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Neuro-EPO by Nasal Route as a Neuroprotective Therapy in Brain Ischemia

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1. Introduction

Cerebral vascular diseases (CVD) are the third leading cause of death in industrialized countries and in Cuba affect the 50% of the population above 60 years old [1-2]. The mortality is exponentially increased with age and doubles every five years. A total of 22,000 annual cases are estimated in Cuba, a country where life expectancy should increase to 80 years in the near future [1].

CVD are often followed by a high social and individual cost as a consequence of disability 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 [3].

Prioritized attention has been granted to CVD prevention in Cuba for reduction of mortality and morbidity indexes associated with these diseases. Risk factors and patients suffering from transient ischemic attacks (TIA) have received special consideration [4].

While most strategies, such as thrombolysis, are aimed at CBF recovery, neuroprotection intends to increment cell survival through the modification of the ischemic cascade [4, 5]. The most important therapeutic strategy in patients with ischemic stroke is directed to improve CBF and to reduce or block sub cellular and cellular metabolic consequences [6].

Some proposals point to more than just a partial solution to the problem of ischemic cascade and call for combined therapies and the use of molecules involved in endogenous neuroprotection. [5-7]. 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 [8-14].

Given the systematic failures in clinical trials using proven neuroprotection ability molecules in animal models [16], it seems logical to reevaluate the therapeutic strategy for therapeutic treatment in the acute phase of stroke.

Searching within selected biological mechanisms for endogenous neuroprotection appears to be the most promising. Today this is possible by the advances science and technology have achieved through biotechnology, which allows us to obtain proteins similar to Erythropoietin to autologously use our brain to maintain homeostasis at a stroke.

Helping this process may be the way to a successful therapeutic strategy for neuroprotection against stroke and general neurological diseases and inherited degenerative diseases too.

EPO had been reported as a neuroprotective molecule in animal models with perspectives for its clinical use. However, systemic administration has shown potential side effects. For that reason, recently researchers have demonstrated neuroprotective effects in different animal models of stroke using EPO as a neuroprotector or other different types of EPO without erythropoiesis-stimulating activity. These new molecules retain their ability to protect neural tissue against injury and they include Asialoerythropoietin (asialoEPO) carbamylated EPO (CEPO), and rHu-EPO with low sialic acid content (Neuro-EPO). [15, 16]

On the other hand, some of these derivatives of EPO or rHu-EPO have been shown to have no neuroprotective effect during *in vivo* studies. [17, 18]

Indeed, nasal administration presents advantages treating the brain compared with intravenous and intraventricular routes, especially for treatments utilizing Neuro-EPO. [16]

In this chapter, the authors focus on the neuroprotective effect from treatment of nasal Neuro-EPO in the acute stroke animals' models and the security and efficacy of this treatment from the perspective of the stroke therapies.

2. Neuroprotection and endogenous erythropoietin

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 [19]. A strategy to circumvent this problem can be the use of the same molecules expressed in the brain after different lesions, [9, 20,] helping in the maintenance of homeostasis.

The use of endogenous biomolecules with therapeutic activity is a recent proposal in neuroscience research [20]. An example of this type of molecule is erythropoietin, a glycoprotein produced in the kidney and involved in the proliferation, differentiation, and maturation of erythrocyte progenitors, increasing oxygen supply to the tissues [21]. Indeed, EPO is one of the molecules more conserved evolutionarily [22]. This can be interpreted, as the EPO molecule is affective and safe in their biological activity.

The observation that EPO and its receptor (EPO-R) are expressed in the brain has stimulated the development of studies related to the neuroprotective effect of this molecule in different models of stroke [23, 24]. EPO expression increases during cerebral ischemia, suggesting its role in the endogenous neuroprotector system of the mammalian brain [25].

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 this drug is normally expressed within the brain and is regulated by hypoxia inducible factor 1 (HIF-1) (**Figure 1**), which is in turn activated by a wide variety of stress factors.

Indeed, in the last 5 years several reports of applications of rHu-EPO in cytoprotection were done (**Table 1**)

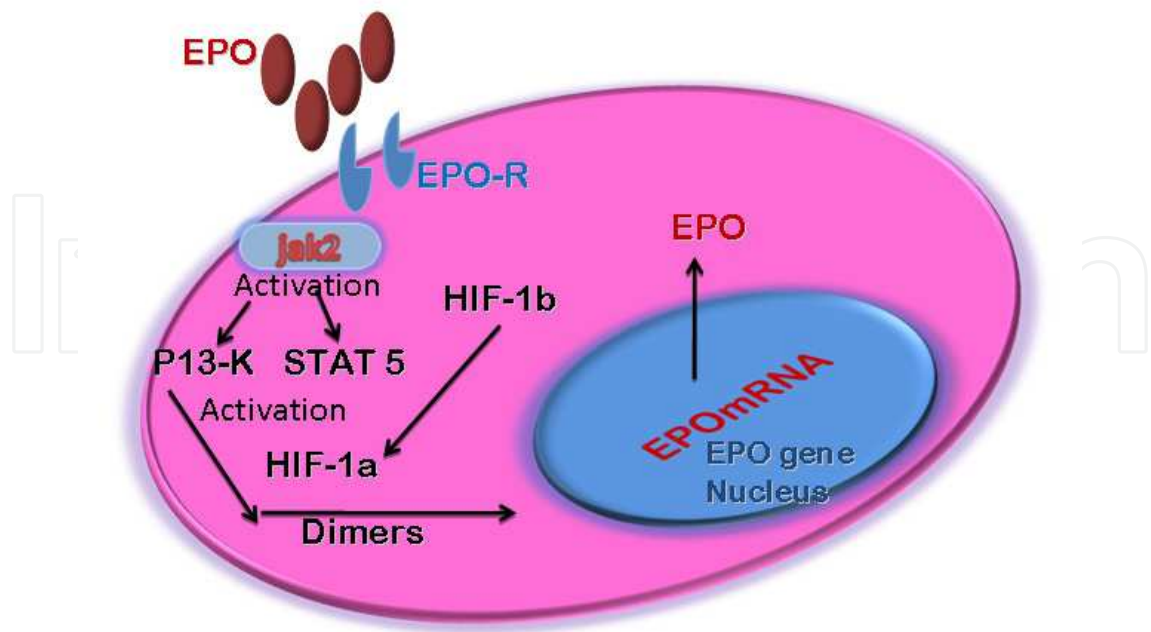


Fig. 1. Simplified diagram showing the main actors associated with neuronal protective response mediated by HIF-1. Where participating EPO, EPOR, HIF1a dimerizes with HIF-1b is the signal for transcription in the nucleus and results in EPO mRNA and finally to the synthesis of erythropoietin (EPO).

