Chapter

Neuroprotective and Neurorestorative Properties of Copolymer-1: Its Immunomodulating Effects on Ischemic Stroke

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Abstract

Stroke is a pathology of great relevance worldwide as it currently occupies the second motif of death and the third reason of disability. Although exits some therapies that are used successfully in the clinic, a very high percentage of patients do not have the opportunity to benefit from them; therefore, it is imperative to propose other alternatives that may favor more patients. In this chapter, we briefly review the inflammatory response induced by stroke and also its deleterious and protective effects. We will describe the characteristics of copolymer-1 and the effects that this compound has shown in models of cerebral ischemia.

Keywords: cerebral ischemia, copaxone, neurogenesis, protective autoimmunity, neuroregeneration, neuroprotection

1. Introduction

Cerebral ischemia is the main disorder of cerebrovascular diseases; currently, according to data from the World Health Organization, it is the second main cause of death worldwide [1] and the third principal cause of disability. In the last 40 years alone, the incidence of this condition has more than doubled in people from low and middle-revenue countries [2].

The increment in the incidence of this condition is due to increased risk factors as diabetes mellitus, hypertension, obesity, hyperlipidemia, and increased longevity of the population [3]. These factors allow the development of atherosclerosis, which is the main cause of ischemia [4]; thereby, it is considered that for the coming years this scenario will be maintained while strategies to reduce these factors are progressing.

Stroke is distinguished by the brusque reduction of blood flow; therefore, the levels of oxygen and glucose are also reduced significantly, to the point of altering the metabolic activities of the neural tissue [5]. As a consequence of the latter, the low production of ATP and the acidification of the environment induce the

depolarization of the membranes causing the intracellular increase of Ca^{2+} that is added to the one released by the endoplasmic reticulum and mitochondria [6].

Neuronal depolarization causes the release of glutamate which, when bound to its ionotropic N-methyl-D-aspartate (NMDA) and -amino-3-hydroxy-5-methyl-4-isoxazolpropionic (AMPA) receptors, achieves greater depolarization and, as a consequence, conditions of excitotoxicity [7]. These conditions are coupled with the production of free radicals [8] and lead to cell death by the activation of molecules that induce necrosis and apoptosis [9].

Along with the lesion caused by the decrease in blood flow, the immune response is added to the events involved in both the detriment of the tissue and its protection.

2. Immunological response in stroke

Inflammation is usually present before the development of arterial obstruction that gives rise to the ischemic event. The development of atherosclerosis is accompanied by the production of oxygen free radicals (ROS), expression of cell adhesion molecules, and production of proinflammatory cytokines as IL-1β and tumor necrosis factor-α (TNF-α) by endothelial cells [10].

Shortly after occlusion, endothelial cells express a greater amount of intercellular adhesion molecules (ICAM), deposition of mannose binding lectin molecules that trigger activation of the complement pathway [11], producing higher amounts of ROS. The overproduction of ROS activates the prostaglandin pathway that stimulates the production of matrix metalloproteinases (MMP) that even though degrading constituents of the extracellular matrix, reshape the vascular endothelium seeking to protect of the blood brain barrier (BBB) [12].

The release of chemokines such as CCL2 allows endothelial permeability [13], leading to the translocation of P-selectin from Weibel-Palade bodies, as well as the expression of ICAM-1 and vascular cell adhesion molecule (VCAM)-1 and E-selectin, on the endothelial surface [14]. Theses phenomena, together with the damage of the extracellular matrix facilitate the extravasation of macromolecules and water, which causes the development of vasogenic edema [15]. Peripheral immune cells then enter the injured cerebral parenchyma [16] facilitating the loss of the integrity of the BBB.

Neutrophils are the first leukocytes that migrate to the cerebral parenchyma; they have been detected since the first hour after ischemia and reach their maximum peak in 1–3 days [17]. In the clinic, it has been observed that the higher blood neutrophil count is associated with higher infarction volumes in patients with acute stroke [18].

The second cell type that enters the neural tissue are monocytes, these infiltrate within 24 h of the onset of the ischemic event reaching its peak on day 3 [19]; their differentiation process toward macrophages and their activation will be determined by the molecular environment to which they arrive. This process is similar to that experienced by T lymphocytes, which reach the parenchyma 24–96 h post-ischemia [20].

