Impact of dexmedetomidine on amino acid contents and the cerebral ultrastructure of rats with cerebral ischemia-reperfusion injury[1]

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Abstract

Purpose: To investigate the effects of dexmedetomidine (DEX) on amino acid contents and the cerebral ultrastructure of rats with cerebral ischemia-reperfusion injury (I/R).
Methods: Thirty-six, male, Wistar rats were randomly divided into three groups: the sham operation group (group C), the ischemia-reperfusion group (group I/R), and the DEX group (group D). The middle cerebral artery occlusion model was prepared by the modified Longa method. The time of ischemia was 180 min, and 120 min after reperfusion, the amount of glutamate (Glu), and γ-aminobutyric acid (GABA) in the brain were measured, and the ultrastructure-level changes in the cerebral cortex were examined using electron microscopy.
Results: Compared to group C, Glu contents in group D, and I/R significantly increased. Compared to group I/R, Glu contents in group D significantly decreased. Compared to group C, GABA contents in group D, and I/R significantly increased, and those in group D significantly increased, as compared to group I/R. The cerebral ultrastructure was normal in group C. Vacuolar degeneration in the plastiosome and nervous processes, was more critical than in group D. Vascular endothelial cells (VEC) were damaged. On the contrary, these changes in group D significantly improved.
Conclusion: Dexmedetomidine is capable of decreasing glutamergic content, and increasing GABAergic content, in order to decrease the injury of the cerebral ultrastructure, following cerebral ischemia-reperfusion injury.

Key words: Medetomidine. Amino Acids. Brain Ischemia. Reperfusion. Rats.

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■ Introduction

The central nervous system (CNS) has a large number of amino acids, which play important roles in the conduction process of nerve functions, by working as neurotransmitters. Based on the excitatory or inhibitory effects of postsynaptic neurons, amino acids could be divided into two types: excitatory amino acids (EAAs), and inhibitory amino acids (IAA). The maintenance of excitability, or the lack thereof in the CNS, depends on the relative proportions of these two types of neurotransmitters. EAAs mainly refer to glutamate (Glu), and aspartate (Asp), and IAA mainly refer to γ-aminobutyric acid (GABA), and glycine (Gly).

EAAs, especially Glu, play important roles in cerebral ischemia-reperfusion injury (I/R) injury. Glu is an important neurotransmitter responsible to mediate rapid synaptic responses within the CNS, and only a small amount of Glu exists in the synaptic cleft of brain tissues, under normal circumstances. Its metabolic balance is mainly achieved by a normal release of Glu from the presynaptic membrane, and the subsequent reuptake by neurons, and glial cells. Imbalances in this process, of Glu release and uptake, could aggravate cerebral I/R injury[1,2]. In cerebral ischemia, the presynaptic membrane would be in a depolarized state, leading to a massive release of Glu. Meanwhile, due to a lack of adenosine triphosphate (ATP), the neuron-glial cell signaling transduction would be disrupted, followed by a reuptake disorder of glial cells, the accumulation of Glu among cells, and an excessive activation of Glu receptors on the postsynaptic membrane, of which the most important is the N-methyl-D-aspartate (NMDA) receptor. An excessive activation of the NMDA receptors causes cell death, primarily through mechanisms such as Ca^{2+}-overload associated secondary mitochondrial dysfunctions, an increasing amount of reactive oxygen species, abnormal transcription, and protease and endonuclease activation[3]. Previous experiments, conducted in vivo or at the cellular level have also shown that in addition to cerebral ischemia, Glu is also responsible for the activation of Glu receptors on the postsynaptic membrane, generation of excitotoxicity, and causing neuronal damage in the pathological processes of trauma, and CNS chronic degenerative diseases[4].

Dexmedetomidine (DEX) is a highly selective α2-adrenoceptor agonist5. A study found that DEX has neuroprotective effects, as it could improve oxygenation following cerebral I/R, reduce the cerebral infarction area, and reduce the apoptosis of neuronal cells[6-8]. Some in vitro studies have also found that DEX could inhibit astrocytes and thus increase the oxidative metabolism of the astrocytes towards Glu, thus preventing Glu to be metabolized to glutamine[9,10]. However, the direct effects of DEX on brain Glu were not reported. In order to investigate this, we hypothesized that DEX could potentially reduce the content of Glu in rats with cerebral I/R injury, thus playing neuroprotective effects.

■ Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Yantaishan Hospital.

