1. Introduction

The World Health Organization has reported that around 15 million people suffer from stroke (Hoffmann et al., 2012), which is the leading public health concern for both developing and developed countries. Stroke presents itself with very high mortality and morbidity rates and also is documented with high relapse rates (Doyle et al., 2008; Hong and Saver, 2009). Tissue-plasminogen activator (t-PA), administered within 3 h of onset of stroke, is the currently employed FDA-approved therapy. However, t-PA therapy reportedly increases the risk of haemorrhage and cerebral injury currently employed FDA-approved therapy. However, t-PA therapy reportedly increases the risk of haemorrhage and cerebral injury. Adverse effects of current therapies are imposing the need in development of neuroprotective agents that are very effective. To explore this we experimentally induced ischemic brain injury and investigated the effects of plumbagin. Induction of cerebral infarction and ischemia-reperfusion (I/R) was done by middle cerebral artery occlusion (MCAO) in Sprague-Dawley rats. Plumbagin (50, 100 or 200 mg/kg b. wt) was intragastrically administered for 9 days before ischemia induction and an hour prior on the day of ischemic insult. Plumbagin treatment attenuated pulmonary edema, neuronal apoptosis and reduced cerebral infarct volume. Cleaved caspase-3 and apoptotic cascade protein expressions were regulated. Overproduction of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and nitric oxide (NO) following I/R were reduced. Prior plumbagin administration had down-regulated NF-κB signalling and MMP-2 and MMP-9 expression. Overall, the results reveal the potent neuroprotective efficacy of plumbagin against I/R-induced brain injury via effectively modulating apoptotic pathways, MMPs and neuro-inflammation cascades.

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illustrated the elevated levels of cytokines following sustained activation of NF-κB following 24 h of reperfusion following ischemia (Jin et al., 2015). Studies have also illustrated that matrix metalloproteinases (MMPs) exert major roles in ischemia/reperfusion induced brain injury (Rosell and Lo, 2008). MMPs as MMP-2, alloproteinases (MMPs) exert major roles in ischemia/reperfusion of cerebral ischemia in animal experimental models (Chang et al., 2003). Further, TNF-α and IL-1β have been reported to induce the production of MMPs (Gottschall and Yu, 1995; Kauppinen and Swanson, 2005; Crocker et al., 2006). Thus targeting inhibition of the NF-κB and MMPs could be beneficial in ischemia/ reperfusion-induced brain injury.

Recent researches have been focused on the neuroprotective effects of plant-derived compounds in stroke and ischemic injury (Tu et al., 2014; Lv et al., 2015; Antonisamy et al., 2015; Balamurugan, 2015; Rathi et al., 2015). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a naphthoquinone derivative identified from the roots of Plumbago zeylanica (Sakamoto et al., 2009) has been demonstrated to possess extensive bioactive properties such as antioxidant (Tilak et al., 2004), anti-microbial (De Paiva et al., 2003); anti-cancer (Kawamura et al., 2014), anti-inflammatroy (Checker et al., 2010) and neuroprotective (Luo et al., 2010) effects. Here, neuroprotective efficacy of plumbagin following ischemia/reperfusion-induced brain injury was investigated.

2. Materials and methods

2.1. Animals

Sprague-Dawley male rats that weighed 280–300 g were got from laboratory animal house of the University and were retained in sterile acrylic cages and monitored carefully under standard laboratory conditions (12 h: 12 h day/night cycle, 21–22 °C, humidity 55–60%). For 4–5 days prior experiment schedule, the animals were acclimatized to the new in-house conditions. The animals were fed on regular pellet diet and water at ad libitum. Experimental protocols were approved by the Animal Studies Committee of the University. Animal handling in this study were conducted in compliance with the guidelines of the National Animal Welfare Law of China and international guidelines for the care of laboratory animals (Garber, 2011).

