

INTRANASAL ADMINISTRATION OF RECOMBINANT HUMAN ERYTHROPOIETIN EXERTS NEUROPROTECTIVE EFFECTS ON POST-ISCHEMIC BRAIN INJURY IN MONGOLIAN GERBILS.

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Summary

Erythropoietin had been reported as a neuroprotective molecule in animal models with perspectives for its clinical use. Nasal administration presents advantages treating the brain compared with intravenous and intraventricular routes, especially for the treatment with the low-content sialic acid erythropoietin (rHu-EPOb). It could be considered better for neuroprotection than the full-sialilated molecule. Our experiments were designed to demonstrate whether rHu-EPOb reach the brain after its nasal administration and exerts therapeutic efficacy after brain ischemia. I¹²⁵ labeled rHu-EPOb was administered nasally in Mongolian gerbils. Radioactivity was detected in olfactory bulb and cerebellum at 5 min. Brain ischemia was induced by permanent occlusion of the right common carotid artery. Clinical signs of ischemia were recorded at 24 h. Functional integrity was measured through the spontaneous exploratory activity. Brain edema was determined by a gravimetric method. Histological changes were also assessed. Gerbils treated with 30 µg of rHu-EPOb daily during 4 days showed a lower expression of clinical signs of ischemia, edema and a better functional integrity at 24 h compared with vehicle-treated animals. Seven days after surgery, a lower incidence of pathologic changes was observed in the brain tissue.

Body weight loss, observed in vehicle-treated animals was prevented by the intranasal rHu-EPOb treatment. Results suggest that rHu-EPOb reach the brain when is administered in the nasal cavity, and exerts neuroprotective effects in the acute and chronic stage after brain ischemia.

Key words: erythropoietin, brain ischemia, neuroprotection, nasal.

Hitherto, there are no drugs sufficiently effective, specific and safe administered to the CNS, to be used as neuroprotectant in the acute or chronic stages of the brain ischemia. Main used therapeutic agents, demonstrated effective in animal models, are not well tolerated by patients or they lack therapeutic efficacy (15, 20, 47). Administration of the endogenous molecules to prevent brain damage after different injuries could be a strategy to obtain better outcome (19).

The finding that erythropoietin is expressed in the brain, induced by the hypoxia inducible factor-1 (18), has encouraged studies directed to evaluate the effect of the recombinant human erythropoietin [rHu-EPO] in different models of brain ischemia (13, 23). In these studies, rHu-EPO has showed potent neuroprotective effects *in vitro*, as well as *in vivo* (4, 5, 8, 13). Action of rHu-EPO is mediated by receptors located mainly in the membrane of endothelial cells and astrocytes (7, 17, 45). These effects involve mechanisms inhibiting apoptosis and inflammation, potentiating neurotrophic factors and modulating the neuronal excitability (38, 49).

A body of evidences about the protective effect of rHu-EPO in different paradigms of brain damage has been reported recently (5, 8, 24, 28, 29, 30, 33, 50). A clinical trial of rHu-EPO in the acute brain ischemia showed that the biomolecule is safe and shows therapeutic effects. Nevertheless, main investigations used the intra ventricular or the intravenous routes for treatments to the CNS. Intraventricular administration is highly invasive and clinically unfeasible. Intravenous route requires a long time to reach enough amounts of rHu-EPO to exert a significant therapeutic effect. Since the time is a critical factor to protect the brain during the acute stroke, any therapeutic agent has to reach the brain as soon as possible, to minimize the ischemia-induced injury. A recently reported route to administrate rHu-EPO to the brain is into the nasal cavity (50). This approach could be more safe, invasiveless and 10 times more rapid than the intravenous via.

On the other hand, when erythropoietin is administrated intravenously, it constitutes a potential risk for increasing the blood viscosity due to the stimulation of erythropoiesis. Thus, it would be preferable to use an erythropoietin with a low content of sialic acids (rHu-EPO_b), similar to that produced in the brain during hypoxia (24). The rHu-EPO with an incomplete oligosaccharide chain has no erythropoietic properties. A treatment designed for this molecule must avoid its crossing through the liver, where it is degraded. It is an additional advantage for the intranasal administration.

