

ANTIOXIDANT ACTIVITY-MEDIATED NEUROPROTECTIVE EFFECTS OF AN ANTAGONIST OF AT1 RECEPTORS, CANDESARTAN, AGAINST CEREBRAL ISCHEMIA AND EDEMA IN RATS

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We examined the effects of post-ischemic blockade of angiotensin AT1 receptors by candesartan on cerebral infarction and formation of edema. Male Sprague–Dawley rats were divided into three groups, sham, control ischemic, and candesartan-treated (0.3 mg/kg) ischemic. Transient focal cerebral ischemia was induced by 90-min-long occlusion of the left middle cerebral artery followed by 24-h-long reperfusion. Neurological deficit score was evaluated at the end of the reperfusion period. Thereafter, the animals were randomly selected and used for three projects: (i) Measurement of the infarct volumes, (ii) investigation of ischemic brain edema formation using a wet/dry method, and (iii) assessment of the malondialdehyde (MDA) and reduced glutathione (GSH) concentrations using a HPLC technique. Induction of cerebral ischemia in the control group produced considerable infarctions in the cortex and striatum in conjunction with severely impaired motor functions. Candesartan treatment significantly reduced the infarct volumes and improved the above functions. The water content in the left (lesioned) hemisphere was considerably elevated in the control ischemic group. Candesartan treatment significantly lowered the water content in the ischemic lesioned hemisphere, retained tissue GSH level, and led to a lower MDA production. AT1 receptor blockade by candesartan treatment can noticeably decrease ischemic brain injury and attenuate edema formation, likely via increasing the antioxidant activity.

Keywords: focal cerebral ischemia, brain edema, candesartan, malondialdehyde, glutathione.

INTRODUCTION

With an around 30% mortality rate, stroke remains the third leading cause of death in industrialized countries. Ischemic brain injury results from a complex sequence of pathophysiological events developing over time and space [1]. The extent of the tissue damage depends on both intensity and duration of focal cerebral ischemia [2]. Ischemic brain edema is a life-threatening complication of cerebral infarction that significantly aggravates the primary ischemic injury of the brain [3] via increased intracranial pressure and herniation [1]. Therefore, prevention from the development of brain edema may decrease cerebral injury and reduce stroke-related mortality.

Experimental and clinical studies allowed

researchers to suggest that inhibition of the renin-angiotensin system (RAS) by inactivation of ACE or angiotensin 1 (AT1) receptors might be effective not only in reducing the occurrence of stroke, but also may attenuate neuronal injury [4]. Furthermore, long-term pretreatment with ACE inhibitors or AT1 receptor blockers was reported to prevent the occurrence of cerebral ischemia in stroke-prone spontaneously hypertensive rats [5-7]. Other reports also indicated that inhibition of ACE [8] or AT1 receptors [9] prior to the induction of ischemia improve neurological recovery from cerebral ischemia. There are a few studies of the effects of post-ischemic AT1 receptor blockade on brain ischemic/reperfusion (I/R) injuries and their mechanisms.

Oxygen free radicals may play a noticeable role in brain I/R injuries. The amount of oxygen provided during reperfusion exceeds the capabilities of mitochondrial utilization. Therefore, a shift towards high production of free radical-related compounds, such as NO, superoxide, hydrogen peroxide, and

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hydroxyl, is formed [10]. These agents are constantly scavenged by superoxide dismutase, glutathione peroxidase, and catalase. Other antioxidants, including reduced glutathione (GSH), ascorbic acid, and vitamin E, are also likely to be involved in detoxification of the free radicals. When endogenous antioxidant mechanisms are suppressed or the production of free radicals outweighs these mechanisms, a chain of reactions (denaturation of proteins, inactivation of enzymes, and breaking down of carbohydrates) starts. This leads to intense lipid peroxidation in the inner and outer mitochondrial and cell membranes [11].

As the RAS and inflammatory responses play important roles in I/R injuries, our study was designed to evaluate the neuroprotective effects of post-ischemic treatment with candesartan, an AT1 receptor antagonist, and the mechanisms of these effects.

METHODS

Fifty-four male Sprague-Dawley rats (280-320 g) were obtained from the Central animal house facility of the Ardabil University of Medical Sciences (Ardabil, Iran). Anesthesia was made by i.p. injections of chloral hydrate (400 mg/kg). Body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a heating feedback control system.

