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PERSPECTIVE

Immunization with Cop-1 promotes neuroprotection and neurogenesis after ischemic stroke

Cerebrovascular diseases are considered to be amongst the most serious public health issues, since they are the third leading cause of death (WHO, 2014) and the most common cause of disability worldwide. Its monetary significance is evidenced by the economic burden imposed on health care systems, given that the cost of medical care for a patient that has suffered a stroke is around $25,741 US dollars every 5 years (Luengo-Fernandez et al., 2012). A stroke occurs as a result of a disturbance or interruption of cerebral blood flow that significantly reduces the supply of oxygen and glucose to the neural tissue. Consequently, several cell death mechanisms (secondary lesion mechanisms) such as necrosis, excitotoxicity, free radical production and inflammation are triggered (Castillo, 2000). Over the last decades, a variety of therapies with thrombolytic, neuroprotective, and restorative properties have been investigated. However, the results of these studies appear to be limited. That is the case of the tissue plasminogen activator (tPA) – the first line of treatment for decades – which is associated with low rates of recanalization and high rates of morbidity. Also, endovascular intervention, particularly mechanical thrombectomy, has been proposed as a promising therapeutic adjunct to tPA for the treatment of stroke; however, until recently, the efficacy of this therapeutic approach has been controversial (Ding, 2015). Innovative theurapeutic options are currently being developed in order to restore affected neuronal circuits following a cerebral ischemic event. Some of these innovative therapeutic approaches are based on stem cell transplantation and/or induction of neurogenesis.

After stroke; astrocytes, microglia, and endothelial cells induce an early response in gene expression through the activation of nuclear factor-κB (NF-κB). This event promotes a pro-inflammatory environment characterized by the expression of interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α) and several chemokines (Fumagalli et al., 2015). These proteins activate adhesion molecules and produce a subsequent infiltration of inflammatory cells, especially T lymphocytes specific to neural constituents. Recruitment and activation of immune cells increase the presence of lytic enzymes and neurotoxic mediators (*e.g.*, free radicals) which in turn, cause secondary damage to the neural tissue.

The immune system plays an essential role in the pathophysiology of some neurodegenerative diseases. It has previously been associated with disease exacerbation (Castillo, 2000). Nevertheless, recent work suggests that inflammatory cells and even autoimmune T lymphocytes could have the ability to promote neuroprotection (Schwartz and Shechter, 2010). These findings provide the basis to conceive a new therapeutic paradigm: Protective autoimmunity (PA), a physiological phenomenon that develops after central nervous system (CNS) damage (Hauben et al., 2000). Paradoxically, the beneficial effect of this immune response is exerted by autoreactive T cells directed against neural contituents (Schwartz and Shechter, 2010). In this light, PA might have beneficial effects over the secondary mechanisms of stroke and cerebrovascular diseases, nonetheless in order to exert these, it must be modulated. Evidence suggests that PA could be modulated by active inmunization with neural-derived peptides (NDP) in favor of protecting neural tissue after CNS damage (Cruz et al., 2015). Copolymer-1 (Cop-1; Copaxone, glatiramer acetate) is a synthetic polypeptide consisting of four amino acids: L-alanine, L-glutamic acid, L-lysine, and L-tyrosine in a fixed molar ratio of 6.0:1.9:4.7:1.0 and a molecular weight ranging from 4.7 to 11 kDa. Cop-1 has demonstrated to positively modulate PA and induce a strong effect over the immune response by binding to the MHC class II molecules on the surface of antigen-presenting cells, without being processed. Vaccination with Cop-1 stimulates T cells, which are activated by determinants common to Cop-1 and myelin basic protein (MBP); suggesting that it has a strong cross-reaction with MBP peptides. Cop-1 increases Th2/3 cytokine secretion patterns, regulatory T cells (Aharoni et al., 2003), IL-4, IL-10, and transforming growth factor-β (TGF-β), a cytokine type that by itself possesses immunomodulatory properties and inhibitis the production of inflammatory cytokines such as INF-γ, TNF-α and IL-12. Moreover, Cop-1 immunization has the ability to exert neuroregenerative properties. It has proven to increase the production of brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1) and neurotrophin 3 and 4 (NT-3 and 4) in models of experimental autoimmune encephalomyelitis (EAE) and schizophrenia (Aharoni et al., 2003; Kipnis et al., 2004). Together, these findings suggest that modulation of PA –through Cop-1 inmunization– could promote a neuroprotective and neurorestorative environment. Therefore, our group decided to investigate the neuroprotective and neuroregenerative effects of this strategy in a focal cerebral ischemia/reperfusion model.

