

Recombinant human erythropoietin as a neuroprotective therapy in brain ischemia

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ABSTRACT

Current therapeutic strategies against ischemic cerebral vascular diseases are directed towards both, the recovery of cerebral blood flow and the protection of nerve cells. The search of neuroprotective agents has been focused to blocking some of the molecular events occurring in the nerve cells after brain ischemia. At present, none of them have met the efficacy and safety criteria in controlled clinical trials. Human recombinant erythropoietin has been recently proposed for neuroprotection. It has shown to have more than one mechanism of action, and appears to be a promising choice in the future, although its action on erythropoiesis could be inconvenient for either chronic treatment or secondary prevention. The use of an erythropoietin with low sialic acid content, without erythropoietic but with neuroprotective activity, could be preferable. Such molecule might be administered by a non systemic route, such as the intranasal route, to prevent its hepatic degradation. The Intranasal administration of a human erythropoietin with low sialic acid content has showed to be safe; the molecule reaches rapidly the brain; does not stimulate erythropoiesis after acute treatments and shows efficacy in some rodent models of brain ischemia. This proposal could become a therapeutic option for brain vascular diseases.

Keyword: cerebral ischemia, neuroprotection, erythropoietin, intranasal

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RESUMEN

La eritropoyetina humana recombinante como terapia para la neuroprotección en la isquemia cerebral. Las estrategias terapéuticas actuales para las enfermedades cerebrovasculares se encaminan al restablecimiento del flujo sanguíneo cerebral y a la protección de las células nerviosas. Se buscan agentes neuroprotectores para el bloqueo de alguno de los eventos moleculares que acontecen en las células nerviosas como consecuencia de la isquemia. Hasta el momento, ninguno de ellos ha satisfecho los criterios de seguridad y eficacia en ensayos clínicos controlados. La eritropoyetina recombinante humana constituye una propuesta reciente, que ha mostrado mecanismos de acción neuroprotectores en más de un nivel, y que parece ser una opción prometedora a corto plazo; aunque su acción eritropoyética puede representar un inconveniente para tratamientos crónicos o en la prevención secundaria. El uso de una eritropoyetina con bajo contenido en ácidos siálicos con actividad neuroprotectora pero no eritropoyética, puede ser una buena opción. Esta molécula debiera administrarse por una vía no sistémica como la vía intranasal, para prevenir su degradación hepática. La administración intranasal de una eritropoyetina recombinante humana con bajo contenido en ácidos siálicos, ha mostrado ser rápida y segura en su acceso al encéfalo, no estimula la eritropoyesis en tratamientos agudos y muestra eficacia terapéutica en varios modelos de isquemia cerebral en roedores. Esta propuesta puede convertirse en una opción terapéutica para la enfermedad cerebrovascular.

Palabras clave: isquemia cerebral, neuroprotección, eritropoyetina, intranasal

Introduction

Cerebral vascular diseases (CVD) are the third cause of death in industrialized countries and Cuba, affecting the 50% of the population above 60 years old [1-4]. The mortality is exponentially increased with age and doubles every five years. A total of 22000 annual cases are estimated in Cuba, a country where life expectancy should increase up to 80 years in the next future [3, 4]. CVD are often followed by a high social and individual cost as a consequence of invalidity and family affectation.

The most problematic among such diseases, is ischemic cerebral vascular disease, characterized by the reduction of cerebral blood flow (CBF) below a critical level. From this initial event several processes take place to induce the clinical symptoms of cerebral ischemia [5].

The most important therapeutic strategy in patients with ischemic stroke is directed to improve CBF, and to reduce or block subcellular and cellular metabolic consequences [6]. While most strategies, such as thrombolysis, are aimed at CBF recovery, neuroprotection intends to increment cell survival through the modification of the ischemic cascade [7, 8].

Prioritized attention has been granted to CVD prevention in Cuba for reduce mortality and morbidity indexes associated with these diseases. Risk factors and patients suffering from transient ischemic attacks (TIA) have received special consideration [9].

Some recent proposals point out to more than just a partial solution to the problem of ischemic cascade

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and claim for combined therapies and the use of molecules involved in endogenous neuroprotection. [10-13]. A good candidate could be human recombinant erythropoietin (rHu-EPO), which has been employed in the treatment of renal insufficiency associated anemia, and in cancer patients suffering anemia as a consequence of chemo and radiotherapy. The effect of rHu-EPO in the protection of brain cells from ischemic injury has been investigated during the last decade [14-16].

