molecular mechanisms responsible for edema which strongly necessitates and suggests that fulminating cerebral edema needs to be intervened by some pharmacological molecule [2].

Aquaporin-4 (AQP4) are integral membrane proteins which plays a key role in maintaining water homeostasis in the central nervous system, and its dysfunction may lead to brain edema [2]. The bidirectional water channel AQP4 has been found to play a

determining role in brain water homeostasis [3]. AQP4 protein is expressed strongly in astroglia at the BBB and CSF-brain interfaces [4], involved in water movement between fluid compartments (blood and CSF) and brain parenchyma. It has been suggested that AQP4 deletion markedly reduced brain swelling of cytotoxic brain edema, including water intoxication and focal cerebral ischemia [5,6]. AQP4 levels are markedly altered in experimental models of brain injury and swelling in response to ischemic neuronal insult [2]. In middle cerebral artery occlusion (MCAO) animal model of focal cerebral ischemia which represents a model for brain edema, and AQP4 deficient mice subjected to MCAO show better neurological and functional outcome than normal control mice. Evaluating cerebral edema by calculating the percentage of hemisphere enlargement at 24 hr

after injury was 35% lower in AQP4 deficient mice than in normal control mice [6]. As, AQP4 appears to facilitate water movement in cytotoxic edema, so detection of expression level of AQP4 can indirectly determine the brain swelling extent in cerebral ischemia Therefore a pharmacological molecule targeting AQP4 represent potential therapeutics for the treatment of brain edema [2].

Free radical mediated injury has been proved to be one of the prominent factors during pathological condition like ischemic stroke. They play a critical role in ischemic brain damage by exacerbating membrane damage leading to neuronal cell death. There are many therapeutic strategies which have been reported in past studies which reduce free radical induced damage process following acute ischemic stroke. [7–9]. The end result of cerebral ischemia is brain injury, associated with neurological and neurobehavioral deficits that depend on the areas of brain or networks in the brain that are disrupted [10]. Hence, a pharmacological molecule is necessitated which can act in a multifaceted dimension.

Recent studies have showed that non steroidal anti-inflammatory drugs (NSAIDs) like flurbiprofen inhibit inflammation and acidotoxicity by acting against mediator of inflammations and acid sensing ion channels but no such effect on cognitive function and AQP4 by NSAID have been reported yet to best of our knowledge [11]. Although, from the past in-silico studies from our lab we have hypothesized that Piroxicam may be one of the molecules of choice to combat brain stroke mediated edema, acid sensing ion channel 1a(ASIC1a) mediated acidotoxicity, μ-calpain and matrix metalloproteinases inhibition mediated neuroprotection [12] and stroke mediated cognitive deficits concomitantly [13,14]. Hence, the present molecular study was undertaken with Piroxicam as a candidate NSAID, whose neuroprotective efficacy is yet to be explored in vivo targeting AQP4 expression, however some positive results were reported by in vitro studies regarding its neuroprotective action on neuronal cells [15,16]. We have tried to determine the neuroprotective efficacy spectrum of Piroxicam in rodent model of focal cerebral ischemia and have also explored its neuroprotective effects with AQP4 channel as one of the targets to inhibit stroke mediated brain edema.

Materials and Methods

Ethics Statement

The approved standard procedures and the institutional animal ethical committee (Central Animal House of Banaras Hindu University (Registration No- 542/AB/CPCSEA) guidelines were followed throughout the experiments. The study was approved by the Institutional Animal Ethical Committee of the Central Animal House of Banaras Hindu University.

Chemicals

Piroxicam, 2,3,5- triphenyltetrazoliumchloride (TTC) and most of the other chemicals were purchased from Sigma unless otherwise specified. Molecular biology grade chemicals were used wherever necessary. All the chemicals and enzymes were used as per manufacturer's instructions.

Animals

Male Charles Foster rats (6 weeks, 250 ± 10 g) were in-bred at the Central Animal House of Banaras Hindu University which were used for the experiments. Animals were kept under standard laboratory conditions maintained with the highest standards of animal care and housing. Animals were provided with commercial diet having standard ingredients. Animals were fasted overnight with free access to water and maintained at 12 h day/night cycle.

Focal cerebral ischemia

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCA) with modified intraluminal technique [17]. Rats were anesthetized by the administration of a combination of ketamine (75 mg/kg b.w.) and xylazine (10 mg/kg b.w.) and then transferred to the surgical table with a heating pad to maintain a constant body temperature of $37\pm0.5°C$. Rat was placed in a supine position with forelimbs fixed on the table by tape and the fur on the ventral neck was shaved and the skin was cleaned by 0.5% Butadiene and 75% alcohol. Eye cream was applied to protect corneal drying. Midline incision in the neck was done to expose the left common carotid artery (CCA). A 5.0 cm length 3-0 monofilament nylon suture (Ethicon) was introduced into the CA lumen through a small nick and gently pushed from ICA lumen to block the origin point of MCA. Approximately 18–22 mm length of nylon filament was inserted to reach the MCA blockade site from the bifurcation point. The ECA stump was clamped around the intraluminal nylon suture to prevent bleeding. Reperfusion for 24 hrs was done by gently removing the filament after 1 hr of ischemia. Animals were allowed to recover from anesthesia and on regaining the righting reflex, were transferred to polypropylene cages in the animal room with temperature maintained at $26°\pm2.5°c$ with food and water supply in ad-libitum. In sham-operated animals, all the procedures were carried on except the insertion of nylon filament. Doppler monitoring showed that all rats subjected to MCAO, cortical cerebral blood flow was reduced by at least 70% of pre-ischemic values within 5 min of advancing the filament and induction of MCAO (Data not shown). Rats not exhibiting significant reduction in cerebral blood flow by 70% were excluded from the study.

Dose optimization of Piroxicam and experimental design

Piroxicam dissolved in normal saline and were put to physiological pH before i.p. administration 30 min before inducing focal cerebral ischemia and 2 hr and 4 hr post ischemia. Piroxicam was administered i.p in four doses of 5 mg, 10 mg, 20 mg and 40 mg/kg b/w. to determine the minimum effective neuroprotective dose. A total of 73 animals were divided into six groups consisting of sham (n = 12), vehicle (n = 12) and Piroxicam (drug) treated (n = 7) for each dose group. Further, biochemical and molecular studies were conducted with the effective dose in three more groups viz. sham, vehicle (n = 12 in each group) and Vehicle+Drug (n = 7). All experiments were conducted at the same time in the morning to avoid diurnal or circadian changes. Room temperature and humidity kept constant. The experimenter was blinded to the treatments the rats had received prior to all subsequent analyses. Pictograph of the experimental design is in **Figure 1**. The total animals reported are less than the total animals considered for the study taking into account of the co-morbidity factors.

Rectal temperature of the rats was measured at an ambient temperature of $21.5\pm1°C$ with a lubricated digital thermometer probe inserted 3 cm into the rectum, the rat being lightly restrained by holding in the hand. Temperature was recorded before any drug treatment and thereafter every 60 min up to 8 h. Probes were re-inserted from time to time until the temperature stabilized. Arterial blood parameters (pH, $PaCO_2$, PaO_2) and MAP were monitored in all the animals starting at 30 min pre ischemia and continuously throughout the experiment until 120 min of post ischemia in those rats which underwent MCAO and drug treatment, but no significant differences between the experimental groups were observed as shown in Table 1.
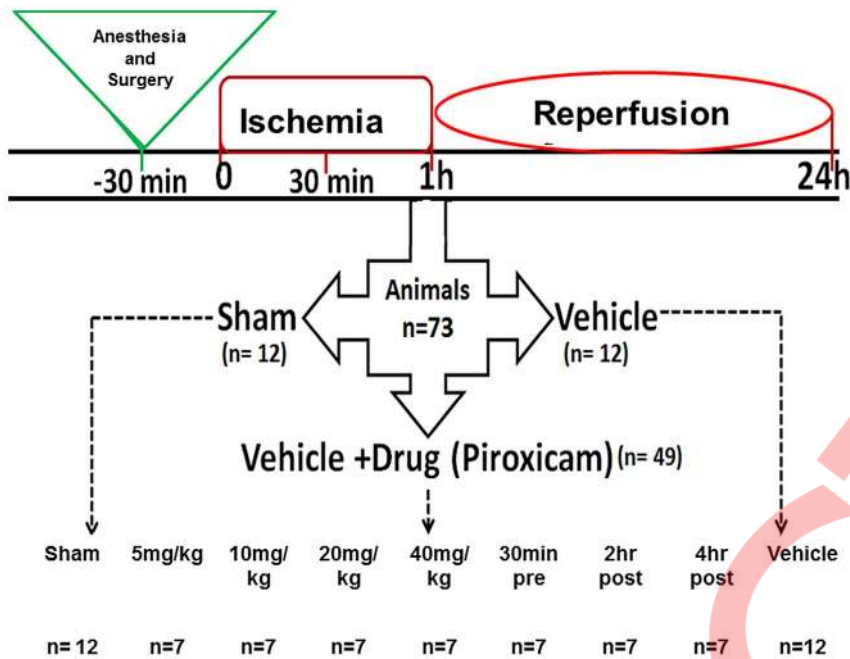
Figure 1. Experimental Design.
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Evaluation of motor coordination and neurological scoring

A performance examination was performed 24 h post reperfusion [17]. Rotarod [IITC Life Science, USA] has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod [18]. The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for five minutes. Each rat was given five trials for 2 days before the actual reading was taken. The readings were taken at 10, 15, 20 and 25 rpm after 24 hr, 48 hr and 72 hr post MCAO in all groups of rats [19].

