Therapeutic Effects of Policosanol and Atorvastatin against Global Brain Ischaemia-Reperfusion Injury in Gerbils

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Stroke is the third cause of death and the first of permanent adult disability. Pretreatment with policosanol and atorvastatin has been effective in experimental models of cerebral ischaemia in rodents. The objective was to compare the therapeutic effects of policosanol and atorvastatin in a model of global cerebral ischaemia in gerbils. Gerbils were distributed into seven groups, a negative control and six with ischaemia-reperfusion-induced global cerebral ischemia (one vehicle positive control, two policosanol (100 and 200 mg/kg), two atorvastatin (10 and 20 mg/kg) and one aspirin (60 mg/kg) group). Treatments were given 4 h after ischaemia induction. Effects on ischemia-reperfusion-induced symptoms, hyperlocomotion, damage of pyramidal hipoccampal neurons and increased plasma oxidative markers were investigated. Positive, not negative controls, exhibited clinical symptoms, hyperlocomotion, neuronal damage and increased plasma oxidative markers. Policosanol (100 and 200 mg/kg) reduced significantly ischemia-reperfusion-induced symptoms, the frequency of symptomatic animals, histological scores of neuronal damage and plasma oxidative markers as compared with the positive control group. Atorvastatin (10 and 20 mg/kg) decreased significantly the symptoms and histological scores, but unchanged the frequency of symptomatic gerbils and oxidative variables. Only the highest dose of policosanol (200 mg/kg) and atorvastatin (20 mg/kg) reduced significantly ischemia reperfusion-induced hyperlocomotion, policosanol being the most effective. Aspirin 60 mg/kg lowered significantly symptom score, the rate of symptomatic gerbils and hyperlocomotion versus the positive controls, but failed to modify oxidative parameters. In conclusion, postreperfusion treatment with policosanol and atorvastatin was effective for ameliorating symptoms, hyperlocomotion and neurological damage of hippocampal CA1 neurons in gerbils with ischemia-reperfusion-induced global cerebral ischemia, but only policosanol reduced increased plasma oxidative variables.

Key words: Atorvastatin, policosanol, cerebral ischaemia, gerbils, statins

Stroke is a prevalent disease with a large burden on the health system since it represents the leading cause of disability, the second of dementia and the third of death worldwide in adulthood[1-3]. Among the various types of stroke, ischaemic stroke is the most prominent and accounts for most of the long term disability[4,5].

Conceptually, stroke is an acute neurological injury emerging from the interruption of blood and oxygen supply to the whole brain (global ischaemia) or to specific brain territories (focal ischaemia) in dependence of the brain artery occluded whose underlying cause lies in the damage of the arterial endothelial cell layer[6,7]. In turn, global brain ischaemia, a potential outcome of clinical conditions that produce drastic reduction of blood flow to the brain selectively damages vulnerable brain regions, mainly the CA1 hippocampal neurons, so that ischaemia-reperfusion (IR) injury produce a serious loss of these neurons[8,9].

The pathogenesis of neuronal injury in ischaemic stroke involves multiple mechanisms. Excessive releases of excitatory neurotransmitters trigger the metabolic cell death chain, and other factors induce secondary reactions within the central nervous system, like the release or activation of free radicals, eicosanoids, lipid degradation products, inflammation and/or immune responses that may act after the primary ischaemic insult either sequentially or in parallel to cause cell death[10,11].

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Preclinical models of ischaemic stroke support the key role of oxidative stress and inflammation in the ischaemic brain injury. IR injury induces the production of reactive oxygen species (ROS) in the brain and the subsequent oxidative damage of membrane lipids, proteins and nucleic acids, key factors that mediate the delayed neuronal death of the pyramidal neurons in the hippocampal CA1 area in global brain ischaemia[12-14].

