

# 6-Shogaol Attenuates Traumatic Brain Injury-Induced Anxiety/Depression-like Behavior via Inhibition of Oxidative Stress-Influenced Expressions of Inflammatory Mediators TNF- $\alpha$ , IL-1 $\beta$ , and BDNF: Insight into the Mechanism

Muhammad Afzal,\* Imran Kazmi, Anwarulabedin Mohsin Quazi, Shah Alam Khan, Aameeduzzafar Zafar, Fahad A. Al-Abbasi, Faisal Imam, Khalid Saad Alharbi, Sami I. Alzarea, and Neelam Yadav



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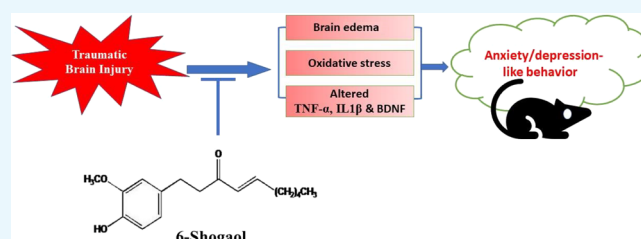
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**ABSTRACT:** Anxiety and depression are among the major traumatic brain injury-induced psychiatric disorders in survivors. The present study was undertaken to investigate the beneficial effects of 6-Shogaol against depression-like behavior and anxiety, induced by traumatic brain injury (TBI), in mice. The mice were administered either fluoxetine, vehicle, or three different doses (10, 20 and 30 mg/kg/day, i.p.) of 6-Shogaol after 10 days of impact-accelerated TBI. The treatment was continued for 14 consecutive days. Elevated plus maze test, marble burying test, staircase test, and social interaction test were employed to investigate the effect of 6-Shogaol on anxiety-like behavior. The impact of treatment on depression-like behavior was assessed using hyper-emotionality behavior or open-field exploration test. The expressions of brain-derived neurotrophic factor (BDNF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and malondialdehyde (MDA) levels in brain tissue and brain water were measured to elucidate possible mechanisms involved. 6-Shogaol treatment (higher dose) was able to attenuate anxiety/depression-like behaviors in mice with TBI. 6-Shogaol treatment also altered MDA formation and expressions of TNF- $\alpha$  and IL-1 $\beta$  that act as major inflammation-inducing cytokines in brain tissue. Additionally, brain BDNF levels were also affected by 6-Shogaol treatment. Although the lower dose of 6-Shogaol was able to rectify inflammation and BDNF expression in brain tissue, it was unable to improve anxiety/depression-like behaviors. 6-Shogaol treatment produced beneficial effects for TBI-induced anxiety/depression-like behaviors in mice, which could be attributed to the reduction of lipid peroxidation, inflammation, and enhanced BDNF expression.



## INTRODUCTION

Traumatic brain injury (TBI) is characterized as a disturbance of brain function due to an external physical force. It is a key cause of disorder and death among children and elderly people around the world. According to an estimate, approximately 10 million people per year experience TBI across the globe, of which 52,000 people die from TBI and almost 100,000 people develop new disabilities from the injury.<sup>1–3</sup> The survivors of TBI often develop various psychiatric complications.<sup>1–4</sup>

Among the psychiatric complications developed after TBI, major depression- and anxiety-related disorders are highly prevalent psychiatric complications.<sup>5</sup> The staggeringly high prevalence of depressive disorders after TBI (~60%) is worrisome as it not only leads to physical disability, unemployment, and functional dependence but also causes poor psychosocial functioning and community participation and a suicidal tendency among the survivors.<sup>5,6</sup> Many patients also develop anxiety disorders characterized by exaggerated anxiety, delayed-stress disorder, social phobias, obsessions, and

compulsions.<sup>7</sup> Although the mechanism is still non-elusive, it is hypothesized that chronic inflammation following TBI is a critical issue in the progress of nervousness and depression diseases. As a result, malondialdehyde (MDA) and brain-derived neurotrophic factor (BDNF) are altered in patients who develop post-TBI depression.<sup>8–10</sup> Recent research also identified a close association between post-TBI depression and inflammatory cytokines interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and IL-6.<sup>11,12</sup>

The rhizome of *Zingiber officinale* L. (Zingiberaceae), referred to as ginger in everyday language, is a widely used spice in food preparation and health practices. Among the

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active constituents found in ginger, 6-Gingerol and 6-Shogaol (dehydrated form of 6-Gingerol) possess a wide array of pharmacological properties that are responsible for analgesic, anti-inflammatory, antipyretic, antitussive, and hypotensive effects. 6-Shogaol is also potent against neuroinflammation and is associated with cell protection.<sup>13</sup> Herein, we examined the efficacy of 6-Shogaol against depression- and anxiety-like behaviors induced by TBI in an animal model. We further explored the underlying mechanism through which 6-Shogaol exerts antidepressant- and anti-anxiety-like effects in mice with TBI.

