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Research Article

Effect of zonisamide and *Nigella sativa* on blood-brain barrier permeability and neurological severity in traumatic brain injury-induced mice

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ABSTRACT

Traumatic brain injury (TBI) is a primary public health issue that has resulted in millions of deaths and disabilities worldwide. There are a variety of drugs available to help with TBI-related consequences, but they do not prevent further impairment. As a result, new therapeutic drugs that protect against neuronal damage caused by trauma and its implications, particularly secondary injury, are needed. Swiss albino mice (25-30 g) of either sex were utilized in the investigation. The weight-drop method was used to cause TBI. Following the treatment of zonisamide (100 mg/kg) and *Nigella sativa* (NS) (300 mg/kg) separately and in combination, blood-brain permeability was assayed. The albumin content in CSF and the level of Evan's blue in the brain diminution significantly in drug-treated groups. The neurological severity score in the co-administered group was found similar to that of the control group (no significant difference compared to the control group) on days 7 and 21. The results affirmed the potential of both drugs in preventing TBI-induced blood-brain barrier damage and reducing neurological severity.

Keywords:
Blood-brain barrier, *Nigella sativa*, Neurological severity score, Traumatic brain injury, Weight drop model, Zonisamide

1. INTRODUCTION

Traumatic brain injury (TBI) is typically characterized as a disruption in normal brain functioning induced by a blunted or strident head impact. It is also a leading cause of death and long-term disability all around the world[1]. TBI is projected to impact over half of the world's population once in their lifetime, with a yearly prevalence of around 50 million cases. Severe TBI has a death rate of more than 30% in underdeveloped nations like India, and it can cause significant morbidity in 60%-70% of patients[2].

TBI can cause several pathologic processes, including neuroinflammation and the disruption of the blood-brain barrier (BBB), according to growing data[3]. The BBB, which controls the movement of blood elements into and out of the extracellular brain matrix, protects the central nervous system. In reality, BBB breakdown is becoming more common in various central nervous system (CNS) illnesses, including acute injuries like TBI and stroke, as well as chronic neurodegenerative disorders[4]. BBB dysfunction is seen frequently in TBI patients and is a primary cause of high mortality and morbidity. More importantly, clinical data showed that BBB damage in TBI patients might last for years and is strongly linked to long-term neurological impairments[5]. As a result, they assess the level of BBB breakdown following TBI and elucidate the fundamental molecular pathways in animal models. Additional measurements such as BBB testing may benefit the diagnosis and long-term management, while these approaches focus on the clinical neurological score.

Zonisamide is a novel broad-spectrum antiepileptic drug that works well for refractory partial seizures[6-8]. Zonisamide has a structural, molecular, and pharmacokinetic profile apart from other antiepileptic drugs[8]. By blocking voltage-dependent Na^+ channels, reducing voltage-dependent T-type inward Ca^{2+} currents, binding to the GABA-benzodiazepine receptor complex, and

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inhibiting voltage-dependent Na^+ channels, zonisamide is thought to aid dopaminergic and serotonergic neurotransmission. Ca^{2+}-dependent K^+ triggered extracellular glutamate release is reduced by zonisamide.

Recently, plants and herbs' low toxicity and cost-effectiveness have gained attention to investigate potential pharmacological activities[9]. In several human and animal investigations, *Nigella sativa* (NS) and its active component, thymoquinone, have been shown to have antioxidative properties[10]. Immunomodulatory[11], neuroprotective[12], antibacterial[13], hypertensive[14-15], and hypoglycemic[16] effects.

2. MATERIALS AND METHODS

2.1. Animal

The study used Swiss albino mice (25-30 g) of either sex, kept under natural day and night cycles in polypropylene cages with rodent food and water ad libitum. All experimental procedures were permitted by the Institutional Animal Ethics Committee of Maharshi Dayanand University Rohtak, Haryana, India.

2.2. Induction of TBI by weight drop method

Mice were anesthetized with 2% isoflurane and were allowable to breathe naturally without tracheal intubation. After that, mice were placed on a sponge using surgical tape, and a small longitudinal incision was given to the overhead of mice exposed to the skull. The metallic disc was centrally fixed on the exposed skull, and adequately placed the mice under the metallic pipe. Then the metallic spherical weight (60 g) freely falls through the metallic pipe over the head of the mice. After that metallic disc was removed, and the exposed skull was sutured. Finally, Neosporin powder was spread over the surgery site and returned to its home case for recovery.

