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Measurement of Inflammatory Mediators at Different Time Intervals after Neuronal Injury Induced by Bilateral Common Carotid Artery Occlusion Model.

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Abstract

The purpose of the study was to assess the influence of ischemia reperfusion injury on various inflammatory mediators that participate in cerebral damage. Analysing the time dependent change in the inflammatory markers will help in therapeutics as well as to understand the interrelationship. Bilateral common carotid arteries (2-VO; BCCAO) were occluded in mice and the expression of the inflammatory mediators were studied at different time points after reperfusion injury (I/R) (2 hr, 6 hr, 12hr, 24 hr, 72hr, and 7day). Behavioural studies after I/R in BCCAO mice showed reduced locomotor activity till 7th day and in rota rod test functional recovery was observed. Gene expression studies for inflammatory mediators (NF-κB, iNOS, COX2, TNF-α, IL-1β and IL-6) were done by semi quantitative PCR. Results confirmed early elevation of iNOS and COX 2 at 2hr and peaked at 24hr and 6hr respectively. The expression level of TNF-α and IL-1β was found to be peak at 24 and 72hr respectively. IL6 levels were found to be unaltered up to 72hr. The NF-κB level was found to be at peak at 12hr. Western blot analysis revealed that cytosolic fraction of NF-κB protein was found to be maximum at 24hr. The nuclear NF-κB level was found to be elevated from 2hr and lasted till 72hr. The study can be concluded that time dependent changes in the expression of cytokines and different inflammatory markers. This study might help the selection of drugs to treat cerebral ischemia.

Keywords: Cerebral ischemia, 2-VO, NF-κB, iNOS, COX2, TNF-α, IL-1β

1. INTRODUCTION

Cerebral ischemic injury has high incidence of severe morbidity in surviving victims and unbearable burden both psychologically and economically. Ischemic stroke is the most common type of stroke (85% of cases) caused by a transient or permanent occlusion of a cerebral artery most often by a thrombus or an embolus. During ischemic stroke blood flow to the brain is reduced which leads to production of oxidative stress, activation of inflammatory events, and the neuronal cell death [1]. Several animal models have been developed to resemble the human global cerebral ischemia. Among them, in global ischemic/reperfusion injury, induced with 2-VO, will reduce the blood flow throughout the brain and produce marked injury to hippocampus, neocortex, and striatum neurons [2]. Reperfusion injury is associated with multiple pathways such as inflammatory, apopotic and prosurvival processes. Immense evidences state that oxidative stress and neuroinflammation play pivotal roles in the progression of cerebral ischemia injury. Inducible NOS (iNOS) is perhaps the most relevant to inflammation and its expression is limited almost exclusively to immune cells. Activation of microglia will release cytokines, astrocytes, which participates in the complex linking of nuclear signaling pathways such as NF-κB that can ultimately regulate the inflammatory mediators. This will lead to loss of neuronal functions and cell death due to apoptotic as well as necrotic pathways [3].Activation of NF-κB in neurons during cerebral ischemia can cause acute phase of inflammatory responses and potentiate ischemic injury [4] by activating genes involved in the pathogenesis of cerebral ischemia. NF-κB activates iNOS, COX-2, interleukin (IL-1β), tumor necrosis factor (TNF-α) and IL-6. The COX-2 expression or the levels of PGs have been extensively investigated in different cerebral ischemia animal models [5]. It is also evidence that TNF-α and IL-1β has both neurotoxic and neuroprotective roles after ischemic stroke in rats and in mice [6]. IL-6 has been demonstrated to be crucial for neuron survival in culture and serves a key function in the regeneration of peripheral nerve cells [7]. Previous study has shown iNOS levels were elevated in early phase i.e., with in 12hrs of reperfusion and COX 2 was found persistent till 15 days in ischemic stroke [8]. The inflammatory mediators with respect to NF-κB are poorly studied based on their time dependent expression. Hence, in the present study, the time course expression profile of inflammatory genes in bilateral common carotid artery occlusion reperfusion injury (I/R) model has been studied.