## RESULTS AND DISCUSSION

**Elevated Plus Maze Test.** Mice with TBI showed significantly increased close arm entries and time spent (Table 1). Pretreatment with 6-Shogaol at a dose of 10 mg/

**Table 1. Performance of Mice in the Elevated Plus Maze Test after 6-Shogaol Treatment<sup>a</sup>**

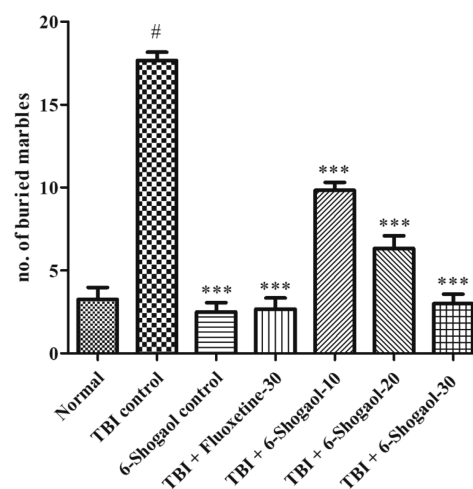
| group ( <i>n</i> = 6)      | no. of open arm entries    | no. of close arm entries   | total arm entries |
|----------------------------|----------------------------|----------------------------|-------------------|
| normal control             | 9.87 ± 0.32 <sup>#</sup>   | 1.11 ± 0.31 <sup>#</sup>   | 11.15 ± 0.46      |
| TBI control                | 2.10 ± 0.11                | 5.68 ± 0.35                | 7.69 ± 0.43       |
| 6-Shogaol control          | 9.85 ± 0.36 <sup>***</sup> | 1.12 ± 0.23 <sup>***</sup> | 10.94 ± 0.48      |
| TBI + fluoxetine           | 9.70 ± 0.34 <sup>***</sup> | 1.02 ± 0.25 <sup>***</sup> | 10.75 ± 0.55      |
| TBI + 6-Shogaol (10 mg/kg) | 6.00 ± 0.37 <sup>*</sup>   | 2.15 ± 0.31                | 8.15 ± 0.65       |
| TBI + 6-Shogaol (20 mg/kg) | 6.60 ± 0.43 <sup>**</sup>  | 2.00 ± 0.20 <sup>*</sup>   | 8.85 ± 0.64       |
| TBI + 6-Shogaol (30 mg/kg) | 7.70 ± 0.43 <sup>***</sup> | 1.82 ± 0.15 <sup>***</sup> | 9.50 ± 0.59       |

<sup>a</sup>Values indicate mean ± S.E.M. (*n* = 6). <sup>#</sup>*P* < 0.001 compared with normal control and \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 compared with TBI control mice (one-way ANOVA followed by Tukey's post hoc test).

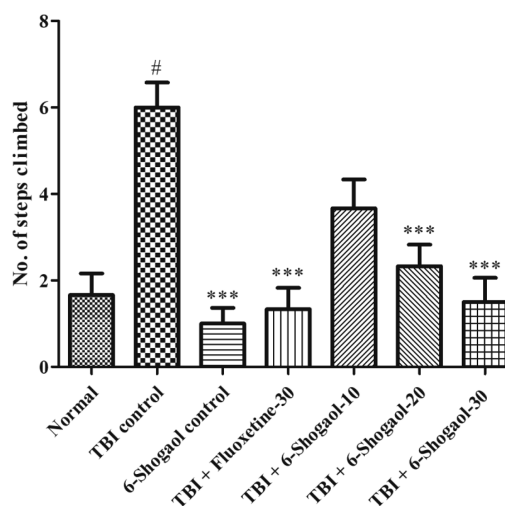
kg insignificantly prevented the TBI-induced alteration, compared to the TBI control animals. On the other hand, fluoxetine (30 mg/kg, i.p.) and 6-Shogaol (20 and 30 mg/kg, i.p.) significantly improved the open arm activity (both the parameters) compared to TBI control mice. Administration of 6-Shogaol at the dose of 30 mg/kg to sham mice did not show significant changes compared to the normal control animals.

**Marble-Burying Behavior.** Chronic treatment of mice with 6-Shogaol at 10, 20, and 30 mg/kg dose-dependently suppressed marble-burying behavior, *i.e.*, a considerable difference between control and 6-Shogaol-treated mouse behaviors was confirmed by ANOVA Tukey's test. Compared to mice treated with vehicle, pretreatment with fluoxetine also caused a favorable reduction in the marble-burying animal as revealed by the post hoc test (Figure 1).

**Staircase Test.** The number of stairs climbed by mice upon chronic treatment with different doses of 6-Shogaol was significantly less compared to that by TBI control mice. The significance of this finding was confirmed by sequential analysis using a post hoc test. Additionally, fluoxetine treatment also caused a significant decrease in the number of steps climbed by mice (Figure 2). Administration of 6-Shogaol at the dose of 30 mg/kg to sham mice did not show significant changes compared to the normal control animals.



