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EXPERIMENTAL STUDY

Is ceftriaxone effective in experimental brain ischemia/ /reperfusion injury?

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ABSTRACT

OBJECTIVE: The aim of the present study was to investigate the neuroprotective effect of ceftriaxone in a rat brain ischemia/reperfusion injury model.

METHODS: The oxidative stress parameter, malondialdehyde (MDA) levels with or without ceftriaxone treatment in brain ischemia/reperfusion injured rats as well as in controls were measured in serum and brain tissue. Motor examinations of the rats were also performed. One-way Analysis of Variance (ANOVA) test was used for analysis. Duncan's Multiple Range Test was performed in multiple comparisons. $p < 0.05$ were considered statistically significant.

RESULTS: The data of this study showed that ceftriaxone treatment reduced the MDA levels in brain tissues in ischemia/reperfusion injured rats. Moreover, Bederson motor scores were higher in the ceftriaxone treated group as compared to the ischemia group ($p = 0.092$).

CONCLUSION: These results suggest that ceftriaxone could be beneficial for the prevention of brain ischemia/reperfusion injury caused by acute arterial occlusion through reducing the tissue MDA level *(Tab. 2, Fig. 5, Ref. 24).* Text in PDF *www.elis.sk*

KEY WORDS: ceftriaxone, ischemia/reperfusion injury, antioxidant, neuroprotective, stroke.

Introduction

Ischemia involves cell death through a shortage of oxygen in tissue, exhaustion of stored intracellular energy, and formation of toxic metabolites due to the reduction of arterial or venous blood flow, occlusion, bleeding of the vessel (1). Reactive oxygen radicals (ROS), mainly formed by the rapid introduction of free oxygen into the cell, are responsible along with several mechanisms for reperfusion-induced damage; and proteins, deoxyribonucleic acids, nucleic acids and membrane lipids are the most sensitive structures among cellular structures (2). Damage in reperfusion is much more serious than ischemia itself (3). Cerebral ischemia (stroke) can be defined as permanent or temporary neurological deficit formation due to ischemia, when focal or global cerebral perfusion decreases or ceases (4, 5). Cerebral ischemia is one of the most common causes of disability and death in the world (6). 80–85 % of stroke cases are due to ischemia and 15–20 % are due to haemorrhage. Although an acute stroke is the most common cause of mortality after coronary diseases and cancer, it is among the common causes of epilepsy, dementia and depression in the elderly. In the USA, there are 795,000 new stroke cases per year and one person undergoes a stroke every 40 seconds, and one person is lost for this reason every 4 minutes (7). Cerebrovascular ischemia appears to be an important pathology that seriously affects the quality of life of people, who do not die and causes them to need care in their daily lives (8).

There are many studies and experimental models on ischemic stroke as well as studies to better understand it, to be able to reverse the cerebral damage, and to create treatment models due to the mortality, morbidity, economic and psycho-social losses of ischemic stroke. Currently, there is no clear treatment for ischemia reversal.

The residual blood flow around the ischemic region is dysfunctional in the acute period while neurons maintain their short-term morphological and biochemical integrity. The areas in this process, in which these areas maintain their structural integrity and progress towards infarct, depending on the severity and the length of the ischemia are called *penumbra*. This process is associated with the residual blood flow and can take from minutes to hours. Penumbra can be saved in the early period by providing a blood flow again and / or using neuroprotective agents (9). This recoverable tissue is the target of today's treatment approaches. In order to save the penumbra, which has serious consequences in case of progressing to ischemia, today, treatment methods such as: glutamate antagonists, calcium channel blockers, dimethylsulfoxide, mannitol, steroids, isoproterenol, cerebrospinal fluid pressure reduction, vitamins E and C, thyroid releasing hormone,