Our investigation was focused to demonstrate the reaching of the low content-sialic acid rHu-EPO administered intranasal and its therapeutic efficacy after brain ischemia in Mongolian gerbils.

Methods

Animals: one hundred fifty eight Mongolian gerbils were used, produced in the National Center for Laboratory Animal Breeding. Each protocol was discussed and approved by the Institutional Ethic Committee, considering the international statements established by ICLAS.

Drug: the rHu-EPOb was supplied by the Center of Molecular Immunology (CIM, Havana, Cuba). The preparation was buffered in PBS to pH 7 at 0.15 mM.

Transport of rHu-EPOb from the nasal cavity to the brain: rHu-EPOb was labelled with I¹²⁵, according to the method described by Bolton (6). A formulation of 1 mg/ml in a PBS (pH 7,0) buffered solution was prepared for nasal application. Eighteen male Mongolian gerbils weighting 70 - 90 g were used. All the animals received from 100 to 120 µl of the formulation. Animals were sacrificed under anesthesia at 5, 30 and 60 mins after the end of the administration. Brains were removed in less than 70 seconds. Olfactory bulbs and cerebellum were dissected. Radiative counting of the brain regions was done in a gamma counter Pracitronic, 4π.

Therapeutic efficacy: Fifty male and female Mongolian gerbils, weighting 60 - 70 g were used. They were fed ad libitum and maintained in a 12 h light-dark cycle during the experiment.

Gerbils were anesthetized with ketamine-atropine-diazepam (47 mg/Kg; 0.02 mg/Kg; 5 mg/Kg, respectively). Lesions were performed according to the Butterfield and McGraw method [9]. Briefly, right common carotid artery was isolated, double ligated and sectioned. In sham operated animals, the artery was isolated only.

For the clinical and histopathological evaluation, animals were randomly distributed in three experimental groups: controls (shame operated gerbils), n=10; ischemic vehicle-treated gerbils, n=20; and ischemic rHu-EPOb -treated gerbils, n=20.

Treatment consisted in the nasal application of 10 µg of rHu-EPOb or a corresponding volume of vehicle 3 times a day during 4 days, beginning into the first hour after surgery.

Each animal was evaluated to determine its neurological state according to a scale from 2 to 5 involving the body tone, grasping strength and disturbances in the posture and gait. An animal without pathological signs had a value of 5 in this scale.

For the functional evaluation, it was used the spontaneous exploratory activity, where the rearing during the first 3 minutes in a 30 cm diameter and 25 cm high open field, was counted. Seven days after surgery, gerbils were cardiac perfused with saline solution and a subsequent buffered 4% formaldehyde solution, pH 7.0. Brains were carefully removed and maintained for several days in the fixative.

Then, they were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Sections were blindly evaluated in a light microscope with a magnifying power of 10x and 40x. A score including the incidence of pyknosis, edema and necrosis was established for the comparison between ischemic groups, using the Mann-Whitney U test.

Brain edema: Ninety female Mongolian gerbils, weighting 60 - 70 g were used. Animals were randomly allocated either in a group of sham operated (n = 20), in a group of vehicle-treated ischemic gerbils (n = 35) or in a group of rHu-EPOb -treated ischemic gerbils (n = 35).

At 3, 12 and 24 hours after surgery, the animals were perfused with saline solution. Forebrains were removed and hemispheres were separated. Determination of the water content was performed according to the gravimetric method described by Danica et. al. (15) through the following equation:

$$\% \text{ of water} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100$$

In the data analysis, there were established differences between groups for each variable using the Student's t test for related samples.

Body weight progression: Thirty male Mongolian gerbils, weighting 60 - 70 g were divided in 3 groups of 10 gerbils each, following the same treatment schedule of the experiments described above.

Animals were weighted immediately before surgery and once in a week for the five subsequent weeks. Differences among experimental groups were established by the one way ANOVA test and the Student's t test for independent samples.