The Mongolian gerbil is an animal species useful to study global ischaemia due to its specific Circle of Willis that makes these animals susceptible to experimental cerebral ischaemia by unilateral (permanent) or bilateral (transient) occlusion of the common carotid artery[15] and suitable for evaluating the efficacy of potential treatments for ischaemic stroke[16]. Brain ischaemia induced by occlusion of the common carotid artery in gerbils is associated to significant increase of malondialdehyde (MDA) level and a decreased antioxidant activity of superoxide dismutase and catalase in the ischaemic brain[17]. Also, antioxidant substances have been shown to exert potent neuroprotective effect against neuronal damage in hippocampus after transient global cerebral ischaemia (GCI) in gerbils, associated with antioxidant effects on brain oxidative markers[18,19].

In contrast, experimental studies show that statins have cytoprotective actions, like reduction of clot formation, increase of clot lysis, upregulation of endothelial nitric oxide synthase, downregulation of inducible nitric oxide synthase, antioxidant effects and modulation of the inflammatory response that may support a neuroprotective effect[20]. In particular, atorvastatin (5 or 15 mg/kg) given at reperfusion and 12 h later reduced the infarct size and improved the neurological outcome in experimental stroke in Wistar, spontaneously hypertensive rats (SHR) and type 2 diabetic Goto-Kakizaki rats[21]. In addition to its well-known cholesterol-lowering effects, atorvastatin has been reported to produce antiplatelet effects[22].

Policosanol, a mixture of high molecular weight alcohols purified from sugarcane wax, has been shown to produce antiplatelet and antioxidant effects[23-25] and to protect against local and GCI-induced by carotid ligation in gerbils[26-28].

There are no reports in literature which compared the neuroprotective effects of policosanol and atorvastatin on experimental brain ischaemia. In light of these issues, this study was aimed to compare the therapeutic effects of oral single doses of policosanol and atorvastatin on IR-induced GCI in 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° 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 centre approved the use of animals and the study protocol.

The policosanol batch containing: 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%) was supplied by the Plants of Natural Products from the National Centre for Scientific Research, atorvastatin by Laboratorios Novatec, Havana, Cuba and aspirin by the Chemical Pharmaceutical Industry (Quimefa), Havana, Cuba.

Treatment and experimental design:

Once concluded their quarantine, gerbils were randomised into seven groups, a sham group without GCI treatment with the vehicle (negative control), and six groups with IR-induced GCI, one treated with the vehicle (positive control), two with policosanol (100 or 200 mg/kg, respectively), two with atorvastatin (10 or 20 mg/kg, respectively) and other with aspirin 60 mg/kg. All treatments were given by oral gastric gavage (0.5 ml/70 g of body wt) 4 h after ischaemia induction. Pretreatment with the selected doses of policosanol (100 or 200 mg/kg), atorvastatin (10 or 20 mg/kg) and aspirin (60 mg/kg) had been shown to protect against brain ischaemic injury in rodents[21,26-29].

Induction of GCI:

Transient GCI was induced in the gerbils by occlusion of both common carotid arteries for 5 min under anaesthesia in ether 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. Four hours after ischaemia induction treatment were given as single oral doses. 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 4 h 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 hindlimb) and 5 (seizures)[30]. Locomotor activity was assessed as follows, at 24 h after IR, gerbils were weighed and placed in a motor activity monitor (50×50×30.5 cm), with a 12-square grid floor of 14.5 cm on each side. Then the times of crosses through the squares were counted for 6 min.

Histological studies:

After concluding the locomotor activity test, gerbils were anesthetised with ether, euthanized by decapitation and their brains were immediately removed, fixed in 10% formalin, 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.*, 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)[31].

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. Total plasma protein concentrations were assessed by a modification of the Lowry method[32].

Assay for plasma lipid peroxidation markers:

Plasma products of the lipid peroxidation were determined as thiobarbituric acid-reactive substances (TBARS)[33]. 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 millilitre 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.

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[34]. 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.

Statistical analyses:

Continuous data were compared by the two sided Kruskal–Wallis followed by pair wise comparisons (Mann–Whitney U test); categorical data (frequency of symptoms) with the Fisher's Exact Probability test. Difference levels at $P<0.05$ were considered statistically significant. Statistical analysis was performed using the statistical package program Statistic for Windows (Release 4.2; Copyright StatSoft, Inc. US).