By analyzing recent results on cerebral ischemia, this article discusses the possibility of using rHu-EPO modified to display low sialic acid contents as a neuroprotector for cerebral ischemia, as well as the benefits of nasal administration of this drug.

Some aspects related to the physiopathology and therapy of cerebral ischemia

The interruption or reduction of CBF in a given vascular region affects the availability of oxygen and glucose required for the energetic metabolism, preventing the cells from preserving their resting membrane potential, and neurons from conserving their properties of electrically excitable membranes [5]. Depending upon the conditions of the ischemia, the molecular consequences can be more drastic: uncontrolled liberation of neurotransmitters, influx of calcium and sodium ions to cytoplasm, cellular edema, as well as the activation of lysosomal hydrolases, events leading to acute cell death at the focus of ischemia, characterized by tissue necrosis [17, 18]. This area of infarction is surrounded by a region of ischemic penumbra, which remains viable for some time, depending upon the efficacy of collateral circulation [4, 5]. This residual flow, however, is insufficient to maintain cell functionality.

In areas of ischemic penumbra, mostly after reperfusion, a mitochondrial failure takes place. This event aggravates the energy insufficiency, the increment of oxygen reactive forms leading to oxidative stress, micro vascular injury preventing the normalization of cerebral blood flow, and selective expression of genes related to either cell survival or death [3, 19]. Selective gene expression includes the expression of immediate-early response genes, encoding for transcription factors, stress related proteins such as heat shock proteins, neurotrophins and growth factors; as well as apoptosis associated effector proteins (Figure 1) [5, 20]. Apoptosis is considered to be the predominant mechanism of cell death during the chronic stage of cerebral ischemia [21]. The identification of the different stages of apoptosis after ischemia has stimulated numerous investigations for searching of therapeutic agents with anti-apoptotic properties [22].

The normalization of SBF leads to a complete recovery only when it occurs during the first three to six hours after the transient ischemic attack (TIA), usually due to spontaneous thrombolysis [10, 23]. A TIA is indicative of a vascular alteration, which often reflects that conditions have been created for the occurrence of an ischemic lesion in the future.

Therefore, TIA events are the primary subject of attention in the so called secondary prevention [21, 24]. As it has been demonstrated in animal models,

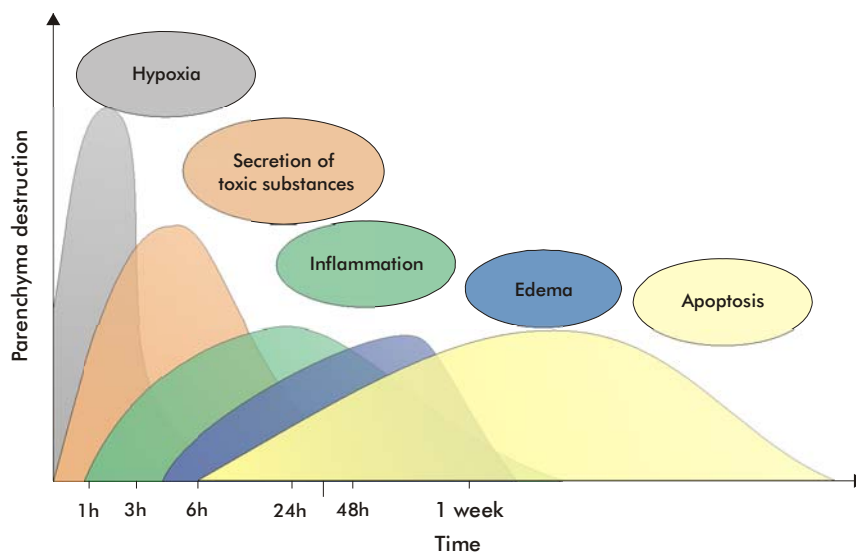


Figure 1. Diagram of the ischemic cascade

early reperfusion is capable of increasing the capacity of the cellular response against later ischemic insults. This phenomenon has been named tolerance to ischemia. Otherwise, the ischemic cascade would be unstoppable and even reinforced by reperfusion, which is itself responsible for other injuries [20, 25].

