Effect of policosanol on cerebral ischemia in Mongolian gerbils

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Abstract

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Policosanol is a mixture of higher aliphatic primary alcohols isolated from sugar cane wax, whose main component is octacosanol. An inhibitory effect of policosanol on platelet aggregation and cerebral ischemia in animal models has been reported. Thus, the objective of the present study was to evaluate the effect of policosanol on cerebral ischemia induced by unilateral carotid ligation and bilateral clamping and recirculation in Mongolian gerbils. Policosanol (200 mg/kg) administered immediately after unilateral carotid ligation and at 12- or 24-h intervals for 48 h significantly inhibited mortality and clinical symptoms when compared with controls, whereas lower doses (100 mg/kg) were not effective. Control animals showed swelling (tissue vacuolization) and necrosis of neurons in all areas of the brain studied (frontal cortex, hippocampus, striatum and olfactory tubercle), showing a similar injury profile. In the group treated with 200 mg/kg policosanol swelling and necrosis were significantly reduced when compared with the control group. In another experimental model, comparison between groups showed that the brain water content of control gerbils (N = 15) was significantly higher after 15 min of clamping and 4 h of recirculation than in sham-operated animals (N = 13), whereas policosanol (200 mg/kg) (N = 19) significantly reduced the edema compared with the control group, with a cerebral water content identical to that of the sham-operated animals. cAMP levels in the brain of control-ligated Mongolian gerbils (N = 8) were significantly lower than those of sham-operated animals (N = 10). The policosanol-treated group (N = 10) showed significantly higher cAMP levels (2.68 pmol/g of tissue) than the positive control (1.91 pmol/g of tissue) and similar to those of non-ligated gerbils (2.97 pmol/g of tissue). In conclusion, our results show an anti-ischemic effect of policosanol administered after induction of cerebral ischemia, in two different experimental models in Mongolian gerbils, suggesting a possible therapeutic effect in cerebral vascular disorders.

Introduction

Policosanol is a mixture of higher aliphatic primary alcohols isolated from sugar cane wax (*Saccharum officinarum* L) whose main component is octacosanol, followed by triacontanol and hexacosanol, while the other alcohols (tetracosanol, heptacosanol, nonacosanol, dotriacontanol and tetracontanol) are minor components.

Policosanol is a new cholesterol-lowering drug used to treat type II hypercholesterolemia with hypocholesterolemic effects proved in experimental models (1-3), healthy volunteers (4) and type II hypercholesterolemic patients (5-8). Previous results have shown that policosanol (25-200 mg/kg) inhibits platelet aggregation, reduces thromboxane A_2 (Tx A_2) and increases prostacyclin (Pg I_2) levels in serum of rodents (9,10). Also, an antiplatelet effect of policosanol (10-20 mg/day) has been demonstrated in healthy volunteers (11,12) and has been related to a reduction of serum Tx A_2 levels (13).

It is well known that the absence of posterior communicating arteries is the most often quoted reason for the unique susceptibility of the Mongolian gerbils to develop cerebral infarction after unilateral ligation of the common carotid artery (14,15), supporting the use of these animals in the screening of anti-ischemic drugs. In this regard, pretreatment with policosanol (200 mg/kg) significantly protected against cerebral ischemia induced by unilateral ligation of the common carotid artery in Mongolian gerbils, and this result was related to a significant reduction in serum Tx A_2 levels and an increase in Pg I_2 (16).

On the other hand, Mongolian gerbils submitted to bilateral clamping of the common carotid arteries and recirculation develop cerebral edema in which cyclic nucleotides (cAMP and cGMP) are involved as a consequence of traumatic insults to the brain (17).

On the basis of the inhibitory effect of policosanol on platelet aggregation and cerebral ischemia in animal models, the objective of the present study was to investigate the possible effect of policosanol on cerebral ischemia induced by unilateral ligation and by bilateral clamping and recirculation in Mongolian gerbils.