At the same time, the cells of the injured cerebral parenchyma release damage associated molecular patterns (DAMPs) that activate the microglia. Depending on the activation environment, the microglia can acquire a proinflammatory (M1) or anti-inflammatory (M2) phenotype [21]. In the M1 phenotype, the microglia acquires phagocytic capacity, produces NO, free radicals, and proinflammatory cytokines (e.g. TNF-α, IL-12 and IL-6) [22]. Some regions in the ischemic penumbra present an activation of M2 microglia distinguished by the production of anti-inflammatory and repair molecules, such as insulin growth factor 1 (IGF-1), IL-10, and arginase 1 [23].

Some researchers suggest that the M2 phenotype is initially activated during the acute phase in the peripheral zone to the infarction [24], since it has been determined that the levels of IL-10, TGF-β, and CD206 increase from the first day after the lesion and reach the maximum point between 4 and 6 days, possibly trying to keep the viability of tissue. In addition, TGF-β induces the anti-inflammatory phenotype of microglia, related with enhanced proliferation and neuroprotection [25, 26].

In contrast, some authors suggest that the first response is proinflammatory [27], due to the loss of regulatory mechanisms; when a stroke occurs there is an important activation of the M1 microglial phenotype [28].

Although contradictory, both positions could be correct. The fact is that, M1 and M2 phenotypes actively participate in the response observed after ischemic event; however, in normal conditions, there is an important prevalence of the M1 phenotype leading the response to a proinflammatory reaction that, instead of helping, promotes more damage.

On the other hand, perivascular macrophages and monocytes of peripheral origin that arrive at the injured parenchyma induce the synthesis of chemokines like CXCL1 and CXCL2, which are fundamental for recruiting more neutrophils to the injury site [29, 30]. The dendritic cells (DC) present a greater expression of the major histocompatibility complex II (MHCII) and the co-stimulant molecule CD80. This causes an important enhance in the interaction of T cells around and within the damaged areas inducing then a stronger immune response [31].

When T lymphocytes are activated by antigen-presenting cells (APCs) toward a Th1 phenotype, the secretion of proinflammatory cytokines like as IFN-γ, TNF, and LT-α [lymphotoxin] increases. This cytokine profile, intensify proinflammatory response and thereby, tissue damage. Contrarily, when T cells are activated toward a Th2 phenotype they produce anti-inflammatory cytokines such as IL-4 and IL-10 [32]. These cytokines have been associated with tissue protection mechanisms and even increased neurogenesis. This immune response that exerts protective effects and limits the damage caused by ischemia [19] can be stimulated by immunomodulatory molecules such as copolymer-1.

3. Copolymer-1

Copolymer-1 [Cop-1], also known as glatiramer acetate (GA) or copaxone [trade name], is a blend of peptides formed by random sequences of four amino acids: glutamic acid, lysine, alanine, and tyrosine; these have a variable length from 45 to 200 amino acid residues and a molecular weight of 4000–9000 Da [33].

Cop-1 was originally synthesized from mylelin basic protein (MBP) to identify the precise immunogenic sequence and provoke experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS); however, it did not present encephalitogenic characteristics [34]; on the contrary, it has suppressive and protective effects on EAE [35]. In the clinic, copaxone is able to diminish the relapse rate and improve the disability of patients with relapsing-remitting MS [36]. Copaxone obtained its approvement by the Food and Drugs Administration [FDA] of U.S.A. in 1996 and in Europe in 2001 [33].

At this time, the exact mechanism by which Cop-1 exerts its protective effects is not known at all. Studies carried out in EAE suggest that Cop-1 has greater affinity for the MHCII binding site of APC when competing with peptide complexes derived from the MBP, specifically with the epitope 82–100 [37]. This competition may also be present among the complexes for the TCR binding site of the lymphocytes [38] that, when activated, induces a Th2 response [39].

The Cop-1 response is distinguished by increased synthesis of IL-4, IL-5, IL-10, IL-13, and TGF-β [33, 40–43]. Cop-1 has also been observed to increase the presence of regulatory T lymphocytes [44] and regulatory CD8+ T lymphocytes in patients with multiple sclerosis [45, 46].