2.3. Treatment schedule

The experimental study was designed to determine albumin content in cerebrospinal fluid (CSF), Evan's blue in the brain at 24h, and Neurological severity score on days 1, 7, and 21. The zonisamide and NS were administered per se and in combination 30 min after TBI induction. Mice were divided into five groups, each consisting of 6 mice. Group 1, the control group, did not receive any injury or drug treatment, whereas TBI was induced in all other four groups then Group 2 received vehicle, Group 3 received zonisamide (100 mg/kg), Group 4 received NS (300 mg/kg) and Group 5 received combination of both zonisamide (100 mg/kg) and NS (300 mg/kg).

2.4. Estimation of blood-brain barrier (BBB) permeability

2.4.1. Measurement of albumin content in cerebrospinal fluid (CSF)

CSF was collected 24h post TBI induction. Firstly, the capillary made up of borosilicate glass, length of 10 cm, an outer diameter of 1 mm, and an internal diameter of 0.75 mm, were divided into two parts using flam. Then, the capillary tip was trimmed with scissors to its inner diameter of about 0.5 mm. The mice were then anesthetized. The neck area was shaved and cleaned with a cotton swab using 70% ethanol. The longitudinal incision was given on the neck and removed the subcutaneous muscles to expose the dura mater of the cisterna magna. Then, the mouse was laid down at a 135° angle to the body for CSF collection. The prepared capillary was penetrated to the cisterna magna through the dura mater. After collecting CSF in the capillary, the capillary was connected with a 3 mL syringe using polyethylene tubing with a 1 mm internal diameter. CSF was collected into a 2 mL tube[17]. After CSF sampling, the suture was inserted and injected with the 1 mL normal saline subcutaneously to prevent dehydration, and albumin content was estimated using the following method. In this method following solutions were prepared:

Working standard albumin solutions: 50, 100, 150, 200, 250, and 300 mg/dL solutions were prepared by dissolving bovine serum albumin powder (Loba Chemie Pvt. Ltd., Mumbai, India) in 0.9% NaCl solution.

Bromocresol green working reagent: 12.5, 2.5, 1.25 and 0.125 mg/dL solutions of bromocresol green dye (Loba Chemie Pvt. Ltd., Mumbai, India) were prepared in distilled water.

After CSF collection, the 50 µL CSF was mixed with 5 mL of 2.5 mg/dL strength of bromocresol green working reagent, which produced the green color. Then, the absorbance was measured at 630 nm using a spectrophotometer with the same procedure, 50 µL of each working albumin standard solution was prepared above, and absorbance was checked at 630 nm. Then the concentration of albumin content in CSF was estimated. The data was expressed as mg/dL.

2.4.2. Evan's blue extravasation method

The integrity of BBB was explored using Evan's blue (Loba Chemie Pvt. Ltd., Mumbai, India) extravasation as described by Ding-Zhoue et al. After induction of TBI, mice were injected with 100 µL Evan's blue (2% in PBS) through the tail vein[18]. The mice were sacrificed after 24h, and the whole brain from each mouse was removed and placed at -55°C for some time to fix the enzymatic activity. Then these brains were homogenized in formamide (Rankem Pvt. Ltd., Delhi,

Table 1. Various tests for assessment of NSS.

Task	Description	Success	Failure
Exit circle	Ability to exit a circle of 30 cm diameter	0	1
Mono/hemiparesis	Paresis of the contralateral side's upper and lower limbs	0	1
Straight walk	Activeness and motor capacity to walk straight when placed on a level surface.	0	1
Startle reflex	Ability to bounce when we clap	0	1
Seeking behavior	Indication of 'interest' in their surroundings	0	1
Beam balancing	Ability to balance for at least 10 sec. on a 5 mm diameter stick.	0	1
Round stick balancing	Ability to balance for at least 10 sec. on a round stick with a diameter of 5 mm.	0	1
Beam walk: 3mm	Ability to cross a 30 cm long beam of 3 cm width	0	1
Beam walk: 2mm	The same task with a 2 cm wide beam,	0	1
Beam walk: 1mm	The same task with a 1 cm wide beam	0	1
Maximum score		0	10

India) (1:20 w/v) and incubated at 60°C overnight. Evan's blue concentration in the supernatant was evaluated spectrophotometrically at 620 nm after centrifuging the homogenate at 14,000 rpm for 30 minutes. The results were expressed in µg Evans blue/g tissue[18].