Assay	<i>in vivo</i> or <i>in vitro</i> Models	Route/dose	Reference
In vitro Primary Culture of Astrocytes	Rat	5-20 U/mL	Diaz Z, (2005)
Focal and global cerebral ischemia	Mouse	IP 25-100U	Marti HH (2004)
Focal Ischemia MCA	Rat	IN 4.8, 12, 24 U/kg	Yu YP, et al (2005)
Focal Ischemia MCA	Rat	IP 100, 1000, 5000 U/kg	Belayev L et al (2005)
Retinal Ischemia	Mouse and rat	IP 5000 U	Grimm C, et al 2006
Neonatal brain hypoxia	Rat	IP 1000 U/kg	Yamada M, et al (2011)
Spinal cord injury	Mouse and rat	IP 5000 U	Mofidi A et al (2011)
Focal cerebral ischemia	Gerbils	IN 249.4 U	Rodríguez Y et al (2010)

Table 1. Some reports of applications of rHu-EPO in cytoprotection.
U=Unit; IP= Intraperitoneal Injection; MCA=Middle Cerebral Artery; IN= Intra Nasal

3. Brain protection by erythropoietin

It has been demonstrated that EPO and its receptor are expressed in brain tissue and their expression increases during ischemia, suggesting that they are involved in an endogenous neuroprotective system in mammalian brain [25].

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 (**Table 1**), 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 [26]. The neuroprotective mechanism of rHu-EPO seems to be multifactor. 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 [27] and the nuclear factor NFkB [28]. 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 [29] and also EPO plays an important role in protecting against brain ischemia/reperfusion through inhibiting lipid peroxidation and decreasing blood brain barrier (BBB) disruption [30].

It has been demonstrated that rHu-EPO also displays neurotrophic activity [31], which implies an effect of larger latency than the inhibition of apoptosis [32] and reduces neuronal exitotoxicity, involved in many forms of cerebral injury. rHu-EPO has been also identified as a potent mediator of tolerance to ischemia [33].

Like other HIF-1 induced cytokines, this glycoprotein promotes angiogenesis as a response to hypoxia and neuronal injury [34] by stimulating the generation of microvessels through the interaction with its receptor in the blood vessels [35, 36].

Its antiapoptotic action is given through the EPOR mediated activation of JAK2, which in turns leads to the activation of NF-kB and to the overexpression of the apoptosis inhibiting genes XIAP and c-IAP2 [34]. rHu-EPO protects neurons from ischemic injury by overexpression of Bcl-x in the hippocampus of gerbils [29].

At the same time it stimulates cell survival by inhibiting the MAPK and PI3K/Akt complex which promotes apoptosis [29]. These data suggest that rHu-EPO acts by controlling the balance of the expression of either pro apoptotic or antiapoptotic molecules [37].

The neuroprotective effect attributed to rHu-EPO can be also derived from its anti-inflammatory effect signaling in neurons, glial and cerebrovascular endothelial cells and stimulates angiogenesis and neurogenesis. These mechanisms underlie its potent tissue protective effects in experimental models of stroke [38.]

A summary of the different biological activities on cells of the nervous system to explain the cytoprotective capacity of EPO is shown in **Table 2**

Activity/ Cells	Immature Neurons	Neurons	Astrocytes	Microglia	Ependimal	Oligodendrocyte
Neurogenesis	YES	?	?	?	?	?
Anti oxidative	?	YES	YES	YES	?	?
Tropic	YES	YES	?	?	?	?
Anti apoptotic	YES	YES	YES	?	YES	YES
Anti Inflammatory	YES	?	YES	YES	?	YES
Anti-Glutamate	?	YES	?	?	?	?

Table 2. Differents neuroprotective profile for rH-EPO on cells of the nervous system. (?)= Not yet demonstrated activity; (YES) = Demonstrated Activity.

4. Delivery of drugs to CNS and BBB

The deficiency in this first decade of the century to have efficient and safe drugs to counter neurodegenerative diseases and cerebrovascular accidents are, without a doubt a pressing need for the development of pharmacology and neuroscience in general.

Some molecules have been developed with proven ability in different biomodels of neuroprotective stroke; however, none of them managed to overcome the barrier of Phase III clinical trials [35]. In this respect, several negative factors are limiting the success. At this point, we refer to the strong obstacles that represent the BBB.

We focus our commentary on the possibilities for EPO to be one of the safest and most effective drugs that has developed global biotechnology. EPO has been used in millions of peoples for over 20 years with very few adverse effects reported.

The application of EPO and its non-erythropoetic variants like Neuro-EPO through nasal delivery to the central nervous system target an area of great interest right now [39-41]. In *cynomolgus monkeys*, intra nasal route is relatively well known anatomically, physiologically as well as the transport mechanism of low molecular weight molecules and proteins to the brain [42].

In a simplified form the nasal cavity can be divided into three parts. They are: 1) Nasal vestibule, 2) Olfactory region and 3) Respiratory region (**Figure 2**).

Of these three regions, drugs released into the nostril in contact with the nasal epithelium, which has several types of cells through the *lamina propria*, and a thin layer off loose connective tissue containing blood. Vessels, lymphatic vessels, axons and glands are involved.

