Circulatory Levels of C-X-C Motif Chemokine Ligands 1, 9, and 10 Are Elevated in Patients with Ischemic Stroke

İskemik İnmeli Hastalarda 1, 9 ve 10 CXC-Kemokin Ligandlarının Dolaşımdaki Seviyelerinde Artış

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ABSTRACT

Objective: Inflammation plays a significant role in the development of ischemic stroke. CXC chemokines play pleiotropic roles in prolonged leukocyte locomotion, astrocyte migration/activation, and neural attachment/sprouting in response to focal stroke. In this study, we aimed to explore the changes in serum levels of three chemokines, C-X-C motif chemokine ligand 1 (CXCL1), C-X-C motif chemokine ligand 9 (CXCL9), and C-X-C motif chemokine ligand 10 (CXCL10), in ischemic stroke patients at the time of admission and before discharge from the hospital ward.

Materials and Methods: In this study, we recruited 43 unrelated ischemic stroke patients using an easy convenience method or accidental sampling which is a type of non-probability sampling that involves the sample being drawn from that part of the population that is close to hand. We also enrolled 50 genetically unrelated healthy controls showing no history of neurologic, cardiovascular, or inflammatory diseases. Serum levels of the considered chemokines were measured by enzyme-linked immunosorbent assay (ELISA) in patients and healthy controls.

Results: No significant difference was observed in ischemic stroke patients following hospitalization and prior discharging from the hospital; however, there was a significant difference in serum levels of CXCL9 and CXCL10 between patients and healthy controls. We also found that the level of the chemokine was not related to gender or medical therapy. It appears that CXCL9 and CXCL10 are more predisposing factors and play a direct role in stroke considering that they were higher in patients than in healthy controls.

Conclusion: We believe that this study might be used as a basis for further studies on more effective medication regimens to prevent the onset and subsequent complications of stroke. However, these mediators are useful diagnostic and prognostic tools rather than therapeutic tools.

Keywords: Chemokine, ischemia, stroke

ÖZ

Amaç: Enflamasyon iskemik inme gelişiminde önemli bir rol oynar. CXC kemokinlerinin fokal inmeye yanıt olarak gelişen nöral bağlanma/filizlenme, astrosit migrasyon/aktivasyon ve uzamış lökosit lokomosyonunda pleiotropic etkileri vardır. Bu çalışmada iskemik inmeli hastalarda, C-X-C motif kemokin ligand 10 (CXCL10), C-X-C motif kemokin ligand 1 (CXCL1) ve C-X-C motif kemokin ligand 9'u (CXCL9) içeren üç kemokinin serum düzeyindeki değişikliklerinin hastaneye kabul ve taburculuk sırasında araştırılması amaçlanmıştır.

Gereç ve Yöntem: Bu çalışmada, kolay uygunluk yöntemi kullanılarak birbiriyle ilişkisi olmayan 43 iskemik inmeli hasta incelendi. Ayrıca herhangi bir nörolojik, kardiyovasküler veya inflamatuvar hastalık öyküsü olmayan, genetik olarak birbiriyle bağlantısız 50 sağlıklı denek de çalışmaya dahil edildi. Hastalarda ve sağlıklı kontrollerde kemokinlerin serum düzeyleri enzime bağlı bağışıklık deneyi (ALISA) ile ölçüldü.

Bulgular: Mevcut çalışmada, iskemik inme geçirdiği için hastaneye yatırılan ve taburcu edilen hastalarda anlamlı bir farklılık izlenmedi. Ancak, hastalar ve sağlıklı kontroller arasında CXCL9 ve CXCL10 serum düzeyleri açısından anlamlı bir farklılık bulundu. Ayrıca, kemokin düzeylerinin cinsiyet ile ilişkili olmadığı ve tıbbi tedavinin hastalardaki kemokin seviyelerini etkilemediği görüldü. CXCL9 ve CXCL10 seviyelerinin kontrol grubu ile kıyaslandığında hasta grubunda daha yüksek olduğu düşünüldüğünde, bunların daha çok yatkınlık oluşturan etkenler oldukları ve inmede doğrudan bir rollerinin olduğu görülmektedir.

Sonuç: Bu çalışmanın, inmenin ortaya çıkmasını ve sonraki komplikasyonlarını önleyebilecek daha etkili tedavi rejimleri planlamada kullanılacak çalışmalar için bir temel oluşturabileceğine inanmaktayız. Ancak, bu medyatörler terapötik olmaktan ziyade, faydalı tanısal ve prognostik gereçlerdir.