In order to evaluate the neuroprotective effect of Cop-1, a model of transient middle cerebral artery occlusion (tMCAo) was developed in our laboratory. Animals were injected with 200 μg of Cop-1 dissolved in saline solution and emulsified in an equal volume of complete Freund's adjuvant. Immunization was applied subcutaneously at the interscapular space immediately after reperfusion (acute phase). In a first study, Cop-1 immunized animals presented a significant neurological recovery when compared to controls 7 days after ischemia (Ibarra et al., 2007). Additionally, histopathological findings had a significant correlation with neurological recovery: rats receiving Cop-1 immunization presented a smaller infarct volume after stroke. Such reduction in infarct volume could be related to increased neuroprotection, resulting in less tissue necrosis or inhibition of growth of the ischemic core (**Figure 1A**). According to recent evidence, these results suggest that Cop-1 specific immune modulation could be the primary source of neuroprotection after ischemia.

The neurological recovery observed in treated animals led us to investigate the neurorestorative effects of Cop-1 over focal cerebral ischemia. During a second experiment (Cruz et al., 2015), immunization with Cop-1 promoted neurological recovery in treated rats (**Figure 1B**). In addition, a significant reduction of infarct volume in Cop-1 immunized animals was observed once again.

In the same work, biochemical studies revealed that treatment with Cop-1 increases the concentration of NT-3 but not BDNF *in vivo*. It is known that Cop-1 immunization increases the production of BDNF *in vitro*. However, our findings *in vivo*, showed that Cop-1 augments NT-3, but not BDNF. Additionally, we counted the number of newly formed neurons since existing evidence confirms that the presence of neurotrophic factors enhances neurogenesis. As a result, we found that Cop-1 increases neurogenesis in the cerebral cortex (CC), the subventricular zone (SVZ) surrounding the ventricles, and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (**Figure 2A**). The presence of neural progenitor cells (NPCs) and the increased number of new neurons after immunization could explain, at least in part, the neurological recovery observed in treated animals. Hence, allowing to consider that these newly formed neurons could be able to establish neural connections.

The choroid plexus (CP) plays an important role in modulating the immune response, especifically PA; thus, it could modulate neurogenesis (Baruch and Schwartz, 2013). Therefore, in order to establish whether Cop-1 is able to modify the microenvironment of the CP, we analyzed the expression of growth factors and citokines 15 days after tMCAo and Cop-1 immunization. Gene expression analysis revealed a considerably higher expression of genes associated with neurogenesis such as NT-3, IGF-1 and also anti-inflammatory genes like IL-10 (unpublished data).

The promising results provided by Cop-1 led us to examine its effect in a model that comprises memory and learning (unpublished data). Aging brings forth an impairment in memory and learning that is clearly associated with a functional deficit of the hippocampal system. This deficit is accompanied by alterations in growth factors (especially BDNF), neurogenesis, and in the hippocampal synapse. We tested whether Cop-1 had a positive effect on memory and learning restoration in aged rats. To achieve our goal, 6 month old rats were immunized with Cop-1. Two months later, the spacial memory

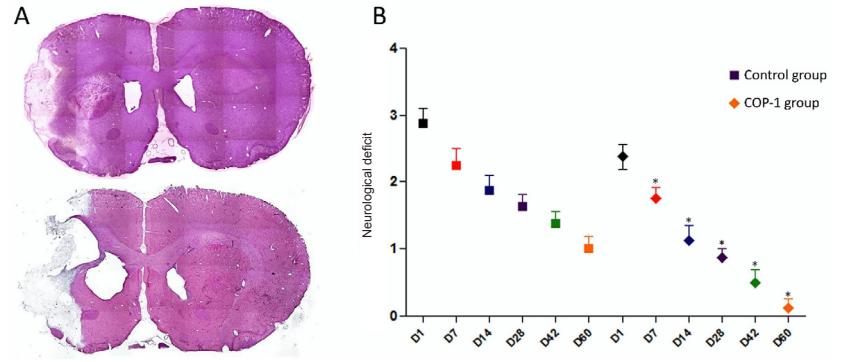

Figure 1 Beneficial effects of copolymer-1 (Cop-1).
(A) Neuroprotective effect of Cop-1. Cop-1-treated rats had a smaller infarct volume (upper image) compared to untreated rats (lower image). (B) Neurological deficit after treatment with Cop-1. Evaluations were performed at 1, 7, 14, 28, 42 and 60 days post-ischemia using the Longa EZ scale, (n = 8 per group; mean ± SD). Two-way repeated measures ANOVA and Sidak's *post hoc* multiple comparison test. *P < 0.05, *vs.* control group.

