Effects of Conivaptan versus Mannitol on Post-Ischemic Brain Injury and Edema

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ABSTRACT
Objective: The aim of this study was to compare the effects of conivaptan, an arginine vasopressin antagonist, and mannitol, a sugar alcohol, on cerebral ischemia-induced brain injury and edema in rats.

Materials and Methods: Fifty-eight 8-week-old male Sprague Dawley rats were randomly divided into five groups: control, ischemia–reperfusion (I/R)+saline, I/R+mannitol, I/R+10 mg/ml conivaptan, and I/R+20 mg/ml conivaptan. Cerebral ischemia was induced by common carotid artery occlusion for 30 minutes. Saline, mannitol, or conivaptan were administered intravenously at the onset of reperfusion. Blood and brain tissue samples were taken at the 6th hour of reperfusion. The electrolytes (Na⁺–K⁺–Cl⁻), osmolality, arginine vasopressin, albumin, progranulin (PGRN), neuron-specific enolase (NSE), and myeloperoxidase activity were measured in rat serum samples. Brain frontal/hippocampal sections were stained with hematoxylin–eosin and TUNEL techniques to evaluate histopathological changes.

Results: Statistical analyses revealed that conivaptan caused significant changes in the electrolyte, NSE, and PGRN levels and osmolality when compared with mannitol. Conivaptan treatment showed positive effects on serum biochemistry and tissue histology.

Conclusion: Our findings revealed that conivaptan shows more diuretic activity than mannitol and triggers neither any damages nor edema in the brain tissue. This study may provide beneficial information for the development of treatment strategies for ischemia-related cerebrovascular diseases.

Keywords: Brain injury, conivaptan, ischemia–reperfusion, mannitol, neuronal damage

Introduction
The formation of brain edema (BE), a continuous process with an intense intracellular and extracellular ion and water exchange [1], is one of the most important acute/subacute complications of cerebral injuries. Changes in intra- and extracellular volumes may threaten the regional or global cerebral blood flow and cell metabolism; given the fixed volume of the skull, this can lead to critical consequences due to the compression of vital brain structures [2].

The osmotherapy applied as a part of the medical treatment algorithms is of great importance in the management of cerebral edema and increased intracranial pressure (ICP) following brain injury [3]. Mannitol, a widely used osmotic diuretic agent for the treatment of BE and high ICP for many years [4], cannot meet the anticipated efficacy because it can cause serious side effects and more fluid to be drawn into the tissues [5]. Therefore, the need for further research is strongly emphasized in the literature to detect an ideal anti-edema agent that has a low number of side effects [2].

Arginine vasopressin (AVP), a neurohypophysial antidiuretic hormone, has many functions, such as the regulation of free-water reabsorption and body fluid homeostasis, and it acts upon its specific G protein-coupled receptors defined as V₁a (V₁, vascular), V₂ (renal), and V₃ (V₃, pituitary) [6]. It has been suggested that the AVP hypersecretion plays a critical role in the BE formation, leading to vasospasm, water retention, dilutional hyponatremia, and low plasma osmolality [7]. AVP receptors might be also preferred as a crucial therapeutic target [8].

Vaptans, a new group of drugs that blocks the AVP receptors, were recommended for the treatment of diseases accompanied by water retention [9]. Conivaptan, one of these V₁a/V₂
receptor antagonists, was approved by the Food and Drugs Administration in 2005 for the treatment of patients with clinical hyponatraemia [10]. Conivaptan has aquarectic effects, and as such, it has a high affinity for the V2 receptors in renal collecting ducts, promoting renal free-water excretion without having a significant effect on electrolyte excretion [11]. However, the benefits of AVP receptor antagonists on the brain edema process, when applied at the onset of reperfusion, have not been clarified.

This study, designed with the purpose of obtaining the above-mentioned scientific data, was aimed to compare the effects of conivaptan and mannitol treatments on the post-ischemic brain injury and BE in the acute phase. In the literature, to the best of our knowledge, there are no studies that compare the effects of mannitol and conivaptan with this regard.