Anahtar Kelimeler: Kemokin, iskemi, inme

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Introduction

Ischemic stroke is defined as a vascular brain disease that occurs because of a reduction in blood flow to the brain caused by acute occlusion of the intracranial blood vessels [1]. Ischemic stroke is considered as the third leading cause of death in industrial countries and the most frequent cause of adult permanent disability worldwide [2]. Approximately, 15%-30% of stroke survivors are permanently disabled, and almost 20% require institutional care [3]. Several severe deficits varying from partial paralysis, memory difficulties, and thinking, language, and speech problems to movement and locomotion disabilities are reported following ischemic stroke. Various aspects of the disease, such as severity and magnitude of symptoms, signs, and disabilities as well as mortality and survival rates, are mostly affected by the artery and region, duration of occlusion, postponement in appropriate medical interventions, and collateral blood flow; of these, the last aspect depends on the individual vascular anatomy and the site of the occlusion.

A wide spectrum of risk factors have been described for ischemic stroke, including age, cardiac diseases and anomalies (arrhythmias, septal defects, valvular diseases, etc.), vascular diseases (atherosclerosis of the brain or carotid arteries and deep vein thrombosis), and coagulopathies [2, 4].

Inflammatory response is considered one of the first immune processes occurring following injury, and it is believed that inflammation has a significant role in the commencement and further development of ischemic stroke.

As mentioned previously, by Braunersreuther et al. [5] the most prevalent reason for blood vessel occlusion and subsequent ischemic stroke is thrombosis and/or embolism. One of the most important diseases responsible for thrombosis and embolism is atherosclerosis, in which the inflammatory system plays a paramount role in disease development and progression [4, 5].

Subsequent to the event of ischemic stroke, the death of the neurons and particularly, the release of necrotic cell debris trigger an inflammatory response. During ischemic stroke, the production of various molecules involved in the initiation of immune cell recruitment to the lesion sites is induced. Similar to that was observed in other circumstances of acute brain trauma clinical state, inflammation also may occur during ischemic stroke to clean up the lesion and limit its spread [6]. Furthermore, ischemic reactions in the affected brain region result in dynamic and widespread activation of inflammatory cytokines and chemokines in the peripheral immune system [7].

Chemokines are a subclass of the larger cytokine superfamily that direct the migration of blood inflammatory cells, such as neutrophils and macrophages, toward the source of chemokine-expressing tissues and they play important roles in cellular communication and inflammatory cell recruitment [2, 8-10]. Concerning the position of the first two cysteine residues at the N-terminal end, chemokines are further subdivided into CC, CXC, XC, and CX3C [11]. They are also functionally described as "homeostatic" (meaning that they are secreted constitutively and involved in immune surveillance and mostly lymphocyte traffic) or "inflammatory" (meaning that they mediate pro-inflammatory signals and induce leukocyte-oriented locomotion to the damaged or infected tissue). Chemokines have also been shown to influence the phenomena of angiogenesis and cellular differentiation [12].

CXC chemokines are described as classical neutrophil chemoattractants in humans and mice. The interferon-inducible protein 10 (IP-10)/CXCL10 is a potent chemoattractant for monocytes, T-cells, and smooth muscle cells. It binds the CXCR3 receptor, which is shared with CXCL9, and is expressed during focal stroke in animal models [13]. CXCL10 plays a pleiotropic role in prolonged leukocyte recruitment, astrocyte migration/activation, and neural attachment/sprouting following focal stroke [14, 15]. In recent studies, CCL11, CCL5, and CXCL10 are associated with ischemic stroke independent of traditional cardiovascular risk factors [16].

CXCL1 is a potent neutrophil chemoattractant that is involved in the pathophysiology of stroke through inflammatory reactions during the immediate early phases of ischemic stroke [17].

CXCL9 is also involved in inflammatory disorders, such as atherosclerosis and rheumatoid arthritis, but its role in ischemic stroke has yet to be clarified [3]. However, because CXCL-9 belongs to the family of interferon-gamma-inducible chemokines (like CXCL10) which these are critical molecules in T-cell trafficking and the generation of effector T-cells.