RESULTS AND DISCUSSION

All positive control animals, none negative control, exhibited the characteristic clinical symptoms induced by IR. Oral treatment with policosanol (100 and 200 mg/kg) significantly reduced the mean symptom score (58.3 and 73.3%, respectively) and the

frequency of animals with symptoms (50% reduction with both doses) as compared with the positive control group (Table 1). Atorvastatin treatment (10 and 20 mg/kg) also decreased significantly the symptom score (54.3 and 62.6%, respectively), but not the frequency of symptomatic gerbils. Aspirin (60 mg/kg), the reference substance, lowered significantly and markedly symptom score (87.6%) and the frequency of animals with symptoms (87.5%) versus the positive control group.

The data of the locomotor activity displayed 24 h after IR are presented in Table 2. Hyperlocomotion was seen in positive controls gerbils as compared with the negative ones. While the treatment with aspirin 60 mg/kg and the highest doses of policosanol (200 mg/kg) or atorvastatin (20 mg/kg) significantly decreased IR-induced hyperlocomotion by 74.5, 84.2 and 74.2%, respectively, the lowest dose of policosanol and atorvastatin failed to significantly ameliorate such behaviour. Comparison between treatment groups did not show significant differences.

Histological examination revealed the damage and loss of numerous pyramidal cells from the CA1 region in the positive control but, not in the negative controls. Consequently, the histological score of the IR-challenged control group increased significantly as compared with the negative control (Table 2). IR-induced hippocampal neuronal damage was significantly (P<0.05) lowered by aspirin 60 mg/kg (31.3% decrease), policosanol 100 and 200 mg/kg (26.4 and 66.2% reduction, respectively), or atorvastatin 10 and 20 mg/kg (19.7 and 26.4% reduction, respectively), as compared with the positive control groups. Only the highest dose of policosanol, however, produced a marked reduction of the score, which was significantly lower than in the other treatment groups (Table 3).

Table 4 shows the data of plasma oxidative variables after 24 h of IR. Plasma levels of MDA and SHG significantly increased in positive control as compared with the negative control group, effects significantly and markedly attenuated by policosanol (49.5 and 64.6% reductions of plasma MDA with 100 and 200 mg/kg, respectively; and 58.8 and 76.5% decreases of plasma SHG values with 100 and 200 mg/kg, respectively). Aspirin 60 mg/kg and atorvastatin 10 and 20 mg/kg, however, failed to

TABLE 1: EFFECTS OF TREATMENTS ON CLINICAL SYMPTOMS IN MONGOLIAN GERBILS WITH IR-INDUCED GCI

Treatment	Doses (mg/kg)	Clinical symptoms (CS) score	Inhibition (%)	Frequency (%) of gerbils with CS	Inhibition (%)
Negative control (vehicle)	0	0±0*	--	0+	--
Positive control (vehicle+IR)	0	3.00±0.56	---	100	---
Policosanol+IR	100	1.25±0.55*	58.3	50+	50
Policosanol+IR	200	0.8±0.32*	73.3	50+	50
Atorvastatin+IR	10	1.37±0.41*	54.3	75	25
Atorvastatin+IR	20	1.12±0.35*	62.6	75	25
Aspirin+IR	60	0.37±0.37*	87.6	12.5+	87.5

IR=Ischaemia-reperfusion, All the values are expressed as mean±standard error (SEM); Statistical analysis of CS score was done by the two sided Kruskal-Wallis followed by Mann-Whitney U test wherein *P<0.05 was considered vs positive control group; Frequency (%) of gerbils with CS was compared by the fisher's exact probability test wherein+P<0.05 was considered vs positive control group, GCI=global cerebral ischaemia