Materials and Methods

All research protocols in this study were approved by the Institutional Ethics Committee (Protocol Number: 560-1/2018).

Chemicals and kits

The following chemicals were used in the study: conivaptan hydrochloride (cat. #TRC-C384700) purchased from Toronto Research Chemicals (Canada), dimethyl sulfoxide (cat. #D5879), D-mannitol (cat. #M4125), hydrogen peroxide (cat. #I8312), and 3,3',5,5'-etramethylbenzidine dihydrochloride hydrate (cat. #846110) were purchased from Sigma-Aldrich (USA). Ketamine HCl was purchased from Pfizer (USA); xylazine HCl from Egevet (Turkey); lidocaine–fentanyl (cat. #109844) and hematoxylin (cat. #861510) were purchased from Roche Diagnostics (Mannheim, Germany), and eosin Y (cat. #105174) solutions from Merck (Germany); rat commercial enzyme-linked immunosorbent assay (ELISA) kits from AVP (cat. #CEBI39Ra); and NSE (cat. #SEA537Ra) from Cloud-Cloud Corp. (USA). The progranulin (cat. #CSB-EL009939RA) ELISA kit was purchased from Cusabio Biotech Comp. (China). The albumin kit (cat. #3183688122) was supplied from Roche Diagnostics (Mannheim, Germany), and ApopTag Plus peroxidase in situ apoptosis detection kits (Cat. #57101) were from Merck (Germany).

Animals and housing

Fifty-eight 8-week-old male Sprague Dawley rats weighing 250–300 g were purchased from KOBAY Laboratories (Ankara, Turkey) and housed at room temperature (21±1°C) and under the 12/12 h light/dark cycle in a humidity-controlled (45%-50%) environment with a free access to standard rodent food pellets and tap water ad libitum.

Anesthesia

Prior to surgical procedures, the animals were deprived of food for approximately 12 hours. On the day of surgery, their body weight was measured. The anesthesia was performed by intramuscular injection of ketamine HCl (50 mg/kg), which is an anesthetic/analgesic agent for painful procedures, and xylazine HCl (10 mg/kg), which is a muscle relaxant. At the end of the reperfusion process, the animals were anesthetized intraperitoneally again with the same agents and doses, and blood and brain tissue specimens were taken immediately.

Experimental design

The rats were randomly divided into the following five groups: control (sham; n=10), ischemia–reperfusion (I/R)+saline (I/R; n=12), I/R+mannitol (MAN; n=12), I/R+conivaptan 10 mg/ml (CON10; n=12), and I/R+conivaptan 20 mg/ml (CON20; n=12). One animal in the I/R group was eliminated from the study due to respiratory arrest during reperfusion.

Preparation and analysis of serum samples

All blood samples were centrifuged at 1000 x g for 5 minutes. Serum electrolytes (Na+, K+, Cl-), albumin, and osmolality measurements were conducted immediately on the same day, and the remaining serum samples were maintained at −80°C until the other biochemical analysis. The electrolyte levels in serum samples were measured using the Auto Analyzer System (Roche Cobas ISE modular; ISE-indirect) and expressed as mmol/L. Serum albumin was measured using the Roche Cobas C modular and was expressed as g/dL. Serum osmolality was measured using a vapor pressure osmometer (Vapro Model 5600, Wescor Inc., USA) and expressed as mmol/kg.

Preparation and analysis of tissue samples

The serum AVP, neuron-specific enolase (NSE), and progranulin (PGRN) levels were measured with the help of the commercial ELISA kits, according to the manufacturer’s instructions, using a Plate Reader (VICTOR X3, PerkinElmer Inc., USA) at an optical density of 450 nm. The AVP ELISA kit was based on a competitive inhibition enzyme immunoassay technique for the quantitative measurement of AVP in rat serum samples. The other two ELISA kits were based on a quantitative sandwich enzyme immunoassay technique. The detection range, minimum detectable dose, and intra- and inter-assay precisions of all the ELISA kits were as follows, respectively: 12.35–1.000 pg/mL, 4.63 pg/mL, and CV<10% and CV<12% for the AVP kit; 0.625–40 ng/mL, 0.237 ng/mL, and CV<8% and CV<10% for the NSE kit; and 7.8–500 ng/mL, 1.95 ng/mL, and CV<10% and CV<12% for the PGRN Kit.

