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Combination effect of Aspirin and *N*-acetylcysteine against global cerebral ischemic reperfusion injury in rats

Ravi Chandra Sekhara Reddy Danduga[a,*], Subba Reddy Dondapati[b], Phani Kumar Kola[a], Sanith Satya Has Kavati[a], Meenakshi Seshadri Singapalli[a]

[a] Department of Pharmacology, University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Nagarjuna Nagar, Andhra Pradesh, 522510, India
[b] Department of Pharmacology, Nirmala College of Pharmacy, Atmakur, Andhra Pradesh, 522503, India

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ABSTRACT

Aim: The study was intended to investigate the combined influence of Aspirin and *N*-acetylcysteine on global cerebral ischemic reperfusion injury in rats.

Materials and methods: The ischemic reperfusion injury was induced by bilateral common carotid artery ligation (BCCA) for 20 min and reperfused for 48 h. The treatment groups Aspirin, *N*-acetylcysteine and combined treatment groups were administered respective treatments, one week prior to the global ischemia and continued for the next two days. After 24 h of reperfusion, the animals were observed for behavioral assessments and after 48 h, all the animals were sacrificed to estimate oxidative stress parameters, acetylcholinesterase levels, neurochemical analysis in the brain homogenates. The proinflammatory cytokines in serum and histopathological alterations in the cortex and hippocampus (CA1 and CA3) were performed.

Key findings: The combined treatment group improved all the behavioral performances in the ischemic reperfuion injured rats than the individual treatment group of animals. Further, decreased the oxidative stress in the combined treatment group than the individual treatment groups. The acetylcholinesterase levels in the brain homogenates and the proinflammatory cytokines in the serum were significantly reduced in the combined treatment group than the individual treatment groups. The neurochemical alterations in the brain were significantly mitigated in the combined treatment group than the individual treatment groups. Further, the neuroprotection of the combined treatment group was confirmed by the histological studies in the cortex and hippocampus (CA1 and CA3).

Significance: Hence, the study suggested that the additive effect was observed in the combined treatment group of animals.

1. Introduction

Worldwide ischemic stroke is the leading cause of death and disability that might be due to global or focal cerebral ischemia (Heiss & Kidwell, 2014). In the tissues, the imbalance in nutrition supply to the metabolic demands leads to cellular injury becoming ischemic while subsequent reperfusion to the same ischemic tissue leads to an augmentation of cellular injury (Eltzschig & Eckle, 2011). Global cerebral ischemia is the condition in which an extensive area of the central nervous system is compromised for blood flow due to various reasons such as cardiac arrest, pulmonary embolism, and severe hypotension. The immediate rescue for ischemia is the restoration of blood flow to the ischemic regions. Ischemic brain injury frequently causes irreversible neuronal injury (Xu et al., 2010). The pathogenesis of ischemic reperfusion neuronal injury involves multiple mechanisms like glutamate excitotoxicity, the release of excessive free radicals, and stimulation of inflammatory responses (Amantea, Nappi, Bernardi, Bagetta, & Corasaniti, 2009; Mansoorali, Prakash, Kotresha, Prabhu, & Rao, 2012). At present we have very few treatment approaches for cerebral ischemia is to recanalization to salvage ischemic penumbra by the administration of tissue plasminogen activator (tPA) for tolerable patients or endovascular interventions and administration of anti-platelet drugs (Ramos-Cabrer, Campos, Sobrino, & Castillo, 2011). Restoration of oxygen levels by reperfusion exuberates oxidative stress by promoting the formation of reactive oxygen species and aggravates proinflammatory responses in the brain (Kalogeris, Baines, Krenz, &

* Corresponding author at: Department of Pharmacology, University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Nagarjuna Nagar, Andhra Pradesh, 522510, India.
E-mail address: ravichandra.pharma2262@gmail.com (R.C.S.R. Danduga).

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Korthuis, 2012). Because of the multiple factorial influences in cerebral ischemic reperfusion injury, aiming at a single factor is not a prompt therapeutic approach. So, in the present study, we try to evaluate the combined effect of Aspirin and *N*-acetylcysteine against global cerebral ischemia in rats.

Aspirin (ASP) is a nonsteroidal anti-inflammatory agent having a wide range of pharmacological activities with diverse mechanisms, especially in the treatment and prevention of stroke as anti-thrombotic and neuroprotective agent (Castillo et al., 2003). There are some scientific reports stating that aspirin and its metabolite, sodium salicylate was proved to have neuroprotective potential against glutamate neurotoxicity in rat neuronal cultures and hippocampal slices (Riepe, Kasischke, & Raupach, 1997). *N*-Acetylcysteine (NAC) is an FDA approved mucolytic agent with greater safety for the last few decades (Eakin et al., 2014). Previous literature also supporting the neuroprotective activity of NAC because of its potential antioxidant property and by promoting the regeneration of endothelial dependent relaxation factor (Cuzzocrea et al., 2000).

In the present study, we try to examine the combined effect of ASP and NAC on global cerebral ischemic reperfusion injury by bilateral common carotid artery ligation method in rats by assessing the behavioral, biochemical and histological parameters.

2. Materials and methods

2.1. Chemicals

Chemicals such as aspirin (gift sample from Lifeline Pharmaceuticals, Vijayawada, India), *N*- acetylcysteine (Cystey ampule, 200 mg/ml, Bangalore, India), TBA (Otto chemicals, India) GABA (Himedia, India), Glutamate (Himedia, India), acetylthiocholine iodide (Himedia, India), 5,5′- dithiobis-(2-Nitrobenzoic Acid) (Loba chemicals, India), ELISA kits for TNF-α, IL-1β (Krishgen Biosystems, India) and all other chemicals were analytical grade.

2.2. Animals

Male Sprague-Dawley rats weighing 220–280 were procured from Mahaveer Enterprises, Hyderabad. The animals were maintained in the animal house facility provided by Acharya Nagarjuna University with standard temperature (22 ± 5 °C) and humidity (44%–60%) with a 12 h light and dark cycle. All the animals were allowed to have free access to rat chow and water ad libitum. The Institutional Animal Ethical Committee of Acharya Nagarjuna University College of Pharmaceutical Sciences approved the experimental protocol and all the experiments were conducted according to the committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines, Govt. of India.

2.3. Experimental design

Animals were divided into six groups, each consists of ten animals.

