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Effects of conivaptan and mannitol on serum cytokine levels (TNF-α, IL-15 and IL-35) following bilateral carotid artery occlusion

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Abstract: This study aimed to investigate the post-ischemic effects of aquaretic conivaptan and diuretic mannitol on serum tumor necrosis factor-α (TNF-α), interleukin-15 (IL-15) and interleukin-35 (IL-35) levels in an experimental cerebral ischemia-reperfusion (I/R) rat model. Healty male Sprague-Dawley rats were randomly divided into five groups: Control (Sham), I/R (I/R+Saline), MAN (I/R+mannitol), CON10 (I/R+conivaptan 10 mg/ml), and CON20 (I/R+conivaptan 20 mg/ml). Cerebral ischemia was conducted using bilateral common carotid artery occlusion technique. At the onset of reperfusion, saline, conivaptan or mannitol were administrated intravenously. The blood samples were taken at 6th hours of reperfusion. Rat serum TNF-α, IL-15 and IL-35 levels were measured by using commercial ELISA Kits. The biochemical analyses showed that I/R method led to an increase in serum TNF-α and IL-15 levels when compared with the control. Conivaptan treatments decreased TNF-α and IL-15 levels significantly compared to the I/R group ($p<0.001$). IL-35 levels were high in all ischemia groups, but 10 mg/ml conivaptan brought its levels close to the control ($p<0.001$). The results of Spearman's correlation analyses showed that serum TNF-α levels were positively correlated with IL-15 levels ($p<0.001$, $r=0.596$) and IL-35 levels ($p<0.05$, $r=0.319$). According to our findings on pro-inflammatory and anti-inflammatory cytokine levels, conivaptan was more effective than mannitol in balancing inflammatory response in a dose-dependent manner. This study may provide useful information in the development of treatment/follow-up strategies for ischemia and inflammation related diseases such as stroke and brain edema.

Keywords: brain edema, conivaptan, interleukin, mannitol, serum cytokines, vasopressin

Bilateral Karotis Arter Oklüzyonu Sonrasında Conivaptan ve Mannitol'ün Serum Sitokin (TNF-α, IL-15 ve IL-35) Düzeyleri Üzerine Etkileri

Özet: Bu çalışmada, deneysel bir serebral iskemi-reperfüzyon hayvan modelinde akuaretik Conivaptan ve diüretik Mannitol tedavilerinin serum tümör nekroz faktör-α (TNF-α), interlökin-15 (IL-15) ve interlökin-35 (IL-35) düzeylerine post-iskemik etkilerinin araştırılması amaçlanmıştır. Sağlıklı erkek Sprague-Dawley sıçanlar rastgele 5 gruba ayrılmıştır: Kontrol (Sham), I/R (I/R+Salin), MAN (I/R+Mannitol), CON10 (I/R+Conivaptan 10 mg/ml), and CON20 (I/R+Conivaptan 20 mg/ml). Serebral iskemi, bilateral kommon karotis arter oklüzyonu tekniği kullanılarak gerçekleştirilmiştir. Reperfüzyonun başlangıcında salin, conivaptan veya mannitol intravenöz yolla uygulanmıştır. Kan örnekleri reperfüzyonun 6. saatinde alınmıştır. Sıçan TNF-α, IL-15 ve IL-35 serum düzeyleri ticari ELISA kitleri kullanılarak ölçülmüştür. Biyokimyasal analizler, I/R uygulaması TNF-α ve IL-15 düzeylerinde kontrole göre artışa neden olmuştur. Conivaptan tedavileri TNF-α ve IL-15 düzeylerini I/R grubuna göre anlamlı düzeyde azaltmıştır ($p<0.001$). IL-35 düzeyleri tüm iskemi gruplarında yüksek bulunurken, 10 mg/ml Conivaptan tedavisi IL-35 düzeylerini kontrol grubuna yaklaştırmıştır ($p<0.001$). Yapılan Spearman korelasyon analizleri, serum TNF-α düzeylerinin IL-15 ($p<0.001$, $r=0.596$) ve IL-35 ($p<0.05$, $r=0.319$) düzeyleri ile pozitif yönde ilişkili olduğunu göstermiştir. Sonuç olarak, pro-inflamatuar ve anti-inflamatuar bu sitokin düzeylerine dair bulgularımız, Conivaptan'ın doza bağlı olarak inflamatuar yanıtın dengelenmesinde Mannitol'den daha etkili olabileceğini göstermektedir. Bu çalışma, inme ve beyin ödemi gibi iskemi ve inflamasyon ile ilişkili hastalıkların tedavi ve takibine yönelik stratejilerin geliştirilmesinde yararlı bilgiler sağlayabilir.

