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Research Article

Ascorbic acid-containing compound efficacy in ischemic brain damage

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Abstract

Introduction: Ischemic brain injury remains one of the main causes of disability and mortality worldwide. Protection of cellular population, depriving from oxygen supply and nutrients, is of extreme importance for further both clinical and health outcomes of timely implemented intravascular intervention. **The aim:** to assess anti-ischemic activity of 3-hydroxypyridine ascorbate in the *in vitro* and *in vivo* models of brain cell and tissue response to ischemia and reoxygenation.

Materials and Methods: 3-hydroxypyridine ascorbate (laboratory code 3-EA) was assessed as chemical substance (purity 99.8%) diluted in sterile phosphate-buffered saline. Intracellular Ca^{2+} response to glutamate excitotoxicity (GluTox), ischemia and reoxygenation as well as cellular viability was evaluated on NMRI murine fresh cortical neuro-glial cell culture incubated with 2-EA by registering intracellular Fura-2 and propidium iodide fluorescence respectively. Expression of apoptosis regulating genes *BCL-2, STAT3, SOCS3*, inflammation regulating genes *TRAIL, MLKL, Cas-1, Cas-3, IL-1β u TNFα*, and genes *MAO-A and MAO-B* was determined by real-time PCR. The substance neuroprotection was studied in male Sprague-Dawley rats with intraluminal middle cerebral artery (MCA) occlusion/reperfusion treated with 18 mg/kg of 2-EA along with neurological deficiency evaluation and morphological assessment of brain sections.

Results: Preincubation of cortical cells with 10-100 µM of 3-EA leads to inhibition of $[Ca^{2+}]i$ in cytosol of neurons and astrocytes under GluTox and oxygen-glucose deprivation (OGD) conditions. Reducing $[Ca^{2+}]i$ inhibits necrotic cell death in an acute experiment. Incubation of cerebral cortex cells with 3-EA leads to an overexpression of anti-apoptotic genes *BCL-2, STAT3, SOCS3*, along with downregulation of genes *TRAIL, MLKL, Cas-1, Cas-3, IL-1β* and *TNFα*. Intraperitoneal administration of 3-EA reduces the volume of necrotic areas, perinecrotic edema, cell damage, and neurological deficits in rats with MCA occlusion.

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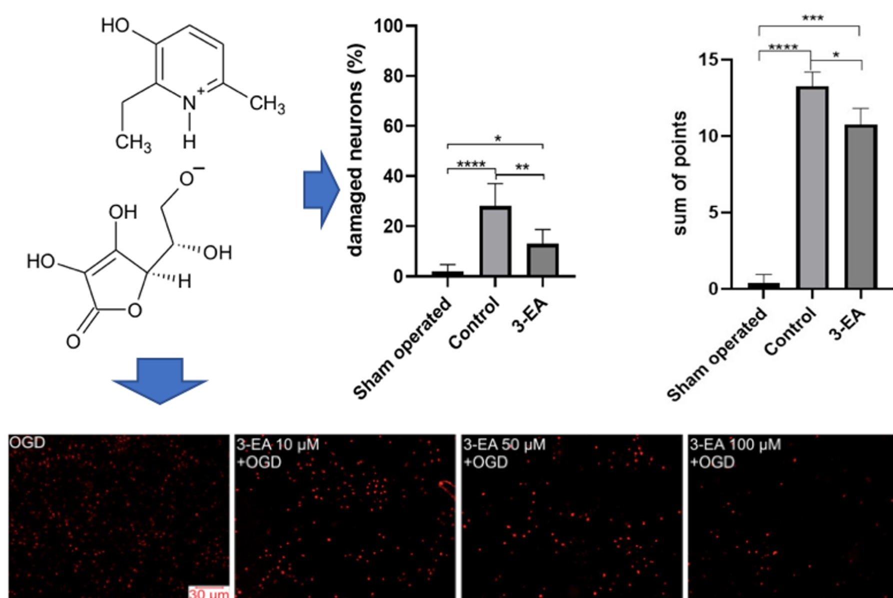
Conclusion: 3-EA dose-dependently suppresses the death of cerebral cortex cells under the excitotoxic effects of glutamate and ischemia/reoxygenation. Cell-protective effect of 3-EA involves changes in the basal and ischemia/reoxygenation-induced expression of genes encoding anti-apoptotic proteins and oxidative status proteins, which leads to inhibition of the late irreversible stages of apoptosis. A course administration of 3-hydroxypyridine ascorbate at a dose of 18 mg/kg per day reduces the severity of damage both by preserving the population of neurons in the penumbra zone and by limiting the local stress response.