As a predictor of leukocyte infiltration and inflammation, the serum myeloperoxidase (MPO) activity was measured according to the previously reported method [14]. The method is based on the measurement of the oxidation of a synthetic substrate, 3,3’,5,5’-tetrathylbenzidine (TMB), by MPO-catalyzed reaction. The reaction was initiated by the addition of hydrogen peroxide to the reaction mixture at 37°C. The increase in absorbance during the reaction was monitored using a spectrophotometer (UV-1601; Shimadzu, Tokyo, Japan) at 655 nm for 5 minutes, and the serum MPO activity in a minute was calculated as IU/mL.
the fixative reaches all regions of the tissue, to reduce the mechanical damage, and to evaluate the tissue histology more accurate [15]. For this purpose, the anesthetized rats were perfused first with 20 mL of saline followed by approximately 100 mL of freshly prepared neutral formaldehyde solution. The brain tissues were removed meticulously, post-fixed in 10% paraformaldehyde solution, and kept in a dark and cool environment until the analyses.

For the histological analysis, tissue sections were obtained from the frontal cortex and hippocampus using a rat brain slicer according to the stereotaxic coordinates. Tissue sections were fixed in 10% formaldehyde, exposed to ascending graded ethyl-alcohols, and embedded in paraffin. The obtained paraffin blocks were cut to slices 4–5 µm in thickness. These slices were stained with the hematoxylin–eosin (HE) technique to evaluate the histopathological changes and with TUNEL to display apoptotic cells using a commercial apoptosis detection kit. After the staining, images were captured using a DP70 digital camera (Olympus Corp., Tokyo, Japan) attached to a BX51 light microscope (Olympus Corp., Tokyo, Japan).

To detect the BWC, fresh brain tissues obtained from non-perfused rats (other half of the groups) weighed immediately (wet weight), dried at approximately 100°C for 24 hours using an incubator (Binder BD 53, Binder GmbH, Germany) and reweighted (dry weight). The obtained paraffin blocks were cut to slices 4–5 µm in thickness. These slices were stained with the hematoxylin–eosin–eosin (HE) technique to evaluate the histopathological changes and with TUNEL to display apoptotic cells using a commercial apoptosis detection kit. After the staining, images were captured using a DP70 digital camera (Olympus Corp., Tokyo, Japan) attached to a BX51 light microscope (Olympus Corp., Tokyo, Japan).

Statistical Analysis
Data were assessed for the normality using the Shapiro–Wilk test. Comparisons for variables that consisted of independent measurements and showed normal distribution were evaluated with the one-way analysis of variance (Tukey's post-hoc analysis) test, and the comparisons for non-normally distributed variables were analyzed by the Kruskal–Wallis test by a blinded statistician. The data were shown as a sample size (n), the mean±SD, or median, 25th and 75th percentile values. The statistical significance level was set at p<0.05. Statistical analysis was performed using the The Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM Corp., Armonk, NY, USA).

Results
Results of the serum analyses are summarized in Table 1, and the frontal and hippocampal micrographs are presented in Figures 1 and 2.

Serum electrolyte (Na⁺, K⁺, Cl⁻) levels and osmolality
According to our results, cerebral I/R caused a significantly decrease in the serum Na⁺ levels when compared with the control (p<0.001). Although both mannitol and conivaptan increased the Na⁺ levels, the conivaptan group apparently had higher Na⁺ levels than the MAN group (p<0.001). There was no significant difference in K⁺ levels among the groups (p>0.05). In addition, serum Cl⁻ levels decreased in the I/R group compared to the control significantly (p<0.001). However, the conivaptan group had higher Cl⁻ levels than other groups (p<0.001). In addition, serum osmolality showed a decline after the treatment with mannitol and 20 mg/ml conivaptan when compared to the I/R group (p<0.001). The CON10 group had the highest osmolality (p<0.001).