Material and Methods

Animals

A total of 216 adult female Mongolian gerbils (*Meriones unguiculatus*) weighing 60-80 g were obtained from the National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba) and randomly divided into different groups. The animals were kept at room temperature (25 ± 2^oC) on a 12-h light/dark cycle and had free access to a standard pellet diet (CENPALAB) and tap water.

Administration and dosage

Policosanol (100 and 200 mg/kg) suspended in Tween 20-water (2%) vehicle was administered orally by gastric gavage (0.5 ml/70 g body weight) immediately after ischemia induction. Suspensions were freshly prepared before use.

Effect of policosanol on cerebral ischemia induced by unilateral ligation

The following experimental groups were used: 1) Positive controls (ligated animals orally treated with the vehicle/12 h) (N = 19); 2) ligated and policosanol-treated animals (100 mg/kg/12 h) (N = 11); 3) ligated and policosanol-treated animals (200 mg/kg/12 h) (N = 19); 4) positive controls (ligated animals orally treated with the vehicle/24 h) (N = 27); 5) ligated and policosanol-treated animals (200 mg/kg/24 h) (N = 24). All treatments were performed over a period of 48 h.

Induction of unilateral ischemia. Animals were anesthetized with ether and the left common carotid artery was exposed at the neck and doubly ligated with surgical thread. The time needed for surgery never exceeded 3 min. During the postsurgical period, the gerbils were placed in temperature-controlled (25°C) transparent cages and observed for neurological symptoms. The incidence of circling behavior, rolling fits, seizures and mortality occurring during the 48-h period in the control and treated groups was compared by the Fisher exact probability test.

Histological study

Unilateral cerebral ischemia was induced in the gerbils as described above, and the animals were divided into the following groups for histological analysis: 1) control (ligated animals orally treated with vehicle) (N = 7); 2) ligated and policosanol (100 mg/kg)-treated animals (N = 7); 3) ligated and policosanol (200 mg/kg)-treated animals (N = 7).

After 4 h of ligation the gerbils were sacrificed with excess ether and the brains were removed and fixed in 10% buffered formaldehyde. Serial sections were obtained from 4 brain regions: frontal cortex, hippocampus, striatum and olfactory tubercle. Sections were dehydrated in a graded alcohol series and embedded in paraffin. Coronal sections were stained with hematoxylin and eosin and examined with a light microscope.

Histologic swelling. The degree of tissue vacuolization was measured by the method of Plum et al. (18): grade 0, mild or no focal vacuolization; grade 1, slight diffuse or moderate focal vacuolization; grade 2, moderate diffuse or severe focal vacuolization; grade 3, severe diffuse vacuolization.

Histological necrosis. The degree of necrosis was estimated on the basis of cytoplasmic eosinophilia and nuclear pyknosis as follows: grade 0, no cellular change; grade 1, slight diffuse or moderate focal cellular changes; grade 2, moderate diffuse or severe focal cellular changes; grade 3, severe diffuse cellular changes.

Effect of policosanol on cerebral ischemia induced by bilateral clamping and recirculation. Quantification of cerebral edema and cyclic AMP determination

The experimental groups used were the following: 1) sham (N = 13); 2) positive controls orally treated with vehicle immediately after clamp removal (N = 15); 3 and 4) animals orally treated with policosanol (100 mg/kg (N = 12) and 200 mg/kg (N = 19)) immediately after clamp removal.

Gerbils were anesthetized with ether. 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 anesthesia for 30 min. The catheter was then removed, the arteries were dissected and a clamp was placed just behind the catheter preventing blood flow for 15 min. The clamp was removed and cut and the necks of the gerbils were sutured. The sham-operated animals were submitted to the same procedure, except for clamping. Reflow was verified by visual inspection of blood flowing past the point of occlusion. After a 15-min occlusion and 4 h of reflow animals were killed by decapitation. The whole brain was removed from the skull, weighed and dried (48 h at 70°C) for water determination. Percent water content was calculated as follows: water (%) = (wet weight - dry weight/ wet weight) x 100.