Graphical abstract



Explanation: 3-hydroxypyridine ascorbate increases neurons survival and protects animals' brain in acute ischemia *in vitro* and *in vivo*.

Keywords

3-hydroxypyridine ascorbate (3-EA), ischemia, brain damage, cell culture, excitotoxicity, Ca^{2+}, cerebral artery occlusion, neurological deficits

Introduction

Stroke is a leading cause of disability and cognitive impairment and the fifth leading cause of death in developed countries (Benjamin et al. 2017). In 2015, ischemic stroke accounted for 5.2% of all deaths worldwide (GBD 2015, 2016). The main pathophysiological cause of ischemic stroke is intravascular thrombosis, which can lead to brain tissue necrosis and focal neuronal deficits. There are three leading causes of ischemic strokes: in half of cases it is due to atherosclerosis of cerebral vessels and rupture of atherosclerotic plaque, in 20% of cases –due to cardiogenic cerebral infarction and in 25% of cases – due to lacunar infarctions because of damage to small vessels (Bailey et al. 2012). The remaining 5% are due to other rare causes such as vasculitis and extracranial arterial dissection (Warbow et al. 2012).

Acute stroke is a type of ischemic attack that can cause severe damage to the brain and neurons within a very short time after the ischemic episode (Balch et al. 2020). According to numerous studies on the mechanisms and clinical course of ischemic stroke and cerebral infarction, there are three main mechanisms underlying neuronal damage caused by the pathological process. First, neuronal death caused by ischemia and infarctions is one of the most direct causes of neuronal damage (Chen et al. 2020). Regarding pharmacological strategies related to this mechanism, researchers have focused on the processes of neuroprotection and regeneration, as well as their associated biomarkers and molecular pathways (Chen et al. 2020). Second, vascular obstruction caused by ischemia leads to excessive production of reactive oxygen species (ROS), and oxidative stress has been shown to exacerbate neuronal damage and lead to severe functional deficits (Katan and Luft 2018). Possible ways

to effectively respond to oxidative stress and reduce it have been widely studied in world science for more than half a century. Inflammation caused by ischemia is an additional factor that leads to further neuronal damage after stroke (Ren et al. 2020).

For more than 30 years, researchers have been focusing on the neuroprotective effects of derivatives of endogenous compounds – metal-containing sulfoketoacids, hydroxypyridines, etc. 3-hydroxypyridine ascorbate (3-EA) is one of the compounds of the group. Previous studies have shown that this substance obtained lipid-regulating and anti-diabetic activity (Blinova et al. 2022), which makes it a promising molecule for further research as an brain-protective agent.

Aim of the study was to assess anti-ischemic activity of 3-hydroxypyridine ascorbate in the *in vitro* and *in vivo* models of brain cell and tissue response to ischemia and reoxygenation.

Material and Methods

The studied substance

A compound, 2-ethyl-6-methyl-3-hydroxypyridinium-gammalactone-2,3-dihydro-L-gulonate (3-hydroxypyridine ascorbate, 3-EA) was studied. The compound was synthetized in the form of creamy-tinted white crystalline substance (purity more than 98%), highly soluble in water. The substance working solution for *in vitro* experiments was prepared immediately before the study by dissolving a sample of the compound in a sterile 0.9% phosphate buffered saline (PBS) heated to a temperature of 37^0C.