2. METHODS AND MATERIALS

2.1 Essential Chemicals and Reagents

Enhanced avian RT-PCR Reaction kit (Sigma aldrich, USA), PCR primers (Sigma, India), PCR tubes and desired plastic wares (Tarsons, INDIA), PMSF (Sigma, USA) , ECL plus Western detection kit (Amersham Pharmacia Biotech, USA), Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ), antibody phospho-p65(NFκβ), antibody β-actin, antibody Histone (Santa Cruz Biotech Inc, USA).

2.2 Animals

All animal protocols were approved by Institutional Animal ethical committee, PSG IMS&R. swiss albino male mice, 8-10 weeks old and weighing about 20-25 grams were obtained from from the central animal house facility at,

PSGIMS&R, Coimbatore and were housed at 25 ± 1 °C with 12 h light and 12 h dark cycle; water and food were available ad libitum. The rats were randomly divided into 7groups (n = 8 each): a sham-operated group, and 6 operated groups were divided as per time points at 2 hr, 6 hr, 12hr, 24 hr, 72hr and 7th day after I/R respectively

2.3 Bilateral common carotid artery occlusion (BCCAO)

Mice (25-30g) were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg). A midline incision in the neck region was made and a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea, both the common carotid arteries were exposed [9]. Then arteries were isolated and occluded both common carotid arteries with aneurysm clips for 15 min. After the cessation of blood flow in the bilateral artery was visually confirmed. Ischemic duration was measured from the application of the last clip to the left common carotid artery. After 15 minutes, both clips were removed, followed by reperfusion of blood flow was confirmed in every case by direct inspection of each artery under a microscope. After sutured, antibiotics were applied to the mice and returned to their home cage [10]. Post BCCAO, Brain was dissected at different time points such as 2, 6, 12, 24, 72 hr and 7th day for PCR and western blot analysis.

2.4 Behavioral studies

Locomotor activity: The locomotor activity was studied by actophotometer, mice were individually placed in behavioral chambers and the basal activity score of all the animals were recorded for 5 mins. Locomotor activity was calculated using the number of beams broken by the animals after placement in the chamber.

Rota rod test: Rota rod is used to evaluate motor coordination by testing the ability of mice to remain on a revolving rod. The rate of rotation of the rod was adjusted such that the normal mouse was able to stay on the rotating rod for a period of five minutes. The difference in the fall off time from the rotating rod between the control and treated animals is taken as an index of motor in coordination. The mice that are able to stay on the rotating rod for a period of five minutes before global cerebral ischemia were selected and the test was again performed 24 hr, 72 hr and 7th day after global cerebral ischemia and reperfusion [11].

2.5 Gene expression studies using semi quantitative PCR

Total RNA was isolated by Trizol method as per manufacturer protocol.The pellet was washed once with 75% ethanol, centrifuged again for 5 min, air-dried and dissolved in 50 µl DEPC treated water. Total RNA was stored at -80ºC. 1µg of RNA was reverse-transcribed (RT) using Enhanced Avian HS RT-PCR Kit (Sigma Aldrich,USA). After initial denaturation for 10 min at 95 °C, thirty five amplification cycles were performed for iNOS (30s of 95 °C denaturation, 1min of 56 °C annealing and 30s 72 °C extension), COX-2 (15s of 95 °C denaturation, 1 min of 60 °C annealing and extension), NFκB (15s of 95 °C denaturation, 1 min of 60 °C annealing and extension), β-actin (15s of 95 °C denaturation, 1 min of 60 °C annealing), TNF-α(30s of 95 °C denaturation, 1 min of 60 °C annealing and extension), IL1-β (15s of 95 °C denaturation, 1 min of 60 °C annealing and extension),IL6 (15s of 95 °C denaturation, 1 min of 60 °C annealing and extension) followed by a final extension of 72°C for 5min.The primer sequences used are listed below.