2.2. Chemicals and antibodies

Plumbagin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Primary antibodies against MMP-2, MMP-9, cleaved caspase-3, Bcl-2, Bax, β-actin, Bad, Bcl-xL were procured from Abcam, USA. TNF-α, NF-κBp65, IκBα, p-IκBα, p-IKKβ, IKKβ, and horseradish peroxidase-labeled IgG secondary antibodies were procured from Cell Signaling Technology Inc. (Danvers, MA, USA). ELISA kits for determination of cytokines - TNF-α, IL-1β and IL-6 were obtained from Biolegend (San Diego, CA, USA). Cell lysis buffer for Western blotting analysis was purchased from Beyoime Institute of Biotechnology (Beijing, China). All the other chemicals used for analysis were from Sigma–Aldrich, if otherwise are specified.

2.3. Study design

The rats were allocated as 5 separate groups randomly (n = 18/group). Plumbagin was dissolved in saline and was administered intragastrically to rats at dose of 50, 100 or 200 mg/kg b.wt for 9 days prior ischemia/reperfusion induction. On the day of ischemic insult, a single dose of plumbagin was given 1 h prior induction of ischemia.

The rats were fasted overnight prior ischemic insult, however, had free access to water. The following day, animals were anesthetized by administering zoletil (Virbac Laboratories, Carros, France) at 25 mg/kg b.wt; i.p. The induction of cerebral infarction and ischemia/reperfusion (I/R) was performed by middle cerebral artery occlusion (MCAO) method as previously described by Leonardo et al. (2010). In brief, the left common carotid arteries (CCA) were open through a ventral midline neck incision. The internal carotid artery (ICA) was isolated and its extra cranial branch was ligated adjacent to its origin. Ischemia was induced with a 3–0 nylon monofilament suture interleaved via external carotid artery (ECA) into ICA in order to block MCA. The filament was maintained in place for 120 min. The suture was then withdrawn to reinstate the ICA-MCA blood flow. Reperfusion was allowed for about 24 h. Body temperature of the animals was kept constant at 37 °C using a thermostatically controlled heating pad until the animals recovered from surgery.

2.4. Animal grouping

- Group 1 - Control (normal saline, (2 mL) intra–gracically);
- Group 2 - MCAO model; Group 3 - Plumbagin (50 mg/kg) + MCAO; Group 4 - Plumbagin (100 mg/kg) + MCAO; Group 5 – Plumbagin (200 mg/kg) + MCAO.

Rats that were administered equal volume of normal saline intragastrically instead of plumbagin and not subjected to MCAO, served as control. Rats that received saline and subjected to MCAO served as MCAO model. The rats from each experimental group were sacrificed following 24 h following reperfusion by transcardial perfusion of ice-cold saline followed by paraformaldehyde (4%) in 0.1 M phosphate buffer. Blood samples were collected and the brain tissues were removed immediate for analysis.

2.5. Neurobehavioral deficit evaluation

After reperfusion for 24 h the rats were evaluated for any neurobehavioral deficits. The assessment was based on - spontaneous activity, balance, symmetry of movements, symmetry of forelimbs, climbing ability, muscular co-ordination, reaction to touch, and response to vibrissae touch. The animals were scored on a five point scale as follows: 0 (no deficit) -normal; 1- mild deficit; 2- moderate deficit; 3-severe deficit; and 4- serious deficit (Longa et al., 1989).

2.6. Determination of levels of cytokines and NO

Inflammatory cytokines - TNF-α, IL-1β and IL-6 in the plasma were determined using ELISA kits (Biolegend). Levels of NO were assessed using NO assay kit (ab65328, Abcam), where utilizing nitrate reductase nitrate are converted to nitrates that gets converted to a deep purple azo compound with Griess Reagent. The intensity of the chromophore accurately reflects nitric oxide levels were measured at 540 nm using a 96-well microplate reader (Spectra MAX 340PC, Molecular Devices). The absorbance values were further analyzed using the software (Softmax Pro). The amount of NO was calculated using sodium nitrite (0–150 μM) as standard.