Laser Doppler Flowmetry. Regional cerebral blood flow (rCBF) was monitored in the cerebral cortex of the left hemisphere within the territory supplied by the middle cerebral artery (MCA) using a laser Doppler flowmeter pencil probe (MNP100, AD Instrument, Australia). Following dissection of the left *m. temporalis* between the eye and the ear, a burr hole was drilled 5 mm lateral and 1 mm posterior to the bregma [12]. To prevent displacement of the probe, rCBF was continuously measured from before MCA occlusion, during such occlusion, and during the first 10 min of reperfusion time. Baseline CBF values measured before occlusion were defined as 100%. Occlusion of the MCA was documented by the decrease in laser Doppler signals to lower than 20% of the baseline.

Experimental Protocol. Effects of post-ischemic candesartan treatment on brain injury and neurological score were investigated in the following randomly divided three groups of animals: group 1 (sham group, $n = 6$), rats underwent surgery at the neck region with no occlusion of the MCA, group 2 (control ischemic group, $n = 6$), rats experienced

brain ischemia by 90-min-long MCA occlusion followed by 24-h-long reperfusion and received the vehicle (normal saline, 1 ml/kg) at the beginning of the reperfusion period, and group 3 (candesartan post-treated ischemic group, $n = 6$), rats experienced ischemia and reperfusion similarly to group 2, but received i.p. injections of candesartan (0.3 mg/kg, LKT laboratories, USA) at the beginning of reperfusion.

Assessment of the effects of post-ischemic candesartan treatment on the formation of brain edema and measurement of the level of tissue malondialdehyde (MDA) as a marker of lipid peroxidation and that of tissue reduced glutathione (GSH) as a marker of the antioxidant capacity were done in another six parallel groups ($n = 6$ in each) of animals under the same conditions as in groups 1-3.

Induction of Transient Focal Cerebral Ischemia. Ninety-minute-long occlusion of the MCA and 24-h-long reperfusion of the left cerebral hemisphere were carried out using an intraluminal filament method described by Longa et al. [13] and modified by Panahpour et al. [14]. Briefly, the left common carotid artery was exposed through a midline neck incision. Through this artery, a surgical nylon silicone-coated thread (4-0 Ethilon) was placed into the internal carotid artery and gently advanced up until seeing a sharp decline in the blood flow trace is seen. Occlusion was terminated by gently pulling out the thread. After re-establishment of the blood flow, all the incisions were sutured; the animals were allowed to recover from anesthesia and returned to a warm cage for recuperation during the 24-h-long reperfusion period.

Behavioral tests were performed by a blinded observer 24 h after surgery in the sham group or 24 h after MCA occlusion in the ischemic groups. As was described previously, the five-point scale grading neurological deficit score (NDS) test was carried out to evaluate the neurological outcome [14]. Rats with normal motor function were graded 1. Rats with contralateral flexion of the body or forelimb upon lifting by the tail were graded 2. Grade 3 was assigned to dysfunctional rats circling to the side contralateral with respect to occlusion. Grade 4 was assigned to rats with loss of the righting reflex and decreased resistance to lateral push; finally, grade 5 characterized rats having no spontaneous motor activity. After the NDS test, animals were sacrificed under deep anesthesia; the brain was removed, cleaned, and solidified by immersion in cold saline (4°C). Frontal 2-mm-thick slices were prepared using a brain matrix and stained with triphenyltetrazolium chloride (TTC), as was described previously

[14, 15]. After staining, the slice images were digitized using a Cannon camera, and the cerebral infarction areas were measured with computer-based NIH image analyzer software [14, 15].

Ischemic brain edema was assessed using a dry/wet method according to the technique presented by Gerriets et al. [16]. The normalized brain water content (WC) for each hemisphere was calculated by measuring the wet weight (WW) and dry weight (DW) of the ipsilateral (lesioned) and contralateral (non-lesioned) hemispheres by the following equation:

$$\text{WC (\%)} = [(WW - DW)/WW] \cdot 100\%.$$

Preparation of Tissue Samples. Twenty-four hours after the beginning of reperfusion, the animals were decapitated. The brain was removed, and the ischemic area (core and penumbra regions) of the ipsilateral hemisphere was dissected according to the well-known protocols for rodent models with unilateral MCA occlusion [17, 18]. Briefly, the brain was sectioned into three slices beginning 3 mm from the anterior tip of the frontal lobe. In section 2 (4 mm thick), the ischemic area was dissected with a longitudinal cut approximately 2 mm from the midline. The tissue was weighed and homogenized in PBS with a weight-to-volume ratio 1:5. The homogenate was centrifuged (10,000g, 4°C) for 30 min. The supernatants for each sample were separated in Eppendorf tubes and kept at -80°C until analysis.