Cell culture

Cells for a mixed cortical culture of neurons and astrocytes were isolated from the brain of newborn NMRI mice using the method described earlier (Turovsky et al., 2021; Varlamova et al., 2021). The animals were purchased at SPF animal breeding facility of Bioorganic Chemistry Institute of The Russian Academy of Sciences (Pushchino, Russia). Trypsinization of samples was carried out in Versene solution in the absence of magnesium and calcium. Isolated cells were incubated in neurobasal medium containing 0.5 mM glutamine, 2% B-27, and 20 µg/mL gentamicin sulfate. After 5 hours of incubation in a CO_2 incubator at a temperature of 37^0C, a culture medium was added, 2/3 of which was changed every 3 days. For the experiments, a 10-day (10 DIV) culture with a density of 15×10^3 cells/cm^2 was used. Half of the cell culture wells were used for biophysical and pharmacological experiments, the other half – for RNA extraction and real-time polymerase chain reaction (qPCR).

Modelling of ischemia-like condition

Ischemia-like conditions, oxygen-glucose deprivation (OGD), were modelled according to the method in (Turovsky et al. 2021; Varlamova et al. 2021) by using a glucose-free HBSS medium and replacing oxygen with argon. To induce acute excitotoxicity (up to 50 min), 100 mM of glutamate and 20 µM glycine were added to the magnesium-free cultivation medium. To study the contribution of apoptosis to cell death, the mixed culture was incubated in the presence of glutamate and glycine for 24 hours. Short-term (30 s) application of KCl (35 mM) and ATP (10 µM) was performed to detect neurons and astrocytes, respectively.

Cell death assessment

Cell death was assessed in a cytochemical reaction after treating cells with 1 µM propidium iodide (Merck Sigma-Aldrich, Germany) before and after pharmacological treatment in the same field of view of the microscope. Fluorescence of dying cells was recorded in 5 random fields of view using an Axio Observer Z1 microscope (Carl Zeiss, Germany) equipped with a Hamamatsu ORCA-Flash 2.8 high-speed monochrome camera (Japan). The ImageJ and Origin 8.5 (USA) software was used for image processing.

Gene expression assessment

RNA was extracted using a Mag Jet RNA Kit (Thermo Fisher Scientific, USA). We used electrophoresis in 1 mg/mL ethidium bromide to determine the quality of the isolated RNA; the concentration of nucleic acids was estimated by spectrophotometry. Reverse transcription of RNA was performed using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Real-time PCR was carried out in 25 µL of a mixture consisting of 5 µL qPCRmix-HS SYBR (Evrogen, Russia), 1 µL primer solution, 17 µL water (RNase-free), and 1 µL cDNA. Amplification was performed using the Dtlite Real-Time PCR System (DNA-technology, Russia). Primers produced by Evrogen (Russia) were used (Table 1).

Table 1. Primers for qPCR used in the study imidazotetrazine 1 in four cell cultures