Anahtar Kelimeler: beyin ödemi, conivaptan, interlökin, mannitol, serum sitokinleri, vazopressin

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1. Introduction

Inflammation which is defined as an immunological reaction involving natural and adaptive immunity mechanisms, is a protective response in which exogenous and endogenous harmful agents that initiate cell damage are reduced, destroyed, or neutralized and attempted to be removed to maintain tissue homeostasis (Kumar et al., 2012; Chen and Xu, 2015). During this response, the process of repairing and cleaning of damaged cells and tissues is also initiated. It is generally considered that a controlled inflammatory response has beneficial effects, but it could be detrimental if triggered in excessive manner (Medzhitov, 2008).

The progressive inflammation process can be induced by ischemic conditions, and depends on the expression, release and activation of inflammatory mediators such as cytokines from cerebral and peripheral cells (Amantea et al., 2014). Cytokines are small signal molecules that can act on picomolar or nanomolar concentrations and defined as immunomodulators which are in form of polypeptide, protein and/or glycoprotein that regulate the activities of the target cells (Ramesh et al., 2013; Dembic, 2015). Various cytokines that are released following ischemic brain injury have been detected in blood, cerebrospinal fluid, and variety of brain regions both in humans and experimental animal models (Mizuma and Yenari, 2017). Tumor-necrosis factor (TNF, TNF-α) is one of the most widely studied cytokine in experimental models with ischemia-related diseases (Lambertsen et al., 2012). It was suggested that the increase in levels of TNF-α released from active microglia in response to brain damage or neuroinflammation induced and promoted BBB destruction (Nishioku et al., 2010). Interleukin-15 (IL-15) is defined as a neuroprotective cytokine that has various effects on and outside the immunity system (Patidar et al., 2016), and is a new mediator that enhances and regulates TNF signaling (Pan et al., 2009; Pan et al., 2013). Interleukin-35 (IL-35) has been described as a new cytokine in 2007 that has anti-inflammatory and immunosuppressive properties (Banchereau et al., 2012). However, there is no sufficient information about the roles and molecular mechanisms of IL-15 and IL-35 in the post-ischemic inflammatory process.

It is suggested that ischemia-reperfusion (I/R) process which is continued with damage of the blood-brain barrier (BBB) and with cerebral edema, is becoming more complicated by the hypersecretion of arginine vasopressin (AVP). AVP is a neurohypophysial antidiuretic hormone and has many functions such as regulation of free water reabsorption, body fluid homeostasis, and acts upon its specific G protein-coupled receptors called V_{1A} (V_1, vascular), V_2 (renal), and V_{1B} (V_3, pituitary) (Thibonnier et al., 2001; Palmer, 2015). In some brain injuries such as ischemic stroke (Barreca et al., 2001) and traumatic brain injury (Szmydynger-Chodobska et al., 2011), have been estimated that AVP levels increase in plasma and a variety of brain regions, and AVP hypersecretion stimulates platelet aggregation and coagulation factor release (Thibonnier et al., 2001). AVP receptors might also prefer as an important therapeutic target (Zhao et al., 2015).

Vaptans, a new group of drugs produced in order to block AVP receptors, were recommended for the treatment of diseases accompanied by water retention (Peri 2013). Conivaptan (V_{1A}/V_2 receptor antagonist), one of these AVP antagonist agents, is approved by the FDA in 2005 for the treatment of patients with clinical hyponatremia (Palmer et al., 2016). Conivaptan has aquaretic effects such as promoting renal free-water excretion without a significant change in electrolyte excretion (Wada et al., 2002; Murphy et al., 2004; Ali et al., 2007).