Neurological scores were derived on five point scale with 10 grading scores: a score of 0 indicated no neurological deficit; a score of 1 means failure to extend opposite forepaw fully, a score of 2 was assigned when contralateral circling was seen. While the rat

Table 1. General physiological parameters observed during experiment.

	Pre-Ischemia	Ischemia	Post Ischemia		
			30 min	**60 min**	**120 min**
maBP mmHg	124±2.0	119±3.0	123±3.0	126±3.2	117±2.3
PaCO$_2$ mmHg	39±0.5	39.3±0.5	38.5±1.57	38.2±1.37	37.9±0.31
pH	7.371±0.004	7.374±0.014	7.343±0.012	7.388±0.018	7.41±0.002
PaO$_2$ mmHg	158±13.0	160±9.0	152±6.0	149±10.0	155±12.0

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which was not able to grip the wire meshes and fell on the contralateral side of brain damage, a score of 3 was assigned. Further when the rats were unable to walk spontaneously and had a depressed level of consciousness was given score of 4. The neurobehavioral scores obtained after testing on each scale were averaged to denote the degree of neurological deficit. Importantly, rats not exhibiting neurological score, having small infarct sizes and inconsistent physiological parameters were excluded from the study.

Estimation of cerebral infract volume and brain swelling

After neurological examination, rat brain was perfused with normal saline by transcardiac perfusion and isolated in chilled conditions. Six coronal sections (2 mm thick) were taken from the region beginning 1 mm from the frontal pole and ending just rostral to the corticocerebellar junction and were transferred at $-20°C$. The brain slices obtained were incubated in TTC (0.5% in 0.1M PBS) at 37°C for 30 min. TTC stains, viable brain tissue to brick red whereas unstained brain tissue i.e. infarcted portion of tissue remains unstained and appears as white. The infarct area thus obtained was measured and quantified by image analysis software (NIH image J). Further, infarct volume was calculated by linear integration of the infarct area of each slice multiplied by average thickness of brain section and expressed in mm^3. Brain swelling was calculated according to the following formula [20]:

$$\left(\frac{InfractVolume - IpsilateralUndamagedVolume - ContralateralVolume}{ContralateralVolume} \right) \times 100$$

Biochemical Analysis

To perform biochemical analysis we measured nitrite, malondialdehyde and calcium influx, 20 min and 60 min post ischemia respectively in cortex and striatal regions of ipsilateral rat brain. The above brain parts of the model were quickly

removed by decapitation after cervical dislocation and homogenized (5:1 v/w) in ice cold 0.1M phosphate buffer, pH 7.4. The tissue homogenate thus obtained was used for estimation as per protocol given below.

Measurement of Nitrite

Nitrite and nitrate are generally the markers for nitric oxide (NO) production in a damaged cell or tissue. The nitrite levels were estimated in affected brain regions using Griess reaction [21]. Briefly, the tissue homogenate was centrifuged at 1600 g for 15 minutes at 4°C and the supernatant thus obtained was deproteinized by mixing with an equal amount of 4% sulfosalicylic acid. Further 350 µl of this reaction mixture was made to react with 350 µl of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1- naphtyl) ethylenediamine in distilled water) and incubated for 10 minutes in dark at room temperature. The absorbance of the assay samples was measured at 540 nm in spectrophotometer (Perkin Elmer, Germany). Nitrite concentrations were calculated using a calibration curve prepared from sodium nitrite and expressed as pmole/mg of protein in tissue homogenate [60].

Estimation of Malondialdehyde (MDA)

The MDA is a by-product of lipid peroxidation and is a biomarker of membrane damage. It was determined based on its reaction with thiobarbituric acid (TBA). Briefly, the tissue homogenate (500 µl), 30% trichloroacetic acid (300 µl), 5M HCl (150 µl) and 2% (w/v) TBA in 0.5M NaOH (300 µl) were thoroughly mixed up. After each addition, the total volume was made 3 ml with distilled water. The above mixture was heated in a water bath at 80°C for 20 min and centrifuged at 4000 g for 10 min. The resultant pink chromogen formed was measured at 532 nm in spectrophotometer. MDA concentration in brain was determined using standard curve and values expressed as nmole/mg tissue protein in homogenate [22].

Estimation of Ca^{2+} influx

The levels of Ca^{2+} in cortical and striatal region of all groups were estimated by using Fura 2AM with minor modification of previously mentioned method of Pokorski et al.(1999) [23]. The tissue homogenate in sample buffer pH 7.4 (5.3 mM KH_2PO_4; 137 mM NaCl, 4.2 mM $CaCl_2$; 0.1% BSA) incubated with Fura 2 AM for 1 h at 37°C. Later, the fluorescence was measured at excitation wavelengths of 340–380 nm, with an emission wavelength at 510 nm using Spectrofluorimeter. The ratio between the fluorescent intensities of Fura 2-Ca^{2+} complex and the unchealated Fura 2(F340/F380) reflected the Ca^{2+} concentration.

Molecular Studies

On the basis past studies which reported maximum and stable AQP4 expression 24 hr post ischemia, we performed molecular studies 1/24 hr reperfusion injury [24–26].

Isolation of Total RNA

Expression of the gene for AQP4 in the striatum and cortex were studied by semi-quantitative RT–PCR using a MJ Research Thermal Cycler. Total RNA was isolated using TRI reagent (Sigma) according to its user manual and dissolved in DEPC-treated water. DNase I (DNA free ambion) digestion of total RNA was done according to manufacturer's guideline to remove any DNA contamination. The RNA was quantified by spectrophotometry.

Reverse Transcription

The first strand of cDNA was synthesized from 2.0 µg of total RNA, by using random hexamer primer and MuLV reverse transcriptase (Revert AidTM H Minus, 200 units, MBI Fermentas). Briefly, 2.0 µg of RNA was mixed with 200 ng of random hexamer in sterile water in the reaction volume of 11 µl. It was incubated at 70°C for 5 min and chilled on ice. After that the following components were added in the order indicated: 5× Reaction buffer 4.0 µl, 10 mM dNTP mix 2.0 µl RNase inhibitor (human placenta) 0.5 µl (20 units) Deionized water 1.5 µl. The tube was incubated for 5 min at 25°C and 1.0 µl of MMuLV reverse transcriptase (RevertAidTM H Minus, 200 units) was added. Further, the tube was incubated for 10 min at 25°C and then at 42°C for 1 h. The reaction was terminated by heating at 70°C for 10 min, and after chilling on ice the tube was stored at −70°C or directly used for the PCR reaction.