Gene	Primers
GAPDH	Forward 5'- aaggagccctatgagaatgtc -3' Reverse 5'- acatcctcttctacatattcttc -3'
RIPK1	Forward 5'-ggaggatgaggcggggacc-3' Reverse 5'-gcatgctcttcgggaggcttcaaaac-3'
TRAIL	Forward 5'- ctaaccacaacacggaacctg -3' Reverse 5'- cagcagatggttgatggaggc -3'
MLKL	Forward 5'- caaagagcactaaagcagagag -3' Reverse 5'- ggcaatcctgacccactgg -3'
Cas-1	Forward 5'- ttattcaggcatgccgtggag -3' Reverse 5'- tcctccaagtcacaagaccag -3'
Bcl-2	Forward 5'- ctacgagtgggatgctggagatg-3' Reverse 5'- tcaggctggaaggagaagatgc-3'
Bcl-xL	Forward 5'- tggccacagcagcagtttg -3' Reverse 5'- tctccggtaccgcagttcaa -3'
Bax	Forward 5'- taaagtgcccgagctgatcagaac -3' Reverse 5'- cttcccagccaccctggtctt -3'
Caspase3	Forward 5'- tcagaggcgactactgccggag -3' Reverse 5'- cgtgagcatggacacaatacacgggt -3'
Stat3	Forward 5'- ttctgggcacgaacacaaaagt -3' Reverse 5'- gcctccattcccacatctctg -3'
Socs3	Forward 5'- aagaacctacgcatccagtgtga -3' Reverse 5'- atgtagtggtgcaccagcttgag -3'
NF-kB	Forward 5'- aagtgcaaaggaaacgccagaa -3' Reverse 5'- actaccgaacatgcctccacca -3'
IL1b	Forward 5'- aatctcgcagcagcacatcaaca -3' Reverse 5'- tccacgggaaagacacaggtagc -3'
TNFα	Forward 5'- acggcatggatctcaaagacaac -3' Reverse 5'- tcctggtatgagatagcaaatcgg -3'
Catalase	Forward 5'- gctgacacagttcgtgaccctcg -3' Reverse 5'- acaggcaagtttttgatgccctggt -3'
Mao-A	Forward 5'- ggcggcatctcaggattggct -3' Reverse 5'- tatgccaaggggttccacacaggt -3'
Mao-B	Forward 5'- gcctcagtgtggtggttctggaag -3' Reverse 5'- cactgggaatctcttggcccatctcatc -3'
Sod1	Forward 5'- tcgagcagaaggcaagcggtg -3' Reverse 5'- cggccaatgatggaatgctctcctgag -3'
Sod2	Forward 5'- ctcccggcacaagcacagc-3' Reverse 5'- tcctttgggttctccaccaccctt -3'

The results were analyzed by Dtlite software (DNA-technology, Russia) using the method in (Bederson et al. 1986). The expression of the studied genes was normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Animal model of cerebral ischemia

Animal study protocol was reviewed and approved by Local Etic Committee of Sechenov University (Minutes. No. 12/53, 19.09.2023). Cerebral ischemia was modelled by middle cerebral artery (MCA) intraluminal occlusion according to (Ren et al. 2020) in our modification in rats (Blinova et al. 2022). Twenty-four Sprague-Dawley male rats, weighing 200-240 g, were randomly divided into four groups of six rats each. Animals in the experimental group were administered intraperitoneally (i.p.) 18 mg/kg 3-EA in 0.5 mL PBS 5 min after onset of the experimental ischemic cerebral disorder, and then daily for 6 days at the same time, while sham-operated rats and control animals with MCA occlusion received an only equivalent volume of saline in the same regimen. In the comparator group, animals received 15 mg/kg 3-hydroxypyridine succinate in the same regimen as 3-EA. Neurological deficits was assessed 1, 3 and 7 days after MCA occlusion using a modified Bederson scale (Bederson et al. 1986).

Morphological examination

On Day 8, brain of the experimental animals was extracted; 4-µm-thick sections of formaldehyde-fixed, paraffin-embedded brain tissue samples were stained with hematoxylin and eosin (BioVitrum, Moscow, Russia) and 0.1% toluidine blue (BioVitrum, Moscow, Russia) by the Nissl method. We used Leica DM4000 B LED microscope equipped with Leica DFC7000 T digital camera with LAS V4.8 software (Leica Microsystems, Germany) to examine the sections. We used semi-quantitative analysis of Nissl-stained specimens to objectify the results of histological examination.

Statistical analysis

All values are given as Mean ± standard deviation (SD) or as individual neuron responses. Data were analyzed to assess normality of distribution and then statistically compared using a paired t-test; the results were considered significant at $p \leq 0.001$. Animal data were also presented as Mean ± SD. Time-dependent intergroup comparisons were performed using ANOVA followed by Tukey's test, and the significance of the difference between the experimental and control groups on Days 3 and 7 was confirmed using an independent two-tailed t test using STATA 17.0 software (Stata Corp. LLC, USA).