The time frame between the establishment of symptoms and clinical intervention in an ischemic patient leads to the concept of therapeutic window, a variable period in which the restoration of CBF and the inhibition of mediators of cellular ischemic injury would prevent the death of the cells at risk. However, beyond this time frame, any therapeutic intervention will be useless [10, 17]. In medical practice, the therapeutic window is a critical factor determining the efficacy of neuroprotection. The most currently accepted value is 12 hours. However, some experiences demonstrated that the viability of part of the brain tissue under ischemic penumbra can extend up to 48 to 72 hours [22, 25-27].

The rHu-EPO as neuroprotector

A drug sufficiently effective and with safe access to the central nervous system (CNS) has not been developed yet for neuroprotective treatment of neurological diseases in either chronic or acute stages. Besides, most of neuroprotective therapeutic agents, effective in ischemia biomodels, have failed to be clinically tolerated [17, 28]. A strategy to circumvent this problem can be the use of the same molecules expressed in the brain after different lesions, [11, 29] helping in the maintenance of homeostasis. One of these molecules is erythropoietin.

Erythropoietin is a kidney produced glycoprotein involved in proliferation, differentiation and maturation of erythrocytes and other hematopoietic cells, increasing the oxygen supply to tissues [30-32].

Human recombinant erythropoietin (rHu-EPO) is one of the ten best selling products of world biotechnology. The study of the neuroprotective effect of erythropoietin has been stimulated by the fact that

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this drug is normally expressed within the brain and is regulated by hypoxia inducible factor 1 (HIF-1) [11, 33], which is in turn activated by a wide variety of stress factors. Different *in vitro* models and several models of cerebral infarction have been used for this purpose (Table 1) [33-37].

It has been demonstrated that this glycoprotein and its receptor (r-EPO) are expressed in brain tissue and their expression increase during ischemia, suggesting that they are involved in an endogenous neuroprotective system in mammalian brain [38-40]. The neuroprotective efficacy of rHu-EPO has been tested in several animal *models of nervous system injury* in mouse, rat, gerbil, and rabbit, including focal and global cerebral ischemia [41-44], showing a reduction of neuronal death.

Although the neuroprotective mechanism of rHu-EPO is still being investigated, it is known that this effect is mediated by receptors located at the walls of the vascular endothelia and astrocytes [41, 45]. The neuroprotective mechanism of rHu-EPO seems to be multifactorial. rHu-EPO may indirectly mediate neuroprotection by restoring the blood supply to the injured tissue or acts directly over the neurons by activating multiple molecular signaling pathways.

The rHu-EPO molecule positively modulates the expression of antioxidant enzymes and reduces nitric oxide mediated formation of free radicals, by a mechanism involving JAK2 and the nuclear factor NF- κ B [30, 46]. Its antioxidant action is also sustained by restoring the cytosolic catalase and glutathione peroxidase activities in erythrocytes, which protects against the oxidative stress by reducing lipid peroxidation [47].

It has been demonstrated that rHu-EPO also displays neurotrophic activity, which implies an effect of larger latency than the inhibition of apoptosis [46, 48] and reduces neuronal excitotoxicity, involved in many forms of cerebral injury. rHu-EPO has been also identified as a potent mediator of tolerance to ischemia.

As other HIF-1 induced cytokines, this glycoprotein promotes angiogenesis as a response to hypoxia and neuronal injury [48-51] by stimulating the generation of microvessels through the interaction with its receptor in the blood vessels [41, 52].

Its antiapoptotic action is given through the r-EPO mediated activation of JAK2, which in turns leads to the activation of NF- κ B and to the overexpression of the apoptosis inhibiting genes XIAP and c-IAP2 [48, 50, 51, 53]. rHu-EPO protects neurons from ischemic injury by overexpression of Bcl-x in the hippocampus of gerbils [47]. rHu-EPO inhibits the expression of Bax in PC12 cells and increases the expression of Bcl-XL, a member of the group of Bcl-2 antiapoptotic proteins [47, 50, 54]. At the same time it stimulates cell survival by inhibiting the MAPK and PI3K/Akt complex which promotes apoptosis [47]. These data suggest that rHu-EPO acts by controlling the balance of the expression of either proapoptotic or antiapoptotic molecules [32].