2.5. Neurological severity score

A 10 points NNS was employed to assess post-traumatic neurological damage, which includes 10 different types of tests, as mentioned in Table 1[18]. This test was performed after 1, 7, and 21 days and the drug was administered after half an hour of TBI induction and continued for 21 days after every 24h.

2.6. Statistical analysis

The data were analysed using one-way ANOVA, followed by Bonferroni's multiple comparison post hoc test. Data were denoted as mean±SEM, and statistical significance was denoted as **or *** for $p<0.01$ and 0.001, respectively. p values were shown with '#' representing $p<0.05$ when compared with the vehicle.

3. RESULTS

3.1. Effect of zonisamide and NS on albumin content in CSF

The albumin content was observed at 24h, and the results are described in Figure 1. The albumin content in CSF was drastically increased in the vehicle-treated group. However, co-administration of both zonisamide (100 mg/kg) and NS (300 mg/kg) showed a more considerable reduction in the albumin content in CSF than in vehicle-treated groups.

3.2. Effect of zonisamide and NS on the level of Evan's blue in the brain

The amount of Evan's blue in the brain was observed at 24h, and the result is described in Figure 2. The amount

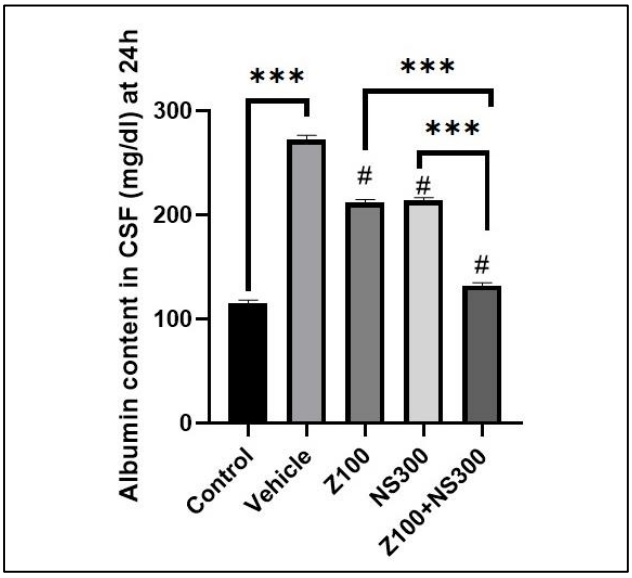

Figure 1. Effects of zonisamide, NS, and their combination on albumin content in CSF at 24h
The data were analyzed using one-way ANOVA, followed by Bonferroni's multiple comparison post hoc test. Data were denoted as mean±SEM, and statistical significance was denoted as **or *** for $p<0.01$ and 0.001, respectively. p values were shown with '#' representing $p<0.05$ when compared with the vehicle.

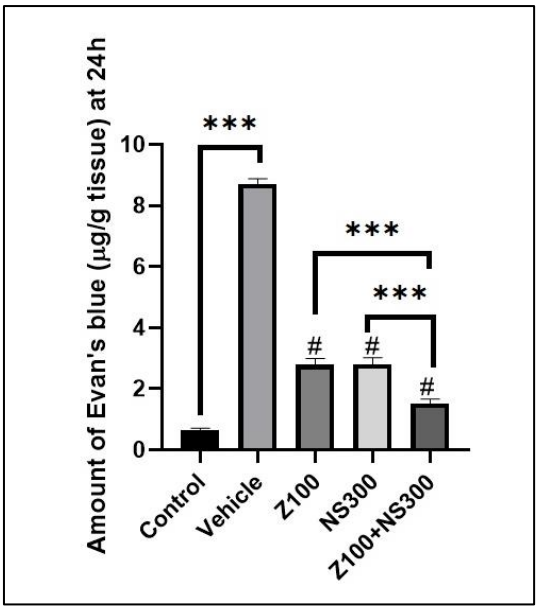

Figure 2. Effects of zonisamide, NS, and their combination on the level of Evan's blue in the brain at 24h
The data were analyzed using one-way ANOVA, followed by Bonferroni's multiple comparison post hoc test. Data were denoted as mean±SEM, and statistical significance was denoted as **or *** for p<0.01 and 0.001, respectively. p values were shown with '#' representing p<0.05 when compared with the vehicle.