In this study, we aimed to explore the changes in serum levels of three chemokines of the CXC subfamily CXCL10, CXCL1, and CXCL9 in ischemic stroke patients at the time of admission and before discharge from the hospital ward.

Materials and Methods

Subjects

In this study, we recruited 43 unrelated ischemia cerebrovascular accident (CVA) patients with ischemic stroke and not transient ischemic attack using an easy convenience method (Table 1). The mean age was 74.6±2 (range 39-93) years. Women who were pregnant and patients having a history of inflammatory or infectious diseases other than CVA were excluded from the study. The occurrence of ischemia CVA was confirmed by an expert neurologist. We also enrolled 50 genetically unrelated healthy controls showing no history of neurologic, cardiovascular, or inflammatory diseases. The study protocol was approved by the ethics committee at our institution (Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran) and written informed consent was obtained from all participants (or their legal guardian) prior to sample collection.

Sample collection

After diagnosis of ischemic CVA is confirmation by a neurologist, peripheral blood specimens were collected in ethylene diamine tetra acetic acid (EDTA) pre-coated tubes for hematological analysis. A clotted sample was also collected (clotted blood tube) and was further subjected to serum isolation. Another similar peripheral blood specimen was collected before discharging the patient from the hospital ward. Blood samples were immediately carried to the Molecular Medicine Research Center laboratory.

Chemokine assay

Serum levels of the considered chemokines were measured by ELISA (R&D systems, UK) in patients and healthy controls. Assays were performed as per the manufacturer's guidelines. The sensitivity of the kits was 2 pg/mL, and inter-assay and intra-assay assessments of the reliability of the kits were conducted.

Statistical analysis

Results are presented as the mean±standard error of mean (SEM) for numeric variables and as absolute frequencies and percentages for categorical variables. Student's t-test was employed for analysis of continuous variables that contain CXCL1, CXCL9, and CXCL10 chemokines, and the normality of the distribution of data was checked with the Kolmogorov-Smirnov test. Statistical Package for the Social Sciences software version 20.0 (IBM Corp.; Armonk, NY, USA) was used for all analyses. All p values were two-tailed, with statistical significance defined as $p \leq 0.05$.

For data without normal distribution, we used the Mann-Whitney U test to analyze the difference between healthy controls and patients and the Wilcoxon signed-rank test for comparing patients at the time of admission and before

discharge and effect of medication on the difference. p<0.05 was considered as significant.

Results

Demographics

Of the 43 patients, 25 were females and 18 were males, and their age ranged from 39 to 93 years (mean 74.6±2 years). The mean ages of the male and female patients were 74.8±3.2 and 74.4±2.7 years, respectively. We also included 50 healthy controls with a mean age of 48±12 years. Demographic data are presented in Table 1. A combination of ASA, citicoline, and clexane was the most frequent medication used, and other formulations were negligible. Hence, we can use statistical tests for this regimen only.

CXCL1

The obtained results demonstrated that the mean serum levels of CXCL1 in healthy controls and patients at the time of admission and before discharge were 122.67±13.54 pg/mL, 34.31±1.33 pg/mL, and 37.89±1.88 pg/mL, respectively (p<0.027) (Figure 1).

The expression of CXCL1 was not significantly different at the time of admission and before discharge in patients (p>0.223); however, it was significantly lower in patients than in healthy controls (p<0.004).

CXCL9

Serum levels of CXCL9 were 212.57±12.65 pg/mL, 557.82±35.35 pg/mL, and 571.64±132.08 pg/mL in healthy controls and patients at the time of admission and before discharge, respectively (p<0.001) (Figure 2). The expression of CXCL9 was not significantly different at the time of admission and before discharge in patients (p>0.525), but it was significantly higher in patients than in healthy controls (p<0.05).

CXCL10

Serum levels of CXCL10 were 75.52±4.21 pg/mL, 89.55±3.98 pg/mL, and 83.12±3.39 pg/mL in healthy controls and in patients at the time of admission and before discharge, respectively (p<0.729) (Figure 3). The expression of CXCL10 was also not significantly different between healthy controls and patients at the time of admission and before discharge, but there was a significant difference between the expression at the time of admission and before discharge time depending on the medication regimen (p=0.014).