For a drug to travel from the olfactory region in the nasal cavity to the CSF or brain parenchyma has to go through the olfactory epithelium, depending on the path followed, also the arachnoid membrane. In principle you can consider three paths through the olfactory epithelium:

- Via endocytic pathway, primarily through sustentaculares cells, where endocytosis processes occurring receptor-mediated endocytosis of liquid phase or by passive diffusion. This path corresponds especially to small lipophilic molecules or large molecules.
- Via extra cellular through the tight junctions or open cracks in the membrane between sustentacular cells and olfactory neurons. This pathway is particularly suited to small hydrophilic molecules.
- Via axonal: The drug can be transported through the olfactory neurons (where endocytic mechanisms enters or pinocytotic) to the olfactory bulb by axonal transport intracellular

Possible mechanisms by which macromolecules applied to the nasal cavity transport to the brain are under investigation.

These mechanisms are involved in several possible anatomical structures such as olfactory and trigeminal nerves, vasculature, cerebrospinal fluid (CSF), and the lymphatic system. Possible routes for the molecules of the nasal cavity to the brain may involve several mechanisms as bulk flow and diffusion within perineural channels, perivascular spaces, or lymphatic channels directly connected to brain tissue or CSF [40].

It is known that the trigeminal nerve innervates the nasal cavity and provides a direct connection to CNS, a description of these pathways and Genc (2011) in an excellent Expert Opinion [41] has recently reported processes.

We note that there are now enough experimental evidences and clinical practice show that the olfactory BBB level can be considered highly permeable to many molecules, including proteins of medium molecular weight such as erythropoietin.

This possibility is challenges to use this route to deliver drugs to the CNS that do not allow access the BBB. What are the advantages and disadvantages offered by the nasal route to deliver molecules to the CNS will discuss below.

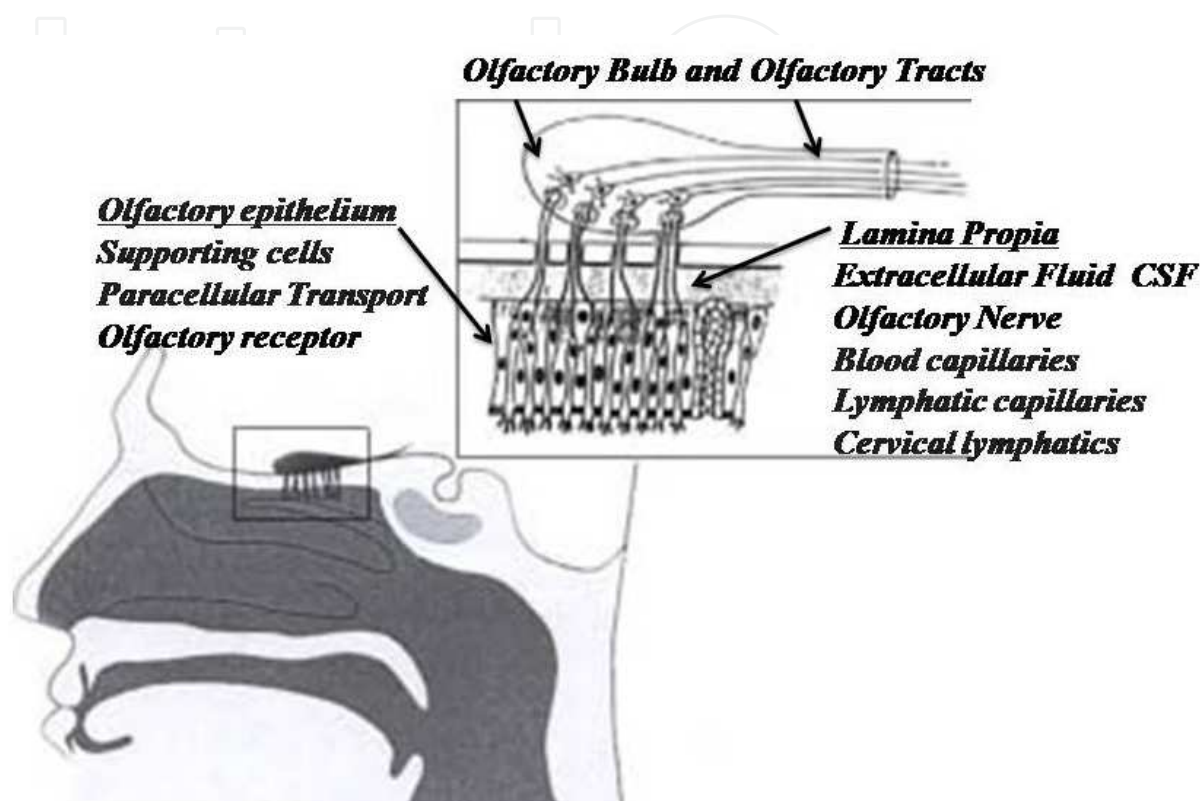


Fig. 2. Diagram of the nasal cavity. Structure of olfactory epithelium with the communication proposed for the circulation of substances by intranasal route to the CNS and possible routes to come from the Neuro-EPO to the brain when applied nasally.

5. Advantages and disadvantages of the nasal route for drug delivery to CNS

In this section, we would point out those aspects that we believe according with our experience should always be taken into account for proper projection of work with a formulation of nasal application.

It is truly remarkable to consider the anatomical differences between rodents and humans with respect to the nasal system. These become more important since most drug studies are conducted in rodents.

We must also take into account the experimental general procedure in trials where it has demonstrated the efficacy and safety of a nasal preparation. The possible influence of anesthesia methods employed (mostly placed in the supine position) and experimental factors to try to find optimal conditions, far removed from real life human beings.

Among the advantages of nasally is low cost, as opposed to systemically, this product does not require needles or syringes, which represents a negligible cost for a drug. For the same reason your application is easier and less traumatic and allows smooth, self-application.

Perhaps the biggest advantage is its rapid nasal application release to the CNS, concomitantly with a considerable less amount of drug applied and therefore a lower risk of side effects from excessive drug.

Studies carried out in nonhuman primates using rH-EPO systemically and Neuro-EPO nasally, showed that Neuro-EPO was more readily to the lumbar CSF and for more longer time and with a considerably lower dose of Neuro-EPO.

This study shows the distribution levels of Neuro-EPO content in CSF obtained by lumbar puncture at different time intervals after application intranasal (Neuro-EPO 1000 IU) or intravenously (rHu-EPO, 5000 IU).