Another important effect of copolymer-1 is the production of growth factors, among which stand out; the brain derived neurotrophic factor [BDNF] [47, 48], IGF-1, [49] and neurotrophins NT-3 and NT-4 [47]. It is known that, in addition to inducing neuroprotection and neurorestoration, these growth factors are related to mechanisms such as memory and learning.

The molecular basis by which Cop-1 exerts its neuroprotective effect has been evaluated in several *in-vitro* assays. The most explanatory results have been obtained in the analysis of the effect of Cop-1 on APC such as monocytes, microglia, and astrocytes.

It has been showed that through the blockade of the nuclear factor kappa B [NF-kB], Cop-1 reduces the expression of the chemokine CCL5 [RANTES], which is upregulated by the presence of IL-1β [50] and TNF-α in human astroglial cells [51]. A similar effect has also been observed on the monocyte chemotactic protein-1 [MPC-1] and adhesion molecules VCAM-1 and selectin E in endothelial cells as well as COX2 and iNOS [52].

It has also been observed that Cop-1 induces differentiation of type II monocytes independently of the binding of Cop-1 to MHCII. Weber et al. demonstrated that this differentiation is due to the fact that Cop-1 reduces the phosphorylation of the transcription factor STAT-1 by stimulating the expression of IL-10 and TGF-β [53].

On the other hand, it has also been observed that Cop-1 has a direct effect on glial cells [microglia and astrocytes] which are activated in conjunction with T cells reducing STAT-1 and STAT-3 phosphorylation through increased expression of cytokine signaling suppressor (SOCS-1) and independently of IFNϒR, accompanied by a reduction of IL-12 by CD4+ T lymphocytes [54].

Even though the molecular pathways by which Cop-1 acts are not yet completely established, the microenvironment induced by this compound is capable of allowing neuroprotection since it reduces the deleterious scenario that leads to neural death. Additionally, the new conditions could facilitate tissue restoration through the synthesis of growth factors.

4. The effect of copolymer-1 on inflammatory diseases

The beneficial effects showed by copaxone in patients with MS, even though the knowledge of its immunomodulatory mechanisms is partial, encouraged the evaluation of its effect in other experimental models.

In the model of optic nerve lesion—which tries to reproduce the characteristics of secondary degeneration—Cop-1 demonstrated an interesting neuroprotective effect. Kipnis et al. [55] evaluated the effect of adoptive anti-Cop-1 T cell transfer and immunization with Cop-1 immediately after causing optic nerve contusion in Lewis rats; their results were very encouraging as they observed reduction in axonal degeneration, accumulation of T lymphocytes in injured areas and obtained a significant increase in IL-10 and BDNF *in-vitro*. In contrast, using a model of axon transection of the optic nerve, Blair and coworkers [56] found no beneficial effects of Cop-1. The difference in the results may be due to the different inflammatory response evoked by the type of injury (contusion or transection). Inflammation is more pronounced after contusion as compared to the one observed after a transection. It should be an issue to be studied by future investigations.

Parkinson's disease presents gradual reduction of dopaminergic neurons in the region of the substantia nigra and the striatum in the brain, it is not known the reason that causes the death of these neurons, but the pathology is characterized by a significant increase in oxidative stress, mitochondrial dysfunction, neuroinflammation, and cell death [57]. Patients with PD present an increase in TNF-β, IL-1β, and IL-6 and other inflammatory cytokines resulting from the activation of the macrophages and microglia towards a proinflammatory phenotype capable of releasing NO and superoxide radicals that further damage neural tissue facilitating disease progression [58].

In the traditional model to induce Parkinson's disease in mice [induction by 1-1-methyl-1,2,3,6-tetrahydropyridine], it was observed that Cop-1 reduces the degeneration of dopaminergic cells. This effect is achieved since Cop-1 induces the up-regulation in the protein expression of tyrosine hydroxylase [59, 60]. Additionally, it has been reported an increase in glial cell-derived neurotrophic factor (GDNF), reduction of activated microglia markers, and restoration of BDNF [61]. Based in these findings, several research groups consider COP-1 as a pharmacological alternative for this pathology which should be deeply studied [62].