When the expression of chemokine was compared with respect to the type of ischemia, there was only a slightly significant difference in major stroke (p=0.048), and no significant difference was found in the

Table 1. Demographic data and some clinical features of ischemic stroke patients

Demographics		
Gender	**Age range (years)**	**Number**
Female	<40 (young)	0
	40-59 (middle aged)	5
	60-79 (old)	9
	>80 (very old)	11
	Total	25
Male	<40 (young)	1
	40-59 (middle aged)	2
	60-79 (old)	6
	>80 (very old)	9
	Total	18
Total		43
Clinical Features		
Ischemia	**Gender**	**Number**
Lacunar Infarct	Male	2
	Female	8
	Total	10
Minor Stroke	Male	6
	Female	3
	Total	9
Major Stroke	Male	10
	Female	14
	Total	24
Total		43

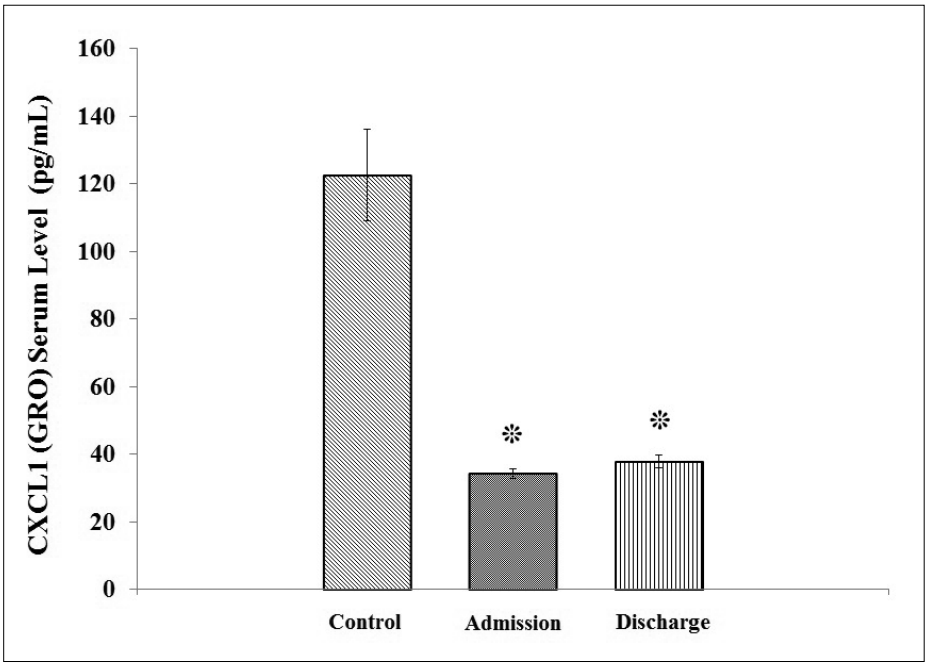

Figure 1. The serum level of CXCL1 obtained by ELISA (pg/mL).
Results are presented as mean±SEM
*Significant difference with healthy controls

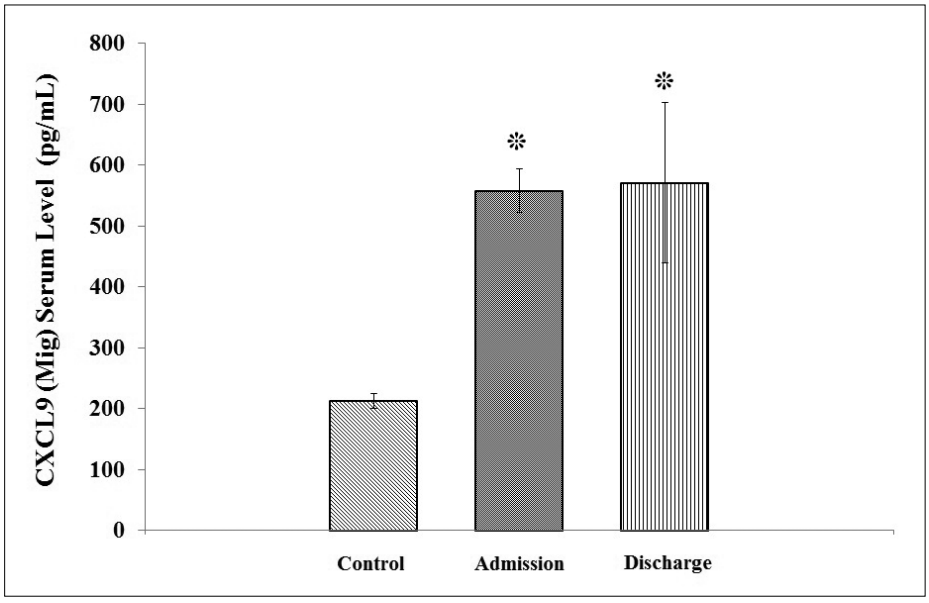

Figure 2. The serum level of CXCL9 level obtained by ELISA (pg/mL).
Results are presented as mean±SEM
*Significant difference with healthy controls

