

Research Article



Effects of Policosanol Pre-treatment on Blood-brain Barrier Damage Induced by Ischemia-reperfusion in Rats

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ABSTRACT

Blood-brain barrier (BBB) disruption following ischemia-reperfusion (I/R) is associated with poor outcomes in stroke patients. Policosanol, a mixture of sugarcane wax alcohols, has been shown to protect against cerebral functional and histological disturbances induced by I/R in gerbils. Nevertheless, the effects of policosanol on experimentally induced BBB disruption have not been explored. The objective of this study was to investigate the effects of policosanol on BBB disruption following the induction of cerebral I/R in rats. Rats were randomized into a negative vehicle control and five I/R groups: a positive vehicle control, three policosanol (50, 200 and 400 mg/kg), one aspirin (150 mg/kg). Treatments were given orally 1 h before ischemia induction. Brain ischemia was induced by 30 min bilateral occlusion of carotid arteries, followed by 24 h reperfusion. BBB permeability was evaluated according to Evans blue (EB) dye extravasation. Also, myeloperoxidase (MPO) activity in brain homogenates was measured. EB concentration and MPO values after 24 h of reperfusion were significantly increased in positive controls when compared with the negative controls. Oral pre-treatment with policosanol (50 - 400 mg/kg) significantly decreased EB extravasation (60.4% - 71.7%) as compared to the positive controls. Aspirin reduced significantly BBB leakage by 92.5%. Also, the I/R-induced increases of MPO activities were lowered significantly with policosanol (50 - 400 mg/kg) (46.3% - 65.3) and aspirin (68.4%). In conclusion, oral policosanol pre-treatment (50 - 400 mg/kg) protected against BBB damage induced by cerebral I/R in rats, and attenuated the increase of MPO, a marker of inflammation, in the brain tissue.

Keywords: Aspirin, Blood-Brain Barrier, Brain Ischemia, Myeloperoxidase, Policosanol.

INTRODUCTION

Ischemic stroke represents the leading cause of disability, the second of dementia and the third of death worldwide in adults,^{1,2} resulting from a transient or permanent reduction in cerebral blood flow in a major brain artery.³ The interruption of blood and oxygen supply may affect the whole brain (global ischemia) or specific brain territories (focal ischemia) in dependence of the brain artery occluded.^{4,5}

The pathogenesis of acute neuronal injury in ischemic stroke involves multiple mechanisms, including inflammatory responses in the brain,⁶ which starts with the movement of leukocytes across the blood-brain barrier (BBB) prior entering the parenchyma.⁷ Under normal conditions, the BBB protects the brain against harmful insults. Ischemic damage, however, makes the BBB more permeable which allows that deleterious stimuli may move across the BBB.⁸ Indeed, BBB disruption is a signature of brain ischemia with or without reperfusion that has been linked with vasogenic edema, hemorrhage and poor outcomes in stroke patients.^{9,10}

The pathogenesis of brain ischemia that accompanies the BBB injury involves the excessive release of excitatory neurotransmitters that triggers the metabolic cell death chain, and other factors induce secondary reactions like the quick release or activation of microglia mainly resident cells, inflammatory and/or immune responses, free radicals, eicosanoids and lipid degradation products in the ischemic brain tissue.¹¹

There are several models of brain ischemia that may be classified into models of focal and global brain ischemia.^{12,13} The widely used rat middle cerebral artery occlusion (MCAO) stroke model has demonstrated a biphasic opening of the BBB after ischemia/reperfusion (I/R), with an early increase in BBB opening after 2–3 h of reperfusion following MCAO,¹⁴ after which the BBB restores its functions and remains stable until 24 to 48 h after reperfusion, followed by a drastic BBB breakdown. Although these earlier reports pointed out a biphasic opening, discrepancies occurred among what happens at to the second opening,^{15,16} so that serial studies of BBB leakage after cerebral induction of I/R in rats have shown that following transient focal cerebral ischemia, BBB leakage is continuous and during 1 week post I/R no BBB closure occurs.¹⁶

On its side, the injury induced by the bilateral occlusion of common carotid arteries and further reperfusion in rats, a model of global transient brain ischemia, is also commonly used to assess the effects of anti-ischemic strategies, in which the conditions of I/R induce a remarkable functional and structural damage.¹⁷⁻¹⁹

Policosanols, a mixture of eight higher aliphatic alcohols purified from sugarcane wax, has been shown to produce antiplatelet effects in rodents,²⁰ and to protect against focal and global cerebral ischemia induced by carotid ligation in gerbils.²¹⁻²⁵ A recent study demonstrated that policosanol inhibits cyclooxygenase (COX)-1 enzyme activity *in vitro*,²⁶ an effect that may support its antiplatelet



action and a potential effect on inflammatory events. Despite previous studies have shown the protective and therapeutic effects of oral treatment with policosanol against experimental brain ischemic injury in rodents,²¹⁻²⁵ none had explored, however, its effects on BBB integrity.

