Research Article

INFLUENCE OF TIME DEPENDENT ISCHEMIC DURATION ON PATHOLOGICAL CASCADES FOLLOWING TRANSIENT GLOBAL CEREBRAL ISCHEMIA

Varun Gupta and Anil Kumar *

Pharmacology Division, University Institute of Pharmaceutical Sciences, UGC Centre of Advanced Study, Panjab University, Chandigarh, India

*Corresponding Author Email: kumaruijs@yahoo.com

Article Received on: 26/04/19 Approved for publication: 31/05/19

DOI: 10.7897/2230-8407.1007228

ABSTRACT

Aim: The present study has been designed to elucidate the effect of time dependent ischemic duration on transient global cerebral ischemia (TGCI) induced oxidative stress, neuroinflammation, mitochondrial dysfunction, apoptosis and neuronal damage in bilateral common carotid artery occlusion (BCCAO) model. **Methods:** Male Wistar rats were subjected to BCCAO for 20, 30 or 40 minutes followed by 48 hours of reperfusion. Various behavioural, biochemical, mitochondrial and histological assessments were done. **Results:** No significant difference was observed in behavioural, biochemical and histological assessments in sham treated animals as compared to Naive group. However, 20, 30 and 40 min of BCCAO significantly altered behavioural function (locomotor activity, rota rod performance and beam balance score), increased oxidative stress (malondialdehyde, nitrite, glutathione and superoxide dismutase), neuroinflammation (TNF- α), mitochondrial dysfunction in cortex and hippocampus, increased apoptosis (caspase-3), infarct area and neuronal damage as compared to sham treated animals. Effect with 30 min BCCAO was significantly greater as compared to 20 min BCCAO. Further, 40 min BCCAO was found to cause pronounced cerebral injury in some of the parameters but was associated with a higher mortality. **Conclusion:** These findings suggest that 20, 30 and 40 minutes of TGCI time dependently produces moderate, severe and very severe brain injury in Wistar rats respectively but rate of mortality increases with increase in duration of ischemia.

Keywords: Global cerebral Ischemia; reperfusion injury; oxidative stress; neuroinflammation; neuronal damage

INTRODUCTION

Stroke is currently the second leading cause of death and third leading cause of disability worldwide^{1,2} and therefore presently a major healthcare challenge. More than 70% of stroke cases are of ischemic origin³. Stroke can occur due to an emboli or a thrombus in a distal cerebral artery, which causes focal ischemia. It may also occur as a result of reduction in generalized cerebral blood flow which usually leads to global ischemia. Global ischemia may occur due a major occlusion in the carotid arteries (carotid artery stenosis) or during cardiac arrest and certain surgical procedures (cerebrovascular surgery, carotid endarterectomy, intra-cardiac surgery including valvular procedure) where blood supply to the brain is impaired⁴.

Primary ischemic insult induces various motor deficits and leads to altered behavioural function including paralysis, compromised sensorimotor ability, altered locomotion, speech, vision and cognition. Following a primary ischemic insult, depletion in energy, hindered metabolism of brain cells and reperfusion lead to the initiation of complex biochemical and cellular cascades including glutamate mediated excitotoxicity, neuronal inflammation, oxidative and nitrosative damage and mitochondrial dysfunction⁵. This further leads to the exaggeration of neuronal injury and causes a progressive and irreversible damage.

Amongst various animal models used for stroke or cerebral ischemia⁶, bilateral common carotid artery occlusion (BCCAO) is an established model for the induction of transient global cerebral ischemia (TGCI) which has been shown to induce

oxidative stress, neuroinflammation, excitotoxicity and apoptosis in experimental animals⁷⁻⁹. However, different research groups choose different durations of ischemia for the induction of clinically comparable brain injury using this model¹⁰⁻¹³. Studies with BCCAO for different time intervals makes it difficult to identify the optimum duration of experimental ischemia required in this model to cause significant cerebral injury without causing very high mortality. This also makes it difficult to compare and evaluate the effectiveness of various interventional research studies on cerebral ischemia since the effect produced by such interventions is subjectively dependent on the neuronal damage caused by the model in the first place. The present study was therefore designed to study the influence of time dependent ischemic duration on cerebral injury caused by experimentally induced transient global cerebral ischemia using BCCAO in Wistar rats.