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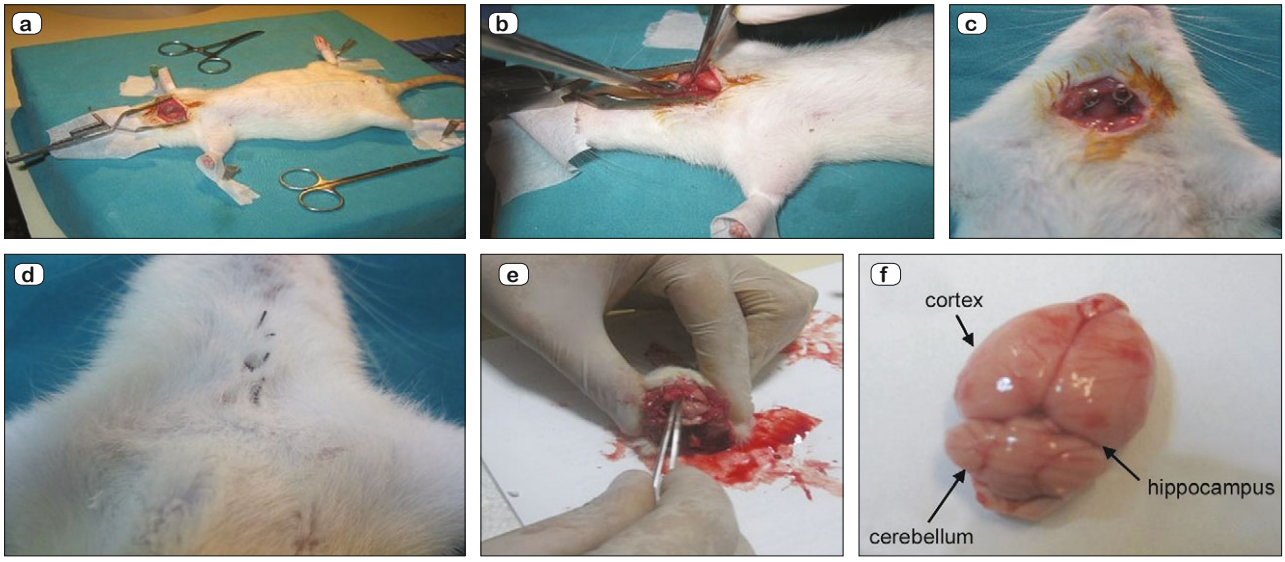

Fig. 1. Surgical procedures performed on rats (a) Dissection of bilateral paratracheal areas to reveal A. Carotis Communis, (b) After dissecting A. Carotis Communis from N. Vagus, clipping of these arteries by Yasargil aneurysm clips, (c) Yasargil aneurysm clips were placed for 30 min, (d) The tissue layers were sutured properly, (e) The brains of the rats were removed, (f) The hippocampus, c. striatum, corpus callosum, and thalamus parts of the brain were dissected.

aminophylline, sodium channel blockers, hypothermia and local cooling are used (10, 11).

Ceftriaxone is the third-generation cephalosporin that is effective against H. influenzae, N. gonorrheae, meningococci and pneumococci, and is 90% bound to plasma proteins, penetrates to cerebrospinal fluid well, is partially metabolised in the liver and partially eliminated from the kidney, and has the longest half-life among cephalosporins. In addition to its calcium binding properties, it also has a neuroprotective effect by protecting neurons from glutamate toxicity by causing an increase in glutamate-bearing protein levels (12). A reduction and a complete blockage of cerebral flow by clipping bilateral artery carotis communis in rats is a widely used method to understand the pathophysiology of focal cerebral ischemia (13).

Considering the studies, we aimed to investigate the neuroprotective effects of ceftriaxone on ischemia and reperfusion injury by creating an experimental focal brain ischemia in rats.