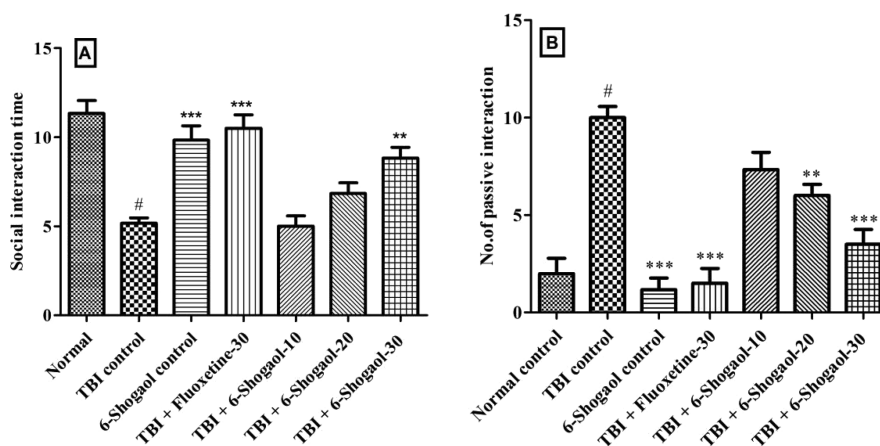
**Figure 1.** Effect of 6-Shogaol on marble-burying behavior in TBI mice. Values are expressed as mean ± S.E.M. (*n* = 6). Values represent mean ± S.E.M. (*n* = 6). <sup>#</sup>*P* < 0.001 vs normal control and \**P* < 0.05 and \*\*\**P* < 0.001 vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).



**Figure 2.** Effect of 6-Shogaol on staircase test in TBI mice. Values are expressed as mean ± S.E.M. (*n* = 6). Values represent mean ± S.E.M. (*n* = 6). <sup>#</sup>*P* < 0.001 vs normal control and \**P* < 0.05 and \*\*\**P* < 0.001 vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).

**Social Interaction Test.** The summary of the social interaction test results is shown in Figure 3. These studies were performed with pairs of mice that belonged to the same experimental treatment. Active social interaction behavior (time spent in grooming, following, sniffing, kicking, jumping over the partner, or crawling under) and social interaction behavior were evaluated. The total interaction time was found to be significantly increased by 6-Shogaol at doses of 20 and 30 mg/kg and fluoxetine treatment compared to TBI control mice. However, the number of passive interactions decreased significantly upon treatment with these chemicals in comparison with the TBI control group. Administration of 6-Shogaol at the dose of 30 mg/kg to sham mice did not show significant changes compared to the normal control.

**Open-Field Exploration.** Traumatic brain injury significantly increased open-field explorative activity (Table 2). In TBI mice, compared to the TBI control group, the number of

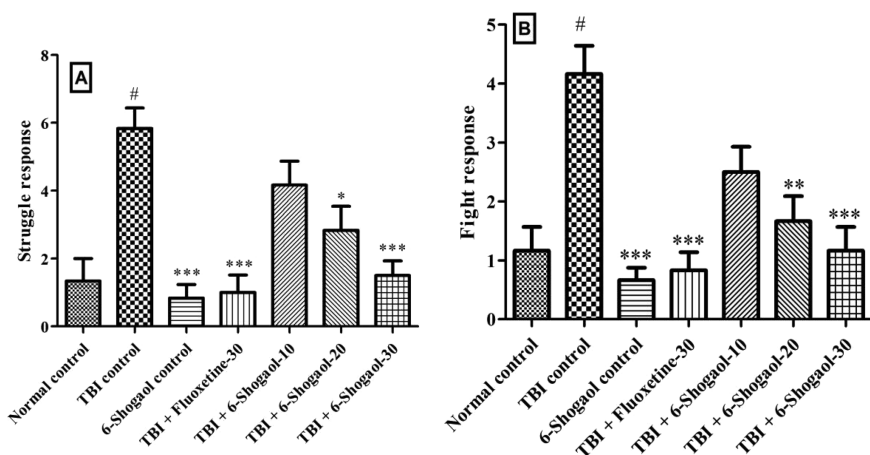


**Figure 3.** Effect of 6-Shogaol on social interaction behavior in TBI mice. (A) Social interaction time and (B) number of passive interactions. Values represent mean  $\pm$  S.E.M. ( $n = 6$ ). # $P < 0.001$  vs normal control and \* $P < 0.05$  and \*\*\* $P < 0.001$  vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).

**Table 2. Assessment of Mice in the Open-Field Exploration Test after 6-Shogaol Treatment<sup>a</sup>**

| group ( $n = 6$ )          | ambulation                    | rearing                      | defecation                   |
|----------------------------|-------------------------------|------------------------------|------------------------------|
| normal control             | 35.00 $\pm$ 1.26 <sup>#</sup> | 6.62 $\pm$ 0.72 <sup>#</sup> | 0.49 $\pm$ 0.16 <sup>#</sup> |
| TBI control                | 107.3 $\pm$ 0.88              | 35.33 $\pm$ 0.49             | 4.33 $\pm$ 0.21              |
| 6-Shogaol control          | 35.01 $\pm$ 1.08***           | 6.59 $\pm$ 0.53***           | 0.50 $\pm$ 0.09***           |
| TBI + fluoxetine           | 40.00 $\pm$ 1.18***           | 7.73 $\pm$ 0.66***           | 0.55 $\pm$ 0.12***           |
| TBI + 6-Shogaol (10 mg/kg) | 77.50 $\pm$ 0.84*             | 14.50 $\pm$ 0.95*            | 2.33 $\pm$ 0.21*             |
| TBI + 6-Shogaol (20 mg/kg) | 55.50 $\pm$ 0.56**            | 11.17 $\pm$ 0.65**           | 1.33 $\pm$ 0.33*             |
| TBI + 6-Shogaol (30 mg/kg) | 45.67 $\pm$ 1.43***           | 8.83 $\pm$ 0.30***           | 0.83 $\pm$ 0.30**            |

<sup>a</sup>Values represent mean  $\pm$  S.E.M. ( $n = 6$ ). # $P < 0.001$  compared with normal control and \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  compared with TBI control mice (one-way ANOVA followed by Tukey's post hoc test).