There was a peak of 430 mIU / mL for rHu-EPO at 10 min after application, representing approximately 0.01% the total injected dose, whereas the total volume of CSF in this species is 15 mL approx. This distribution of rHuEPO from blood to the CSF is consistent with those reported by other authors in non-human primates [42] and human [43]. This finding is characteristic of those components of protein origin not permeable to the BBB, but in small amounts or traces is present in CSF, as albumin [44]. It should be noted that these small amounts physiological concentrations achieved much higher baseline levels of rHu-EPO in the CSF action ensuring already reported neuroprotective.

An analysis of the results in the model of *M. fascicular* is shows that after the first hour the behavior of both approaches (intranasally and intravenous) is very similar, suggesting rapid elimination of the molecule. This makes it possible to avoid side effects caused by an excess of circulating Neuro-EPO long time in the CNS [45].

In a study by Brines et al (2000) [46] demonstrated an application of 5000 IU / kg via intraperitoneal mouse EPO levels in CSF was highest 3.5 hours from application.

Among the disadvantages we can point to the nasal route is that we can not determine with absolute precision the dose that each nasal application is delivered to the CNS. Another aspect is the exclusion of people with allergies, deviated nasal septum, the mucus and clearings may reduce the passage of product to the CNS.

Among the side effects of intranasal application of erythropoietin, is the fact that most of the application moves into the bloodstream and therefore has the capacity to induce erythropoiesis, at least, increases the number of red blood cells. Any increase in these cells will increase the viscosity of blood, which is undesirable for patients in the acute stroke. Therefore, the development of non-erythropoietic variants from the ability to maintain protective capacity shown for erythropoietin is an emerging field within neuroscience comment below as variants not erythropoietic of erythropoietin and its neuroprotective action.

6. Non-erythropoietic variants of erythropoietin and their neuroprotective action

Recently, several different types of new EPOs without erythropoiesis-stimulating activity have been developed. These new molecules retain their ability to protect neural tissue against injury; they include Asialoerythropoietin (asialoEPO) [47,48], carbamylated EPO (CEPO)[49-51], and rHu-EPO with low sialic acid content (Neuro-EPO)[16, 39,45].

6.1 AsialoEPO

The need for nonerythropoietic rHu-EPO derivatives that still retain neuroprotective action has led to the discovery of asialoEPO, generated by total enzymatic desialylation of rHu-

EPO. AsialoEPO has the same EPO-R affinity and neuroprotective properties as EPO, but an extremely short plasma half-life [50].

The ability to dissociate the tissue-protective actions of EPO from its erythropoietic actions may eventually be applied in the clinic to promote neurological regeneration without increasing red blood cell formation [52]. Erbayraktar and coworkers [53] have shown protective activities of the nonerythropoietic asialoEPO in models of cerebral ischemia, spinal cord compression, and sciatic nerve crush. Additionally, asialoEPO protects against neonatal hypoxia-ischemia as potently as EPO in hypoxic-ischemic brain injury in 7-day-old rats [52] and also in short-term changes in infarct volume, penumbra apoptosis and behaviors following middle cerebral artery occlusion in rats [48].

6.2 CEPO

Another modified EPO molecule that solely manifests tissue-protective action without erythropoietic activity may have a more targeted effect. As an example, transformation of lysine to homocitrulline by carbamylation gives rise to carbamylated EPO (CEPO) [50].

CEPO, similar to asialoEPO, lacks erythropoietic effect, but still shows neuroprotective effects in animal models of stroke, diabetic neuropathy, and experimental autoimmune encephalomyelitis to an extent comparable to that of EPO [54]. It is important to note that CEPO has a minimal affinity for EPO-R and that its effects are mediated via a different EPO receptor. It is thought that this receptor consists of the EPO-R monomer together with a dimer of the common β chain (CD131). Recently, an investigation carried out in a rat model of focal cerebral ischemia showed that post ischemic intravenous treatment with CEPO led to improved functional recovery [52]. In 2007, a study by Mahmood et al. [55] assessed the effect of intraperitoneally infused rHu-EPO and CEPO in a traumatic brain injury rat model, and they concluded that rHu-EPO and CEPO are equally effective in enhancing spatial learning and promoting neural plasticity, but hematocrit was significantly increased only with rHu-EPO. Similarly, a recent study by Wang et al [52], demonstrated equivalent effects of rHu-EPO and CEPO in the reduction of neurological impairment in rats subjected to embolic middle cerebral artery occlusion (MCAO). As expected, rHu-EPO, but not CEPO, produced a transient increase in hematocrit levels.

Another advantage of EPO over CEPO was demonstrated in rodents. Short-term treatment with EPO at doses optimal for neuroprotection caused significant alterations in platelet function and composition with in vivo haemostatic consequences, while CEPO treatment had no effect on these parameters [56].

6.3 Neuro-EPO

During the biotechnological production of rHu-EPO, various isoforms with different contents of sialic acid are obtained. When the sialic acid content is 4–7 mol/mol protein, it is considered a low sialic acid-containing EPO, modified to display low sialic acid content (Neuro-EPO) is very similar to the one that occurs in the mammalian brain. Low sialic acid-containing EPOs are rapidly degraded by the liver. Thus, this molecule could be administered by a non-systemic route, such as the intranasal route, to prevent its hepatic degradation. The intranasal administration of Neuro-EPO has been shown to be safe; the molecule reaches the brain rapidly, does not stimulate erythropoiesis after acute treatments, and shows efficacy in some rodent models of brain ischemia and in nonhuman primates. This proposal could be considered a therapeutic option for stroke and others neurodegenerative illness. (See review García Rodríguez and Sosa Testé, 2009) [3]

There have been two strategies followed so far by different research groups. Some have postulated the safer use of erythropoietin as a neuroprotectant in cerebral ischemia. Their works have been based on existing knowledge of primary and secondary structure of the protein erythropoietin (**Figure 3**) and knowing there are differentially placed amino acid sequence with erythropoietic capacity and cytoprotective property in this cytokine. From this knowledge has been postulated by different chemical modifications to inactivate the capacity of erythropoietic region, leaving only the resulting molecules with neuroprotective capacity. This variant has the potential disadvantage of the molecule obtained in this way; it is not similar to that naturally produced by the CNS, which may bring difficulties in recognition or neurotoxicity. These topics have not yet been fully studied in works of neurotoxicology.

