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Original Article

Effect of BIBN4096 in Neurobehavioural Changes of Nitroglycerin-induced Migraineous Rat Model

Anson Sunny Maroky, Varadarajan Parthasarathy*
Immunology Laboratory, Department of Pharmacy, Annamalai University, Annamalainagar, Chidambaram, Tamil Nadu, INDIA.

ABSTRACT

Background: The pathogenesis of migraine pain has not yet been adequately explained. The incidence of cognitive dysfunction and psychological symptoms, as well as their reciprocal relationships in migraine patients, is still under consideration. The study aims to characterise the neurobehavioral and molecular changes that occur during a migraine condition. **Methods:** For the present study, nitro-glycerine (NTG)-induced rats were treated with antimigraine drugs such as ergotamine, sumatriptan, as well as BIBN4096. The pain was measured by the hot plate method, and the motor activity was assessed using actophotometer. The neurobehavioural activities were tested by using open field, elevated plus-maze and forced swim test. Vasoactive substances such as NO and CGRP were detected in the plasma and CGRP was detected in the isolated parts of the rat's brain. Analysis of the proinflammatory marker such as TNF-α was carried out in the serum. Histopathological changes of the animal brain were identified using Cresyl violet (Nissl body) staining. **Results:** A significant analgesic activity was observed with BIBN4096 ($p<0.01$). Significant ($p<0.05$) regaining of motor and neurobehavioural changes was observed in the NTG-induced migraneous animal after treatment with BIBN4096 as compared to the NTG-treated animal. But there was a non-significant difference observed in the forced swim test. Vasoactive and inflammatory markers such as NO, CGRP and TNF-α were significantly reduced on treatment with BIBN4096 ($p<0.05$) when compared to the model group. On Cresyl violet staining, less damage of neurons was observed with BIBN4096 treatment and a near-normal morphology of the rat's cerebral cortex was observed. **Conclusion:** Thus, the present study exhibited a remarkable antimigraine effect with a CGRP antagonistic agent, BIBN4096 than the other tested drugs such as ergotamine and sumatriptan.

Key words: Migraine, Nitroglycerin, CGRP, BIBN4096, Open field task, Elevated plus-maze.

Correspondence

Dr. Varadarajan Parthasarathy

Professor, Department of Pharmacy, Annamalai University, Chidambaram-608002, Tamil Nadu, INDIA.

Phone: +91 9443512724

Email: vapartha@yahoo.com

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INTRODUCTION

Migraine is the most disabling neurovascular disorder characterised by unilateral throbbing head pain associated with various neurological symptoms, including hypersensitivity to light, sound, and smells, leading to nausea and vomiting. In addition to these, a variety of autonomic, cognitive, emotional and motor disturbances have also been recorded.[1] The development of a migraine attack is also associated with a broad range of internal and external stimuli, such as stress, hormonal changes, sleep disturbances, meal skipping, or sensory overload.[2,3] Furthermore, the overuse of medication is also known to lead to headaches. Migraine affects 12% of the world's population,[4,5] particularly individuals who are less than 50 years of age.[6] The disorders that are commonly comorbid with migraine are neck pain, depression, as well as anxiety, which are the top ten causes of disability worldwide, placing migraine in a central position among the world's most disabling disorders.

Migraine primarily affects the sensory nervous system[7] and is known to be linked with autonomic phenomena in the face, typically reddening of the eyes, tearing, flushing, or pallor.[8] Some patients experience premonitory signs, such as cognitive changes, hunger/ thirst, euphoria, or irritability up to 72 hr before the attack. The sensory function does not usually return to normal immediately following the attack-milder pain and sensory enhancement can continue for hours to days. The chronification mechanism results in a continuous alteration in such a way that the sensory network reacts to the environment causes and unstable plasticity of the sensory system.[8,9]

The calcitonin gene-related peptide (CGRP) is almost exclusively found in neurons and is most abundantly expressed in sensory nerves.[10,11] The secretion of CGRP from the trigeminal nerves plays a crucial role in migraine pathogenesis. CGRP is a potent cerebral and dural dilator and is involved in the transfer of nociceptive information from intracranial vessels to the central nervous system (CNS).[12-14]

Activation of the the Trigeminovascular System (TS) plays a central role in the pathophysiology of migraine pain and its related symptoms.[15] Extensive evidence has shown that the chemical activation of TS by the use of NTG, a nitric oxide (NO) donor, is a safe and reliable model for the study of migraine.[16-19] The cause of NTG-mediated migraine pain is thought to be the result of vasodilation of the cranial blood vessels and activation of the pathways involved in nociception.[20] Several studies have shown that CGRP promotes the synthesis and release of NO, which in turn supports the CGRP as well. Both of these substances can enhance the activity of each other.[21,22] A nonpeptide CGRP-receptor antagonist, BIBN4096BS, is highly specific towards human CGRP receptor[23] and is the first CGRP antagonist developed. *In vitro* studies with human cephalic arteries have shown that it potentially blocks the CGRP effect.[24]