Materials and methods

The study was conducted in the Karadeniz Technical University (KTU) Faculty of Medicine Experimental Research Center Laboratory. All experimental protocols were approved by the Laboratory Animals Ethics Committee of Karadeniz Technical University (with the decision of the Animal Experiments Local Ethics Committee of Karadeniz Technical University numbered 2016/55). The biochemical analyses were carried out in the the Research Laboratory of the Department of Biochemistry.

Laboratory animals

38 female Spraque Dawley rats, each weighing on average 220–280 g, were used in the study. The general health of the rats was examined before the study and they were monitored under standard conditions and in individual cages without water and feed restrictions, and each rat was marked with the appropriate method according to the group in which it was included.

The rats were divided into 4 main groups:

Group 1 (Ceftriaxone): This group of twelve rats received daily intraperitoneal injections of 200 mg/kg ceftriaxone following 30 min of bilateral carotid artery clipping + hypotension. They underwent neurological examination at the end of postoperative days 1, 4, 7 and 10. At the end of day 10, they were sacrificed, and the brain tissue was removed.

Group 2 (Ischemia): Clipping of the bilateral carotid arteries + hypotension induction was performed for 30 minutes in this group consisting of 12 rats. They underwent neurological examination at the end of postoperative days 1, 4, 7 and 10. At the end of day 10, they were sacrificed, and the brain tissue was removed.

Group 3 (Sham = Control): This group of 7 rats was anesthetized only and neurological examinations were performed at the end of day 1, day 4, day 7, and day 10. At the end of day 10, they were sacrificed, and the brain tissue was removed.

Group 4 (Pure Control Group): This group of 7 rats underwent skin laceration after anaesthesia and ceftriaxone was given every day intraperitoneally at a dose of 200 mg / kg / day. Neurological examinations were performed at the end of days 1, 4, 7 and 10. At the end of day 10, they were sacrificed, and the brain tissue was removed.

Anaesthesia and surgical procedure

The rats were fasted for 24 hours prior to surgery and only water was given during this period. In order to provide anaesthesia, the rats were given 10 mg/kg xylazin hydrochloride (Rompun; Bayer Healthcare) intraperitoneally and 30 mg/kg ketamine

hydrochloride (Ketalar; Pfizer) intraperitoneally. While the anesthetized rats were in the supine position, the surgical site was shaved and stained with 10 % povidone iodine solution (Batticon; Adeka). After making a midline skin incision, a retractor was placed. Bilateral paratracheal areas were dissected by blunt dissection. A. Carotis Communis were revealed (Fig. 1a). After dissecting A. Carotis Communis from N.vagus, Yaşargil aneurysm clips were placed (Fig. 1b). There was a 30 min wait period after placing Yaşargil aneurysm clips in the bilateral A. Carotis Communis (Fig. 1c). Meanwhile, approximately 3cc (10 ml/kg) of blood was taken intracardially and hypotension was induced simultaneously. After the clips were removed at the end of 30 minutes, the arterial flow was reviewed, and the layers were sutured properly (Fig. 1d). Rats were placed in separate cages, where they could easily access food and water. Neurological examinations were performed on rats on the 1st, 4th, 7th, and 10th days. Ceftriaxone was prepared at a dose of 200 mg/kg/day and injected intraperitoneally for 10 days. Ten days later, intracardiac 4cc blood was taken from the 10 surviving rats and the rats were sacrificed, and their brains were removed (Fig. 1e). The hippocampus, c. striatum, corpus callosum, and thalamus parts of the brain were dissected (Fig. 1f). They were kept in a deep freezer at –76 °C for biochemical analysis. Changes in the brain were evaluated biochemically by measuring malonaldehyde (MDA) levels in both blood and tissue.

Tab. 1. Bederson motor scale.

Grade 0	No neurological deficits	5 point
Grade 1	Flexion in the front legs	4 point
Grade 2	Reduced resistance to lateral pushing motion without Rolling motion	3 point
Grade 3	Addition of rotational motion in addition to Grade 2	2 point
Grade 4	Exitus	1 point

Determination of malondialdehyde in tissues

The determination was made with the modification of Mihara and Uchiyama method (14). The method is based on measuring the absorbance at 532 nm of the colour of the molecule made by MDA with thiobarbituric acid (TBA) in an acidic environment.

