Original Article

Exploring the Neuroprotective Effects of Mechanosensitive Channel Blocker, GsMTX4 in Intracerebral Hemorrhage Model in Rats

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ABSTRACT

Aim: The current study explored the usefulness of a mechanosensitive channel blocker, GsMTX4 in intracerebral hemorrhage (ICH)-associated deleterious effects along with the possible mechanisms. **Materials and Methods:** Type VII collagenase was administered in the right basal ganglia using stereotactic apparatus to induce ICH in rats. The ICH-associated injury was assessed using corner turn and forelimb placement tests (behavioral test); Evans blue extravasation test (blood-brain barrier damage), brain edema, apoptotic markers (caspase-3 and Bcl-2 levels). The levels of TNF-α (neuroinflammation), reactive oxygen species, and Brain-derived neurotrophic factor (BDNF) were also measured in ICH-subjected rats. GsMTx4, as a mechanosensitive channel blocker, and ANA-12, as a selective BDNF receptor blocker were employed as pharmacological agents. **Results:** Administration of GsMTx4 attenuated ICH-associated behavioral changes, preserved blood-brain barrier, prevented brain edema, and decreased apoptosis. GsMTx4 also decreased neuroinflammation and oxidative stress, while it increased the levels of BDNF. Moreover, administration of ANA-12 attenuated the neuroprotective effects of GsMTx4 suggesting that BDNF may be important in inducing neuroprotective effects of GsMTx4. **Conclusion:** GsMTx4 exerts neuroprotective effects in intracerebral hemorrhage-associated deleterious effects, which may be possibly due to an increase in BDNF levels along with a decrease in neuroinflammation and oxidative stress.

Key words: Neuroinflammation, Oxidative stress, Intracerebral hemorrhage, Mechanosensitive channels, BDNF.

INTRODUCTION

Stroke is a neurological emergency, which is characterized by sudden focal neurologic deficit lasting for at least 24 h and it mainly arises due to problems related to blood flow. Ischemic stroke, characterized by a decrease in blood supply to the brain, is the more prevalent form of stroke. However, the prognosis is much better in patients with cerebral ischemia in comparison to the hemorrhagic form of stroke.[1] The characteristic feature of intracerebral hemorrhage (ICH) involves the rupture of cerebral blood vessels, which leads to very high mortality, around 40% in stroke-affected population.[2] The major focus on the management of ICH has been on effectively and rapidly lowering blood pressure, controlling the hemostasis by transfusing platelets and clotting complexes, and employment of invasive techniques to reduce clot volume.[3,4] In recent years, there have been studies exploring new neuroprotective interventions to limit neuronal damage associated with ICH.[5,6]

Recent studies have shown the neuroprotective actions of mechanosensitive ion channel blockers. There have been studies showing the blood-brain barrier protecting actions of a pharmacological inhibitor of transient receptor potential vanilloid 4 (TRPV4), a

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mechanosensitive channel, in ICH.[7] Gadolinium, a non-selective mechanosensitive channel blocker, has also been shown to produce neuroprotection in intracerebral hemorrhage.[8] Piezo channels are the relatively new members of mechanically activated ion channels.[9] These channels are widely distributed in the body including the lungs, gastrointestinal tract, bladder, and skin.[10] Moreover, these channels are also abundantly present in the central nervous system.[11,12] GsMTx4 is a 35 amino acid peptide derived from *Grammostola spatulata* and it has been found to selectively inhibit mechano-gated piezo channels.[9,13] Apart from the Piezo 1 and 2 channels, GsMTx4 has also been shown to block mechanosensitive channels, such as TRPC1 and TRPC6.[14] Studies have shown the beneficial effects of GsMTx4 in different conditions, including ischemia-reperfusion-induced neuronal injury;[15] demyelination in the central nervous system[16] and muscular dystrophy[17] etc. However, the neuroprotective role of GsMTx4 in ICH models still needs to be identified. Accordingly, the current study was aimed to explore the therapeutic potential of a mechanosensitive channel blocker, GsMTX4 in ICH-associated deleterious effects along with the possible mechanisms.

