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Research Article

A NOVEL BIOMARKER IN EXPERIMENTAL CEREBRAL ISCHEMIA: JUNCTIONAL ADHESION MOLECULE-A

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ABSTRACT

Objective: To investigate the role of blood brain barrier biomarkers for the detection of experimental cerebral ischemia in rats.

Materials and Methods: Forty adult male Wistar albino rats with a mean age of 4–6 months and an average weight of 350–400 g were used in the study. The rats were divided into five ischemia groups (control, 1.5 h of ischemia, 4.5 h of ischemia, 6 h of ischemia, and 24 h of ischemia). Cerebral ischemia was achieved by unilateral ligating of CCA and ECA at the same time. After surgical preparation and awaiting for appropriate ischemia time we collected blood and brain tissue samples. Then we investigated serum occludin, claudin-5 and JAM-A levels from blood samples and the apoptotic index and percentages of pycnotic nucleus from brain tissues histologically. The obtained data were analyzed using IBM SPSS Statistics software package version 18 and the Jamovi software package.

Results: Serum JAM-A level showed a statistically significant difference in all ischemia groups compared with the control group ($p<0.05$). Serum claudin-5 level, a statistically significant difference was found between the control group and the 6-h ischemia group ($p<0.05$), while no significant inter-group differences were determined for the serum occludin level. As a result, in our experimental focal cerebral ischemia model, serum JAM-A showed more significant and rapid increases compared to occluding and claudin-5.

Conclusion: Serum JAM-A might be successfully used in the early stages of ischemic stroke. The presence of hemiparesis or hemiplegic effects in all rat ischemia groups and the significant increases in pyknotic and apoptotic cell counts histologically suggest that our model is successful for focal cerebral ischemia.

Keywords: Cerebral ischemia, junctional adhesion molecule-A, occludin, claudin-5

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INTRODUCTION

Stroke is the most common cause of presentation and stays in hospital among neurological disorders. According to the 2019 statistical data of the World Health Organization, stroke ranks second among the worldwide mortality causes and is the third most common cause of disability among those who have to lead a disabled life (1).

In addition to neural injury, cerebral ischemia causes impairment of the blood–brain barrier (BBB), which may lead to vasogenic edema and hemorrhagic transformation, resulting in permanent brain damage. The BBB is a neurovascular structure consisting of endothelial cells, pericytes, astrocytes, microglia, and the extracellular matrix, together with tight-junction (TJ) proteins, such as occludin, claudin-5, junction adhesion molecule-A (JAM-A), and membrane-associated proteins (2). The BBB impairment severity depends on the ischemia's duration (3). As a result of ischemia in the nervous system, oxygen and glucose deficiency causes excitotoxicity. Neuronal cells lose their normal ionic gradient and become depolarized, resulting in the release of glutamate, which causes intracellular calcium (Ca) influx. This causes the activation of apoptosis, autophagocytosis and necrotic cell death pathways (4). Therefore, the delayed provision of reperfusion increases the extent of reperfusion injury, facilitating hemorrhagic transformation (5). Several recent experimental and clinical studies have indicated that TJ proteins could show biomarker characteristics and that the most studied biomarkers are occludin and claudin-5 (6-12).

Several intricately related members of junction adhesion molecules (JAMs) are named JAM-A, JAM-B, and JAM-C. JAM-A and JAM-C are expressed in the TJs of the BBB. JAM-A contributes to the brain endothelial junction complex by participating in paracellular permeability and leukocyte migration/adhesion (13).

To our knowledge, serum JAM-A level has not yet been studied in ischemic stroke. Our study investigated JAM-A, occludin, and claudin-5 levels in the serum of rats with induced ischemic stroke, their interrelationships with each other, and their characteristics as biomarkers.

MATERIALS AND METHODS

This study was approved by the Adnan Menderes University School of Medicine Ethics Committee (08/27/2020-077) and supported by Aydin Adnan Menderes University Research Fund (Project Number: 2020/TPF-20047). Forty adult male Wistar albino rats with a mean age of 4–6 months and an average weight of 350–400 g were used in the study. The animals used in the study were kept in transparent cages, with a maximum of four animals in the same cage, in a semi-climatized laboratory environment with an ambient temperature of 22±1ºC, 12/12 h light/dark cycle, relative humidity of 40%–50%, and controlled aeration. The experimental procedures were carried out in the Experimental Animal Laboratory of Adnan Menderes University. All animals were allowed to eat standard pellet rat chow (ad libitum) until 24 h before the procedure. Drinking water was not restricted.