Sample preparation

Approximately 50 mg cuts were made from each tissue. These tissues were then homogenized in 2 mL of PBS at 9500 rpm (4x10s, 40 °C) with a homogenizer (Jane and Kunkel, Germany). The homogenates were centrifuged at 4000 rpm for 10 minutes. The supernatants at the end of the centrifugation were diluted at 1:10 with PBS and an MDA measurement was performed.

MDA measurement in tissues

3 mL of 1 % H_3POI_4 was added to the 500 µL homogenate and mixed. 1 mL of 0.672 % TBA was added to the mix. After mixing, it was incubated in a water bath for 60 minutes. After 60 minutes, it was allowed to cool at room temperature and centrifuged at room temperature at 4000 rpm for 10 minutes. As the result of centrifugation, 200 µl of supernatants were taken and loaded into 96-well plates, and absorbances at 532 nm wavelength were read in the microplate reader spectrophotometer (Versamax, Molecular Devices, California, USA). The standard absorbance results obtained were plotted against concentration and the MDA standard plot was drawn. Using this graph, the amount of tissue MDA was calculated as nmol MDA / gram wet tissue (Fig. 2a).

Determination of malondialdehyde in plasma

Rat serum samples were kept at –80 °C until biochemical analyses were performed. The amount of malondialdehyde in serum samples was determined using the Thiobarbituric Acid Reactive Substance (TBARS) method developed by Yagi (15). The red colour formed as the result of the reaction between the lipid peroxidation product (MDA) and thiobarbituric acid (TBA) was measured spectrophotometrically. Serum lipids were precipitated together with the protein with the phosphotungstic acid/sulfuric acid system to remove water-soluble substances that react with thiobarbituric acid and give the same colour.

Measurement of Serum MDA

150 µL serum, 1200 µL H_2SO_4 and 150 µL phosphotungstic acid were added in test tubes, and after the tubes were mixed, they

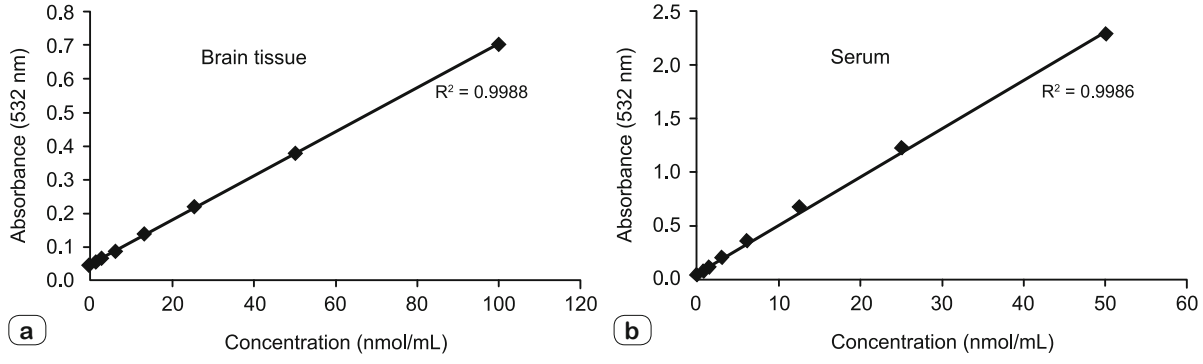

Fig. 2. The graphs of standard absorbance results against brain tissue (a), and serum (b) MDA concentraions.

Tab. 2. Median and Min–Max Values of the groups in the 1st, 4th, 7th and 10th days according to the Bederson Motor Scale.