The neuroprotective effect attributed to rHu-EPO can be also derived from its antiinflammatory effect [53]. The administration of rHu-EPO to rats with focal ischemia reduces notably the migration of inflammatory cells to the ischemic tissue, attenuating

Table 1. Reports of applications of rHu-EPO in cytoprotection

Model	Models <i>in vivo</i> and <i>in vitro</i>	Route/dose	Reference
<i>In vitro</i> Primary culture of astrocytes	Rat	5-20 U/mL	[55]
Focal and global cerebral ischemia	Mouse	IP 25-100	[35]
Focal Ischemia MCA	Rat	IN 4.8, 12, 24 U/kg	[56]
Focal Ischemia MCA	Rat	IP 100, 1000, 5000 U/kg	[43]
Blood test	Non human primates	IV 500, 2000, 4000 U/kg SC 400, 1000, 2400, 5000, 20000 U/kg	[38]
Retinal Ischemia	Mouse and rat	IP 5000 U	[57]
Neonatal brain hypoxia	Mouse	IP 1000-5000U	[58]
Spinal cord injury	Rat	IP 100-5000 U	[59]
Subarachnoid hemorrhage	Rabbit	IP 1000 U	[60]
Cerebral Inflammation	Rat	IP 5000 U	[61]
Injury in peripheral nerves	Rat	SC 1000-5000 U	[62]
<i>In vitro</i> Anoxia mediated injury	Hippocampus neurons	10 ng/mL	[51]
<i>In vitro</i> NO mediated injury	Hippocampus neurons	10 ng/mL	[63]
Glutamate induced toxicity	Neurons from cortex Hippocampus and cerebellum	50 ng/mL	[64]
Disruption of blood brain barrier	Brain endothelial cells	10 U/mL	[65]

the production of proinflammatory cytokines and limiting the size of the lesion [5, 12, 42, 47]. The expression of erythropoietin is markedly reduced by proinflammatory cytokines and by oxygen reactive species [49, 66, 67]. This could contribute to the action of these mediators in the pathogenesis of ischemia and explain why exogenous administration of rHu-EPO can be especially beneficial [19].

It has been also proposed that rHu-EPO could exert its anti-inflammatory effect by inhibiting molecular signaling in the injured neurons [19]. The anti-inflammatory protecting cell activity of rHu-EPO has been demonstrated in *in vitro* experiments with glial and neuronal cell co-cultures, where neuronal death is associated to the release of TNF by glial cells. [51, 68, 69].

Considering the properties of rHu-EPO, Ehrenreich and co-workers carried out the first clinical trial with this drug in acute cerebral ischemia, with a therapeutic window within the first eight hours. These authors reported a significant reduction of the infarcted area in treated patients, associated with a noteworthy neurological and clinical improvement one month after ischemia [70]. The increment of rHu-EPO levels in serum and cerebrospinal fluid (CSF) suggests that rHu-EPO can cross the damaged blood brain barrier and protect from cerebral ischemic injury, as postulated by other investigators [71]. No severe side effects have

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been reported after rHu-EPO treatment in ischemia or other neurodegenerative diseases [72]. The results of Ehrenreich and co-workers reveal that rHu-EPO improves the oxygenation of the brain tissue and reduces the volume of the infarcted area. This is an excellent result to support its use as a neuroprotective agent in cases of cerebral ischemia [73].

The use of rHu-EPO in animal models of subarachnoid hemorrhage [74], intracranial hemorrhage [75], craniocerebral trauma [76, 77] and spinal cord injuries [78, 79] has been reported. It has been also reported that this drug reduces neuronal functional damage in models of encephalitis and multiple sclerosis [80, 81], improves diabetic neuropathy [82] and retinal ischemia [56]. Its cardioprotective action in dogs has been also recently described [77]. Moreover, rHu-EPO has been used to treat cases of schizophrenia [83], perinatal hypoxia [84-86] and in collateral treatments for improvement the quality of life in cancer patients [13, 87]. Due to its stimulatory effects on neuronal plasticity, the use of rHu-EPO could have effects on long term recovery of patients with disabilities, ECV or neurodegenerative disorders [88].

Intranasal administration of rHu-EPO with low sialic acid contents

Most of the neuroprotective drugs, effective in biomodels of cerebral ischemia, are not clinically tolerated. In addition to this fact, no drug with an appropriate level of efficacy, specificity and with safe access to the CNS has been developed so far to justify its use as neuroprotector during the acute phase of cerebral ischemia [18, 89-93].

Several authors agree that the administration of protective molecules such as rHu-EPO is a potential therapeutic alternative to treat acute ischemic injuries [10, 11]. It has been demonstrated that, additionally, this molecule exhibits neuroprotective effect *in vitro* and *in vivo*, indicating its antiexcitotoxic, antiapoptotic, angiogenic and neurogenic activities.

