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Search for Stroke-Protecting Agents in Endothelin-1-Induced Ischemic Stroke Model in Rats

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Key Words: *endothelin-1; ischemic stroke; neurodegeneration; protection; cerebrocrast; mildronate.*

Summary. *Background and Objective. Ischemic stroke may initiate a reperfusion injury leading to brain damage cascades where inflammatory mechanisms play a major role. Therefore, the necessity for the novel stroke-protecting agents whose the mechanism of action is focused on their anti-inflammatory potency is still on the agenda for drug designers. Our previous studies demonstrated that cerebrocrast (a 1,4-dihydropyridine derivative) and mildronate (a representative of the aza-butyrobetaine class) possessed considerable anti-inflammatory and neuroprotective properties in different in vitro and in vivo model systems. The present study investigated their stroke-protecting ability in an endothelin-1 (ET-1)-induced ischemic stroke model in rats.*

Material and Methods. Male Wistar rats were pretreated (for 7 days, per os) with cerebrocrast (0.1 mg/kg), mildronate (100 mg/kg), or their combination, followed by the intracerebral injection of ET-1. Functional and behavioral tests were carried out up to 14 days after the ET-1 injection. Ex vivo, the number of degenerated neurons and the infarction size in the cerebral cortical tissue were assessed histologically.

Results. Cerebrocrast and mildronate effectively normalized ET-1-induced disturbances in neurological status, improved the muscle tone, and decreased the number of degenerated cortical cells. Both drugs also reduced the infarction size, and cerebrocrast showed at least a 2-fold higher activity than mildronate. The combination of both drugs did not cause a more pronounced effect in comparison with the action of drugs administered separately.

Conclusions. The 1,4-dihydropyridine and aza-butyrobetaine structures may serve for the design of novel stroke-protecting agents to prevent severe neurological poststroke consequences.

Introduction

Ischemic stroke is one of the most common causes of disability and death worldwide (1). Especially older people display more severe neurological deficits and a reduced ability to recover from them (2). Ischemic stroke may initiate a reperfusion injury leading to brain damage cascades, including events from energy depletion to cell death, where inflammatory mechanisms play a major role (3). Ischemia induces the expression of cytokines, adhesion molecules, and other inflammatory mediators, including prostanoids and nitric oxide (4), and activates microglia (5). Although inflammation under certain circumstances could promote a functional recovery by supporting neurogenesis and plasticity (6), there is evidence that inflammation, which persists long after an initial injury, can contribute to a secondary ischemic injury and worsen the neurological status by chronically-released inflammatory mediators (4, 7). Therefore, there is a strong rationale for targeting postischemic neuroinflammation by the drugs possessing an anti-inflammatory action, but having no side effects typical of nonsteroidal anti-inflammatory drugs and/or glucocorticoids.

In this context, the present study explored the hypothesis that cerebrocrast (a 1,4-dihydropyridine derivative) and mildronate (the representative of the aza-butyrobetaine class) (Fig. 1) could be relevant in protecting against stroke-induced pathologies. The rationale behind this hypothesis was, firstly, that cerebrocrast and mildronate acted as anti-inflammatory and neuroprotective agents (8–13). For instance, cerebrocrast reduced inflammation in the rat paw edema model and inhibited the secretion of neurotoxic cytokines interleukines IL-1β and IL-6 in human monocyte (THP-1) cell line (9). It also effectively reduced azidothymidine-induced overexpression of molecules involved in inflammatory and apoptotic events, such as NF-κBp65 and caspase-3 in the mouse brain cortex or the myocardium (11).