Control group I: animals were not allowed for any surgical intervention and treatment was given with normal saline (5 ml/kg, *i.p*)

Sham control group II: animals were allowed for surgical intervention, without occlusion of bilateral common carotid arteries and treatment was given with saline (5 ml/kg, *i.p*)

Occlusion control III: animals were allowed for surgical intervention with occlusion of bilateral common carotid arteries and treatment was given with saline (5 ml/kg, *i.p*)

NAC treatment group IV: animals were allowed for surgical intervention with occlusion of bilateral common carotid arteries and treatment was given with NAC with a dose of 150 mg/kg/day, *i.p*.

Aspirin treatment group V: animals were allowed for surgical intervention with occlusion of bilateral common carotid arteries and treatment was given with ASP with a dose of 60 mg/kg/day, *p.o*.

Combination (NAC + ASP) Treatment group VI: animals were allowed for surgical intervention with occlusion of bilateral common carotid arteries and treatment was given with NAC and ASP with a dose of 150 (*i.p*) and 60 (*p.o*) mg/kg/day respectively.

All Treatments were given for one week prior to the surgery and continued for two days after surgery.

2.4. Procedure for the bilateral occlusion of common carotid arteries

Rats were anesthetized with a combination of ketamine and xylazine 50 mg/kg, *i.p* and10 mg/kg, *i.m* respectively. After anesthesia, animals were laid on their ventral side to expose the neck region. A midline incision of 2 cm was made after shaving the neck region. At sternocleidomastoid and sternothyroid regions were dissected to expose common carotid arteries. Both the common carotid arteries identified were separated from the vagus nerve to ligate with a silk thread for 20 min to produce ischemia. After 20 min ligature was removed to allow the perfusion. The reperfusion was confirmed by visual observation. The incision was closed with sutures and iodine ointment was applied to prevent infection (Naderi, Sabetkasaei, Parvardeh, & Zanjani, 2017). After 24 h of reperfusion, animals were allowed to assess the behavioral performances and after 48 h of reperfusion, animals were sacrificed for biochemical and histopathological examinations.

2.5. Behavioral assessments

2.5.1. Neurological scoring

Neurological scoring was done based upon the summation of the Garcia neurobehavioural score, through six individual tests which are; spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception and response to vibrissae touch. The neurological scale was graded from 0 to 18 (Garcia, Wagner, Liu, & Hu, 1995). The neurological scoring was done on 24 h after the surgical procedure. The scoring and analysis of data were done by two individuals who were trained and blinded to the treatment.

2.5.2. Locomotor activity

Locomotor activity is generally used to study sensitivity to the locomotor activating or depressing properties of a drug. After post-surgical treatment, all the animals were allowed to get acclimatized to Photoactometer for 5 min. After acclimatization, animals were observed for the locomotor activity for 10 min and locomotor activity count/10 min was calculated (Kulkarni, 1999).

2.5.3. Hanging wire test

Rats were assessed for grip strength by using a hanging wire test. In this test, animals were allowed to hang on a wire of measurements 45 cm long and 0.3 cm diameter with its forelimbs. Measured the time of fall and 90 s was kept as cut off time (Hunter et al., 2000).

2.5.4. Rotarod test

Rotarod test was used to analyze the motor coordination in rodents. In the present study, the rats were allowed to train on the rotarod apparatus of diameter 5 cm at a speed of 25 rpm, before surgery for 3 days. Each training session includes 3 trials with an interval of 5 min. After 24 h of the surgery, the animals were observed for motor coordination by noting the fall time by keeping the animals on a rod and cut off time was maintained at 180 s (Hong, Belayev, Khoutorova, Obenaus, & Bazan, 2014).

2.5.5. Elevated plus maze test

The elevated plus maze test is one of the paradigms to assess cognitive performance in animal studies. It has four arms with the dimensions (50 cm × 10 cm) of which two arms were closed with 40 cm height wall and two arms were open. The arms were connected to a central platform with the dimensions of (10 cm × 10 cm). Memory

acquisition was done on the day before surgery and observed for transfer latency (TL) by placing the animal opposite to the central arm (Gaur, Aggarwal, & Kumar, 2009). Transfer latency is the time taken by the animal to move from the open arm to the closed arm. The same procedure was repeated after 24 h of the surgery.

2.5.6. Y-maze test

Short term spatial working memory will be assessed using the Y-maze test by observing spontaneous alterations. Y-maze consists of 3 arms labeled as A, B & C. Animals were placed in one arm of the maze and allowed to explore the maze for 5 min and alterations are counted manually. (Consecutive entries in three different arms). Percentage of spontaneous alternations (%SA) were calculated using the formula (Wolf, Bauer, Abner, Ashkenazy-Frolinger, & Hartz, 2016).

$$\%\,SA = (Actual\ number\ of\ alternations) /(maximum\ number\ of\ alternations)\ X\ 100$$

Maximum number of alterations = Total number of arm entries – 2

2.6. Biochemical estimations

After scarification, the brain of animals was isolated and washed with ice-cold saline solution. The brain tissue was minced into small pieces and homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4). The homogenate obtained was centrifuged (8000 rpm, 20 min, −4 °C) and the supernatant was collected for further estimations of biochemical parameters (Bora & Sharma, 2010). The malondialdehyde (MDA) was estimated quantitatively by thiobarbituric acid reactive substance method (Ohkawa, Ohishi, & Yagi, 1979), total thiol and reduced glutathione (GSH) content was estimated by Ellman's reagent at a wavelength of 412 nm (Ellman, 1959; Sedlak & Lindsay, 1968), autoreduction of pyrogallol inhibition method was used to estimate superoxide dismutase (SOD) (Marklund & Marklund, 1974) and Acetylcholinesterase (ACHE) was estimated by measuring the yellow colored thiocholine at 412 nm (Ellman, Courtney, & Andres, 1961).

2.7. Estimation of proinflammatory cytokines in serum

After 2 h of global cerebral ischemia, blood samples were collected for the estimation of cytokines like TNF-α, IL-1β. Blood samples were allowed to clot and centrifuged for 20 min at −4 °C to collect the serum. Then the serum was stored at −80 °C until the time of estimation. Krishgen Biosystems TNF-α, IL-1β Elisa kits were used to estimate the cytokine levels in the animals by following the manufacturer's catalogs (Seo et al., 2013).