Serum arginine vasopressin levels
Because an AVP receptor antagonist was used in this study, the serum AVP levels were also measured to examine the effects of the treatment on the circulating hormone levels. Serum AVP levels were higher in the MAN, CON10, and CON20 groups compared to the control (p<0.001). The CON10 group had the highest AVP level, and it was significantly higher than the I/R group (p<0.001).

Serum NSE, progranulin, and albumin levels
Our statistical analyses showed that I/R led to an increase in NSE levels compared to the control, whereas conivaptan caused a significant decrease (p<0.001). Serum progranulin levels in the I/R and MAN groups was nearly similar to the control, but progranulin levels after conivaptan treatment increased more than in mannitol-treated rats, which was statistically significant (p<0.01). The serum albumin levels showed no significant difference among the groups (p>0.05).

Serum MPO activity
Although the MPO activity was higher in the I/R group than in others, there was no significant difference detected among the groups (p>0.05).

BWC
The percentage of water content of the whole-brain tissues was very close between the groups. However, a decline was observed in other groups when compared with control (p<0.01).

Table 1. Summary of Statistical Data for Blood Serum Parameters of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>CONTROL (n=10)</th>
<th>I/R (n=11)</th>
<th>MAN (n=12)</th>
<th>CON10 (n=12)</th>
<th>CON20 (n=12)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na (mmol/L)</td>
<td>145.60±2.55</td>
<td>141.55±3.56</td>
<td>142.92±3.90</td>
<td>149.17±3.38</td>
<td>148.00±4.57</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>6.40 (6.04-7.45)</td>
<td>6.46 (5.87-6.69)</td>
<td>6.42 (6.04-6.94)</td>
<td>6.09 (5.93-6.29)</td>
<td>6.82 (6.25-7.54)</td>
<td>ns**</td>
</tr>
<tr>
<td>Cl (mmol/L)</td>
<td>103.3±12.40</td>
<td>101.17±1.81</td>
<td>100.13±4.01</td>
<td>107.93±3.75</td>
<td>106.60±4.72</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
<td>3.72±0.18</td>
<td>3.58±0.28</td>
<td>3.63±0.25</td>
<td>3.76±0.27</td>
<td>3.84±0.31</td>
<td>ns*</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg)</td>
<td>345.55±20.58</td>
<td>360.00±14.62</td>
<td>321.08±12.85</td>
<td>376.13±41.61</td>
<td>319.17±18.74</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>AVP (pg/mL)</td>
<td>28.07±5.73</td>
<td>31.53±8.51</td>
<td>35.82±6.82</td>
<td>39.67±8.76</td>
<td>36.40±4.65</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Progranulin (ng/mL)</td>
<td>13.74 (10.41-19.26)</td>
<td>13.95 (10.74-18.25)</td>
<td>12.13 (10.70-13.21)</td>
<td>15.87 (13.90-18.90)</td>
<td>17.08 (14.22-22.41)</td>
<td>&lt;0.01**</td>
</tr>
<tr>
<td>NSE (ng/mL)</td>
<td>3.96±0.80</td>
<td>4.39±0.68</td>
<td>4.45±0.80</td>
<td>3.25±0.67</td>
<td>3.41±0.86</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MPO activity (U/mL)</td>
<td>0.76±0.30</td>
<td>0.82±0.66</td>
<td>0.56±0.39</td>
<td>0.46±0.28</td>
<td>0.72±0.68</td>
<td>ns*</td>
</tr>
<tr>
<td>Brain water content (H₂O%)</td>
<td>78.47±4.29</td>
<td>77.93±0.39</td>
<td>77.71±0.47</td>
<td>77.61±0.21</td>
<td>77.47±0.21</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

- One-way analysis of variance (data are expressed as the mean±standard deviation)
- Kruskal–Wallis one-way analysis of variance (data are expressed as the median, 25th and 75th percentiles)
- n: sample size; ns: not significant; AVP, arginine vasopressin; NSE, neuron-specific enolase; MPO, myeloperoxidase
- a–c: Significant difference versus the control, I/R, MAN, and CON10 group, respectively.
Hematoxylin–Eosin staining of the frontal cortex and hippocampus