Mildronate was found as capable of suppressing neuroinflammatory and apoptotic processes in the brain cortical tissue of azidothymidine-treated

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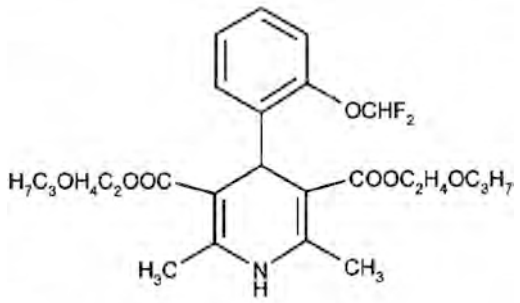

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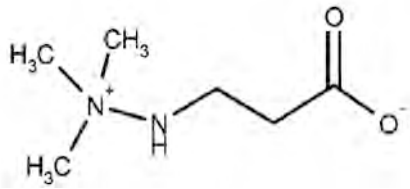

Fig. 1. Structures of cerebrocrast (A) and mildronate (B)

mice by preventing an abnormal expression of cytochrome oxidase c, caspase-3, inducible nitric oxide synthase (iNOS), cellular apoptosis susceptibility (CAS) protein, and glial fibrillary acidic protein (GFAP) (12). Furthermore, in a rat model of Parkinson's disease, mildronate protected against 6-OHDA-induced changes in the expression of nigrostriatal proteins that are involved in inflammation, such as GFAP, iNOS, IBA-1, and markers of microglia (13).

The aim of the present study was to investigate the potency of the drugs, cerebrocrast and mildronate, in endothelin-1 (ET-1)-induced ischemic stroke model in rats. ET-1 is a peptide, which causes vasoconstriction and produces a transient focal cerebral ischemia if exposed to the middle cerebral artery (14). Moreover, it is shown that ischemic stroke is associated with an acute and marked increase in plasma levels of ET-1 (15, 16). We assessed the ET-1-induced neurological and behavioral alterations in vivo and histopathological changes ex vivo in the cortical tissue.

Material and Methods

Material. The following equipment was used: a stereotactic apparatus (Stoelting, USA), a microscope (World Precision Instruments, WPI, USA), an infusion pump (WPI, USA), a pH meter (Eutech Instruments, Singapore), and a rota-rod apparatus (Ugo Basile, Italy).

Animals. Male Wistar rats weighing 250–300 g were obtained from the Laboratory of Experimental Animals, Riga Stradins University (Riga, Latvia). The environment was maintained at a temperature of 22°C±0.5°C with a 12-hour light/dark cycle, and animals were fed a standard laboratory diet.

Ethics. All the experimental procedures were carried out in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments and were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia).

Drugs. Cerebrocrast, 4-[2-(difluoromethoxy) phenyl]-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid di(2-propoxyethyl) diester), was obtained from the Latvian Institute of Organic Synthesis (Riga, Latvia); mildronate, [3-(2,2,2-trimethylhydrazinium) propionate dihydrate] from the JSC "Grindeks" (Riga, Latvia); endothelin-1 from Sigma Aldrich (USA); and ketamine and xylazine from Alfasan (Holland).

Surgery. Stereotactic surgery was made under ketamine (75 mg/kg) and xylazine (10 mg/kg) anesthesia implanting cannulas in the piriform cortex, 2 mm dorsal from the middle cerebral artery, according to Paxinos and Watson's (1986) stereotaxic coordinates atlas: AP, +0.9 mm; L, –5.2 mm; DV, –6.7 mm.

Drug Administration. After the postoperation period, on day 4 (i.e., 3 full days after the cannula implantation on day 0), the drugs were administered daily for 7 days per os: mildronate (100 mg/kg), cerebrocrast (0.1 mg/kg), or a combination of them. The doses of the drugs were selected as the most active from previous experiments (11–13). Then, 24 hours after the last drug administration (day 11), ET-1 was administered intracerebrally at a dose of 240 pmol/3 μL. The control group received 3 μL of artificial cerebrospinal fluid (aCSF). The nonoperated, saline-treated group also served as a control. The rats were divided into 9 groups each consisting of 8 animals: group S+ET-1 (saline, 7 days+ET-1); group C+ET-1 (cerebrocrast, 7 days+ET-1); group M+ET-1 (mildronate, 7 days+ET-1); group C+M+ET-1 (cerebrocrast+mildronate, 7 days+ET-1); group S+aCSF (saline, 7 days+aCSF); group C+aCSF (cerebrocrast, 7 days+aCSF); group M+aCSF (mildronate, 7 days+aCSF); group C+M+aCSF (cerebrocrast+mildronate, 7 days+aCSF); and group S (nonoperated, saline, 7 days).