2.8. Neurochemical analysis

Estimation of GABA and glutamate was done as described by Maynert et al with minor modifications. Brain samples were isolated from the animals and washed with ice-cold 80% ethanol. Brain samples were homogenized with 80% ice-cold ethanol to prepare 10% homogenate. Homogenate was centrifuged at 8000 rpm to separate supernatant and sediment was collected. Sediment was used to extract GABA and glutamate with repeated extraction with 80% ice-cold ethanol. 3 ml of extract was used to evaporate ethanol completely and the residue was reconstituted with 100 ml of distilled water. Standard concentrations (2 mM) of glutamate and GABA were prepared to spot on chromatography paper along with test samples. The spotted chromatography papers were placed in a chromatography chamber saturated with a solvent composition; butanol:acetic acid:water (12:3:5 v/v). After development, chromatography papers were dried and repeat the process and dried papers were sprayed with ninhydrin reagent and dried in an oven for 4 min at 100 °C. The sample spots were identified corresponding to standards and cut the spotted areas to be used to elute the contents with the help of 0.005% $CuSO_4$ in 75% ethanol. The absorbance of elutes was observed at 515 nm using a spectrophotometer (Danduga, Dondapati, Kola et al., 2018).

2.9. Histopathological study

After 48 h of ischemic reperfusion, the animals were anesthetized. The brain samples of animals were isolated, collected, and stored in 10% formalin solution. They were allowed to embed in paraffin wax, sliced coronally into 5 µm thick slices. The slices were stained with eosin and hematoxylin to observe the cortex and hippocampus (CA1 and CA3) viable neuronal cell density, along with the histological changes (Seo et al., 2013).

2.10. Statistical analysis

The values were expressed as Mean ± SEM. The data were analyzed by using one way ANOVA followed by Tukey's test using Graph pad prism software. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Behavioral analysis

3.1.1. Neurological scoring

The ischemic control group of animals showed a significant ($P < 0.001$) neurological deficit compared with the sham control group of animals. The individual treatment groups, NAC and ASP showed improvement in the neurological scoring, but the improvement was not significant. The combination treatment group showed a significant ($P < 0.05$) elevation in the neurological deficit score compared with the ischemic control group of animals (Fig. 1).

3.1.2. Locomotor activity

BCCA ligation control group hampered the locomotor activity significantly as compared to the sham control group ($P < 0.001$). The individual treatment groups, NAC and ASP showed improvement in locomotor performance significantly ($P < 0.05$), as compared with the BCCA ligation control group and the combination treatment group showed marked improvement in locomotor performance as compared with the BCCA ligation control group ($P < 0.001$) and also with the individual treatment groups NAC ($P < 0.05$), ASP ($P < 0.05$)

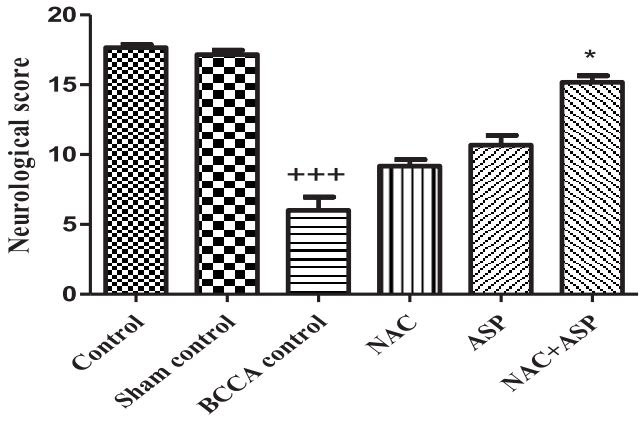


Fig. 1. Effect of NAC, ASP and combination therapy on neurological score in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). + + + indicate $P < 0.001$, Vs sham control; * indicate $P < 0.05$, Vs BCCA control.

Table 1
Effect of NAC, ASP and combination therapy on behavioral assessments in ischemic reperfusion injury.

Groups	Locomotor activity count/5 min	Hanging latency time (s)	Latency in fall of time (s)	% of Spontaneous alterations (% SA)
Group-I	286 ± 24.6	28.8 ± 2.66	64.8 ± 5.35	77.2 ± 2.99
Group-II	226 ± 12.6	25.2 ± 1.82	47.7 ± 6.69	67.5 ± 2.43
Group-III	61.7 ± 6.06^{+++}	8.17 ± 0.70^{+++}	11.8 ± 1.38^{+++}	31.3 ± 3.43^{+++}
Group-IV	129 ± 8.31*	10.3 ± 1.41	19.0 ± 1.53	47.5 ± 4. 90*
Group-V	142 ± 8.10*	12.7 ± 1.33	21.2 ± 2.55	49.0 ± 3.34*
Group-VI	204 ± 16.5***,#,@	20.5 ± 1.61***,##,@	37.2 ± 4.28**, #	65.2 ± 4.69***,#,@

Results are expressed as mean ± SEM (n = 6). + + + indicate P < 0.001, Vs sham control; *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, Vs BCCA control; @ indicate P < 0.05, Vs ASP alone treatment; #, ## indicates P < 0.05, P < 0.01, respectively, Vs NAC alone treatment.

(Table 1).

3.1.3. Hanging wire test

Hanging wire test was done to assess the effect of combination drugs on motor neuromuscular impairment induced by global cerebral ischemia. The BCCA ligation control group showed significant loss of grip strength as compared to the sham control group of animals (P < 0.001). Individual and combination treatment groups attenuated loss of grip strength, but the significant difference was observed only with the combination treatment as compared with the BCCA ligation control group (P < 0.001) and the combination treatment group was also showed significant difference with individual treatment groups NAC (P < 0.01) and ASP (P < 0.05) (Table 1).

3.1.4. Rotarod test

The motor coordination was hindered significantly in the BCCA ligation control group as compared to the sham control group (P < 0.001). The individual treatment groups were not shown significant improvement in fall of time, whereas the combination treatment group showed a significant increase in fall of time as compared to the ligation control group (P < 0.01). The combination treatment group showed significant (P < 0.05) difference with NAC individual treatment group but not with the ASP alone treatment group in elevating the fall of time (Table 1).

3.1.5. Elevated plus maze test

The animals with BCCA ligation showed a significant (P < 0.001) increase in transfer latency by hampering spatial memory when compared to a sham control group of animals. The individual treatment groups and combination treatment group showed significant (P < 0.01 and P < 0.001) decrease in transfer latency, compared to the BCCA ligation control group of animals. The individual treatment groups, NAC and ASP significantly (P < 0.01 and P < 0.05) differ with the combination treatment group (Fig. 2).

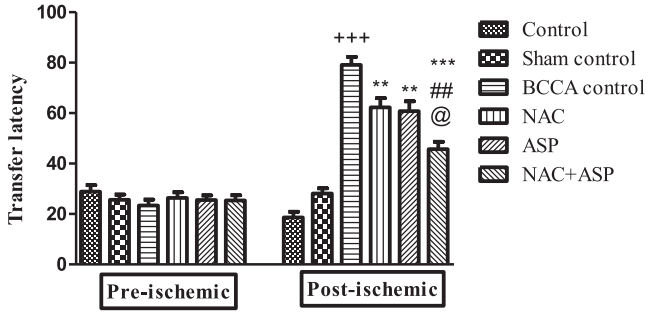


Fig. 2. Effect of NAC, ASP and combination therapy on transfer latency in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). + + + indicate P < 0.001, Vs sham control; **, *** indicate P < 0.01, P < 0.001, respectively, Vs BCCA control; @ indicate P < 0.05, Vs ASP alone treatment; ## indicate P < 0.01, Vs NAC alone treatment.