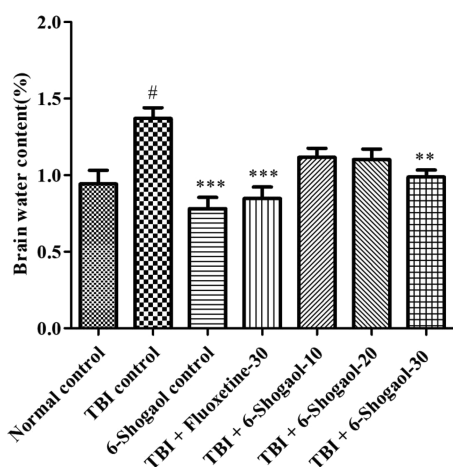
**Figure 4.** Effect of 6-Shogaol on hyper-emotionality behavior in TBI mice. (A) Struggle response and (B) fight response. Values represent mean  $\pm$  S.E.M. ( $n = 6$ ). # $P < 0.001$  vs normal control and \* $P < 0.05$  and \*\*\* $P < 0.001$  vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).

ambulations, rearings, and defecations increased significantly in the 6-Shogaol group at doses of 10, 20, and 30 mg/kg and the fluoxetine-treated group. Administration of 6-Shogaol at the dose of 30 mg/kg to sham mice did not show significant changes compared to the normal control animals.

**Hyper-emotionality Behavior.** Sequential post hoc analysis showed dose-dependent reduction in the total hyper-emotionality score upon chronic treatment with 6-Shogaol at doses of 10, 20, and 30 mg/kg when compared to the TBI control group (Figure 4A,B). In line with the above findings, post hoc test results revealed that the pretreatment of mice

with fluoxetine lowered the hyper-emotionality score when compared to TBI control mice (Figure 4A,B). Administration of 6-Shogaol at the dose of 30 mg/kg to sham mice did not show significant changes compared to the normal control animals.

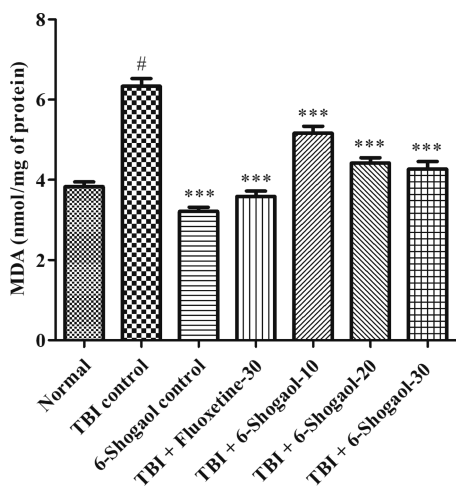
**Brain Water Content.** We observed the suppression of cerebral edema at higher doses of 30 mg/kg 6-Shogaol and fluoxetine. However, statistical significance ( $P < 0.05$ ) was seen for the treatment of fluoxetine and 6-Shogaol treatment at 20 and 30 mg/kg when compared with the TBI control group (Figure 5).



**Figure 5.** Effect of 6-Shogaol on brain water content (%) in TBI mice. Values represent mean  $\pm$  S.E.M. ( $n = 6$ ). <sup>#</sup> $P < 0.001$  vs normal control and  $*P < 0.05$  and  $***P < 0.001$  vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).

## BIOCHEMICAL STUDIES

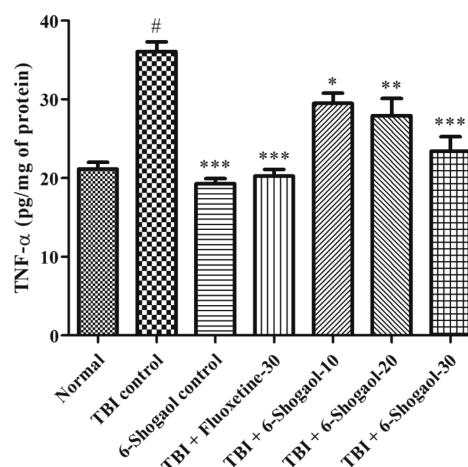
**MDA Levels.** TBI is characterized by significant biochemical alterations such as increased lipid peroxidation (Figure 6). 6-Shogaol at a dose of 30 mg/kg and fluoxetine treatment led to a significant ( $P < 0.05$ ) decrease in MDA levels as compared to TBI control mice.



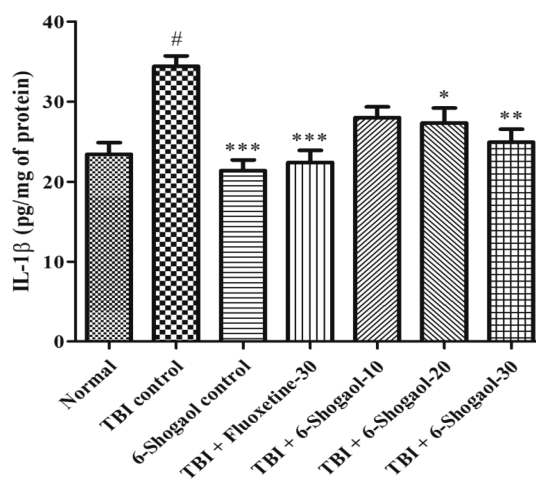
**Figure 6.** Effect of 6-Shogaol on malondialdehyde levels in TBI mice. Values are expressed as mean  $\pm$  S.E.M. ( $n = 6$ ). <sup>#</sup> $P < 0.001$  vs normal control and  $*P < 0.05$  and  $***P < 0.001$  vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).

