Research Article

Therapeutic Effects of Policosanol and Omega-3 Fish Oil against Global Brain Ischemia-reperfusion in *Mongolian gerbils*.

Vivian Molina Cuevas*, Yazmín Ravelo Calzado, Licet Mena Valdes, Miriam Noa Puig, Yohani Pérez Guerra, Ámbar OyarzábalYera, Maikel Valle Clara, Sonia Jiménez Despaigne.
Center of Natural Products, National Centre for Scientific Research, Havana, Cuba.
*Corresponding author's E-mail: vivian.molina@cnic.edu.cu

ABSTRACT

Stroke is the third cause of death, the second of dementia and the first of disability. Policosanol and omega-3 fish oil (FO) haveshown protective effects on ischemic stroke in experimental and clinical studies. A previous comparative study compared the preventive effects of policosanol and FO on global cerebral ischemia in *Mongolian gerbils*, showing greater protective efficacy of policosanol. This study was aimed to compare the therapeutic effects of policosanol and FO in the global cerebral ischemia (GCI) model induced by ischemia-reperfusion (I/R) model in *Mongolian gerbils*. Gerbils were randomized distributed in 7 groups: a negative control group and 6 with GCI: a positive control (vehicle), policosanol (100 and 200 mg/kg), FO (1.25 and 2.5 g/kg) and aspirin (60 mg/kg). All treatments were given after 1 hour of reperfusion initiation and during the following 7 days' post-ischemia. Policosanol (100 and 200 mg/kg) and FO (1.25 and 2.5 g/kg) markedly and significantly reduced neurological symptoms score assessed at 1, 3 and 7 days of induced bilateral ischemia, without statistical differences between both treatments, although greater percentages of inhibition with policosanol were reached. Policosanol (100 and 200 mg/kg) moderately and significantly reduced the histological score of brain damage (35.9 and 43.9% inhibition, respectively), while only the highest tested dose of FO (2.5 g/kg) produced a modest and significant reduction (19.9%), being significantly more effective the policosanol treatment. Policosanol (100 and 200 mg/kg) and FO (1.25 and 2.5 g/kg) markedly and significantly reduced the plasma concentrations of MDA and SH groups in *Mongolian gerbils* with GCI, without significant differences between both treatments, although policosanol produced a slightly higher inhibition percentage on both variables assayed. In conclusion, therapeutic repeated dose of policosanol (100 and 200 mg/kg) and FO (1.25 and 2.5 g/kg) moderately and significantly reduced GCI in *Mongolian gerbils,* with greater policosanol efficacy.

Keywords: Ischemic stroke, policosanol, omega-3 fish oil, therapeutic treatment.

INTRODUCTION

Ischemic stroke is a complication of atherosclerosis consisting of a neurological deficit that is associated with ischemic damage on CA1 neurons of the hippocampus.[1,2] Its most frequent cause is the sudden occlusion of a blood vessel through a thrombus or embolism which leads to the immediate loss of oxygen and glucose to the brain tissue.[3]In addition, its pathogenesis involves other factors such as the release or activation of free radicals, eicosanoids, lipid degradation products, inflammation and/or immune response which may act after the primary ischemic insult either sequentially or in parallel to cause cell death.[3,4]

This disease is very frequent in the adult world population constituting the third cause of death, the second of dementia and the first of disability, and therefore represents a major health problem.[5-8] It is the most frequent of the different types of stroke and it is the most frequently leads to disability.[9]

Thus, from 15 to 30% of patients who survive suffer permanent ischemic stroke disability (partial paralysis and difficulties with memory, reasoning, language and movements), so many require care in medical institutions.[10, 11]Therefore, as part of the management of ischemic stroke one of the most important strategies is to achieve a recovery that reduces the resulting disability. In this sense, the first-line pharmacological therapy has been the use of antiplatelet agents,[12-14] which have only reduced the recurrence of ischemic events by 15-20%[15, 16] and produce adverse effects such as bleeding of lower or higher amount.[17]

Another alternative treatment has been the use of HMG CoA reductase inhibitors, such as atorvastatin, to reduce the risk of ischemic stroke in patients who have previously suffered a stroke[18] or a transient ischemic attack (TIA),[19] although its use is associated to a slight increase in hemorrhagic stroke.[20]

In addition, neuroprotective [21] and antioxidants agents [22] have shown promising effects in experimental studies of ischemia, regrettably not confirmed in clinical studies of ischemic stroke.[23-25]

Taking into account that ischemic stroke therapy is a key factor in the management strategy of the disease and that pharmacological treatments have not resulted in relevant clinical advances, the search for new substances with potential therapeutic effect on ischemic stroke is a current problem.