Table 1.The primer sequences

Gene	Forward (5′-3′)	Reverse (5′-3′)	Amplicon size
β- Actin	GGCTGTATTCC CCTCCATCG	CCAGTTGGTAAC AATGCCATGT	154
iNOS	GCAAGCTGATG GTCAAGATCCA GAG	GCTGTGTGTCAC AGAAGTCTCGA ACTC	236
COX-2	TGCTGTACAAG CAGTGGCAA	AGCCATTTCCTT CTCTCCTGT	139
NF-κB	CTTCCGAATTT GGCGTCCTTC	GGGGACAGCGA CACCTTTTA	219
TNF-α	CCTGTAGCCCA CGTCGTAG	GGGAGTAGACA AGGTACAACCC	148
IL-1β	GAAATGCCACC TTTTGACAGTG	TGGATGCTCTCA TCAGGACAG	116
IL6	CACAAGTCCGG AGAGGAGAC	TGCCATTTGCAC AACTCTTTTCT	135

2.6 Subcellular Fractionation and Western Blotting

To obtain subcellular fractions, we used the protocol of Nada and Madineni et al., [12][13]. Briefly, brain cortices were dissected outweighed and homogenized using ice cold lysis buffer [250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1.5 MgCl2, 10 mM KCl, 20 mM HEPES (pH 7.5), 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 10 mM sodium orthovanadate, 20 mM sodium pyrophosphate and protease inhibitor cocktail. Cell homogenates were kept on ice for 15 min and then centrifuged at 1000×g for 15min to sediment nuclear pellets. Nuclear pellets were resuspended in NET buffer [20 mM HEPES pH 7.9, 20 % glycerol, 0.5 M NaCl, 1.5 mM MgCl2, 1 % Triton-X-100, 1 mM DTT, 0.1 mM PMSF, 50mM sodium fluoride, sodium orthovanadate, 20 mM sodium pyrophosphate and protease inhibitor cocktail(sigma Aldrich,USA). Protein concentration was determined by Bio-Rad Bradford reagent (Bio-Rad Laboratories, Hercules, CA), and protein samples were analyzed by loading equivalent amounts of total cytoplasmic and nuclear proteins (40µg) onto 10 % SDS-polyacrylamide gels. Proteins were transferred from the gel to PVDF membrane (Biorad,USA) and blocked with 5 % dry nonfat milk and/or 3 % BSA for 1 h at RT followed by overnight incubation at 4 °C with the following antibody. Rabbit NFkB p65 (1:1000)

2.6 Statistical Analysis

The experimental results are expressed as the mean ± SD. ANOVA test was used to compare the control group to different treatment groups. The statistical calculations were performed by using graph pad prism software version 5, statistical significance was considered at ($p < 0.05$).

3. RESULTS

3.1 Effect of I/R on behavior in BCCAO mice

Locomotor activity

The locomotor activity of ischemic mice is given in Figure.1.A. The ischemic mice exhibited decreased locomotor activity in comparison to control mice at 24 and 72hr ($p<0.001$). On 7th day the activity was found to be increased but was not equivalent to control mice activity.

Rota rod test

The muscle grip strength of ischemic mice was evaluated by rotarod experiment and the data is represented in Figure1.B. In comparison to control mice ischemic mice have shown poor muscle grip strength as noted by shorten holding time of the rotating rod. The muscle grip strength was found to be reduced at 24hr ($p<0.001$) and 72hr ($p<0.05$). On 7th day, the muscle grip strength was gained in comparison to 24 and 72hr.

