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Original Investigation

Evaluation of the Efficacy of Sildenafil Citrate Following Severe Head Trauma in an Experimental Rat Model

Bilal KILICARSLAN[1], Emel KILICARSLAN[2], Ceren KIZMAZOGLU[3], Hasan Emre AYDIN[4], Ismail KAYA[4], Ayca Ersen DANYELI[5], Hamit Selim KARABEKIR[3]

[1]Bitlis Tatvan State Hospital, Department of Neurosurgery, Bitlis, Turkey
[2]Bitlis Tatvan State Hospital, Department of Pathology, Bitlis, Turkey
[3]Dokuz Eylul University, School of Medicine, Department of Neurosurgery, Izmir, Turkey
[4]Dumlupinar University, Evliya Celebi Training and Research Hospital, Department of Neurosurgery, Kutahya, Turkey
[5]Acibadem Mehmet Ali Aydinlar University, School of Medicine, Department of Pathology, Istanbul, Turkey

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Corresponding author: Bilal KILICARSLAN ✉ drkilicarslan@gmail.com

ABSTRACT

AIM: To investigate the acute effects of sildenafil citrate in an experimental model of severe head trauma, and to compare it with the efficacy of mannitol, which is an osmotically active agent frequently used in clinical treatment of traumatic brain injury (TBI).

MATERIAL and METHODS: Twenty-eight Wistar-derived albino strain female rats were randomized into four groups comprising seven rats each. These groups were designated as follows: Group I: sham; Group II: TBI; Group III: TBI + mannitol (20% 1 gr/kg, intraperitoneal); and Group IV: TBI + sildenafil citrate (10 mg/kg, intraperitoneal). Sections prepared following the tissue processing of samples obtained from the right prefrontal cortex and right hippocampal regions of the brains of sacrificed rats were histopathologically evaluated. Fractionator method via the Stereo Investigator software program (Micro Bright Field) was used to count the neurons. Pyknotic neuron count and pyknotic / total neuron count were compared between the groups.

RESULTS: In the comparison of Group II and IV, pyknotic neuron count (prefrontal; group II: 116.00 ± 30.50, group IV: 80.00 ± 19.47) and pyknotic/ total neuron count (prefrontal; group II: 0,30 ± 0.08, group IV: 0.21 ± 0.02) were significantly lower in Group IV in both regions ($p<0.05$). Similarly, in the comparison of Group II and III, the values in Group III were lower in both regions ($p<0.05$).

CONCLUSION: Sildenafil citrate decreases neuronal death in the acute phase and produces similar results with mannitol. Therefore, we believe that sildenafil citrate can be a useful adjunct or alternative agent for the clinical treatment of patients with acute TBI.

KEYWORDS: Sildenafil citrate, Mannitol, Severe head trauma, Rat

ABBREVIATIONS: TBI: Traumatic brain injury, **PDE-5:** Phosphodiesterase type 5, **SAH:** Subarachnoid hemorrhage, **cGMP:** Cyclic guanosine monophosphate, **NO:** Nitric 0xide, **µm:** Micrometer

INTRODUCTION

Head trauma, a fatal and disabling pathology requiring long-term treatment and care, is a leading cause of death (12,21,34,37). Consecutive primary and secondary injuries lead to fatal and disabling effects of traumatic brain injury (TBI) (8).

Brain swelling following head trauma is one of the major problems that lead to neurological damage (16). Increased intracranial pressure and decreased cerebral perfusion due to cerebral edema result in tissue hypoxia and its harmful effects on neuronal axis (9). Osmotherapy is commonly used to treat cerebral edema and intracranial hypertension in TBI.

Bilal KILICARSLAN : 0000-0002-4938-5197
Emel KILICARSLAN : 0000-0003-0243-2694
Ceren KIZMAZOGLU : 0000-0001-6146-0842
Hasan Emre AYDIN : 0000-0002-8932-1542
Ismail KAYA : 0000-0002-5117-8066
Ayca Ersen DANYELI : 0000-0001-8015-9916
Hamit Selim KARABEKIR : 0000-0003-1173-4483

Mannitol, which reduces vasogenic, cytotoxic and interstitial brain edema, is also an antioxidant agent (15,20,42).