MATERIALS AND METHODS

Animals and drugs

Male Wistar albino rats (200-250 g) were obtained from Nanjing Junke Bioengineering Co, Ltd, Nanjing, China. The animals were kept in the animal house in the standardized laboratory conditions (25± 2°C, 50-60 % relative humidity, 12 h light/12 dark cycle). In total, 105 animals were used for this study. Animal Ethical Committee of Tangshan Gongren Hospital, China with Ethic Number: TGH0924 approved the experimental protocol. The ELISA kits for the estimation of TNF-α (ab46070), BDNF (ab213899) and caspase-3 (ab39401) were obtained from abcam, Cambridge, USA. The ELISA kit for the estimation of Bcl-2 (E-EL-R0096) was obtained from Elabscience Houston, TX 77079, USA. The drugs employed in this study i.e. GsMTx4 and ANA-12 along with collagenase VII, Evans blue, 2',7'-dichlorofluorescin diacetate (DCFH-DA), isoflurane, ketamine, xylazine and acepromazine were procured from Sigma-Aldrich, USA. The doses of GsMTx4[18] and ANA-12[19] were chosen based on earlier reports.

ICH Model

The animals were anesthetized using injecting ketamine (50 mg/kg), xylazine (5 mg/kg) and acepromazine (1 mg/kg) intraperitonially. In anesthetized rats, type VII collagenase (0.5 U in 2 µL saline/rat) was injected into the brain at a rate of 0.4 µL/min in a stereotactic frame. Briefly, a hole of 1 mm was made into the skull to insert the 26-gauge needle in the right basal ganglia. The stereotaxic coordinates were 0.2 mm anterior, 3 mm right lateral to the bregma, and 6 mm ventral to the skull. In the sham group, the same procedure was followed except for infusion of normal saline instead of collagenase VII.[20]

Behavioral Testing

After 24 h of ICH, behavioral testing was done, which included the followings:

Corner turn test

In this behavioral test, the rats were kept at a 30° corner and these were allowed to make 10 turns in total (either right or left), with a time of 15 s between each turn. Amongst these, the total number of right turns made by the rats was recorded. Since there is an injury to the right side of the brain, therefore, there is more tendency to make a turn on the right side (ipsiversive) (n =15 rats/group). The results of the corner turn test were expressed as percentage.[20-22]

Forelimb Placing Test

It is a vibrissae-elicited forelimb placing test (paw whisker test) and the damage to the motor system elicits impairment in paw placing. It is a very useful test to assess the development of asymmetry in the brain regions of the sensorimotor cortex and striatum. In this test, the rat was held in such a way that its four limbs were hanging freely. The testing of the forelimb was induced by brushing the vibrissae of the rat on the corner edge of the cornerstone. The injured rat quickly placed the forelimb on the cornerstone in response to the brushing of vibrissae on the corner edge, while there was impairment in the forelimb placing on the top of the table in injured rats. Ten trials were done on each side of the rat and the number of successful placements (placing the forelimb on the table) was recorded and expressed in percentage of total trials (n =15 rats/group).[20-22]

Evans blue extravasation assay

This test was employed to check the integrity of the blood-brain barrier after 24 h of ICH. In this test, 2% Evans blue dye (2 ml/kg) was injected into a lateral tail vein. After 2 h, animals were sacrificed by allowing rats to inhale vapors of 8% isoflurane in a closed chamber. Thereafter, the brains were removed. The weights of these brains were noted and thereafter, treatment with formamide was done for 24 h at 37°C. Afterward, the brain samples were subjected to centrifugation at 2,500 g

for 15 min to obtain clear supernatant, whose absorbance was recorded at 632 nm[20,21] (n =5 rats/group).