Results

Transport of rHu-EPOb from the nasal cavity to the brain. Nasal administration of I^{125} rHu-EPOb labeled reach the brain in at least 5 minutes, and gradually its content diminishes from frontal to caudal region (Fig 1). Five min after application, I^{125} rHu-EPOb related radioactivity in the olfactory bulb is a 25% of that in cerebellum. At 30 min after application, the relative contents in both regions are approximately the same. At 60 min in the olfactory bulb was found about the 30% of the radioactivity found in cerebellum.

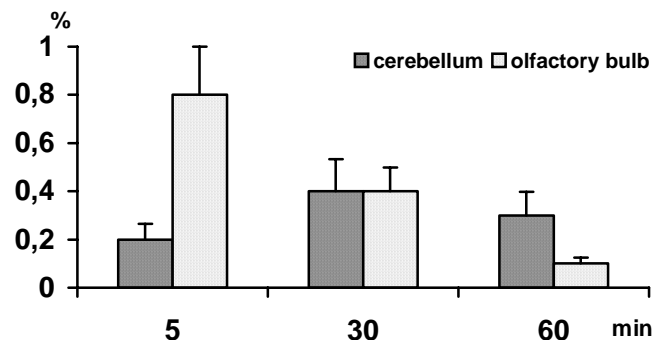


Fig.1. Radioactivity associated to the molecule of I^{125} rHu-EPOb in the olfactory bulb and cerebellum at 5, 30 and 60 minutes after nasal application. In y axis, the percent of radioactivity administered nasally. Each bar represents the average of six animals.

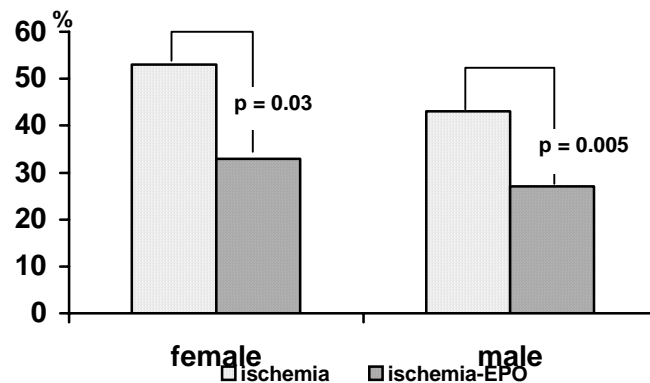


Fig. 2. Mortality expressed in percent, in Mongolian gerbils with permanent occlusion of the right common carotid artery. The p values comparing vehicle and rHu-EPO-treated groups were established with the analysis of proportions.

Therapeutic efficacy. A lower post surgical mortality in both genders was found, evidenced by the analysis of proportions (Fig. 2)

Twenty four hours after surgery, a fraction of the animals showed impairments in the neurological state, expressed in the described score (Fig. 3a). In vehicle-treated ischemic animals was found a significant functional damage, expressed also in a depressed exploratory-motor activity (Fig. 3b) while in rHu-EPOb-treated animals both variables remain similar to controls.