In the present study, we the authors are attempted to provide the insight of neurobehavioural, molecular, and histological changes produced by BIBN4096, a CGRP antagonist in the NTG-induced migraine model.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 180–250 g, were purchased from the Central Animal House, Annamalai University. The Institutional Animal

Ethics Committee approved all protocols (Registration No.: 160/199/CPCSEA and proposal no. 1152). The study was performed as per the health guidelines for the care and use of laboratory animals. All rats had free access to food and water except during behavioural observations, and a constant temperature (25±1°C) and a 12:12 hr cyclic lighting schedule were maintained.

Drugs

Experiments were performed using a commercially available preparation of nitroglycerin at 5 mg/ml (Neon Laboratories Ltd., Mumbai, India), intraperitoneal (i.p) at a dose of 10 mg/kg. The drugs ergotamine and sumatriptan were received as a gift sample from Dr Reddy's Laboratory, Hyderabad. BIBN4096 was purchased from Tocris Bioscience (Bristol, UK), which was diluted in saline with dimethyl sulfoxide (DMSO) and slowly injected subcutaneously (1mg/kg).

Grouping of migraneous animal

Twenty-five Wistar rats were randomly divided into the following five groups consisting of five animals each:

(A) Control group, where the Wistar rats received an i.p injection of normal saline with DMSO; (B) NTG group, where the Wistar rats received an i.p injection of 10 mg/kg body weight of NTG in alternative days consecutively for the first week and followed by weekly once up to four weeks; (C) NTG + Ergotamine; (D) NTG + Sumatriptan; (E) NTG + BIBN4096.

A nociceptive study using the hot plate method

The hot plate method was used to evaluate the pain thresholds of the tested animals. The rats were placed in a hot plate covered with glass chamber, and the temperature was maintained at 55°C. The latency time for the pain sensation was kept at a maximum of 20 sec to prevent burning of the skin. The time taken for the rats to respond to the thermal pain, shown by licking their paws or jumping from the basement, is considered as the latency period.

Neurobehavioral assays

Behavioural assays were performed in a calm environment and the same condition and time were maintained for all the experimental days. All animals were brought to the testing room 30 min before the study to habituate.

Locomotor assay

The behaviour of the animal locomotive was tracked using an actophotometer. Animals were individually put in the actophotometer and the basal activity score was reported over 5 min. Every animal was treated with the respective drugs and the activity score was calculated.

Open field test

Open field test was performed to assess the anxiety and explorative behaviour of the animals in a quiet and undisturbing environment. This test was carried out in an open area arranged in a wooden square box measuring 100 × 100 × 40 cm, coloured with black paint on the floor as well as the inner walls. The wooden box was divided into 25 squares each measuring 20 × 20 cm; among these, 16 squares were in the peripheral region and nine squares were in the central area. The experiment was started by initially placing the rats induced with various experimental conditions at the corner portion of the open field facing towards the opaque sidewall and allowing them to move freely in the area for 5 min. The behavioural changes of the animals were analysed by measuring their rearing and grooming behaviour and counting the number of squares they crossed at the central and the peripheral region.

Elevated plus maze (EPM) test

The test for anxiety-like behaviour was used for the screening of anti-anxiety drugs. The apparatus employed for this study was "elevated plus maze," with a configuration of a plus (+) and comprising of two open arms (50 × 10 × 1 cm) across from each other and perpendicular to two closed arms (50 × 10 × 32 cm) with a centre platform (10 × 10 × 1 cm). The open arms consist of a very small (1 cm) wall to decrease the number of falls, whereas the closed arms have a high (32 cm) fence to enclose the component. The entire apparatus was kept at a height of 50 cm above the floor and placed in the empty circular tank (100-cm diameter, 35-cm tall; normally used for the Morris water maze task)—it must be placed 50 cm above the floor to protect the rat from falling or attempting to escape during the experiment. The number of entries and the time spent in each arm was calculated.

Forced-swim test (FST)

This test was employed to evaluate the antidepressant effect of a drug by keeping the rat in an unusual, stressed environment. The rats were induced with various treatment conditions and were placed into a vertical glass cylinder (50 × 28 × 40 cm) filled with water at 30°C. It was ensured that the hind limbs and tail of the animal do not touch the base. Duration of the test procedure for each rat placed in a cylinder was scheduled for 3 min. Following the subsequent series of efforts made by the rat to get away, it finally stayed in a static position and the floating time was calculated as the immobility time in seconds.