In another group of 36 animals, the whole brains were rapidly removed and homogenized in 10% ethanol solution (w/v) and allowed to stand for 5 min at room temperature before centrifugation. The homogenate was centrifuged for 10 min at 1000 *g* and the supernatant was stored. The precipitate was

washed with ethanol-water solution (2:1; v/v) and centrifuged for 10 min at 1000 g. The supernatant obtained was added to the first. The solution was evaporated to dryness under nitrogen and reconstituted with 500 µl of phosphate buffer. cAMP was quantified using the cAMP [³H] assay system (TRK 432) purchased from Amersham Laboratories (Buckingham, UK). Data concerning brain water and cAMP content of controls and treated groups were compared by the Mann-Whitney U-test.

Results

Effect of policosanol on cerebral ischemia induced by unilateral ligation

Policosanol (100 mg/kg) administered immediately after unilateral carotid ligation and at 12-h intervals thereafter did not change mortality or clinical symptoms (Table 1). Nevertheless, policosanol administered at 200 mg/kg each 12- or 24-h significantly reduced mortality and clinical symptoms in this experimental model. There was no difference in effectiveness between the 12- and 24-h interval regimen of administration (Table 1).

Histological study

In this study, control animals showed swelling (tissue vacuolization) and neuronal necrosis. The different areas of the brain (frontal cortex, hippocampus, striatum and olfactory tubercle) showed a similar injury profile. Animals treated with policosanol at 200 mg/kg (but not at 100 mg/kg) showed a significant reduction of swelling and necrosis compared with the control group (Tables 2 and 3).

Effect of policosanol on cerebral ischemia induced by bilateral clamping and recirculation

Comparison between groups showed that the brain water content of the control Mongolian gerbils after 15 min of clamping and 4 h of recirculation was significantly higher than that of the sham-operated animals, while policosanol (200 mg/kg) administered post-occlusion significantly reduced the edema compared with the control group with a cerebral water content statistically similar to that of the sham-operated animals (Table 4).

On the other hand, cAMP levels in the brain of control-ligated Mongolian gerbils were significantly lower than those of sham-operated animals. The policosanol-treated group (200 mg/kg) showed significantly higher cAMP levels than the positive control and similar to those of nonligated gerbils (Table 4).

Comparison between positive control and policosanol-treated groups at 100 mg/kg showed no significant difference in water or cAMP content.

Discussion

The occurrence of clinical symptoms such as circling behavior and rolling fits after unilateral ligation of the common carotid artery in the control groups agrees with previous reports of the prevalence of an incomplete circle of Willis in 60% of these animals (14,19). Since the intensity of damage is very difficult to evaluate, mortality is a pa-

Table 1 - Effect of policosanol on unilateral ischemia in Mongolian gerbils.

Animals were anesthetized with ether. The left common carotid artery was exposed at the neck and doubly ligated with surgical thread. The time needed for surgery never exceeded 3 min. The incidence of circling behavior, rolling fits, seizures and mortality was compared between control and treated groups during a period of 48 h. *P<0.05; **P<0.01 compared to control (Fisher exact test).

Treatment	N	Symptoms (%)	Mortality (%)
Control (vehicle/12 h)	19	52.6	47.3
Policosanol (100 mg/kg/12 h)	11	54.5	45.4
Policosanol (200 mg/kg/12 h)	19	10.5**	10.5**
Control (vehicle/24 h)	27	51.8	40.7
Policosanol (200 mg/kg/24 h)	24	25.0*	12.5*

rameter that can be easily determined, indirectly indicating the intensity of lesion and representing an important parameter in the evaluation of anti-ischemic drugs (20).