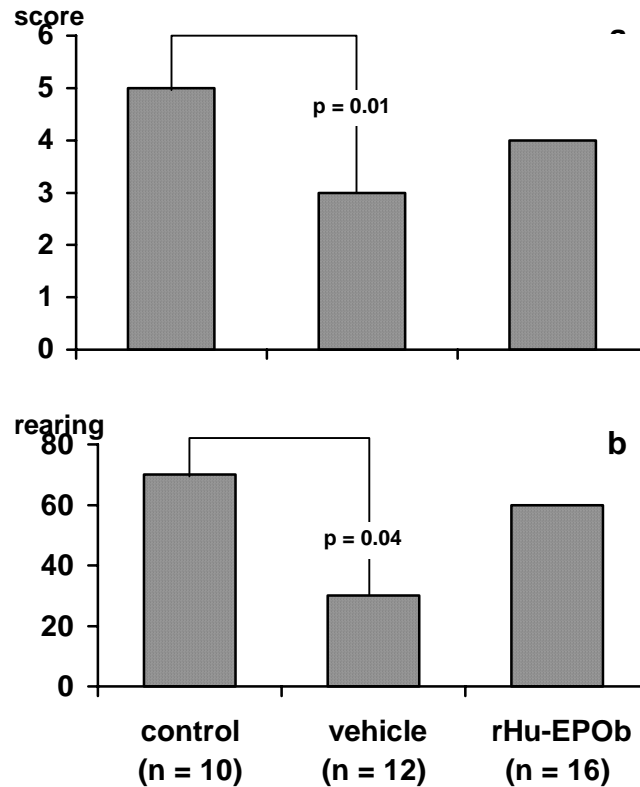


Fig. 3. Effect of intranasal Hu-EPOb treatment on the neurological score (a) and the rearing counts of the animals (b), 24 h after permanent unilateral common carotid occlusion. p values obtained for the Mann-Whitney U test.

The effect of ischemia and rHu-EPOb treatment on the animal body weight is shown in the Figure 4. Ischemic rHu-EPOb treated and control groups showed similar curves, whereas in vehicle-treated ischemic gerbils, a loss of body weight was noted. This group did not recover the initial average values at the end of the experiment.

Nasal administration of rHu-EPOb, protected against the increase of the percentage of water in the right hemisphere (Figs. 6a and 6b). Differences in the water content were not found between rHu-EPOb-treated gerbils and controls. In saline-treated gerbils, the water content in the right hemisphere was found increased at 24 h after surgery ($p < 0,001$).

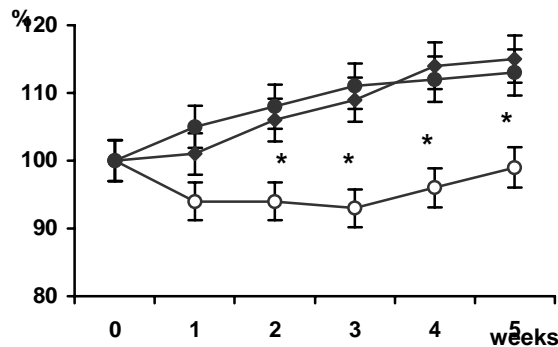


Fig. 5. Effect of the rHu-EPOb treatment on the body weight, during the five weeks subsequent to the permanent unilateral occlusion of the right common carotid artery. In the y axis is the percent of the body weight before surgery. Solid squares, controls (n = 18); open circles, vehicle (n = 16); solid circles, EPO (n = 21). Asterisk (*) means a p < 0,05 in the Student's T test.

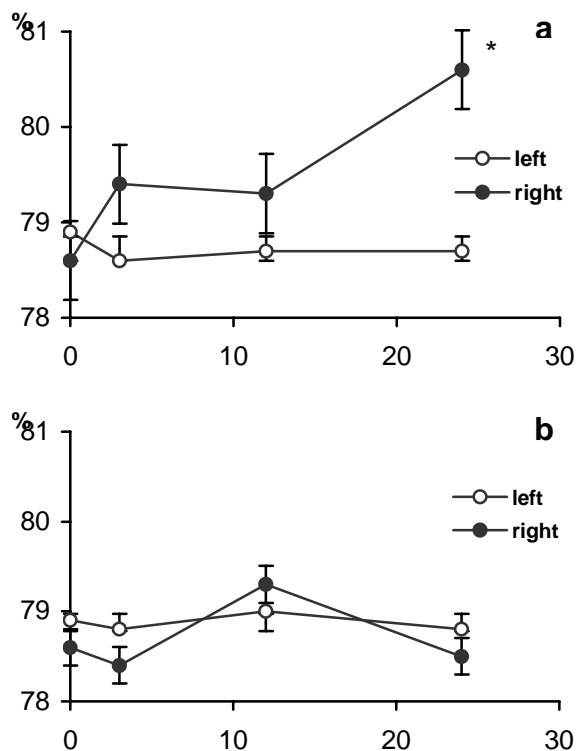


Fig. 6. Percentage of water in both hemispheres after the occlusion of the right common carotid artery a) with rHu-EPOb b) with vehicle. Asterisk (*) means a difference respect to the left hemisphere, p < 0.01 of the Student's T test.