Methods were used according to (17).

1. Functional Assessment

1.1. Neurological scores (posture disturbances and hemiplegia) were assessed in all the animals before the surgery (day 0), before the administration of drugs (day 4), before the ET-1 or aCSF administration (day 11), and 24 hours, 48 hours, and 72 hours after the ET-1 or aCSF administration, i.e., on days 12, 13, and 14 according to the following scale: 0, no deficit; 1, forelimb weakness; 2, circling to the affected side; 3, partial paralysis on the affected side; and 4, no spontaneous motor activity.

1.2. Initiation of walking (movement ability) was assessed on days 0, 4, 11, and 12–14. Each animal

was placed on a flat surface, and the time in seconds for the rat to move one body length was recorded.

1.3. Visual placement (motor impairments of the affected side) was assessed on days 0, 4, 11, and 12–14. The rat was suspended by its tail and slowly lowered toward a tabletop. If the animal extended both its forelimbs toward the surface, then the animal was considered as +; if the animal extended only one forelimb toward the surface, then it was considered as –; if neither forelimbs were extended toward the surface, the animal was considered as 0.

2. Behavior Test

The rota-rod test (motor coordination and muscle tone) was performed 24, 48, and 72 hours after the administration of ET-1 or aCSF (days 12, 13, and 14, respectively). The time spent on the rota-rod (speed of 32 rpm) was registered in seconds (0–180) until the rat fell from the rod.

3. Histology

After the termination of in vivo tests, the rats were euthanized with a pentobarbital overdose (intraperitoneally) and perfused through the ascending aorta with 50 mL of isotonic saline followed by 250 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) The brains were removed and fixed at –80°C. The cortical tissue was fixed in 10% neutral buffered formalin. Then, 24–48 hours after the fixation, the brain tissue was embedded in paraffin. Brain sections (4 μm) were deparafinized in xylol and ethanol (96° and 70°), stained with cresyl violet for 2 minutes, and washed in ethanol. Normally, neurons were stained in light and dark violet color; however, degenerative neurons did not stain with cresyl violet.

All the sections were analyzed using the Motic Image software applied to a light microscope. The results were expressed as the number of degenerative neurons per mm^2 (cells/mm^2).

The cerebral cortical infarction size was evaluated histopathologically, and the results were expressed in percentage calculating the necrotic area per the total cortical tissue area in a high-power field (n=20).

Statistical Analysis. The data were analyzed using the GraphPad Prism 4 software. The results were expressed as mean±SEM; a P value of <0.05 was considered statistically significant. The Kruskal-Wallis test with the Dunn post test and 1-way or repeated-measures ANOVA with the post hoc Bonferroni test were used.

Results

1. Influence on Neurological Scores (Posture Disturbances/Hemiplegia)

On day 12, ET-1 caused slight neurological disturbances; however, the neurological scores of the rats treated with cerebrocrast or mildronate, or their combination were on the same levels as the control (saline+aCSF) and nonoperated groups (Fig. 2A). Similar results were obtained on days 13 and 14 (data not shown). The drugs administered separately (without ET-1) did not influence neurological scores.