Brain Edema Test

The brain edema was measured by determining the amount of water in the brain (after sacrificing the rats using 8% isoflurane). For this test, the brain was divided into two halves. The right half of the brain was weighed as the wet weight. Then, the brain portion was dried at 100°C for 24 h and weighed again as the dry weight. The brain water content was calculated of right half as a percentage (n =5 rats/group).[20,22]

Biochemical Analysis in brain homogenates

The brains were removed after sacrificing the rats (using 5% isoflurane) and then homogenized in the phosphate buffer solution, pH=7.4. The brain homogenate was centrifuged at 4000 × g for 15 min and the clear supernatant obtained was used to measure biochemical parameters (n=5 rats/group). The supernatants were stored at -70°C in a deep freezer till biochemical testing was performed.

Apoptosis Markers

The levels of caspase-3 and Bcl-2 as markers of apoptosis were measured using commercially available ELISA kits. The values were expressed in terms of ng/mg of protein.

TNF-α, BDNF and reactive oxygen species (ROS) levels

The quantitative analysis of TNF-α and BDNF was performed using ELISA kits, and instructions to conduct analysis were followed as described in these commercial kits. The measurement of ROS levels was done by the treatment of brain homogenate with 2',7'-dichlorofluorescin diacetate (DCFH-DA) for 30 min at 37°C. The intensity of fluorescence was checked at 484 nm (excitation splitter) and 530 nm (emission splitter), respectively.[20-23]

Study Design

The study included seven groups and each group comprised of 15 animals. The study design as explained below (Figure 1):

(i) Group I (Normal control): Animals were not subjected to any treatment and all tests were performed

(ii) Group II (Sham control): Animals were administered normal saline stereotactically and after 24 h, all tests were performed

(iii) Group III (ICH control): Animals were administered collagenase VII in the brain and after 24 h, all tests were performed

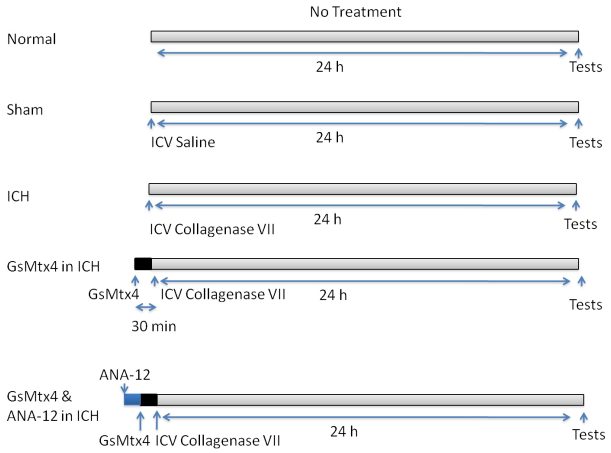

Figure 1: Diagrammatic representation of experimental design.

(iv) Group IV [GsMTx4 (5 mg/kg) in ICH]: GsMTx4 (5 mg/kg *i.v.*) was administered 30 min before subjecting rats to ICH and after 24 h of ICH, all tests were performed

(v) Group V [GsMTx4 (10 mg/kg) in ICH]: GsMTx4 (10 mg/kg *i.v.*) was administered 30 min before subjecting rats to ICH and after 24 h of ICH, all tests were performed

(vi) Group VI [GsMTx4 (10 mg/kg) and ANA-12 (0.25 mg/kg)]: ANA-12 (0.25 mg/kg *i.p.*) was administrated 1 h before, while GsMTx4 (10 mg/kg *i.v.*) was administered 30 min before subjecting rats to ICH. After 24 h of ICH, all tests were performed

(vii) Group VII [GsMTx4 (10 mg/kg) and ANA-12 (0.50 mg/kg)]: ANA-12 (0.5 mg/kg *i.p.*) was administrated 1 h before, while GsMTx4 (10 mg/kg *i.v.*) was administered 30 min before subjecting rats to ICH. After 24 h of ICH, all tests were performed.

Statistical Analysis

The GraphPad Prism 7 was employed for statistical analysis. The results were reported in the form of mean ±SD. The comparison of experimental data was done using One Way ANOVA. This statistical test was followed by Tukey's multiple range test. The value of p<0.5 was considered to be statistically significant.