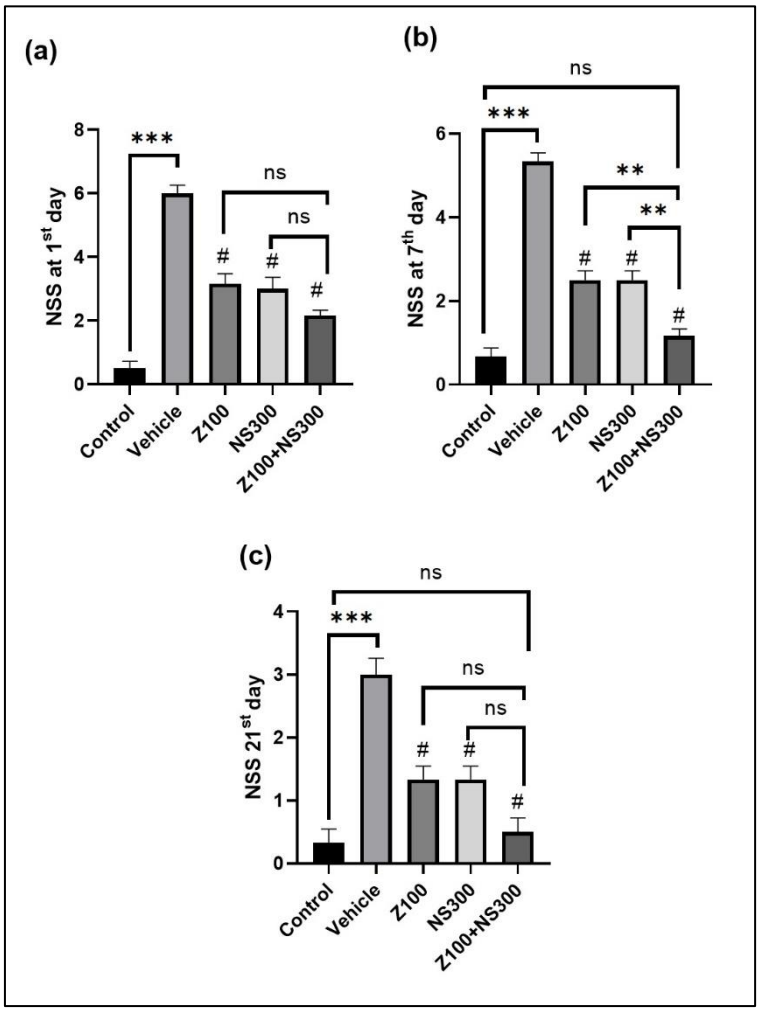

Figure 3. Effects of zonisamide, NS, and their combination on NSS on (a) day 1, (b) day 7, and (c) day 21
The data were analyzed using one-way ANOVA, followed by Bonferroni's multiple comparison post hoc test. Data were denoted as mean±SEM, and statistical significance was denoted as **or *** for p<0.01 and 0.001, respectively. p values were shown with '#' representing p<0.05 when compared with the vehicle.

of Evan's blue in the brain was drastically increased in the vehicle-treated group. However, co-administration of both zonisamide (100 mg/kg) and NS (300 mg/kg) showed a more considerable reduction in Evan's blue level in the brain than in vehicle-treated groups.

3.3. Effect of zonisamide and NS on Neurological severity score

The neurological severity score was observed on days 1, 7, and 21; the results are described in Figure 3. The neurological severity score was drastically increased in the vehicle-treated group. However, co-administration of both zonisamide (100 mg/kg) and NS (300 mg/kg) showed a more considerable reduction in neurological severity score than in vehicle-treated groups. Although, on days 7 and 21, the neurological severity score of the co-administered group showed no significant difference compared to the control group.