Estimation of Lipid Peroxidation. Lipid peroxidation was evaluated by measuring the MDA concentration in brain samples. The MDA levels were measured using a high-performance liquid chromatography (HPLC) method [19] with some modifications. Briefly, a 100 ml aliquot of the supernatant was placed in a 1.5-ml Eppendorf tube, and 50 ml of 6 M NaOH was added. Alkaline hydrolysis of protein-bound MDA was achieved by incubating this mixture in a 60°C water bath for 30 min. Then, protein was precipitated with 50 ml of 35% (v/v) perchloric acid, and the mixture was centrifuged at 2,800g for 10 min. A 100 ml volume of the supernatant was transferred to an Eppendorf tube and mixed with 10 ml 2,4-nitrophenylhydrazine (DNPH) prepared as a 5 mM solution in 2 M hydrochloric acid. Finally, this reaction mixture was incubated for 30 min at room temperature (protected from light). A 50 ml aliquot of the reaction mixture was injected into the HPLC system equipped with a C18 column (4.6'250 mm, 5 µm particle size). Results were expressed in nanomoles per one milligram of wet

tissue weight.

Estimation of GSH. The concentration of reduced GSH as an important biomarker of the antioxidant defense capacity was also measured using the HPLC method [20] with some modifications. Briefly, a 100 ml volume of the sample supernatant was placed in an Eppendorf vial and diluted with an equal volume of trichloroacetic acid (5% final concentration w/v) and centrifuged at 10,000g for 15 min. A 100 ml volume of the supernatant was transferred to a new Eppendorf vial. After alkalization, the sample was reacted with an equal volume of 2,4-dinitrofluorobenzene solution (1.5% in ethanol v/v) for 1 h at room temperature in the dark. After acidification with 10 ml HCl (37% initial concentration v/v), 50 ml of the sample was loaded onto the HPLC. Results were expressed in millimoles per one milligram of wet tissue weight.

Statistical Analysis. Numerical values are expressed as means ± s.e.m. The independent *t*-test and one-way ANOVA with the *post-hoc* Holm-Sidak test were used for comparisons. The statistical significance was accepted at $P < 0.05$.

RESULTS

Cerebral Blood Flow Recording. The rCBF value was reduced to less than 20% baseline in the control and candesartan-treated ischemic groups after MCA occlusion. There was no significant difference between rCBF values in the groups during occlusion and the first 10 min of reperfusion (Fig. 1).

Evaluation of NDS. The mean NDS of control ischemic rats (4.3 ± 0.6) was dramatically higher than that of sham-operated rats (1.00; $P < 0.01$). The NDS of ischemic rats receiving 0.3 mg/kg candesartan post-ischemically (1.67 ± 0.2) was significantly lower (less than 40%) than that of control ischemic rats ($P < 0.05$).

Assessment of the Cerebral Infarct Volumes. Sham-operated rats had no infarctions. The total infarct volume in ischemic rats receiving 0.3 mg/kg candesartan was significantly smaller than that in control ischemic rats ($P < 0.01$). When compared to control ischemic animals, candesartan-treated ischemic rats had significantly smaller cortical and striatal infarct volumes ($P < 0.05$; Fig. 2).

Assessment of Ischemic Brain Edema. There was no statistically significant difference between the right-side water content in the brains of animals of the experimental groups. Moreover, there was no significant difference between the left-side and

