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Copolymer-1 (Cop-1) improves neurological recovery after middle cerebral artery occlusion in rats

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Abstract

The damage in ischemic stroke is caused by two events: (i) the ischemic phenomenon by itself; (ii) the self-destructive mechanisms developed as a consequence of ischemia. The inflammatory response is one of these destructive phenomena that accompanies and exacerbates the developing injury. Since it has been suggested that immune cells participate in neuroprotective and restorative processes, modulation rather than elimination of this inflammatory response could be a strategy to improve the neurological outcome. The immune modulator copolymer-1 (Cop-1), a synthetic basic random copolymer of amino acids, is a potent inducer of Th2 regulatory cells which, aside from exerting modulatory actions, is capable of releasing neurotrophic factors. There is evidence that Cop-1-specific T cells exert neuroprotective and even restorative effects in diverse neurodegenerative diseases. In order to test the ability of Cop-1 to prevent ischemic injury in a model of transient middle cerebral artery (MCA) occlusion, two groups of rats were treated either with Cop-1 or with saline solution (SS). Seven days after occlusion, Cop-1 treated rats presented a significant improvement in neurological function compared to SS-treated animals (1.2 ± 0.4 and 2.8 ± 0.5 mean $\pm$ S.D., respectively; $p = 0.008$). Histological findings showed that the percentage of infarct volume was smaller in Cop-1 treated rats (4.8 ± 1.5), in comparison with those receiving SS (32.2 ± 8.6; $p = 0.004$). Cop-1 constitutes a promising therapy for stroke; thereby, the enforcement of further experimental investigation is encouraged in order to be able to formulate the best strategy.

Keywords: Copaxone; Focal cerebral ischemia; Glatiramer acetate; Middle cerebral artery occlusion; Neuroprotection; Protective autoimmunity

Central nervous system (CNS) injury causes diverse neurological disabilities that partially or totally impair the physical, emotional, and economical stability of patients. Therefore, pathologies involving CNS damage are, at the moment, the goal of numerous experimental studies. In this context, stroke is perhaps one of the most studied neurological diseases.

After artery occlusion, the ischemic process activates a wide number of other destructive phenomena (i.e. excitotoxicity, apoptosis, inflammation, etc.) which contribute to increase tissue damage [11]. These mechanisms are currently the target of diverse therapeutic interventions; however, although experimental data have provided encouraging results, until now, the only treatment for acute ischemic stroke that has a proven efficacy is the administration of tissue recombinant plasminogen activator (tPA) within 3 h of onset. Nevertheless, only a small percentage of patients are eligible for this therapy. That is why, a wide range of other therapies are now being evaluated.

Since inflammatory response is one of the most implicated self-destructive mechanisms, several strategies are being evaluated to diminish its harmful effect.

Copolymer-1 (Cop-1), is a synthetic amino acid that consists of four amino acids: L-alanine, L-lysine, L-glutamic acid, and L-tyrosine in a fixed molar residue ratio of 6.0:1.9:4.7:1.0. Cop-1 has demonstrated to suppress experimental allergic encephalomyelitis (EAE), an autoimmune neurological disease whose pathophysiology is characterized by a strong inflammatory response. EAE is induced by myelin basic protein (MBP) and represents the animal model for multiple sclerosis [17,23,24].

The basis for the biological activity of Cop-1 lies in its immunological cross-reactivity with MBP which was estab-

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lished at the level of T cell-mediated immunity [6]. Cop-1 was found to induce suppressor T cells specific to MBP that alleviate the symptoms of EAE and block the *in vitro* response to MBP of murine T cell lines and clones [22]. Cop-1 administration has shown to exert neuroprotective actions in other animal models of CNS damage, specifically in those where the immune response has been proposed as one of the factors contributing to the neurodegenerative process [15,16]. In such cases, the modulation of the immunological reaction is the main mechanism by which Cop-1 exerts its protective action.

Since in stroke the immunological response also plays an important role in the destructive/restorative processes developed after ischemia, the activation of a Cop-1-specific response could also favorably modulate the immunological reaction to promote a better neurological recovery.

In order to test this hypothesis, 12 Sprague–Dawley male rats weighing 350 g each, were subjected to cerebral ischemia. The ischemic procedure was performed by using the middle cerebral artery occlusion (MCAO) model reported by Longa et al. [19]. For a brief time, animals were anesthetised with 3% halothane using a face mask. Body temperature was maintained at 37 °C with a warm pad during the surgical procedure and afterwards until their recovery from the anesthesia. Before surgery and 30 min after reperfusion, the mean arterial blood pressure (MABP), blood partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), and pH were measured.