Thirty-six, healthy, male, Wistar rats (clean-grade, body weight 250-300g, provided by Shandong Luye Pharmaceutical Co., Ltd., animal Certificate of Conformity: SYXK (Lu) 20090013) were selected one week before surgery; they had free access to water, and a standard diet. The laboratory was maintained at a constant temperature (~23°C). Rats were made to fast, with no prohibitions on drinking water, 12 h before the surgery; then, the animals were randomly divided into three groups: 1) the sham operation group (group C): we only exposed the external carotid artery surgically, not causing ischemia; 2) the I/R group: we intraperitoneally injected 1 mL of sterile saline, 30 min before the ischemia operation, followed by cerebral ischemia operation; 3) the DEX group (group D): we intraperitoneally injected DEX 100 μg/kg (Jiangsu Hengrui Medicine Co., Ltd.), 30 min before the ischemia operation, followed by cerebral ischemia operation. The ischemia time was the same as in the I/R group, and 120 min after reperfusion, all rats were decapitated, and their hippocampi were sampled for the content detection of Glu, and GABA.

Model preparation

The I/R model was prepared in the left middle cerebral artery using the modified Longa method11, which could induce focal lesion in the MCA feeding area. Briefly, mice were subjected to anesthesia using 5.0% Isoflurane, and this was maintained by inhalation of 1.5% to 2% Isoflurane, driven by 100% oxygen flow. Mice were ventilated (110 breaths/min with volume 0.5 mL), and body temperature was regulated at 37°C, by surface water heating. We cut the neck fur, prepared the skin, and performed the median incision, then, transversely incised the skin and carefully separated tissue layers to expose the sternocleidomastoid. Following this, we bluntly dissected the sternocleidomastoid, and sternohyoid, using surgical retractors. After exposing the left common carotid artery (CCA), we gently separated upwards along the common carotid artery until it was 1 cm away from the distal end of the left external carotid artery (ECA), then ligated the ECA branches, and ligated ECA at the CCA crotches. This was followed by clamping the proximal end of CCA using artery clamps, penetrating a suture near the bottom of the ECA crotches, and then tying the knot without tightening it. Then, we prepared a small incision between the proximal end of the ECA ligation, and the CCA crotch, gently inserted the nylon suture prepared in advance through the incision, and gently tightened the knot. Following this, we loosened the artery clamp to restore the blood flow, sent the nylon suture along the ECA into the brain, until we felt slight resistance, at which point the nylon suture may have been inserted about 17~19 mm inside. At this point, the tip of the nylon suture had already reached the MCA end; hence, we gently tightened the suture head to completely block the blood supply of MCA. When the iridal color of the rat became lighter, and Horner's syndrome (characterized by ptosis, miosis, "upside-down ptosis" [or elevation of the lower lid], and facial anhydrosis) occurred, we considered that the model preparation was successful. Then, we sutured the incision layer by layer, and left approximately 1 cm of the nylon suture out of the incision. The whole process was completed within 10 min. After the 180 min ischemia, we re-injected 10% chloral hydrate to anesthetize the rat, lightly withdrew the reserved nylon suture until we felt resistance, at which point we considered that its head had returned to the CCA crotch. In this case, the brain was restored with blood supply, and we achieved reperfusion. Finally, we cut the excessive suture to complete preparing the MCAO model. Group C was only anesthetized with 10% chloral hydrate, and in this case, we separated the left ECA until 1 cm away from the distal end, while not performing vessel ligation, and suture introduction. The remaining part of the operation was the same as the I/R group. The DEX group was pre-medicated according to experimental design, and the remaining operations were the same as the I/R group. We maintained the rats' body temperatures at 37°C, postoperatively, and the animals had free access to food and water. We also observed their vital signs, and maintained airway patency.

Specimen collection

After 120 min I/R, rats was re-injected with 10% chloral hydrate (300 mg/kg), intraperitoneally, for anesthesia, and we quickly cut the chest, exposed the heart, cut the left ventricle, and cannulated into the ascending aorta level. After quickly injecting 200 mL physiological saline for flushing, we fixed samples with 4% paraformaldehyde, and cut the head to sample the brain. Then, fixed the brain in 4% paraformaldehyde for 24 h, followed by conventional alcohol dehydration. Following this, we soaked the brain tissues in a paraffin box; after full fixation, we placed the embedding instrument on ice, adequately cooled the brain tissues, and performed continuous brain coronal frozen slicing, with the thickness set as 5 µm. Thereafter, we stored the samples at 4°C for future use.