The rats were divided into five groups as follows: control group, Group A (1.5 h of ischemia), Group B (4.5 h of ischemia), Group C (6 h of ischemia), and Group D (24 h of ischemia). Ischemia durations were determined as follows: 1.5 h for group A, which is the average time of arrival to the hospital and access to

treatment after ischemia; 4.5 h for group B, which is the time that thrombolytic treatment can be received after ischemic stroke; 6 h for group C, which is the time specified for thrombectomy; and the 24 h for group D, which is the most vulnerable period regarding hemorrhagic transformations after thrombolytic treatment and when most complications are experienced (14).

In the surgical procedure of the rats in the ischemia group under ketamine and xylazine anesthesia, the right common carotid artery (CCA) was dissected after making a cervical median incision, and the trachea was exposed. After dissecting the CCA, the dissection was extended superiorly, and the internal carotid artery/external carotid artery (ECA) bifurcation was exposed. Then, ECA and CCA were ligated with a number 1/0 silk suture.

The determined ischemia duration for each group was awaited, with no reperfusion. The rats in the control group were subjected to the same surgical procedures, except for the occlusion of ECA and CCA, and blood and tissue samples were collected after a waiting period of 1.5 h. Tissue samples taken from the right hemisphere of the rats were cut in coronal plane and the anterior part was spared for histological and the posterior part was spared for biochemical examination. Rats in all groups were sacrificed through cervical dislocation after collecting blood samples. Blood samples' serum occludin, claudin-5, and JAM-A levels were measured using a Fine Test (Fine Biotech Co. Ltd., Wuhan, China) rat ELISA kit.

Immediately after blood samples were taken, serum samples were centrifuged at 2000xg for 15 minutes and the serum was transferred to Eppendorfs and stored at -80 degrees for biochemical measurements. Serum occludin, claudin-5, and JAM-A, and levels were measured with the help of an Elisa kit and measurements were made in accordance with the user manual of the kit used. F11R levels in tissue were determined using a commercial kit (Fine Test, ER6476) in accordance with the manufacturer's procedure.

Histopathological evaluation

After tissue samples were homogenized in PBS (pH 7.4), they were centrifuged at 3000 rpm at +4°C and the upper phase was used for kit measurements. Brain tissues were fixed with 10% formalin solution for 72 hours. After routine histological follow-up procedures with alcohol, xylene and paraffin, serial sections of 5µ thickness were taken from the brain tissues embedded in paraffin blocks with a microtome (Leica RM2235, Leica Instruments, Nussloch, Germany). After routine deparaffinization, sections taken from each sample (four sections from each sample) were processed with hematoxylin-eosin. They were stained with and evaluated under a light microscope (Olympus BX50) and pictures were taken. The morphologies of the cerebral cortex and striatum at the same level in control and study groups were examined under a light microscope. The percentage of pyknotic cells was calculated [(number of pyknotic nuclei/total number of cells) × 100]. Pyknotic cells differ from healthy neurons by having a dense cell body, irregular nuclei, and being shrunken cells with prominent vacuoles (Figure 1).

TUNEL method

The degree and presence of damage were determined by the TUNEL (TdT-mediated dUTP nick end labeling) method using the In Situ Cell Death Detection, POD kit (Roche, Germany). In summary, the sections were incubated at 60 °C for one night and then deparaffinized by soaking in xylene for 3x5 min. Then, the

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sections were rehydrated by passing through decreasing alcohol series (100, 96, 80, 70%). After washing the sections with distilled water, they were incubated in 3% H_2O_2 prepared with methanol for 5 minutes. After washing 3 times with PBS, it was incubated in a microwave oven at 750W in citrate buffer (pH: 6) for one minute. After the sections were washed with PBS, the surrounding area was marked with a pap-pen and 50µl of reaction mixture was added onto the tissue. The reaction mixture was prepared by adding 50µl enzyme solution to 450µl marking solution. The sections were incubated with the reaction mixture for 1 hour in a dark and humid environment at 37 °C. After the sections were washed 3 times with PBS, the POD enzyme was distributed homogeneously on the tissue and incubated at 37 °C in a humid environment for 30 minutes. After the sections were washed with PBS, 50µl of DAB (Diaminobenzidine) substrate was dropped homogeneously onto each tissue and incubated for 10 minutes at room temperature. Sections washed with distilled water were stained with Mayer's Hematoxylin for 30 seconds for counterstaining. After the dye was removed by washing, the sections were kept in xylene for 15 minutes and covered with entellan and viewed on an Olympus BX50 light microscope. Coronal sections of the cerebral cortex at the same level randomly selected different areas, at 20x magnification were counted. Apoptotic and normal total cells stained positively with TUNEL were counted. The apoptotic index (number of apoptotic cells/total normal cell count) was calculated. The apoptotic index, which is the ratio of TUNEL positive neurons to all neurons, was expressed as a percentage (15; Figure 2).