Mannitol which an alcohol derivative of mannose sugar, is a widely used diuretic agent (Grande and Romner, 2012) and has therapeutic effects such as neuroprotection by free radical scavenging, improving perfusion to the ischemic area and immunomodulation (Korenkov et al., 2000; Rabinstein, 2006). However, serious cerebral and systemic side effects of mannitol were reported in clinical studies (Wykes and Vindlacheruvu 2015; Rabinstein 2006). In recently presented meta-analyses, the need for an appropriate osmotherapeutic agent with low side effects has been overemphasized (Grande and Romner, 2012).

There is no sufficient and detailed information in the literature about changes and correlations of TNF-α, IL-15 and IL-35 levels during the post-ischemic treatment with conivaptan versus mannitol. This study was aimed to investigate the effects of treatment with aquaretic conivaptan and diuretic mannitol on serum TNF-α (pro-inflammatory), IL-15 (pro-inflammatory) and IL-35 (anti-inflammatory) cytokine levels following an experimental cerebral I/R rat model formed by bilateral carotid artery occlusion.

2. Materials and Method

All research protocols which used in this study were approved by the Animal Experiments Local Ethics Committee of Eskisehir Osmangazi University (Approval date: 14.03.2018; Protocol no: 560-1/2018).

2.1. Chemicals and Kits

The chemicals which was used in this study are as follows: Conivaptan hydrochloride (Cat. no. TRC-C384700) was purchased from Toronto Research Chemicals (Canada). D-mannitol (Cat. no. M4125) and Dimethyl sulfoxide (Cat. no. D5879) were purchased from Sigma-Aldrich (USA). Ketamine HCl (50 mg/mL) was from Pfizer (USA); Xylazine HCl was from Egevet (Turkey). Rat commercial ELISA Kits for Tumor Necrosis Factor Alpha (Cat. no. SEA133Ra) and Interleukin-15 (Cat. no. SEA061Ra) were purchased from Cloud-Clone Corp. (Houston, USA). Rat Interleukin-35 (Cat. no. CSB-E13652r) Kit were purchased from Cusabio Biotech Company (Wuhan, China).

2.2. Animals and Housing

Eight-week-old male Sprague-Dawley rats weighing 250-300 g, were purchased from KOBAY Laboratories (Ankara, Turkey), and housed in the temperature- (21 ± 1 °C), light- (12/12 h light/dark cycle) and humidity-controlled (45-50%)

environment with free access to standard rodent food pellets and tap water at the Medical and Surgical Experimental Research Center (TICAM) of the University.

2.3. Experimental Design

The animals were deprived of food for about 12 hours without any other kind of restriction, prior to the surgical operations. On the day of surgery, rats body weights were recorded, and anesthesia was occured with Ketamine HCl (50 mg/kg) and Xylazine HCl (10 mg/kg) by intramuscular injection. At the sixth hours after the onset of reperfusion, blood samples were obtained immediately under anesthesia which is applied with the same chemicals and the doses, by intraperitoneal route.