Groups	1st day		4th day		7th day		10th day	
	Med.	Min–Max.	Med.	Min–Max.	Med.	Min–Max.	Med.	Min–Max.
Ceftriaxone	2.66	2–5	3	2–5	3.33	2–5	3.75	2–5
Ischemia	2.83	2–5	3.16	2–5	3.08	2–5	3.16	2–5
Sham	4.42	1–5	4.42	1–5	4.42	1–5	4.42	1–5
Pure Control	3.85	3–5	4.71	4–5	5	5–5	5	5–5

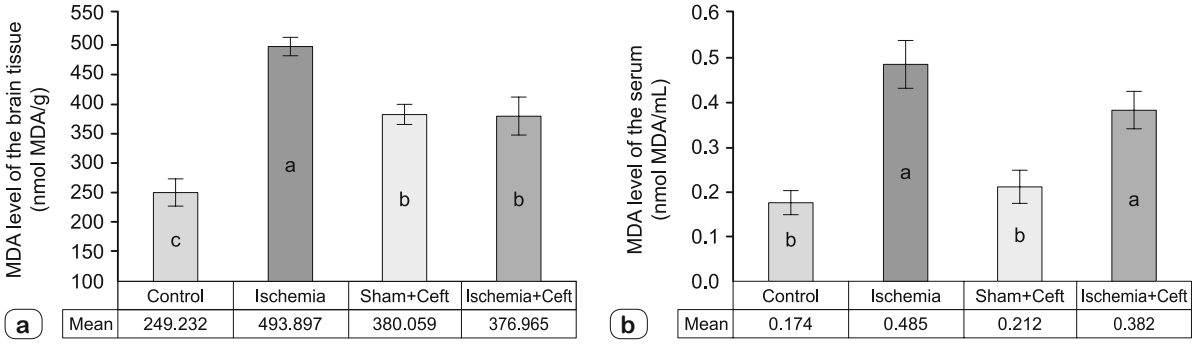

Fig. 3. MDA levels of the brain tissue (a), and serum (b) between groups.

were kept in room temperature for 5 minutes. The mixtures were centrifuged at 1500 g for 10 minutes and the supernatants were discarded. 2 mL of distilled water was added to the precipitate and vortexed until dissolved. 500 µL TBA was added to the tubes and incubated for 1 hour at 100 °C. After the incubation, the tubes were centrifuged at 1000 g for 10 minutes. 200 µL of the clear sections on top and loaded onto 96-well plates, and absorbances at 532 nm wavelength were read on the microplate reader spectrophotometer (Versamax, Molecular Devices, California, USA). The standard absorbance results obtained were plotted against concentration and the MDA standard plot was drawn. Using this chart, the serum MDA amount was calculated as nmol/mL (Fig. 2b).

Motor examinations

Motor scores of the rats in each group were recorded on the 1st, 4th, 7th and 10th days according to the Bederson scale (16) (Tab. 1).

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 22.0 package program was used to analyse the data. In all measurements, statistical differences and significance levels were determined by completing a "One-way Analysis of Variance (ANOVA)" test, and the results at $p < 0.05$ were considered statistically significant. Duncan's Multiple Range Test was performed in multiple comparisons.

Results

When the control and the ischemia group were compared in terms of the MDA levels in the brain, the MDA level in tissue of ischemia group was found to be significantly increased ($p < 0.05$). Significant elevation in tissue MDA level in ischemia group was an indication that brain ischemia had been done adequately. While there was no significant difference between the MDA levels of the pure control and ceftriaxone groups ($p > 0.05$), it was found that ceftriaxone application significantly decreased the tissue MDA level compared to the ischemia group ($p < 0.05$) (Fig. 3a).

Serum MDA level was observed to increase significantly, when the ischemia group was compared to the control group ($p < 0.05$). It was determined that ceftriaxone applied in ischemia did not provide a significant decrease in serum MDA level ($p > 0.05$) (Fig. 3b).

