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

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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 neurobehavioural 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) rearing 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.

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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. 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. Furthermore, the overuse of medication is also known to lead to headaches. Migraine affects 12% of the world’s population, particularly individuals who are less than 50 years of age. 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 and is known to be linked with autonomic phenomena in the face, typically reddening of the eyes, tearing, flushing, or pallor. 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.

The calcitonin gene-related peptide (CGRP) is almost exclusively found in neurons and is most abundantly expressed in sensory nerves. 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 noxious information from intracranial vessels to the central nervous system (CNS). Activation of the trigemino-vascular system (TS) plays a central role in the pathophysiology of migraine pain and its related symptoms. 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. 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. 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. A nonpeptide CGRP-receptor antagonist, BIBN4096BS, is highly specific towards human CGRP receptor and is the first CGRP antagonist developed. In vitro studies with human cephalic arteries have shown that it potentially blocks the CGRP effect.

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 puncturing, 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 acetae (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 concentration of TNF-α.
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<0.001, ##p<0.01, *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<0.01 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.
NO is known to be directly linked with pain processing and associated symptoms of migraine headache. NO donor NTG-induced animals are reported to be a good experimental model to demonstrate several behavioural patterns similar to human migraine. The stimulation of the trigeminovascular system; 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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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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