In this study, rats were randomly divided into the five groups as follows: Control (Sham; n=10), I/R (I/R+saline; n=12), MAN (I/R+mannitol; n=12), CON10 (I/R+conivaptan 10 mg/mL; n=12) and CON20 (I/R+conivaptan 20 mg/mL; n=12). One animal in the I/R group eliminated from the study due to the respiratory arrest which occurred during the reperfusion process. The surgical protocols were based on two-vessel occlusion model with a slight modification (Smith et al., 1984). In this model, cerebral ischemia was induced by clamping arteria carotid communis bilaterally, without any added hypotension procedures. In all groups except the control group, the carotid arteries were clamped by a vascular clamp (Vascu Stop Bulldog Clamp, Istanbul, Turkey) for 30 minutes. Saline (0.9% NaCl), mannitol (20% wt/vol; dissolved in saline; MW=182.17) and conivaptan (10 or 20 mg/mL dissolved in 5% final DMSO vol/vol; MW=535.04) were applied to the related groups by the infusion into the jugular vein for 30 minutes (1 mL/h) with the onset of reperfusion (Taya et al., 2008). Immediately after skin-suturing, 1 mL of saline was injected intraperitoneally to the rats which were treated with mannitol and conivaptan to prevent of hypovolemia, and animals were maintained in separate cages. In the control group neck incision without clamping was performed and surface skin was sutured. The cardiac blood samples were taken at sixth hours of the reperfusion (at sixth hours after skin-suturing in control group) under anesthesia, and serum samples were analyzed as detailed below.

2.4. Preparation and Analyses of Serum Samples

The blood samples were centrifuged in 1000 x g for five minutes and obtained serum samples were maintained at -80 ºC until the biochemical analysis. The serum TNF-α, IL-15, and IL-35 levels were measured with the commercial ELISA Kits according to the manufacturer's instructions using a plate reader (VICTOR X3 Multilabel, PerkinElmer Inc., USA) at an optical density of 450 nm. The TNF-α, IL-15, and IL-35 ELISA Kits were based on a quantitative sandwich enzyme immuno-assay technique. The detection range, the minimum detectable dose, the intra- and inter-assay precisions of TNF-α, IL-15 and IL-35 Kits were as follows, respectively: 15.6-1,000 pg/mL, 6.1pg/mL, CV<10% and CV<12%; 15.6-1,000 pg/mL, 5.8 pg/mL, CV<10% and CV<12%; and 15.6-1,000 pg/mL, 3.9 pg/mL, CV<8% and CV<10%.

2.5. Statistical Analysis

All data were assessed by the Shapiro–Wilk test for the normality. Comparisons for normally distributed variables were shown as n (sample size) and mean ± standard deviation (SD) and were analyzed by the One-Way Analysis of Variance (Tukey's post-hoc analyses) test. The comparisons for non-normally distributed variables were analyzed by the Kruskal-Wallis test. The correlations among non-normally distributed variables were determined by using Spearman correlations analysis. The statistical significance level was accepted for P value less than 0.05 ($p<0.05$). All statistical analysis was performed using SPSS Version 21.0 (IBM Corp., Armonk, NY, USA) by a blinded statistician.

3. Results

To analyze whether conivaptan has a role in balancing pro- and anti-inflammatory cytokines following cerebral injury, TNF-α, IL-15 and IL-35 levels were measured in rat serum samples. Serum TNF-α levels were increased in the I/R group compared to the control group ($p<0.001$), whereas there was a decrease in the CON10 and CON20 groups compared with the I/R group ($p<0.001$) (Fig. 1A). Serum IL-15 levels in the CON10 group was lower than the control, I/R and MAN groups ($p<0.001$). In the CON20 group, a decrease was detected in IL-15 levels compared to I/R and MAN groups ($p<0.001$), but there was no significant difference between the I/R and control group (Fig. 1B). The statistical analysis showed that serum IL-35 levels were increased in all groups (I/R and treatment groups), however, this increase was significant only in the CON20 group compared to the control ($p<0.001$) (Fig. 1C).