In this context, different studies have documented the therapeutic effects of fish oil (FO),rich in omega-3 fatty acids [Eicosapentaenoic acid (EPA) and docosahexaenoic

acid (DHA)] on experimentally induced ischemic stroke as well as in the secondary prevention of stroke patients.[26-28] Thus, there is evidence of an inverse correlation between plasma levels of these acids and the development of stroke.[28,29]

The most commonly used experimental models for the evaluation of substances with a potential beneficial effect on ischemic stroke include the use of *Mongolian gerbils* (*Meriones unguiculatus*), species sensitive to ischemia because it presents anatomical abnormalities in the circle of Willis, [30] and so that temporary bilateral occlusion of their common carotid arteries causes damage to CA1 neurons of the hippocampus.[2, 31]

Policosanol, purified mixture of high molecular weight aliphatic alcohols from sugar cane wax, has lipid-lowering, antiplatelet and antioxidant effects demonstrated in experimental and clinical studies.[32-35]

In addition, previous experimental studies have demonstrated the efficacy of policosanol in models of focal cerebral ischemia in a hemisphere by permanent ligation of a carotid artery and global cerebral ischemia by ligation and reperfusion of both carotids, [36-38] which has been corroborated clinically in patients with ischemic stroke.[39] These anti-ischemic effects of policosanol have been associated with its ability to reduce markers of oxidative stress [38, 40, 41] and inflammation.[42]

Taking into account experimental and clinical evidence of the protective effects of policosanol [36-39] and FO [26-29] on cerebral ischemia, which involve inhibitory effects on platelet aggregation, inflammation and oxidative stress, it is interesting to compare the effects of both substances on the global cerebral ischemia model in *Mongolian gerbils*.

A previous study compared its preventive effects in a model of global cerebral ischemia in *Mongolian gerbils* induced by bilateral ischemia and reperfusion of the common carotid arteries, demonstrating greater efficacy of policosanol on histological damage and equal efficacy on indicators of neurological evaluation and oxidative stress.[43]

The present study aims to compare the therapeutic effects of policosanol and FO in the global cerebral ischemia model induced by ischemia-reperfusion (I/R) model in *Mongolian gerbils.*

MATERIALS AND METHODS

Adult male *Mongolian gerbils* (*Meriones unguiculatus*) (60-80 g), 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 h light/dark cycles) for 7 days. Animals were provided free access to tap water and laboratory chow (rodent pellets from CENPALAB). Experiments were conducted in accordance to the Cuban guidelines for Animal Handling and the Cuban Code of Good Laboratory

Practices (GLP). The independent ethical board of the Center approved the use of animals and the study protocol.

Administration and dosage

Policosanol (batch 031061211) obtained at the Natural Products Production Plant (CNIC, Havana, Cuba) and aspirin (lot: 10091705, Cuban Pharmaceutical Industry, Havana, Cuba) were prepared in a suspension in acacia/water vehicle (1%), while omega-3 fish oil (batch: 117052, Rainbow and Nature Pty Ltd, Australia) was prepared as an emulsion in Tween 65/H_2O (2%).

Once concluded their quarantine, gerbils were randomized into seven groups: a negative control group without global cerebral ischemia receiving only vehicle, and six groups with I/R-induced global cerebral ischemia: a positive control treated with the vehicle, two groups treated with policosanol (100 and 200 mg/kg), two with FO (1.25 and 2.5 g/kg) and one with aspirin (60 mg/kg).

All treatments were given after 1 hour of reperfusion initiation and during the following 7 days post-ischemia (0.5 mL/70 g body weight).

A treatment regimen was selected for 7 days as an increase in oxidative stress and deficiency of CA1 area neurons in the hippocampus was documented at 7 days post-I/R.[44] In addition, the therapeutic efficacy of DHA in rodent cerebral ischemia models has been demonstrated after this period of administration.[26, 27]

Induction of global cerebral ischemia

Induction of global cerebral ischemia was performed 1 h after the last administration of all treatments. Transient global cerebral ischemia was induced in the gerbils by occlusion of both common carotid arteries for 5 min under anaesthesia in halothane atmosphere. A ventral midline incision was made and the common carotid arteries were exposed. A 3 cm length catheter loop was placed around them and the extremities of the catheter were flame-welded. Animals were housed singly after surgery and allowed to recover from anaesthesia for 10 min. Then, the catheter was removed, the arteries dissected and a clamp was placed just behind the catheter preventing blood flow for 5 min. The clamp was removed and gerbil necks were properly sutured. 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.