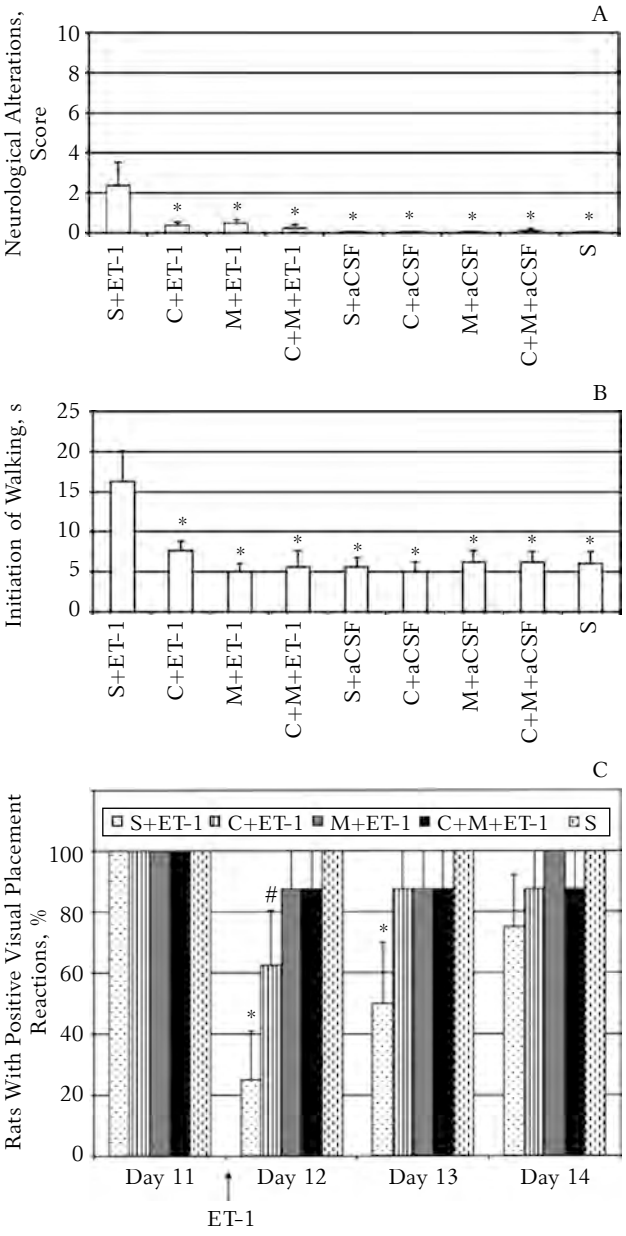

Fig. 2. Influence of cerebrocrast (C), mildronate (M), C+M, artificial cerebrospinal fluid (aCSF), and saline (S) on functional neurological responses in ET-1-induced ischemic stroke in rats

A, neurological scores (day 12), *P<0.05 vs. S+ET-1, the Kruskal-Wallis test with the Dunn post test; B, initiation of walking (day 13), *P<0.05 vs. S+ET-1, 1-way ANOVA with the post hoc Bonferroni test; C, number of rats with visual placement reactions (days 12-14), *P<0.05 vs. S+ET-1 (day 11), #P<0.05 vs. C+ET-1 (day 11), repeated-measures ANOVA with the post hoc Bonferroni test.

2. Effects on Initiation of Walking

On days 12–14, ET-1 caused an impairment in

the movement ability by increasing the walking time (15 seconds). Cerebrocrast, mildronate, and the combination of cerebrocrast and mildronate normalized the ET-1-induced alterations in the walking time (by about 2-fold) to the values (5–7 seconds) of the aCSF and nonoperated groups, as well as of those treated with drugs only (Fig. 2B, day 13).

3. *Effects on Visual Placement Reaction*

Before the administration of ET-1 (day 11), all the animals demonstrated comparable visual placement reactions, which corresponded to a normal reaction (+). After the ET-1 injection on day 12, a normal visual placement reaction was observed in 25% of the animals in the ET-1 group, indicating a motor impairment of the affected brain side. In the cerebrocrast-treated group, the ET-1 effect was reduced, and 62.5% of the animals showed a normal visual placement reaction, while in the mildronate and cerebrocrast+mildronate groups, 87.5% of the animals showed a (+) reaction (Fig. 2C). The drugs similarly normalized the ET-1 effects to the control level on day 13; the effect of ET-1 totally disappeared on day 14 (data not shown).

4. Influence on Motor Coordination (Rota-Rod Test)

The ET-1 group animals showed a significant decrease in the time spent on the rota-rod (about 10–15 seconds) on day 12 as compared with the data from the aCSF (75–80 s) and nonoperated (90–100 seconds) animal groups (Fig. 3). Cerebrocrast, mildronate, and their combination caused a total normalization of these responses up to the control levels (Fig. 3). The same was observed on day 13; on day 14, the effects of all the groups did not differ between each other (data not shown). The data observed after the administration of cerebrocrast and mildronate alone, as well as their combination, were comparable with the control group data.