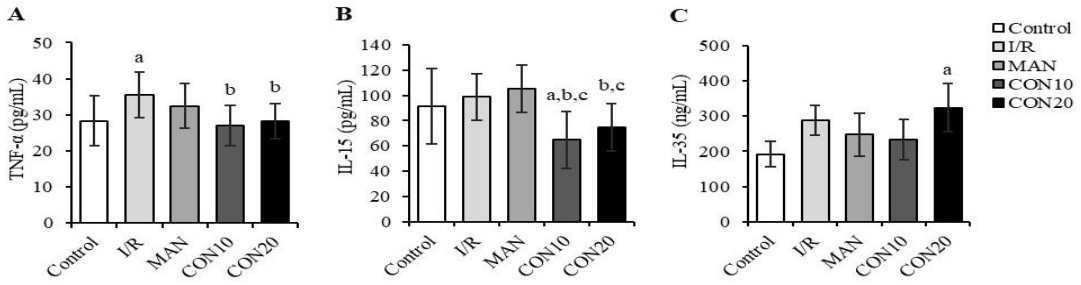

Figure 1. Comparison of the serum cytokine levels among the study groups. **(A)** Tumor necrosis factor alpha (TNF-α) levels, **(B)** Interleukin-15 (IL-15) levels, and **(C)** Interleukin-35 (IL-35) levels. Control, Sham; I/R, I/R+saline; MAN, I/R+mannitol; CON10, I/R+conivaptan 10 mg/ml; CON20, I/R+conivaptan 20 mg/ml. I/R, ischemia-reperfusion; Data are shown as mean ± standart deviation. [a]$p<0.001$ versus control group; [b]$p<0.001$ versus I/R group, and [c]$p<0.001$ versus MAN group.

Correlations between the serum cytokine levels were calculated by using the Spearman's correlation analyses, both in all and each group separately (Table 1). According to the findings, serum TNF-α levels were positively correlated with IL-15 levels ($p<0.001$, $r=0.596$) and IL-35 levels ($p<0.05$, $r=0.319$) in all groups. Scatter plot diagrams of all groups were represented in Figure 2. There was a statistically positive correlation between serum TNF-α and IL-15 levels in the control ($p<0.05$, $r=0.745$), the MAN ($p<0.001$, $r=0.727$) and the CON20 ($p<0.005$, $r=0.615$) groups (Figure 2A). Serum TNF-α and IL-35 levels were positively correlated only in the CON20 group ($p<0.001$, $r=0.748$) (Figure 2B).

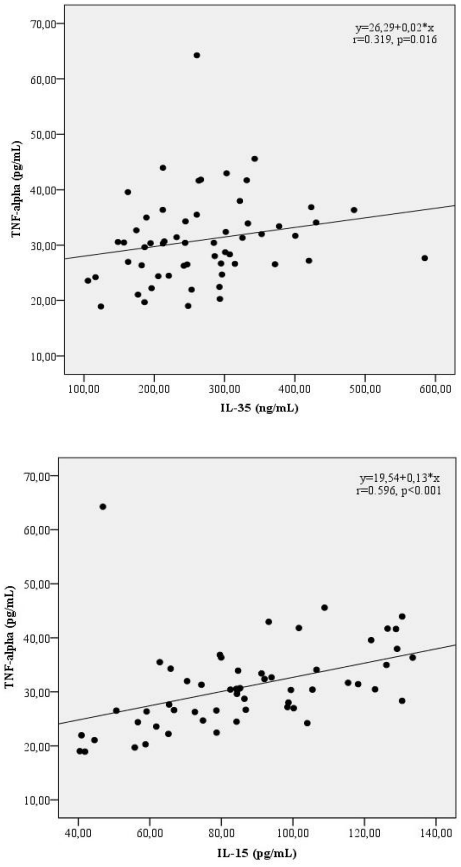

Figure 2. Scatter-plot diagrams representing correlations between serum TNF-α levels (pg/mL) and serum IL-15 (pg/mL) (A) or IL-35 (ng/mL) levels (B), respectively. The r and p values showed in the figure represent the results of Spearman's correlation analysis. TNF-α, tumor necrosis alpha; IL-15, interleukin-15; IL-35, interleukin-35.

Table 1. Spearman's correlation coefficients of the study groups.

