Neuroprotective Effects of *Thalassia Testudinum* Leave Extract BM-21 on Global Ischemia in Mongolian Gerbils

Roberto Menendez[1+]*, Teidy Garcia[1+], Gisela Marrero[1], Ruth A Morales[1], Anoland Garateix [1], Erik Regalado[1], Abilio Laguna[1], Olga Valdés[1], Teresita del Vallin[1], Odalys Valdes[2], María Rodriguez[1]

[1]Center of Marine Bioproducts (CEBIMAR). Calle Loma entre 35 y 37, Alturas del Vedado, Plaza de la Revolución, La Habana, Cuba.
[2]Drug Research and Development Center.CIDEM. 17 No. 6208 e/ 62 y 64, Playa, La Habana, Cuba.
[+]These authors equally contributed to this work.
***CorrespondingAuthor**
Dr. Roberto Menendez Soto del Valle
Department of Pharmacology
Laboratory of Neuropharmacology
Center of Marine Bioproducts (CEBIMAR)
Calle Loma entre 35 y 37, Alturas del Vedado
Plaza de la Revolución
La Habana, CP. 10400
Cuba
Emails: roberto.menendez@infomed.sld.cu; rmenendez@cebimar.cu; rmenendezsoto@gmail.com

Abstract

The aqueous ethanolic extract of the marine plant *Thalassia testudinum*, named BM-21, has powerful antioxidant and antilipoperoxidative activities. The extract also has anti-inflammatory, antinociceptive and neuroprotective effect against acrylamide-induced neurotoxicity. Excessive generation of free radicals and decreased levels of the antioxidant enzymes have been observed either during the brain ischemia or following reperfusion. In the present work we studied the neuroprotective potential of BM-21 against brain damage induced by transient bilateral carotid artery occlusion model of global cerebral ischemia in Mongolian gerbils. Oral administration of BM-21 (400 mg/kg, once a day for 8 days) prior to ischemic insult provides significant neuroprotection with respect to mortality, neurological symptoms, infarct volume and brain edema after. We also found that BM-21 reduces hippocampal neuronal death in the CA1 region and attenuates the increase of lipid peroxidation products (MDA). The extract also improves the activity of SOD and GSHPx and increases the content of GSH in brain homogenates. BM-21 administered at a dose at which the extract showed to be effective as anti-ischemic agent *in vivo* also reduces susceptibility of brain homogenates of non-ischemic gerbils against metal and non-metal lipid peroxidation *in vitro*. Taken

together, our results suggest that BM-21 shows neuroprotective effect on global cerebral IR injury at least partially by inhibiting brain oxidative stress.

Keywords: Global ischemia; Neuroprotection; Oxidative stress; BM-21; *Thalassia testudinum*.

1. Introduction

Stroke/cerebral ischemia resulting from interruption of blood supply to the brain is a severe neurological damage that is a leading cause of mortality and morbidity in many countries, behind cancers and heart disease [1]. However, clinically effective drugs are still unavailable. There is considerable evidence in favor of the important contribution to cell damage in ischemic brain of reactive oxygen species (ROS) produced either during the ischemia itself or following reperfusion [2-5]. Agents that prevent free radical production may therefore, prevent ischemic brain damage. In this context, the beneficial effects of polyphenolic compounds from various kinds of land plants on stroke occurrence and impact have been thoroughly documented [6, 7]. However, little information is available on the pharmacological effects of polyphenols of marine plants with the exception of those of brown seaweeds that exhibit neuroprotective effects [8-12] thought antioxidant and antiinflammatory actions.