Copolimer-1 has also been tested in models of Alzheimer's disease (AD). AD is a pathology that produces deposits of the β-amyloid protein, dystrophic neurites, loss of synapses and neurons, and elevated gliosis [63]. From the early stages of the pathology, it has been observed microgial activation toward a M2 neuroprotective phenotype that is modified as the disease progresses [64]. In advanced stages, a proinflammatory microenvironment characterized by the presence of cytokines such as IL-1β, TNF-α, IL-6 has been reported [65].

After Cop-1 administration, microglia modulation toward a M2 phenotype is observed, in such a way as to promote neuronal survival and neural tissue repair in AD models [66]. Butovsky and coworkers showed that Cop-1 immunizations lead to enhanced infiltration of monocyte-derived macrophages into neural tissue with an anti-inflammatory profile expressing minor levels of TNF-α and high levels of IL-10, TGF-β1, and IGF1. In this scenario, phagocytosis of preformed fibrillar amyloid-β by bone marrow-derived macrophages increased dramatically after the administration of Cop-1. Also, to demonstrate benefits on the preservation of cognitive function, the investigation showed an important synaptic protection, plaque removal, restriction of astrogliosis, and modulation of the immune molecular environment [67, 68].

Another pathology that evidenced the beneficial effects produced by Cop-1 is amyotrophic lateral sclerosis (ALS). This is a neurodegenerative disease known by the progressive depletion of the upper and lower motor neurons [69]. During pathogenesis, glutamate excitotoxicity, structural and functional anomalies of mitochondria, damaged axonal structure, and oxidative stress conducted by free radicals are strongly observed [70].

In this case, Angelov and colleagues showed—in mouse models—that the administration of Cop-1 promotes the survival of motor neurons [71].

The beneficial effects of Cop-1 on ALS have been assessed in a Phase II trial conducted by Mosley. This investigation evaluated the cytokine response of ALS patients treated with copaxone and showed that copaxone is capable of inducing a temporary change in cytokines from Th1 to Th2 phenotype [72].

Copaxone is also tested in other pathologies at clinical settings. For instance, a phase III study on optic neuritis is now being conducted to evaluate the thickness of the layer of nerve fiber of the retina after 6 months of treatment. The results of this study have not been published. Finally, copaxone has been tested in Crohn's disease and various types of carcinomas, studies where copaxone is in evaluation processes [73].

The ability of Cop-1 to modify the proinflammatory milieu and to stimulate the production of growth factors encourages the idea of testing this compound on other pathologies with characteristics of secondary degeneration caused by inflammation. In line with this, the use of Cop-1 after stroke envisions an optimistic result.

5. Effect of copolymer-1 on stroke

As copolymer-1 has been shown to have beneficial effects in various models where neuroinflammation is a detrimental determinant, our group decided to evaluate its neuroprotective effect on cerebral ischemia. For this purpose, we used the median transient cerebral artery obstruction (tMCAO) model. Sprague-Dawley male rats were used. After being subjected to ischemia for 90 min, the rats were immunized in the interscapular region with a dose of 200 μg of Cop-1.

In the first study, the animals were evaluated for neurological deficit at day 1 and day 7 post-ischemia using the Zea Longa scale [74]. Then, a histological analysis was performed using hematoxylin and eosin staining to determine neuroprotection. The results indicated that Cop-1 is able to avoid up to 85.1% increase in infarct size (4.8 ± 1.5 for Cop-1 vs. 32.2 ± 8 for control group; p = 0.004 mean ± SD; **Figure 1A**) and is able to reduce neurological deficit on day 7 post-ischemia.

This neuroprotective effect may be due to the reduction of the proinflammatory cytokines TNF-α and IL-1β and the increase of IL-4, as was observed by the Manguin group in a model of ischemia in diabetic mice [75]. Additionally, the production of neurotrophic factors—known to be implicated in the processes of neural survival and proliferation of neuron precursor cells—could also be involved [76].

On the other hand, recovery from neurological deficit can be achieved by diverse mechanisms; for instance, neuroprotection exerted by Cop-1 could be limiting tissue damage caused by inflammation, this could allow the proper functioning of remaining neuronal connections. Functional recovery could also be the result of neurogenesis induced by Cop-1. Neurogenesis is a phenomenon that can replace neurons that died during the ischemic insult by allowing the substitution of neuronal circuits and thus neurorestoration.