Statistical analysis

Biochemical and histological data were analyzed using the IBM SPSS Statistics software package version 18 and the Jamovi software package. Variables within normal distribution were expressed as mean ± standard deviation (SD), and variables not within normal distribution were expressed as median [IQR]. Groups conforming to a normal distribution were selected using the Shapiro–Wilk analysis and then analyzed using one-way analysis of variance (ANOVA). The groups were evaluated using the post-hoc Tukey or Games–Howell test to identify the groups from which the differences originated. In groups not conforming to a normal distribution, analyses were performed using robust ANOVA or Kruskal–Wallis test with post-hoc Dwass–Steel–Critchlow–Fligner pair wise comparison test. To investigate any presence of correlation, Pearson or Spearman tests were used according to the distribution of normality of variables. A p-value < 0.05 was considered significant in all analyses.

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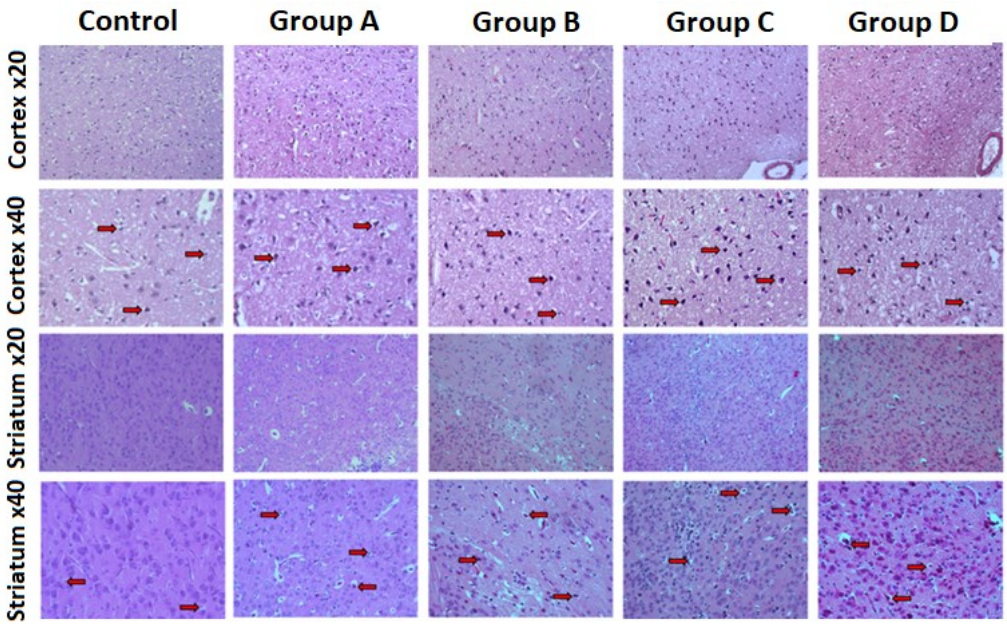

Figure 1. Apoptotic cells indicated by arrows by TUNEL staining (Mayer's hematoxylin was used as counterstaining; Olympus BX50 light microscope at 20x and 40x magnification)

RESULTS

As four rats in Group D died before the ischemia period of 24 h was completed, this group was excluded from the statistical analysis. While there was no death in the other groups, the data of the 4 surviving rats in group D are as in the table below (Table 1).