3.1.6. Y-maze test

The % SA of the BCCA ligation control group decreased significantly as compared to the sham control group (P < 0.001). Whereas, the individual treatment groups and combination treatment group showed significant (P < 0.05 and P < 0.001) increase in % SA respectively as compared to the BCCA ligation group. The combination treatment group was also shown a significant difference with the individual treatment groups in increasing the % SA as compared to individual treatment groups (P < 0.05) (Table 1).

3.2. Biochemical estimations

The BCCA ligation control group caused a significant increase in MDA (P < 0.001) and significantly reduced the total thiols, GSH, SOD levels as compared to a sham control group of animals (P < 0.001). The individual treatment groups, NAC, ASP showed a significant decrease in MDA levels and increased the SOD levels as compared to a BCCA ligation control group of animals. Whereas the elevation of total thiols and GSH levels of individual treatment groups were not significant in comparison to BCCA ligation control group of animals except in NAC alone treatment group for the elevation of total thiol, it showed a significant elevation in total thiol (P < 0.01). The combination treatment group showed a decrease in MDA levels and increased the levels of total thiol, GSH, SOD significantly (P < 0.001) as compared to a BCCA ligation control group of animals. The reduction of MDA levels (P < 0.001, P < 0.001) and elevation of total thiol (P < 0.05, P < 0.01), GSH (P < 0.05, P < 0.05), SOD (P < 0.01, P < 0.01) of the combination treatment group was more significant than individual treatment groups NAC, ASP respectively (Fig. 3).

ACHE levels were significantly (P < 0.001) elevated in a BCCA

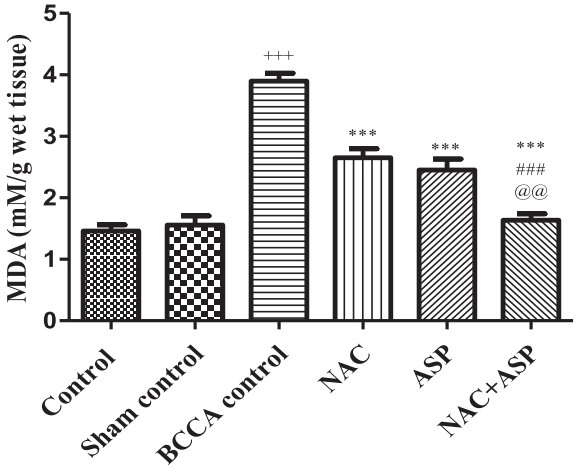


Fig. 3. Effect of NAC, ASP and combination therapy on MDA levels in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). + + + indicate P < 0.001, Vs sham control; *** indicate P < 0.001, Vs BCCA control; @@ indicate P < 0.01, Vs ASP alone treatment; ### indicate P < 0.001, Vs NAC alone treatment.

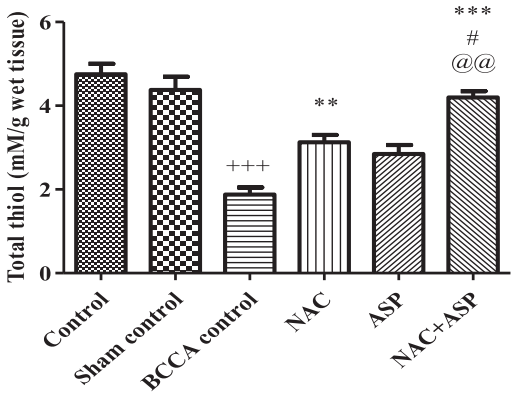


Fig. 4. Effect of NAC, ASP and combination therapy on total thiol levels in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). + + + indicate P < 0.001, Vs sham control; ** indicate P < 0.01, Vs BCCA control; @@ indicate P < 0.01, Vs ASP alone treatment; # indicate P < 0.05, Vs NAC alone treatment.

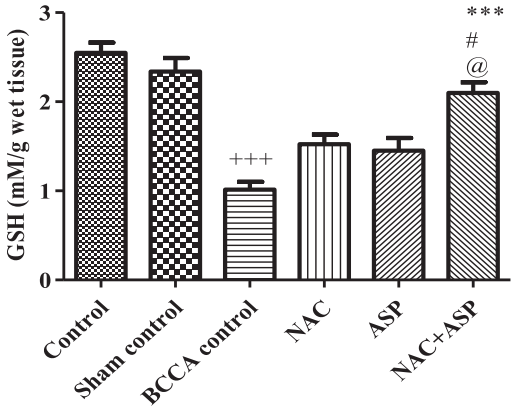


Fig. 5. Effect of NAC, ASP and combination therapy on GSH levels in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). + + + indicate P < 0.001, Vs sham control; *** indicate P < 0.001, Vs BCCA control; @ indicate P < 0.05, Vs ASP alone treatment; # indicate P < 0.05, Vs NAC alone treatment.

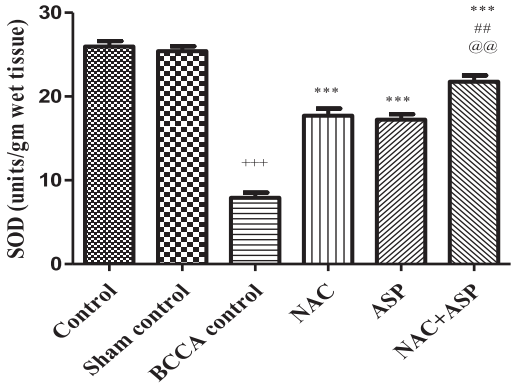


Fig. 6. Effect of NAC, ASP and combination therapy on SOD levels in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). + + + indicate P < 0.001, Vs sham control; *** indicate P < 0.001, Vs BCCA control; @@ indicate P < 0.01, Vs ASP alone treatment; ## indicate P < 0.01, Vs NAC alone treatment.

ligation control group as compared to a sham control group of animals. The individual treatment groups, NAC (P < 0.01), ASP (P < 0.001) and combination treatment group significantly (P < 0.001) reduced the levels of ACHE as compared to a BCCA ligation control group of animals. The reduction of ACHE levels of the combination treatment group was more significant (P < 0.05) than individual treatment groups (Figs. 3–7).