The right common carotid artery (CC), external carotid artery (EC), and internal carotid artery (IC) were exposed through a midline incision. The EC was ligated, coagulated, and cut. The IC was then isolated to avoid damage to the vagus nerve.

A 3-0 monofilament nylon suture (Ethicon, Johnson and Johnson, Mexico City, Mexico) with a flame-rounded head was inserted through the IC via a small incision in the external carotid artery stump. The distance from bifurcation of the CC to the tip of the suture was approximately 18 mm in all rats, consistent with published descriptions of the MCAO model.

To verify MCAO severity, regional cerebral blood flow was determined by laser-Doppler flowmetry (Moor Instruments, England) using a flexible 0.5-mm fiber optic extension connected to the master probe fixed to the intact skull over the ischemic cortex. A reduction in regional cerebral perfusion of 85% or more from the baseline value was taken to indicate the achievement of focal ischemia.

After a 2 h occlusion the suture was withdrawn and cerebral blood flow recovered. The skin was sutured and the rats were allowed to recover. Thirty minutes after reperfusion, six animals, selected at random manner, were injected with 200 μg of Cop-1 (Sigma, St. Louis, MO) dissolved in saline solution (SS) and emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 5 mg/ml of *Mycobacterium tuberculosis* H37 RA (Difco).

The other six rats were only injected with SS emulsified in CFA. The emulsion (total volume 0.15 ml) was injected intramuscularly into the flank.

Rats were supplied by the Animal Breeding Center of Camina Research Project. The rats were age-matched and housed in a light and temperature-controlled room. Efforts were made to minimize the number of animals used and their suffering. All procedures were in accordance with the National Institutes of Health (US) Guide for the Care and Use of Laboratory Animals and the Mexican Official Norm on Principles of Laboratory Animal Care.

Animals were tested for neurological deficits 24 h and 7 days after reperfusion by an observer blind to the treatment. The evaluation was performed using the neurological scale proposed by Longa et al. [19]. Briefly, 0, no deficit; 1, failure to extend left forepaw fully; 2, circling to the left; 3, failing to the left; 4, no spontaneous walking with a depressed level of consciousness. To analyze the size of infarct area, rats were anesthetized 7 days after ischemia, and perfused via the ascending aorta, with 50 ml physiological saline, followed by 500 ml 10% formaldehyde, using a peristaltic pump at 30 ml/min.

The brains were removed, placed in the same fixing solution for 24 h and then cryoprotected in a 30% sucrose solution for at least 3 days. Three coronal cryosections 25-μm thick, were cut every 200 μm and collected onto gelatin-coated slides.

To determine the area of lesion, 30 evenly spaced sections of the brain were observed by a pathologist blind to the treatment group after staining them with hematoxylin–eosin.

A computer image analysis system (Image-Pro 3D Discovery) was used for the evaluation. In order to correct the influence of edema, infarct area in each slice was calculated as follows: measured infarct area × (total contralateral hemisphere area/total ipsilateral hemisphere area). Infarct volumes of each rat were then computed by integrating infarct areas of sequential brain sections.

Total ischemic volume in the ipsilateral hemisphere was determined in percentage of the volume of the contralateral (control) hemisphere.

All data were analyzed using the Mann–Whitney *U*-test. Statistical significance was considered relevant when $p \leq 0.05$.

There were no significant differences in MABP, pH, pO_2, and pCO_2 between pre-and post-MCAO (30 min after reperfusion), physiological parameters (see Table 1).

Twenty four hours after MCAO, animals showed a significant functional deterioration which was similar in both groups (Cop-1-treated: 3.0 ± 0.7, mean ± S.D. and SS-treated: 3.2 ± 0.4; Fig. 1). Nevertheless, seven days after ischemia, Cop-1 significantly improved the neurological outcome of MCAO-animals (1.2 ± 0.4) as compared to SS therapy (2.8 ± 0.5; $p = 0.008$).

Table 1
Comparison of blood parameters of rats subjected to MCAO

Parameters	Groups	Pre-MCAO	Post-reperfusion (30 min)
pH	MCAO + Cop-1	7.35 ± 0.7	7.34 ± 0.5
	MCAO + SS	7.31 ± 0.6	7.36 ± 0.3
pO_2	MCAO + Cop-1	159.90 ± 10.3	148.78 ± 9.2
	MCAO + SS	163.35 ± 14.7	145.89 ± 11.2
pCO_2	MCAO + Cop-1	48.34 ± 5.8	51.72 ± 6.8
	MCAO + SS	47.98 ± 7.3	50.94 ± 8.4
MABP	MCAO + Cop-1	105 ± 14.7	117 ± 10.3
	MCAO + SS	113 ± 12.6	120 ± 14.8

Values are mean ± S.D. of six rats per group.