The marine plant *Thalassia testudinum* popularly known as turtle grass occurs frequently in Cuban coastline. The aqueous ethanolic extract of the leaves plant named BM-21 markedly reduces skin UVB-induced damage by topical application and poses antioxidant effects [13, 14]. In addition, the extract administered by oral route, displayed antinociceptive effects mediated in part by the inhibition of acid-sensing ionic currents (ASICs) [15]. Thalassiolin B (chrysoeriol 7-b-D-glucopyranosyl- 2"-sulphate), the most abundant active component of the extract [15] is likely to be responsible of the above mentioned effects [13, 15]. Beside this compound, several otherphenolic phytochemicalshave been identified in the extract including apigenin 7-O-β-D-glucopyranosyl-2″-sulfate (thalassiolin C), chrysoeriol 7-O-β-D-glucopyranoside, apigenin 7-O-β-D-glucopyranoside, dihydroxy-3',4'-dimethoxyflavone 7-O-β-D-glucopyranoside, luteolin-3'-sulphate, chrysoeriol and apigenin[14]. Recently we observed neuroprotective effects of the extract standardized to thalassiolin B against acrilamide (ACR)-induced central and peripheral distal axonopathy in mice [16]. In view of these results, in the present work we investigate whether or not the oral administration of BM-21 is able to exhibit neuroprotective effects on cerebral ischemia. With this purpose, we examined the effects of BM-21 on the model of transient bilateral common carotid artery occlusion (BCCAO). In this model, we studied the effects of BM-21 on mortality, neurological symptoms, volume of infarct and hippocampal CA1 neuronal damage. Additionally, we investigate the antioxidant potential of BM-21 in gerbil's brain.

2. Materials and Methods

2.1 Materials

All chemicals were from Aplychem (Darmstadtz, Germany) except 2,3,5-triphenyltetrazolium chloride (TTC) that was from Sigma-Aldrich Co. (St Louis, MO). Reagent kits for the determination of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) were from Randox (Crumlin, U.K.). All the other chemicals and reagents were of the highest purity available. Spectrophotometer UV/visible-120-2 was from Shimadzu Corporation (Tokyo, Japan).

2.2 Plant material and preparation

Fresh *Thalassia testudinum* Banks &Soland ex König leaves were collected in the month of March 2012 from "La Concha" beach (22° 05' 45''N, 82° 27' 15'' W). The plant was authenticated by Dr. Areces J.A. (Institute of

Oceanology, La Havana, Cuba) with a standard sample preserved in the Cuban National Aquarium (No. IdO39). The extract was prepared and thalassiolinB was quantified through the standard method previously reported (Garateix et al., 2011). The percentage of thalassiolinB was within the range before described (5.9 ± 0.9%).

2.3 Animals

Adult male Mongolian gerbils (*Merionesunguiculatus*) weighing 60–70 g were purchased from the Centro para la Producción de Animales de Laboratorio (CENPALAB, Havana, Cuba). Before (for at least 1 week of the beginning of the experiment) and after ischemia, they were housed, four in a cage, at a constant room temperature of 24–26^{0}C under a light cycle of 12x12 h. The animals were allowed free access to food and drinking water. Food was standard rodent chow also purchased from CENPALAB. The adaptation and experiments were carried out under strict agreement of the procedures for handling animals and their care conformed to guidelines compliant with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996). A minimum number of animals were used to obtain reliable results.

2.4 Transient Bilateral Common Carotid Artery Occlusion (BCCAO)

Under ether anesthesia, the neck area was shaved and a midline ventral incision was made in gerbil neck. Both common carotid arteries (CCA) were exposed carefully by blunt dissection and then clamped for 7 min with micro aneurysm clips. After the clips were removed, reperfusion was confirmed by visual inspection and then the skin was sutured. In sham-operated animals both CCA were exposed but not occluded. Corporal temperature was maintained during ischemia by the use of a temperature controlled plate. Three experimental groups were used; group 1 control non-ischemic (sham+vehicle); group 2 ischemic control (ischemia+vehicle) and group 3 ischemic treated (ischemia+BM-21 400 mg/kg). Freshly prepared BM-21 in distilled water was administrated by gastric gavage (0.01 mL/g). The extract was administered at 400 mg/kg, once a day, 8 days prior to ischemia.The doses of BM-21 were selected based on earlier studies on the antinociceptive [15] and the neuroprotective [16] effect of this extract. After ischemia, Gerbils were allowed to recover in their home cages for observations. The number of deaths was recorded the first 12 h of recirculation and subsequently at 24, 48 h and 72 h after reperfusion. A neurological assessment was recorded within the first 12 h after perfusion according to McGraw[17]. As neurological signs were considered; hair roughed up, tremor, ptosis, convulsions, head cocked, splayed hindlimb, rolling seizure and coma. The neurological signs were tabulated as the "stroke index" such that a higher score indicated a more severe post-ischemic neurological deficit.