The second variant, through which we get the Neuro-EPO, is characterized not by changes to chemical molecules produced by biotechnology, but selects those molecular populations with a low profile isoforms sialic acid content that make the Neuro-EPO easily degraded as it passes into the bloodstream. In addition, recent study has demonstrated in neurotoxicological study its safety and low toxicity when applied nasally [57]. Therefore, studies of effectiveness and efficiency are top priority. A synthetic form below and discussion of the work with the Neuro-nasally EPO in models of cerebral ischemia.

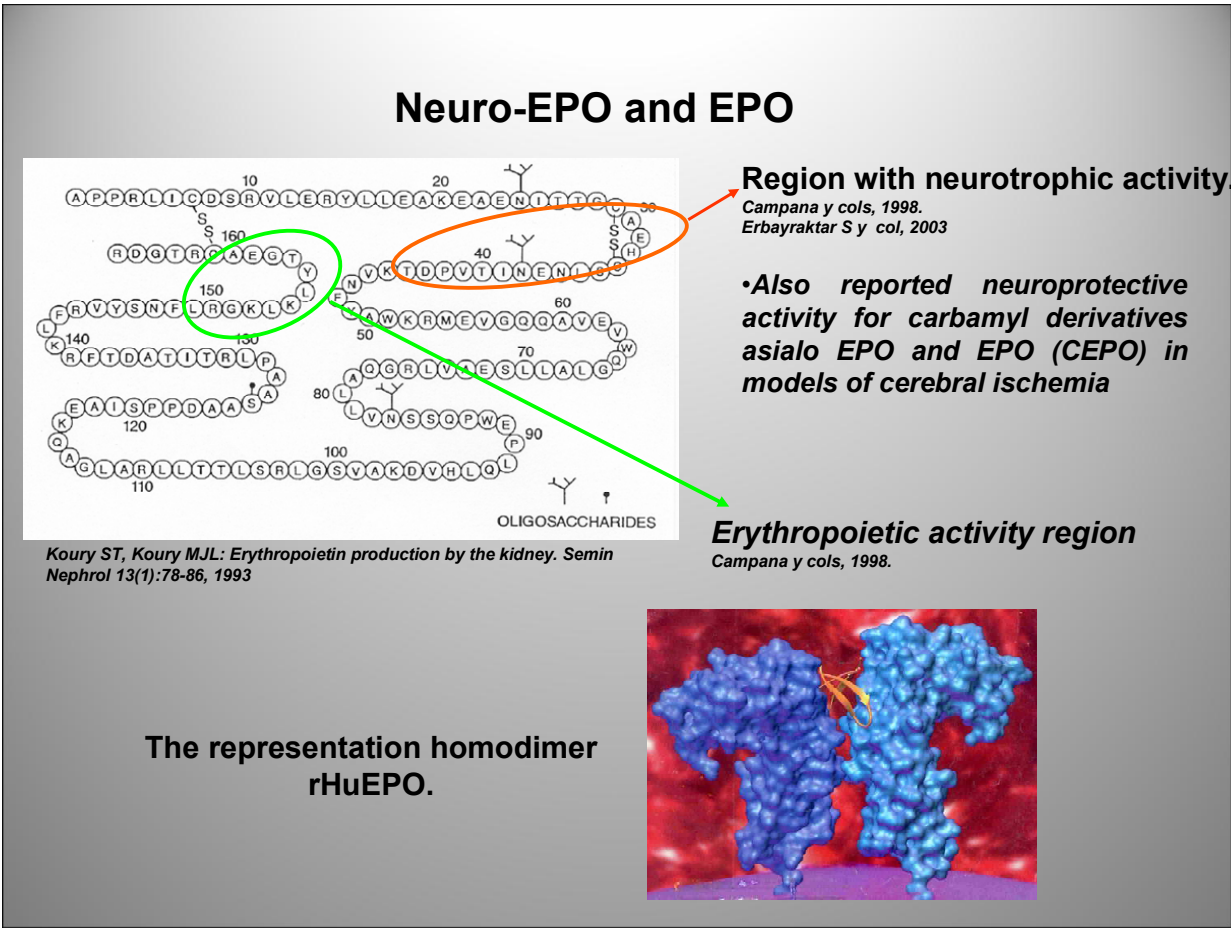


Fig. 3. *Top* Show the representation of EPO protein AA sequences and Regions with Neurotrophic and Erythropoietic activity. *Down*, Show the representation homodimer rHuEPO.

7. Security and efficacy of the nasal Neuro-EPO intra nasally preclinical studies of brain ischemia

Evaluation of whether the formulation produced allowed the arrival of the Neuro-EPO nasally applied to the CNS was one of the first tasks accomplished. The animal model was the Gerbil Mongolia. **(Figure 4)**

Activity detected molecule Neuro-EPO labeled I^{125} was detected in the olfactory bulb and cerebellum. In the CSF concentrations was in the range of physiological and therefore support an adequate therapeutic concentration.

The nasal opening is generally favored for rodents. The nasal olfactory mucosa covers approximately 50% of total nasal epithelium in the rat [58], while in human's covers only 5% [59]. Another important difference is the volume of cerebrospinal fluid (CSF) in the rat is 3.5 ml and in humans is 160 ml, therefore, the replacement time of the CSF in the rat is 1.5 hours while in humans is 5 hours, making it difficult to infer the uptake of molecules of one species to another. However studies conducted on the passage of molecules by the intranasal route in species such as nonhuman primate *Macaca fascicularis* species are recommended for pharmacokinetic studies of the nasal [45, 60].



Fig. 4. Neuroprotective effects of EPO Neuro-nasal model of focal cerebral ischemia in the Mongolian Gerbils. In the group of animals treated with EPO Neuro-applied by nasal way only animals died within 24 hours. This behavior continued until 5 weeks, which demonstrates the powerful protective effect achieved by intranasal application of Neuro-EPO.