The brain frontal cortex sections stained with HE had a normal histology finding in the control group (Figure 1). In the I/R group, some neuron degenerations were observed, whereas in the MAN and CON20 groups, the number of degenerative neurons was lower than in the I/R group. The CON20 group had a nearly normal histology in the frontal sections, and neuron degenerations were minimal than in other treatment groups. In the brain hippocampal sections stained with HE, the tissue histology was normal, and no edematous areas, neuron degenerations, or inflammatory cells were detected. There was no significant difference with regard to the hippocampal histology between the groups.

TUNEL staining of the frontal cortex and hippocampus

In the TUNEL staining of the brain frontal cortex sections, no apoptotic neurons were detected in the control group (Figure 2). In the I/R group, many apoptotic neurons were observed, and their number was lower in the MAN group compared to the I/R. The CON10 group had a normal histology, and no apoptotic cells in the frontal sections were observed. The CON20 group had fewer degenerated neurons with acidophilic cytoplasm with no apoptosis. In addition, the tissue histology of the brain hippocampus was almost normal, and there was no difference between the groups (not shown).

Discussion

In contrast to mannitol, conivaptan caused an increase in the Na\(^+\) and Cl\(^-\) levels

In this study, in which electrolyte levels were examined to assess the fluid–electrolyte balance, it was observed that the serum Na\(^+\) and Cl\(^-\) levels in the I/R group decreased significantly compared to the control. This may be because the decrease in the Na\(^+\)–K\(^+\)–ATPase activity in the I/R process leads to the Na\(^+\) uptake into the cell and also the Cl\(^-\) uptake to provide an osmotic balance, as described in previous studies [17]. A significant increase in the serum Na\(^+\) and Cl\(^-\) levels in the CON10 and CON20 group suggests that electrolytes may have had vascular involvement, increasing the renal excretion of free water due to the aquaretic nature of conivaptan. This approach was supported by the following findings: the Na\(^+\) and Cl\(^-\) levels were high, and the brain histology was relatively close to the control in the CON10 group. These findings suggest that conivaptan, depending on the dosage, may be more effective in the I/R process than mannitol under the same application

Figure 1. a–j. Micrographs taken from the frontal cortex and hippocampal sections of the rat brains, stained with hematoxylin–eosin (HE). (a) The control group showed normal histological appearance. There were many degenerated neurons with acidophilic cytoplasm in the I/R group (b, arrows) and a few of degenerated neurons with acidophilic cytoplasm in the MAN group (c, arrow). (d) Normal histological appearance in the CON10 group. A very few degenerated neurons with acidophilic cytoplasm in the CON20 group (e, arrows). In the HE staining of the brain hippocampus sections (f–j), all of the experimental groups were observed to have a normal tissue histology and no edematous areas, inflammatory cell migrations, or neuronal degenerations. Scale bars for the frontal micrographs are 50 µm and for hippocampal 100 µm.
time period is needed for an apparent edema formation. Interestingly, a decrease in BWC in the I/R group was remarkable. It could be due to the minimal diuretic effect of saline, since the infusion of saline was equal to that of the other groups in the I/R group. In histological examinations, neuron degenerations and a large number of neurons presenting apoptosis (TUNEL-positive cells) were observed in the I/R group. In the frontal cortex of the CON10 group, the brain histology was found to be close to the control in general. This demonstrated that 10 mg/mL conivaptan might be efficient in preventing brain tissue damage, and also the positive effects of mannitol treatment in cerebral injury were clearly more limited.

**Conivaptan increased AVP levels due to its receptor antagonism**

It has been reported that AVP plays an important role in various studies on brain injury, such as cerebral ischemia [18, 24] and trauma [25]. It has been shown that AVP plays an important role in increasing the vascular tone through $V_1$ receptors and in the free-water uptake via $V_2$ receptors [11]. In our study, AVP levels in the MAN and the conivaptan groups were significantly increased compared to control. This increase in the MAN group occurred probably due to the stimulation of water uptake via the mannitol diuresis effect. The reason for the high AVP levels observed in conivaptan groups could be due to the AVP receptor antagonism, which means that AVP was not able to bind to its receptors, the water excretion continued due to the aquaretic effects of conivaptan, and thus the AVP release was stimulated much more.