4. DISCUSSION

TBI remains a significant clinical and economic concern, with limited treatment options matched to its clinical and neurobiological course. The pathophysiology of the events that lead to subsequent brain injury after a TBI is complicated and multifactorial. TBI is accompanied by neuroinflammatory processes that contribute to brain edema and cell death, and BBB disruption is one of the pathological hallmarks of the injury. BBB comprises a continuous layer of astrocytes, pericytes, and basal lamina with brain capillary endothelial cells. To maintain cerebral homeostasis, tight connections between endothelial cells provide a metabolic and physical blockade that prevents macromolecules from moving from the blood to the brain[19]. The therapeutic window for BBB regulation after TBI is uncertain, and the biphasic disruption of the BBB by direct endothelial cell destruction, which develops a few days after TBI, further complicates the injury[20]. After, BBB breakdown can disrupt brain homeostasis and the clearance of toxic compounds like -amyloid, speeding up neuronal damage and contributing to TBI-related late-life neurodegeneration[21-22]. The generation of ROS and inflammatory mediators is one of the mechanisms driving BBB failure following injury. After a TBI, the BBB permeability increases quickly, following a local immunological response that stimulates astrocytes and microglia[23]. This immune-mediated response modifies extracellular homeostasis and excitability by altering glutamate and potassium buffering or by producing or releasing inflammatory mediators locally or extravagantly[24].

Although inflammation and disruptions in extracellular homeostasis are the leading causes of acute neuronal dysfunction, long-term healing is influenced by the brain's degree of neuronal remodeling and plasticity. BBB malfunction may also alter synaptic reconfiguration directly or cause neuronal toxicity and death, impacting local network activity[25]. This BBB dysfunction subsequently leads to edema formation and ultimately increases intracranial pressure. The elements of the neurovascular unit, including neurons, astrocytes, pericytes, microglia, and the extracellular unit, are significantly de-arranged due to increased intracranial pressure because it mechanically impairs cerebral blood flow, which causes an ischemic zone to develop and secondary lesions to progress. In the current study, the vehicle-treated group displayed more albumin content in CSF and a level of Evan's blue in the brain, affirming the destruction of BBB integrity. Intervention with zonisamide (100 mg/kg) and NS (300 mg/kg) per se significantly diminish the albumin content in CSF and the quantity of Evan's blue in the brain, indicating the ability of both experimental drugs to preserve BBB integrity. A recent study affirmed that thymoquinone, a major bioactive constituent of NS, preserves BBB permeability after polysaccharide-induced sickness behaviour[26].

Neurological deficiency is one of the significant secondary outcomes of disability after TBI, responsible for disabilities and immobility. Assessment of neurological function after drug administration help to evaluate the recovery of therapies in the initial phase of TBI. In the present investigation, NSS was also drastically increased in the vehicle-treated group. Co-administration of both experimental drugs showed a more considerable reduction in NSS than in vehicle-treated groups, demonstrating the progression of secondary injury. More interestingly, the NSS of the co-administered group showed a similar score (non-significant) on days 7 and 21 compared to the control group. The study by Yu et al. also demonstrated that combined treatment of lithium and valproate (antiepileptic drug) recover motor coordination from Day 7 and persevere till 21 days after TBI[27], consistent with the results attained in our study.

Similarly, in our previous studies, both zonisamide and NS per se and combination significantly improves behavioral pattern and diminishes oxidative stress and neurotransmitters alteration in fruit flies following TBI[28], along with a significant reduction in oxidative stress, glucose level, and blood pressure in mice following traumatic damage[29-30], indicating that combination of both drugs can be a possible therapeutic intervention to reduce secondary damage triggered by traumatic injuries in the brain by preserving BBB integrity.

5. CONCLUSION

TBI disrupts the normal physiology of BBB and alters various molecular pathways. According to the above observations, it was concluded that the administration of zonisamide and NS per se significantly prevents the destruction of BBB following TBI-induced neuro-

degeneration. Furthermore, it was also concluded that co-administration of both zonisamide and NS exhibited comparatively more neuroprotective effects in mice than their per se administration against TBI-induced neurodegeneration these neuroprotective properties could aid in developing these medications as a therapy for neuronal death caused by traumatic injuries.

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Conflict of interest

There are no conflicts of interest in this article's content.

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Ethics approval

Animals used for the present experiment were approved from the Institutional Animal Ethics Committee (IAEC) of Maharshi Dayanand University Rohtak, Haryana, India (Approval No. 153164/17-12-18).

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SK: Data curation, writing-original draft. GS: Conceptualization, supervision, validation, proofreading. All the authors have read and approved the manuscript for publication.

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