A pilot study in humans initiated in 2002, demonstrated the beneficial effects of intravenous application of rHu-EPO in patients with acute cerebral stroke [70]. In current studies with cerebral ischemia biomodels to evaluate the neuroprotective effect of rHu-EPO, this molecule has been administered by intracerebroventricular, intraperitoneal and intravenous routes [55, 94].

The administration of rHu-EPO by systemic route involves the potential risk of stimulation of erythropoiesis, with a consequent increment of the number of cells and blood viscosity, an undesirable effect in patients with cerebral stroke. Thick blood leads to worse cerebral hemodynamia in the affected region and introduce an additional complication [95]. Therefore, the search for rHu-EPO derivatives lacking erythropoietic activity, but retaining its neuroprotective properties is the proposal of several research groups. Asialylated and carbamylated derivatives of this molecule, lacking hematopoietic activity but retaining its neuroprotective properties after acute cerebral injuries, have been reported [96].

The results obtained with this with low sialic acid content rHu-EPOb (very similar to EPO synthesized inside the brain under ischemic conditions), have been

very similar to those reported for the original rHu-EPO or its asialylated and carbamylated derivatives [97, 98] in ischemia models in mongolian gerbil and rat. The attenuation of the learning disabilities induced by ischemia could be explained by the effect of rHu-EPOb of improving synaptic transmission during ischemia. This has been demonstrated by using *in vitro* models [99]. It has been also postulated that rHu-EPO stimulates neuronal functionality by the activation of Ca^{++} and the release of neurotransmitters [35].

Some researchers have proposed that EPO can facilitate certain mechanisms of restoration and neuroplasticity in ischemic animals [88]. If true, this would open new therapeutic possibilities to stimulate tissue regeneration and recovery of brain areas by using a safe and noninvasive method, such as the intranasal route. These authors propose this route as a novel solution to bring rHu-EPOb to the brain. As has been described before, the olfactory region has unique physiological and anatomic attributes which define extracellular and intracellular routes to CNS, evading the HEB [100, 101]. Several substances penetrate into the CNS by inhalation and the pass of tropic and neuroprotective molecules through this route has been demonstrated [102].

The olfactory region display unique anatomical and physiological attributes which defines extracellular e intracellular routes toward the CNS evading the blood brain barrier [100, 103]. In the upper part of the nasal cavity are the nervous terminals responsible of conducting odor related information. The bundle of nerve cords which constitutes the olfactory tract of CNS pass through the holes of the cribiform plate and extends from the base of the brain to different subcortical regions (Figure 2). Consequently, small amounts of rHu-EPOb administered by this route quickly go into the brain and diffuse through the

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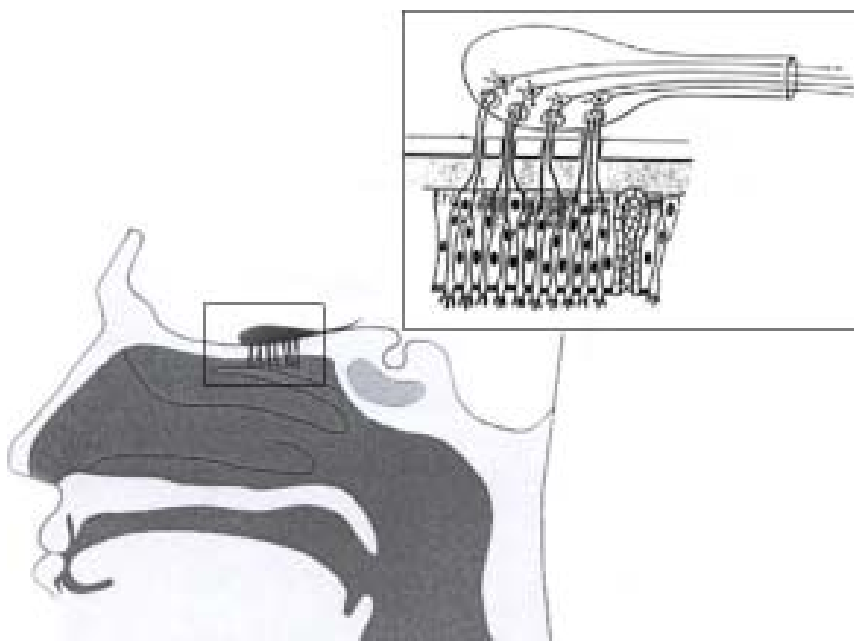


Figure 2. Diagram of the nasal cavity. Structure of olfactory epithelium with the communication proposed for the circulation of substances by intranasal route to the CNS.

interstitial liquid. Therefore, the intranasal route can be used for prophylaxis and therapy of the CNS [104].