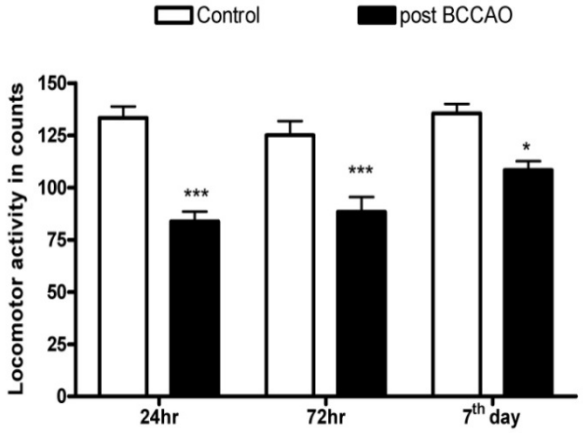


Fig 1A. Locomotor activity

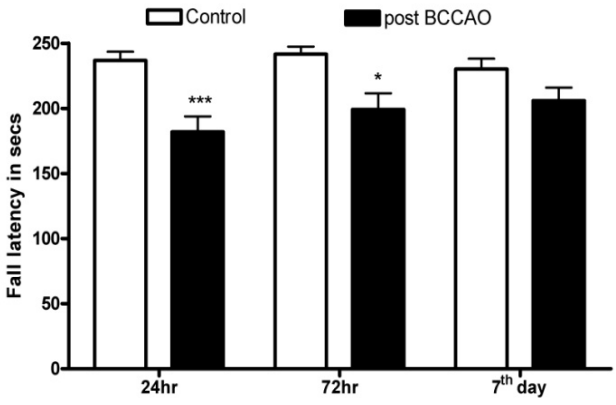


Fig 1B. Rotarod test

Fig.1. Effect of I/R in Global cerebral ischemia mouse model on A) Locomotor activity B) Rotarod test. All data expressed as mean± SD and statistical significance *, **, *** denotes $p<0.05$, $p<0.01$ as compared to control.

3.2. Effect of I/R on iNOS mRNA expression in BCCAO mice

After global ischemia for 15 min, the iNOS level was found to be increased significantly at 2 hr ($p<0.01$), and attained the peak concentration at 24 hr ($p<0.001$), thereafter gradually the iNOS level was found to be decreased up to 7th day (Fig.2.A).

3.3. Effect of I/R on COX 2 mRNA expression in BCCAO mice

COX2 expression was found to be increased at 2hr ($p<0.01$) and peaked at 6 hr ($P<0.001$) in ischemic mice. At 24hr ($p<0.05$) and 72hr ($p<0.05$), the COX2 level was found to be significant higher than the control mice. Further, reduction in COX2 level was found on 7thday decreased in comparison to control mice as well as 72hrs time point (Fig.2.B).

3.4. Effect of I/R on TNF-α mRNA expression in BCCAO mice

The TNF-α mRNA expression was found to be increased after I/R injury at 12 ($p<0.05$) and 24hr ($p<0.01$). At 72hr and on 7th day, TNF-α level was found to be equivalent to control mice (Fig.2.C).

3.5. Effect of I/R on IL-1β mRNA expression in BCCAO mice

The IL1-β mRNA expression was found to be elevated significantly at 6, 24 and 72hrs in ischemic mice as compared to control mice. However the IL-lβ expression was found to be insignificant at 12th hr. On 7th day, IL-1 β mRNA expression was decreased but has not reached to the base line ($p<0.05$) (Fig.2.D).

3.6 Effect of I/R on IL-6 mRNA expression in BCCAO mice

IL-6 expression was found to be unaltered as compared to control mice in all the time intervals studied. On 7th day, IL-6 level was found to be gradually reduced in comparison to control mice but the effect was not significant (Fig.2.E).