Neurological assessment

Neurological symptoms were assessed at 1, 3 and 7 days after IR by using a score system wherein higher values corresponded to higher ischaemic insult: 0 (no symptoms), 1 (torso curvature or hair roughed up), 2 (ptosis), 3 (circling behaviour), 4 (splayed-out hind limb) and 5 (seizures). [45]

Plasma oxidative variables

Immediately after the sacrifice, and simultaneously of taking brain samples for the histopathological study, blood samples were drawn from vena cava for assessing plasma oxidative markers.

Assay for plasma lipid peroxidation markers

Plasma products of the lipid peroxidation were determined as thiobarbituric acid-reactive substances (TBARS).[46] In brief, 1 ml of plasma was added to 0.2 ml of 8.1% sodium dodecyl sulphate (SDS) plus 1.5 ml of 20% acetic acid solution adjusted to pH 3.5, 1.5 ml of thiobarbituric acid (TBA) solution and 1 mM butylated hydroxytoluene, heated at 95° for 45 min and cooled. One milliliter of distilled water plus 5 ml of a mixture of n-butanol: pyridine (15:1 v/v) was then added to the mixture, shaken and centrifuged. The organic layer was used for TBARS determination at 535 nm using freshly diluted MDA-bis (dimethyl acetal) as standard. TBARS concentrations were expressed as nmol of MDA/mg of protein. Total plasma protein concentrations were assessed by a modification of the Lowry method. [47]

Plasma protein oxidation markers

Plasma levels of sulphydrile groups (SHG) were assessed as protein oxidation markers by using the 5',5'-dithio-bis (2-nitrobenzoic acid) (DTNB) assay.[48] Briefly, plasma aliquots (50 ml) plus 950 µl of 10 µM DTNB were incubated for 20 min at 25°. A blank with DTNB was run in parallel. The optical densities of the supernatants at 412 nm were measured using a 13,600/cm/M coefficient of absorptivity. The numbers of SHG were expressed in mM.

Histological studies

Brains were extractad and fixed in 10% buffered formaldehyde, dehydrated and paraffin embedded. Brain coronal sections (5 µm) at level of 1.5-1.7 mm posterior to the bregma were taken with a microtome, and stained with haematoxylin and eosin. Then, the sections of each gerbil were examined bilaterally by light microscopy according to Bartus*et al.*,[49] to score the damage of the pyramidal cells in the CA1 area of the hippocampus as follows: 0 (normal stained cells, densely packed, with rounded soma and a well stained central nuclei); 1 (some shrinkage and irregularly shaped cells, with a pale chromatolytic region surrounded by a peripheral rim of cytoplasm), 2 (some apparent cell loss with pyknotic cells areas), 3 (more moderate cell loss and pyknosis), 4 (lack of Nissl substance, indicating depletion of neurons and only occasional neurons among microglia).

Statistical analyses

Comparisons among groups were done with the Kruskal Wallis test; paired comparisons between each treated and control groups with the Mann-Whitney U test. Statistical significance was chosen for α = 0.05. Data were processed with the Statistics Software for Windows (Release 6.1 Stat Soft Inc, Tulsa OK, USA).

RESULTS AND DISCUSSION

The results of the therapeutic effects of policosanol and FO on neurological symptoms assessed at 1, 3 and 7 days of induced global cerebral ischemia in *Mongolian gerbils* are shown in Table 1. Bilateral occlusion of both carotid arteries for 5 min and 24 h of reperfusion produced neurological symptoms in the positive control group with a score that was significantly higher than the negative control group, which had no symptoms at all times evaluated. Oral administration with aspirin (60 mg/kg), the reference substance, markedly and significantly reduced the neurological symptoms score (92.1, 90.4 and 90% inhibition, respectively) at 1, 3 and 7 days respectively, which demonstrates the validity of the model in our experimental conditions.

Table 1: Effects of policosanol and FO on neurological symptoms score in *Mongolian gerbils* with IR- induced global cerebral ischemia.