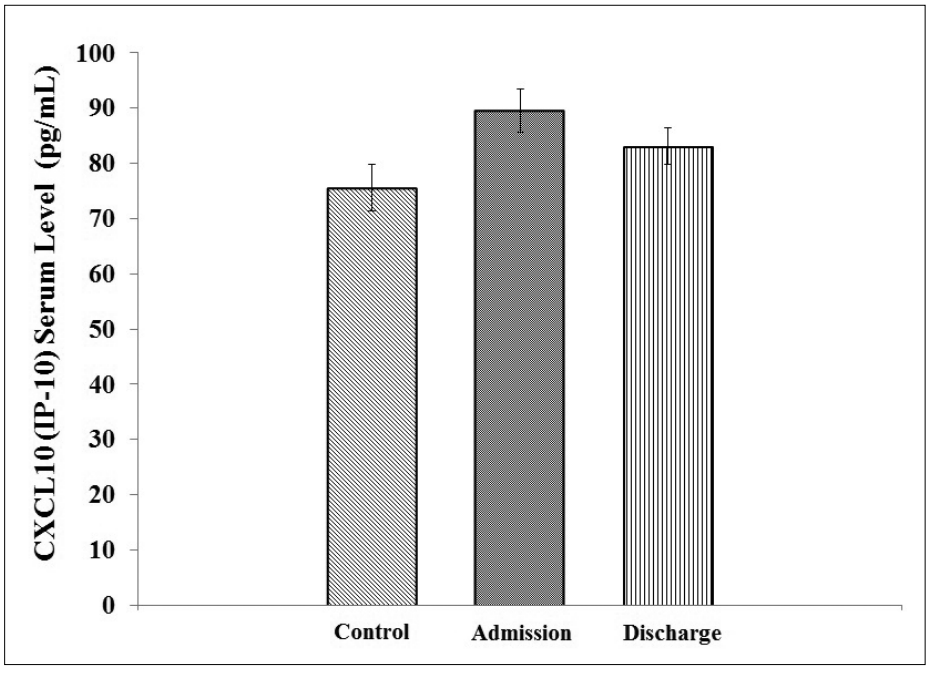

Figure 3. The serum level of CXCL10 obtained by ELISA (pg/mL).
Results are presented as mean±SEM
*Significant difference with healthy controls

expression between lacunar infarct and minor stroke (p>0.05). There was no significant change in any of the studied chemokines based on gender, medication regimen, or type of ischemia.

Discussion

The present study was undertaken to determine circulating levels of some CXC chemokines, such as CXCL1, CXCL9, and CXCL10, in ischemic stroke patients at the time of diagnosis and hospitalization and before discharge from the hospital ward of neurology and to compare these with those in healthy controls. We showed that serum levels of CXCL9 were increased in ischemic stroke patients, but serum levels of CXCL1 was decreased and those of CXCL10 were unchanged. We used ELISA to assess serum levels of chemokinesin participants. In this study, we also assessed the impact of the most routine therapeutic regimen used for stroke patients on circulating levels of the three CXC chemokines. The routine regimen for treatment of patients was ASA, citicoline, and clexane.

CXCL10 and its specific receptor CXCR3 are expressed in focal stroke in animal models [13]. As previously mentioned, CXCL10 plays a pleiotropic role in prolonged leukocyte recruitment, astrocyte migration/activation, and neuron attachment/sprouting after focal stroke [14, 15]. A previous study demonstrated the focal enhanced serum levels of CXCL10 in rat brains peaking at 1 h, a followed by a second peak at 3-6 h, and a third peak at 15 days after stroke [16]. In recent studies, CCL12, CCL5, and CXCL10 were associated with ischemic stroke independent of traditional cardiovascular risk factors. Braunersreuther et al. [5] demonstrated that CXCL10 plays a fundamental role in atherosclerosis along with some other CC and CXC chemokines.