3.7 Effect of I/R on NFκB mRNA and protein expression in BCCAO mice

The NFκB mRNA level in the brain after I/R injury at different time points showed an increases trend from 2hr onwards and peaked at 12 hr ($p<0.01$). After 12hr it gradually reduced. At 24hr ($p<0.05$) the level was found to be higher than the control mice (Fig.3.A). Western blot analysis of NFκB expression profile in cytosolic and nuclear levels, after I/R in BCCAO has been done. Cytosolic NFκB level was increased at 2hr ($p<0.05$) and then gradually reduced till 12hr. At 24hr, again the cytosolic NFκB level was found to be elevated in comparison to 12th hr thereafter the NFκB level started declining ($p<0.01$). However at 72hr, the cytosolic NFκB level was found to be significantly higher than the control mice ($p<0.05$). Nuclear NFκB expression was found to activated from 2hr ($p<0.01$) and elevated at 12th hr ($p<0.01$) afterwards it was found to be reduced. At 72hr, the nuclear NFkB level was found to be increased as compared to control mice ($p<0.01$) (Fig.3.B).The NFkB activity was found to be biphasic in both cytosolic and nuclear fraction.

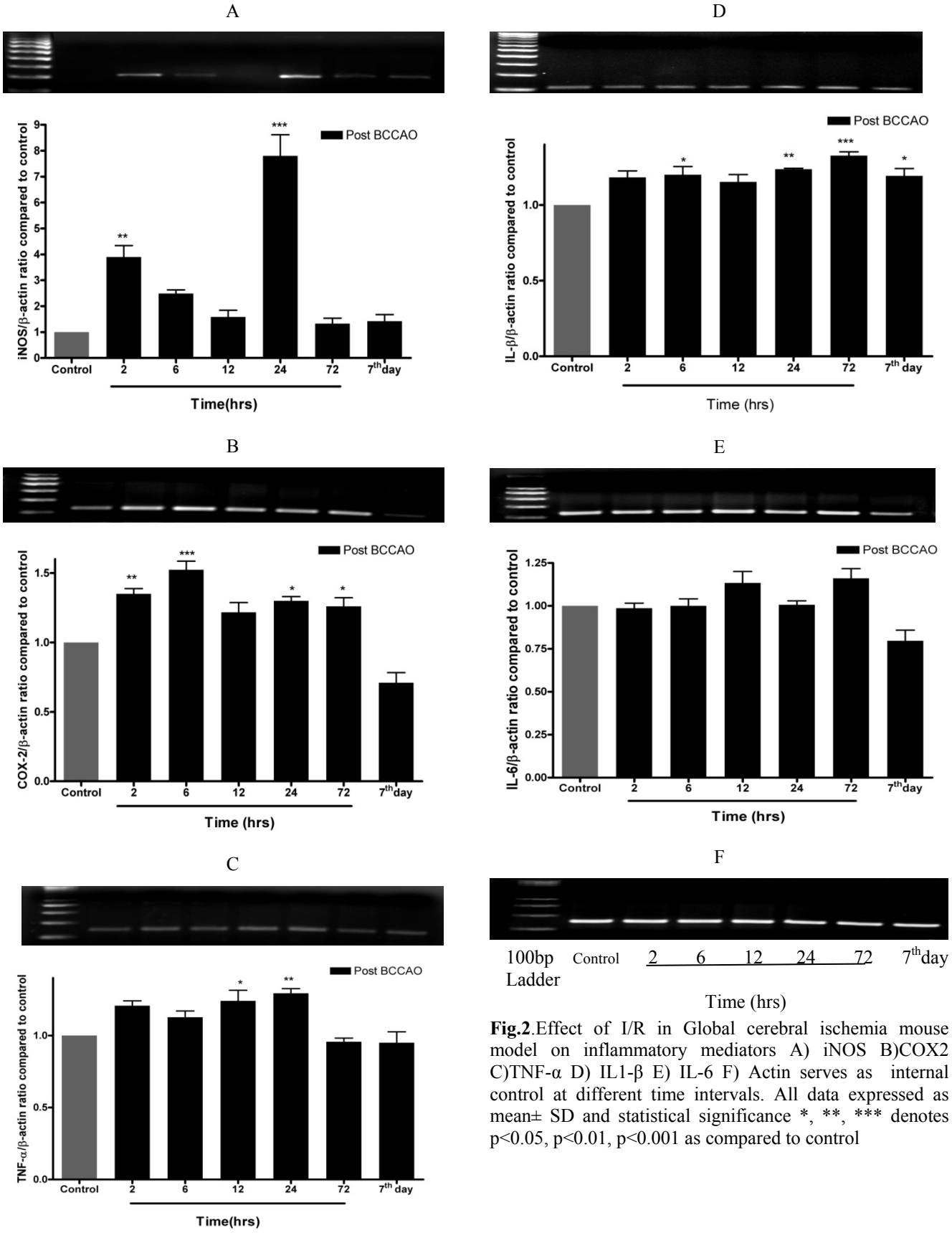
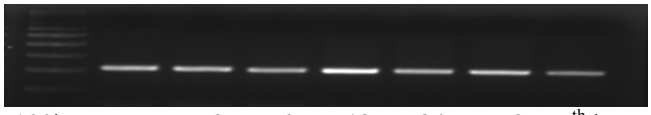