**TNF- $\alpha$  Levels.** TNF- $\alpha$  levels in TBI mice, upon 6-Shogaol and fluoxetine treatments, were analyzed and are depicted in Figure 7. TBI-induced mice showed significantly higher levels of TNF- $\alpha$ . One-way ANOVA indicated that 6-Shogaol (10, 20, and 30 mg/kg) and fluoxetine treatments led to a considerable ( $P < 0.05$ ) decrease in the TNF- $\alpha$  levels when compared with TBI control mice.

**IL-1 $\beta$  Levels.** Data of IL-1 $\beta$  analysis are represented in Figure 8. One-way ANOVA suggested elevated IL-1 $\beta$  levels in the hippocampus of TBI-induced mice. The post hoc test showed that the hippocampal IL-1 $\beta$  levels were significantly decreased upon 6-Shogaol and fluoxetine ( $P < 0.05$ ) treatments of TBI-induced mice. Administration of 6-Shogaol



**Figure 7.** Effect of 6-Shogaol on TNF- $\alpha$  levels in TBI mice. Values represent mean  $\pm$  S.E.M. ( $n = 6$ ). <sup>#</sup> $P < 0.001$  vs normal control and  $*P < 0.05$  and  $***P < 0.001$  vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).



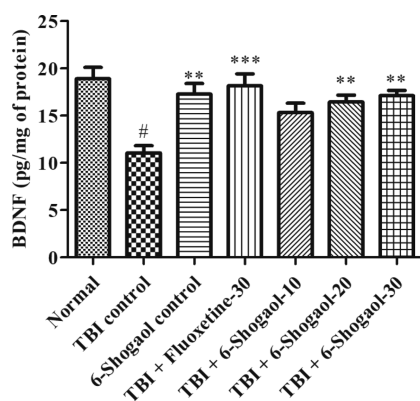
**Figure 8.** Effect of 6-Shogaol on IL-1 $\beta$  levels in TBI mice. Values represent mean  $\pm$  S.E.M. ( $n = 6$ ). <sup>#</sup> $P < 0.001$  vs normal control and  $*P < 0.05$  and  $***P < 0.001$  vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).

at the dose of 30 mg/kg to sham mice did not show significant changes compared to the normal control animals.

**BDNF Levels.** As illustrated in Figure 9, post hoc analysis indicated that the TBI mice showed a significant reduction in hippocampal BDNF levels ( $P < 0.05$ ). However, fluoxetine and 6-Shogaol treatments promoted an increase in the hippocampal BDNF levels of TBI-induced mice.

## DISCUSSION

The main findings of the study described here are as follows: (1) 6-Shogaol ameliorated anxiety- and depression-like behaviors developed post-TBI in the mice; (2) 6-Shogaol dose-dependently suppressed the TBI-induced overproduction of lipid peroxidation and IL-1 $\beta$  and TNF- $\alpha$  levels in the mice brain; (3) 6-Shogaol treatment significantly increased the production of BDNF in mice that experienced TBI. Several *in vitro* and animal studies documented neuroprotective effects of 6-Shogaol in multiple neurodegenerative diseases including dementia, senile dementia, multiple sclerosis, and Parkinson's disease.<sup>14–19</sup> As per the literature review, the study presented



**Figure 9.** Effect of 6-Shogaol on BDNF levels in TBI mice. Values represent mean  $\pm$  S.E.M. ( $n = 6$ ). # $P < 0.001$  vs normal control and \* $P < 0.05$  and \*\*\* $P < 0.001$  vs TBI control mice (one-way ANOVA followed by Tukey's post hoc test).

here is most likely the first that investigates the efficacy of 6-Shogaol as a therapeutic agent for depression- and anxiety-like behaviors in an animal model.<sup>8</sup>

Pandey et al. demonstrated that TBI exacerbates both anxiety- and depression-like behaviors in an animal paradigm. In the present study, the mice treated with vehicle after TBI were found to have depression-like activities as evident by hyper-emotionality behavior (increased struggle and fight response) and open-field exploration test (increased ambulation, rearing, and defecation).<sup>20</sup> Likewise, the results of the elevated plus maze test (increased close arm entries and decreased open arm entries), marble-burying test (increased burying behavior, indicative of neophobia and compulsiveness), staircase test (increased stair climbing), and social interaction test (decreased social interaction period and increased passive interactions) were also in accordance with the anxiety-like behavior of the animals that experienced TBI.<sup>20</sup> Treatment with 6-Shogaol for 14 consecutive days following TBI in mice decreased anxiety- or depression-related symptoms at 20 and 30 mg/kg doses. Therapeutic effects occurred in a dose-dependent manner and were identical to those that occurred after 14 day treatment with fluoxetine.