Biomarker analysis in blood and various regions of the brain

After the establishment of the experimental model, blood was collected from the rats by retro-orbital punching, and the concentration of NO was measured colorimetrically. Rats were sacrificed after the behavioural studies, and the skull was removed. The brain of the animals was rapidly removed and dissected. Various regions of the brain such as brain stem, cerebral cortex, as well as trigeminal ganglion were quickly removed and placed over dry ice. The expression levels of TNF-α in serum and the CGRP were determined at different sites of the brain as well as plasma using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Bioassay Technology Laboratory, Shanghai, China).

Histopathological study

Cresyl violet (CV) staining

The whole brain of randomly selected rats from each group was used for histopathological examination. Nissl body staining was performed to assess the extent of neuronal damage that occurred in the cerebral cortex of the rat's brain. Coronal brain sections were stained with (0.1% w/v) Cresyl violet acetate (Nissl stain) for 5 min, followed by dehydration through graded concentrations of ethanol, and cleared using xylene. The stained sections were visualized under a light microscope (BX40 Olympus, Melville, NY). The appearance of Nissl-stained dark neurons (NDN) was analysed in the cerebral cortex region of the rat's brain of control and experimental groups. The digital images were recorded using a microscope coupled with a digital camera [Nikon D70 DSLR (6.1 megapixels)].

Statistics Analysis

The statistical analysis of the data was carried out using one-way ANOVA followed by *post hoc* Tukey's multiple comparisons test using GraphPad Prism (Version 7). All the results are expressed as Mean ± SEM. Where $P<0.05$ was considered as statistically significant.

RESULTS

Effect of thermal hypersensitivity in NTG-induced model

The pain threshold of NTG-induced animals was observed to be significantly decreased ($p<0.001$) all throughout the four weeks when compared to the control animal. In thermal hypersensitivity of ergotamine, the first week ($p<0.53$) and second week ($p<0.10$) were non-significant as compared to the NTG-induced group. Whereas, the third ($p<0.05$) and fourth weeks ($p<0.05$) showed a significant effect. The sumatriptan-treated group showed an insignificant impact in the first week (p<0.10) but a substantial impact throughout the remaining weeks ($p<0.05$). Interestingly, the BIBN4096 group showed a statistically significant effect in four weeks ($p<0.01$).

Neurobehavioural investigations

Locomotor activity of NTG-induced model

The locomotor activity of NTG-induced animals was compared with control, NTG, NTG + ergotamine, NTG + sumatriptan, and NTG + BIBN4096 treated animals. The data showed that the locomotor activity of the NTG-treated group significantly reduced ($p<0.001$) when compared to the control group. The ergotamine group showed a non-significant ($p<0.15$) effect. A statistically significant effect was observed with sumatriptan ($p<0.05$) and BIBN4096-treated groups ($p<0.05$) as compared to the NTG-induced group.

Open field test

As shown in Figure 3, all the peripheral ($p<0.001$), central (p<0.01), grooming ($p<0.01$) and rearing ($p<0.001$) activities of the NTG-induced group significantly reduced ($p<0.01$) when compared with control animals. Ergotamine showed a non-significant increase in peripheral ($p<0.98$), central ($p<0.89$) and rearing ($p<0.99$) activities. Treatment with sumatriptan showed a non-significant increase in peripheral ($p<0.70$), central ($p<0.69$) and rearing ($p<0.56$) behaviours. Sumatriptan ($p<0.05$) and BIBN4096 ($p<0.05$) were observed to significantly decrease grooming activity when compared with the NTG-induced group.

Elevated plus maze (EPM) test

The EPM test was used to assess the anxiety-like behaviour of rodents. From Figure 4, it is evident that the time spent in the open ($p<0.001$) and closed ($p<0.001$) arms in the model group significantly differed as compared to control. BIBN4096 showed a significant difference in the time spent in open ($p<0.05$) and closed ($p<0.05$) arms. Ergotamine-treated animals were observed to spend less time available in open ($p<0.76$) and closed ($p<0.75$) arms when compared with the NTG-induced group. Sumatriptan-treated animals showed a nonsignificant difference in the time spent at open ($p<0.17$) and closed ($p<0.16$) arms as compared to NTG-treated animals.

Forced-swim test

The forced-swim test is gold standard for assessing depression activity. As per the result shown Figure 5, NTG injection induced an increase of immobility time of model rats, which was statistically significant ($p<0.001$) as compared to control rats. However, insignificant immobility was observed with ergotamine ($p<0.35$), sumatriptan ($p<0.27$) and BIBN4096 ($p<0.09$) treated animals when compared with the model group. The immobility time of all treated groups was comparable, but the result was not statistically significant.