Observation of cell ultrastructure

After 120 min I/R, we sampled l mm3 brain tissues at the junction of the striatum, and the cortex (the penumbra region), from the fresh rat brain coronal slices. Then, we stored samples in 2.5% glutaraldehyde at 4°C, rinsed with phosphate buffer 15 min × 3 times, fixed with 0.1% osmium tetroxide for 1h, and dehydrated with 90%, followed by 95%, and finally 100% ethanol. This dehydration process was repeated with acetone, going from 90%, to 95%, and finally 100%. Samples were further impregnated with epoxy resin, embedded, and fixed. We prepared 5 nm ultrathin sections with LKB ultramicrotome (Hubei Huida Instrument Co., Ltd. Wuhan, China), and observed ultrastructural changes using a staining transmission electron microscope (Hitachi, Japan).

Detection of Glu and GABA

We centrifuged the prepared brain tissues in a low-temperature, high-speed (4□ □12000r/min) centrifuge for 10 min at low temperature. Then, we diluted the supernatant of the tissue homogenate with saline, detected and calculated the contents of Glu and GABA in strict accordance with the kit instructions, using a spectrophotometer (Shanghai Optical Instrument Factory; Glu and GABA Kit: Nanjing Jiancheng Bioengineering Institute).

Statistical analysis

All data were processed using SPSS (version 13.0) software; the measurement data were expressed as mean ± standard deviation (±s). We used ANOVA for intra- and inter-group comparisons, at different time points, and the q test for pairwise comparison among the averages, with P<0.05 considered as statistically significant.

■ Results

Comparison of Glu and GABA content

Compared to group C, the Glu contents in group D, and I/R significantly increased (P<0.05), and those in group D were significantly lower than in group I/R (P<0.05). Compared to group C, the GABA contents in group D, and I/R were significantly reduced, and those in group D were significantly higher, compared to group I/R (Table 1).

Table 1 - Comparisons of Glu and GA B A content among the groups (n=12, µmol/gprot).

Group	Glu	GABA
C	176.6±23.4	467.4±33.5
I/R	434.5±46.4*	166.6±12.2*
D	367.6±34.2*#	258.4±16.2*#

*Compared with group C, P<0.05, # compared with group D, P<0.05.

Ultrastructural changes

The ultrastructure of the brain tissues in group C was normal (Figure 1A). The neurons, nuclei, and nuclear membrane were complete, without edema around the nuclei. Peri-nuclear gaps were not widened, the nucleoli were obvious, and chromatin was distributed evenly. No vacuolar degeneration was seen in the mitochondria. The cytoplasm of the neurons, oligodendrocytes, and microglia in the cerebral cortex of the samples in the I/R group exhibited edema, and membrane structures were not complete, apart from being swollen, and deformed. The cytoplasm exhibited vacuolization, and the nucleoli had shrunk and dissolved, along with peri-nuclear regions exhibiting obvious edema, and widened perinuclear gaps. In addition, majority of the cell mitochondria exhibited vacuolar degeneration, the vascular endothelial cells were damaged, congestion was seen in capillaries, and the endothelial cells were damaged with visible congestion (Figure 1B). The neurons, oligodendrocytes, and microglia in group D were intact, and the membrane structures were complete. Few mitochondria exhibited vacuolar degeneration; the gaps between the blood vessels and nervous processes (V-R gap) were slightly changed, the continuity of the vascular endothelial cells was complete, and no congestion was seen in the capillaries (Figure 1C).

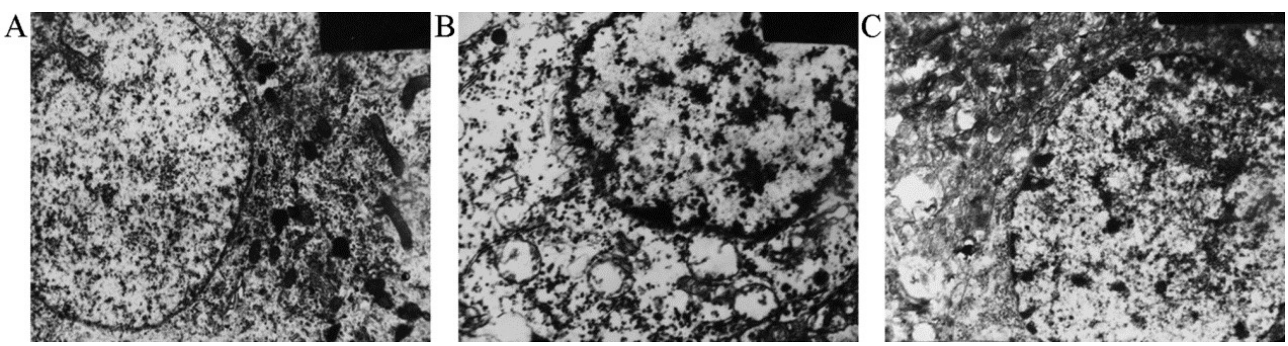

Figure 1 - **A:** Normal neurons. **B:** Neurons in the I/R group. **C:** The lesions in group D were significantly reduced (x7200).