When rHu-EPOb is administered by intranasal route it does not pass through the liver, where it would be rapidly degraded by proteases before reaching the CNS because its sialic acid content is low (values below 10 molecules of sialic acid/mol of protein).

The transit of rHu-EPO to the CNS after intranasal administration and its effect were confirmed in this study [55, 105]. The detection of the molecule either in the olfactory bulbs or in the cerebellum demonstrated its contact with the CRF. This was further confirmed by the significant increase of rHu-EPO in CLF of *Macaca fascicularis* five minutes after its administration by intranasal route. [106]. Therefore, the intranasal route allows the rapid and effective access of rHu-EPOb to different regions of the brain.

When rHu-EPOb was administered by intranasal route in the Mongolian gerbil, the edema of the hemisphere corresponding to the permanent occlusion of the carotid was not observed. In the same model, a significant reduction of mortality, as well as the clinical symptoms of stroke at 24 h was documented. A survival of 66% in females and 73% in males was achieved with the administration of rHu-EPOb, contrasting with 47 and 57%, respectively in placebo treated animals [107].

The incidence of histopathological events in brain tissue seven days after unilateral occlusion of the carotid was significantly lower in the animal treated with rHu-EPOb by intranasal route during the four days following the occlusion. In the model of bilateral occlusion of the carotids during 6, 8 and 10 minutes, the treatment with rHu-EPOb reduced the retarded neuronal death in the CA1 sector of hippocampus [108]. In animals treated with rHu-EPOb a preservation of the habituation behavior in the spontaneous exploratory activity was observed in both models, which demonstrated the conservation of the structural integrity of the brain regions related to learning and short and long-term memory [105].

In the model of middle cerebral artery occlusion for two hours in rats, animals treated with rHu-EPOb by intranasal route showed the smaller volumes of ischemic tissue and a better clinical condition at 48 hours (unpublished results). The results of this study in rodents show therapeutic efficacy in either the acute or chronic phases of ischemia, as well as in reperfusion ischemia models, suggesting neuroprotective effects

in brain structure and function. Those are indirect evidences of the access of rHu-EPOb administered by intranasal route in the amounts equivalent to the therapeutic dose recommended for ischemia.

Safety is one of the major requirements nowadays for all biotechnology products. rHu-EPO has proved to be very safe, with a large experience of administration to patients for more than 20 years [109]. In a recent experiment rHu-EPOb administered by intranasal route did not stimulate hematopoiesis in B6D2F1 mice, while blood parameters (hemoglobin and hematocrit) of *M. fascicularis* were not modified 14 days after the administration of intranasal rHu-EPOb (unpublished data). Those results suggest additionally advantages for the intranasal route, which could be safer and faster than the intravenous route [110].

A general survey carried out by a group of investigators about the use of the intranasal route to administer drugs for treatment of diseases affecting the CNS, indicated that in the last decade roughly the 11% of the new drugs generated by the industry are administered by this route. Patients prefer intranasal administration due to its efficacy and safety of these formulations. This report explain that this type of drugs require a rigorous dose study to demonstrate safety, since it is a very rapid route to the CNS. The characteristics of the excipients, vehicles, preservatives and packaging for this type of formulations also required further studies, because they can improve the stability of the formulation and maintain the efficacy of the drug previously demonstrated in preclinical studies [111].

The results in animal models with the application of rHu-EPOb by intranasal route in equivalent amounts to the therapeutic dose in preclinical studies show the transit of the molecule to the CNS. Its therapeutic effect on cell death and cerebral function, as well as the safety in acute phase of cerebral stroke, in either animal models of ischemia and ischemia reperfusion, demonstrate the neuroprotective effect of this drug [107].

Conclusions

The clinical neuroprotective strategy is currently debatable. However, the application of small molecules as rHu-EPOb has strong theoretical support and showed very promising preclinical results in cerebrovascular and neurodegenerative disorders. Thus, the rHu-EPOb is an excellent cytoprotective candidate in humans.

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