Biomarkers in blood and various regions of rat's brain

As shown in Figure 6A, a significant reduction of plasma nitric oxide level in both sumatriptan ($p<0.01$) and BIBN4096 ($p<0.01$) treated animals was noted when compared with the NTG-induced group. A significantly increased NO level was observed with NTG-treated animals as compared to control animals. Figure 6B revealed the plasma concentration of CGRP in treated groups, where sumatriptan ($p<0.05$) and BIBN4096 ($p<0.05$) significantly reduced the concentration of CGRP when compared with the NTG-treated group. The serum concentration of TNF-α significantly ($p<0.01$) increased in NTG-treated animals as compared with control animals. BIBN4096 showed a significant ($p<0.05$) decrease in the concentration of TNF-α when compared to NTG but an insignificant difference was observed in the ergotamine ($p<0.24$) and sumatriptan-treated ($p<0.08$) animal groups (Figure 6C). In the isolated regions of rat's brain such as brain stem, cerebral cortex, and trigeminal ganglion, a significant elevation of CGRP was observed in NTG-treated animals ($p<0.01$) when compared with control animals. Whereas, the BIBN4096-treated ($p<0.05$) group showed a significant decrease in the

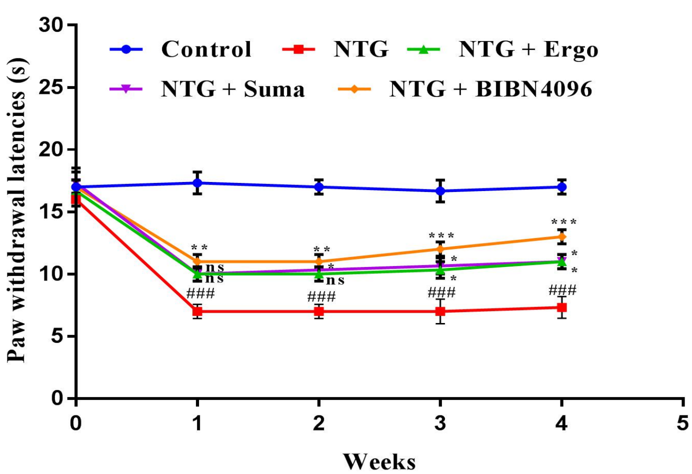

Figure 1: Effect of nitroglycerin in thermal hypersensitivity of rats. The results are the Mean ± SEM. Where, #comparing with control and NTG, *comparing NTG with treated groups. Where, ###$p<0.001$, ***$p<0.001$, **$p<0.01$ and *$p<0.05$. $p<0.05$ considered as statistically significant; ns denotes non-significant. One-way ANOVA with Bonferroni's *post hoc* test was used for statistical analysis. Ergo-Ergotamine, Suma-Sumatriptan.

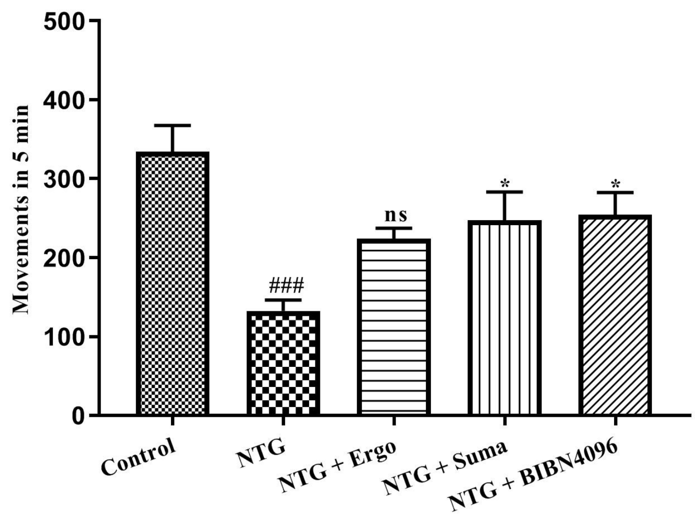

Figure 2: Effect of locomotor activity of NTG induced rats. The data represents the Mean ± SEM. #denotes comparing with control vs NTG, *comparing NTG vs treated groups. Where, ###$p<0.001$, *$p<0.05$. and $p<0.05$ are considered as statistically significant; ns-denotes non-significant. Ergo-Ergotamine, Suma-Sumatriptan. One-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