RESULTS

Development of behavioral abnormalities, brain edema, and disruption of the blood-brain barrier in ICH-subjected rats.

In response to collagenase VII administration in the right portion of the brain, there was a significant development of behavioral abnormalities assessed in

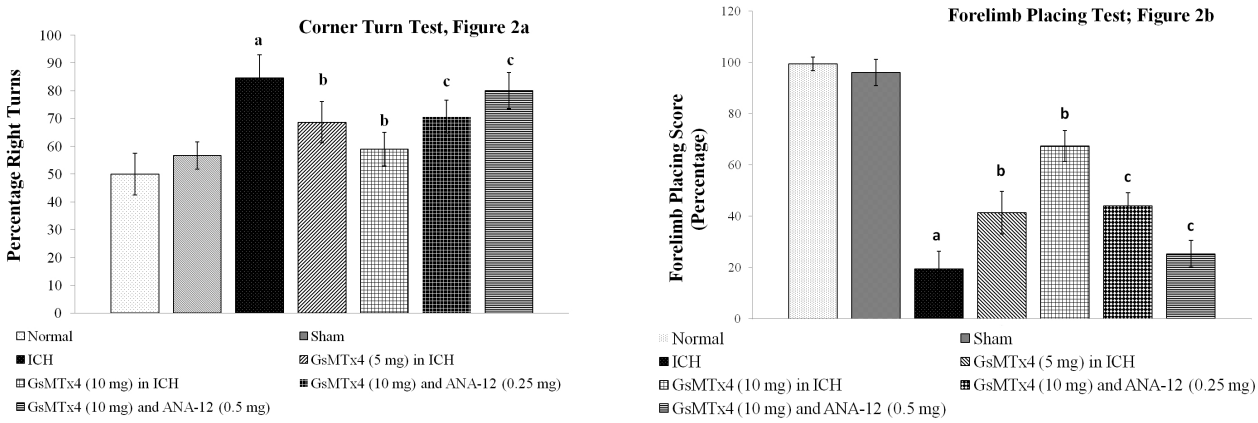

Figure 2: Effect of different treatments on corner turn test (2a) and forelimb placement test (2b). a = $p < 0.05$ vs. sham; b = $p < 0.05$ vs. ICH; c = $p < 0.05$ vs. GsMTx4 (10 mg/kg) in ICH.

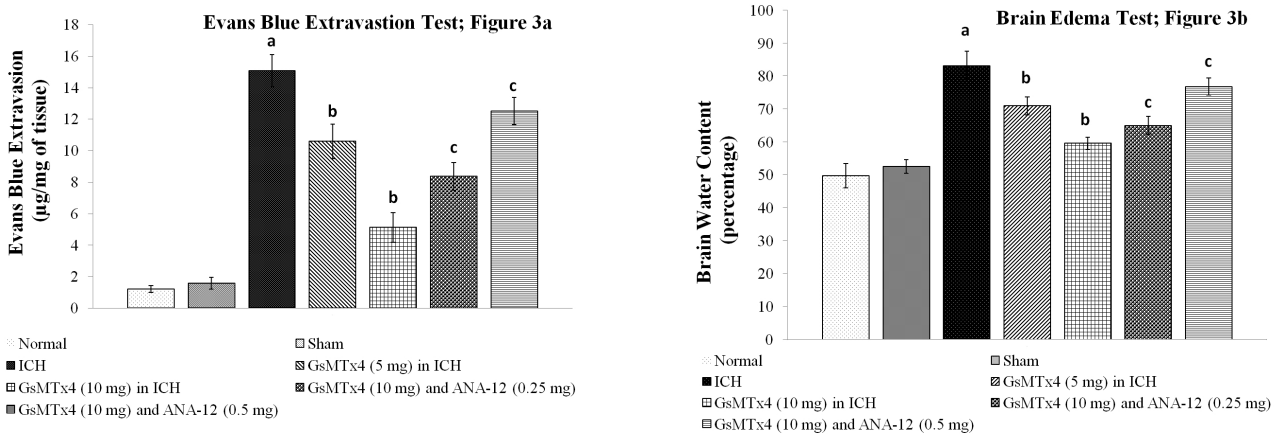

Figure 3: Effect of different treatments on Evans blue extravasation test (3a) and brain edema test (3b). a = $p < 0.05$ vs. sham; b = $p < 0.05$ vs. ICH; c = $p < 0.05$ vs. GsMTx4 (10 mg/kg) in ICH.