In the tissue examination, the score involving pyknosis, edema and necrosis was lower in vehicle-treated gerbils (Fig. 7). In ischemic brains was observed edema, pyknosis in the right hemisphere, hemorrhages in the fimbria hippocampi and the parietal cortex of the right hemisphere, dense and diffuse chromatin in the right hemisphere and pycnotic neurons in the hippocampus.

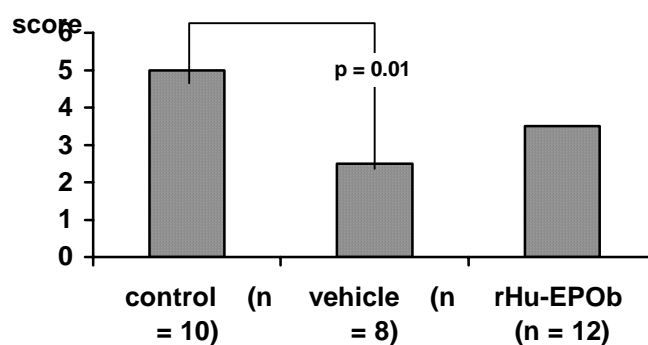


Fig.7 Histologic score involving pyknosis, edema and necrosis, 24 after the common carotid occlusion in Mongolian gerbils. P value for the Mann-Whitney U test.

Discussion

The attractive strategy of favouring the endogenous neuroprotective mechanisms in the injured CNS has been suggested recently (27). According to the conclusions stated by Shingo, 2001 (43) administration of brain-protecting molecules, such as rHu-EPO is a potential therapeutic alternative to counteract acute injuries in the CNS.

It is well known that different substances penetrate into the brain when they are inhaled (2, 14). The olfactory neuroepithelium is the unique place in the mammalian body entering directly in contact with the external milieu (34). It has been demonstrated the crossing of other trophic or neuroprotective molecules through the nasal cavity (3, 26).

It could be used for the prophylactic or therapeutic treatment with rHu-EPOb in acute or chronic brain injury (17, 50). Detection of 125 I-rHu-EPOb in the brain after its nasal administration is a strong evidence of the rHu-EPOb crossing. Detection of the radiolabelled molecule in regions unrelated to olfactory bulb, such as cerebellum, suggests that rHu-EPOb could diffuse into the mucus and permeates through discontinuities of the olfactory epithelium, where the molecule takes contact with the cerebrospinal fluid (CSF). The rHu-EPOb could broadly spread through the CSF (26).

This finding has a practical importance, because if rHu-EPOb freely diffuses across the CSF, it could reach zones with impaired blood supply. According to our results, rHu-EPOb reaches the CNS in at least 5 minutes after its nasal administration. Some substances, transported through the nasal via, reach the brain rapidly, according to reported findings

(3, 42). Furthermore, the amounts of administered rHu-EPOb were significantly lower than those used in intravenous administration (50), avoiding unnecessary higher dosage. These facts represent advantages compared with the endovascular route.

In the upper part of the nasal cavity are the nerve terminals conducting the smell information through the cribiform plate (3, 14). Terminal axons form the olfactory tract, extended from the basal brain to different subcortical regions. Thus, applying the rHu-EPOb through the nasal allows reaching the brain, circumventing the blood brain barrier.

Permanent unilateral occlusion of the common carotid artery has been used for studying the physiopathology of the brain ischemia and for the evaluation of putative neuroprotective agents (9, 11, 25, 31, 32, 35, 47). In this model, it has been reported a mortality rate from 25 to 50% (39, 40). In our experiment were found evidences of ischemia in the right hemisphere. Mortality rate was in females and males of 53% and 42%, respectively. Nevertheless, it was observed a mortality rate of 33% in females ($p = 0,03$), as well as a 24% in males ($p = 0,005$) treated with rHu-EPOb. Moreover, a greater functional integrity at day 7 in rHu-EPOb-treated gerbils was noted, when compared with vehicle-treated gerbils. Motor and exploratory activities expressed by rearing counts in a new recipient, was preserved in rHu-EPOb-treated animals. Results demonstrate the effectiveness of the rHu-EPOb to counteract the deleterious effects of the acute brain ischemia, and potentially could increase in at least two hours the therapeutic window for erythropoietin. It is important to remark that using this via, the rHu-EPOb was detected in the brain in only five minutes.