MATERIALS AND METHODS

Animals

Male Wistar Rats (240-280g) bred in the Central Animal House facility of Panjab University, Chandigarh were used. The animals were maintained on 12 hour light/dark cycle and were provided food and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (Approval No. PU/IAEC/S/14/124) and conducted according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSCEA) guidelines for the use and care of experimental animals. Care was taken to minimize the discomfort caused to experimental animals throughout the study.

Grouping and Induction of cerebral ischemia

Rats were randomly divided into 5 groups consisting of 8 animals per group. Group 1 animals were Naïve and did not receive any treatment. Group 2 animals were sham operated while group 3 (IR (20)), 4 (IR (30)) and 5 (IR (40)) were subjected to 20, 30 and 40 minutes BCCAO respectively. Cerebral Ischemia and reperfusion injury was induced by BCCAO as previously reported¹². Animals were anaesthetized with 80mg/kg ketamine & 10mg/kg xylazine and a dissection between sternocleidomastoid and sternohyoid muscles parallel to the trachea was made to expose both common carotid arteries. Each carotid artery was carefully separated from its adventitial sheath and vagus nerve closely attached to it. The bilateral common carotid arteries were occluded for 20, 30 or 40 minutes using aneurysm clamps followed by 48 hour reperfusion to induce IR injury. The skin was closed with waxed surgical sutures. Sham control animals received surgery but without any occlusion of the common carotid arteries. Saline was injected intraperitoneally in all animals subjected to surgical procedure. After the completion of reperfusion period, the animals were assessed for behavioural outcomes and then sacrificed for biochemical, molecular and histological studies.

Behavioural Parameters

i) Measurement of locomotor activity

Locomotor activity was recorded by using an actophotometer (IMCORP, India). Before the locomotor activity was assessed, each animal was individually placed in the activity meter for acclimatization for a period of 3 minutes. Thereafter, rearing and ambulation for each animal was recorded for a period of 5 minutes. The locomotor activity was expressed in terms of total photo beam counts per 5 minutes¹⁴.

ii) Rotarod performance

Rotarod performance was checked to evaluate the motor coordination and grip performance of animals. Rats were pre-exposed to the rota rod apparatus (IMCORP, India) under a training protocol for the purpose of acclimatization to the apparatus. Rats were then placed on a rod rotating at a speed of 25 rpm. Cut off time of 180s was maintained throughout the experiment. Three separate trials at a gap of 5 minutes were performed and the average fall off time was recorded¹⁴.

iii) Beam balance test

Beam-balance test was used to assess gait abnormality and gross vestibular motor function. The rats were placed in the centre of a 2 cm wide wooden bar which was kept inclined at an angle of 60° from the horizontal platform. Balancing, gripping and locomotive performance of rats was scored on a scale ranging from 0 to 4. A score of 0 was assigned to animals that could readily traverse the beam whereas score 1, 2 and 3 were assigned to the animals demonstrating mild, moderate and severe impairment, respectively. Animals were scored 4 if they were completely unable to balance on the beam^{15, 16}.

Biochemical estimations

i) Dissection, homogenization and sample preparation

The animals were sacrificed by cervical dislocation after assessment of behavioral function. The brains were surgically removed; cortex and hippocampus were isolated on ice and weighed. 10% (w/v) tissue homogenates were prepared in ice-cold isolation buffer and post mitochondrial supernatant (PMS; cytosolic fraction) was used for biochemical and molecular assessments.

ii) Measurement of oxidative stress parameters

Estimation of malondialdehyde (MDA) concentration as a measure of lipid peroxidation was done by Thio Barbituric Acid Reactive Substances (TBARS) assay according to the method of Wills¹⁷. Accumulation of nitrite in the tissue as an indicator of nitric oxide production was estimated colorimetrically with

Griess Reagent as per the method described by Green¹⁸ using a sodium nitrite standard curve. Amongst endogenous antioxidants, reduced glutathione (GSH) in the supernatant was estimated according to the method described by Ellman¹⁹ and superoxide dismutase activity was assayed according to the method of Kono²⁰. Protein content was measured by Bradford method using bovine serum albumin as standard²¹.