Treatments	Doses	NS Score (1 day)	I (%)	NS Score (3 day)	I (%)	NS Score (7 day)	I (%)
Negative control (Vehicle)	0	0 ± 0 ***	--	0 ± 0 ***	--	0 ± 0 ***	--
Positive control (Vehicle + I/R)	0	3.18 ± 0.50	--	2.60 ± 0.47	--	2.50 ± 0.47	--
Policosanol + I/R	100 mg/kg	1.55 ± 0.37 *	51.2	1.11 ± 0.35 *	57.3	1.11 ± 0.35 *	55.6
Policosanol + I/R	200 mg/kg	0.88 ± 0.45**	72.3	0.66 ± 0.33**	74.6	0.66 ± 0.33**	73.6
FO + I/R	1.25 g/kg	1.00 ± 0.36 **	68.5	0.90 ± 0.37 *	65.4	0.90 ± 0.37 *	64
FO + I/R	2.5 g/kg	1.30 ± 0.39 *	59.1	1.20 ± 0.41 *	53.8	1.10 ± 0.43 *	56
ASA + I/R	60 mg/kg	0.25 ± 0.25 ***	92.1	0.25 ± 0.25 **	90.4	0.25 ± 0.25 **	90

NS: Neurological symptoms; I (%): inhibition percent; *p<0,05; ** p<0,01; *** p<0,0001 Comparison with the positive control (Mann Whitney U test)

Oral therapeutic administration with policosanol (100 and 200 mg kg) and FO (1.25 and 2.5 g/kg) markedly and significantly reduced neurological symptoms score assessed at 1, 3 and 7 days of induced bilateral ischemia. Thus, the lowest tested dose of policosanol (100 mg/kg) caused inhibitions on neurological symptoms score of 51.2, 57.3 and 55.6% at 1, 3 and 7 days, respectively, whereas the highest dose tested at 200 mg/kg resulted in inhibitions of 72.3, 74.6 and 73.6%, respectively. Treatment with FO (1.25 g/kg) reduced these neurological symptoms score by 68.5, 65.4 and 64% at 1, 3 and 7 days, respectively, while the

higher dose of 2.5 g/kg caused inhibitions of 59.1, 53.8 and 56%, respectively. The statistical comparison between both treatments did not show significant differences, although the percentages of inhibition achieved with policosanol were slightly higher than those obtained with FO.

The results of the histological study are shown in Table 2. The brains of the negative controls showed no alterations (Fig. 1), while all of the brains of the positive controls exhibited disappearance and damage of numerous pyramidal cells of the CA1 region, such as irregularity of the cellular form, pale regions, chromatolytics, with a peripheral ring of cytoplasm and abundant cellular picnosis as shown in Fig.2.
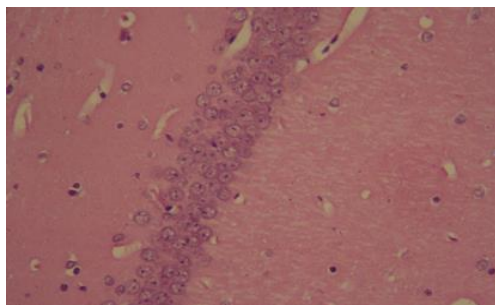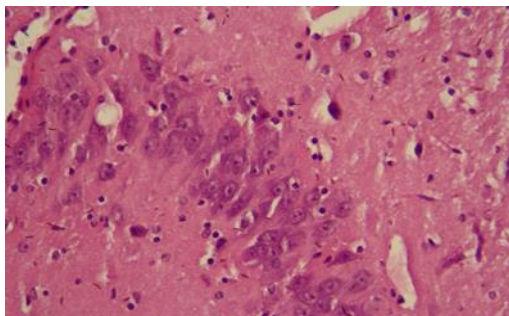