Polymerase Chain Reaction

Expression of AQP4 and β-actin was assessed by polymerase chain reaction (PCR) using the following gene-specific primers: 5′GGAAGGCTAGGTTGGTGACTTC3′ and 5′TGGTGACT-CCCAATCCTCCAAC 3′ for AQP4; 5′ATCGTGGGCCGC-TCTAGGCACC 3′ and 5′CTCTTTGATGTCACGCAC-GATTTC 3′ for β-actin [26]. The PCR reactions were carried out in a 25 µl reaction mixture containing 2 µl cDNA, 10× Taq polymerase buffer with $MgCl_2$, 0.2 mM of each dNTP (MBI Fermentas, USA), 1.0 unit of Taq DNA polymerase (Banglore Genei, India), and 10 pmol of appropriate primers. Reactions were performed in a Mini- CyclerTM thermal cycler (MJ Research). The samples were denatured at 94°C for 5 min and amplified using the following amplification parameters: AQP4 (denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, elongation at 72°C for 45 s and number of cycles 29) β-actin (denaturation at 94°C for 30 s, primer annealing at 57°C for 30 s, elongation at 72°C for 30 s and number of cycles 26). The number of cycles used for PCR was chosen to be within the exponential phase of amplification. All PCR experiments included negative controls, in which template cDNA was omitted. The PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide. The resulting gel bands were visualized in a UV-transilluminator and photographed by Nikon digital camera. The relative amount of the AQP4 was expressed as optical density relative to that of the β-actin.

Tissue Lysate Preparation

Both cortex and striatum were homogenized in 50 mM Tris–Cl, (pH 7.4), containing 0.2% triton X-100, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF,5 mM benzamidine, 2 mM b-ME, and protease inhibitor cocktail (Sigma–Aldrich). Protein was estimated by Bradford method using BSA as standard [27].

SDS–Polyacrylamide Gel Electrophoresis

For Western blotting, 50 µg of crude sample was denatured in 5× Laemelli gel loading buffer (100 mM Tris–Cl (pH 6.8), 2% sodium dodecyl sulfate, 2% b-ME, 20% glycerol and 0.2% bromophenol blue) in boiling water bath for 5 min. The samples were cooled on ice and loaded on 10% SDS–polyacrylamide gel along with prestained protein marker (MBI Fermentas). Electrophoresis was carried out in gel running buffer containing 250 mM glycine, 25 mM Tris, and 0.1% SDS. For proper stacking, the samples were run at 15 mA in stacking gel and resolved at 30 mA in resolving gel.

Immunoblotting

After electrophoresis, proteins were transferred onto the PVDF membrane (Millopore) overnight at 4°C with constant power supply of 50 V. After transfer, the membrane was blocked in 5% nonfat milk in phosphate buffered saline (PBS) (pH 7.4) for 4 h at room temperature. The blot was then incubated with rabbit polyclonal anti- AQP4 (1:1,000 dilution, Santa Cruz) or rabbit monoclonal anti-β-actin (1:20,000, Sigma-Aldrich) in 5% nonfat milk and 0.05% Tween- 20 in PBS (pH 7.4) overnight at 4°C. After three washes with PBST (PBS+0.05% Tween-20) for 5 min each, the blot was incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000 dilution, Bangalore Genei) in PBS (pH 7.4) containing 5% nonfat milk and 0.05% Tween 20 for 4 h at room temperature. After washing with PBST (pH 7.4), immunoreactive proteins were revealed with ECL super signal west pico kit (Pierce Biotechnology) in X-ray film, and their expression level was measured by densitometry. β-actin was used as control for immunoblotting. Band density values were normalized to β-actin.

Protein Estimation

Protein content was estimated by the method of Bradford using bovine serum albumin as standard [27].

Statistical analysis

The mean ± SEM was analyzed by Origin analysis software. All the data were examined by one-way ANOVA followed by Student–Newman–Keuls test. $P<0.05$ and <0.01 was taken as statistically significant. For RT-PCR, the signal intensity of the target band, and for immunoblots, monomeric as well as higher oligomeric bands were measured after normalization with β-actin and expressed as relative densitometric value (RDV).

Results

Dose optimization of Piroxicam

We determined the minimum effective neuroprotective dose of Piroxicam on the basis of reduction in neurological deficit and cerebral infarct volume of rats subjected to 1/24 h of I/R injury. In our studies, 1/24 h I/R injury consistently produced marked infarcts in both cortical and subcortical ipsilateral regions of rat brain as evidenced in TTC stained coronal brain sections (**Figure 2a**). The mean of infarct volume was found to be 230.32±19.32 mm^3 in vehicle rats whereas pretreatment with Piroxicam at 5, 10, 20, 40 mg/Kg i.p. doses produced marked reduction in infarct volume, ranging from 198±32.2, 65.36±25.3, 73.87±26.2, 128.37±29.4 mm^3 respectively ($P \leq 0.05$) (**Figure 2b**). Neurological deficit was analyzed on the basis of neurological scores obtained post 1/24 h of I/R injury in all experimental groups. The vehicle group of rats shows significantly higher neurological deficit as compared to sham group of animals while significant ($P<0.05$) improvement in neurological deficit score was found in Piroxicam pretreated rats at all doses except lowest dose as compared to vehicle group (**Figure 2c**). Thus 10 mg/kg Piroxicam i.p. showed significant ($P<0.01$) improvement in neurological deficit and infarct size, therefore 10 mg/kg Piroxicam was selected as optimum dose for further studies.

Effect of Piroxicam on pre and post treatment on cerebral infarct volume and neurological deficit

In our studies, 1/24 h I/R injury consistently produced marked infarcts in both cortical and subcortical ipsilateral regions of rat brain as evidenced in TTC stained coronal brain sections (**Figure 3a**). The mean of infarct volume was found to be

240.34±21.3 mm^3 in vehicle rats whereas treatment with Piroxicam at 30 min pre, 2 h post and 4 h post produced marked reduction in infarct volume, ranging from 67.78±22.2, 170.37±30.4, 185.36±33.9, mm^3 respectively (**Figure 3b**). Neurological deficit was analyzed on the basis of neurological scores obtained 1/24 h of I/R injury in all experimental groups. The vehicle group of rats shows significantly higher neurological deficit as compared to sham group of animals while significant ($P<0.05$) improvement in neurological deficit score was found in10 mg/kg b.w Piroxicam treated rats at all doses except lowest dose as compared to vehicle group (**Figure 3c**). Thus 30 min pretreatment with Piroxicam i.p. showed significant ($P<0.05$) improvement in neurological deficit and infarct size, therefore 10 mg/kg b.w. Piroxicam was selected as optimum dose for further studies.

One of the major outcomes of ischemia/reperfusion is brain swelling due to increase in brain water content. This may be attributed to differential expression of brain water channels. Brain swelling leads to increase in cranial pressure which further can leads to brain compression. 30 min pretreatment of Piroxicam significantly reduced brain swelling as compared to vehicle (**Figure 4**).

Analysis of Rota rod Test

Rotarod experiment showed a significant decrease in the retention time on the rotating rod in the vehicle and vehicle+Drug administered rats at 10, 15, 20 and 25 revolutions per minute (rpm) when compared to sham. Piroxicam treatment in ischemic rats significantly ($p<0.05$) reversed the retention time near to control at 10, 15,20 and 25 rpm(**Figures 5a,5b and 5c**). Long term outcomes up to 72 hrs were assessed in view to consider the STAIR criteria.

### Effect of Piroxicam on brain nitrite levels

As we know that calcium dependent activation of neuronal nitric oxide synthase (nNOS) has always been associated with early rise in post ischemic nitrite levels in rat brain. Therefore, nitrite levels were measured at 20 minutes post ischemia in ipsilateral cortex and striatal regions of rat brain. In both brain loci the nitrite levels were found to be significantly higher ($P<0.05$) in the vehicle group as compared to sham group of animals while Piroxicam pretreatment significantly ($P<0.05$) attenuated the increase in nitrite levels in both striatal and cortical regions of ischemic rat brain (**Figure 6a**).

Effect of Piroxicam on brain MDA levels

MDA is a marker of lipid peroxidation, therefore the MDA levels were measured post 1 h of ischemia in cortex and striatal regions of rat brain. MDA levels in brain were significantly ($P<0.05$) increased in vehicle rats compared to sham rats. The MDA levels in vehicle rats were found to be 5.48±0.32 & 5.58±0.82 nmol/mg proteins whereas 10 mg/kg b.w Piroxicam i.p. treated rats were 3.32±0.52 & 3.46±0.75 nmol/mg proteins, in cortical and striatal regions, respectively (**Figure 6b**). Thus Piroxicam treatment resulted in significant ($P<0.05$) reduction in post ischemic brain MDA levels.