Our results indicate that policosanol at 200 mg/kg, administered immediately post-ligation and at 12- or 24-h intervals thereafter, significantly reduced clinical symptoms and mortality in this model. Policosanol at 100 mg/kg was ineffective. In the histological study performed in this model, representative areas of the brain were chosen. Many authors have studied the hippocampus because of the marked sensitivity of some neurons to brief ischemia and because the selectivity of damage is most clearly seen in this region (21,22). In our results, no qualitative histopathological differences were noted in the four regions examined, with each region showing the same pattern, as also reported by Christie-Pope et al. (23). This study showed that policosanol at 200 mg/kg (but not 100 mg/kg) administered post-ligation significantly reduced swelling and necrosis of cerebral tissue compared to control. It can be seen that the results of the histological study confirmed the clinical symptoms and mortality obtained for Mongolian gerbils with unilateral cerebral ischemia, since in both experiments policosanol at 200 mg/kg administered immediately post-ligation significantly protected against cerebral damage.

Clinical evidence supports the view that platelet activation is a primary event in the occurrence of stroke (24). Progressive ischemic cell damage may be preceded by an imbalance between the production of Tx A_2 and Pg I_2. While the former is a vasoconstrictor which enhances platelet aggregation, the latter is a vasodilator and antiplatelet aggregator (25). Thus, an absolute or relative (increased Tx A_2/Pg I_2 ratio) excess in Tx A_2 might enhance the pathophysiological events initiated by the ischemic insult.

Previous data have indicated anti-platelet (9), antithrombotic (26) and anti-ischemic effects of policosanol (16) which have

Table 2 - Histological study of brains of Mongolian gerbils with unilateral ischemia for the determination of the extent of tissue vacuolization (histological swelling).

H: Hippocampus; FC: frontal cortex; S: striatum; OT: olfactory tubercle. The extent of tissue vacuolization was measured by the method of Plum et al. (18). Score: grade 0, slight or no focal vacuolization; grade 1, slight diffuse or moderate focal vacuolization; grade 2, moderate diffuse or severe focal vacuolization; grade 3, severe diffuse vacuolization. *P<0.01 compared to the other groups (Mann-Whitney U-test).

Treatment	N	H	FC	S	OT
Control (vehicle)	7	2.7 ± 0.48	2.7 ± 0.48	2.7 ± 0.48	2.5 ± 0.2
Policosanol (100 mg/kg)	7	2.5 ± 0.2	2.7 ± 0.18	2.5 ± 0.2	2.8 ± 0.1
Policosanol (200 mg/kg)	7	0.4 ± 0.2*	0.8 ± 0.1*	0.7 ± 0.1*	0.5 ± 0.2*

Table 3 - Histological study of brains of Mongolian gerbils with unilateral ischemia for the determination of the extent of neuronal necrosis (histological necrosis).

H: Hippocampus; FC: frontal cortex; S: striatum; OT: olfactory tubercle. The extent of neuronal necrosis was estimated on the basis of cytoplasmic eosinophilia and nuclear pyknosis as follows: grade 0, no cellular change; grade 1, slight diffuse or moderate focal cellular change; grade 2, moderate diffuse or severe focal cellular change; grade 3, severe diffuse cellular change. *P<0.01 compared to the other groups (Mann-Whitney U-test).

Treatment	N	H	FC	S	OT
Control (vehicle)	7	2.8 ± 0.1	2.8 ± 0.1	3.0 ± 0	2.7 ± 0.18
Policosanol (100 mg/kg)	7	2.5 ± 0.2	2.8 ± 0.1	2.7 ± 0.18	2.7 ± 0.18
Policosanol (200 mg/kg)	7	0.57 ± 0.2*	0.4 ± 0.2*	0.57 ± 0.2*	0.28 ± 0.18*

Table 4 - Effect of policosanol on bilateral ischemia in Mongolian gerbils.

After a 15-min occlusion and a 4-h reflow, animals were killed by decapitation. The whole brain was removed from the skull, weighed and dried (48 h at 70ºC) for the determination of water content. In other animals, the whole brain was rapidly removed and homogenized. The homogenate was centrifuged and the supernatant was stored. The precipitate was washed with ethanol-water and centrifuged. The supernatant obtained was added to the first. The solution was evaporated to dryness under nitrogen and reconstituted. cAMP was quantified using the cAMP [3H] assay system. ns, Nonsignificant. *P<0.01 compared to control animals (Mann-Whitney U-test).