Our study provided evidences about the neuroprotective effect of Cop-1; however, the fact that Cop-1-induced T cells are able to produce neurotrophic factors, led us to think that, it was imperative to investigate if behind the clinical recovery there was also a possible neurogenesis phenomenon.

In the following study, we evaluated whether Cop-1 induces neurogenesis in the two neurogenic niches of the adult brain: in the subventricular (SVZ) and the

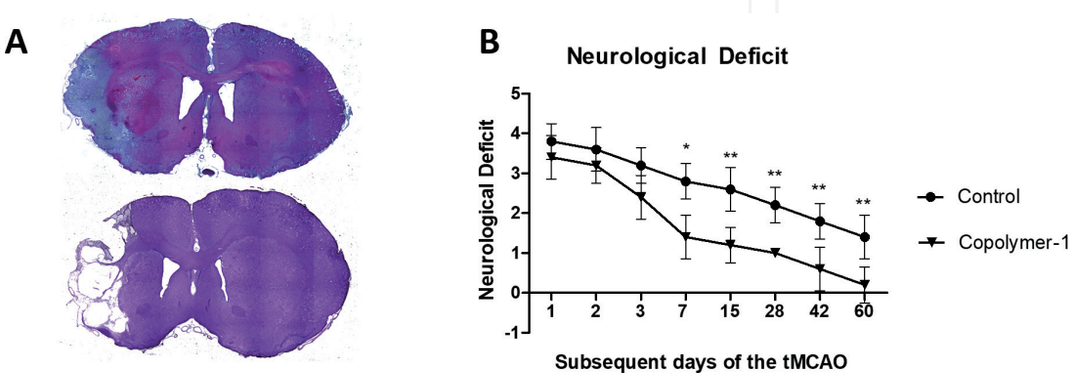

Figure 1.
*Neuroprotective effect of the copolymer-1. (A) Infarction size reduction. (B) Effect of the copolymer-1 on the neurological deficit. n = 8. Each bar represents mean ± SEM. Two-way repeated measure ANOVA. Sidak's post hoc multiple comparisons test. *p < 0.05; **p < 0.0.*

subgranular zone of the dentate hippocampus gyrus (SGZ) [77]. To accomplish the evaluation, we performed an immunofluorescence technique using a double marking of 5-bromo-2′-deoxyuridine (BrdU) and doublecortin (Dcx) at 7 and 60 days after ischemia.

The results were very encouraging, the neurological recovery caused by Cop-1 was observed from day 7 post-ischemia as in the first experiment [78] and was improving even in the chronic phase at 60 days (**Figure 1B**). The number of neuroblasts in the groups treated with Cop-1 was significantly higher in the two neurogenic niches at both 7 and 60 days in the SVZ and SGZ (**Figure 2**). This neurogenic phenomenon correlated with the clinical recovery of treated rats. Simultaneously, an important increase of NT-3 was observed in the area of the ischemic penumbra [79].

Cop-1-induced neurogenesis has been evaluated in other animal models such as EAE [47], Alzheimer [66], and recently in the model of permanent cerebral ischemia in diabetic male mice C57Bl6 [75]. Regarding the latter, it is important to mention that, in a previous experiment carried out by the same group, they did not observe improvement in the neurological function nor reduction in the volume of the infarction. These findings could be the result of the use of inappropriate evaluation techniques [80] as in their most recent study, they observed a reduction in infarct size of up to 30–40% and an increase in neurogenesis 7 days after permanent ischemia in the SVZ. In addition, they found a reduction of proinflammatory cytokines such as TNF-α, IL-1β, and a significant increase of IL-4 and IL-10 [75].

Neurogenesis is a mechanism widely regulated by signals that stimulate the stem cells of neurogenic niches [81]; many of these signals are produced by the choroid plexus (CP), which is a complex structure of cells considered an interface that mediates communication between the immune system and the cerebral parenchyma [82]. Therefore, trying to analyze the mechanism by which Cop-1 induces neurogenesis, we evaluate whether Cop-1 modifies the microenvironment of CP, 14 days after tMCAO.