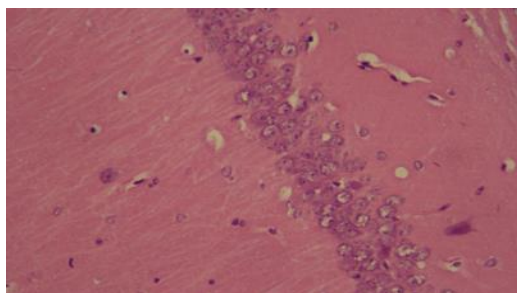**Figure 1:** Negative control

Figure 2: Positive control
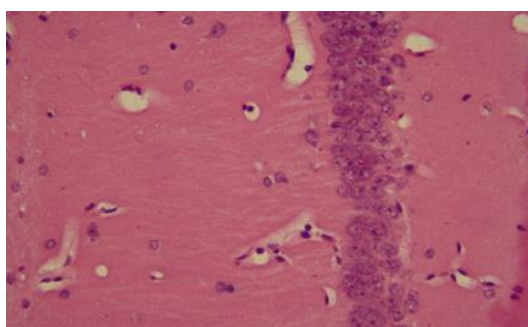

Figure 3: Policosanol 100 mg/kg

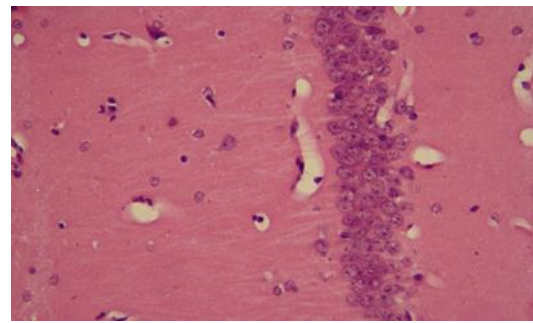

Figure 4: Policosanol 200 mg/kg
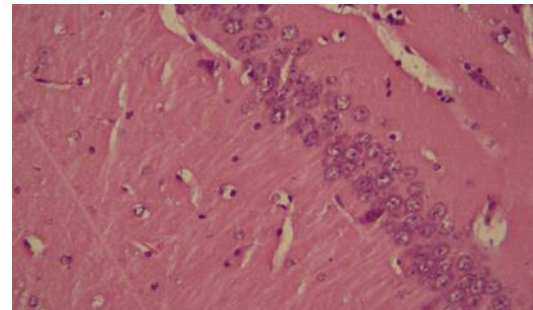

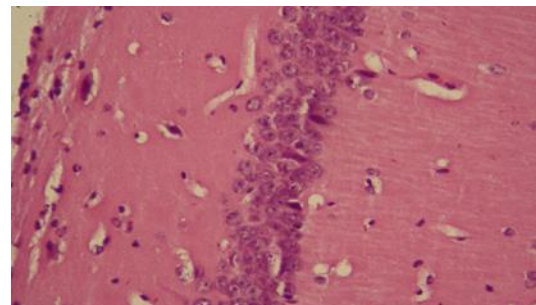**Figure 5:** FO 1.25 g/kg

Figure 6: FO 2.5 g/kg

Figure 7: ASA

Table 2: Effects of policosanol and FO on histological score of brains from *Mongolian gerbils* with IR- induced global cerebral ischemia.

Treatments	Doses	Histological score	I (%)
Negativo control (Vehicle)	0	0 ± 0***	--
Positive control (Vehicle + I/R)	0	3.12 ± 0.12	--
Policosanol + I/R	100 mg/kg	2.00 ± 0.18***Δ	35.9
Policosanol + I/R	200 mg/kg	1.75 ± 0.16 ***ΔΔ+	43.9
FO + I/R	1.25 g/kg	2.75 ± 0.16	11.85
FO + I/R	2.5 g/kg	2.5 ± 0.18*	19.9
ASA + I/R	60 mg/kg	2.12 ± 0.22 **	32.05

I (%): Inhibition percent; ** $p<0,01$; *** $p<0,0001$ Comparison with the positive control; Δ $p<0,05$; ΔΔ $p<0,01$ Comparison with FO 1.25 mg/kg; + $p<0,05$ Comparison with FO 2.5 mg/kg (Mann Whitney U test)

Therapeutic treatment with policosanol (100 and 200 mg/kg) for one-week post-ischemia reperfusion moderately and significantly reduced the histological score of brain damage (35.9 and 43.9% inhibition, respectively), reducing cell depletion in this area and the picnosis of the cells (Fig 3 and 4), while only the

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highest tested dose of FO (2.5 g/kg) produced a modest and significant reduction (19.9%) (Fig 6), being ineffective the lowest dose of FO (1.25 g/kg) (Fig.5).

The statistical comparison between each dose of policosanol with the lowest dose of FO (1.25 mg/kg) was significant, while only the higher dose of policosanol (200 mg/kg) resulted in a significantly higher inhibition of histological damage than that achieved with the higher FO dose (2.5 mg/kg). Thus, the comparison

between the effects of both substances on histological damage showed greater efficacy with policosanol (43.9% inhibition) than with FO (19.9% inhibition).