**Conivaptan decreased the serum NSE levels**

In clinical trials, it was emphasized that the majority of circulatory biomarkers, which reflect post-injury dysfunction in the brain tissue, have an independent diagnostic and prognostic significance in ischemic conditions [26]. One of these important clinical biomarkers is NSE (2-Phospho-D-Glycerat-Hydrolase; EC 4.2.1.11). In another study, it was reported that MCAO-induced cortical infarction and neurological dysfunction were associated with neuronal depletion and vascular distribution of NSE in the brain, causing an increase in NSE in the plasma [27]. In this study, mannitol treatment did not result in a significant difference in NSE levels compared to the I/R group, consistent with the studies that serum NSE levels did not change after mannitol infusion. Conivaptan treatments caused a decrease in NSE levels compared to the I/R and MAN groups, bringing tissue histology close to the control, and they did not have a negative effect on tissue damage. Therefore,
it may be considered that conivaptan causes all these changes due to its free-water excretion and prevents neuronal NSE losses more than mannitol.

Conivaptan increased PGRN levels, contrary to mannitol

In a comprehensive study, it was found that PGRN had a protective effect against ischemia with various mechanisms such as the mitigation of BBB destruction, suppression of neuroinflammation, and neuroprotection [28]. But there is no study about the relationship of PGRN levels with mannitol or with those of the vaptans. In our findings, there was a significant increase in progranulin levels in the conivaptan groups compared to the MAN group. Conivaptan could be more beneficial against the neuronal damage than mannitol; however, the high standard deviations observed in the groups make it difficult to establish whether conivaptan could really be neuroprotective.

MPO activities did not change after conivaptan treatment

Myeloperoxidase, which is an important enzyme classified as hydrogen peroxide oxidoreductase (EC 1.11.1.7), is secreted as the inflammatory response to tissue damage and is widely distributed in ischemic tissues [29]. In our study, there was no significant difference between the groups in MPO activities, and no histological findings proved that the infiltration of the immune cells occurred in the tissue, which was not in compliance with the previous study. The migration of the inflammatory cells probably did not occur in the determined time-point in this study. But in our recent study, we showed that conivaptan decreased pro-inflammatory cytokine (TNF-α and IL-15) levels significantly compared to the I/R group [30]. In addition, serum MPO activities in the control group showed that the model we used caused no additional infection.

As a result, an early use of conivaptan in post-ischemic conditions did not cause any necrotic/apoptotic effects on the brain tissue or had any inflammatory triggering effects. Due to its ability to cause renal free-water excretion, conivaptan did not lead to the serum electrolyte loss, contrary to mannitol. The beneficial effect of mannitol on the brain tissue damage was more limited than conivaptan.

In the literature, no studies comparing the effects of mannitol and conivaptan have been found in this respect. We concluded that conivaptan, an arginine vasopressin antagonist, might be considered as an alternative to mannitol for the treatment in the early phases of ischemia-related diseases, but further experimental studies are required. This study can also contribute to the literature providing useful information to design new comprehensive studies.

Ethics Committee Approval: Ethics Committee approval was received for this study from the Ethics Committee of Eskişehir Osmangazi University (5601/2018).

Peer-review: Externally peer-reviewed.

Author contributions: Concept – B.C., Ö.A.; Design - B.C., Ö.A.; Supervision - Ö.A.; Resource - B.C., Ö.A.; Materials - B.C., Ö.A.; Data Collection and/or Processing - B.C.; Analysis and/or Interpretation - B.C., Ö.A., S.O., vi̇, A.M.; Literature Search - B.C.; Writing - B.C.; Critical Reviews - B.C., Ö.A.

Conflict of Interest: The authors have no conflict of interest to declare.

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