Results

Ischemia-like conditions *in vitro* for 50 min cause a biphasic increase in the concentration of calcium ions ([Ca2+]i) in neurons (Fig. 1A) and astrocytes (Fig. 1B) of the cerebral cortex, which correlates with necrotic cell death, recorded by the appearance of fluorescence in the nuclei (Fig. 1B). After 24-hour preincubation of cells with different concentrations of 3-EA, a concentration-dependent suppression of OGD-induced [Ca2+]i increase in neurons (Fig. 1A) and astrocytes (Fig. 1B) was observed in the second phase, with no effect on the first reversible phase of [Ca2+]i increase. In this case, a decrease in the number of necrotic cells is observed after 50 min of the experiment, with the most pronounced effect when using 100 µM 3-EA (Fig. 1B).

A more severe model of ischemia-like conditions, a combination of 2 hours of ischemia followed by reoxygenation in a CO_2 incubator for 24 hours (OGD/R), led to necrotic death of about 70% of cells (compared to 34% in the case of ischemia only), and the remaining part of the cells was in the late stages of apoptosis (Fig. 2). Preincubation of cortical cells with 3-EA resulted in a concentration-dependent reduction in the number of cells in late stages of apoptosis and prevention of necrotic cell death after OGD/R by increasing the percentage of viable cells and cells in early stages of apoptosis (Fig. 2). The most effective concentration of 3-EA in inhibiting necrosis was 100 µM.

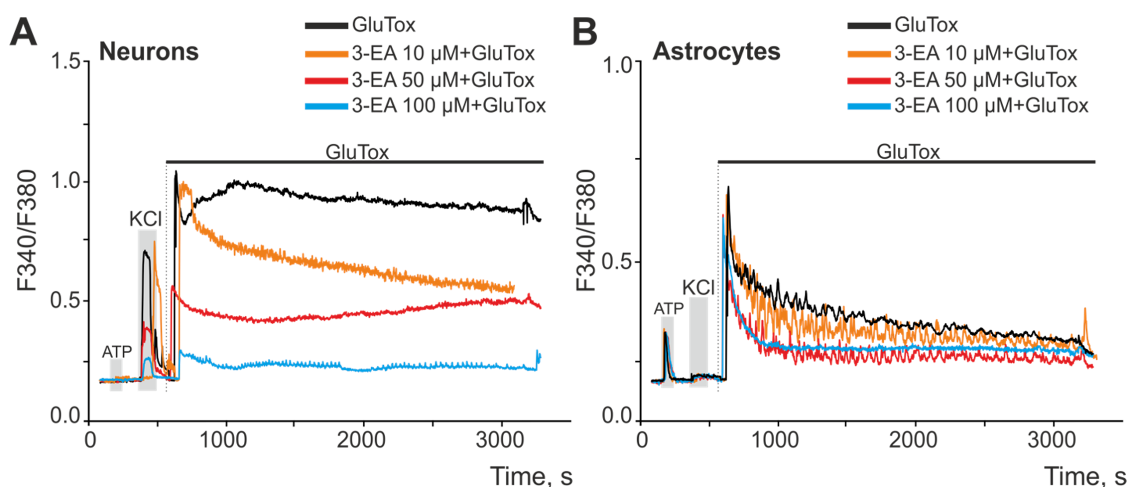


Figure 1. Effect of 24-hour preincubation of mouse cerebral cortex cells with 3-EA on the intracellular [Ca2+]i under ischemia-like conditions (50 min): A, B – averaged Ca^{2+} signals of neurons (**A**) and astrocytes (**B**), n = 9; GluTox – glutamate excitotoxicity, 3-EA – 2-ethyl-6-methyl-3-hydroxypyridinium-gammalactone-2,3-dihydro-L-gulonate (3-hydroxypyridine ascorbate).