After permanent unilateral occlusion of common carotid, the administration of rHu-EPOb intranasal promotes a significant reduction of the rearing counts when comparing with the vehicle-treated group ($p < 0.05$). It indicates the efficacy of the nasal administered rHu-EPOb.

Study of body weight lasts for a relative long period (5 weeks), where it was demonstrated that in conditions of free water and food intake, the rHu-EPOb-treated gerbils had a body weight curve similar to controls. These results could be explained arguing at least two related explanations. First, motor impairment, evident in vehicle-treated gerbils, could affect the feeding behaviour. Brain regions associated to the appetite, such as the hypothalamus and the limbic system, were affected by the ischemia. Second, ischemia-induced stress must be reflected in the body weight as a remarkable loss. Whatever was the main reason, it was counteracted by the intranasal administration of rHu-EPOb.

Another important marker in brain ischemia is edema (46). Brain edema was undetectable when rHu-EPOb was applied to ischemic animals. In the ischemia processes, they are well known the presence both the vasogenic and the cytotoxic edema (10). Experimental evidence indicates that at least in the first 24 h edema was inhibited in the rHu-EPOb-treated animals. It could

be related with the mechanisms of regulation of the intracellular calcium, reported for erythropoietin (37). These results agreed with that reported by other authors about the role of the erythropoietin in the reduction of brain edema (41).

It has been reported by other authors an anti-inflammatory effect of the rHu-EPO_b in different models, such as the experimental autoimmune encephalitis (1) and the spinal cord injury (7, 12). This protection is explained by the antiapoptotic, antioxidant and angiogenic actions described for erythropoietin, which justifies its neurotrophic and neuroprotective potential.

To propose an explanation of the mechanisms by which the short- and long-term protective effects were obtained, exceed the research frame of this work. Nevertheless, we can speculate that rHu-EPO_b could act favouring the mechanisms of restoration and neuroplasticity in the ischemic animals. Erythropoietin regulates production of neural progenitors from stem cells in the hindbrain of mammals, suggested by Shingo et. al. 2001 (43). It opens new therapeutic possibilities to stimulate endogenous tissue autoregeneration and recovery of brain areas, using a safe, as well as non invasive method.

A significant protection of the tissue in the rHu-EPO_b-treated ischemic animals was evidenced. It suggests that intranasal administered rHu-EPO_b could protect at least to 24 h after the arterial occlusion, where the mechanisms leading to cell necrosis are present. Siren and coworkers report recently that acid rHu-EPO inhibits the apoptotic neuronal death after brain ischemia (44). According to our results, nasal administration of rHu-EPO_b appears to be also effective against the development of cell necrosis.

During the 90's it was demonstrated that erythropoietin was also produced in the CNS and had neuroprotective action in different models of brain ischemia. Recent investigations had demonstrate, that rHu-EPO is able to cross the blood-brain-barrier, inhibiting excitotoxicity, free radical-induced damage, inflammation and apoptosis (5, 16, 48), counteracting the mechanisms leading to cell death.

At the moment, there are no available data from clinical results about the rHu-EPO action on the brain ischemia. The first clinical data with hypoxic patients indicates an improvement of the oxygenation of brain tissue (21, 36). Furthermore, there are reports of very good results in the treatment of schizophrenia (22). Current knowledge about rHu-EPO up to date is an excellent endorsement for its utilization as neuroprotector in the brain ischemia.

These results suggested that nasal drops of rHu-EPO_b reach the brain and exert a neuroprotective effect in a model of acute ischemia, reflected in a significant improvement of the assessed morphological and functional variables. Then, the intranasal via constitutes an alternative of access of the rHu-EPO_b to the brain, and the low content-sialic acid rHu-EPO has enough therapeutic efficacy to deserve future investigations.

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