5. Effects on Histopathological Changes

A histopathological examination of the brain tissue showed a significantly increased number of degenerated cortical neurons (~40 cells/mm^2) in the ET-1 group as compared with the aCSF group (~20 cells/mm^2) and nonoperated group (~10 cells/mm^2) (Fig. 4A). The effects of compounds per se, i.e., cerebrocrast, mildronate, and the combination of cerebrocrast and mildronate, were comparable with the effect of aCSF. The treatment with cerebrocrast in the ET-1 group significantly (by about 2.5-fold) decreased the effect of ET-1, and that was comparable with the data of the nonoperated animal group. Mildronate decreased the ET-1-induced effect to the level of the aCSF group. The combination of cerebrocrast and mildronate did not influence the ET-1-induced increase in the number of degenerated cells (Fig. 4A). Fig. 4B demonstrates the cerebrocrast effect in the ET-1-induced morphological changes. Fig. 4C shows that both drugs reduced the infarction size. Particularly high activity (reduction of the infarction size by about 4-fold) was demonstrated by cerebrocrast, and its effect was about 2-fold greater than that of mildronate. The drug combination did not enhance the effect observed after the drug administration separately.

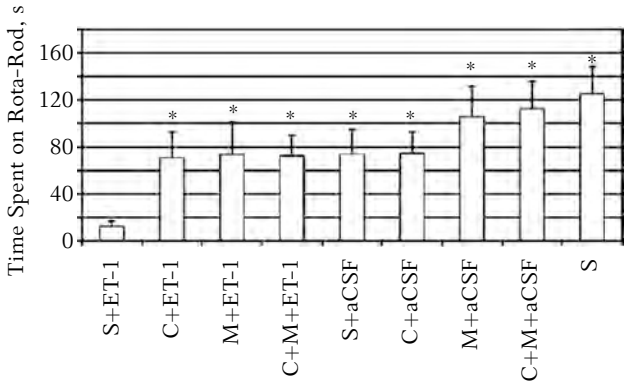

Fig. 3. Influence of cerebrocrast (C), mildronate (M), C+M, artificial cerebrospinal fluid (aCSF), and saline (S) on time spent on the rota-rod on day 12 in ET-1-induced ischemic stroke in rats

*$P<0.05$ vs. S+ET-1, 1-way ANOVA with the post hoc Bonferroni test.

Discussion

The present study investigated two drugs with different chemical structures – cerebrocrast and mildronate – in a rat model of stroke induced by ET-1, which caused considerable neurological dysfunction and changes in behavior and cortical tissue morphology. Cerebrocrast is a derivative of 1,4-dihydropyridines; mildronate represents the aza-butyrobetaine class. Nevertheless, in functional tests, they comparably normalized (to the control levels) the poststroke functional neurological reactions, i.e., the drugs reduced hemiplegias and improved walking and motor ability. These drugs also normalized behavior in the rota-rod test by increasing the time spent on the rotating rod in the ET-1-treated animals, which was 5- to 6-fold lower in comparison with the data from the aCSF and nonoperated groups. Therefore, our obtained data demonstrated a pronounced beneficial protection of the neurological ability, motor activity, and muscle tone in cerebrocrast- and mildronate-treated animals after an ET-1-induced ischemic stroke. The concomitant administration of both drugs did not potentiate these effects. The drugs administered alone as the controls (when aCSF was injected instead of ET-1) did not affect functional or behavioral reactions.

As to degenerative alterations in cortical neurons caused by ET-1, they were successfully (about 2.5-fold) protected by both tested drugs. Moreover, both drugs also significantly reduced the infarction size, and cerebrocrast showed about a 2-fold higher activity (reduction by about 4-fold) than mildro-