No statistically significant difference in Bederson motor scores was found between ceftriaxone and ischemia groups ($p > 0.05$). But the p value was very close to the limit of significance ($p = 0.092$) (Tab. 2, Fig. 4).

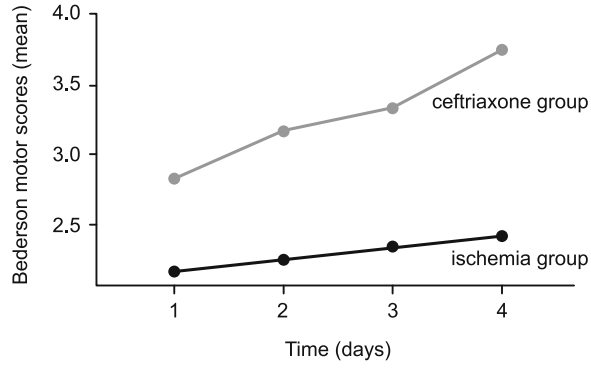

Fig. 4. The graph of the Bederson motor scores between ceftriaxone and ischemia. groups.

Discussion

Many experimental methods have been tried in order to understand the pathophysiology of cerebral ischemia and to create treatment models (17). Among those methods, temporary occlusion by means of clipping in the bilateral main carotid arteries, which is commonly preferred, and performing hypotension at the 30–35 mmHg level by removing the blood simultaneously, followed by performing reperfusion by removing the clips were used in our study. In our study, we created an ischemia/reperfusion injury in the brain by applying the bilateral carotid arteries clipping + hypotension model, and used ceftriaxone antibiotics that were shown to have neuroprotective effects in previous studies, which is one of the surgical prophylaxis preferences that also has a penetration to cerebrospinal fluid.

Following cerebrovascular ischemia, a damage occurs as the result of the interaction of multiple complex pathways including excitotoxicity, acid-base imbalance, ion imbalance, peri-infarction depolarization, oxidative and nitrative stress, inflammation, and apoptosis (18). After the breakdown of adenosine 5'-triphosphate (ATP) production and Na^+/K^+ carrier, cellular depolarization provides Ca^{2+} flow resulting in the activation of the intrinsic apoptosis pathway and cell death. The accumulation of glutamate in the extracellular space results in the activation of N-methyl-d-aspartate (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and the cainate glutamate receptors mediating the Ca^{2+} flow. With an increased oxidative stress, the reduction of energy dependent scavenging enzymes during stroke, free radical formation occurs with superoxide, hydroxyl radical, nitric oxide, and peroxynitrite (1). Free radical production also consists of arachidonic acid metabolism, infiltration of peripheral leukocytes, and upregulation of inducible nitric oxide synthase caused by microglia and monocytes, all occurring during an acute stroke. Free radicals and Ca^{2+} induce inflammatory cytokines and chemokines, as well as endothelial cell adhesion molecules and proinflammatory genes (19).

Free radicals also result in lipid peroxidation, apoptosis induction, and affect the production of 4-hydroxynonenal, which covalently modifies Na^+/K^+ ATPase, glucose and glutamate carriers (20). Endothelial changes may also result in a dysfunction in the blood brain barrier and increased inflammatory response. Increased cerebral oedema after ischemic stroke also plays a role in the deterioration of patient prognosis (21).

Briefly, the excessive release of glutamate, an excitant neurotransmitter, in ischemia due to multiple complex cascades including excitotoxicity, acid-base imbalance, ion imbalance, peri-infarction depolarization, oxidative and nitrative stress, inflammation, apoptosis, can overly stimulate N-methyl-d-aspartate (NMDA) receptors, causing a calcium overload and triggering an apoptotic cell death in neurons (Fig. 5). Glutamate released in the synaptic cleft is taken up by the glial cells via glutamate transporter-1 (GLT-1), thereby terminating the glutamate function in the synapse. An increased clearance of glutamate from synapse helps to prevent glutamate excitotoxicity. Ceftriaxone (CEF), a beta-lactam antibiotic, enhances GLT-1 expression and the uptake of released glutamate and improves glutamate excitotoxicity and can be an alternative strategy to protect neurons from an excitotoxic cell death (18, 22–24). Our study was done by measuring MDA values, an indicator of ischemic stress, in brain tissue and serum.