In light of these issues, this study was undertaken to investigate the effect of policosanol oral pre-treatment on BBI disruption following the induction of cerebral I/R in rats. Also, policosanol effects on myeloperoxidase (MPO) activity in brain homogenates were also assessed as a preliminary approach to explore its effects on inflammatory markers.

MATERIALS AND METHODS

Animals

Male Wistar rats (180 – 200g), acquired in the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba), were quarantined and adapted to laboratory conditions (22 ± 2°C of temperature, 60 ± 5% of relative humidity, 12:12 hour light/dark cycles) for 7 days. Animals were provided free access to tap water and lab chow (rodent pellets from CENPALAB).

Experiments were conducted according to the Cuban guidelines for Animal Handling and the Cuban Code of Good Laboratory Practices (GLP). The independent ethical board of the centre approved the use of animals and the study protocol.

Administration and dosage

The policosanol batch (Plants of Natural Products from the National Centre for Scientific Research), used after corroborating its quality specifications, had the following composition: tetracosanol (0.17%), hexacosanol (5.5%), heptacosanol (0.98%), octacosanol (61.2%), nonacosanol (0.51), triacontanol (14.4%), dotriacontanol (7.3%) and tetratriacontanol (1.9%) (purity 91.0%). Aspirin was supplied by the Chemical Pharmaceutical Industry (QUIMEFA) (La Habana, Cuba). For dosing both policosanol and aspirin were suspended in 1% acacia gum vehicle

Rats were randomized into 6 groups (10 rats/group): a negative vehicle control and five subjected to I/R: a positive vehicle control, three treated with policosanol (50, 200 and 400 mg/kg), one with aspirin (150 mg/kg). All treatments were given by gastric intubation (5 mL/kg bodyweight) 1 h before ischemia induction. The doses of policosanol chosen for the study are within the range reported to protect effectively against cerebral ischemia in gerbils studies.²¹⁻²⁵

Induction of global cerebral ischemia by ischemia and reperfusion (I/R)

Transient global cerebral ischemia was induced in the rats by the occlusion of both common carotid arteries for 30 min under anaesthesia with sodium thiopental (ip). A ventral midline incision was made and carotid arteries were exposed, carefully separated from the vagus nerve,

occluded bilaterally for 30 min with micro-vascular clamps, and then carefully removed to allow reperfusion for 24 h. The negative control group was submitted to the same procedure, except to the occlusion of common carotid. Reflux was verified by visual inspection of blood flowing past the point of occlusion.

First experiment: evaluation of blood-brain barrier (BBB) leakage with Evans blue extravasation

One (1) hour before the rats were euthanized, 2% Evans blue (Sigma) in normal saline was injected into the femoral vein (4 mL/kg) of each animal. All rats were anesthetized and perfused with normal saline (≅ 200-250 ml) until the perfusion fluid was colorless. Brains were removed and 2 mm sections were taken from each hemisphere and incubated with 5 mL of formamide in water bath (60 ° C) for 24 hours. The samples were centrifuged at 12000 rpm for 10 min. The absorbance was measured at 620 nm in the supernatant. The concentration of Evans blue was determined by using a standard curve of the dye.²⁷

Second experiment: measurement of myeloperoxidase (MPO) in rat brain tissue

Immediately after the sacrifice blood samples from the abdominal aorta were taken, and brains were dissected and then frozen at -20 ° C up to use.

MPO activity was quantified according to the procedure described in the Worthington manual.²⁸ For this purpose, rat brains were homogenized in 50 mmol/L phosphate buffer (pH = 6) containing 0.5% hexadecyl trimethylammonium bromide (HTBA) (100 mg of tissue/mL buffer), sonicated for 10sec, frozen and thawed (-20 to - 30 ° C) for 3 successive times. Upon completion of the final thaw, samples were centrifuged at 12000 rpm for 25 min. at 4 ° C and MPO was quantified in the supernatant. To this, 625 µL of 50 mmol/L phosphate buffer (pH = 6) containing 0.167mg/mL of O-dianisidine dihydrochloride were mixed with 250 µL of the sample and 125 µL of 0.0005% hydrogen peroxide. Finally, the changes in absorbance at 460nm occurred during the next 2 min were followed in a spectrophotometer. MPO activity is referred as units (U)/g tissue, one MPO U being defined as the degradation of a 1 µmol of peroxide/min at 25 ° C.