Sildenafil citrate, a potent phosphodiesterase type 5 (PDE-5) inhibitor, is used to treat erectile dysfunction and pulmonary hypertension (4,40). In addition, it has been studied for its effect on diseases involving the central nervous system, such as stroke, Alzheimer's disease, subarachnoid hemorrhage (SAH), hypoxic–ischemic damage, and neurodegenerative diseases, because it can cross the blood–brain barrier (2,24,30,38,41,43). These studies have demonstrated that PDE-5 inhibitors, including sildenafil citrate, induce vasodilatation by increasing the level of cyclic guanosine monophosphate (cGMP) in the brain through the glutamate - nitric oxide (NO)- cGMP signaling pathway, thus increasing the cerebral blood flow and accelerating functional recovery by acting on inflammatory cells and neurons at the cellular level. Some experimental studies have shown that cGMP also activates anti-apoptotic mechanisms in the brain (3,11). Another experimental study on rats has shown that sildenafil citrate reduces brain edema and infarct volume following stroke (7).

However, in the literature, no study has yet investigated the neuroprotective activity of sildenafil citrate in acute head trauma. Here, we aimed to investigate the acute effect of sildenafil citrate in head trauma and compare it with the efficacy of mannitol, which is an osmotically active agent frequently used in the clinical treatment of head trauma.

MATERIAL and METHODS

This study was approved by the Experimental Research Ethics Committee of the Faculty of Medicine, Dokuz Eylul University, Turkey (January 12, 2016; Approval No.: 59/2015).

Twenty-eight Wistar-derived albino strain female rats (age: 6–8 weeks; weight: 300–400 grams), which were not previously used in any other study, were obtained from the Faculty of Medicine, Experimental Animal Research Laboratory, Dokuz Eylul University. The rats were fed with standard rat feed (pellets) and were monitored before and during the experiment at room temperature (20°C ± 2°C) under 12-hour light/dark conditions, with free access to water for a week. The rats were then randomized into four groups comprising 7 rats each, designated as follows: Group I, sham (rats anesthetized but not induced to trauma); Group II, TBI (rats induced to trauma, no treatment has given); Group III, TBI + mannitol (rats induced to trauma, %20 mannitol, 1 gr/kg intraperitoneal, has given as treatment); and Group IV, TBI + sildenafil citrate (Viagra®- Pfizer, U.S.), (10 mg/kg, intraperitoneal) (6,27,35). For anesthesia, Xylazine HCl (Rompun®-Bayer, Turkey)(5–10 mg/kg; intraperitoneal); and Ketamine (Ketalar®-Eczacibasi, Turkey) (50 mg/kg; intraperitoneal;, were used (33). During the experiment, the "Marmarou's weight drop model" was used to induce severe head trauma in rats (1,22). No rats exhibited head fracture, nasal bleeding or mortality. The rats were sacrificed 4 hours after TBI.

Histopathological Examination

The tissue samples were fixed using 10% formaldehyde and were embedded in paraffin blocks following routine tissue processing. Crystal violet-stained sections sized 4 µm prepared following the routine tissue processing of samples obtained from the right prefrontal cortex and right hippocampal region were evaluated using the Stereo Investigator Software (Micro Bright Field). This software generates unbiased virtual counting spaces in all the related fields and performs the counting process through the "fractionator" method by automatically stepping on the x-y axes. For each section, the related field was selected at 40× (low) magnification (Figure 1). The counting process was performed at 400× (high) magnification in 20 counting frames sized 253 × 176 micrometer (µm), which were randomly determined by the abovementioned software (Figure 2). Small, dense, irregularly shaped pyknotic nuclei and normal neurons were marked using different markers to obtain the number of pyknotic, normal, and total neurons in both the prefrontal and hippocampal regions of each rat.

Statistical Analysis

All data were statistically analyzed using the SPSS Statistics v15.0 for Windows. $p<0.05$ was considered statistically significant. Between groups comparisons were made using the non-parametric Mann–Whitney *U*-test.