Effect of Piroxicam on Ca^{2+} level

One of the major outcomes of ischemia/reperfusion is Ca^{2+} influx. Interestingly, treatment of Piroxicam (10 mg/kg) inhibited the focal ischemia mediated increase in Ca^{2+} in cortex and striatum. The significant inhibition of calcium was observed in Piroxicam treated group which advocate the calcium channel blocking capacity of Piroxicam ($P \leq 0.05$). This underlies the
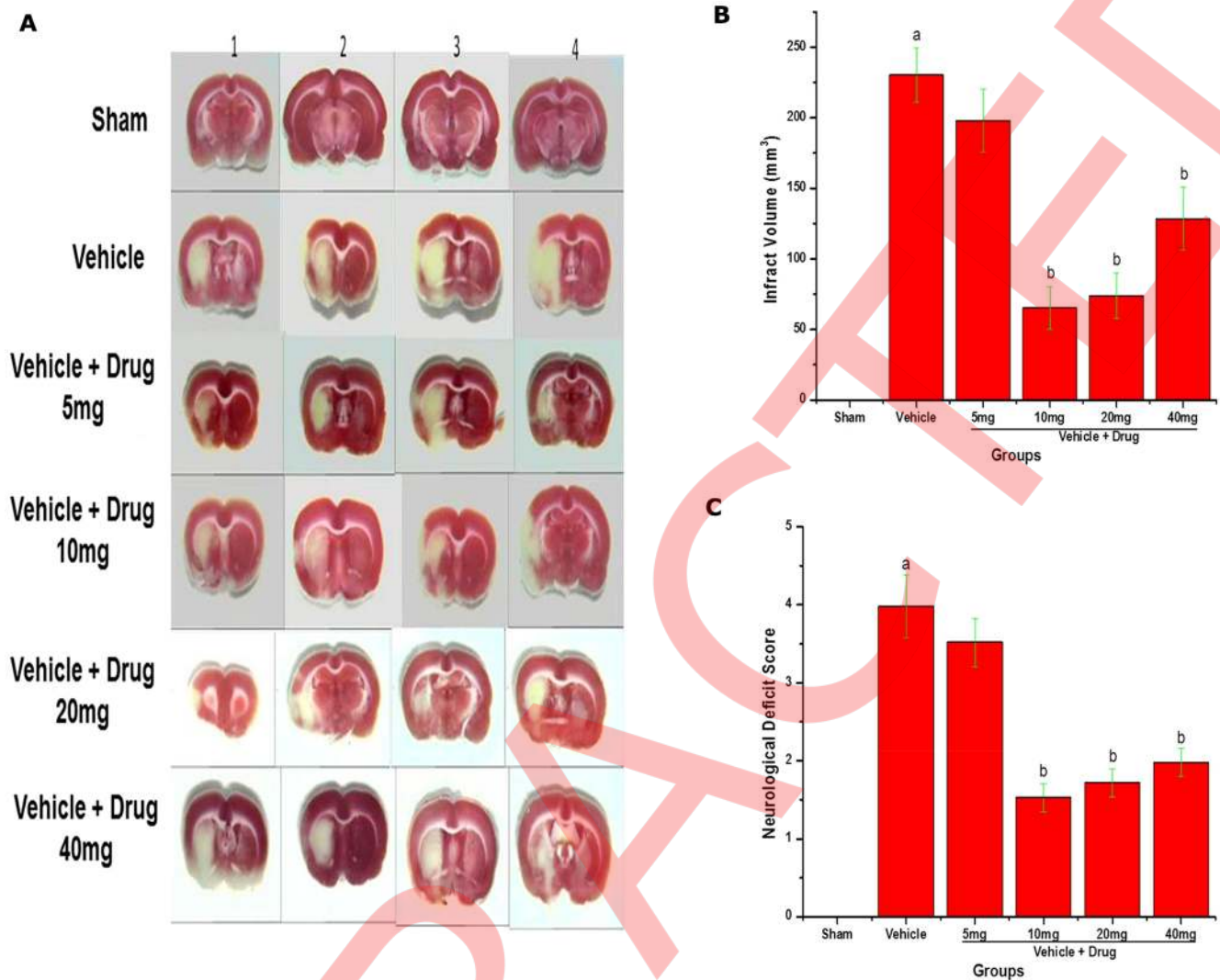
Figure 2. Effect of different doses of Piroxicam pretreatment on (a) TTC stained coronal brain sections of sham, vehicle and Piroxicam treated rats (b) cerebral infarct volume of rats ($P \leq 0.05$)(c) neurological deficit score following I/R injury ($P \leq 0.01$). ([a] vs sham & [b] vs vehicle). The following panels of Figure 2a are excluded from this article's CC-BY license: first and fourth Sham panels; first and fourth Vehicle panels ; first, third, and fourth Vehicle + Drug 5mg panels; first, third, and fourth Vehicle + Drug 10mg panels; third and fourth Vehicle + Drug 20mg panels. See the accompanying retraction notice for more information.
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specificity in reducing the Ca 2+ mediated excitotoxicity (**Figure 6c**).

Semi- quantitative RT-PCR of AQP4

For getting amplification in linear range for semi-quantitative RT–PCR, the number of cycles considered for AQP4 was 29 and for β-actin, it was considered 26 as previously reported by Gupta et al., (2011) [25]. Semi-quantitative RT–PCR of AQP-4 of the cortical and striatal region (**Figure 7a and 7b**) shows that its expression is significantly highest in vehicle ($P \leq 0.05$) as compared to that of sham, and its expression is significantly low in vehicle with drug as compared to that of vehicle ($P \leq 0.05$).

Western Blot Analysis of AQP4

To detect the expression of aquaporin-4 protein, a rabbit polyclonal antibody specific to AQP-4 was used. It detects the expression of 34 kDa monomer AQP-4. Its expression in the vehicle was found to be significantly ($P \leq 0.05$) higher than the sham in both striatal and cortical region of the brain (**Figure 8a**

and 8b). Piroxicam supplemented vehicle cortex and striatum showed significant decrease in AQP4 level and proves the AQP4 inhibitory property of Piroxicam.