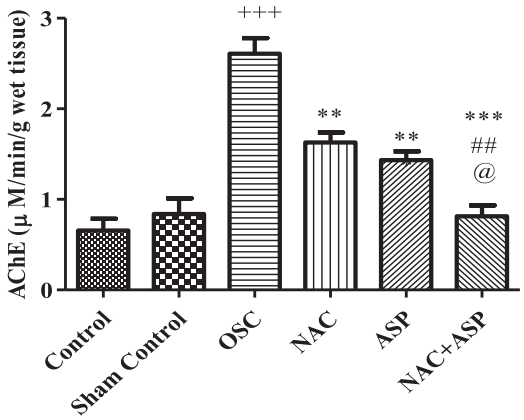


Fig. 7. Effect of NAC, ASP and combination therapy on AChE levels in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). + + + indicate P < 0.001, Vs sham control; **, *** indicate P < 0.01, P < 0.001, respectively, Vs BCCA control; @ indicate P < 0.05, Vs ASP alone treatment; ## indicate P < 0.01, Vs NAC alone treatment.

3.3. Estimation of proinflammatory cytokines in serum

The proinflammatory cytokines were significantly (P < 0.001) increased in the blood samples of the BCCA ligation control group when compared with the sham control group of animals. The individual treatment group of NAC and ASP showed a significant decrease in the cytokine levels in comparison with the BCCA ligation control group. A marked decrease in the cytokine levels was observed with the combination treatment group than the individual treatment groups when compared with the BCCA ligation control group of animals. There is a significant difference between the NAC individual treatment group to the combination treatment group in lowering the cytokine levels, but not with ASP alone treatment group (Fig. 8).

3.4. Neurochemical analysis

Systemic administration of 3-NP showed a significant decrease in the levels of GABA and elevated the levels of glutamate in the brain when compared to a sham control group of animals. The administration of NAC and ASP alone reduced the alterations in neurotransmitters induced by 3-NP, but they were not significant when compared to a BCCA ligation control group of animals. The combination treatment group significantly mitigated the 3-NP induced alterations in neurotransmitters in the brain as compared to the BCCA ligation control group (Fig. 9).

3.5. Histological study

The histological changes in the cortex and the hippocampus (CA1 and CA3) were observed with H&E staining. The coronal sections of the brain were assessed for the viable neuronal cell count in the cortex and hippocampus. The BCCA ligation control group of animals showed a marked increase in the intracellular space in the cortex region of the brain. The BCCA ligation group significantly decreased the viable cell count in the cortex and hippocampus as compared with the sham control group of animals. The individual treatment groups increased the viable neuronal cell count, but not significant as compared to a BCCA ligation control group of animals. Whereas, the combination treatment group showed a significant increase in the viable neuronal cell count, in the cortex and hippocampus as compared to the BCCA ligation control

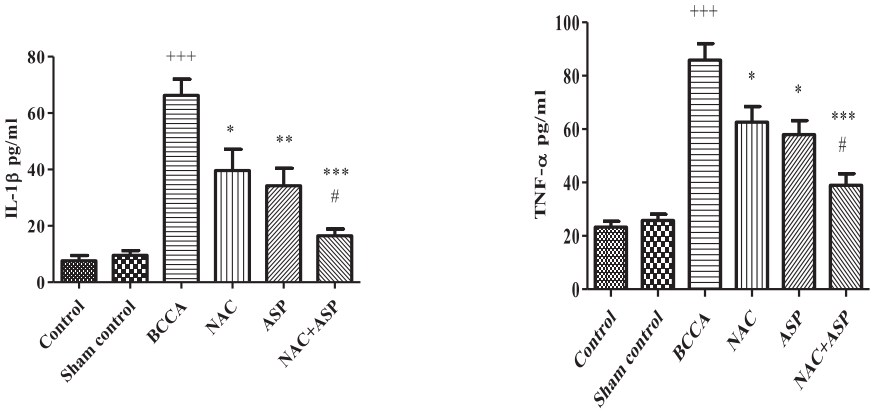


Fig. 8. Effect of NAC, ASP and combination therapy on proinflammatroy cytokine levels in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). + + + indicate P < 0.001, Vs sham control; *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001 respectively, Vs BCCA control; # indicates P < 0.05, Vs NAC alone treatment.

group of animals. The individual treatment groups and the combination group showed a marked decrease in the intercellular space in the cortex region (Figs. 10–12).

4. Discussion

The present study was designed to compare the combination and individual treatments of NAC and ASP in global cerebral ischemic reperfusion injury in rats and behavioral changes. The results showed that the combination treatment group improves behavioral performances significantly than individual treatment groups that were associated with oxidative stress, neuro-inflammation, excitotoxicity, and pyramidal cell death in the hippocampus.

Ischemic stroke is an unmet clinical condition having limited therapeutic options for the acute ischemic stroke. Recombinant tissue plasminogen activator (tPA) is an approved drug for acute ischemic stroke, must be administered before 4.5 h after stroke (Tajiri et al., 2014). However, tPA has limited clinical use because of its narrow therapeutic window, hemorrhagic complications and it also promotes reperfusion injury (Lakhan, Kirchgessner, & Hofer, 2009). Ischemic injury comprises of multiple molecular mechanisms includes excitotoxicity, oxidative damage which in turn causes blood brain barrier dysfunction and inflammatory response followed by neuronal death (Dirnagl, Iadecola, & Moskowitz, 1999; Moskowitz, Lo, & Iadecola, 2010). Although, reperfusion of the ischemic tissue can reduce extensive brain injury and it is critical for the restoration of normal physiological functioning of the brain. It can paradoxically exacerbate brain injury, leads to secondary damage in some patients, called cerebral ischemic reperfusion injury (Aronowski, Strong, & Grotta, 1997). The cerebral ischemic reperfusion injury involves the complex mechanisms includes the excessive release of excitatory amino acids, calcium overloads, neuro-inflammation, and generation of reactive oxygen species (Yang, Chen, & Xiao, 2017).