Fig.2.Effect of I/R in Global cerebral ischemia mouse model on inflammatory mediators A) iNOS B)COX2 C)TNF-α D) IL1-β E) IL-6 F) Actin serves as internal control at different time intervals. All data expressed as mean± SD and statistical significance *, **, *** denotes $p<0.05$, $p<0.01$, $p<0.001$ as compared to control

A. Gene expression of NFκB at different time points



100bp Ladder Control 2 6 12 24 72 7th day

Time (hrs)

B. Western blots analysis of NFκB Cytosol fraction

NFkB p65

Actin

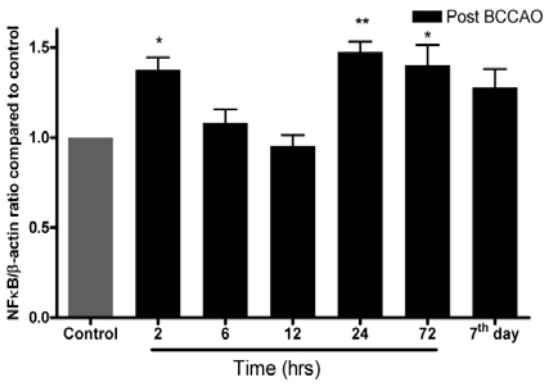
Nuclear fraction

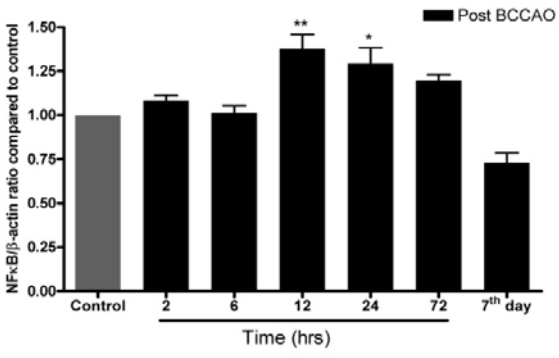
NFkB p65

Histone

Control 2 6 12 24 72 7th day

Time (hrs)





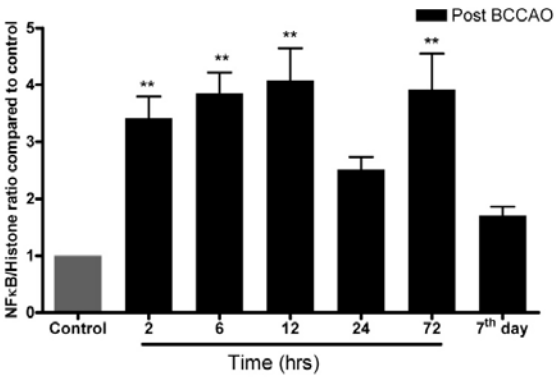


Fig.3.Effect of I/R in Global cerebral ischemia mouse model on A) NFκB mRNA expression B) NFκB protein levels Actin serves as in internal control for cytosolic and Histone serves as internal control for nuclear fraction at different time intervals. All data expressed as mean± SD and statistical significance *, **, *** denotes $p<0.05$, $p<0.01$ as compared to control.