Mitochondrial enzyme assessments

i) Isolation of mitochondria

Mitochondria were isolated by the principle of differential centrifugation using slightly modified method of Brown²².

ii) Mitochondrial Enzyme complexes

NADH dehydrogenase or mitochondrial complex I activity was assessed by the method of King and Howard²³. Complex II (Succinate Dehydrogenase) activity was measured by the method of King²⁴ whereas activity of complex IV (Cytochrome C oxidase) for direct oxidation of cytochrome C was estimated according to the method of Sottocasa²⁵.

iii) Mitochondrial viability (MTT assay)

Reduction of di-methyl-thiazol di-phenyl-tetrazolium bromide (MTT) by hydrogenases present in living mitochondria is believed to reflect the extent of cell viability²⁶. Blue formazan crystals formed after the incubation of sample and MTT were solubilised with dimethyl sulfoxide and absorbance values were recorded by using microplate absorbance reader (Biorad i-Mark, USA) at 595 nm filter.

Molecular Estimations

Caspase-3 colorimetric assay

Enzymatic reaction for the assessment of caspase-3 activity was performed using caspase-3 colorimetric kit (R&D systems, USA) according to manufacturer's instructions.

TNF- α assessment by ELISA

TNF- α measurement was done by using rat TNF- α immunoassay kits (BD OptEIA™; BD Biosciences, San Diego, USA) according to the manufacturer's instructions.

Infarct Area

Infarct area in the ischemic brain tissue was assessed using Triphenyl Tetrazolium Chloride (TTC) staining. TTC stained viable area in deep red colour while the infarcted area remained unstained. The sections were imaged and analyzed using ImageJ software (NIH, USA). Infarct area was expressed as % infarct of the total region examined²⁷.

Histopathological Examination

Histopathological assessment of the cerebral tissue was done using hematoxylin and eosin staining (H&E) following standardized procedure. Slides were allowed to dry and later observed and photographed using a microscope (Nikon Eclipse E200, Japan).

Survival analysis

The animals in all groups were observed for mortality during the entire protocol. Kaplan Meier survival plot was made to assess the mortality rate.

Statistical Analysis

Graph Pad Prism was used for all statistical analysis. All the values were expressed as mean $\pm$ SEM. Behavioural assessments were analyzed by two way Analysis of Variance (ANOVA) followed by Bonferroni post hoc test. Biochemical, mitochondrial, molecular and histological assessments were analysed by one way ANOVA followed by Tukey's post hoc test and Kaplan Meier survival analysis was analyzed using chi-square test. In all the tests, criterion for statistical significance was considered to be $p < 0.05$.

RESULTS

Effect of duration of ischemia on behavioral parameters

- i. Effect on locomotor activity: No significant difference in locomotor activity was observed between all treatment groups at 0 h and between naïve and sham groups after 48 h. However, BCCAO for 20 min (IR (20)) significantly reduced locomotor activity as compared to sham treatment. A time dependent and significant reduction in locomotor activity was observed in IR (30) group as compared to IR (20) group which was further reduced significantly with 40 min BCCAO (IR (40)) as compared to IR (20) and IR (30) groups (Figure 1A).
- ii. Effect on rotarod performance: At 0 h, there was no significant difference in rotarod performance between all treatment groups. Also, no significant difference was observed in the rotarod performance of sham group as compared to naïve after 48 h. However, BCCAO for 20 min (IR (20)) significantly reduced rotarod performance (decreased fall time) as compared to sham treatment. BCCAO for 30 min (IR (30)) reduced the rotarod performance (decreased fall time) significantly as compared to IR (20) group which was further reduced significantly with 40 min BCCAO (IR (40)) as compared to IR (20) and IR (30) groups (Figure 1B).
- iii. Effect on beam balance score: At 0 h, there was no significant difference in beam balance scores between all the groups. Also, no significant difference was observed in beam balance scores of sham group when compared to naïve after 48 h. However, BCCAO for 20 min (IR (20)) significantly increased beam balance scores as compared to sham treatment. A significant increase in beam balance score was observed in IR (30) group as compared to IR (20) which was further increased significantly with 40 min BCCAO (IR (40)) as compared to IR (20) group but not as compared to IR (30) group (Figure 1C).