Free radicals are the reactive oxygen and nitrogen species which can attack the polyunsaturated fatty acids. The brain is the most vulnerable tissue for the free radicals, because of its high content of polyunsaturated fatty acids (Weaver & Liu, 2015). The free radicals promote the lipid peroxidation in the tissues results in the production of MDA. The elevated levels of MDA may indicate the oxidative stress in the tissues leads to neuronal death (Yu & Wang, 2013). A large number of researchers reported that the oxidative stress is involved in the neuronal death of ischemic reperfusion injury (Godinho et al., 2018; Mansoorali et al., 2012; Naderi et al., 2017). As in line with the previous reports, in the present study BCCA ligation control group significantly elevate the levels of MDA, as an indicator of lipid peroxidation. The individual treatment groups, NAC and ASP ameliorated the elevated MDA levels in the brain and the combination treatment group ameliorated the elevated MDA levels in the brain more significant than the individual treatment groups. The innate anti-oxidant defense system like GSH, total thiol, and SOD which protects the neuronal cells from the oxidative stress by preventing the initiation and progression of the free radical chain reactions (Erkut & Onk, 2015; Thippeswamy et al., 2011). There are many reports stating that the oxidative stress induced by ischemic reperfusion, reduces the anti-oxidant defense system in the brain tissue (Ban et al., 2012; Liang, Shi, Luo, & Yang, 2015). In our experimental results showed that the BCCA ligation control group of animals decreased the levels of GSH, total thiol, and SOD in accordance with the previous literature. The individual treatment groups and the combination treatment group showed a significant improvement in the elevation of anti-oxidant defense system in the brain tissue, as compared to a BCCA ligation control group of animals. In our finding, the combination treatment group showed better results, in the elevation of the anti-oxidant defense system, in the treatment groups than that of individual treatment groups. These results demonstrated that the

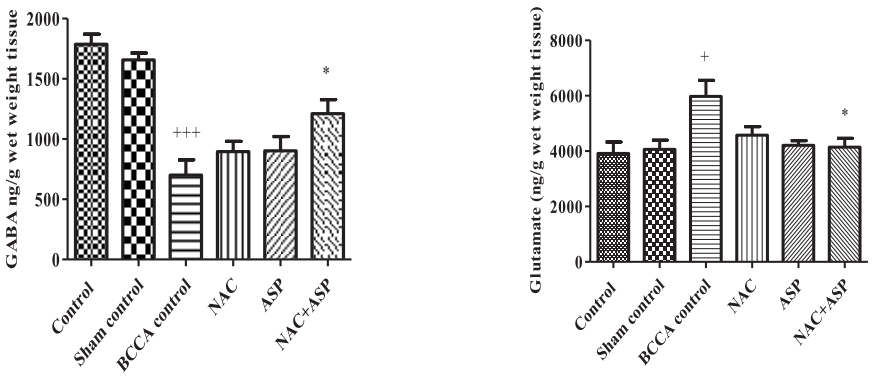
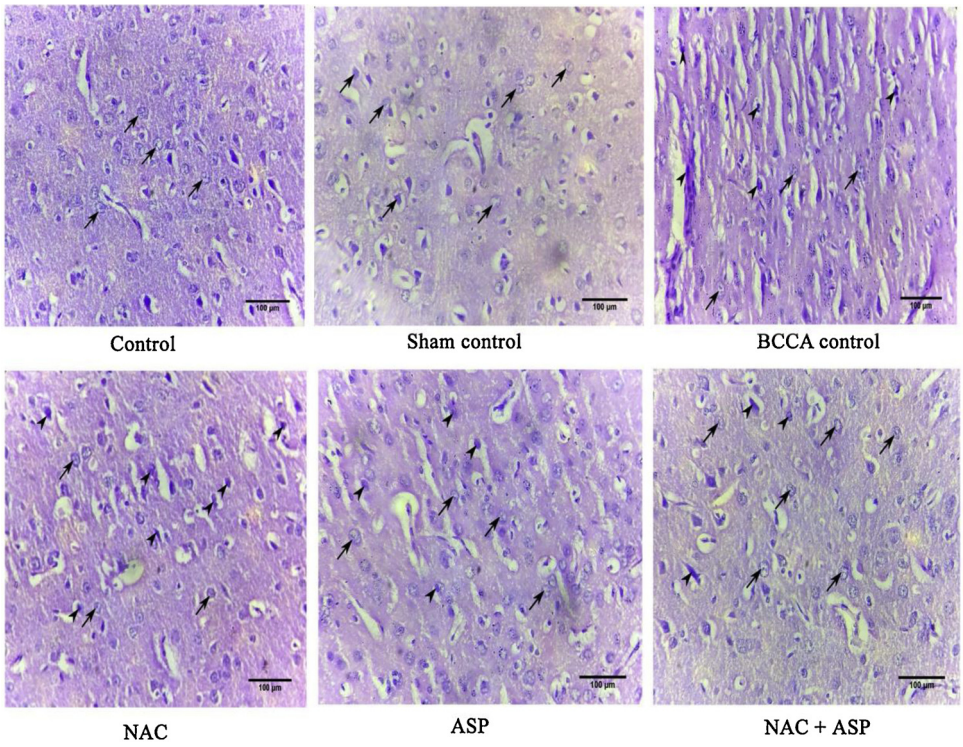


Fig. 9. Effect of NAC, ASP and combination therapy on neurochemical levels (GABA and glutamate) in ischemic reperfusion injury. Results are expressed as mean ± SEM (n = 6). +, + + + indicate P < 0.05, P < 0.001 respectively, Vs sham control; * indicate P < 0.05, Vs BCCA control.



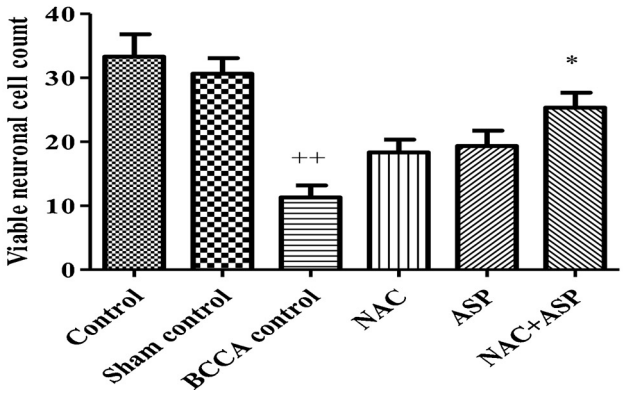
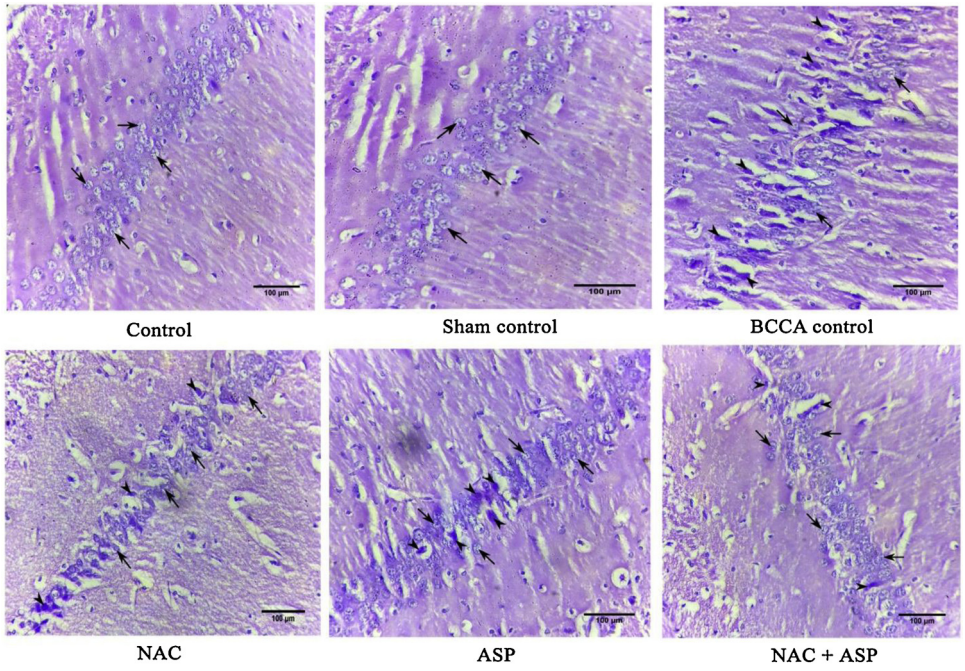