The transit of Neuro-EPO to the CNS after intranasal administration and its effect were reported by us [41]. The detection of the molecule either in the olfactory bulbs or in the cerebellum suggested its contact with the CSF. This was further confirmed by the significant increase of rHu-EPO in the CSF of *M. fascicularis* (Figure 4) 5 min after its administration by the intranasal route. In animals treated with Neuro-EPO, a preservation of the habituation behavior in spontaneous exploratory activity was observed in both models, demonstrating the conservation of the structural integrity of the brain regions related to learning, and short- and long-term memory [61].

In the model of MCAO for 2 h in rats, animals treated with Neuro-EPO by the intranasal route displayed smaller volumes of ischemic tissue and a better clinical condition at 48 h [62]. The results of this study in rodents show therapeutic efficacy in both the acute and chronic phases of ischemia, as well as in reperfusion ischemia models, suggesting neuroprotective effects in brain structure and function.

These are indirect evidences of the access of Neuro-EPO administered in the amounts equivalent to the therapeutic dose recommended for ischemia by the intranasal route.

Those results suggest additional advantages for the intranasal route, which could be safer and faster than the intravenous route, which was recently demonstrated for delivery of Neurotrophic factors BDNF, CNTF, EPO, and an NT-4 to the CNS. [63]

In correlation with these ideas, Fletcher et al. [64] demonstrated that intranasal EPO plus IGF-I penetrate into the brain more efficiently than other drug delivery methods, and could potentially provide a fast and efficient treatment to prevent chronic effects of stroke.

A general survey carried out by a group of investigators concerning the use of the intranasal route to administer drugs for the treatment of diseases affecting the CNS indicated that in the last decade, roughly 11% of the new drugs generated by the industry are administered by this route. Patients prefer intranasal administration due to the efficacy and safety of these formulations.

A recent study assessing the safety of the Neuro-EPO was carried [57] out because the use of human recombinant erythropoietin (EPO) as a neuroprotective agent is limited due to its hematological side effects. Neuro-EPO, similar to that produced in the brain during hypoxia, may be used as a neuroprotective agent without risk of thrombotic events.

The objective of this investigation was to assess the toxicological potential of a nasal formulation with Neuro-EPO in acute, subacute and nasal irritation assays in rats. Healthy Wistar rats (Cenp: Wistar) were used for the assays.

In an irritation test, animals received 15 micro liters of Neuro-EPO into the right nostril. Rats were sacrificed after 24h and slides of the nasal mucosa tissues were examined. Control and treated groups showed signs of a minimal irritation consisting of week edema and vascular congestion in all animals. In the acute toxicity test, the dose of 47,143UI/kg was administered by nasal route. Hematological patterns, body weight, relative organ weight, and organ integrity were not affected by single dosing with Neuro-EPO.

In the subacute toxicity test, Wistar rats of both sexes received 6,600 UI/kg/day for 14 days. The toxicological endpoints examined included animal body weight, food consumption, hematological and biochemical patterns, selected tissue weights, and histopathological examinations. An increase of lymphocytes was observed in males that were considered to reflect an immune response to treatment. Histopathological examination of organs and tissues did not reveal treatment-induced changes.

The administration of Neuro-EPO at daily doses of 6,600 UI/kg during 14 days did not produce hematological side effects.

In conclusion, these results suggest that Neuro-EPO could offer the same neuroprotection as EPO, without hematological side effects.

In fact the great advantage of having a formulation of erythropoietin with neuroprotective capacity but non-erythropoietic enables its possible implementation, not only in the acute phase of stroke but in its later stage, where its behavior will give us useful information, possible for the treatment of chronic neurodegenerative diseases of the CNS.

Therefore, a discussion to determine the effectiveness of the Neuro-EPO treatment in chronic phase of stroke model using Gerbils follows.

8. Nasal delivery of Neuro-EPO. Effect in acute and sub chronic phase post stroke in animal model

We are relatively poorly acquainted with the effects of erythropoietin in the central nervous system. Therefore, our bank of knowledge with the novel nasal formulation of Neuro-EPO is correspondingly miniscule.

Therefore, it is logical to want to know the effect on the CNS by administration of Neuro-EPO by the nasal route, not just over days, but when it is applied for weeks.

Of practical interest, it has been postulated that EPO activates mechanisms of neuroplasticity [65]. The mechanism through which the Neuro-EPO does this neuroprotective effect in the short and medium term is an issue that requires more basic research. In this direction a recently paper from Reitmer et al., (2011) [66] showed that EPO administered intra-cerebroventricularly at 10 IU/day; starting 3 days after 30 min of middle cerebral artery occlusion. The neurological recovery was associated with structural remodeling of ischaemic brain tissue, reflected by enhanced neuronal survival, increased angiogenesis and decreased reactive astrogliosis with expression changes of plasticity-related molecules that facilitated contralesional axonal growth, and establishes a plasticity-promoting effect of EPO after stroke. [66]. In this excellent work, unfortunately the route used to delivery EPO to the brain is not applicable to stroke patients.

If this were verified, it would be invaluable for the treatment of patients, not only in the acute phase, but also for the recovery period of mental and motor activities affected by Stroke, using pathways and molecules safer, such as nasal delivery of Neuro-EPO.

The protective effect of Neuro-EPO was also evaluated at short and medium term [24]. Not evident, at the 24-hr post ischemia time point, when the percentage of deaths in both groups was similar (**Figure 5**). However, the mortality at 48 hr after brain ischemia was greater in the Nasal-Vehicle group than that in the Nasal-Neuro-EPO group ($p=0.02$). Indeed, 50% more of the animals in the Nasal-Vehicle group were dead at 48 hr after ischemia, whereas no animals from the Nasal-Neuro-EPO group died before 48 hr post ischemia. No further mortality was recorded during the five weeks of the study. Among other tests, this result confirms the well-known phrase "*Time is Brain*". This expression is also valid for the nasal application of Neuro-EPO.