the corner turn test and forelimb placing test. There was a marked rise in the number of right turns in the ICH model in the corner turn test (Figure 2a) and a decrease in the percentage of forelimb placing (Figure 2b) in comparison to sham and normal control. Moreover in collagenase VII administered rats, there was a significant increase in the absorbance in the brain homogenate following IV injection of Evan blue through tail vein, suggesting the leakage of blood-brain barrier. Along with the significant Evans blue extravasation (Figure 3a), there was an increase in the brain water content (Figure 3b) in ICH-subjected rats suggesting the significant disruption of the blood-brain barrier and brain edema.

Biochemical alterations in the brains of ICH-subjected rats

In ICH-subjected rats, a marked alteration in the apoptotic markers including an increase in caspase-3 (proapoptotic) (Figure 4a) and a decrease in Bcl-2 (antiapoptotic) (Figure 4b) was observed in the brain homogenates in comparison to sham and normal control. It suggests a significant increase in the apoptotic cell injury in the ICH model. A marked rise in the TNF-α (Figure 5a) and a decrease in the BDNF levels (Figure 5b) were also observed in the brain homogenates of the ICH model. Moreover, there was also a rise in the levels of reactive oxygen species (Figure 5c) in rat brains, suggesting the increase in oxidative stress in response to ICH.

GsMTx4 attenuated behavioral and other alterations in ICH-subjected rats

Administration of GsMTx4 (5 and 10 mg/kg) significantly attenuated ICH-induced alterations in behavior (Figure 2), injury to blood-brain barrier (Figure 3a), increase in brain edema (Figure 3b), development of apoptotic cell injury (Figure 4), increase in TNF-α (Figure 5a) and reactive oxygen species (Figure 5c). Moreover, GsMTx4 also restored the levels of BDNF (Figure 5b) in ICH-subjected rats in a significant manner.

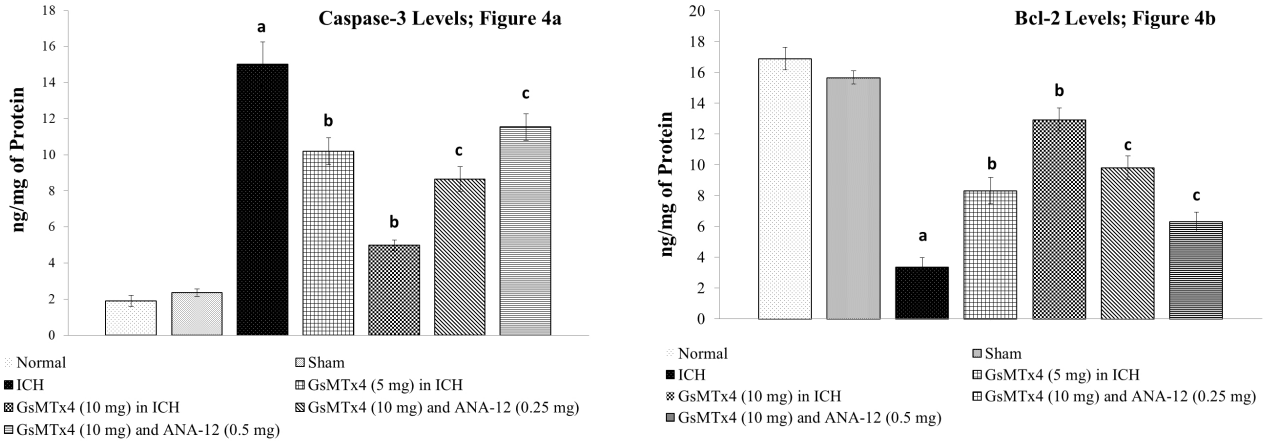

Figure 4: Effect of different treatments on apoptotic markers, caspase-3 (4a) and Bcl-2 (4b). a = $p < 0.05$ vs. sham; b = $p < 0.05$ vs. ICH; c = $p < 0.05$ vs. GsMTx4 (10 mg/kg) in ICH.