Table 1. Data for Group 4

Rats (n=4)	Serum JAM-A (pg/ml)	Serum Occludin (ng/ml)	Serum Claudin-5 (ng/ml)	Pyknotic nucleus %	Apoptotic index
D1	8521.53	31.22	3.67	57	48
D2	21430.72	45.41	12.48	43	41
D3	4809.84	20.42	12.23	49	36
D4	23050.88	42.27	17.35	53	33
Mean value	14453	34.83	11.43	50.5	39.5

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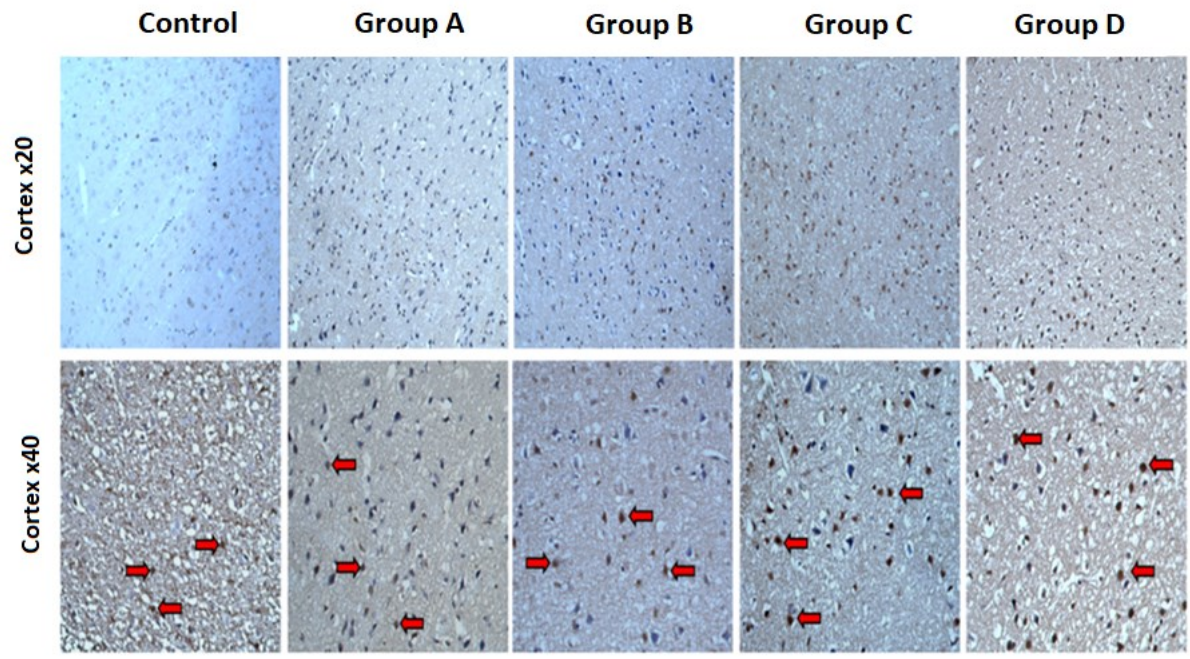

Figure 2. Preparations Stained Using the TUNEL Method. Hematoxylin was used as counterstainig. Apoptotic cells indicated by arrows (Olympus BX50 light microscope at 20x and 40x magnification)

Biochemical investigations

The serum JAM-A level showed a significant difference in all ischemia groups compared with the control group ($p<0.05$). In the serum claudin-5 level, a significant difference was found between the control group and the 6-h ischemia group ($p<0.05$), while no significant inter-group differences were determined for the serum occludin level. The groups' data are shown in Table 2, and the variables' values are presented as box plot graphs in Graphs 1, 2, and 3.

Table 2. Mean values and statistical analysis of the groups' histopathologic data

	Groups				
	Control	**A**	**B**	**C**	***p*-value**
Pycnotic nucleus %	9.13±2.42*	22.3±6.34*	29.8±5.57*	42±2.77*	<0.001
Apoptotic index	11.1±1.46*	20.6±4.57*	25.6±2.92*	37.8±2.38*	<0.001

*Significantly different from the control group in post hoc analysis.

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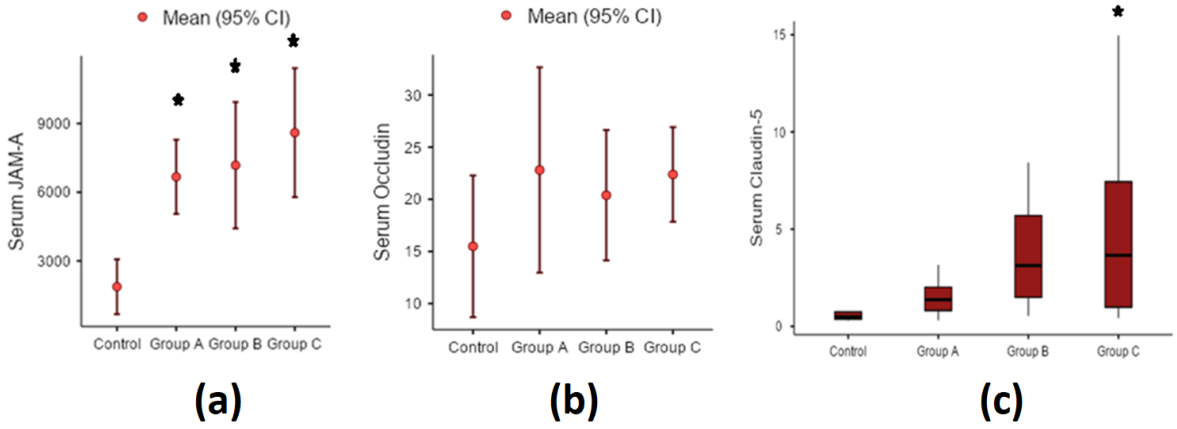