Treatment with aspirin (60 mg/kg) resulted in a significant and modest reduction of histological damage in brain (32.05%) reducing the apparent cellular loss and the damage of the same ones as the picnosis and the change in its morphology (Fig.7), corroborating the validity of this model in our experimental conditions.

Table 3: Effects of policosanol and FO on MDA and sulphydrile groups plasma concentrations in *Mongolian gerbils* with IR- induced global cerebral ischemia.

Treatments	Doses	MDA (nM/ mgPt)	I (%)	SHG (mmol)	I (%)
Negative control (Vehículo)	0	45.12 ± 3.62**	--	0.57 ± 0.05 **	--
Positive control (Vehículo + I/R)	0	67.02 ± 3.01	--	1.07 ± 0.12	--
policosanol+ I/R	100 mg/kg	53.74 ± 3.90 *	60.6	0.58 ± 0.04 **	98
policosanol+ I/R	200 mg/kg	47.43 ± 3.43 **	89.5	0.57 ± 0.02 **	100
FO + I/R	1.25 g/kg	53.55 ± 5.02 *	61.5	0.63 ± 0.04 *	88
FO + I/R	2.5 g/kg	50.71 ± 5.33 *	74.5	0.62 ± 0.04 *	90
ASA + I/R	60 mg/kg	60.63 ± 1.67	29.1	0.98 ± 0.02	18

I(%): Inhibition percent; *p<0,05; ** p< 0,01 Comparison with the positive control (Mann Whitney U test)

The pathogenesis of neuronal damage in ischemic stroke involves multiple mechanisms, in which the increase of oxidative stress and inflammation in ischemic tissue is highlighted. IR induces the production of free radicals in the brain and subsequent oxidative damage to the lipids of membranes, proteins and nucleic acids, key factors mediating neuronal death in the CA1 area of the hippocampus.[50-52]

Table 3 shows the effects of policosanol and FO on plasma concentrations of MDA and SH groups in *Mongolian gerbils* with global cerebral I-R. The I/R process significantly increased both variables in the positive control group relative to the negative control. Administration with aspirin did not modify any of these variables, which does not contradict its effectiveness in this model since its anti-ischemic mechanism is based on its antiplatelet, non-antioxidant effects.[12]

Therapeutic treatment with policosanol (100 and 200 mg/kg) and FO (1.25 and 2.5 g/kg) markedly and significantly reduced the plasma concentrations of both oxidative variables.

Thus, 7-day treatment with policosanol (100 and 200 mg/kg) resulted in reductions in plasma concentrations of MDA in 60.6% and 89.5%, respectively, as well as in SH groups in 98% and 100%, respectively. FO (1.25 and 2.5 g/kg) reduced MDA by 61.5% and 74.5%, respectively, and SH groups by 88% and 90%, respectively. The statistical comparison between the two treatments did not show any significant differences. However, it is worth noting that policosanol produced a percentage of inhibition on MDA values and SH groups slightly higher

than those obtained with FO.

Thus, the fact that policosanol produced a percentage of inhibition slightly higher than that obtained with FO on lipid peroxidation could be contributing, at least in part, to the greater efficacy obtained with policosanol on histological damage. However, other factors must be involved in such a result which should be investigated by further studies.

Previous studies have shown the therapeutic efficacy of policosanol to reduce global cerebral ischemia in *Mongolian gerbils* after administration with single oral doses and evaluating behavioral and histological variables at 24 hours of induced ischemia.[40] The present study not only confirms the efficacy of policosanol in this model, but demonstrates for the first time its anti-ischemic effects with a therapeutic regimen of repeated doses, which also shows the absence of tachyphylaxis with this substance. The higher anti-ischemic therapeutic efficacy of policosanol compared to FO on global cerebral ischemia in *Mongolian gerbils* observed in this study was very similar to the preventive efficacy obtained by both substances on this experimental model, [43] which gives policosanol a potential higher value for both preventive and therapeutic use in patients with ischemic stroke. Nevertheless, clinical studies in patients with stroke should confirm such results.

CONCLUSION

Therapeutic treatment of repeated oral doses for 7 days with policosanol (100 and 200 mg/kg) and FO (1.25 and 2.5 g/kg) moderately and significantly reduced global

cerebral ischemia in *Mongolian gerbils* with greater policosanol efficacy.

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