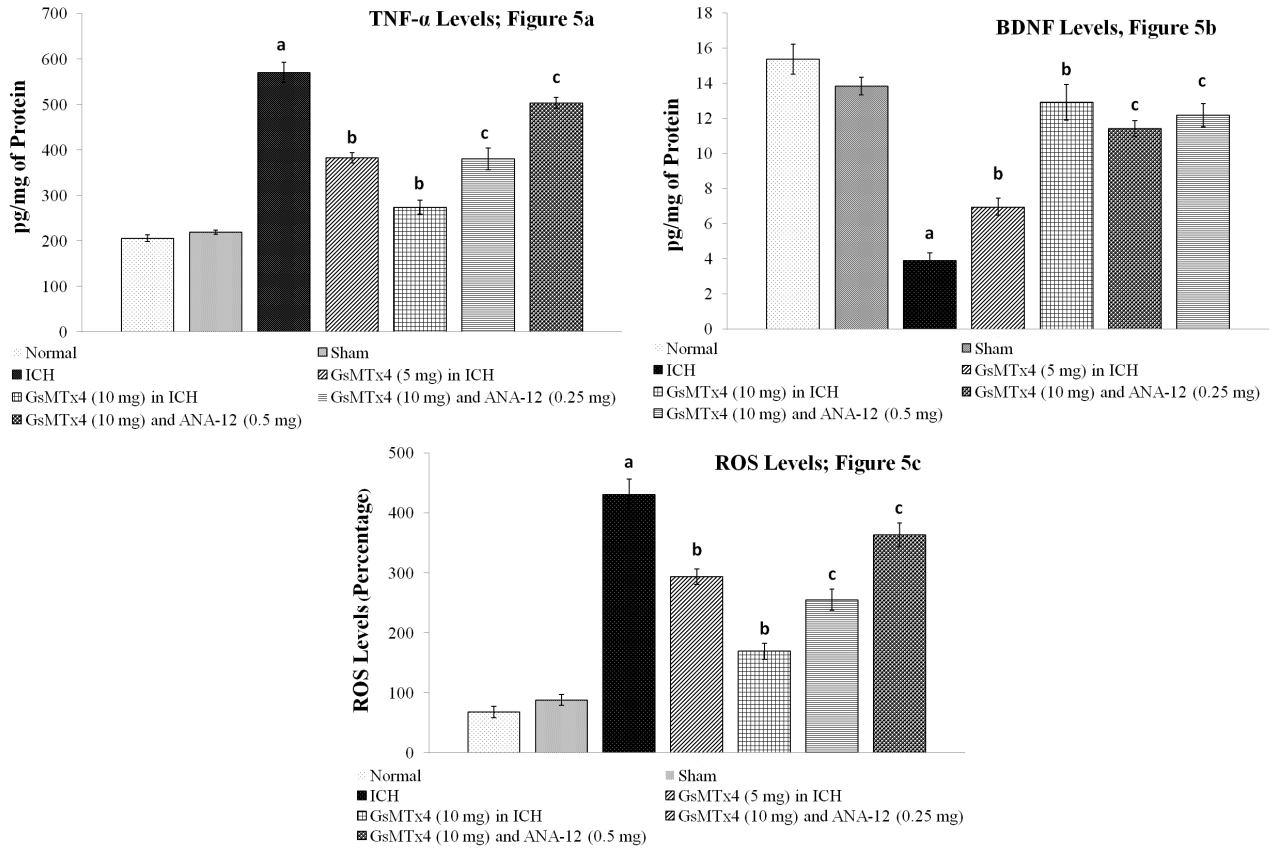

Figure 5: Effect of different treatments on TNF-α (5a), BDNF (5b) and reactive oxygen species (5c). a = $p < 0.05$ vs. sham; b = $p < 0.05$ vs. ICH; c = $p < 0.05$ vs. GsMTx4 (10 mg/kg) in ICH.