Figure 3. (a) Serum JAM-A, **(b)** serum occludin one-way ANOVA, **(c)** serum claudin-5 boxplot graphs. * showed a significant difference compared to the control group.

Table 3. Mean/median values and statistical analysis of the groups' biochemical data

	Groups				*p*-value
	Control	**A**	**B**	**C**	
Serum JAM-A (pg/ml)	1876 ± 1295	6671 ± 1749*	7178 ± 3301*	8598 ± 3365*	<0.001
Serum occluding (ng/ml)	15.5±8.13	22.8±11.8	20.4±7.49	22.4±4.91	>0.05
Serum claudin-5 (ng/ml)	0.49 [0.39]	1.38 [1.22]	3.13 [4.21]	3.66 [6.47]*	<0.05

Histopathologic investigations

The groups' pyknotic nucleus percentages and apoptotic index data are listed in Table 3. Significant differences were found among the groups regarding both variables (Figures 4a and 4b). According to the post hoc analysis, all groups were significantly different from each other.

The serum JAM-A and serum claudin-5 levels were moderately correlated with the apoptotic index and pyknotic nucleus percentage (Table 4).

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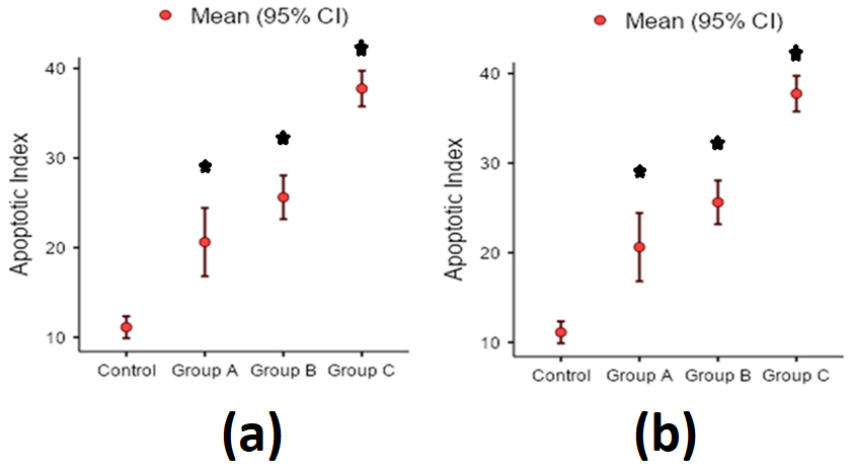

Figure 4. (a) The differences in apoptotic index, **(b)** pycnotic nucleus percentages among experiment groups. * showed a significant difference between the control group and each other.

Table 4. Correlation matrix of the relationships between variables

	Serum JAM-A	Serum occludin	Serum claudin-5	Apoptotic index	Pycnotic nucleus %
Serum JAM-A	-	0.427*	0.445 ***	0.643***	0.614***
Serum occludin	0.427*	-	-	-	-
Serum claudin-5	0.445***	-	-	0.339**	0.429**
Apoptotic index	0.643***	-	0.339**		0.917***
Pycnotic nucleus %	0.614***	-	0.429**	0.917***	-

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

DISCUSSION

We found that the level of JAM-A was elevated during the early ischemia period. Furthermore, the serum JAM-A level significantly increased in all groups, starting from 1.5 h of ischemia, compared with the control group proving that JAM-A may be a novel biomarker that rapidly increases during the early ischemia period. Although there is no ischemic brain injury model in the literature on serum JAM-A levels, JAM-A expression has been reported to decrease in brain tissues in models of cortical cold injury, multiple sclerosis, and traumatic brain injury (16-18).