2.7. TTC staining

The brains were excised immediately after sacrifice and the wet weight was measured. 2,3,5-triphenyltetrazoliumchloride (TTC) staining was performed to assess brain viability and to determine the infarct size. The brain tissues were sectioned (2 mm sections) and were incubated with TTC for 30 min (at 37 °C). The tissue
sections were then immersed in 4% paraformaldehyde in dark (overnight). The infarct area was determined using NIH Image J software (Version 1.42; National Institutes of Health, Bethesda, MD). Normal areas of the brain stain with TTC, while the infarct areas remain unstained. Infarct volume was calculated as follows: - adding the infarct area of every section x thickness of the sections. The results are presented as percentage infarction/ipsilateral hemisphere.

The brain samples (n = 6) were dried in an oven for 24 h (100 ºC) to obtain the dry weight and the water content was derived (wt weight - dry weight)/wt weight × 100%.

2.8. TUNEL analysis

Neuronal apoptosis following I/R injury was assessed using Terminal transferase-mediated dUTP nick end-labeling (TUNEL) staining using DeadEnd TM fluorometric TUNEL system kit (Promega, Madison, WI, USA) as per manufacturer's protocol. The paraformaldehyde brain tissue sections (5-µm) were subjected to analysis and the TUNEL positive cells were visualised and analyzed using NIS-Elements BR imaging processing and analysis software (Nikon Corporation, Japan).

2.9. Immunoblotting

The brain hemispheres (n = 6) following I/R were homogenized on ice using cell lysis buffer and were centrifuged at 4 ºC for 15,000 rpm for 10 min. The total protein concentration of the supernatant was determined using Bicinchoninic acid (BCA) protein assay kit from Bio-Rad (Hercules, CA, USA). Further, to assess NF-κB (p65) expression both in the cytosolic and nuclear fractions, an aliquot of the homogenate was separated into fractions using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL, USA) according to the instructions specified by the manufacturer. Protein samples (50 µg) (from NF-κB (p65) from both the fractions/group) from different experimental groups were electrophoretically separated on SDS-PAGE (2%) and electro transferred on to polyvinylidene difluoride (PVDF) membranes (Invitrogen). The membrane was blocked (2 h at room temperature) with 5% BSA (Fetal Bovine Serum Albumin) in Tris-buffered saline containing Tween-20, 0.1% (TBST). The membranes were then incubated with respective primary antibodies, overnight at 4 ºC. Following which, the blots were washed with TBST and further incubated with HRP-conjugated secondary antibodies (2 h; room temperature). The immunoreactive bands were visualized and scanned using Image Master II scanner (GE Healthcare, Milwaukee, WI, USA). The band densities were further analyzed by ImageQuant TL software (GE Healthcare, Milwaukee, WI, USA). The test protein expression levels were normalized with that of β-actin.

2.10. Statistical analysis

The data observed are expressed as mean ± SD derived from 6 individual experiments. Using SPSS software (version 21.0, SPSS Inc., Chicago, IL) all statistical analyses were performed. Multiple group comparisons were performed using ANOVA (one-way analysis of variance) followed Duncan’s Multiple Range Test (DMRT). Values at p < 0.05 were considered statistically significant.

3. Results

3.1. Plumbagin attenuated neurobehavioral deficits following MCAO

The effects of plumbagin on response and behavior of rats were examined. Prior 1 h of induction of cerebral ischemia, the rats scored normal for behavioral responses and neuromuscular coordination. Following MCAO significant (p < 0.05) neurological deficits were noticed (Fig. 1). The animals hesitated to walk and the left limbs were observed to exhibit considerably less flexibility and movement. The rats also exhibited difficulty to respond to stimuli. The neurological deficit was found to be lower substantially in rats that were treated with plumbagin as compared to MCAO controls. Plumbagin treatment markedly improved behavioral responses and motor coordination. Further 200 mg plumbagin treatment was more effective than 50 and 100 mg doses.