2.5 Morphometric determination of infarct volume

For detection of the infarction volume of the brain, the cross-sectional infarction area on the surfaces of each brain slice was defined by the TTC staining method. After 72 h of reperfusion, a group of 7 survival gerbils for each experimental group were anesthetized with ether and received an intracardiac perfusion of 0.9% buffered saline. The brain was then removed, frozen and cut into 2-mm serial slices starting 1 mm from frontal pole. The coronal slices were then immersed in TTC prepared in 2% phosphate buffered solution for 20 min at 37ºC. After TTC staining, the slices were fixed in overnight 10% phosphate-buffered formalin and then were scanned using a flatbed scanner Hewlett Packard HP Scanjet 3670. The infarction area was then determined by image analyzer software (Image L, version 1.34). Infarct area of each slice was calculated (mm^2) manually by outlining the margins of infarct areas and the infarct volume (mm^3). Infarct volume of all sections was cumulated to get total volume infarct.

2.6 Evaluation of brain edema

Brain edema was measured as describe previously [18]. After the reperfusion period the whole brain of 5 gerbils for each group were removed from the skull, weighed and dried (48 h,

70°C) for water determination. Percent water content was calculated as follows:

water (%) = (wet weight - dry weight/wet weight) x 100.

2.7 Delayed neuronal death

A group of 5 Gerbils from sham, ischemic control and treated group were euthanized under a light anesthesia with ether by decapitation on the seventh day after ischemia and reperfusion. Sham-operated animals served as controls. The brain was dissected from the skull and preserved in 10% buffered neutral formalin for 10 days. Formalin-fixed brain blocks containing dorsal hippocampus were embedded in paraffin. Thick sections (6μm) were sliced at the level of the dorsal hippocampus and stained with cresyl violet acetate in the usual manner.

2.8 *In vivo* antioxidant study

After 24 h of reperfusion, 6 gerbils of each experimental group were euthanized under slight ether anesthesia and brains were quickly removed, rinsed with ice-cold physiological saline and homogenized in Tris–HCl (0.1 M, pH 7.3) in a ratio of 1:4. The homogenate was then centrifuged for 10 min at 10,000 ×g at 4 °C and the resultant supernatant was used for the biochemical determinations. The total protein concentration of the brain sample was estimated according to a modification of the Lowry procedure [19]. The degree of lipid peroxidation in brain tissue was estimated as the concentrations of malondialdehyde (MDA) and was assessed by measuring thiobarbituric acid reactive substance (TBARS) in the tissue homogenate at 532 nm [20] . The concentrations were expressed as nmol MDA/mg protein. Reduced glutathione (GSH) was determined according to by Ellman's method [21]. The superoxide dismutase (SOD) and glutathione peroxidase activity (GSHPx) were assayed according to the methods described by commercial assay kits purchased from Randox Laboratories (Antrim, UK).

2.9 *Ex vivo* antioxidant study. Susceptibility of the brain homogenate of gerbils to the *in vitro* spontaneous and iron/ascorbate-induced lipid peroxidation

Male gerbils weighting 65-70g were also used. They were divided into two groups 6 animals each; a control group treated with the vehicle and a group treated with BM-21 (400 mg/kg), a dose at which the extract showed to be effective as anti-ischemic agent. After administration, animals were euthanized under slight ether anesthesia and brains were similarly processed. Susceptibility of whole brain to lipid peroxidation was determined by measuring TBARS induced by iron-ascorbate[22]. The reaction mixture contained varying amount phosphate buffer (50 mM, pH, 7.4), brain homogenate (0.5 mg protein), 1 mM ferric chloride and 1 mM ascorbic acid. The tubes were incubated at 37 °C for 30 min and the extent of peroxidation was measured.

2. 10 Statistical analysis

Data are presented as mean ± SEM along with the number of animals. Statistical analysis was performed using Graph-pad prism 5.0 software. Comparison was performed using One Way ANOVA and when significant results were obtained, multiple comparisons were done by Turkey's Test. Data of neurological score and *in vitro* lipid peroxidation was analyzed by Student t test. Mortality was examined for significance using the Fisher's Exact Test. $P < 0.05$ was considered statistically significant.