Discussion

Neuronal insult following cerebral ischemia results from the overload of calcium within neurons which is contributed due to activation of NMDA receptors followed by oxidative stress, inflammation and apoptosis which subsequently leads to a sequel of biochemical events leading to ischemic insult. The elevated calcium level inside the neurons activate a group of calcium dependent enzymes like neuronal nitric oxide synthase (nNOS), calpain, phospholipase, xanthine oxidase, ligase and DNAses which are involved in generating free radicals and catabolism of proteins, phoshpholipids and nucleic acids. Following reperfusion after ischemic insult there is further mass generation of free radicals. These altered pathological inputs causes membrane alterations and breakdown leading to severe cellular insult [28,29].
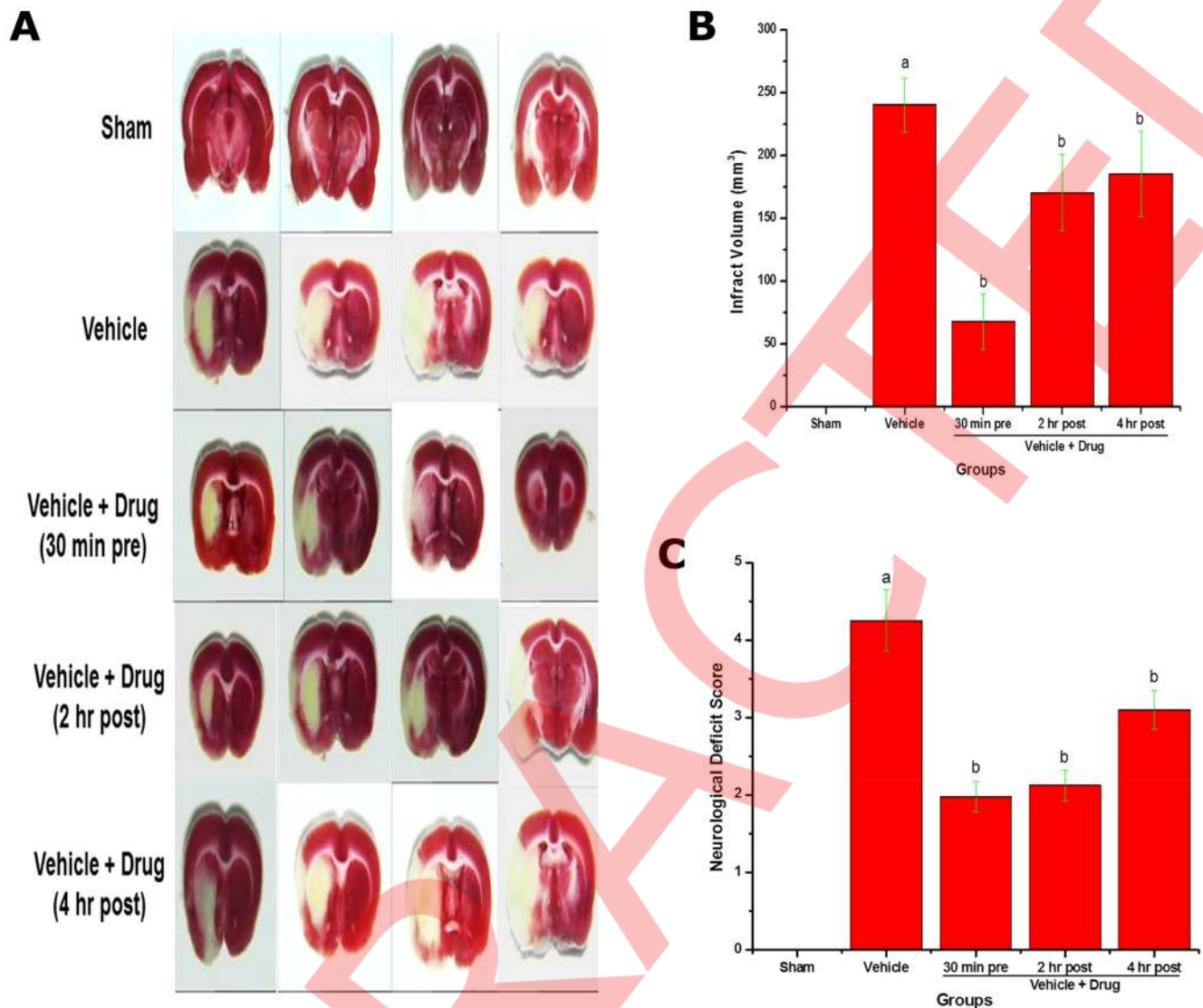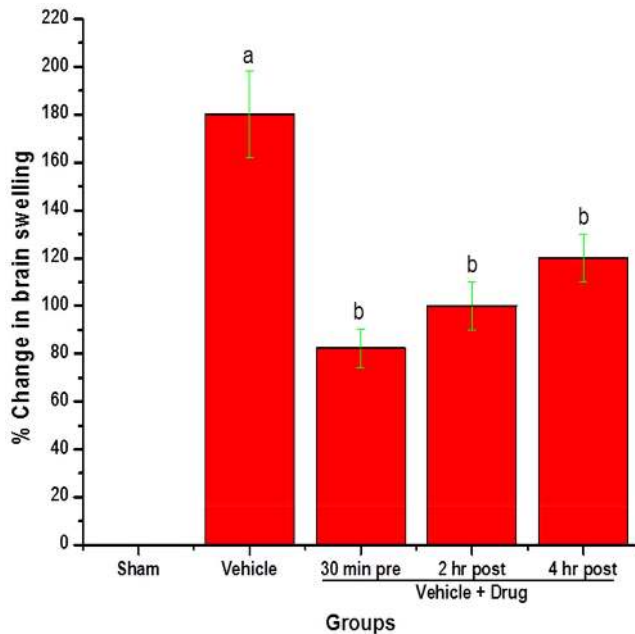
Figure 3. Effect of Piroxicam (10 mg/kg) pre and post treatment on (a) TTC stained coronal brain sections of sham, vehicle and Piroxicam treated rats (b) cerebral infarct volume of rats ($P\leq0.05$) (c) neurological deficit score following I/R injury ($P\leq0.05$). ([a] vs sham & [b] vs vehicle). The following panels in Figure 3a are excluded from this article's CC-BY license: second Sham panel; second, third, and fourth Vehicle panels; third Vehicle + Drug (30 min pre) panel; fourth Vehicle + Drug (2 hr post) panel; third and fourth Vehicle + Drug (4 hr post) panels. See the accompanying retraction notice for more information. **doi:10.1371/journal.pone.0073481.g003**

Numerous research are being conducted in search of a neuroprotective pharmacological composition for cerebral ischemia but none of them could be translated as a promising outcome for an efficient therapy. Eminent researchers and clinicians participated in Stroke Therapy Academic Industrial Roundtable (STAIR) unanimously suggested that due to complex and multifactorial pathology of cerebral ischemia, agents with mixed pharmacology or sequential staging of therapy by a combination of different mechanistic approaches might hold good for stroke therapeutics [30].

Neuronal injury promoted by the cyclooxygenase -2 (COX-2) isoforms are being studied in the rat model of cerebral ischemia since long and administration of COX-2 inhibitor has produced good results in context to reduction in the infarct volume [30,31]. The anti-inflammatory property of most NSAID's, are due to inhibition of cyclooxygenase (COX) mediated prostaglandins synthesis, particularly prostaglandin E2, which plays a major role in inflammation and nociception [31–34].Moreover, COX

independent actions of NSAIDs are also reported, such as flurbiprofen R12 enantiomer doesn't inhibit COX although possess potent antinociceptive and anti-inflammatory effect. Later these effects were found to be mediated by inhibition of NFkB and AP-1 activation [35].

Owing to such, we selected Piroxicam, a NSAID, as a molecule of choice for our study. In past, the neuroprotective role of Piroxicam has already been established in vitro besides its antioxidant and anti-inflammatory property, which is a common characteristics of all NSAIDs [32,33]. Studies by Nakagomi et al reported piroxicam and flurbiprofen, at their higher doses, ameliorated delayed neuronal death in the hippocampal CA sector after forebrain ischemia and also can affect hyperlocomotion [36].However, no study has been undertaken to explore its role in modulating AQP4 water channel and reducing AQP4 mediated brain edema. Thus, we have tried to look upon this aspect besides other neuroprotective properties.

Figure 4. Effect of Piroxicam pre and post treatment on brain swelling ($P \leq 0.05$). ([a] vs sham & [b] vs vehicle).
doi:10.1371/journal.pone.0073481.g004

Our studies involved, use of focal cerebral ischemia rat model and analysis of neuroprotective role of Piroxicam and its effect on the downstream AQP4 expression dependent survival and death mechanisms in striatal and cortical regions of ischemic brain. The reason behind selecting these regions lies in the observation that following MCA occlusion cells in the striatum die immediately [36,37]. It is expected that by administration of Piroxicam, the cell death in these areas should be slow or prevented. Treatment with Piroxicam offered significant neuroprotection at a dose of 10 mg/kg b.w. was found to be optimal in providing neuroprotection as exhibited by the reduction in neurological deficit score, cerebral infarct volume and improvement in motor function. Therefore, it seems that Piroxicam is modulating the mechanism of neuronal damage, reduces inflammation and thus helping in reducing the progression of neuronal infarction.

Owing to ischemic insult in brain there is a rise in intra neuronal calcium which triggers calcium calmodulin dependent kinase and protein kinase C regulated neuronal nitric oxide synthase (nNOS). Activation of nNOS generates nitric oxide (NO) which is a physiological mediator of vasodilation by elevating level of cGMP in vascular smooth muscles. At high concentrations during cerebral ischemia NO causes neuronal insult by combining with the superoxide anion to form peroxynitrite anion, which further degrade to give rise to toxic hydroxyl and nitrogen dioxide radicals [38]. In our study, NO produced following focal ischemia/reperfusion injury was quantified by measuring nitrite which is been a stable end product of NO [39]. A rise in the nitrite level is being evident in the region of ischemic insult of brain after 10 minutes following cerebral ischemia and declined to basal levels after 60 min [40]. A significant decrease in NO levels due to administration of Piroxicam could be associated with decreased calcium influx resulting from ASIC1a inhibition as reported previously by us on the basis of in-silico studies [14,15], thus aiding towards neuroprotection.

MDA is a biomarker for lipid peroxidation and its quantification elucidates the extent of neuronal membrane damage, which

has a high lipid composition. The lipid peroxidation cascade in a post ischemic brain sample is a result of combined action of NO and free radicals generated due to the activation of nNOS and phospholipase A2 [41].Hence, we have looked into the status of lipid peroxidation, which is evident from increased MDA levels in the ischemic rat brain immediately to post reperfusion [42]. Piroxicam administration mediated decrease in MDA level suggests the inhibition of calcium influx involved in activation of nNOS and phospholipase A2 as well.