It has been proven that the primary brain injury often progresses into secondary damages, which are attributed to different factors including oxidative stress, MDA, mitochondrial dysfunction, excitotoxicity, axon degeneration, apoptotic cell death, and neuroinflammation.<sup>21</sup> Induction of TBI has been reported to cause increased oxidative stress in brain tissue.<sup>22</sup> The present study data well support this finding as elevated levels of MDA were observed in TBI control mice compared to the normal control. It is noteworthy that oxidative stress adversely affects brain plasticity, synaptic signaling, and cerebral blood flow and therefore leads to neuronal injury.<sup>21,23</sup> Administration of 6-Shogaol attenuated the levels of MDA in TBI-induced animals, indicating its effects against TBI-induced oxidative stress.

Another major impact of TBI is the dysfunction of the blood–brain barrier (BBB), which permits entry of neutrophils, monocytes, and lymphocytes into the contused brain parenchyma. The impaired BBB permeability ultimately results in parallel upregulation of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  and complement factors.<sup>24–26</sup> Activation of microglia as a consequence of prolonged and delayed neuroinflammation also contributes to the upregula-

tion of TNF- $\alpha$ .<sup>27</sup> Overproduction of various cytokines has been associated with the formation of edema and neurological deficits.<sup>21</sup> The present study data were in agreement with the above findings that the induction of TBI significantly increased the brain tissue water content and levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the mice brain, whereas the administration of 6-Shogaol attenuated TBI-induced brain tissue water content and IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels in the mice brain, indicating its protective actions against TBI-induced BBB impairments in mice. These results are in agreement with reported studies of ginger extracts and 6-Shogaol. The ginger extracts inhibited the lipopolysaccharide-stimulated secretion of pro-inflammatory cytokines in BV-2 microglial cells. Later, an experimental study identified that 6-Shogaol in ginger extract was primarily responsible for the observed anti-inflammatory effects.<sup>28</sup> Moreover, 6-Shogaol has been reported for its dopaminergic neuroprotective action in animals through the inhibition of neuroinflammatory responses (TNF- $\alpha$ , NO, iNOS, and COX-2) of microglia.<sup>13,14</sup>

A neurotrophin family member, brain-derived neurotrophic factor (BDNF), is not only concerned with neuronal growth and its survival but also plays a critical role in various neurodegenerative and psychiatric diseases. Various studies identified a strong association between the increased TNF- $\alpha$  level and the reduced expression of BDNF. Patients with depressive disorders are often found to have decreased levels of BDNF.<sup>29,30</sup> Furthermore, the synthesis of BDNF is increased following chronic antidepressant treatment.<sup>31,32</sup> Hence, recent research suggested BDNF as a possible target for depressive disorders. Shim et al. assessed the outcomes of 6-Shogaol on the inhibition of cell death and BDNF synthesis in LPS-treated murine astrocytes.<sup>33</sup> The results demonstrated that pretreatment with 6-Shogaol decreased the LPS-induced cell death through the reduced expression of Bax protein together with the increased expression of B-cell lymphoma-2 (Bcl-2) and BclxL. As these neuroprotective effects were consistent with those of BDNF, it was concluded that 6-Shogaol enhanced BDNF production. In this study, treatment with increasing 6-Shogaol dose after impact brain injury caused proportionately higher BDNF as compared to TBI control mice. Remarkably, although 6-Shogaol at a lower dose was able to alter pro-inflammatory cytokine and MDA levels and BDNF expression, a higher dose could resolve anxiety- and depression-like behaviors together with improved biochemical parameters.

## CONCLUSIONS

The results described in the current study showed that the chronic treatment with 6-Shogaol following impact-accelerated traumatic injury decreases pro-inflammatory cytokine levels (IL-1 $\beta$  and TNF- $\alpha$ ) together with upregulation of BDNF expression. It also decreases brain water content and attenuates lipid peroxidation as evidenced by decreased levels of MDA in the brain. These together could explain the improvement of depression- and anxiety-related disorders developed after TBI. 6-Shogaol can be considered as a therapeutic candidate for TBI-induced depression- and anxiety-like behaviors.

## MATERIALS AND METHODS

**Animals.** Healthy Swiss albino mice (25–30 g) were kept in a cage (group of six mice/cage) of 28 cm  $\times$  20 cm  $\times$  16 cm at a constant temperature (22  $\pm$  2  $^{\circ}$  C), room humidity (60  $\pm$  5%), and standard lighting (12:12 h light–dark cycle) for at

Table 3. Treatment Schedule and Assessment of Behavior in TBI Mice

| 0th day | 0th to 1st day                          | 1st to 10th day  | 11th to 25th day   | behavioral assessments  |  |
|---------|---|--|--|---|--|
|         |   |  |  | 26th day  | 27th day   |
| surgery | recovery from surgery (continuous care) | rehabilitation period (daily handling and observation) | drug/vehicle treatment (intraperitoneal administration/once a day for 14 days) | <ul style="list-style-type: none"> <li>●elevated plus maze</li> <li>●marble burying</li> <li>●hyper-emotionality</li> </ul> | <ul style="list-style-type: none"> <li>●open-field exploration</li> <li>●social interaction</li> <li>●staircase test</li> </ul> determination of: <ul style="list-style-type: none"> <li>●brain water content</li> <li>●malondialdehyde</li> <li>●TNF-<math>\alpha</math></li> <li>●IL-1<math>\beta</math></li> <li>●BDNF</li> </ul> |

least 1 week before the start of the experiment. Standard laboratory food and water *ad libitum* were given to the animals. The animals were housed and treated with care as per the strategy recommended by the regulatory authorities of animals of the Government of India. Appropriate approvals for the experimental protocol were obtained from the Institutional Animal Ethics Committee, India.