3. Results

Table 1 shows the results of mortality rate. Following 7 min of brain ischemia, only 44.4 % of the animals were alive after 3 days of recirculation. However, in the group of animals treated by BM-21 (400 mg/kg) survival rate for the same time period were 77.7%. This increase in the survival rate in comparison to non-treated was significant ($P < 0.01$). No mortality was observed in the sham-operated group.

Data of morbidity calculated on McGraw's scale are depicted in figure 1. Ptosis, convulsions, tremors, rolling seizures and coma were the most recurrent and prominent neurological signs observed within the first 12 h that demonstrated

the significant impairment in neurological function after artery occlusion and reperfusion. After 12 h of reperfusion a significant smaller score in the neurological morbidity of the animals treated with BM-21 with respect to control group was observed.

Table 1. Effect of oral pretreatment with BM-21 (400 mg/kg, once-a day during 8 days) on accumulated mortality in the 72 h period after 7 min-ischemia caused by the occlusion of the bilateral common carotid artery in gerbils

| Group	Mortality Yes/No	%
Sham control (n = 30)	0/30	0
Ischemic vehicle (n= 54)	30/24	55.5
Ischemic + BM-21 400 mg/kg (n=32)	7/25 **	22.2

** P < 0.01. Comparison *Vs* ischemic control. Fisher probability exact test.

The results of our study using TTC staining of brain slices revealed a consistent infarction volume after 72 h of recirculation in all the control ischemic animals (fig. 2). BM-21 decreased the infarct volume (67.4%) . The infarct volume was 179.10 ± 19.68 mm^3 in the ischemia (7min)/reperfusion (72h) control animals whereas it was 58.29 ± 13.12 mm^3 in the BM-21-treated animals.

Microphotographies of the hippocampal CA1 for each group are depicted in figure 3. Histological observation of the hippocampus showed that neurons in the CA1 pyramidal cell layer kept normal ultrastructure in sham-operated gerbils. In the ischemic animals treated with vehicle, pyramidal neurons exhibited significant reduction, dark staining appearance with minimal cytoplasm or complete loss of neurons and widespread damage. However, these neuropathological signs were suppressed by pretreatment with the extract that reduced the neuronal loss and weakened other morphologic changes.

The brain water contents (fig. 4), as the index of cerebral edema, were higher in the vehicle treated than those in sham operated (P < 0.001). BM-21 pretreatment produced a significant reduction of post-ischemic cerebral edema *vs*. vehicle (38.5%, P <0.01).