Cerebral ischemia has been reported to be accompanied by cognitive, behavioural hormonal and locomotor abnormalities [43]. This is due to the destruction of pyramidal neurons of the hippocampal CA1 region are essential for cognitive functions such as spatial learning and memory. Although, ischemia is a powerful stimulus to enhance cell proliferation in the sub-granular zone, leading to an increase of newborn granule cells in the dentate gyrus [44–47]. But do the newly generated neurons have any substantial role in restoring ischemia-induced behavioral impairments remains to be explored. Rotarod test has been used to examine the motor incoordination [44]. Although, we have no substantial evidence to justify the role of Piroxicam in cognition, however we have hypothesized that Piroxicam may influence cognition by targeting cellular mechanics [14,15]. To evaluate motor coordination, the rotarod experiment was performed which demonstrated impairment of the motor function and coordination in the ischemic rats. Rats with ischemia/reperfusion (vehicle group) showed lower fall off time from the rotating rod when compared to sham. The Piroxicam treated ischemic rats showed an improved motor performance in rotarod test compared to vehicle group. Our findings indicate that Piroxicam helps in lowering their time for spatial recognition.

Cerebral edema mechanism extends over days, and thus therapeutic intervention is necessitated to reduce this. Even, the role of water channels towards edema in the brain endothelial cells is still remains unclear. Alternatively, once the blood-brain barrier is disrupted, water can move paracellularly because of the loss of the tight junctions. Astrocytes swelling during ischemia might precipitate early blood-brain barrier disruption. Landmark results published by Verkman and colleagues using aquaporin-4 knockout mice highlighted the idea that aquaporin-4 channels might be an important player in the development of cerebral edema after ischemic stroke.

The experiments done by Verkman and other with AQP4-null mice and suggest AQP4 provides a principal molecular pathway for water permeability in the brain [48,49]. Conclusions were reached in studies using other genetic knockout mice that showed changes in the normal localization and expression of aquaporin-4 channels such as the alpha syntrophin knockout mice. The task of finding an AQP4 blocker has been a difficult one. No NSAID able to inhibit water permeability through AQP4 has been described prior to our work. We hypothesized that an AQP4 water channel blocker would decrease the formation of cerebral edema after ischemic stroke in rat. The purpose of this study was to determine if pharmacological blockers of aquaporin-4 water permeability can be identified, and if these agents will significantly decrease the formation of cerebral edema after ischemic stroke in rat. Here we report chronic changes in AQP4 expression in focal cerebral ischemia by Piroxicam, which might affect edema formation, consequent tissue damage, glial migration and neuronal excitability-all processes critically involved in defining final functional deficits after cerebral ischemia.

We used a widely accepted strategy where first of all we performed molecular docking studies of AQP4 with its natural substrate water and further with Piroxicam. The binding/docking
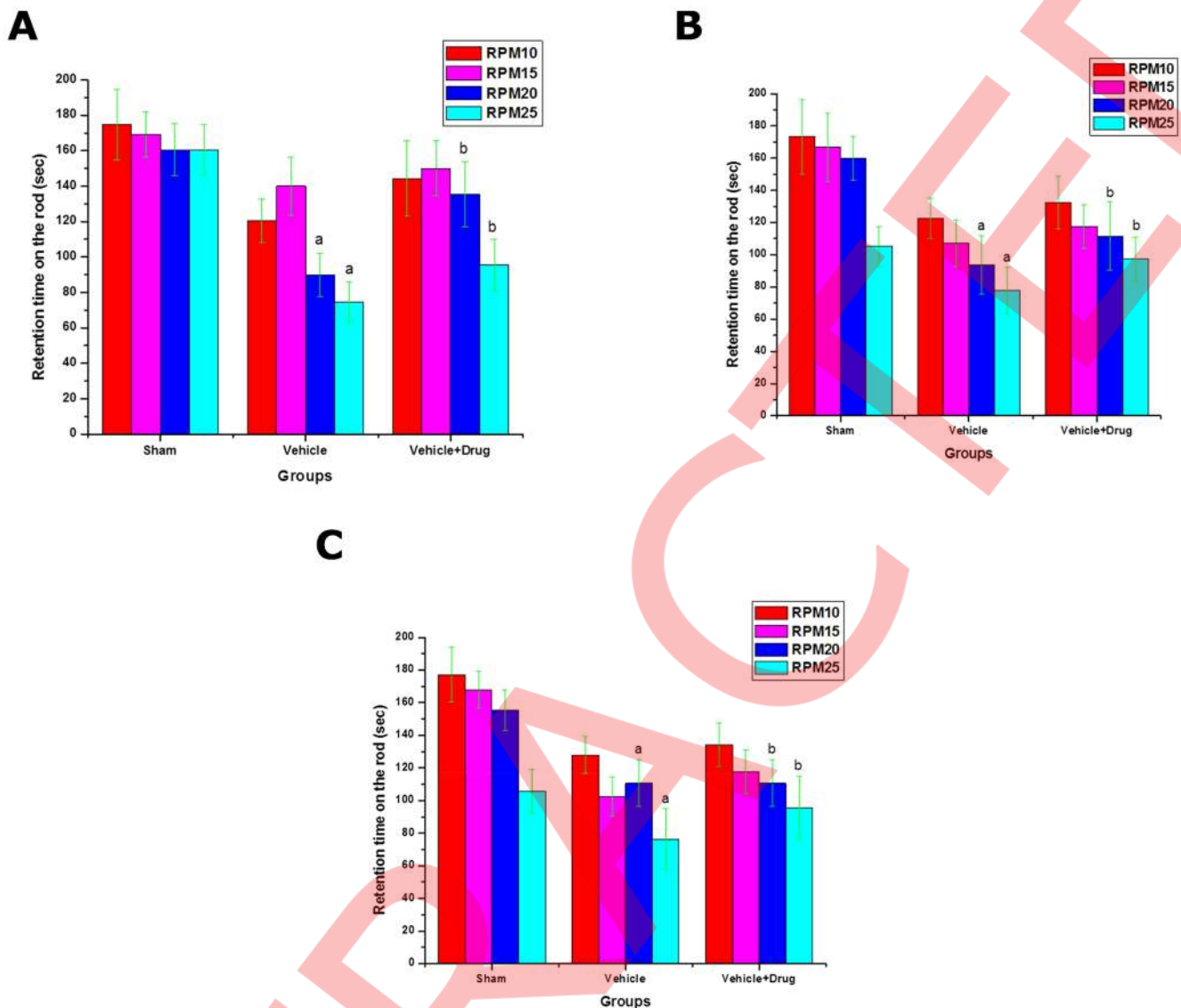
Figure 5. Time spent on metallic rod of control and experimental rats in rota rod experiments after (a) 24 hr (b) 48 hr and (c) 72 hr of MCAo. (^{a} vs sham & ^{b} vs vehicle)($P \leq 0.05$).
doi:10.1371/journal.pone.0073481.g005

energies of both were compared and it was observed that the binding energy of Piroxicam is significantly higher in comparison to its natural substrate [14,15]. Further, on the basis of the previous reported studies which reported maximum and stable AQP4 expression 24 hr post ischemia, we performed molecular studies 1/24 hr reperfusion injury [23,24]. Two brain areas cortex and striatum were considered where we investigated the AQP4 expression. We also considered a study group as Sham+Drug to observe the after effect of Piroxicam in the expression of AQP4 in sham rats. Hence 4 study groups 1) Sham 2) Sham+Drug 3) Vehicle 4) Vehicle+Drug were considered. Our results show that there is significant increase in AQP4 expression in the vehicle group in comparison to both sham and Sham+Drug groups (**Figure 7**). If we look into the comparative outcome of Sham and Sham+Drug we find that Piroxicam does not affect the expression of AQP4 in normal pathology significantly. When the vehicle group administered with Piroxicam seen, AQP4 expression decreases significantly (**Figure 8**). Our result clearly indicates

that Piroxicam downregulates AQP4 expression in focal cerebral ischemia which also justifies the molecular docking results obtained earlier. Further, to explore the comparative AQP4 inhibition property by other NSAID like flurbiprofen whose neuroprotective role is well reported in past [60], we performed a comparative immunoblot. We found that the AQP4 inhibitory property of Piroxicam is optimal in comparison to flurbiprofen. Hence, we conclude that Piroxicam acts on AQP4 mediated brain edema in more effective way than other NSAID (Comparative immunoblot in Information S1).