	TNF-α					
	All (n=57)	Control (n=10)	I/R (n=11)	MAN (n=12)	CON10 (n=12)	CON20 (n=12)
IL-15	0.596**	0.745*	ns	0.727**	ns	0.615*
IL-35	0.319*	ns	ns	ns	ns	0.748**

**p<0.001, *p<0.05. Data are shown as n (sample size) and mean±standart deviation. ns, not significant.

4. Discussion

Brain tissue is characterized by high metabolic rate due to its functions such as preservation of membrane potentials, synthesis/storage of neurotransmitters and renewal of disrupted structural parts. Considering the central role and importance of the brain in the body, it is vital providing a continuous blood flow to the brain through the arterial circulation (Iadecola and Anrather, 2011). The sudden interruption of blood flow to the brain causes tissue hypoxia, induces an inflammatory cascade and can cause irreversible pathological changes due to ischemic death of the affected tissue (Sokoloff, 1997). Interestingly, reperfusion is often associated with an aggravation of tissue injury and a post-ischemic inflammatory response, which is called reperfusion injury (Bonaventura et al., 2016). Although the mechanisms associated with tissue injury such as BBB destruction are multi-factorial, it is suggested that inflammation usually has a pre-dominant role (Witt and Sandoval, 2014).

Tumor necrosis factor (TNF) is a pleiotropic pro-inflammatory cytokine that exhibits a variety of biological activities and is produced by a variety of immune system-related cells in response to several activating stimuli. It is stated that low levels of TNF inhibit neuronal deaths following metabolic exotoxic damage, induce the proliferation of astrocytes and neuronal progenitor cells, and have a critical prescription for repair and regeneration of damaged tissue in the central nervous system (Wang et al., 2003). Although TNF expression is tightly controlled, it is expressed at very high levels in various systemic inflammatory diseases. It is suggested that elevated TNF levels activate injury response and mediate hypotension, diffuse coagulation, and tissue damage (Wang et al., 2003). TNF-α levels in the brain are thought to increase after brain injury or ischemia, as well as in patients with neurodegenerative diseases, and this increase is largely due to activated microglia. In an *in vitro* study in which mouse brain microvascular endothelial cells were co-cultured with microglia, it was suggested that the increase in levels of TNF-α released from active microglia in response to brain damage or neuroinflammation induced and promoted BBB destruction (Nishioku et al., 2010). It is emphasized that TNF-α is a commonly found cytokine which is rapidly triggered in experimental and clinical traumas of CNS (Hallenbeck, 2002), can modulate neuroregeneration after stroke and enters the CNS through the BBB (Pan et al., 2006; Pan et al., 2013). For the first time in 1994 (Liu et al., 1994), induction of TNF-α mRNA in ischemic cortex was reported to occur in permanent MCAO-reconstructed rats. It is reported that induction was presented 1 hour after MCAO ischemic cortex, peaked at 12 hours, and significantly increased from day 3 to day 5. It has been suggested that pro-inflammatory and procoagulant effects of TNF on the endothelium may impair microcirculation perfusion in brain ischemia (Hallenbeck, 2002). In relation to this, it has been shown that inhibition of TNF-α activity in spontaneously hypertensive rats reduces ischemic damage following cerebral ischemia and improves

microvascular perfusion (Dawson et al., 1996), and that these results support the hypothesis that pro-inflammatory cytokines such as TNF-α are important in stroke pathophysiology. In our study, we observed a significant increase in TNF-α levels in the I/R group compared to the control. These findings suggested that neuroinflammation was triggered in the I/R process when pro-inflammatory effect of TNF-α was considered. CON10 and CON20 treatment groups showed a significant decrease in TNF- α levels according to the I/R group. It seems like that conivaptan effects the inflammatory response more efficiently than mannitol thanks to its aquaretic effect.