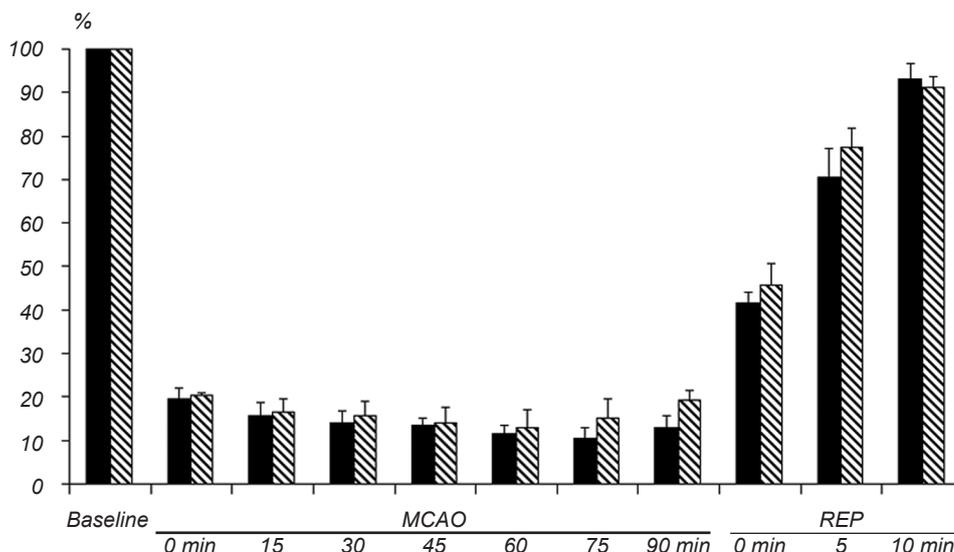


Fig. 1. Normalized values of regional cerebral blood flow in control ischemic rats (filled columns, $n = 5$) and ischemic rats that received 0.3 mg/kg candesartan (dashed columns, $n = 5$) during occlusion of the middle cerebral artery (MCAO) and at the beginning of reperfusion (REP).

Р и с. 1. Нормовані величини місцевого кровотоку в контрольних шурів з ішемією (чорні стовпчики, $n = 5$) та ішемізованих тварин, котрі отримували 0.3 мг/кг кандесартану (заштриховані стовпчики, $n = 5$), протягом оклюзії середньої церебральної артерії та на початку реперфузії.

right-side water contents in the brains of sham-operated rats. The left (ischemic)-side brain water content of control ischemic rats ($83.1 \pm 0.46\%$) was significantly greater than that in sham-operated rats ($P < 0.01$). Post-ischemic treatment with candesartan

(0.3 mg/kg) was associated significantly with the lower left-side brain water content ($80.9 \pm 0.81\%$) than that in control ischemic rats ($81.6 \pm 0.45\%$, $P < 0.05$; Fig. 3).

Assessment of MDA and GSH. Ninety-minute-

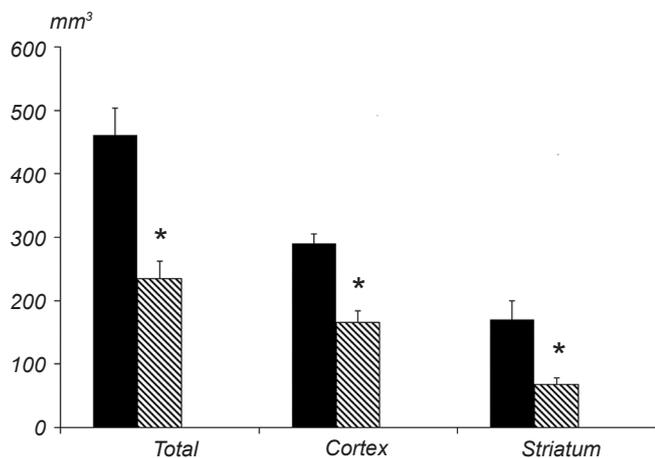


Fig. 2. Total, cortical, and subcortical infarct volumes, mm^3 , in control ischemic rats and ischemic rats that received candesartan at the beginning of reperfusion (filled and dashed columns, respectively). Asterisks indicate significant differences ($P < 0.05$) from control rats.

Р и с. 2. Сумарні, кортикальні та субкортикальні об'єми зон інфаркту (mm^3) у контрольних шурів з ішемією та ішемізованих тварин, котрі отримували кандесартан, на початку реперфузії (чорні та заштриховані стовпчики відповідно).

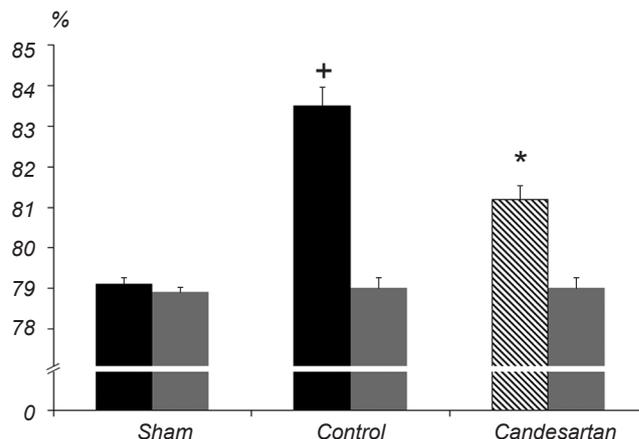


Fig. 3. Normalized values of the brain water content in the left and right hemispheres (filled/dashed and gray columns, respectively) in sham-operated animals, control ischemic rats, and ischemic rats that received candesartan (0.3 mg/kg). Asterisk indicates significant difference ($P < 0.05$) from the control group; cross indicates significant difference ($P < 0.05$) from the sham group.