Effect of duration of ischemia on oxidative and nitrate stress

Sham treated animals did not show any significant difference in oxidative stress parameters as compared to naïve treatment group. BCCAO for 20 min (IR (20)) significantly increased MDA levels, nitrite concentration and depleted endogenous antioxidant enzymes (GSH and SOD) activities in both cortex and hippocampus as compared to the sham treatment. BCCAO for 30 min (IR (30)) significantly increased oxidative stress (increased MDA and nitrite; decreased GSH and SOD) in both hippocampus and cortex as compared to IR (20) and sham group. Further, BCCAO treatment for 40 min (IR (40)) significantly caused oxidative damage as compared to IR (20) and IR (30) groups. However, BCCAO treatment for 40 min did not influence GSH significantly as compared to IR (30) (Figure 2).

Effect of duration of ischemia on mitochondrial enzyme activities and mitochondrial viability

Mitochondrial enzyme activities (I, II and IV) and mitochondrial viability did not change significantly in the sham group as compared to naïve treatment. However, BCCAO for 20 min followed by 48 hours reperfusion significantly impaired mitochondrial enzyme complex (I, II and IV) activities and decreased mitochondrial viability in both cortex and hippocampus as compared to sham group. BCCAO for 30 min (IR (30)) significantly increased the mitochondrial dysfunction (lowered complex I, II and IV) and decreased mitochondrial viability in both hippocampus and cortex as compared to IR (20) group. However, BCCAO for 40 minutes significantly lowered mitochondrial enzyme activities and mitochondrial viability as

compared to IR (20) group but not as compared to IR (30) group (Figure 3).

Effect of duration of ischemia on caspase-3 activity

A significant increase in caspase-3 activity of both cortex and hippocampus of IR (20) animals was observed as compared to sham animals. BCCAO for 30 minutes (IR (30)) induced a time dependent increase in caspase-3 activity as compared to sham and IR (20) groups. This effect was also found to be significantly enhanced with IR (40) group as compared to IR (20) and IR (30) groups (Figure 4).

Effect of duration of ischemia on TNF- α levels

Cortex and hippocampus of IR (20) animals showed a significant increase in TNF- α levels as compared to sham animals. BCCAO for 30 minutes (IR (30)) induced a time dependent significant increase in TNF- α levels as compared to sham and IR (20) groups. TNF- α levels were further found to be significantly enhanced with IR (40) group as compared to IR (20) group but remained to be insignificant as compared to IR (30) group (Figure 5).

Effect of duration of ischemia on survival analysis

Naïve and sham groups showed 100% survival; 0% mortality at the end of protocol. IR (20) group also showed 100% survival; 0% mortality after 48 h whereas IR (30) group showed 88.9% survival; 11.1% mortality. However, highest mortality was seen in IR (40) group (72.7 % survival; 27.3% mortality) at the end of protocol (Figure 6).

Effect of duration of ischemia on infarct area by TTC staining

A significant increase in infarct area of IR (20) group as compared to sham treatment was noted. Infarct area was also found to be significantly increased in IR (30) group as compared to IR (20) group. Further, significant increase in the infarct area of IR (40) group was observed as compared to IR (20) group. However, increase in infarct area in IR (40) group was not found to be significant when compared to IR (30) group (Figure 7A and 7B).

Effect of duration of ischemia on histopathological analysis

Histopathological analysis of motor cortex area of the brains of naïve and sham groups showed well preserved cytoarchitecture with healthy neurons bearing clearly distinguishable nucleus and cytoplasm (black arrows). However, IR (20) group showed many pyknotic neurons (red arrows) with condensed chromatin as a result of necrotic and apoptotic neuronal death associated with inflammation. Further, increased pyknosis and enucleated neurons were observed in IR (30) group whereas large number of pyknotic neurons with shrunken chromatin, enucleated and red neurons (eosinophilic neurons) along with vacuolation were seen with IR (40) group suggesting time dependently increased neuronal damage (Figure 8).