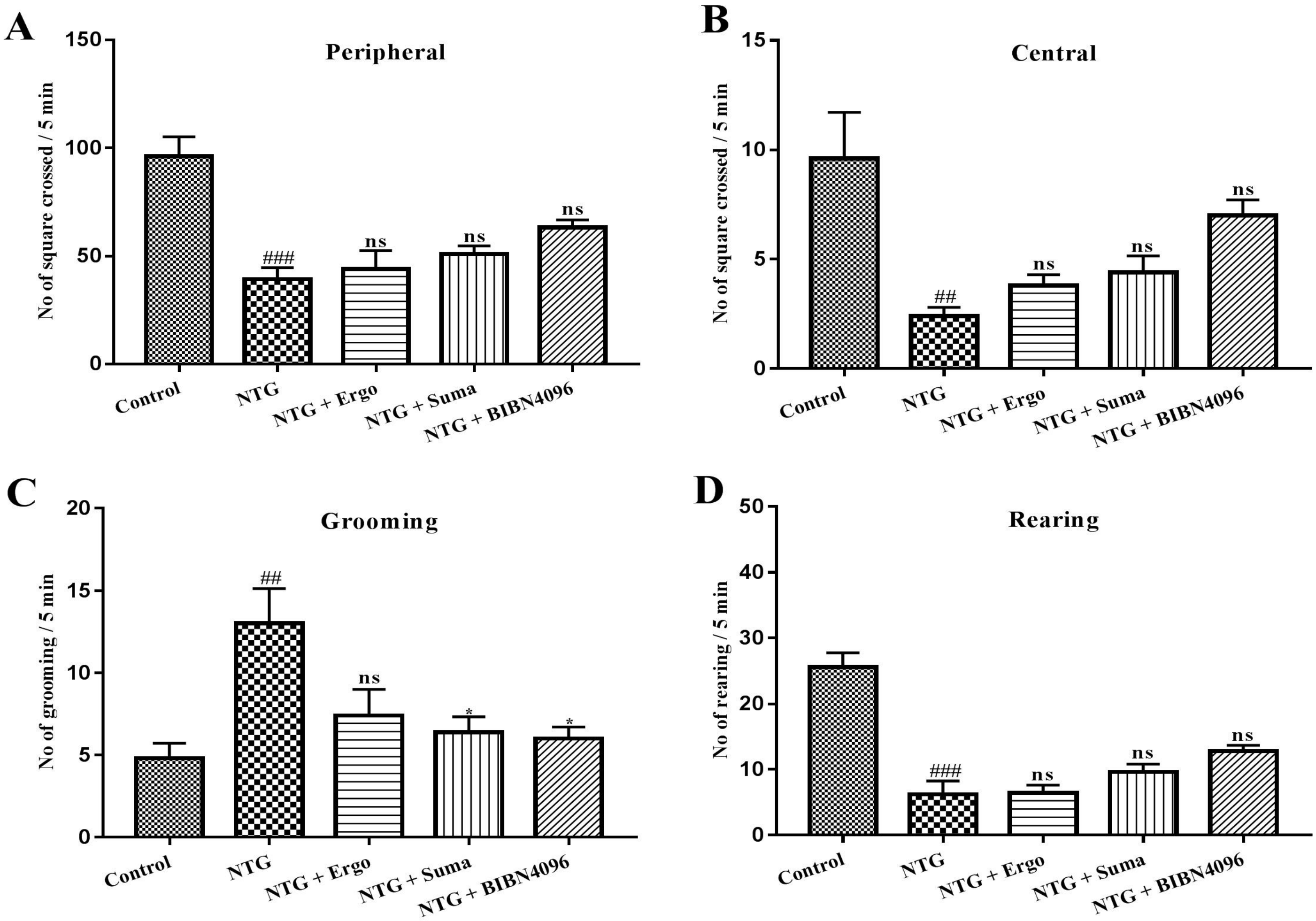

Figure 3: The neurobehavioral assay with rats using the open field test (A) Number of squares crossed peripherally within 5 min; (B) Number of squares crossed in the central region within 5 min; (C) Total number of grooming within 5 min; (D) The total number of rearing within 5 min. The data represented are the Mean ± SEM. #comparing, control and NTG, *comparing NTG and treated groups. Where, ##*p*<0.01, ###*p*<0.001, **p*<0.05. *p*<0.05 are considered as statistically significant; [ns]denotes non-significant. One-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