RESULTS

The pyknotic neuron count and pyknotic / total neuron count were obtained from the samples obtained from both the right prefrontal cortex and right hippocampal regions of all rats. Based on these data, Group I (sham) was compared with Group II (TBI), Group II (TBI) with Group III (TBI + mannitol), Group II (TBI) with Group IV (TBI + sildenafil citrate) and finally, Group III (TBI + mannitol) with Group IV (TBI + sildenafil citrate). Measurements, counting results, average values and *p* values for the right prefrontal region and the right hippocampus are summarized in Table I, II.

In the comparison of Group I and II, the pyknotic neuron count and pyknotic / total neuron count was significantly higher in Group II (TBI) in both prefrontal and hippocampal

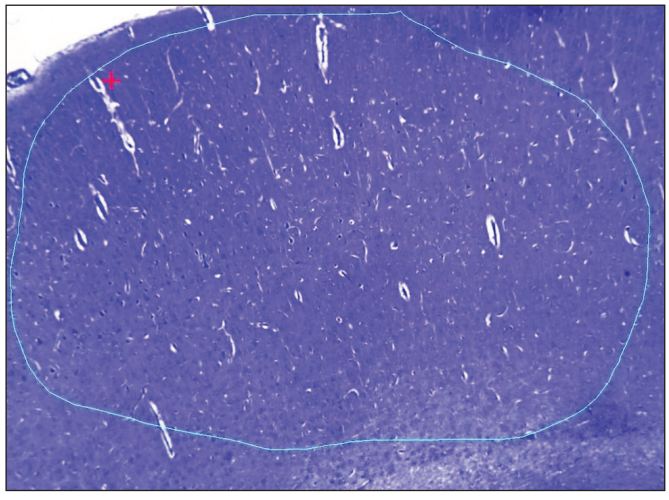

Figure 1: The areas from prefrontal cortex and hippocampus were selected via 40x objective.

regions (p<0.05). In the comparison of Group II and III, the abovementioned values were significantly lower in Group III (TBI + mannitol) in both regions (p<0.05). Similarly, in the comparison of Group II and IV, the values in Group IV (TBI + sildenafil citrate) were lower in both regions (p<0.05). Finally, in the comparison of Group III and IV, no statistically significant difference was found between the two groups (p<0.05).

DISCUSSION

Recently, studies reporting positive effects of sildenafil citrate and other PDE-5 inhibitors on the central nervous system have been frequently encountered in literature. For example, certain studies have reported that PDE-5 inhibitors are beneficial following stroke (7,31,36,44). Ozdegirmenci et al. reported that sildenafil has positive effects in preventing

Table I: The Mean Values of Measurements and Counting Results for the Right Prefrontal Cortex in All Groups

	Group I (n=7)	Group II (n=7)	Group III (n=7)	Group IV (n=7)
Pyknotic neuron count	46.28 ± 12.90	116.00 ± 30.50	68.57 ± 21.96†	80.00 ± 19.47*
Total neuron count	374.42 ± 86.42	400.42 ± 152.81	380.42 ± 44.89	369.00 ± 84.01
Pyknotic / total neuron count	0.12 ± 0.02	0,30 ± 0.08	0.17 ± 0.05†	0.21 ± 0.02*
Total area (μm²)	2050522.85 ± 357292.04	2480398.57 ± 445431.24	2051244.28 ± 328575.07	2868950.00 ± 281475.27

**pyknotic neuron count (p= 0,025), and pyknotic/total neuron count (p= 0,018) were significantly lower in group IV than group II.*
†pyknotic neuron count (p= 0,018) and pyknotic/total neuron count (p= 0,006) were significantly lower in group III than group II.