DISCUSSION

2-vessel occlusion model (BCCAO) has been used since long as a model for TSCI⁶ and induces acute cerebral and hippocampal injury in rat brains²⁸ which manifests as altered sensorimotor function, oxidative & nitrate stress, excitotoxicity, neuroinflammation and apoptosis. However, different research groups employ different duration of BCCAO for the induction of ischemia-reperfusion injury¹¹⁻¹³. Since different ischemic time points are expected to induce varying extent of brain injury, the present study focused on studying the influence of time dependent ischemic duration on behavioral functions, cortical and hippocampal oxidative pathology, neuronal damage, with emphasis on survival analysis at different time points in this model.

Figure 1. Effect of duration of ischemia on (A) Locomotor activity, (B) Rotarod activity and (C) Beam balance score: Values are expressed as mean $\pm$ SEM (n=8). Data was analyzed by two way ANOVA followed by Bonferroni post hoc test. ^aP<0.05 as compared to sham, ^bP<0.05 as compared to IR (20) and ^cP<0.05 as compared to IR (30).

Figure 2. Effect of duration of ischemia on oxidative and nitritive stress in cortex and hippocampus: Values are expressed as mean $\pm$ SEM (n=5). Data was analyzed by One Way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. ^aP<0.05 as compared to sham, ^bP<0.05 as compared to IR (20) and ^cP<0.05 as compared to IR (30).

Figure 3. Effect of duration of ischemia on Mitochondrial Complex I (A), II (B) & IV (C) activity and mitochondrial viability (D) in cortex and hippocampus: Values are expressed as mean $\pm$ SEM (n=5). Data was analyzed by One Way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. ^aP<0.05 as compared to sham and ^bP<0.05 as compared to IR (20).

Figure 4. Effect of duration of ischemia on Caspase-3 activity in cortex and hippocampus: Values are expressed as mean $\pm$ SEM (n=5). Data was analyzed by One Way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. ^aP<0.05 as compared to sham, ^bP<0.05 as compared to IR (20), and ^cP<0.05 as compared to IR (30).

Figure 5. Effect of duration of ischemia on TNF- α levels in cortex and hippocampus: Values are expressed as mean $\pm$ SEM (n=5). Data was analyzed by One Way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. ^aP<0.05 as compared to sham and ^bP<0.05 as compared to IR (20).

Figure 6. Effects of duration of ischemia on survival analysis: Kaplan Meier survival plot as analyzed using chi-square test.

Figure 7 (A) Pictograms of TTC stained brains (B) Effect of duration of ischemia on infarct area: Values are expressed as mean $\pm$ SEM. Data was analyzed by one way ANOVA followed by Tukey's post hoc test. ^aP<0.05 as compared to sham and ^bP<0.05 as compared to IR (20).

Figure 8. Pictograms of H&E stained rat brains

In the present study, BCCAO for 20, 30 and 40 min followed by 48 hour reperfusion produced moderate, severe and very severe alteration in behavioral function respectively. A significant time dependent decrease in locomotor activity, grip strength and motor coordination and an increase in beam balance score as assessed by actophotometer, rota rod and beam balance performance tests respectively was observed in the test groups as compared to sham treated animals. This suggests that increasing the duration of ischemic exposure increases the alteration in behavioral function.

Oxidative stress, characterized by overproduction of ROS, inactivation of defense antioxidant enzymes, and consumption of antioxidants, is believed to be a major contributor of secondary neuronal damage after an event of ischemia reperfusion. In the current study, BCCAO for 20 min followed by 48 h reperfusion significantly induced oxidative and nitrative stress as assessed by increased MDA levels (as a measure of lipid peroxidation) and nitrite levels in both cortex and hippocampus as compared to the sham group. This was also associated with a significant depletion in the levels of endogenous antioxidant enzymes such as glutathione reductase (GSH) and superoxide dismutase (SOD). Further, it was found that BCCAO for 30 min potentiated this effect and a significantly higher degree of oxidative stress was observed when compared to the IR (20) group. When the duration of ischemia was further increased to 40 min, oxidative stress was significantly exaggerated as compared to IR (20) and IR (30) groups, suggesting that increasing the duration of ischemia has a direct relation with oxidative pathology. However, insignificant decrease was seen in the levels of GSH in both cortex and hippocampus of IR (40) group when compared with IR (30) group.