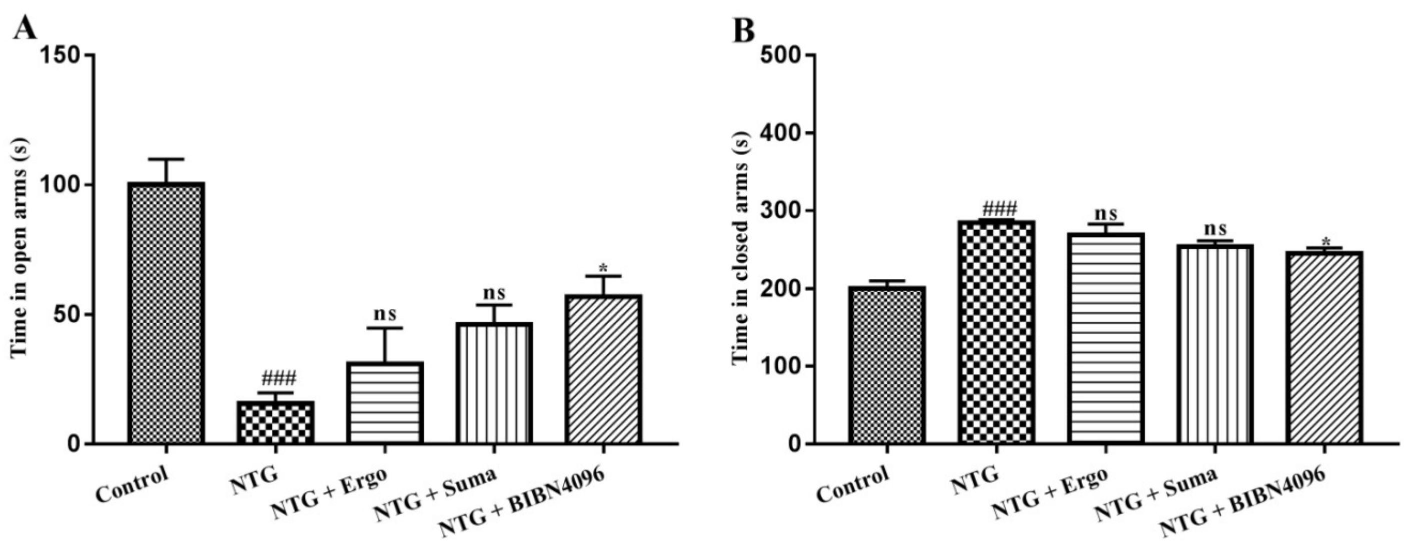

Figure 4: Effect of neurobehavioral characteristics of BIBN4096 in NTG-induced rats. (A) Time spent in open arms of the EPM; (B) Time spent in closed arms of the EPM. The data represents Mean ± SEM. Where, #comparing control and NTG, *comparing NTG with treated groups. ###p<0.001, ****p*<0.001, ***p*<0.01 and **p*<0.05. *p*<0.05 considered as statistically significant; ns-denotes non-significant. One-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

expression of CGRP than NTG model group. All the remaining groups were comparable but statistically insignificant (Figure 6D).

Histological observation of cerebral cortex of the rat

The Cresyl violet staining of cerebral cortex regions of rat's brain showed distinct histopathological changes with ergotamine, sumatriptan, and BIBN4096 in the NTG-induced animal model. Normal morphology was seen with control animals. While NTG treatment caused an extensive neuronal loss, the cytoplasm was shrunken and damaged nuclei known as Nissl-stained dark neurons (N-DNs) were observed. Interestingly, sumatriptan and BIBN4096-treated animals exhibited nearer to normal morphology (Figure 7).

DISCUSSION

Migraine is one of the leading causes of health-related disability worldwide. Prodromal, headache, and postdrome phases of migraine are known to have an impact on productivity at work, apart from causing cognitive impairment. The burden of migraine affects individuals, their families and society.[25,26]

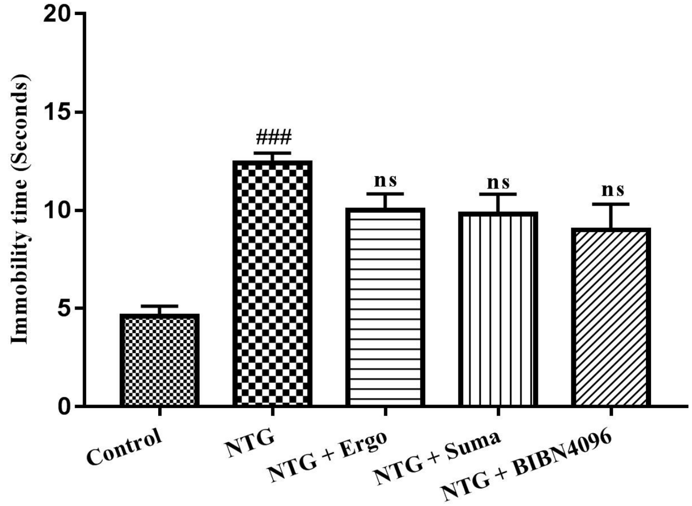

Figure 5: Effect of forced swim test in NTG induced rats. The data represented are the Mean ± SEM. Where #comparing control and NTG. $p<0.05$ considered as statistically significant; ns-denotes non-significant. One-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