4. DISCUSSION

Global cerebral ischemia has been widely used in experimental studies. Temporarily occluding the bilateral common carotid arteries is sufficient to induce brain injury in the striatum, cerebral cortex and hippocampus. In this study we have demonstrated the time course effect of ischemia reperfusion injury in BCCAO model on various inflammatory mediators like NF-κB, iNOS, COX2, TNF-α, IL-1β and IL-6. The locomotor activity was reduced in ischemic mice shows their hypo-reactivity and emotionality. Inducement of global cerebral ischemia in rats has shown hyper-reactivity at day1 and hypo-reactivity at day 5 suggesting the time dependent effects. The extent of neuronal degeneration occurred and how it is associated to locomotor activity remains to be determined [14]. The rotarod is one of the most frequently used tests of motor function after ischemia in the mouse. In our study mice tested on rotarod had reduced motor coordination but later days they showed a functional recovery. This data is accordance with the previous study that showed functional recovery in rat after focal cerebral ischemia [15]. In normal brain iNOS is not expressed. Inflammatory mediators such as LPS and cytokines induces the expression of iNOS in microglia and astrocytes possibly in neurons leading to continuous production of high levels of NO. NO at low concentrations functions as a signaling molecule and high levels it induces neuronal cell death. Previous study in MCAo rats, have shown expression of iNOS mRNA that began early and returned to baseline at later days [16]. In our study we found early up regulation of iNOS and COX 2 expression in brain cortices and the peak concentration was at early and then reduced in later period of the study. In a recent study, the time-dependent upregulation of COX2 mRNA and the production of COX2 protein in rat hippocampus persisted for 7 days after ischemia [8]. In our previous study we have reported activation of NFκB p65 subunit, in LPS induced RAW 264.7 that resulted in the elevation of pro inflammatory mediator TNF α [17]. In the present study, we found increased NFκB expression at

early time points and then reduced gradually. Cytosolic and nuclear NFκB levels were also found to be elevated. The increase in nuclear NFκB levels indicates the translocation of NFκB which leads to the production of inflammatory mediators. In focal cerebral ischemia mouse model, NF-kB DNA binding activity was observed in the ischemic hemisphere after reperfusion which correlates our data though the induction of ischemia is different [18]. After LPS incubation, the inflammatory mediators iNOS, COX-2, and MyD88 and pro-inflammatory cytokine TNFα, IL1β, and IL6 levels were elevated via phosphorylation of p65 (NFkB) in the IMR-32 cell line [19]. The current study demonstrated that in the reperfusion injury BCCAO model, expression of TNF-α and IL-1β was found to be elevated at later stage. The later increase in cytokines, particularly TNF-α in the hippocampus and striatum reflect the contributions of T-cells adhering to the microvascular endothelium [20]. There have been findings regarding the role of IL-6 in clinical ischemic stroke and experimental ischemia models. IL-6 works as a mediator of inflammatory damage or a neuroprotective agent in cerebral ischemia remains undetermined [21]. However, in present study, IL6 was found to be unaltered.

5. CONCLUSION

Both clinical and experimental studies have indicated that cerebral ischemia promotes the marked increase in inflammation. Inflammatory mediator's time dependent profile after I/R injury has shown their marked impact at both early and late stage. The results indicate that inflammatory mediators have biphasic expression profiles that may contribute to the both early and later stage of ischemic brain damage. Thus, inhibition of these cytokine synthesis and release following global ischemia at defined time point may serve to attenuate the neuronal damage following ischemia I/R injury and may be the basis for intervention therapy to treat cerebral ischemia.

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