In the literature, it is pointed out that TNF accelerates the post-translational process of interleukin-15 (IL-15) which acts as a growth factor (Pan et al., 2009). Studies with TNF-α have identified the important functions of IL-15 in BBB (Pan vd., 2013). IL-15 is anti-apoptotic and neurotrophic (Budagian et al., 2006) and is a new mediator of TNF signaling at BBB level and acts to amplify and modulate TNF-α signal (Pan et al., 2009). Interleukin-15 is defined as a neuroprotective cytokine and it has indicated that IL-15 gene expression occurs in many tissue and cell types (Patidar et al., 2016). In many studies, it is stated that IL-15 is expressed in the brain especially from neurons, whereas glial cells are thought to express IL-15 when they undergo activation or transformation following inflammatory response (Gomez-Nicola et al., 2008). It is explained that IL-15 inhibits apoptosis of pro-apoptotic factors by down-regulation, and anti-apoptotic factors by up-regulation and is also required for homeostasis, development and function of various cells related to immun system (Patidar et al., 2016). IL-15 also plays an important role in processes such as neurogenesis, proliferation, differentiation and neuron self-renewal, and neutrophil activation. However, there is no sufficient information yet about its role in the central nervous system (Ransohoff and Benveniste, 2006). In our study, IL-15 levels were increased in the I/R and MAN group compared to the control, but decreased in the conivaptan groups as seen TNF-α. In addition, correlation analysis results showed that serum TNF-α levels were positively correlated with IL-15 levels in all groups together ($p<0.001$, $r=0.596$), and in each of the control ($p<0.05$, $r=0.745$), MAN ($p<0.001$, $r=0.727$) and CON20 ($p<0.005$, $r=0.615$) groups.

Interleukin-35 (IL-35) is a new cytokine that described in 2007 and its encoding gene transcription has been reported to occur in pro-inflammatory cytokines-activated monocytes, vascular endothelial cells and smooth muscle cells (Banchereau et al., 2012). Initially, IL-35 had been thought to be produced only by regulatory T cells, but with the help of further studies was shown that inducing the development of a new T cell population, called iTr35, producing IL-35 (Sun et al., 2015). In recent studies, it has been reported that B cells also regulate IL-35, particularly in autoimmune and infectious diseases, and IL-35 has been acted as an immunomodulator in various diseases (Vignali and Kuchroo, 2014). In the literature, there was insufficient study or data on cerebral I/R model in which IL-35 levels were investigated and associated with ADH antagonist treatments comparing mannitol. In our study, there was an increase in IL-35 levels in all groups when compared with control, but this increase was significant only between control and CON20 treatment group. Additionally, according to our findings, serum IL-35 levels were positively correlated with TNF-α levels ($p<0.05$, $r=0.319$) both in all groups together and in the CON20 group ($p<0.001$, $r=0.748$). However, there is a need for comprehensive and reliable studies of the possible role and utility of IL-15 and IL-35 in the pro-inflammatory, anti-inflammatory or immunomodulatory pathways associated with CNS pathologies.

5. Conclusions

The brain tissue is subject to a continuous support of oxygen and glucose through arterial blood flow. Although the failure of the cerebral blood supply causes to brain damage that can not be rectified, the restoration of blood flow can be lead to a more considerable damage. Thus, I/R damage results in a progressive cellular and molecular changes. Inflammatory signaling which is a progressive process triggered by I/R is contributory closely in all critical events of the ischemic cascade, from the early damage induced by arterial occlusion, to the late regenerative processes. Inflammatory changes can also be observed in the blood, so that, the analysis of cytokines that are included among inflammatory mediators in peripheral blood samples may reflect cerebral cytokine production. In cerebral I/R process, which is continued with damage of the blood-brain barrier and with cerebral edema, which is one of the destructive complications of neurology inpatients, the increased release of AVP can aggravate injury or edema. Mannitol is a widely used diuretic-agent, but its clinical utility is controversial. Instead of mannitol, it is very little known about the benefits of aquaretic conivaptan during these processess. This is the first study to investigate the comparison of the effects of conivaptan and mannitol treatments on serum TNF-α, IL-15 and IL-35 levels and the correlations of these cytokines in an ischemia-reperfusion injury induced by bilateral common carotid artery occlusion. According to our biochemical and statistical findings, conivaptan can be more effective than mannitol in balancing pro-inflammatory/anti-inflammatory response and get the progressive inflammation process induced by ischemia under control. Additionally and importantly, we are of the opinion that this study may provide crucial information to the new treatment strategies especially for ischemia/inflammation related cerebrovascular diseases. It is clear that there is a need for comprehensive experimental studies.

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Authors give final approval of the version submitted. The authors declare that there is no conflict of interest.

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