Kago et al. reported that occludin levels decreased by Western blot in the brain tissue on the 1st, 2nd and 3rd day in the microembolism ischemia model, and Jiao et al. reported that in the MCAO reperfusion model, the decrease in occludin and claudin-5 in brain tissue started at the 3rd hour and continued on the 3rd day (19-20). In another study, Pan et al. stated that hourly blood occludin levels reached their highest values at 4.5 hours, and that the acceleration of the rise increased significantly after 5 minutes of reperfusion (10). However, Shi et al. stated that blood occludin levels increased in their reperfusion model, but they did not find a significant increase in claudin-5 levels (9).

There is only one JAM-A-related ischemia model in the literature. In this model, the effect of JAM-A on inflammation was investigated by administering a JAM-A antagonist peptide, which was reported to reduce the infarct area (13). According to our data, even though not a significant but still a noticeable moderate increase was present in the blood occludin levels, the blood claudin-5 level significantly increased at the 6thhour. Therefore, we consider that significant increases in the claudin-5 level at the 6th hour in wake-up stroke may provide clues to the clinician about the stroke time.

In various experimental cerebral ischemia–reperfusion models conducted in the literature, the occludin and claudin-5 levels in brain tissue decreased for periods of 3 h to 3 days. Conversely, serum occludin and claudin-5 levels increased between 1.5 h and 4.5 h (6-9). Occludin and claudin-5 have also been investigated in human studies. Various publications have reported that occludin and claudin-5 levels were significantly higher in ischemic stroke cases with hemorrhagic transformation, and there was a relationship between the serum occludin level and poor prognosis (11-12). Our data are consistent with the literature. The lack of a significant difference in occludin levels could have been due to our model's absence of the reperfusion process. Therefore, we suggest that JAM-A and claudin-5 will be more successful in predicting complications and prognoses in patients with ischemic stroke whose reperfusion cannot be established.

The Rice–Vannucci model one of the most extensively studied among focal cerebral ischemia models and applied as a combination of extracranial CCA occlusion and recurrent exposure to respiratory hypoxia, was modified by Edward et al. in 2017 (21). They created a new focal cerebral ischemia model by adding ECA occlusion to the CCA occlusion and hypoxia procedure. They attained more severe and standard ischemia levels with this model than the ischemia induced by the Rice–Vannucci method alone (22). Therefore, to avoid hypoxia's global effects, we preferred to occlude both the right CCA and ECA to establish a focal cerebral ischemia model. The fact that hemiparesis or hemiplegic effects were observed in all rats after the termination of the anesthesia's effects and that pyknotic cells and apoptotic cells, which are the indicators of ischemic damage, were significantly more common in the ischemia groups than in the control group indicate that the ischemia model we employed was successful.

Limitations

In this study an experimental cerebral ischemia model was created with rats. For this reason, the data we have obtained may not be applicable in humans. Due to the deaths in the 24-hour ischemia group, the effects of ischemia on this hour could not be investigated.

CONCLUSION

Starting from 1.5 h of ischemia, the serum JAM-A level showed more significant increases than the occludin and claudin-5 levels. This suggests that this biomarker could be successfully used in the early stages of ischemic stroke. The presence of hemiplegic effects in all rat ischemia groups and the significant increases in pyknotic and apoptotic cell counts suggest that our model is successful for focal cerebral ischemia. To shed light on future studies, the usability of serum JAM-A levels as a biomarker should be continuously investigated not only in animal experiments but also in human studies and to clarify its predictive power in

the treatment, prognosis, and complications of ischemic stroke. Thus, this biomarker can be used as a guide to predict the prognosis and complications and to determine the treatment modality of ischemic stroke, which is one of the main causes of mortality and morbidity.

Acknowledgments

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Authorship contributions

GTY and YEO formed the study concept and design, GTY, ÖÇ and EG contributed to the acquisition of the data, YEO, ÖÇ and EG performed analysis and interpretation of the data, GTY, YEO, ÖÇ and EG contributed to the drafting of the manuscript, critical revision of the manuscript for important intellectual content, YEO and ÖÇ provided statistical expertise, and YEO and GTY contributed to the acquisition of funding.

Data availibity statement

The data that support the findings of this study are available from the corresponding author, [YEO], upon reasonable request.

Declaration of competing interest

GTY, YEO, ÖÇ, and EG reports no conflict of interest.

Ethics

This study was approved by the Adnan Menderes University School of Medicine Ethics Committee (08/27/2020-077) and supported by Aydin Adnan Menderes University Research Fund (Project Number: 2020/TPF-20047).

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