Treatment	N	Water content (% of wet weight) (mean ± SEM)	N	cAMP (pmol/g of tissue) (mean ± SEM)
Sham (nonligated)	13	79.17 ± 0.10*	10	2.97 ± 0.10*
Control	15	80.00 ± 0.15	8	1.91 ± 0.30
Policosanol (100 mg/kg)	12	79.83 ± 0.23 ns	8	1.98 ± 0.25 ns
Policosanol (200 mg/kg)	19	79.28 ± 0.07*	10	2.68 ± 0.22*

been related to reduced production of Tx A_2 and increased production of Pg I_2. On the other hand, in the present study policosanol administered post-occlusion at 200 mg/kg significantly reduced the cerebral edema induced by bilateral clamping and recirculation in Mongolian gerbils, and also significantly increased cAMP levels in brain compared to the control group.

The brain is extremely sensitive to ischemia and it is well known that periods of hypoxia or disruption of blood flow result in impairment of neuronal function (27) and in behavioral disorders (28). Among other mediators, catecholamines, serotonin, angiotensin, histamine, thrombin, thromboxane A2, leukotrienes, endothelin-1, platelet-activating factor and oxygen free radicals may participate in the development of ischemic cerebral failure (29). Cyclic nucleotides (cAMP and cGMP) appear to be involved in the consequences of traumatic insults to the brain (17). Schwartz et al. (30) demonstrated that adenylate cyclase activity declines after clamping of the carotid arteries and blood recirculation restored up to 5 h post-ischemia. The damage to central enzymes seen after a period of ischemia followed by recirculation has been termed "secondary ischemia" and has been attributed to various biochemical processes such as release of cellular lysosomes, breakdown of the blood-brain barrier, Ca^{2+} influx, formation of free radicals, rise in extracellular K^+, prostaglandin and thromboxane formation, cellular acidosis and damage to cell membrane components resulting from activation of membrane phospholipases (31,32). This damage is especially evident during reflow following ischemia when the blood source of metabolites is available to the central tissue due to a breakdown of the blood-brain barrier.

Taylor et al. (17) reported that adenylate cyclase damage may result from generation of free radicals during reflow in gerbils. Moreover, drugs that inhibit enzymatic processes associated with free radical formation such as allopurinol and indomethacin were effective in preventing the damage to adenylate cyclase. It is well known that free radicals and related reactive oxygen species have been implicated in cerebral ischemia/reperfusion injury (33). Arachidonic acid is particularly susceptible to oxygen radical attack because of the presence of double bonds that can undergo peroxidation. Through a chain of oxidative reactions, the lipoxygenase and cyclooxygenase pathways generate more reactive free radicals (34). Brain damage and neuronal necrosis develop in the presence of substantial generation of superoxide radicals, hydrogen peroxide, and hydroxyl radicals in ischemia-reperfusion (33).

The protective effects of antioxidants, free radical scavengers and inhibitors of free radical production against damage support a contribution of reactive oxygen species to damage in cerebral ischemia (35,36). In previous studies by our group policosanol inhibited the increase in serum malondialdehyde levels induced by collagen in rats (9). Moreover, Fraga et al. (37) demonstrated an antioxidant effect of policosanol on rat liver microsomal lipid peroxidation.

In conclusion, our results show the effectiveness of policosanol at high doses (200 mg/kg) in protecting against cerebral ischemia induced in two different experimental models in Mongolian gerbils. The fact that policosanol was administered after induction of ischemic event supports the idea not only of a preventive effect, but also of a possible therapeutic effect in cerebral vascular accidents.

The anti-ischemic effect of policosanol on cerebral ischemia could be explained because of its platelet antiaggregating and antioxidant effects, supported by a significant reduction of Tx A_2 and malondialdehyde production, respectively (9,10,13,37). Nevertheless, further studies must elucidate better the possible protective mechanism of policosanol.

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