Р и с. 3. Нормовані величини вмісту води в лівій та правій півкулях мозку (чорні та заштриховані/сірі стовпчики відповідно) в інтактних контрольних шурів, контрольних тварин з ішемією та ішемізованих шурів, котрі отримували кандесартан (0.3 мг/кг).

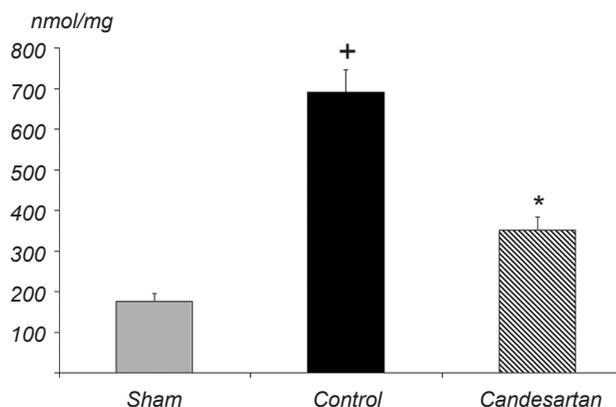


Fig. 4. Concentration of malondialdehyde (nmol/mg brain wet tissue) in sham-operated animals, control ischemic rats, and ischemic rats that received candesartan at the beginning of reperfusion. Asterisk indicates significant difference ($P < 0.05$) from control ischemic rats; cross indicates significant difference ($P < 0.01$) from sham-operated rats.

Р и с. 4. Концентрація малондіальдегіду (нмоль/мг вологої тканини мозку) в інтактних контрольних щурів, контрольних тварин з ішемією та ішемізованих щурів, котрі отримували кандесартан на початку реперфузії.

long ischemia and 24-h-long reperfusion in the control ischemic group resulted in significantly reduced GSH and increased MDA concentrations in the left side of the brain tissue, as compared to the sham group ($P < 0.01$). Post-ischemic candesartan treatment provided a significantly increased GSH concentration and a reduced MDA concentration, as compared to the corresponding figures in the control ischemic group ($P < 0.05$, Figs. 4 and 5).

DISCUSSION

The renin-angiotensin system has been shown to significantly participate in the pathogenesis of ischemic events, including stroke [4, 21, 22]. Most of the actions of Ang II are mediated through AT1 receptors. Since these receptors contribute to stroke-related pathophysiologic mechanisms, such as hypertension, atherothrombosis, and cardiac hypertrophy [23], it is possible that Ang II aggravates I/R injuries through stimulation of AT1 receptors. Previous studies showed that pre-ischemic RAS inhibition provides protective effects with respect to ischemic brain injuries [14, 24, 25]. Our study was carried out to evaluate the effects of post-ischemic candesartan treatment on ischemic brain injuries and edema formation. Candesartan freely crosses

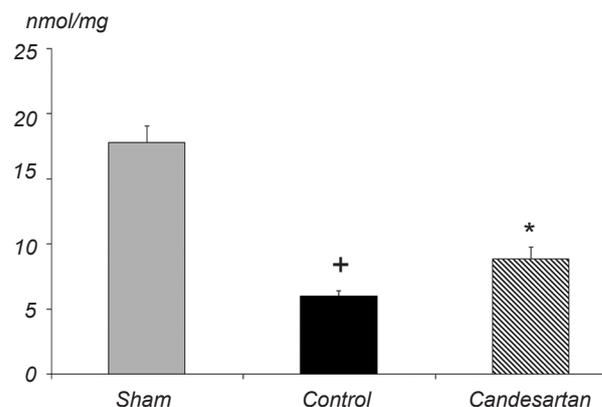


Fig. 5. Concentration of reduced glutathione (mmol/mg brain wet tissue) in sham-operated animals, control ischemic rats, and ischemic rats that received candesartan. Other designations are the same as in Fig. 4.

Р и с. 5. Концентрація відновленого глутатіону (нмоль/мг вологої тканини мозку) в інтактних контрольних щурів, контрольних тварин з ішемією та ішемізованих тварин, котрі отримували кандесартан.

the blood-brain barrier and produces an effective and long-lasting blockade of cerebral AT1 receptors [4, 26].