Fig. 10. Effect of NAC, ASP and combination therapy on histological alterations (400 X) in the cortex, stained with H&E (arrows represent normal viable cells and arrow heads represents the pyknotic cells). The graph represents the viable cell count in the experimental groups. The values are expressed as mean ± SEM (n = 4); ++ indicate P < 0.01 Vs sham control; * indicate P < 0.05 Vs BCCA control.

potential synergistic effect of NAC and ASP in ameliorating the lipid peroxidation and in elevating the anti-oxidant defense system.

Numerous studies have shown that cerebral ischemic reperfusion injury promotes the transcription of proinflammatory mediators and followed by the production of proinflammatory mediators such as cytokines, chemokines and adhesion molecules in the brain, all of which cumulatively contributing to the neuronal death (Ceulemans et al., 2010; Shukla, Shakya, Perez-Pinzon, & Dave, 2017). Proinflammatory cytokines like IL-1β and TNF-α are the major contributing inflammatory mediators of the ischemic reperfusion injury, with potentially deleterious effects on the neurons. Previous reports showed that the mice with the deficient in IL-1β exhibited lesser infarct volume than that of wild animals. Overexpression of TNF-α also observed in the ischemic brain injury, like that of IL-1β. In addition, there are scientific reports stating that the administration of IL-1β receptor antagonists and the inhibition of TNF-α, reduces the infarct size (Pinteaux, Rothwell, & Boutin, 2006; Sun et al., 2011). Our results demonstrated that significantly increased the levels of proinflammatory cytokines like IL-1β and TNF- in BCCA ligation group of animals. The individual treatment groups showed a significant decrease in cytokine levels in the serum as compared to the BCCA ligation control group of animals. The present findings are in good agreement with the previous reports stated that the individual treatments of ASP and NAC showed decreased expression of inflammatory cytokines (Bavarsad, Harrigan, & Alexandrov, 2014; Chang et al., 2008). In addition, the combination treatment group showed marked decreased levels of cytokine in the serum than that of the individual treatment group of animals. The results may indicate the potential influence of combination therapy in reducing the cytokine levels in the serum.

In recent years, the role of amino acids in the central nervous system has gained a lot of research interest, especially in the cerebral ischemic reperfusion injury. There are several amino acid neurotransmitters in



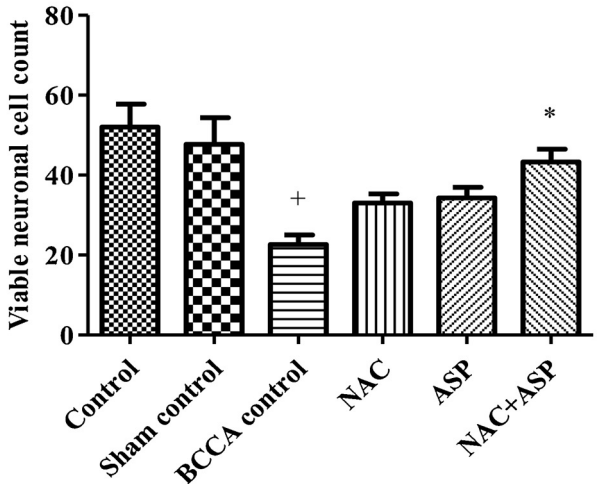


Fig. 11. Effect of NAC, ASP and combination therapy on histological alterations (400 X) in the CA1 region of the hippocampus, stained with H&E (arrows represent normal viable cells and arrow heads represents the pyknotic cells). The graph represents the viable cell count in the experimental groups. The values are expressed as mean ± SEM (n = 4); + indicate P < 0.05 Vs sham control; * indicate P < 0.05 Vs BCCA control.

the central nervous system, in that alteration of GABA and glutamate levels plays a major role in the neuronal injury. Being one of the major excitatory neurotransmitters, glutamate acts as a major contributor for the neuronal excitotoxicity. The excitotoxicity has been identified as a major contributing factor in the pathology of cerebral ischemia. During hypoxic conditions, glutaminergic neurons are activated by the entry of calcium or by the depolarization of the neurons and releases glutamate (Rodrigues et al., 2017). The massive release of glutamate in ischemic state stimulates the glutamate receptors and elevates the calcium levels in the neuronal cells leads to neuronal death. Scientific investigations showed a therapeutic approach for ischemic injury by promoting inhibitory neurotransmitters in the brain. GABA is a major inhibitory neurotransmitter and it inhibits neuronal response by promoting hyperpolarization in the neurons (Danduga, Dondapati, Kola et al., 2018). Thus, modulation of GABA neurotransmitter is a promising therapeutic strategy to ameliorate the neuronal injury during ischemic reperfusion injury. So, it is important to study the neuroprotection of combination therapy in ischemic reperfusion injury, on the concentrations of amino acid neurotransmitters like GABA and glutamate. In our present study, a significantly increased glutamate and decreased GABA levels were found in the BCCA ligation control group. The individual treatment groups mitigated the alteration of glutamate and GABA induced by the ischemic reperfusion but not significantly, whereas the combination treatment group significantly attenuated the neurotransmitters alterations induced by ischemic reperfusion injury. The results are in line with the earlier reports stating that the role of amino acid neurotransmitters in the neuronal protection of the cerebral ischemic reperfusion injury (Rodrigues et al., 2017). Thus, the study strongly supports the neuroprotective potential of combination therapy, attributed to its capacity to modulate the amino acid neurotransmitters in the brain.