In this study, the animals treated with Nasal-Neuro-EPO showed better general states. The gerbils with higher neurological scores not only showed deterioration in the extremities corresponding to the damaged hemisphere but also showed defects on the opposite side, including bilateral palpebral ptosis. The open field-testing to evaluate the exploratory activity of the animals showed significant differences ($p<0.05$) when comparing activity before surgery to after surgery in all groups of ischemic animals, except in the groups treated with Nasal-Neuro-EPO, which showed no difference. A marked behavioral difference existed at 24 hr between the ischemic animals in both treatment groups [24]

These results taken together speak for the effectiveness and safety of nasally administered Neuro-EPO in one of the strongest models of stroke.

The concept of achieving a significantly higher survival in animals treated with Neuro-EPO nasally during the 5 weeks of the study gives clear evidence of its neuroprotective properties. If we attach to this value the protection, which it confirms to the neurological status of animals, and histological protection displayed [24] we can ensure that this molecule has strong neuroprotective effects in the short and medium terms. However, we [24] recently reported its relationship with the EPOR and the expression of another important protein in the CNS, neuroglobin.

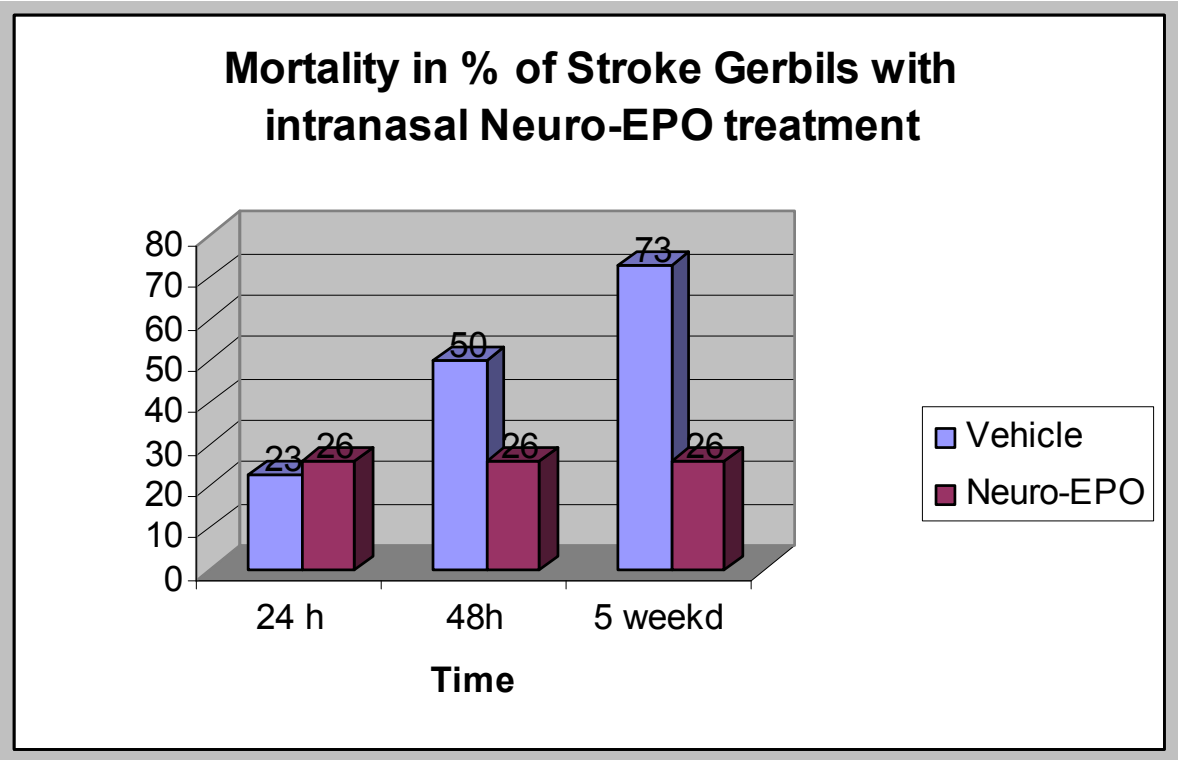


Fig. 5. The protective effect of Neuro-EPO was also evaluated at short and medium term. Not evident at 24-hr post ischemia time point; when the percentage of deaths in both groups was similar. However, no further mortality was recorded during the five weeks of the study. Among other tests, this result confirms establishes a survival-promoting effect of Neuro-EPO in the treatment of acute stroke.

In the next and final section of this chapter, we discuss the effect of Neuro-EPO application by nasal route in Gerbils Stroke model, and the effect on the expression of EPO receptor and Neuroglobin in this animal model.

9. Neuro-EPO, EPO receptor and Neuroglobin in acute phase of stroke animal model

As yet the molecular events, through which EPO makes its neuroprotective effect in the CNS have not been fully described. There are reports where it has been shown that the presence of its receptor in the hypoxic tissue is necessary for expression, which has been interpreted as the over-expression is necessary to trigger the molecular mechanisms of cytoprotection. That is, there have to be over expressed receptors for EPO and EPO existing in the region where these receptors are expressed.

The foregoing, that is the theoretical basis of EPO supplementation to hypoxic tissue as the tissue damage, mainly in the so-called ischemic penumbra region, within the mechanisms of endogen neuroprotection is the expression of new receptors to cells.

In addition, EPO has been shown to have the ability to induce the formation of new receptors. The latter should be a possible tipping point for the analysis of Neuro- EPO optimal dose, to avoid unwanted effects on the CNS in long-term treatment.

This apparent paradox of the EPO receptor should be carefully studied in the future and if in real terms for the stress neurons as a penumbra, on the limited availability to generate

energy by synthesis of new receptors to the EPO. It is a challenge to the classical concept of *cellular economy*. Therefore, obviously there must exist important new functions, the EPOR, which we have not been able to elucidate to the present. Among them may be the establishment of the new homeostasis, given the change of surroundings and intrinsically in those neuron-specific functions in the new contact of damaged tissue.