The results of our study demonstrate that blocking of AT1 receptors significantly reduces the cortical and striatal infarct volumes and improves neurological motor deficits. This study also shows that transient ischemia induces brain edema by increasing the water content in the ischemic hemisphere, while post-ischemic candesartan treatment significantly reduces the water content in the above hemisphere and prevents edema formation. These findings are in agreement with reports of other investigators demonstrating that blocking of AT1 receptors with candesartan reduced the infarct size evoked by transient cerebral ischemia in hypertensive rats [27, 28].

Various mechanisms might be responsible for the beneficial effects of the AT1 receptor blockade in brain ischemia. Such effects might be partly attributed to the stabilizing action on the impaired cerebrovascular autoregulation within the penumbra [29-31]. In addition, anti-apoptotic mechanisms may enhance the protective effects of blocking of AT1 receptors [32]. Furthermore, the beneficial effects of the AT1 blockade might also be attributed to a reduction in the production of ROSs [33].

Cerebral ischemia is associated with excessive

production of ROSs, especially of superoxide [34]. The production of ROSs initiates chain reactions, causing damage of cellular macromolecules and promoting the mitochondrial apoptosis pathway, which ultimately leads to cell death [35]. Our study showed that candesartan inhibits ROS generation, increases glutathione production, and reduces MDA production. These findings are in agreement with other reports that showed that activation of AT1 receptors results in the intense production of superoxide, whereas blockade of these receptors is associated with reductions in the amounts of superoxide [33] and peroxynitrite [36].

The findings of our study also indicated that inhibition of RAS by blocking AT1 receptors reduces ischemic edema formation. Recent evidence suggests that Ang II may be an important stimulus for the production of superoxide and peroxynitrite in blood vessels [36]. Oxygen-derived free radicals are known to increase the permeability of the blood-brain barrier [37]. Our results showed that candesartan treatment suppresses lipid peroxidation and increases the endogenous antioxidant defense capacity. Thus, blocking of AT1 receptors may reduce ischemic edema via protective effects on the blood-brain barrier integrity by reduction of the ROS production.

Therefore, inhibition of RAS by the AT1 receptor blocker candesartan noticeably reduces the cerebral infarction volume and edema formation in rats exposed to transient MCA occlusion. The respective mechanisms may be attributed to inhibition of lipid peroxidation and increase in the endogenous antioxidant capacity.

All protocols of the study were approved by the Institutional Animal Ethics Committee of the Ardabil University of Medical Sciences, which follows the NIH guidelines for care and use of experimental animals.

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ОПОСЕРЕДКОВАНІ АНТИОКСИДАНТНОЮ АКТИВНІСТЮ НЕЙРОЗАХИСНІ ЕФЕКТИ КАНДЕСАРТАНУ ПРИ ІШЕМІЇ ТА НАБРЯКУ МОЗКУ У ЩУРІВ

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Резюме

Досліджували ефекти постішемичного блокування ангіотензивних рецепторів AT1 кандесартаном щодо зони церебрального інфаркту та формування набряку. Самці щурів лінії Спрейг–Доулі були розділені на три групи (інтактних контрольних, контрольних з ішемією та ішемізованих, котрим вводили 0.3 мг/кг кандесартану). Епізод фокальної ішемії мозку створювався за допомогою 90-хвилинної оклюзії лівої середньої церебральної артерії; оклюзія супроводжувалася 24-годинною реперфузією. Неврологічний дефіцит оцінювався в балах після закінчення періоду реперфузії. Потім тварини рандомізовано відбиралися для вимірювань трьох видів: об'єму зони інфаркту, ступеня набряку мозку з використанням методу „суха/волога тканина” та концентрацій малонового діальдегіду (MDA) й відновленого глутатіону (GSH) з використанням методики HPLC. Індукція церебральної ішемії у відповідній контрольній групі призводила до розвитку значних інфарктів у корі та стріатумі, спряженому з важкими порушеннями моторних функцій. Уведення кандесартану забезпечувало істотне зменшення об'ємів інфарктів і послаблення порушень зазначених функцій. Вміст води в лівій (ураженій) півкулі в контрольній групі з ішемією був значно підвищеним. Використання кандесартану призводило до значного зменшення вмісту води в ішемізованій лівій півкулі, відновлення рівня GSH у тканині та зменшення продукції MDA. Отже, блокування AT1-рецепторів кандесартаном може помітно зменшувати ступінь ішемичного пошкодження мозку та послаблювати формування набряку; ці ефекти опосередковуються підсиленням антиоксидантної активності.

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