TABLE 2: EFFECTS OF TREATMENTS ON THE LOCOMOTOR ACTIVITY OF MONGOLIAN GERBILS AT 24 H AFTER IR-INDUCED GCI

Treatment	Doses (mg/kg)	Crosses	Inhibition (%)
Negative control (vehicle)	0	266.8±14.8*	--
Positive control (vehicle+IR)	0	351.4±13.4	---
Policosanol+IR	100	327.5±22.2	28.2
Policosanol+IR	200	280.1±20.1*	84.2
Atorvastatin+IR	10	328.1±32.2	27.5
Atorvastatin+IR	20	288.6±17.3*	74.2
Aspirin+IR	60	288.3±15.2 *	74.5

IR=Ischaemia-reperfusion, All the values are expressed as mean±standard error (SEM), statistical analysis was done by Mann-Whitney U test wherein *P<0.05 was considered vs positive control group,GCI=global cerebral ischaemia

TABLE 3: EFFECTS OF TREATMENTS ON THE HISTOLOGICAL SCORES OF IR-INDUCED NEURONAL DAMAGE

Treatment	Doses (mg/kg)	Histological scores	Inhibition (%)
Negative control (vehicle)	0	0.09±0.06*	--
Positive control (vehicle+IR)	0	2.93±0.07	---
Policosanol+IR	100	2.18±0.08*	26.4
Policosanol+IR	200	1.05±0.15*[ab]	66.2
Atorvastatin+IR	10	2.37±0.06*	19.7
Atorvastatin+IR	20	2.18±0.10*	26.4
Aspirin+IR	60	2.04±0.17*	31.3

IR=Ischaemia-reperfusion; All the values are expressed as mean±standard error (SEM); Statistical analysis was done by Mann-Whitney U test wherein *P<0.05 was considered vs positive control group, [a]P<0.05 vs atorvastatin 10 and 20 mg/kg, and [b]P<0.05 vs aspirin 60 mg/kg

TABLE 4: EFFECTS OF TREATMENTS ON PLASMA OXIDATIVE VARIABLES

Treatment	Doses (mg/kg)	MDA (nmol/mg prot)	Inhibition (%)	SHG (mmol)	Inhibition (%)
Negative control (vehicle)	0	38.92±3.01*	--	0.44±0.02*	--
Positive control (vehicle+IR)	0	72.06±5.88	---	0.61±0.03	---
Policosanol+IR	100	55.65±2.82*	49.5	0.51±0.01*	58.8
Policosanol+IR	200	50.63±3.88*	64.6	0.48±0.02*	76.5
Atorvastatin+IR	10	69.74±4.58	7.0	0.60±0.02	5.9
Atorvastatin+IR	20	70.80±3.42	3.8	0.59±0.02	11.8
Aspirin+IR	60	72.30±4.63	0.0	0.60±0.03	5.9

IR=Ischaemia-reperfusion, MDA=malondialdehyde, SHG=sulfhydril groups, all the values are expressed as mean±standard error (SEM), statistical analysis was done by Mann–Whitney U test wherein *P<0.05 was considered vs positive control group

modify these variables. Final values of plasma MDA and SHG were significantly lower in policosanol than in aspirin- and atorvastatin-treated groups.

Previous data have shown the protective effects of policosanol pretreatment against experimental brain ischaemic injury in rodents[26-28], but this study demonstrates, by the first time, the neuroprotective effects of policosanol (100 and 200 mg/kg) given orally after the induction of global cerebral IR injury in gerbils, which compared favourably with those of atorvastatin (10 and 20 mg/kg).

Mongolian gerbils have an incomplete Circle of Willis, a characteristic that makes them prone to develop global brain ischaemia after the bilateral occlusion of carotid arteries, being a suitable model for evaluating substances or drugs with potential benefit on brain ischaemia[14,15]. IR-induced damage results in selective hippocampal injury, mainly in the CA1 region, as supported by hyperlocomotion and histology[8,9,35].