All the analyses described below were carried out in triplicate.

Statistical analyses

All results are expressed as mean ± SD. For comparison between two groups, statistical significance was determined through the non parametric Mann-Whitney U tests. For comparison among multiple groups, statistical significance was evaluated with the Kruskal Wallis test. A probability value of p<0.05 was considered significant. Data were processed with the statistical software Statistics for Windows (Release 4.2 Stat Soft Inc, Tulsa OK,



US). Dose-effect relationships were assessed by using the dose regression linear analysis of the “Primer of

Biostatistics” (Version 3.01, 1992 McGraw-Hill, Inc).

Table 1: Effect of treatments on cerebral Evans Blue extravasation and MPO activity in brain tissues of I/R rats

Treatments	Damage index to the BBB (ng Evans blue/mL)	I (%)	MPO (U/g de tissue)	I (%)
Negative control (vehicle)	1.16 ± 0.05 **		0.072 ± 0.01 ***	
Positive control (vehicle + I/R)	1.69 ± 0.06		0.225 ± 0.03	
Policosanol (50 mg/kg + I/R)	1.37 ± 0.06 **	60.4	0.154 ± 0.01 *	46.3
Policosanol (200 mg/kg + I/R)	1.31 ± 0.02 **	71.7	0.130 ± 0.01 **	62.1
Policosanol (400 mg/kg + I/R)	1.31 ± 0.04 **	71.7	0.125 ± 0.01 **	65.3
Aspirin (150 mg/kg + I/R)	1.20 ± 0.01 **	92.5	0.120 ± 0.01 **	68.4

BBB blood brain barrier, MPO myeloperoxidase I Inhibition, I/R ischemia/reperfusion.

Data expressed as Mean ± SD; *p<0.05; **p<0.01; ***p<0.001 Comparison with the positive control (Mann Whitney U test)

RESULTS

Table 1 summarizes the data of the experiments. EB extravasation after 24 h of reperfusion was significant in positive controls when compared with the negative control group ($p < 0.01$), an effect that was significantly attenuated by single oral doses of policosanol (50, 200 and 400 mg/kg) (60.4%, 71.7% and 71.7%, respectively) as compared to the positive control group. Acute oral administration of aspirin significantly and markedly (92.5%) decreased the BBB damage as compared to the positive control.

The activity of MPO in the brain tissue of the positive control rats was significantly increased as compared to the negative control group. Such increase was significantly reduced by oral acute pre-treatment policosanol (50, 200 and 400 mg/kg) (46.3%, 62.1% and 65.3%, respectively) or aspirin (68.4%).

DISCUSSION

This study demonstrates, for the first time, that oral pre-treatment with policosanol attenuates BBB disruption and the increase of brain concentrations of MPO in rats with transient global brain ischemia induced by bilateral occlusion of carotids and further reperfusion.

The transient brain hypoperfusion induced experimentally in rats by bilateral carotid artery occlusion and further reperfusion causes microvascular damage characterized by several alterations, like the disruption of the BBB and the increase in brain inflammatory mediators among other disturbances.²⁹ Consistently with such report, our IR-challenge positive control rats exhibited the characteristic EB extravasation that occurs in BBB damage induced by I/R in rats, and supports the usefulness of this model for assessing the effect of policosanol on BBB disruption in the brain of ischemic rats.

BBB destruction has been implicated in many central nervous system diseases, including the stroke,³⁰ and such process is accompanied by the increased expression of matrix metalloproteinases (MMPs), enzymes whose

functions, among others, include the induction of inflammation processes.³¹

We then quantified the extravasation of EB dye into the brain as an indicator of BBB permeability. Our study showed that EB leakage was increased in the positive controls, which demonstrates that BBB integrity is compromised in the transient global brain ischemia induced by I/R in rats, and that policosanol oral pre-treatment protects the BBB. Indeed, policosanol (50 – 400 mg/kg) prevented the extravasation of BE up to 71.7% versus the positive controls, which seems to be the ceiling inhibition, achieved with 200 mg/kg, since 400 mg/kg did not produce a greater effect.