**Drugs and Chemicals.** 6-Shogaol was acquired from Natural Remedies Pvt. Ltd., Bangalore, India. Fluoxetine, ketamine, and xylazine were accepted as a gift sample from Scan Lab, India. Other used chemicals were of analytical grade. All the drugs were prepared with 0.9% saline for intraperitoneal administration. Three different concentrations (10, 20, and 30 mg/kg) of 6-Shogaol were administered to the animals to assess dose-dependent activity.

**Induction of Injury.** To achieve the effect of the TBI model, a ketamine and xylazine mixture (100 and 5 mg/kg, i.p.) was used to anesthetize mice. After achieving adequate anesthesia, approximately 1.5 mm midline scalp incision was made, followed by retraction of tissue to expose the skull. Cyanoacrylate adhesive was used to place round stainless steel of 2 mm in diameter and 3 mm in depth definitely over the head in the center between the bregma and the lambda. A load of 75 g was placed from 10 cm elevation onto the steel disc fixed over the skull, directed by a straight pipe (length, 10 cm) without wobbling.<sup>34</sup> Mice were placed on a 10 cm foam bed, which absorbed the impact of weight. The mouse was positioned at the center of the pipe before weight dropping so that the weight precisely drops on the metal disc placed over the head. Upon the removal of the metal disc, absorbable surgical catgut was used to suture the skin. Two groups ( $n = 6$ ) of mice were used for sham surgery; for those mice, midline scalp incision was made and the skin incision was closed without inducing TBI. Application of undiluted povidone-iodine (10%, w/v) was done postoperatively to mitigate the risk of surgical wound infection. For the next 10 days, regular inspection of the surgical wounds was performed to monitor the healing.

The mice that underwent surgery were divided into the following groups ( $n = 6$ ) and treated as follows: for group I, normal (sham) control was treated with saline; for group II, TBI control was treated with saline; for group III, 6-Shogaol (sham) control was treated with 6-Shogaol at the dose of 30 mg/kg; for group IV, standard control was treated with fluoxetine (30 mg/kg); and groups V–VII served as test

groups and were treated with 6-Shogaol at doses of 10, 20, and 30 mg/kg, respectively.

All the above-mentioned respective treatments were administered once a day intraperitoneally (i.p.) for 14 days. Twenty-four and forty-eight hours post last dose of the above treatment, *i.e.*, 27th and 28th days after TBI induction, mice were evaluated for the behavioral test to assess the anxiety- and depression-like behaviors. On the 29th day, mice were euthanized; brains were collected for the determination of water content and biochemical estimations.<sup>35</sup> The treatment schedule and assessment of behavioral and biochemical tests are represented in Table 3.

**Anxiety-like Behavior Assessment. Elevated Plus Maze Test.** The plus maze model formed a plus sign with two open ( $25 \times 5$  cm) and two enclosed ( $25 \times 5 \times 16$  cm) arms. Both arms radiated from the middle platform ( $5 \times 5$  cm). The maze was made up of black acrylic sheets. Elevation of the plus maze to an elevation of 50 cm over the floor was achieved by a mid-single support. Infrared beams were fitted at a regular distance in all four arms. The light–dark phase of the cycle (9:00 to 14:00 h) was chosen to the experiment. To start the trial, a mouse was placed on the central platform of the plus maze such that it was facing an open arm. The behavior of the mouse was observed during the 5 min experiment time as (i) the mouse's preference for its first entrance into either the open or closed arms, (ii) the total count of entries of the mouse into either of the arms, and (iii) the time spent by the animal in an individual arm. Only when all the four paws were on arm areas were the entries of mice counted to have entered an arm.<sup>36</sup> Wiping with damp towels followed by dry towels between the trials ensured the cleanliness of the apparatus. An observer, blinded to the mice treatment type, carried out all behavioral recordings. The total time spent and open arm entries were calculated in percentage. The open arm was determined in percentage based on the percentage ratio of open arm inputs (open arm 1 + open arm 2) to total arm inputs (open arm + closed arm).

**Marble-Burying Behavior.** This model was studied as previously explained.<sup>37,38</sup> In detail, a single mouse was positioned in a plastic cage ( $21 \times 38 \times 14$  cm) that contained three photocells and sawdust bedding of 5 cm in thickness. Photocells were attached to a digital meter. A total of 20 glass marbles (diameter of 10–12 mm) were evenly placed in four rows on the sawdust bedding. The unburied marbles were calculated after 30 min. The number of buried marbles was

counted when at least two-third of their size was covered by sawdust.

**Staircase Test.** The staircase had five identical steps and a height of 2.5 cm, width of 10 cm, and depth of 7.5 cm for each step.<sup>39</sup> Staircase walls had a constant internal height throughout. The experiment began by placing an animal on the box floor, facing its back to the staircase. The count of steps climbed and treaded by the animal in a 3 min period was recorded. Climbing was deemed successful only when all four paws of the mouse were on the destination step. Steps descended were not counted to simplify the observations. To avoid the presence of olfactory cues from previous animals that might impact the behavior of the next animal, the box was cleaned after each test.