In the third investigation, we evaluated neurological recovery—which was observed according to our previous experiments [78, 79], neurogenesis and the expression of proinflammatory (IL-1β, TNF-α, INF-ϒ, and IL-17) and anti-inflammatory cytokines (IL-4 and IL-10) as well as the concentration of growth factors (BDNF, NT-3 and IGF-1) at the CP (**Figure 3**).

In this experiment, we again proved a significant increase of neurogenesis in the groups treated with Cop-1 in both, the SVZ and SGZ [83]. This data was similar to that previously reported [79]. As for the expression of proinflammatory cytokines, we only found significant differences in the expression of IL-17, which was observed reduced in the groups treated with Cop-1. With respect to anti-inflammatory cytokines, only IL-10 was significantly increased. In this investigation, we also found a significant increase of growth factors (BDNF, NT-3, and IGF-1) in the CP [83].

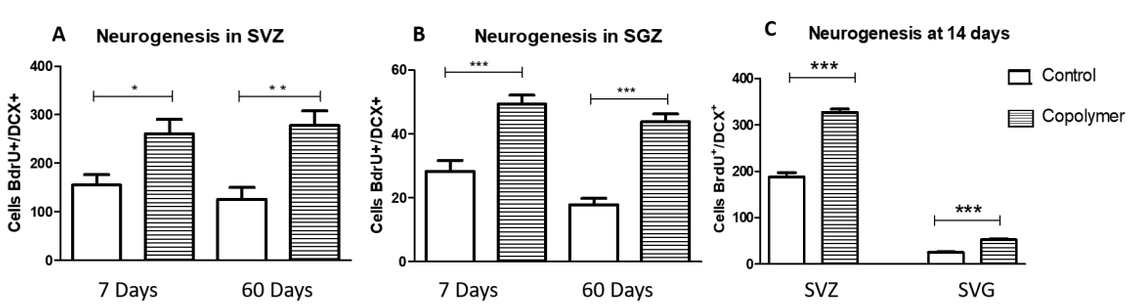

Figure 2.
*Effect of copolymer-1 on neurogenesis at 7, 14, and 60 days. (A) Neurogenesis in SVZ at 7 and 60 days. (B) Neurogenesis in SGZ at 7 and 60 days. (C) Neurogenesis in SVZ and SGZ at 14 days. n = 8 in A and B. n = 5 in C. Each bar represents mean ± SEM. Two-tailed Mann-Whitney U test. Dunn's post hoc multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001. SVZ: Subventricular zone and SGZ: Subgranular zone.*