Previous studies have demonstrated the effect of global cerebral

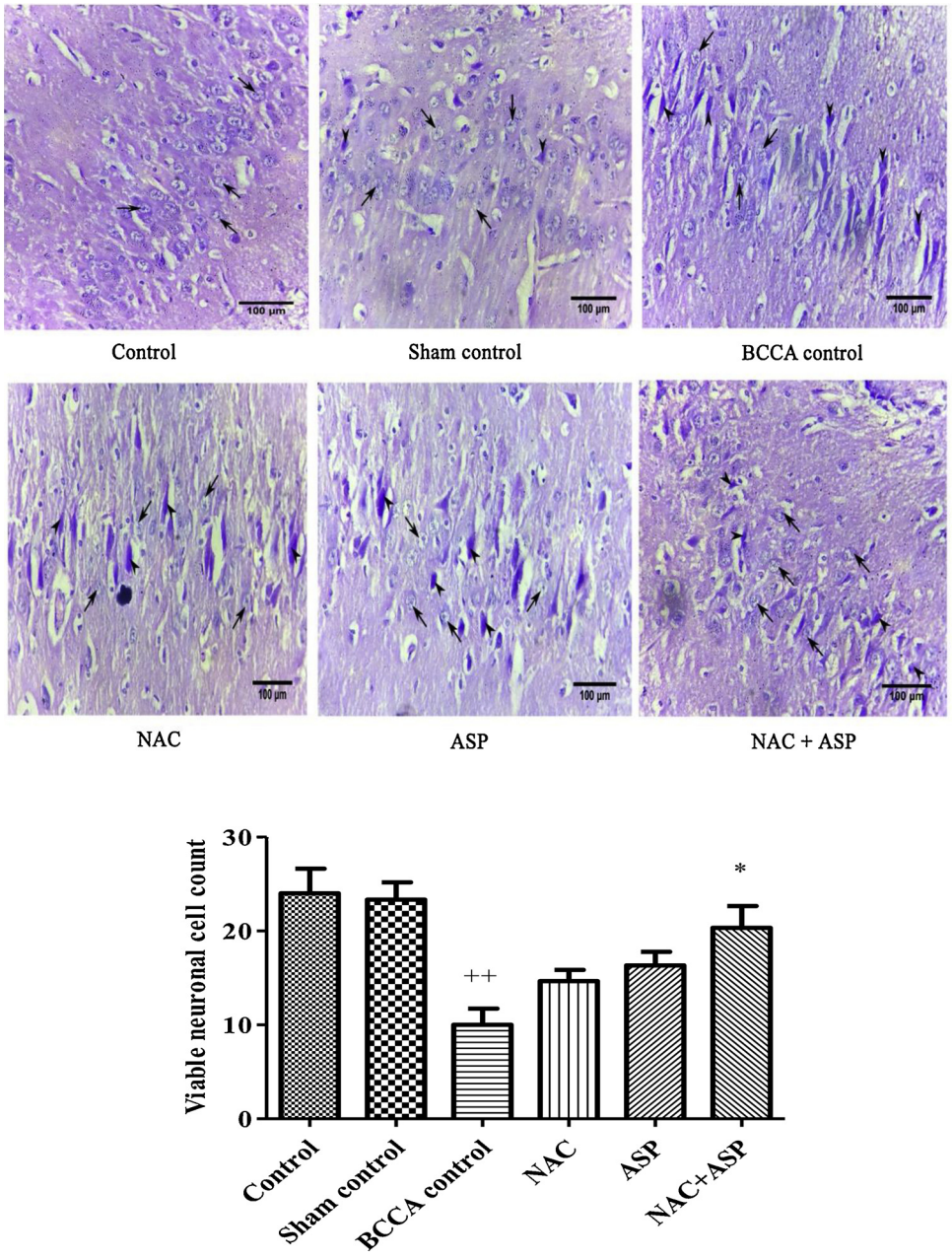


Fig. 12. Effect of NAC, ASP and combination therapy on histological alterations (400 X) in the CA3 region of the hippocampus, stained with H&E (arrows represent normal viable cells and arrow heads represents the pyknotic cells). The graph represents the viable cell count in the experimental groups. The values are expressed as mean ± SEM (n = 4); + + indicate P < 0.01 Vs sham control; * indicate P < 0.05 Vs BCCA control.

ischemic reperfusion injury on behavioral impairment such as motor dysfunction and cognitive impairment in rats (Danduga, Dondapati, Singapalli, Kavati, & Kola, 2018; Naderi et al., 2017). In the present study, motor dysfunction was assessed with neurological scoring, photoactometer, hanging wire and rotarod tests. The results showed that a significant decrease in neurological scoring, locomotor count, time to fall in hanging wire test and the rotarod test indicates the motor dysfunction in the ischemic reperfusion injury. The Y-maze and plus maze tests were performed to assess the cognitive dysfunction in ischemic rats. The ischemic control group showed a significant decrease in the % spontaneous alterations and an increase in transfer latency in Y-maze and plus maze tests respectively. The results are in good agreement with earlier reports (Danduga, Dondapati, Singapalli et al., 2018; Thippeswamy et al., 2011). The individual treatment groups, NAC and ASP showed significant improvement in the locomotor count, decrease in transfer latency and % spontaneous alterations whereas the improvement was not significant in the neurological score, hanging latency and time to fall from the rotarod. The combination treatment group showed significant results in improving behavioral performances with the BCCA ligation control group and the individual treatment groups, except in neurological score in comparison with individual treatment groups, NAC, ASP and time to fall from the rotarod in comparison with the ASP alone treatment group. The results demonstrated the beneficial effect of combination therapy in improving the behavioral performances compared to the individual treatment groups.

To support the potential neuroprotective effect of combination therapy was further confirmed by observing the histological alteration of both hippocampus (CA1 and CA3) and cortex regions of the brain.

5. Conclusion

The study indicates that the concomitant administration of aspirin

and *N*-acetylcysteine showed additive effects in ameliorating the oxidative stress, inflammation, and neurotransmitters alteration in cerebral ischemic reperfusion injury than the individual treatment groups. The histological alteration also mitigated more effectively than the individual treatment group. As a result, the behavioral performances of the combined treatment group showed better improvement than the individual treatment groups.

Ethical statement

The institutional Animal Ethical Committee of Acharya Nagarjuna University College of Pharmaceutical Sciences approved the experimental protocol and all the experiments were conducted according to the committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines, Govt. of India.

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Declaration of Competing Interest

The authors declared no conflict of interest.

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