Table II: The Mean Values of Measurements and Counting Results for the Right Hippocampus in All Groups

	Group I (n=7)	Group II (n=7)	Group III (n=7)	Group IV (n=7)
Pyknotic neuron count	32.00 ± 14.13	71.14 ± 39.57	24.28 ± 10.11†	26.42 ± 6.07*
Total neuron count	349.57 ± 154.47	264.71 ± 109.51	231.42 ± 59.88	269.85 ± 69.20
Pyknotic / total neuron count	0.09 ± 0.03	0.26 ± 0.08	0.10 ± 0.02†	0.10 ± 0.03*
Total area (μm²)	1249415.42 ± 409275.53	1032509.42 ± 270553.04	768854.71 ± 191852.65	767153.00 ± 131385.10

**pyknotic neuron count (p= 0,004) and pyknotic/total neuron count (p= 0,002) were significantly lower in group IV than group II.*
†pyknotic neuron count (p= 0,005) and pyknotic/total neuron count (p= 0,002) were significantly lower in group III than group II.

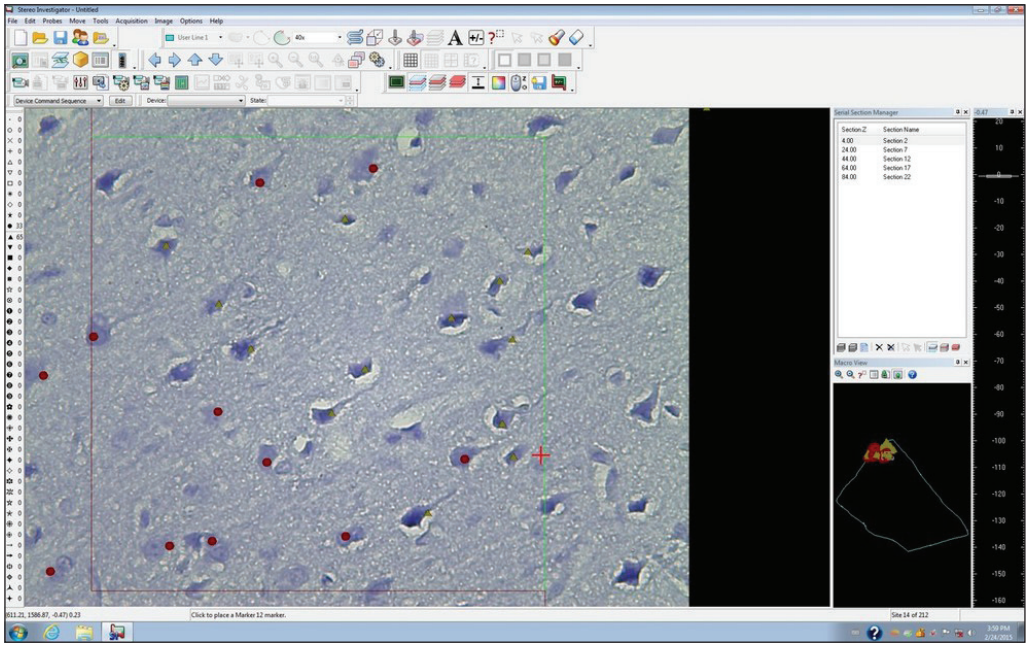

Figure 2: The stereology system allowed us to select and count normal neurons (with red dot), and pyknotic neurons (yellow triangle) in different and automatically randomly selected 20 fields of 400x objective with 253x176 μm size.

ischemia/reperfusion injury in fetal rat brain (24). Another study in neonatal rats reported that sildenafil citrate is very effective in reducing hypoxic–ischemic injury (6). In these studies, these beneficial effects were associated with the fact that sildenafil citrate and some other PDE-5 inhibitors reduce infarct volume by increasing cerebral blood flow and decrease neuron loss by restructuring the extracellular matrix via a number of cellular mechanisms and angiogenesis. Yazdani et al. reported that sildenafil citrate administered at different doses in neonatal hypoxic–ischemic injury in rat pups resulted in increased neuronal counts around the infarct area in the long term (41). Although the just mentioned study did not examine the mechanism of action, it is suggested that sildenafil citrate increases neurogenesis via cellular mechanisms and accelerates brain repair following ischemia. The current study suggests that sildenafil citrate administered following severe head trauma decreases neuronal death in the acute phase.