ANA-12 abolished the neuroprotective actions of GsMTx4 in ICH-subjected rats

Treatment with ANA-12 (0.25 and 0.5 mg/kg) abolished the neuroprotective actions of GsMTx4 in ICH-subjected rats. GsMTx4-mediated improvement in behavior, decrease in blood-brain injury, brain edema and apoptosis was abolished in ANA-12-treated rats. Moreover, the effects of GsMTx4 on TNF-α and oxidative stress were also abolished in ANA-12-treated rats. However, ANA-12 did not modulate the levels of BDNF in GsMTx4-treated rats.

DISCUSSION

In the present study, injection of collagenase VII in the right portion of basal ganglia of rats produced the features of ICH including the increase in the tendency for right turns (corner turn test), decrease in forelimb placing (forelimb placing test), damage to the blood-brain barrier (Evans blue extravasation) and development of brain edema (brain water content test). Moreover, there was also an increase in apoptotic cell injury as assessed by a significant increase in the levels of proapoptotic (caspase-3) and a decrease in the levels of antiapoptotic (Bcl-2) in these rats. The results of this study are supported by the results of the earlier studies.[20,22]

In this study, administration of GsMTx4 produced neuroprotection against ICH-induced brain injury in a dose-dependent manner. In GsMTx4-treated rats, there was a significant improvement in the behavior of animals, a decrease in brain edema, blood barrier damage and markers of apoptosis. As a mechanosensitive channel blocker, GsMTx4 inhibits Piezo 1 and Piezo 2 channels[9] along with TRPC1 and TRPC6.[14] Piezo 1 channels are activated by mechanical pressure and these are widespread in the different parts of the body.[24,25] Moreover, these are also abundantly present in the neurons of the brain.[12,26] Studies have shown the contribution of Piezo 1 channels in the pathology of different diseases.[11,25,27] Moreover, GsMTx4-sensitive other mechanosensitive channels including TRPC1[28] and TRPC6[29] are also present in the brain. Apart from it, GsMTx4 has been shown to attenuate ischemia-reperfusion injury to heart,[19] brain;[15] prevent demyelination in the central nervous system,[16] muscular dystrophy.[17] It is the first report describing the effectiveness of GsMTx4 in producing beneficial effects in rats mimicking intracerebral hemorrhage.

In this study, ICH led to a rise in the levels of TNF-α (neuroinflammation) and reactive oxygen species (oxidative stress) suggesting that an increase in oxidative stress and neuroinflammation may be critical in inducing deleterious changes observed in ICH-subjected rats. There have been studies showing that neuroinflammation and oxidative stress may be critical in the pathogenesis of intracerebral hemorrhage-induced deleterious effects.[30,31] However, administration of GsMTx4 mitigated ICH-associated increase in neuroinflammation and oxidative stress. Previous studies have suggested the potential of GsMTx4 in decreasing inflammation and oxidative stress.[32,33] Accordingly, it may be suggested that the neuroprotective effects of GsMTx4 in the intracerebral hemorrhage may be due to a decrease in inflammation and oxidative stress.