As complicated and not well-recognized biochemical processes of brain endogenous neuroprotection has emerged a relatively new protein that has been investigated by several research groups, we refer to neuroglobin (NGB). In the well-studied evolutionary branch of globins, hemoglobin has played a critical role in all vertebrates and their evolutionary adaptation to the oxygen atmosphere.

At this point, we must not forget that oxygen is the final acceptor in the respiratory chain and the key atom of the vital energy production in mitochondria in the cells. We have long conceived that this step has been free of oxygen and is therefore a thermodynamically favored process.

But what happens in critical conditions where there really is not enough oxygen? A few possibilities can be unwanted interaction with the simple entry of oxygen into a cell under stress or normal. These and many more are questions that motivated us to study the expression patterns of NGB and EPOR in the Cerebral Cortex and Hippocampus revealed distinctive intranasal delivery of therapeutic effects of EPO for neuro-ischemic insults to the gerbil brain. Later, we discuss results of this work [24].

Indeed, our results [24] not only showed that the intranasal delivery of Neuro-EPO is a valuable neuroprotective approach for the treatment of ischemia but also suggested that Ngb and the EPO/EPOR system have a close relationship with ischemic insults and Neuro-EPO treatment.

Ngb may act as a positive neuroprotective biomarker for brain ischemic insults or brain protection in vertebrates, including humans [68]. Thus, the overall expression patterns of Ngb at different time points after ischemic insults suggested that it might be an indicator of the neuroprotective action of Neuro-EPO in the brain (**Table 3**).

Actually, recent studies have also reported that treatment with EPO after brain ischemia could up-regulate Ngb expression in the brain [67-70], concurring with our work. Furthermore, the neuroprotective effects of EPO have various complementary actions, including antagonism of the effects of glutamate, increased expression of antioxidant enzymes, changes in the production of neurotransmitters, and induction of Ngb [70].

In addition, we have recently demonstrated that Ngb has antioxidant and free-radical scavenging activities, providing fundamental evidence for the neuroprotective function of this protein [70]. Therefore, the neuroprotective functions of Ngb and EPO/EPOR in the brain are probably closely related [24].

The dramatic expression pattern of endogenous Ngb and EPOR is possibly important for improving gerbil survival with intranasal Neuro-EPO treatment. EPO played a neuroprotective role mediated by EPOR accompanied with Ngb up regulation.

In addition, the distinctive biphasic expression patterns of Ngb in the gerbil brain are largely associated with ischemic tolerance in the cerebral cortex and ischemic sensitivity in the hippocampus. These data are important for understanding the different reaction properties of different brain regions, which are subjected to ischemic insults at specific time points and also important for improving the therapeutic effects of intranasal Neuro-EPO administration.

Time post ischemic Insults		10min	1hr	12hr	24hr	48r	72hr	1Week	5 Weeks
Protein	Brain Region								
Ngb	CC	-	+	+	+	-	+	+	+
	HP	+	-	-	-	+	ND	+	+
EPOR	CC	ND	-	-	-	+	ND	ND	ND
	HP	+	-	-	-	+	-	-	+

Golden Hour

Silver days

Late recovery Phase

CC= Cerebral Cortex; HP= Hippocampus; EPOR= EPO receptor; ND= not determined;
(+) = Upregulation; (-) = Down regulation. All cases p<0.05. Gold Hours is the practical more important time “Time is Brain”. The Silver day it is critical period of time. The 10 min and 48 hr seemed to be two time points for the brain to switch the expression of both Ngb and EPOR to early and late recovery phase, respectively. In addition, there were two phases, 10 min to 1 hr and 24 hr to 72 hr, respectively, closing to the “golden hour” of about 60 min and the “silver day” of 1 to 3 days, for the brain to recover from stroke onset with intranasal Neuro-EPO treatment.

Table 3. Expression Pattern of Ngb and EPOR in the Cerebral Cortex and Hippocampus of Gerbils in the Nasal-Neuro-EPO Group Compared to Nasal-Vehicle group. Data Represent of 3 independent experiments.

10. Conclusions

There is a significant amount of histologic findings, behavioral, biochemical and pharmacological to support Neuro-EPO by nasal route as a neuroprotective therapy in brain ischemia. Unlike other molecules of EPO, which have no capability for erythropoiesis yet retain their neuroprotective capacity, the Neuro-EPO is not a carrier of chemical alterations that may cause unwanted effects, when in contact with the CNS.

Various studies have not identified adverse effects and it has been shown to be safe and effective in protecting cortical regions and sub cortical hypoxic brain 18 hours after the occurrence of hypoxia in models where the effect has been studied.

Evaluation of its safety and effectiveness in the clinic is the new challenge is to address Neuro-EPO nasally as a new neuroprotective drug to combat ischemic acute stroke.

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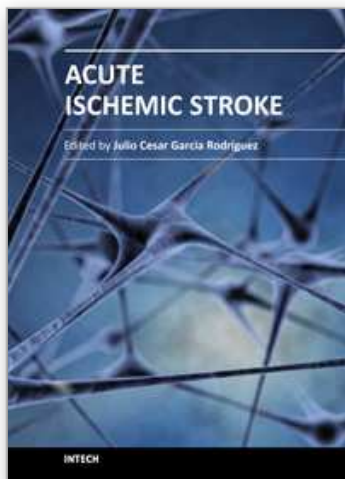
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Despite significant technological advances in recent years, their impact on our overall health and social, well-being is not always clear to see. Perhaps, one of the best examples of this can be highlighted by the fact that mortality rates as a result of cerebrovascular diseases have hardly changed, if at all. This places cerebrovascular diseases as one of the most prominent causes of both disability and death. In Cuba, for instance, a total of 22,000 cases of cerebrovascular diseases are reported each year in a country where life expectancy should increase to 80 years in the near future. In such a situation, to have a book that includes in a clear and summarized way, a group of topics directly related to the preclinical investigations advances and the therapeutic procedures for the cerebrovascular disease in its acute phase constitutes a useful tool for the wide range of the contributors to this affection's problems solution. In this group is included students, professors, researchers, and health policy makers whose work represents one of the greatest social and human impact challenges of the XXI century basic and clinical neurosciences.

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