In the present study, sample collection was performed on the day of admission and before discharge, and the average admission time was 11 days. There was no significant difference between patients and healthy controls or between patients at the time of admission and before discharge. But based on the medication regimen, there was a significant decrease during the time of admission and a weakly significant difference was seen between the time of admission and before discharge in major stroke patients. It seems that CXCL10 is a more predisposing factor that plays a role in the prediction of stroke, and a decrease in serum levels based on the medication regimen might reduce the future risk of stroke.

There are a few studies focused on the relation between CXCL1 and stroke. Consistent with our results, Losy et al. [17] showed that CXCL1 plays an important role in the pathophysiology of stroke and might be involved in inflammatory reactions during the early phase of ischemic stroke. Losy and co-workers reported that there was no significant difference between patients and healthy controls CXCL1 serum levels, however, it was higher in patients' cerebrospinal fluid, which had a positive relation with hypo-density in computed tomography.

In a study performed by Schmerbach et al. [18], the expression of CXCL1 mRNA was decreased in vivo by the anti-atherosclerotic drugs candesartan and pioglitazone. There was not also significant differences between patients and healthy controls or patients at the time of diagnosis and prior discharge as well as medication, gender, or type of ischemia regarding CXCL1 level. However, CXCL1 mRNA was significantly lower in patients in comparison to healthy controls. According to

the study by Losy et al. [17], it seems as though changes in the level of CXCL1 are more focal than systemic. It might be necessary to measure CXCL1 in the cerebrospinal fluid instead.

There are not enough studies considering levels of CXCL9 in stroke; however, it is known to be involved in inflammatory disorders such as atherosclerosis and rheumatoid arthritis, and participation with other inflammatory factors is well studied [3, 12, 14, 19, 20]. Its beneficial role in muscle reconstruction along with other chemokines is also well known [20, 21].

Again, in this study, there were no significant differences between patients and healthy controls, patients at the time of admission and before discharge, and regarding medication, gender, or type of ischemia, however CXCL9 was significantly higher in patients in comparison to healthy controls. It seems like the CXCL10 chemokine is a more predisposing factor than a chemokine and it plays a direct role in stroke considering that it is higher in patients than in healthy controls. To the best of our knowledge, this is the first report to address the role for this set of CXC chemokines in stroke.

In conclusion, the increased levels of CXCL1 as an angiogenesis factor might support the notion that this chemokine is highly involved in the pathogenesis of ischemic stroke. It needs to be evaluated at the beginning of ischemic stroke how CXCL1 is changed so as to delineate which event is the initiating factor (the increase of CXCL1 or the event of ischemic stroke). When looking at the other functions of CXCL1 as an inflammatory chemokine, our data suggest that increase in the level of CXCL1 is in response to other upstream events that occur during ischemic stroke. Surprisingly, CXCL9 was significantly increased while CXCL10 was unchanged following ischemic stroke. The differential pattern of CXCL9 compared with that of CXCL1 suggest that these chemokine changes are independent of their roles in inflammatory responses, and they should be looked at from the view point of angiogenesis/angiostasis functions rather than inflammation, both of which are important in ischemic stroke. To examine this, it is suggested to determine whether receptors of these chemokines are strongly expressed on endothelial cells of the blood vessels in ischemic regions rather than on infiltrated immune cells that were recruited to these regions.

To achieve better oxygenation in the ischemic regions mediated through vessel formation, our results suggest a pathomechanism by which angiogenic chemokines could be employed for rehabilitation after ischemic stroke. In other words, our findings propose that following the first ischemic stroke, we might be able to 1) predict the level of disease severity and 2) apply a cocktail of various recombinant angiogenics or antibodies against angiostatic chemokines to aid in rehabilitation or in prevention of the development of ischemic stroke.

In future studies, we will explore the expression of these chemokines at the mRNA level as well as determine how the chemokine receptors are expressed on the membranes of immune cells. These types of studies are limited because of sampling and ethical issues, and we were unable to perform immunohistochemical studies in stroke tissues in human-derived specimens. Finally, we believe that this study might be used as a basis for further studies seeking to develop effective medication regimens to prevent the onset and subsequent complications of stroke.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Rafsanjan University of Medical Sciences (Decision No: IR.RUMS.REC.1394.274).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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