As detected by Western blot analysis, MCAo-60 min and reperfusion at 24 h caused AQP4 upregulation in rats, however others have also demonstrated changes in AQP4 levels after ischemia using other models. In a model of MCAo-30 min in mice, AQP4 over-expression in the periinfarct zone was not significant at 24 h after reperfusion but was at 48 h. AQP4 expression conversely decreased 23 h after brain transient ischemia 90 min. These varying results may have been from
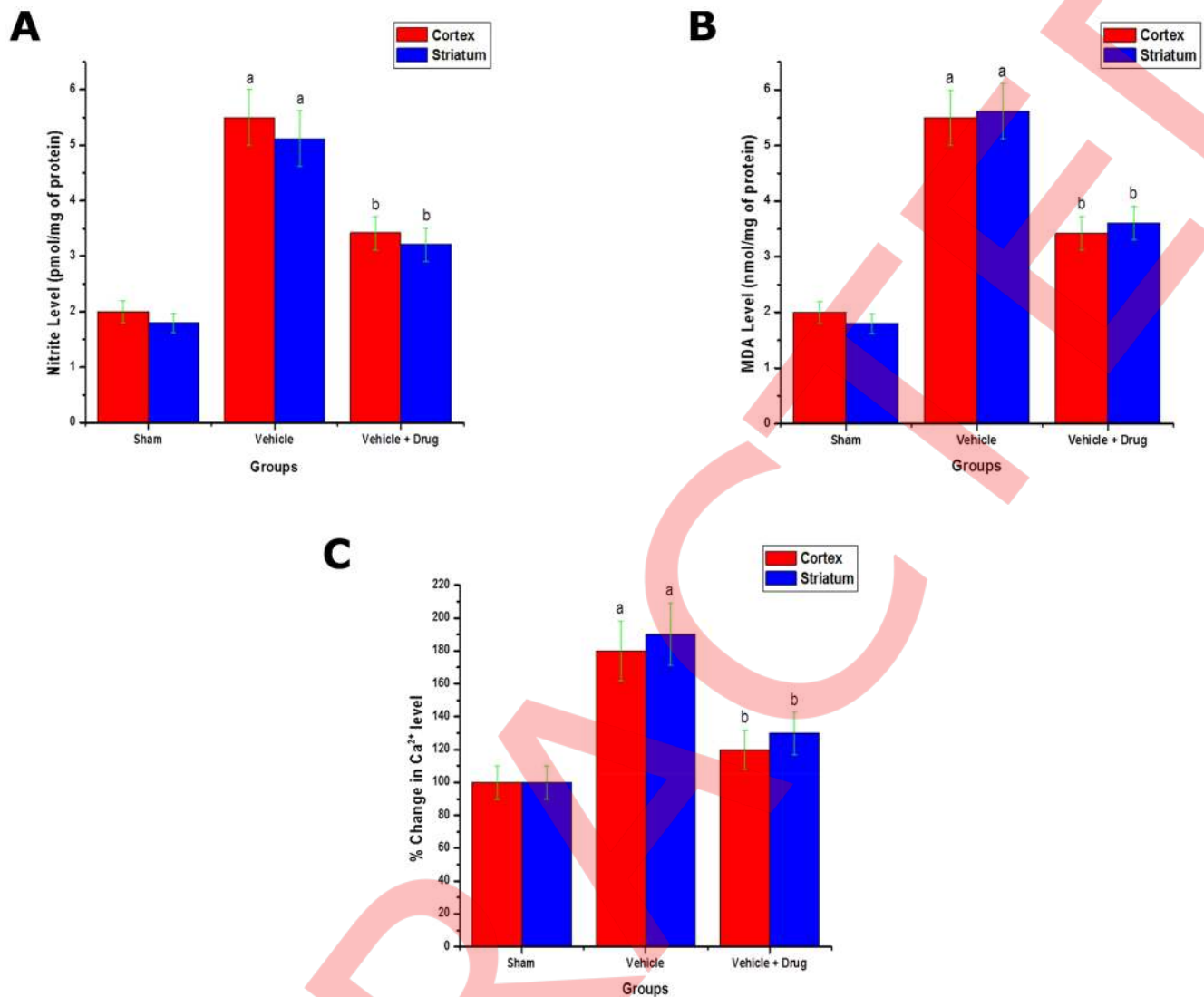
Figure 6. Effect of Piroxicam (10 mg/kg) pretreatment on (a) nitrite ($P \leq 0.05$). (b) MDA levels ($P \leq 0.05$) in cortical and striatal region of rat brain following post 20 min of ischemia (c) Ca^{2+} level in cortical and striatal region of rat brain ($P \leq 0.05$). ([a] vs sham & [b] vs vehicle).
doi:10.1371/journal.pone.0073481.g006

differences in species used (mouse versus rat), age of animals studied (young versus adult), or the severity of the ischemia and reperfusion (different duration in ischemia or reperfusion). Meanwhile, we identified AQP4 down-regulation in a rat MCAo model in the Piroxicam group accompanied by an obvious reduction in cerebral edema. According to the classification of cerebral edema based on the pathophysiologic mechanism, cytotoxic edema develops by the time point of reperfusion at 24 h [50–52]. Others have speculated that AQP4 over-expression enhances a rapid influx of water and is responsible for the development of cytotoxic edema after focal brain ischemia thus, AQP4 downregulation may alleviate cerebral edema [53,54].

The mechanism may involve reversible protein phosphorylation and amino acid neurotransmitters. Protein kinase C is one of the potential phosphorylation sites of AQP4, and water permeability activity alters with its phosphorylation [55]. NSAIDs in general are Protein kinase C activators that inhibited ischemia-induced up-regulation of AQP4 and attenuated brain swelling in a MCAo model [56] Therefore, protein kinase C can reasonably be suggested as a possible key location for Piroxicam regulation of