In our study, when the control and the ischemia group were compared in terms of the MDA levels in the brain, the MDA level in tissue was found to be significantly increased ($p < 0.05$). While there was no significant difference between the MDA levels of the sham and ceftriaxone groups ($p > 0.05$), it was found that ceftriaxone application significantly decreased the tissue MDA level compared to the ischemia group ($p < 0.05$).

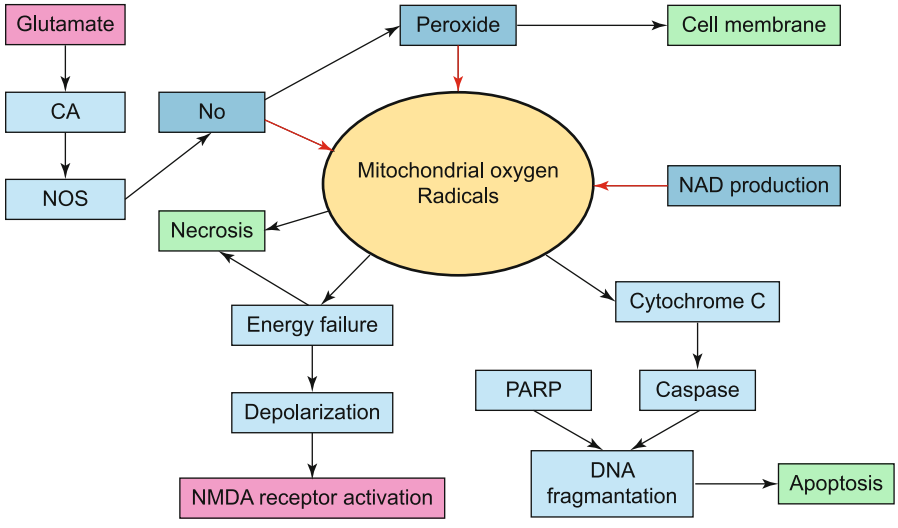

Fig. 5. Diagram of multiple complex cascades after cerebral ischemia/reperfusion injury triggering apoptotic cell death in neurons.

Serum MDA level was observed to increase significantly, when the ischemia group was compared to the control group ($p < 0.05$). It was determined that ceftriaxone applied in ischemia did not provide a significant decrease in serum MDA level ($p > 0.05$).

Here, ceftriaxone certainly did not have an antioxidant characteristic, but it was also seen that it did not reduce the effect of ischemia application on blood. This effect may seem normal due to the weak antioxidant capacity of blood tissue compared to other tissues. The weakness of the antioxidant capacity of the blood tissue is due to erythrocytes containing low levels of defence agents as it lacks mitochondrial activity, the creation of a continuous source of superoxide through oxidative activities such as: nitric oxide (NO) activity around the endothelium, and reflecting the effects occurring in other tissues. In fact, serum MDA levels also indicate a general oxidative stress occurring in the body. We think that the serum MDA level is not only specific for ischemia.

Conclusion

In this study, as ceftriaxone did not have an antioxidant characteristic, it was seen that it did not decrease the oxidative stress effect caused by ischemia. We believe that this result is normal because the antioxidant capacity of blood tissue was weak compared to other tissues. In fact, serum MDA levels also indicate a general oxidative stress occurring in the body. We think that it would not be correct to associate changes with only ischemia as the changes are not specific.

In conclusion, in our study it was shown that ceftriaxone had neuroprotective activity in cerebral tissue in cerebral ischemia/ /reperfusion injury.

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