Data of the *in vivo* oxidative stress study are showed in table 2. When subjected to BCCAO, after 24 h of reperfusion, ischemic animals showed a significant elevation of MDA (P< 0.01) a marker of lipid peroxidation, in comparison with sham operated. BM-21 400 mg/kg showed a significant decrease in the MDA levels (P < 0.01). Also, ischemic animals showed a significant decrease in the brain levels of SOD (P< 0.05) and GSHPx (P < 0.01) and GSH (P < 0.05) as compared with the sham-operated animals. BM-21 treatment showed a significant increase in the brain of SOD (P < 0.01), GSHPX (P < 0.01) and GSH (P < 0.05) in comparison with vehicle. BM-21 restored all parameters towards the normal level.
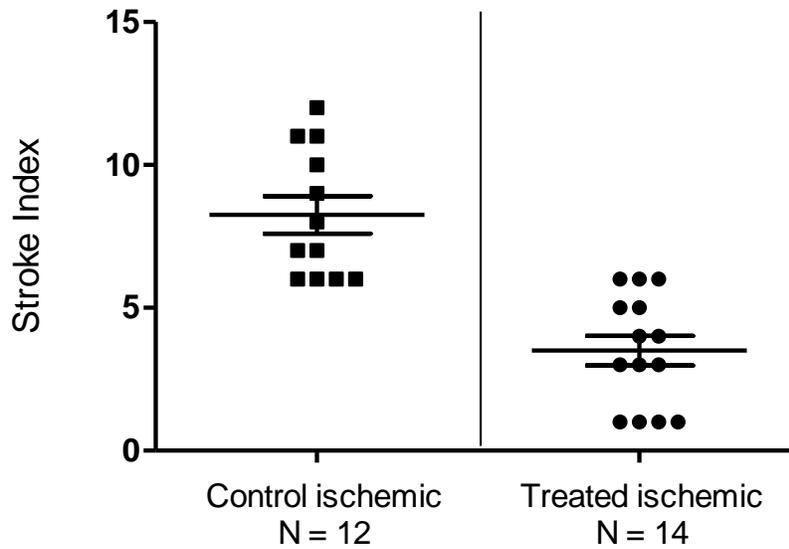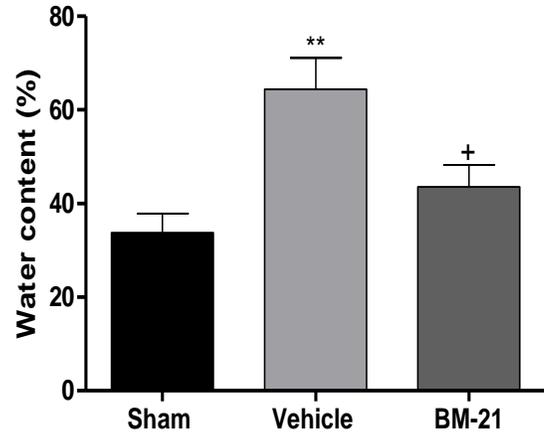
Figure 1. Effects of pre-treatment with BM-21 (400 mg/kg) on stroke index in the 12 h period after 7 min-ischemia caused by the occlusion of the bilateral common carotid artery in Mongolian gerbils. Control: 8.25 ± 0.65; treated: 3.50 ± 0.52. Comparison control ischemic *Vs* Treated, $P < 0.001$; Student t test.

D
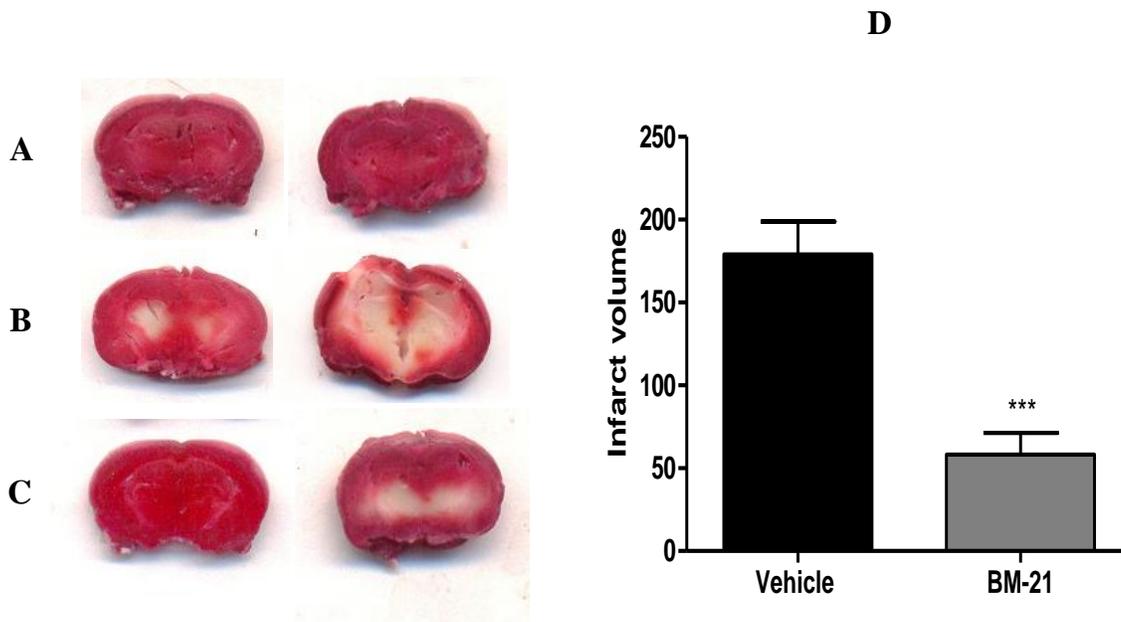
Figure 2. Brain infarction after ischemia/reperfusion induced by the occlusion of the bilateral common carotid artery in Mongolian gerbils. Representative photograph of two brain slices cut at 6 mm away from the frontal pole of two animals stained with 2,3,5-triphenyltetrazolium chloride. (A) sham control, (B) ischemia/reperfusion after vehicle and (C) ischemia/reperfusion after BM-21 treatment for 8 days. (D) Total infarct volume (mean $\pm$ SEM, n = 7). *** $P < 0.001$; BM-21 treated *Vs* vehicle control. Student t test.
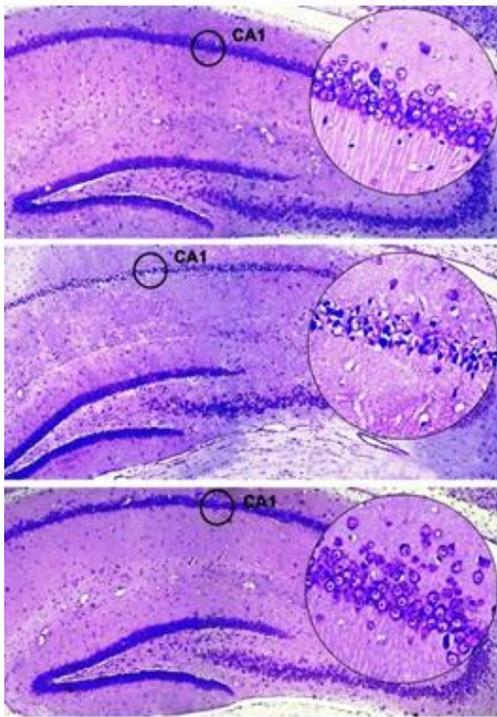
Figure 3. Representative photomicrographs of 6 μm thick coronal sections of the gerbil hippocampal CA1, six days after a global cerebral ischemia stained by cresyl violet viewed by a light microscope. Panel A: Sham operated; B: BCCAO vehicle treated control group; C: BCCAO after BM-21 400 mg/kg.