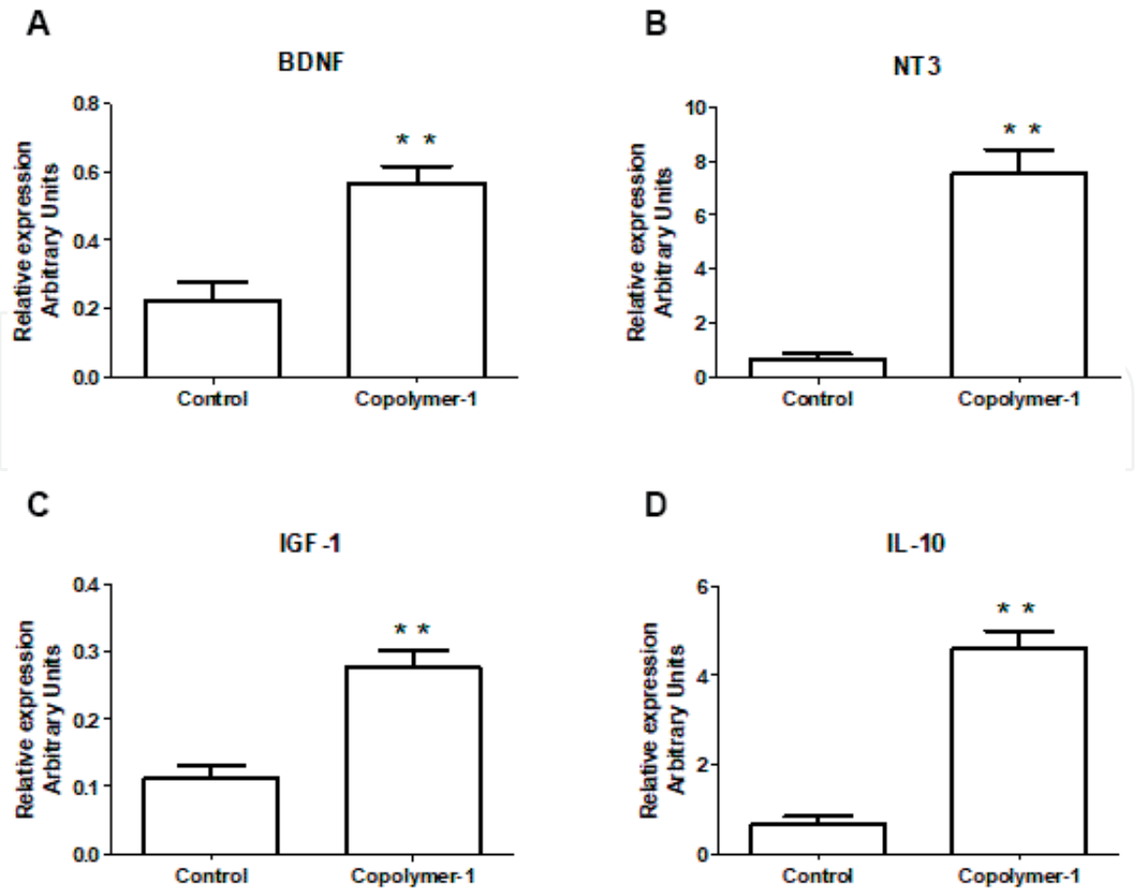

Figure 3.
*Effect of the copolymer-1 on the expression of growth factors and IL-10. Gene expression of: (A) BDNF; (B) NT-3; (C) IGF-1; and (D) IL-10. Bars represent mean ± SEM of 5 rats from each group. *p < 0.05, **p < 0.001. Mann-Whitney U test. Dunn's post hoc multiple comparison test.*

Both growth factors and IL-10 have been reported to be directly involved in the stimulation of SVZ and SGZ stem cells; specifically, IL-10 has been observed to induce stem cell proliferation but not differentiation in primary cultures [84]. Moreover, IL-10 has immunomodulatory capacity as it inhibits the synthesis and release of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β that are known to affect neurogenesis [85]. Moreover, growth factors such as NT-3 maintain viable stem cells from neurogenic niches facilitating plasticity [86]. BDNF promotes the proliferation and survival of neuroblasts [87] and IGF-1 promotes stem cell differentiation and migration of neuroblasts [88]. Therefore, this investigation allowed to demonstrate that Cop-1 is capable of raising the expression of IL-10 and growth factors, which have beneficial effects on neurogenesis.

In order to know if Cop-1 modulates the number of leukocytes in CP and to know if these intervene in the synthesis and release of growth factors and IL-10, we evaluated the cell types present in the cerebrospinal fluid in animals submitted to tMCAO and Cop-1 therapy. The results showed a significant increment in CD8+ T cells, which positively linked with the increase in growth factors and IL-10 [unpublished data].

The increase in CD8+ T lymphocytes has been observed as an effect of copaxone immunization in patients with MS [46]. In addition, experiments performed in the EAE model have considered these cells indispensable for the development of the beneficial effect of Cop-1 [89]. However, it is necessary to identify the nature of these cells and whether the type of CD8 T lymphocytes is of a regulatory phenotype.

Finally, the combination of Cop-1 with other strategies like polyunsaturated fatty acids has shown optimistic results as together, they have a greater capacity to significantly reduce the size of the infarction in the tMCAO model [unpublished data].

6. Conclusion

The existing evidence of the effect of Cop-1 in the tMCAO model has been very encouraging, as it shows a significant neurological recovery. This beneficial effect could be caused by modulatory mechanisms that allow the increase of IL-4 and the reduction of TNF-α and IL-1β at the lesion site, promoting then neuroprotection. Additionally, neurological recovery could also be reinforced by the changes induced by Cop-1 at the CP as the increase of IL-10 and growth factors in this site stimulate neurogenesis after ischemia. We consider that more investigations are needed in order to analyze in greater detail the mechanism by which Cop-1 acts so that in the medium term, it may be considered as a pharmacological alternative for patients suffering from a cerebrovascular event.

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