AQP4 expression during brain ischemia-reperfusion injury. Another related factor may be the amino acid neurotransmitter, glutamate. One study showed that astrocytic swelling that glutamate elicited seemed to correlate with AQP4 [57]. Knock-out AQP4 in mice results in altered glutamate levels in the brain. Nevertheless, NSAID could decrease glutamate levels by many possible mechanisms [49]. Glutamate might therefore be another factor in the mechanism by which Piroxicam regulates AQP4 expression. Detectible increase in AQP4 expression after cerebral ischemia could also be related to the translocation of AQP4 to the endfeet with no alteration in overall AQP4 abundance. Either way, during this early phase, BBB dysfunction allowed an important passage of plasma proteins (IgG) and water, while AQP4 upregulation may handle such opening of the BBB and subsequent water influx. Thus, in the early phase AQP4 upregulation might represent an intrinsic protective mechanism of the brain to facilitate more water movement. But at later point of time a limit to brain swelling is also necessitated, thus AQP4 down regulation may bring the desired effect [58,59]. Piroxicam helps in such neuroprotective action by down regulating AQP4
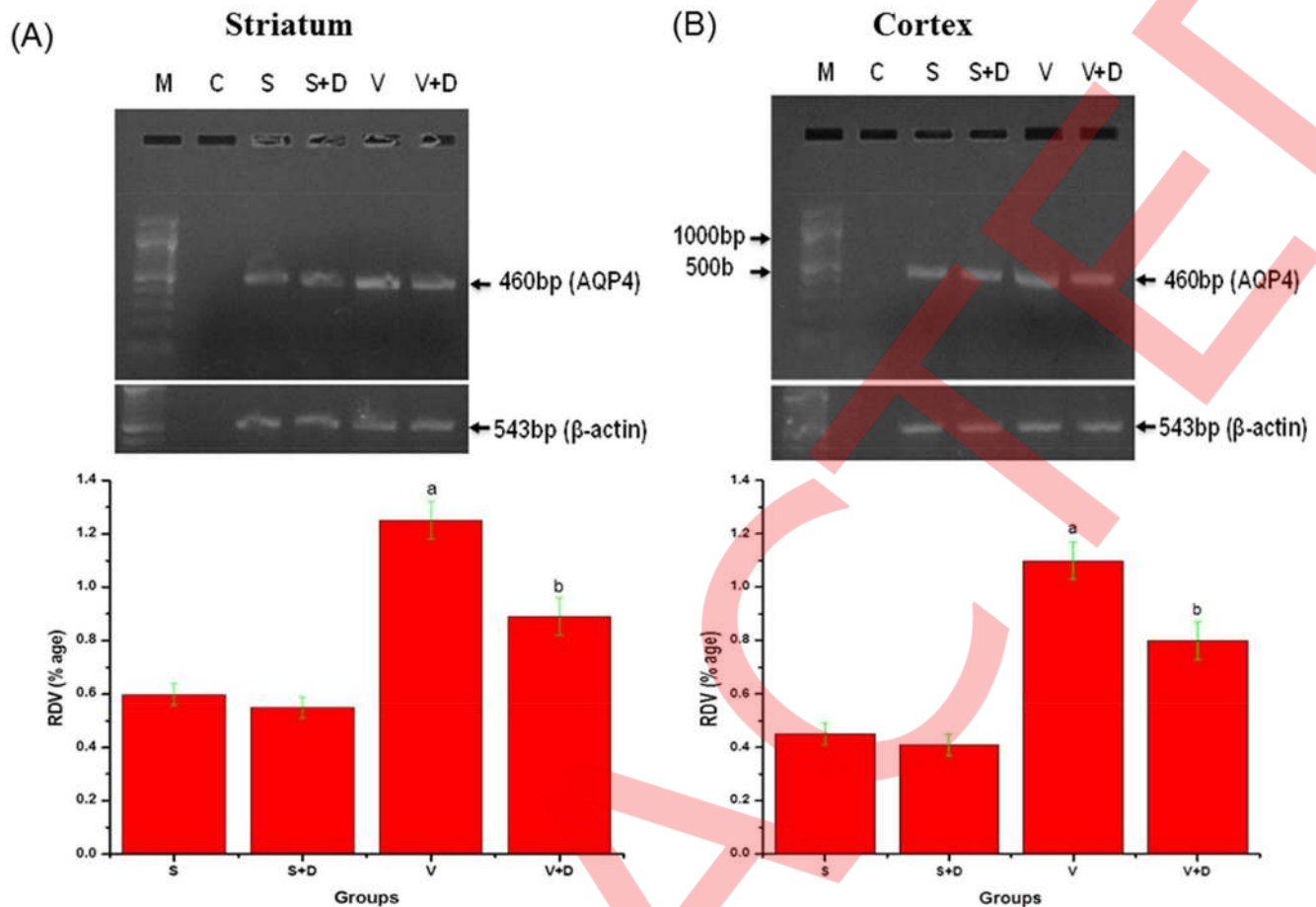
Figure 7. RT–PCR of AQP4 of striatum (a), and of cortex (b) of different set of experimental rats. M denotes marker i.e. DNA ladder. C denotes negative control. S denotes sham groups, S+D denote sham treated with drug, V denotes the vehicle and V+D denote the vehicle treated with drug. Histograms represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments conducted for striatum and cortex. ([a] vs sham & [b] vs vehicle) ($P \leq 0.05$).
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and delaying edema exacerbation. Hence we can infer from our results that Piroxicam effectively inhibits AQP4 expression which subsequently can inhibit brain edema formation and render neuroprotection in rat model of focal cerebral ischemia.

On the basis of our present study, we concluded that Piroxicam improved neurological function, decrease cerebral infarction volume and brain water content, and played a neuroprotective role by regulating expression of AQP4 following by ischemic reperfusion injury. Thus the finding of present studies provide significant evidence that Piroxicam acts as potent AQP4 regulator and gives neuroprotection in MCAo rat model of focal cerebral ischemia. Additionally, post ischemic neuroprotective action of Piroxicam enhances its curative and clinical potential although other mechanisms like inhibition of COX and NF-kB activity may also partly contribute towards neuroprotection as observed with other NSAIDs.

The future directions of this work will be to test and identify novel Piroxicam derivatives able to modulate AQP4 channels as well as co transporters responsible for water movement and that have increased water solubility and membrane permeant characteristics. Our main objective will be to induce neuroprotection and resolution of brain edema and to maintain the normal balance of AQP4 in the brain rather than to either block or to stimulate their action for good therapeutic strategies. Once this novel compound

is identified, it would be necessary to test its protective effect in the formation of cerebral edema after ischemic stroke in rat. If we identify a water soluble and membrane per meant compound that has protective effects in cerebral edema formation, it would be important to evaluate its effects in other transporters (e.g., the Na^+, K^+, Cl^- co-transporter and organic anion transporters) in order to evaluate if its beneficial protection is directly associated with the aquaporin-4 channels. In addition, lately, it would be interesting to evaluate its pharmacological proprieties such as half maximum dose and toxicological effects in animals and humans.

Supporting Information

Information S1 Comparative western blot analysis of AQP4 of cortex in different set of experimental rats. S denotes sham groups, V denotes the vehicle, V+Pir denote the vehicle treated with piroxicam and V+FBP denote the vehicle treated with flurbiprofen. Histograms represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments conducted for cortex ($P \leq 0.05$).([a] vs sham & [b] vs vehicle).
(TIF)
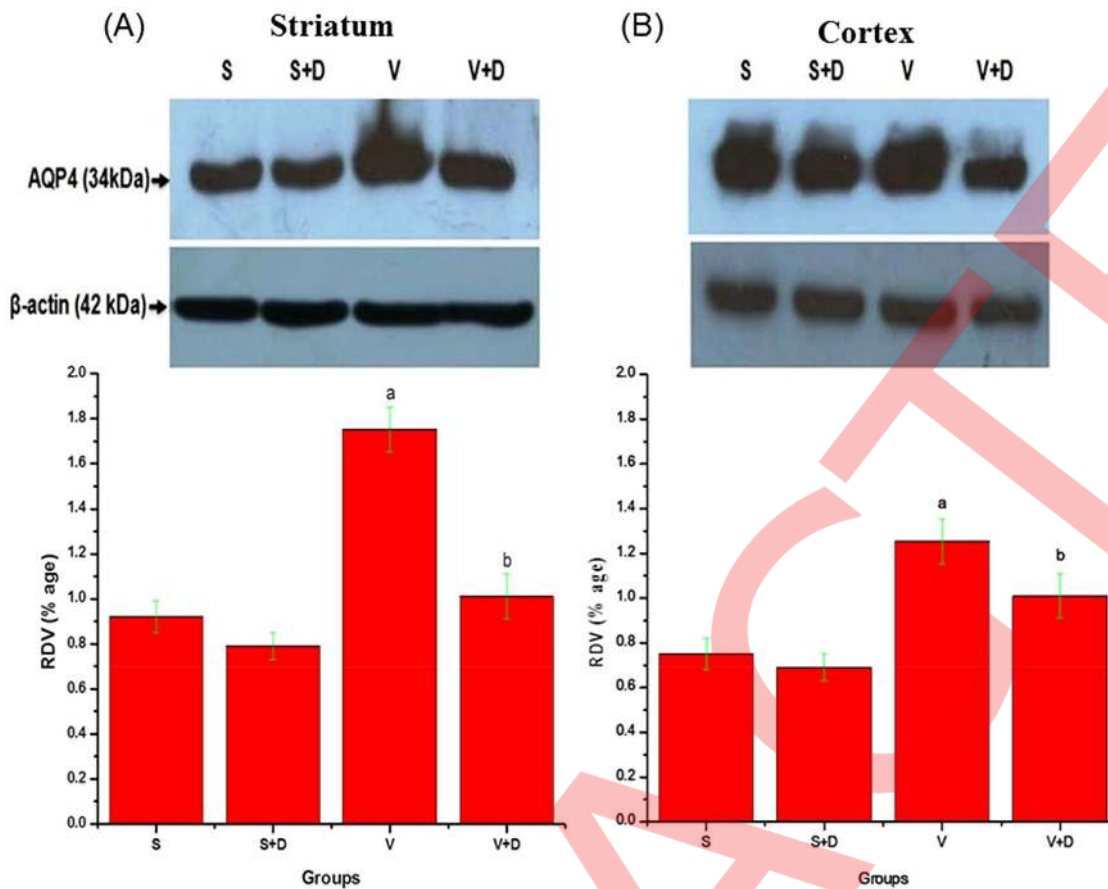
Figure 8. Western blot analysis of AQP4 of striatum (a), and of cortex (b) of different set of experimental rats. S denotes sham groups, S+D denote sham treated with drug, V denotes the vehicle and V+D denote the vehicle treated with drug. Histograms represent cumulative data expressed as mean $\pm$ SEM obtained from three different sets of experiments conducted for striatum and cortex.([a] vs sham & [b] vs vehicle)($P \leq 0.05$).
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Author Contributions

Designed the study: PB AP SP RP. Experiments Conducted: PB AP SP. Result Analysed: PB AP RP DY.

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