Figure 4. Effect of BM-21 (400 mg/kg) pretreatment on water content (%) after ischemia/reperfusion induced by the occlusion of the bilateral common carotid artery in Mongolian gerbils. Each column represents the mean ± SEM. of five animals. **P <0.01, comparison Sham *Vs* vehicle, $^+$ P <0.05, comparison vehicle *Vs* BM-21 treated. One-way analysis of Variance (ANOVA) followed by multiple comparisons Tukey's test.
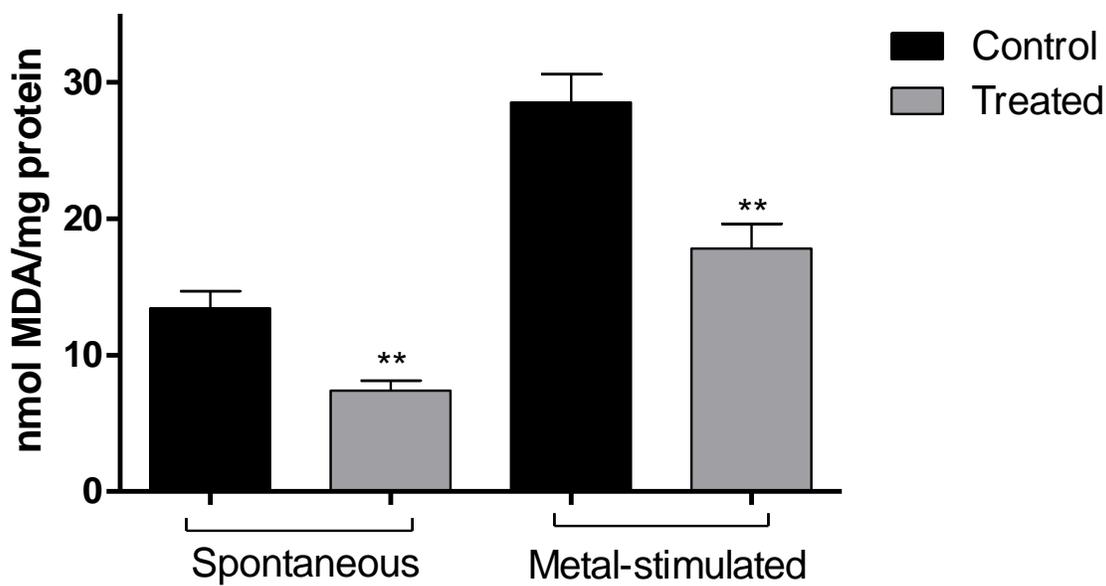
Table 2. Effect of pretreatment with BM-21(400 mg/kg) on MDA, GSH, SOD and GSHPx in brain of ischemic gerbil