3.2. Plumbagin significantly reduced infarct area

TTC staining was performed to determine the extent of infarction following MCAO. The observations revealed the ischemic sections as unstained regions majorly in the fronto-parietal cortex area and in the striatum (Fig. 2). Rats that were prior treated with plumbagin for 10 days exhibited considerably (p < 0.05) lesser infarct volume as compared to MCAO controls. Also plumbagin decreased brain water content as well in a dose-dependent manner. The infarct volume decreased from 29.88 to 10.2 with treatment with 100 mg and 200 mg plumbagin, illustrating its protective effects.

3.3. Plumbagin inhibit neuron apoptosis following Ischemia/ reperfusion injury

To further explore the neuroprotective effects of plumbagin TUNEL assay was performed. The results indicated that pretreatment with plumbagin (50, 100 or 200 mg) significantly (p < 0.05) inhibited ischemia-induced neuronal cell death (Fig. 3a). Further, western blot analysis revealed enhanced cleaved caspase-3 expression along with elevated (p < 0.05) levels of Bax and Bad 24 h following I/R (Fig. 3b and c). Bcl-2 and Bcl-xL expressions were observed to be substantially (p < 0.05) inhibited indicating activation of apoptotic cascade. Interestingly, pretreatment with plumbagin down-regulated cleaved caspase-3, Bax and Bad expression, while it elevated levels of Bcl-xL and Bcl-2. In line with TUNEL positive counts, the results of protein expression also illustrated significant neuroprotective effects of plumbagin against ischemia injury.
Inflammatory responses have been well documented in I/R injury. NF-κB signalling is a key pathway in inflammatory responses (Fan et al., 2001; Tu et al., 2014). Following MACO, we noticed significantly (p < 0.05) increased NF-κB (p65) nuclear expression levels with substantial decrease in the NF-κB (p65) cytosolic fractions, indicating activation of the NF-κB pathway. Also, significantly (p < 0.05) up-regulated expression of TNF-α, IKKα, p-IKKα, IKKβ, p-IKKβ, IκBα and p-IκBα were noticed (Fig. 4) following 24 h of reperfusion. Prior administration of plumbagin caused marked (p < 0.05) inhibition of p- IKKα, p- IKKβ and IκBα expression vs MCAO alone group. The expression of total IKKα, IKKβ and IκBα though reduced were not significantly down-regulated. Further suppression of TNF-α and NF-κB p65 (nuclear fraction) expression were noticed on plumbagin treatment in comparison with MCAO control. These observations indicate down-regulation of the NF-κB signalling on plumbagin treatment.

**3.6. Plumbagin potentially reduced expressions of matrix metalloproteinases -2 and 9**

The MMPs have been implicated in neuro inflammatory responses and play a vital role in I/R -induced brain injury. Here demonstrated to be involved in ischemia/ reperfusion - induced brain injury (Lv et al., 2015). NO is also a major inflammatory mediator. Multi-fold (p < 0.05) rise in the levels of plasma NO, IL-1β, IL-6 and TNF-α were observed in ischemia/reperfusion-induced rats as compared against normal controls (Fig. 5). Plumbagin at all the tested doses significantly (p < 0.05) reduced NO and cytokines in a dose-dependent manner.

**3.5. Plumbagin reduced levels of inflammatory cytokines**

NF-κB signalling pathway crucially regulates the expressions pro-inflammatory cytokines as IL-1β, IL-6 and TNF-α. These cytokines activate inflammatory response cascades and have been

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**Fig. 2.** Plumbagin reduced cerebral infarct area (A) brain edema following I/R injury (B). Values are represented as mean ± SD, n = 6. *p < 0.05 as determined by one-way ANOVA followed by DMRT analysis. † represents p < 0.05 vs control; ‡ represents p < 0.05 vs MCAO control; ‡‡ represents MCAO control vs plumbagin (200 mg) + MCAO at p < 0.05. (a)–(e) represents mean values from different experimental groups that differ from each other at p < 0.05.