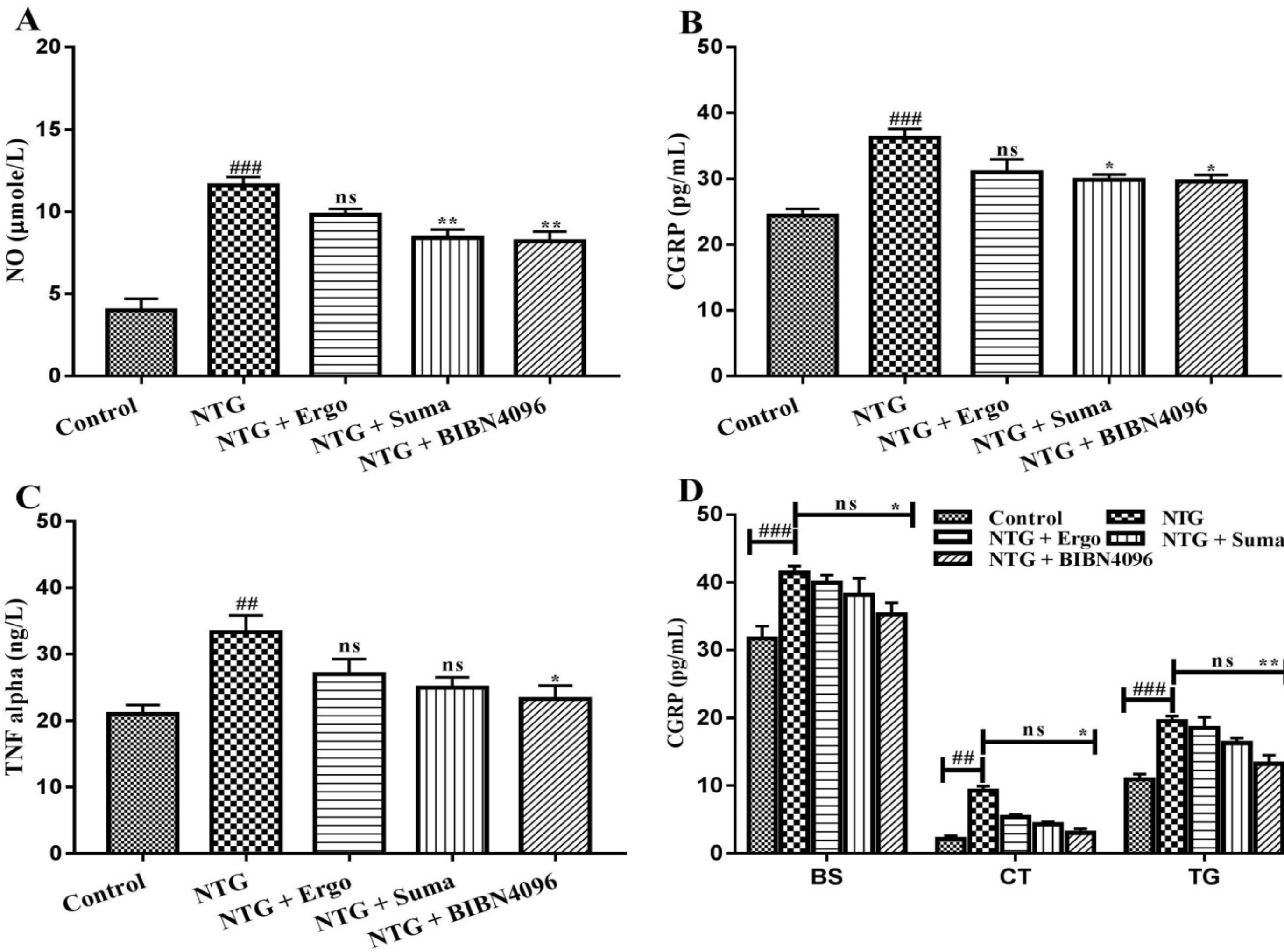

Figure 6: BIBN4096 in nitroglycerin induced biomarkers expression in rat's blood (A) NO, (B) CGRP, (C) TNF-α, and (D) CGRP in various regions of rat's brain. The results are the Mean ± SEM. Where, #comparing with control and NTG, *comparing NTG with treated groups. Where, ###p<0.001, ***$p<0.001$, **$p<0.01$ and *$p<0.05$. $p<0.05$ considered as statistically significant; ns-denotes non-significant. One-way ANOVA with Tukey's *post hoc* test was used for statistical analysis. Where, BS-Brain Stem, CT-Cerebral Cortex, TG-Trigeminal Ganglion.