Ischemia induced oxidative stress occurs primarily as a consequence of mitochondrial ROS production with hypoxia, excitotoxicity and Ca^{2+} overload being the major post ischemic triggers²⁹. In the current study, mitochondrial enzyme activity (complex I, II and IV) was found to be impaired after BCCAO induced injury in IR (20), IR (30) and IR (40) groups as compared to sham. Further, the mitochondrial viability was also found to be significantly decreased after BCCAO as assessed by MTT assay. However, insignificant decrease in these activities was observed in IR (40) group when compared to IR (30) group which may be attributed to insensitivity of the methods to detect below a certain level or to the rapidly changing mitochondrial dynamics after the rush of oxygen and nutrients, post reperfusion.

The above stated mitochondrial impairment along with oxidative stress are two most responsible factors for the initiation of apoptotic mechanisms after an ischemic event primarily in the penumbral regions of the infarct³⁰. BCCAO for 20 minutes in the present study lead to a significant increase in the levels of caspase-3 which is a known marker for apoptosis³¹. This effect was found to be exaggerated with increase in the duration of ischemia to 30 and 40 minutes.

Neuroinflammation is one of the major culprits of secondary neuronal damage and is associated with activation of various pathological cascades in the penumbra following an ischemic insult. As a marker of neuroinflammation, TNF- α was studied in cortex and hippocampus of rat brains in this study and was found to be significantly exaggerated in IR (20), IR (30) and IR (40) groups as compared to sham. However, insignificant increase in TNF- α levels was observed when compared with IR (30) group.

Cerebral ischemia is associated with irreversible neuronal death and measurement of infarct area is one of the major hallmarks in identifying the extent of neuronal death in experimental animals²⁷. TTC staining of the coronal brain sections revealed a significant increase in the infarct area of both IR (30) and IR (40) groups as compared to sham and IR (20) groups suggesting direct relation with the duration of ischemia. However, the slight increase in infarct area of IR (40) group (with high intra group variations) as compared to IR (30) group was statistically insignificant.

In another histopathological examination of the motor cortex areas using H&E staining, BCCAO for 20 min lead to neuronal death as seen by the presence of un-distinguishable cytoplasm and nucleus, and pyknotic neurons in the brain sections of IR (20) animals. In the IR (30) group, presence of more pyknotic neurons and enucleated neurons was observed whereas in IR (40) group increased pyknosis associated with the presence of shrunken and enucleated neurons alongwith eosinophilic neurons (red neurons) and vacuolation suggested an exaggerated neuronal damage as compared to the other test groups.

Survival analysis in the current study revealed the occurrence of higher mortality in IR (30) group as compared to sham and IR (20) groups. Interestingly, mortality was markedly increased in IR (40) group suggesting decreased survival rate of animals after 40 min ischemia. It is important to note here that IR (40) group did have highly exaggerated response of neuronal damage as observed in histopathology (but not in infarct area), oxidative stress, behavioral function (except beam balance) and apoptosis, but not in mitochondrial function as assessed in this study. IR (30) group on the other hand also lead to a consistent and increased neuronal damage but with a markedly low mortality.

CONCLUSION

The present study therefore critically analyses the influence of time dependent ischemic duration on the extent of brain injury in BCCAO model in rats with respect to behavioral function, oxidative pathology, neuroinflammation, apoptosis, histological analysis and mortality rate. The study suggests that in this model, IR for 30 min is most suitable considering the extent of injury and lower mortality rate for the induction of cerebral ischemia-reperfusion (or hypoperfusion) injury in rats.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support from "INSPIRE- Department of Science and Technology (DST), Govt of India" (DST/INSPIRE Fellowship/2014/IF140742) for carrying out this work.

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Cite this article as:

Varun Gupta and Anil Kumar. Influence of time dependent ischemic duration on pathological cascades following transient global cerebral ischemia. *Int. Res. J. Pharm.* 2019;10(7):105-113
<http://dx.doi.org/10.7897/2230-8407.1007228>

Source of support: DST-INSPIRE, Govt of India, Conflict of interest: None Declared

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