**Fig. 3.** Plumbagin decreases neuronal apoptosis and regulates apoptotic pathway proteins (A and C). Expression of pro-apoptotic proteins (B and C). Values are represented as mean ± SD, n = 6. *p < 0.05 as determined by one-way ANOVA followed by DMRT analysis. † represents p < 0.05 vs control; ‡ represents p < 0.05 vs MCAO control; ‡‡ represents MCAO control vs plumbagin (200 mg) + MCAO at p < 0.05. (a)–(e) represents mean values from different experimental groups that differ from each other at p < 0.05. (L1-Control; L2-MCAO control; L3-Plumbagin (50 mg/kg) + MCAO; L4-Plumbagin (100 mg/kg) + MCAO; L5-Plumbagin (200 mg/kg) + MCAO).
we determined the expression of MMP-2 and -9 after MCAO. I/R injury induced a multi-fold (p < 0.05) up-regulated expression of MMPs -2 and -9 (Fig. 4). Plumbagin (50, 100 or 200 mg) administration prior ischemic insult caused significant (p < 0.05) downregulation of MMP expression (Fig. 6), with 100 or 200 mg dose exhibiting more marked suppression than 50 mg.

4. Discussion

Cerebral ischemia-induced brain injury has complex underlying pathophysiological mechanisms such as oxidative stress, inflammatory reaction, and neuronal apoptosis (Moskowitz et al., 2010). tPA therapy in stroke has limitations owing to its neurotoxic effects (Guan et al., 2013). Thus the need for development of clinically effective neuroprotective agents is high. In the present study the effects of plumbagin on experimentally-induced rodent model of cerebral infarction was explored.

Prior treatment with plumbagin (50, 100 or 200 mg) exerted substantial protective effects against I/R injury, as seen by a marked decrease in infarct area and apoptotic cell counts. Brain edema was considerably reduced as well. Plumbagin administration further improved neuromuscular coordination. Apoptosis after I/R is regarded as one of the key mechanisms that lead to neuronal cell death (Yao et al., 2012). Western blott analysis revealed significantly enhanced cleaved caspase-3 expression along with Bad and Bax proteins, suggesting activation of apoptotic pathways. The balance between pro-apoptotic proteins (Bad and Bax) and Bcl-2 and Bcl-xL, the anti-apoptotic proteins, crucially regulates the mitochondrial membrane integrity and the release of cytochrome C and apoptogenic factors that influence cell survival and cell death (Zhao et al., 2003). Interestingly, plumbagin treatment markedly
Plumbagin reduced MMP-2 and -9 expression values are represented as mean ± SD, n = 6. * p < 0.05 as determined by one-way ANOVA followed by DMRT analysis. Relative expression of proteins from different treatment groups with control expressions set at 100%. # represents MCAO control vs plumbagin (200 mg) control, (a)–(e) represents mean values for the same cytokine from different experimental groups that differ from each other at p < 0.05. (L1-Control; L2-MCAO control; L3-Plumbagin (50 mg/kg) + MCAO; L4-Plumbagin (100 mg/kg) + MCAO; L5-Plumbagin (200 mg/kg) + MCAO).

Fig. 6. Plumbagin reduced MMP-2 and -9 expression values are represented as mean ± SD, n = 6. * p < 0.05 as determined by one-way ANOVA followed by DMRT analysis. Relative expression of proteins from different treatment groups with control expressions set at 100%. # represents MCAO control vs plumbagin (200 mg) control, (a)–(e) represents mean values for the same cytokine from different experimental groups that differ from each other at p < 0.05. (L1-Control; L2-MCAO control; L3-Plumbagin (50 mg/kg) + MCAO; L4-Plumbagin (100 mg/kg) + MCAO; L5-Plumbagin (200 mg/kg) + MCAO).

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