In gerbils, global brain ischaemia induces locomotor hyperactivity several hours after reperfusion, with peak at 24 h and persistent for several days, so that hyperlocomotion is a predictive indicator of hippocampus damage[36-39]. Neuroprotective treatments have been shown to ameliorate closely hippocampal delayed neuronal damage and IR-induced locomotor hyperactivity, which suggests that IR-induced CA1 injury and hypermotility share common mechanisms[38].

Consistently with such reports, our IR-challenge positive control exhibited characteristic clinical symptoms, hyperlocomotion and histological features at 24 h post-reperfusion, all significantly reduced by aspirin 60 mg/kg, the reference drug, which supports the validity of this model in our experimental conditions.

Oral treatment with policosanol (100 and 200 mg/kg) and atorvastatin (10 and 20 mg/kg) were effective to lower the mean symptom score, but only policosanol reduced the frequency of animals exhibiting clinical symptoms, while only the highest doses of both treatments decreased significantly IR-induced hyperlocomotion, without differences among them.

All treatments produced significant reductions of the histological scores, which indicates an effective protection against IR-induced injury, but these decreases were mild-to-moderate (<50%) except with the highest dose of policosanol (66% reduction of the neuronal damage). Although a dose-effect relationship was not explored due to the number of experimental groups, the data suggest that the effects increased with the doses, although they were generally modest-to-moderate (<50%). Since atorvastatin effects increased with the doses and higher doses were not investigated, we cannot conclude that the neuroprotective effect of policosanol was superior to that of atorvastatin.

The ability of policosanol and atorvastatin to reduce hyperlocomotion and neuronal damage also favour the hypothesis of a positive correlation between hippocampal function and hyperlocomotion outcome in gerbils. In this model, after a 5 min occlusion the neurons located within the hippocampal CA1 region exhibit extensive and selective damage[38]. After ischaemia, reoxygenation during reperfusion provides an excess supply of oxygen that sustains neuronal viability and contributes produce ROS directly involved in oxidative damage of lipids, proteins and DNA[40], so that oxidative stress predominates in the pathophysiology of this IR injury, and experimental studies have reported the efficacy of antioxidant substances for reducing neuronal damage and oxidative stress in the central nervous system generated by cerebral IR process[41,42].

In such regard, the extent of cerebral lipid peroxidation, a marker of oxidative stress, can be estimated by measuring cerebral MDA levels, meanwhile concentrations of the SHG associated to proteins have been used to assess the extent of protein oxidative damage. We investigated the effects of treatments on plasma, not on cerebral MDA and SHG concentrations, which is a limitation of this study, but we wanted to know whether IR-induced transient GCI in gerbils increased systemic markers of oxidative stress, as occurred. Consistent with our speculation, increased plasma lipid peroxidation and protein oxidation markers were seen in IR-challenged gerbils. We found that policosanol, not atorvastatin or aspirin, significantly reduced MDA and SHG levels after cerebral IR injury, results consistent with previous reports of the antioxidant effects of policosanol[24], but apparently contradictory with data supporting the antioxidant effects of atorvastatin[43-45]. However, keeping in mind that atorvastatin has been shown to produce antioxidant effects when given as repeated[43,44], not as single doses[21], these results are coherent with such background. In turn, the fact that aspirin did not modify the oxidative variables was expected since its antiischaemic efficacy in this model depends of its antiplatelet effects[46].

Previous studies had demonstrated the neuroprotective effects of policosanol and atorvastatin in experimental brain ischaemia, this is the first experimental demonstration of the therapeutic ability of policosanol to ameliorate IR-induced damage. This study did not pretend to investigate the mechanisms of the neuroprotective effect of policosanol and atorvastatin against IR-induced global ischaemia in gerbils, but the present results suggest that the effect of policosanol, not of atorvastatin, could be related, at least partially, to its ability to reduce lipid and protein oxidation.

In conclusion, post-reperfusion treatment with single oral doses of policosanol and atorvastatin attenuated clinical symptoms, hyperlocomotion and neurological injury and decreased increased levels of plasma oxidative markers in gerbils with IR-induced GCI.

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