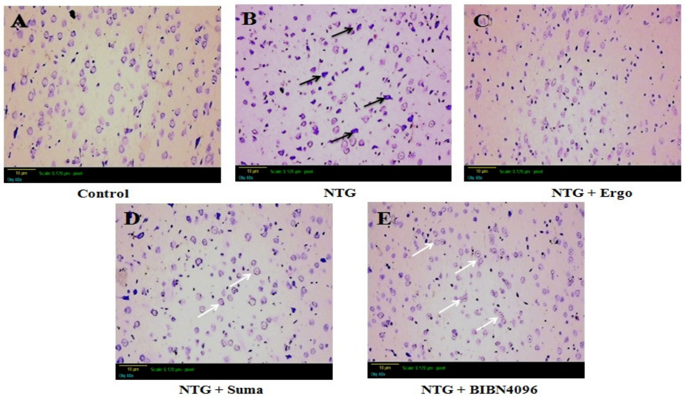

Figure 7: Effect of BIBN4096 on nitroglycerin-induced changes in the cerebral cortex of the rat's brain (A) Control, (B) NTG, (C) NTG + Ergo, (D) NTG + Suma, (E) NTG + BIBN4096. The appearance of NDN in the cerebral cortex sections was visualized under the light microscope in the magnification of × 400 of the control and experimental group of rats analyzed. Normal morphology was seen with control animals, whereas NTG showed increased NDN while NTG + BIBN4096 showed nearer to normal morphology. White arrow indicates regenerated neurons and the black arrow indicates degenerated neurons.

NO is known to be directly linked with pain processing[27] and associated symptoms of migraine headache.[16,28] NO donor NTG-induced animals are reported to be a good experimental model to demonstrate several behavioural patterns similar to human migraine.[29] The stimulation of the TS occurs, which is the most important inducing factor for migraine.[15] Systemic administration of NTG produces deferred sudden headaches through vasodilatation, activation of TS and central sensitization.[30-33]

The presence of NO in the periphery as well as in the major sites leads to the development and maintenance of hyperalgesia.[34] The induction of hyperalgesia by NTG might be through the vasodilatation and activation of TS.[32] In the present study, NTG-induced hyperalgesia was observed following each injection[35] attenuated by sumatriptan, which is in agreement with reports of Bates and co-workers,[20] and we found similar effects with the BIBN4096-treated group as well. This hypersensitivity was accompanied by an elevated CGRP level in plasma (Figure 6B) as well as various brain regions of rat's brain such as brain stem, cerebral cortex and trigeminal ganglia (TG) (Figure 6D). Interestingly, elevated levels of CGRP were found to be attenuated by BIBN4096 treatment.

The primary headache, in particular, migraine, has a bidirectional relationship with depression and anxiety.[36,37] Comorbidities of depression and anxiety may increase headache frequency in migraineurs.[38] Open field and elevated plus-maze tests are used to evaluate the anxiety-like behaviours of animals. The locomotor and rearing behaviours of animals are also known to reflect depression.[39] From the study we found that the locomotor (Figure 2), peripheral, and central movement (Figure 3A&B) and rearing (Figure 3D) activities were drastically reduced in the NTG-induced group and were significantly regained on treatment with BIBN4096. The grooming behaviour of rats was considered as pain perception or anxiety through various stressors.[40] The present study revealed that NTG induced grooming behaviour, which was significantly reduced on treatment with BIBN4096. The elevated plus-maze has been primarily used to determine anxiety of animals.[41] In the present study, we adopted this technique and found that the NTG-induced group showed a significant increase in the time spent in closed arms and a significant decrease in the time spent in open arms. Similarly, the BIBN4096 group also showed a significant difference in the time spent in both the open and closed arms (Figure 4). FST has been most widely used as a model for evaluating depression and antidepressant activity in rodents. In the present study, the data were comparable, but no significant difference among the groups was found regarding the immobility behaviour in FST—this report is in agreement with the previous study.[42]

Proinflammatory cytokine TNF-α plays an important role in migraine pain and promotes the sensitization of the meningeal nociceptors.[43] TNF-α increases the expression of excitatory neurotransmitter and decreases the expression of inhibitory neurotransmitters,[44] leading to the occurrence of cortical spreading depression (CSD). BIBN4096 attenuates the expression of TNF-α and may lead to antidepressive-like activity. The histopathological reports showed a wide range of neuronal damage in the cerebral cortex on treatment with NTG injection, and this effect is similar to the finding of Lipton.[45] This effect was reversed by BIBN4096 treatment by regeneration of neurons. But comparably with ergotamine and sumatriptan, the BIBN4096-treated group changed the damage of neurons and induction of regeneration as well; these results were evident in neurobehavioral and inflammatory studies.

CONCLUSION

From the present study, we found that CGRP antagonistic drug-like BIBN4096 gives a special effect than the other antimigraine drugs such as ergotamine and sumatriptan. Although the study was carried out carefully, there continues to be a lack of significant difference in the behavioural, molecular and histopathological results. Hence, a treatment strategy involving modified housing conditions as an adjuvant along with drugs may help to improve the behavioural and molecular level imbalance in migraine conditions.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ABBREVIATIONS

NTG: Nitroglycerin; **CGRP:** Calcitonin gene related peptide; **TS:** Trigeminovascular system; **NO:** Nitric oxide; **TG:** Trigeminal ganglia; **FST:** Forced swim test; **TNF-α:** Tumour Necrosis Factor alpha; **Ergo:** Ergotamine; **Suma:** Sumatriptan.

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