The effect of aspirin on EB leakage here observed is coherent with its ability to reduce cerebral ischemic injury. Oral aspirin 30 mg/kg given for 7 days prior to inducing brain ischemia in diabetic rats subjected to permanent MAOC reduced the infarct size, neurological deficit and platelet aggregation without modify blood glucose and insulin levels, which suggests that aspirin attenuated cerebral ischemic injury through the decrease of platelet aggregation.³² Other study demonstrated that the neuroprotective effects of aspirin on rats with cerebral I/R induced by MAOC might be attributed to its effects by increasing the ratio of prostacyclin (PGI₂) to thromboxane (TXA₂) and reducing lipid peroxides.³³ Also, aspirin reduced the BBB impairment, early mortality and superoxide production, and the decrease of NO synthase activity in the basilar arteries of salt-loaded stroke-prone rats, which suggested that aspirin may exert protective effects against cerebrovascular inflammation and damage by salt loading through down-regulation of superoxide production and induction of nitric oxide synthesis.³⁴

Like aspirin, policosanol has been shown to produce antiplatelet effects and to increase the PGI₂/TXA₂ ratio,^{20,22} and to produce antioxidant effects in a model of brain ischemia in gerbils,²⁴ so we may hypothesize that these actions should explain the ability of policosanol for lowering the BBB disruption here seen. Nevertheless, since we did not assess these effects, we cannot



demonstrate which mechanism(s) was/were the responsible(s) of the effect of policosanol on BBB integrity.

Stroke triggers an inflammatory response that continues for hours after its onset, which plays a key role in the pathogenesis of neuronal damage in ischemic stroke. Inflammatory events contribute to the late stages of ischemic damage and to trigger poor neurological outcomes through different mechanisms.¹¹

Our results showed that policosanol reduced MPO activity in ischemic cerebral tissue since all doses of policosanol (50 – 400 mg/kg) inhibited the I/R-induced increase of MPO activity (46.3 – 65.3%) in the brain tissue. The dose of 200 mg/kg elicited the maximal inhibition, since that reached with 400 mg/kg was practically the same. These results then indicate that policosanol produced anti-inflammatory effects since MPO activity is a well known enzymatic mediator of several inflammatory cascades.

The inhibition of MPO activity in brain homogenates of ischemic rats indicates that policosanol exhibits an anti-inflammatory effect *in vivo*, besides its inhibitory effect on COX-1 activity *in vitro*,²⁶ which also agrees with the ability of long-chain fatty alcohols to inhibit the release of different pro-inflammatory mediators by cells involved in inflammatory processes.³⁵ The magnitude of the inhibition of MPO ($\cong 65\%$) was near to the reduction of BE extravasation ($\cong 72\%$) achieved with policosanol, which grossly suggests that protective effect of policosanol on BBB integrity correlated with the inhibition of the inflammatory response.

In addition, MPO has been considered an index of neutrophil infiltration that is highly expressed in cerebral ischemia after 24 h, and a correlation between neutrophil infiltration and infarct formation has been demonstrated in a model of cerebral ischemia.³⁶ This suggests that the protective effects of policosanol on BBB integrity here shown might involve a reduction in neutrophil infiltration.

The effect of aspirin on MPO activity also might includes this effects, but in this case the effect on MPO activity was obviously lower ($\cong 68\%$) than its effects on BE leakage ($\cong 93\%$), which indicates that events others than neutrophil infiltration may contribute greatly to its protective effect.

The effects of policosanol on BBB disruption and brain MPO in ischemic-reperfused rats here seen could be considered as beneficial in terms of brain ischemic injury and coherent with its anti-ischemic effects on I/R-induced cerebral ischemia in gerbils.^{21 - 25} Nevertheless, although several agents investigated in animal models of cerebral ischemia have been found to reduce structural and functional injuries, the translation of such benefits from the experimental scenario to the stroke clinics has not been successful, which have lead to accept with caution promising results and to update preclinical recommendations for experimental stroke models.³⁷

The good thing is that previous clinical studies, two of them placebo-controlled, have demonstrated that added to conventional aspirin therapy, oral treatment with policosanol post-stroke has been beneficial for the neurological recovery of patients with ischemic stroke.^{38 - 40} Then, although these studies have not been conducted in acute stroke therapy, they provide enough rationale to continue studies on the effects of policosanol on ischemic stroke. The present study just adds new knowledge about the actions of policosanol for preserving BBB integrity after experimental I/R processes, and to ameliorate inflammatory events that happen after the cerebral assault. Further research is required to elucidate the mechanism whereby policosanol produces these effects, and in particular, its effect on metalloproteinases activity merits extensive investigation.

CONCLUSION

Oral policosanol pre-treatment (50 – 400 mg/kg) protected against BBB damage induced by cerebral I/R in rats, and attenuated the increase of MPO, a marker of inflammation, in the brain tissue.

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