**Social Interaction.** To study the social interaction of animals, an apparatus with a circular square arena consisting of a 25 cm-high wall was used. On the day when the experiment was performed, pairs of mice from different cages but belonging to the same treatment group were taken to the open-field arena and placed into two corners. A battery of social interaction behaviors, such as crawling under other mice, frontward running, mounting, probing, sniffing, and grooming, were recorded for 5 min.<sup>40</sup>

**Depression-like Behavior Assessment. Open-Field Exploration.** This experimental setup consisted of an apparatus with a circular arena of 50 cm in diameter and 25 cm-high aluminum walls. The floor was further divided into equal squares of 10 cm. The illumination during the experiment was provided through a 60 W light bulb placed at the height of 90 cm above the base of the arena. At a time, a single animal was put in the middle of the open field to determine its response. A trained observer who was blinded about the experimental treatment received by the animal noted for 5 min the following parameters: (1) ambulation score (count of squares crossed in total, which implied the hind limb movement into the adjacent square), (2) rearing episode (total count of upright standing of the animal on its hind limbs to investigate the surrounding), and (3) defecation (exact count of fecal pellets dropped by the mouse during the observation period). After each test, the residual odor from the apparatus was eliminated by spraying diluted alcohol and thorough wiping.

**Hyper-emotionality Behavior.** The procedure to assess hyper-emotionality was described previously by Ogushi et al.<sup>41</sup> The analysis consisted of scoring of responses to the following stimuli: (i) struggle response: the response received while handling the mouse with a gloved hand was considered a struggle response; (ii) fight response: the response to tail pinching with blunt forceps was scored as a fight response. A trained scientist carried out all the aforementioned procedures. Obtained responses were graded on a scale from 0 to 4, where 0 indicated no response and 4 indicated an extreme response. The sum of scores was considered as hyper-emotionality scores.

**Measurement of Brain Water Content.** Mice were euthanized under anesthesia and brains were collected and separated. The wet weight of the brain was obtained by weighing it immediately on an electronic analytical balance. Subsequently, brains were dried at 55 °C in an oven for 24 h, followed by weighing to obtain the dry weight.<sup>42</sup> The brain water content was determined using the following formula: (wet weight – dry weight)/dry weight × 100.

**Biochemical Estimations (MDA, TNF- $\alpha$ , IL-1 $\beta$ , and BDNF).** The amount of MDA formation in tissue homogenates was

measured to evaluate oxidative stress. Two milliliters of tissue homogenate was mixed with an equal volume of trichloroacetic acid (10%, w/v). The mixture was allowed to cool for 15 min at room temperature. This was followed by centrifugation to obtain the supernatant. The supernatant (0.5 mL) was transferred to a new tube and mixed with 3 mL of thiobarbituric acid (0.67%). Next, the mixture was heated in boiling water for 10 min, followed by cooling and measurement of absorbance at 535 nm against a respective blank on a Shimadzu 1700 UV spectrophotometer. The amount of MDA formed per mg of protein was expressed as nmol/mg of protein.<sup>43</sup> BDNF, TNF- $\alpha$ , and IL-1 $\beta$  concentrations were measured using commercial ELISA kits (R&D Systems, USA) according to the manufacturer's instructions and expressed as pg/mg of protein.<sup>44</sup>

**Statistical Analysis.** GraphPad Prism for Windows was used to perform all statistical analyses. Analyzed results were shown as mean  $\pm$  S.E.M. One-way ANOVA was used to evaluate significance by Turkey's post hoc test, where the relevant *P*-value of less than 0.05 was considered to be statistically significant.

## AUTHOR INFORMATION

### Corresponding Author

Muhammad Afzal – Department of Pharmacology, College of Pharmacy, Jof University, Sakaka, Aljof 72341, Saudi Arabia; [orcid.org/0000-0003-2570-3223](https://orcid.org/0000-0003-2570-3223); Phone: +966-561272872; Email: [afzalgufran@gmail.com](mailto:afzalgufran@gmail.com)

### Authors

Imran Kazmi – Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah 21589, Saudi Arabia; [orcid.org/0000-0003-1881-5219](https://orcid.org/0000-0003-1881-5219)

Anwarulabedin Mohsin Quazi – Department of Pharmacology, College of Pharmacy, Jof University, Sakaka, Aljof 72341, Saudi Arabia

Shah Alam Khan – Department of Pharmaceutical Chemistry, College of Pharmacy, National University of Science and Technology, Mascot 130, Oman

Ameeduzzafar Zafar – Department of Pharmaceutics, College of Pharmacy, Jof University, Sakaka, Aljof 72341, Saudi Arabia

Fahad A. Al-Abbasi – Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah 21589, Saudi Arabia

Faisal Imam – Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

Khalid Saad Alharbi – Department of Pharmacology, College of Pharmacy, Jof University, Sakaka, Aljof 72341, Saudi Arabia

Sami I. Alzarea – Department of Pharmacology, College of Pharmacy, Jof University, Sakaka, Aljof 72341, Saudi Arabia

Neelam Yadav – Central Council for Research in Ayurvedic Science, New Delhi 110058, India

Complete contact information is available at:  
<https://pubs.acs.org/10.1021/acsomega.1c04155>

### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; IL, interleukin; MDA, malondialdehyde; TBI, traumatic brain injury; TNF, tumor necrosis factor

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