■ Discussion

There are many amino acids acting as neurotransmitters inside the CNS, and they are involved in a variety of nerve information transmissions among the neurons, and the maintenance of the intra- and intercellular distribution of water and ions. When the brain functions are normal, EAA and IAA are in a relatively balanced state. Of these, Glu plays a major role in cerebral I/R injury: in cerebral I/R injury, large amounts of Glu, usually stored in the presynaptic endings are released, causing a sharp increase in the Glu content within the extracellular fluid of the brain cells[12]. The normal brain cell membranes have a large number of highly affiliative excitatory amino acid transporters (EAATs), whose function is to transport EAA from the synaptic cleft into cells, thereby maintaining the amino acid balance within brain tissues. When in cerebral I/R injury, the ion gradient inside and outside the cell membrane is reduced; therefore, the intake capacity of EAAs by EAATs reduces, resulting in EAAs reuptake disorder. The substantially increased Glu then acts on the EAA receptors on the postsynaptic membrane, thus causing an over-activation of the EAA receptors on the postsynaptic membrane, and the secondary neuronal injury[13]. GABA is a major inhibitory neurotransmitter in the brain, and could generate presynaptic or postsynaptic inhibition of neurons, when bound with its receptors[14].

The levels of intra-cerebral EAA following acute cerebral I/R injury are closely related to the severity of the brain injury[15,16]. Studies have shown that Glu produces brain injury primarily through two ways: first, through the non-NMDA receptor, and second, through exciting the NMDA receptor, thus directly or indirectly starting the voltage-dependent Ca2+ channel, leading to an intracellular Ca2+ overload, and thus delayed neuronal degeneration, and necrosis[17]. The neurotoxicity of EAA is mainly expressed in the following ways: 1, inducing lipid peroxidation towards the unsaturated fatty acids of the cell membrane, thus damaging the cell membrane ion channels and causing cell edema; 2, causing a decrease or loss in the biological activities of a variety of enzymes[18,19]; and 3, reducing mitochondrial functions, disturbing energy metabolism, and causing apoptosis in some cells[20,21].

Certain studies have shown[22] that extracellular Glu concentrations are closely related to the severity, duration, organ sizes involved, and ischemic sites of cerebral ischemia, which usually gradually decline with the restoration of the blood flow; however, the concentration significantly increases, again, at approximately 120 min of the infusion recovery. Therefore, our study designed the brain sampling two hours later, after the blood flow is recovered.

DEX is a novel, highly selective α2-adrenoceptor agonist, and its binding ratio with the α2 adrenergic receptor is 1620:1. It has inhibitory effects towards the sympathetic nervous system, such as analgesia, sedation, and antianxiety. Foreign scholars have applied DEX for sedation assistance in neurosurgical procedures, and it is known that its sedative effects can be easily reversed. Additionally, it also makes the patient tolerate the endotracheal tube[23,24]. DEX has a lighter suppression towards respiratory functions, and due to this characteristic, it is now used as a major adjunct for clinical anesthesia. Recent studies conducted on rabbits with subarachnoid hemorrhage found that DEX exhibits protective effects on hippocampal neurons[25]. We also found in our previous experiments that DEX could mitigate the apoptosis of rat brain cells. In this study, we found that DEX could reduce Glu content, while increasing the GABA content in brain tissues with cerebral I/R injury. DEX stimulates astrocyte α-2 adrenergic receptors, which in turn raise astrocyte calcium concentrations. These elevated concentrations then stimulate glutaminase activity, and the ability of astrocytes to eliminate glutamine by oxidative metabolism, thereby reducing the availability of glutamine as a precursor of neurotoxic glutamate. Furthermore, DEX inhibits evoked glutamate release by stimulating α-2 adrenergic receptors though a mechanism involving the suppression of Calcium voltage-gated 2.2, and Calcium voltage-gated 2.1 channels, and mitogen-activated protein kinase activity. The results achieved using the electron microscope also show that DEX is capable of reducing damage in the brain tissues caused due to cerebral I/R injury, indicating that DEX might reduce apoptosis through the reduction of Glu content, and thus exert its neuroprotective effects.

■ Conclusions

Dexmedetomidine has the capability to reduce glutamergic content, and simultaneously increase GABAergic content, in order to reduce the damage to the cerebral ultrastructure, that occurs after cerebral ischemia.

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