Apart from these alterations, the significant role of BDNF was also identified in the ICH model. There was a marked decrease in the BDNF levels in the brains of ICH-subjected rats. BDNF is a neurotrophic factor and its beneficial role in intracerebral hemorrhage has been well documented.[34-36] Moreover, the observed neuroprotective effects of GsMTx4 in this study were associated with the increase in the brain BDNF levels, which suggests that GsMTx4-mediated beneficial effects in the ICH model may be secondary to arise in BDNF levels. The relationship between the inhibition of mechanosensitive channels with GsMTx4 and an increase in the BDNF levels is an interesting and needs further experimental studies. There have been previous studies showing that the inhibition of mechanosensitive channels by GsMTx4 stimulates the neurite outgrowth in the Xenopus spinal cord[37] and PC12 cells.[38] In these studies, GsMTx4 was shown to produce synergistic effects with the nerve growth factor (NGF) in promoting neuritic growth. Jacques-Fricke *et al.* proposed that inhibition of Ca^{2+} influx in the presence of GsMTx4 contributes in accelerating the neurite growth extension.[37] However, more studies are required to explore the mechanisms involved in GsMTx4-mediated increase in the levels of BDNF. The role of BDNF in GsMTx4-mediated neuroprotective effects in the ICH model was further supported by the results showing that ANA-12 (BDNF receptor antagonist) abolished the effects of GsMTx4. ANA-12 abolished GsMTx4-mediated improvement in behavior, neuroprotection, decrease in neuroinflammation, and oxidative stress, which suggests the critical role of BDNF in mediating neuroprotective effects of GsMTx4. It is the first report describing that GsMTx4 may exert beneficial effects by increasing the levels of BDNF in the brain. However, there is a need for future studies to identify the possible signaling pathways involved in GsMTx4-mediated increase in BDNF levels in the ICH model.

From the results of this present study, it is difficult to identify which mechanosensitive channel is particularly involved in GsMTx4-mediated protective actions. Moreover, the mechanisms involved in GsMTx4-mediated increase in the BDNF levels cannot be ascertained from the current study. Accordingly, the future experimental studies are required to overcome the limitations of this study.

CONCLUSION

GsMTx4 exerts neuroprotective effects in intracerebral hemorrhage-associated deleterious effects. The increase in BDNF levels along with a decrease in

neuroinflammation and oxidative stress may contribute to mediating the neuroprotective effects of GsMTx4.

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CONFLICT OF INTEREST

There are no competing interests among authors.

ABBREVIATIONS

BDNF: Brain-Derived Neurotrophic Factor; **DCFH-DA:** 2',7'-dichlorofluorescin diacetate; **ICH:** Intracerebral Hemorrhage; **ROS:** Reactive Oxygen Species; **TRPV4:** Transient Receptor Potential Vanilloid 4.

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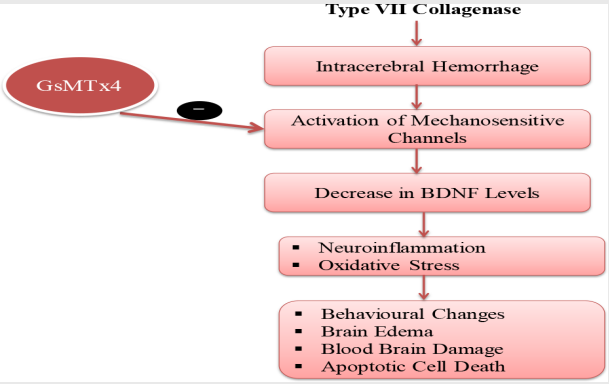
PICTORIAL ABSTRACT



SUMMARY

This study reported the beneficial effects of selective Piezo 1 channel blocker, GsMTX4 in the intracerebral hemorrhage (ICH) model in rats. GsMTx4 reduced ICH-associated behavioral changes, preserved the blood-brain barrier, prevented brain edema and decreased apoptosis. It also increased the levels of BDNF, decreased neuroinflammation and oxidative stress. The blocker of BDNF, ANA-12, abolished the neuroprotective effects of GsMTx4 suggesting the important role of BDNF. It is concluded that GsMTx4 exerts neuroprotective effects in the ICH model due to an increase in BDNF, decrease in neuroinflammation, and oxidative stress.

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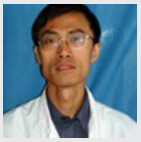

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