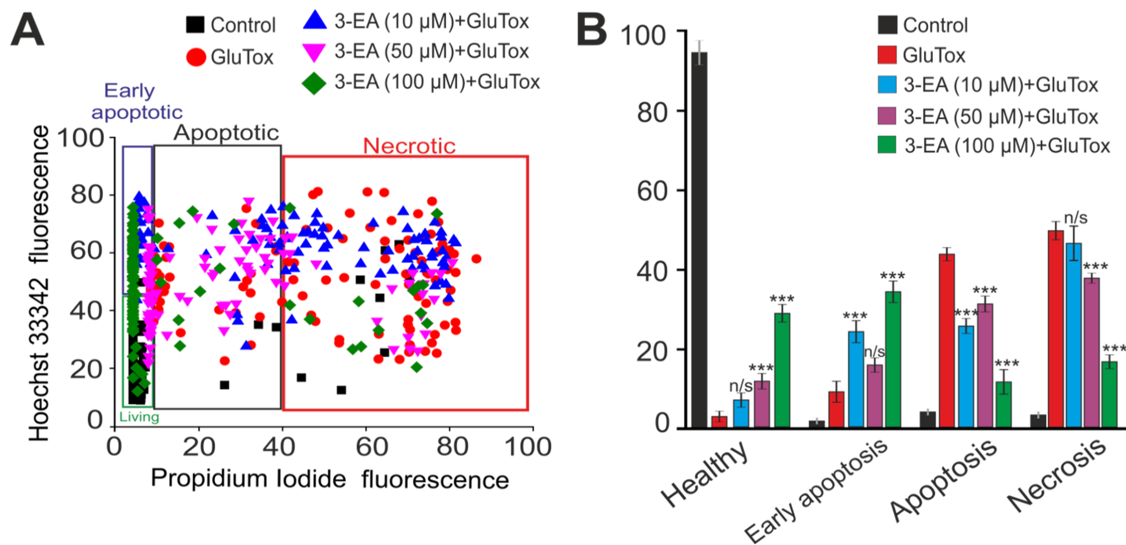


Figure 2. Effect of 24-hour preincubation with 3-EA on the viability of mouse cortical cells after 2 hours of oxygen-glucose deprivation followed by 24 hours of reoxygenation (OGD/R): **A** – cells viability; **B** – proportion of cells depending on vital status (%); n = 5; *p<0.001 compared with OGD/R (ANOVA, Tukey's test); GluTox – glutamate excitotoxicity, 3-EA – 2-ethyl-6-methyl-3-hydroxypyridinium-gammalactone-2,3-dihydro-L-gulonate (3-hydroxypyridine ascorbate).

To determine the effects of 3-EA on gene expression, cortical cell cultures were incubated with 100 μM 3-EA for 24 hours. It turned out that 3-EA reduced the basal expression of the *TRAIL*, *MLKL* and *Cas-1* genes, which encoded proteins involved in the induction of necrotic cell death. In this case, the expression of anti-apoptotic genes *Bcl-2, Stat3* and *Socs3* was induced along with downregulation of the pro-apoptotic gene *Bcl-xL* and pro-inflammatory genes *IL-1β* and *TNFα* (Fig. 3A). Following OGD/R, there was a change in gene expression patterns, but 3-EA promotes a decrease in *MLKL* and *Cas-1* levels, which might lead to inhibition of necrosis. OGD/R induced twofold or more overexpression of the anti-apoptotic genes *Bcl-2, Stat3* and *Socs3* along with a decrease in the transcription of pro-inflammatory genes *Cas-3, IL-1β*, and *TNFα* (Fig. 3B). Interestingly, 3-EA downregulate the basal and OGD/R-induced expression of the *Mao-A* and *Mao-B* genes (Fig. 3B), and an increase in the expression of the catalase gene (Fig. 3D).