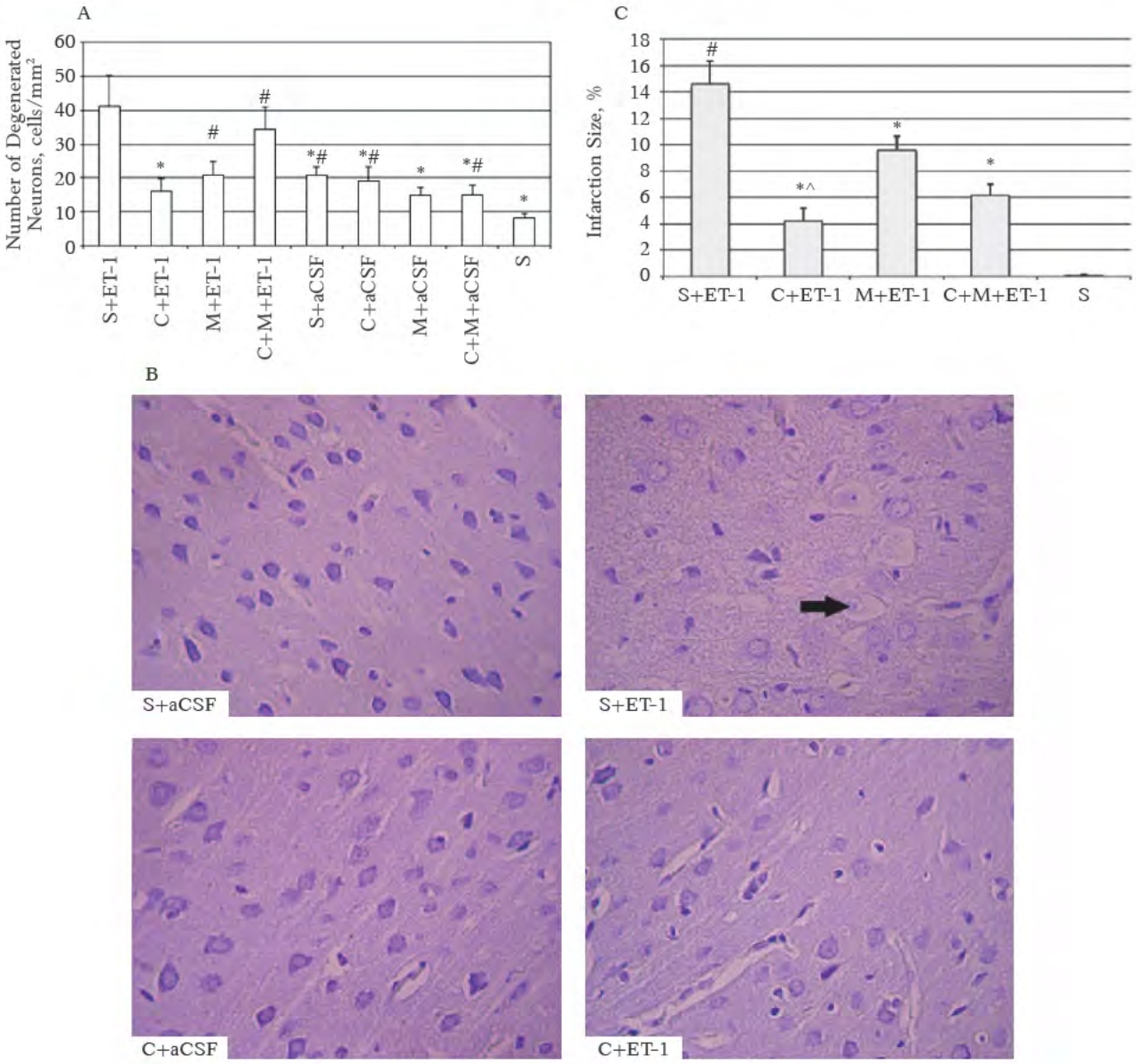

Fig. 4. Influence of cerebrocrast (C), mildronate (M), C+M, cerebrospinal fluid (aCSF), and saline (S) on ET-1-induced histopathological changes in the brain cortical tissue of rats
A, number of degenerated cortical neurons/mm², $^{*}P<0.05$ vs. S+ET-1, $^{\#}P<0.05$ vs. S, the Kruskal-Wallis test with the Dunn post test; B, a photomicrograph showing cortical neurons stained with cresyl violet in the cerebrocrast-treated rat brain cortical tissue (magnification ×400; the arrow indicates degenerated neurons); C, infarction size, $^{\#}P<0.05$ vs. S; $^{*}P<0.05$ vs. S+ET-1; $^{\wedge}P<0.05$, C+ET-1 vs. M+ET-1, 1-way ANOVA with the post hoc Bonferroni test.

nate. However, the coadministration of cerebrocrast and mildronate did not enhance the effects observed after the administration of drugs separately.