Molecular events that alter the balance of the post-SAH NO-cGMP pathway (26), and lead to vasospasm and ischemia, thus worsening prognosis, are targeted by PDE-5 inhibitors, such as sildenafil citrate, and studies have been conducted in this regard (10,14,39). One such study reported that sildenafil reduces vasospasm and neuronal cell death following SAH but does not alter intracranial pressure (14); other studies reported that both low and high doses of sildenafil lead to vasodilatation in patients with SAH, with no effects on intracranial pressure or no other systemic adverse effects (10,39).

The study conducted by Kara et al., wherein the abovementioned positive effects were also reported following spinal cord injury in rabbits, is remarkable (17). Kara et al. reported that gelsolin level, which decreases during apoptosis resulting from conditions such as trauma, begins to increase in the cerebrospinal fluid in the acute phase after administering sildenafil in the early post-traumatic period. This indicates that tissue damage and apoptosis are reduced by sildenafil, which has been histologically proven in this study. Spinal hematoma and necrosis in white and gray matter were lesser in the rabbit group treated with sildenafil after trauma than in the control group; this finding supports the neuroprotective activity of sildenafil (17). In our study showing similar results, sildenafil citrate may have increased cerebral blood flow and decreased neuronal cell death at the cellular level during the acute phase through all these mechanisms, namely the NO-cGMP pathway.

Intracranial pressure is the most important issue in TBI. The fact that sildenafil citrate causes vasodilatation suggests that it may increase intracranial pressure. According to Dhar et al. and Washington et al. sildenafil did not change intracranial pressure in their patient with SAH (10,39). They found that despite acute reduction in mean arteriel pressure, global cerebral blood flow did not change, and vasoconstriction due to SAH was prevented. Although these studies differ in terms of the methodology; the mechanism is similar with respect to TBI.

According to some studies, there is hypoperfusion due to decreased blood flow in the acute period of trauma (23,28). Furthermore, parenchymal bleeding caused by microvessel shearing leads to reflex vasoconstriction in and around the damaged area in early TBI (19). Endothelin-1, a potent cerebral vasoconstrictor, has an important role in early brain injury (25). Studies have reported that sildenafil also reduces endothelin levels (29). Based on these mechanisms, we think that sildenafil citrate decreases neuronal death by increasing blood supply in the acute phase.

Edema following head trauma increases the intracranial pressure and results in decreased cerebral perfusion pressure and cerebral blood flow, thus causing ischemia (9,16,18). Thus, controlling the increased intracranial pressure in trauma patients is the most important step of the treatment. Osmotic therapy is applied in this phase. Although mannitol (20%) is currently considered the gold standard hyperosmolar agent, hypertonic saline solutions are also considered very effective in decreasing the intracranial pressure (5,32). In the current study, we compared sildenafil citrate with mannitol, which is still frequently used in treating head trauma.

To the best of our knowledge, no studies have yet reported the effects of sildenafil on head trauma. In the current study, rats with induced head trauma were administered mannitol or sildenafil in the acute phase to investigate the possible protective effects of these agents against injuries caused by trauma.

Significant inferences can be made based on the results of our study. First, the significant results obtained from the comparison between Group I and II indicate that the trauma model selected by us was successfully applied. Second, the results obtained from the comparison between Group II and III and between Group II and IV indicate that mannitol reduces neuronal death following head trauma and has neuroprotective activity. In the comparison between sildenafil and mannitol, no difference was observed between the two groups in terms of neuronal death.

The Stereo Investigator Software (Micro Bright Field) used for histological evaluation generates unbiased virtual counting spaces and ensures a more reliable counting process (13). In addition, we believe that the statistically significant results obtained for the histological evaluation in both the prefrontal and hippocampal regions in all rats improve the reliability of our results.

■ CONCLUSION

The lack of investigation of biochemical parameters supporting cell death/life in blood or damaged tissues is a limitation of this study. However, histological evaluation enables direct monitoring of neuronal death. Therefore, the false positive/negative results that we encounter during the analysis of biochemical parameters are considerably decreased because of histological evaluation. Another limitation of the present study is that a short period of time (4 hours) has been assessed after TBI. Future researchers should consider evaluating the neuroprotective activity of sildenafil citrate treatment by using different doses and durations.

In conclusion, we believe that sildenafil citrate can be a useful adjunct or alternative agent for the clinical treatment of patients with acute TBI.

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