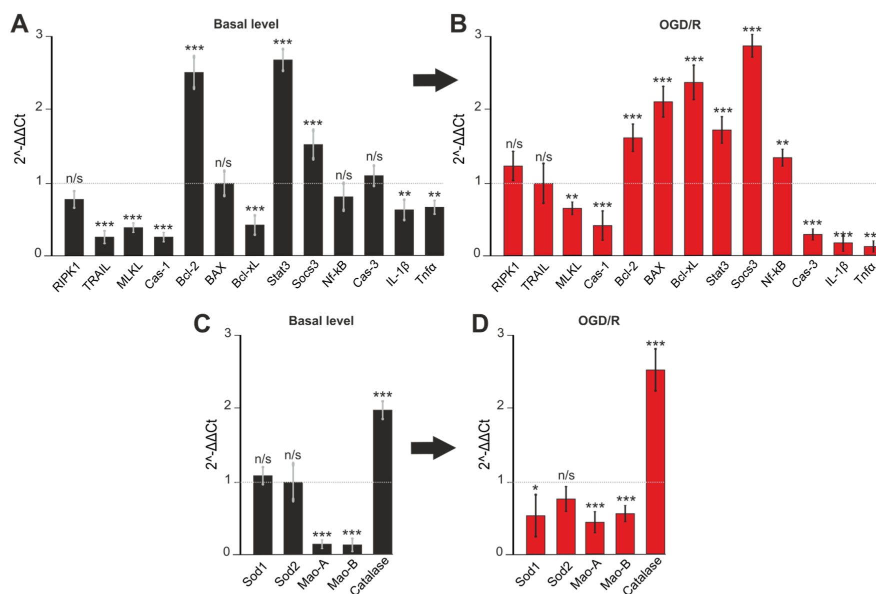


Figure 3. The effect of 24 hours of incubation of mouse cerebral cortex cells in the presence of 100 μM 3-EA on the basal and OGD/R-induced level of expression of genes that regulate cell death and antioxidant status: **A**, **B** – basal and OGD/R-induced levels of expression of genes encoding proteins of necrosis, apoptosis and inflammation in cells; **C**, **D** – basal and OGD/R-induced levels of expression of genes encoding proteins that regulate antioxidant status in cells;*p<0.05; **p<0.01; ***p<0.001 compared with OGD/R (ANOVA, Tukey's test); n/s – differences when compared with gene expression in the absence of 3-EA are not significant; OGD/R – oxygen-glucose deprivation/reoxygenation.

We found that in the control group MCA occlusion resulted in deep depression of the animals' motor and sensory functions with a total score of neurological deficits equal to 4.5, 3.7 and 3.2 on Days 1, 3 and 7 after the experimental pathology onset. Intraperitoneal administration of 18 mg/kg 3-EA for 7 days was accompanied by a decrease in the severity of neurological deficits, which was statistically significant on Days 3 and 7 after the development of the experimental stroke. Thus, by Day 3 the average score was 3.6, and by Day 7 – 1.8 ($p<0.01$ when compared with the control). It should be noted that a week after the experimental exposure to 3-EA, complete restoration of motor and sensory functions was not recorded (when compared with sham-operated animals) (Fig. 4).

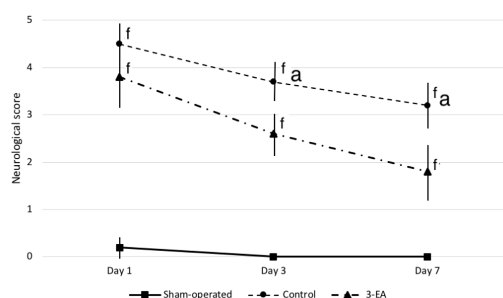


Figure 4. Dynamics of neurological deficits in laboratory rats with MCA occlusion on Days 1, 3 and 7 after the experimental pathology onset and administration of 3-EA: [f] – $p<0.001$ when compared with sham-operated animals; [a] – $p<0.001$ when compared with the control (ANOVA, Tukey's test).