Our results obtained during the present study are in good line with other authors' data demonstrating the effects of cerebrocrast or mildronate in another stroke model, i.e., middle cerebral artery occlusion, in rats. For instance, cerebrocrast completely prevented a fall in the rat brain ATP content (18), and mildronate improved functional responses (19), although it did not influence the infarction size, which contradicts our findings. Mildronate showed an anti-ischemic ability to stimulate the nitric oxide production in the vascular endothelium (20).

The present study demonstrated that the two drugs with different chemical structures showed similar and comparable effects in the ET-1 stroke model. A lot of common features in cerebrocrast and mildronate actions were also found in our previous studies. Firstly, both drugs showed an anti-inflammatory and antiapoptotic action in models of different neurotoxicity (9, 11–13). Secondly, these

drugs demonstrated mitochondria-protecting activity: cerebrocrast normalized oxidative phosphorylation, augmented the ATP-induced contraction rate and amplitude of isolated swollen mitochondria (21), and protected cerebellar granule cells against mitochondria toxin 1-methyl-4-phenylpyridine (MPP^+)-induced cell death, production of reactive oxygen species, and loss of mitochondrial transmembrane potential (10). In turn, in isolated rat liver mitochondria, mildronate protected against azidothymidine-induced hydrogen peroxide generation and inhibition of uncoupled respiration, ADP-to-oxygen ratio, and transmembrane potential (22). Taking into account that mitochondria normally are the major site of the production of energy needed for the cell function, one may consider that mitochondria are strongly involved in the development of stroke-induced tissue injury due to the changes in their major role in supplying ATP and, consequently, due to apoptotic and necrotic cell death (23). The mitochondrial release of multiple apoptogenic proteins has been identified in the ischemic and postischemic brain, mostly in neurons. These components are also often the key players in promoting deleterious changes leading to cell death. Therefore, one may suggest that the mitochondria-protecting, antiapoptotic, and anti-inflammatory properties of cerebrocrast and mildronate play important roles in the maintenance of cell survival.

Moreover, in the context of poststroke therapy, it is also worth stressing the essential importance of neuroregenerative/neurorestorative properties of the drugs, and this approach may be considered as the most effective for the future medicine. Synaptic plasticity-promoting therapy intensifies neurological recovery by enhancing axonal sprouting in lesion-remote brain areas and may prevent secondary postischemic neurodegeneration (24). As it was shown by us previously, cerebrocrast demonstrated neurite outgrowth-promoting activity (25). In a rat model of Parkinson's disease, mildronate showed an ability of neurorestorative/neuroregenerative effects by protecting neurons and promoting the expression of proteins necessary for adult neurogenesis: tyrosine hydroxylase (the key enzyme necessary for dopamine synthesis), ubiquitin (a regulatory peptide involved in the ubiquitin-proteasome degradation system), Notch-3 (a marker of progenitor cells) (13), GDNF (a glial cell line-derived nerve growth factor), NCAM (a neural cell adhesion molecule), and Hsp70 (a molecular chaperone) (26).

One question remains to be elucidated: why did two potent drugs not cause the enhancement of antineurodegenerative effect when administered together? At present, one may suggest that although there are a lot of common cellular targets (mitochondrial, anti-inflammatory, and neuroregenerating processes) for cerebrocrast and mildronate, some distinct signaling mechanisms more or less specific for each drug molecules may shift their action from the beneficial targets if the drugs are used concomitantly.

Conclusions

In the ET-1-induced ischemic stroke model rats, cerebrocrast and mildronate considerably improved stroke-induced functional status and protected against histological changes, indicating that molecules of both drugs comprise pharmacophores beneficial for stroke-protecting activity, i.e., essential for the prevention against reperfusion injury in the brain tissue as well as against recurrent stroke.

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Statement of Conflict of Interest

The authors state no conflict of interest.

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