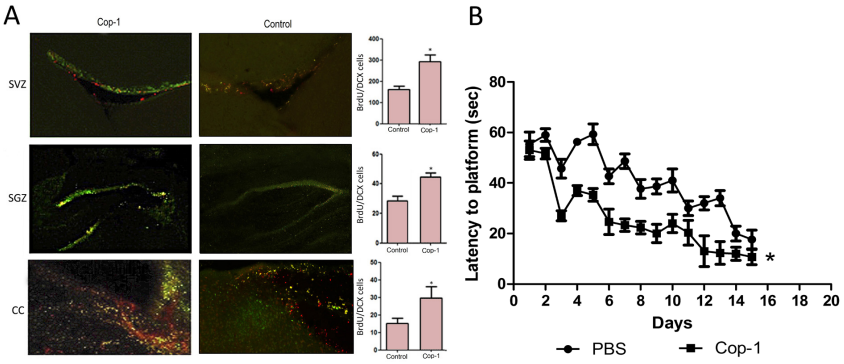

Figure 2 Effect of copolymer-1 (Cop-1) on neurogenesis and upon memory and learning.
(A) Effect of Cop-1 on neurogenesis in the subventricular zone (SVZ), dentate gyrus (DG) and cerebral cortex (CC). Microphotographs show $BrdU^+/DCX^+$ cells in the SVZ, DG and CC of Cop-1-treated and non-treated rats, 7 days after ischemia (n = 8; mean ± SD). *P < 0.05 (two-tailed Mann-Whitney U test). (B) Effect of Cop-1 on memory and learning. The graph shows the latency performed by the rats upon arrival to the platform in the Morris maze test (n = 8 per group; mean ± SD). *P < 0.05 (two-way repeated measures ANOVA).

and learning of the rats were evaluated, revealing that treatment with Cop-1 improved both variables (unpublished data) (**Figure 2B**). Interestingly, Cop-1 immunized rats presented increased levels of BDNF (unpublished data). These results indicate that Cop-1 could be an alternative therapy for cognitive impairment and that the effect of Cop-1 on BDNF production varies with experimental models.

Immunization with NDP (modulation of PA) could be a successful therapeutic strategy to control and diminish the progression of secondary degeneration observed after CNS injury. In the case of neurodegenerative diseases caused by functional alterations associated with age, treatment with Cop-1 could be a preventive alternative. Vaccination with synthetic peptides capable of cross-reacting with self-antigens has proven to be beneficial in several neurodegenerative diseases such as multiple sclerosis, damaged optic nerve, Parkinson´s disease (PD), amyotrophic lateral sclerosis (ALS) and others (Ibarra et al., 2007). Cop-1 is an immunomodulatory drug already approved by the Food and Drug Administration (FDA) for the treatment of MS, implying that the safety of the drug has been validated. As a minimally invasive approach for treating neurological diseases involving an inflammatory response, Cop-1 has the ability to modulate inflammation and increase local neurotrophic factor production. Preliminary data has led us to believe that active immunization with Cop-1 enhances functional recovery by inducing neuroprotection and neurorestoration. These beneficial effects are achieved by avoiding autoimmune disease, local toxicity and by increasing the levels of neurotrophic factors.

In spite of the promising results, a range of new experiments should be designed before Cop-1 therapy can be translated to humans. For instance, the therapeutic window for obtaining the beneficial effects of Cop-1, the use of suitable adjuvants for humans, the administration of Cop-1 without any adjuvant, the optimal number of immunizations and the adecuate amount of Cop-1 are some of the topics that must be explored.

As an FDA-approved treatment, Cop-1 could easily be developed for treatment of clinical cerebrovascular diseases or cognitive disorders, with the objective of decreasing mortality and improving the patients' quality of life. Therapy with Cop-1 represents a promising approach that should be explored in order to optimize the therapeutic strategy for neurodegenerative diseases in the clinical field.

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Yolanda Cruz, Paola Suárez-Meade, Antonio Ibarra *

Facultad de Ciencias de la Salud, Universidad Anáhuac México Norte, Av. Universidad Anáhuac No. 46, Col. Lomas Anáhuac, C.P.52786, Huixquilucan Edo. de México, México (Cruz Y, Suárez-Meade P, Ibarra A)
Proyecto CAMINA A.C., Tlalpan No. 4430 Col. Toriello Guerra, C.P. 14050, México City, México (Ibarra A)

***Correspondence to:** Antonio Ibarra, Ph.D., iantonio65@yahoo.com; jose.ibarra@anahuac.mx.*
***Accepted:** 2015-08-03*
***orcid:** 0000-0003-2489-4689 (Antonio Ibarra)*
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