Morphologically, in the control group, necrotic and dystrophic changes affected the area of the somatosensory cortex and were localized in the caudal parts of the forebrain. According to statistical analysis of the scoring data, they were 26% more pronounced compared with the experimental group (3-EA, 18 mg/kg i.p. for 7 days) ($p<0.01$) (Fig. 5A). Morphometric analysis of Nissl-stained brain sections reviled that in the group of intact rats the proportion of damaged neurons did not exceed 2%, which was due to technical reasons related to the sampling and processing of the brain. In the control, the proportion of damaged neurons increased to 28% ($p = 0.001$ when compared with the sham-operated animals). Experimental exposure to 18 mg/kg 3-EA for 7 days led to statistically significant reduction in the proportion of damaged neurons to an average of 13% ($P= 0.01$ when compared with the control group) (Fig. 5B).

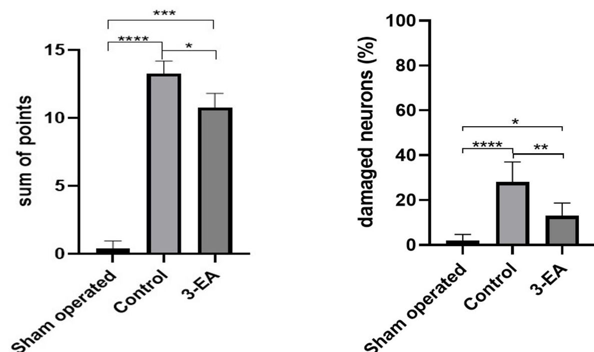


Figure 5. 3-EA reduces the depth of morphological disorders: **A** – morphological scale; **B** – % of dead neurons; *, *** – differences when compared with sham-operated rats are significant at $p<0.05$; ** – differences when compared with the control are significant at $p<0.05$.

Discussion

The new compound of 3-hydroxypyridine, 3-EA, protects cells of the cerebral cortex (neurons and astrocytes) from necrotic death during OGD and OGD/R by suppressing the global increase of intracellular Ca^{2+} signaling, preventing OGD/R-induced overexpression of genes encoding anti-apoptotic proteins *BCL-2, STAT3, SOCS3*, downregulating the genes of necrosis and inflammation *TRAIL, MLKL, Cas-1, Cas-3, IL-1β* and *TNFα*. The effects of 3-EA on the expression of genes encoding monoamine oxidases and catalase may indicate the regulation of the basal and OGD/R-induced oxidative status of cerebral cortex cells through mitochondrial bioenergetics, which is consistent with the previously obtained data on the participation of these signaling systems in the regulation of cell death (Turovsky et al. 2021; Varlamova et al. 2021).

As it has been already shown before, transient intraluminal acute cerebral ischemia develops massive damage to the rats' brain in the area of the MCA blood supply (Mirzoyan et al. 2021). That occurs as foci of necrosis, perinecrotic edema and cell damage with development of a deep neurological deficits. A course administration of 3-hydroxypyridine ascorbate at a dose of 18 mg/kg per day reduces the severity of damage both by preserving the population of neurons in the penumbra zone and by limiting the local stress response.

Conclusions

1. 24-hour preincubation of mouse cortical cells with 3-EA (10, 50 and 100 µM) causes a concentration-dependent suppression of the second phase of OGD-induced [Ca2+]i growth in neurons and astrocytes. At the same time, 3-EA decreases the number of necrotic cells with the most pronounced effect at 100 µM concentration.
2. Preincubation of cortical cells with 100 µM 3-EA leads to a concentration-dependent decrease in the number of cells in the late stages of apoptosis by 34%, and suppression of necrotic death after OGD/R.
3. Intraperitoneal 18 mg/kg 3-EA administration protects brain neurons from ischemic damage, maintains neuronal population and weakens the severity of neurological deficits in rats with MCA occlusion.
4. Assuming mechanisms for 3-EA preventing both neurons' and astrocytes' death in the ischemia models are an growth basal and OGD/R-induced expression of genes encoding the anti-apoptotic proteins *BCL-2, STAT3, SOCS3* alongside with a suppression of transcription of the genes regulating necrosis and inflammation *TRAIL, MLKL, Cas-1, Cas-3, IL-1β* and *TNFα*.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgements

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Data availability

All of the data that support the findings of this study are available in the main text.

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Author Contributions

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