Group	MDA (nmol/mg protein)	GSH (mM)	SOD (U/mg protein)	GSHPx (U/min/mg protein)
Sham	4.57 ± 0.43	9.08 ± 1.93	3.04 ± 0.08	30.82 ± 2.90
Vehicle	9.96 ± 1.67 **	2.34 ± 0.90 *	2.47 ± 0.14 *	15.43 ± 2.72**
Treated	3.73 ± 0.62 $^{++}$	8.22 ± 1.20 $^+$	3.18 ± 0.17 $^{++}$	31.53 ± 2.59 $^{++}$

Values are expressed in mean ± SEM, n=6. * P < 0.05; ** P < 0.01, comparison Sham *Vs* Vehicle group. $^+$P < 0.05; $^{++}$ P < 0.01, comparison vehicle *Vs* treated group. One-way analysis of Variance (ANOVA) followed by multiple comparisons Tukey's test.

Data of our *ex vivo* study (fig. 5) demonstrate that after the oral administration of BM-21 gerbil´s brain homogenates was less prone to oxidative stress as evident by the significant reduction of TBARS after *in vitro* generated metal and non-metal stimulated peroxidative damage to brain membranes of animal previously treated with BM-21 (400 mg/kg).

Figure 5. Effect of oral treatment with BM-21 (400 mg/kg, once a day during 8 days) on the *in vitro* susceptibility of brain homogenates to spontaneous and iron/ascorbate-induced lipid peroxidation. Bars represent mean ± SEM value (n = 6). ** $P < 0.01$, comparison control Vs treated group. Student t test.

4. Discussion

Experimental models of cerebral ischemia have allowed the evaluation at the preclinical level of neuroprotective therapies. Among the different animal models available Mongolian gerbils have been extensively used. Mature gerbils lack posterior communicating arteries between the carotid arteries and vertebral arteries so there is an incomplete Circle of Willi[23]. Due to this exclusive anatomical structure, global ischemia can be produced by occlusion of common carotid arteries. Hence, the focus of the present work was to evaluate the effect of *T. testidinum*BM-21 extract (400 mg/kg) against ischemic neuronal damage in a gerbil model of transient global forebrain ischemia.

The injury produced in our ischemic model was severe, as indicated by the great infarct area and the prominent worsening of neurological signs observed within the first 12 h.This is very similar to the pattern observed by others [24-27]. Our results showed that the administration of BM-21 (400 mg/kg) prior to ischemic insult provided significant neuroprotection with respect to mortality, neurological symptoms, infarct volume and brain edema. The attenuation patters observed in death rate, stroke index is consistent with the amelioration of the volume of brain infarction and brain edema.

Oxidative stress is one of the most important events in ischemia/reperfusion-induced cerebral damage. Ischemia/reperfusion causes an increase in free radical generated from the respiratory chain in mitochondria and ischemia-activated xanthine oxidase [22, 28]. Ischemia also triggers inflammation increasing the formation of superoxide anion ($O_2^{\bullet-}$) [29] that is also increased by the action of NADPH oxidases during NADPH oxidation [30]. In addition, the activation of the N-methyl D-aspartate (NMDA) receptors also activates NO synthase [31], which leads to excessive production of $NO^{\bullet}$ that may interact with $O_2^{\bullet-}$ producing the highly toxic peroxynitrite anion ($ONOO^-$) [32]. Due to its the high content of polyunsaturated fatty acids, the high rate of oxidative metabolic activity as well as the relative low antioxidant capacity [33, 34],

brain is remarkably vulnerable to ischemia/reperfusion-induced oxygen free radicals. SOD and GSH-PX are considered the major endogenous antioxidant enzymes of the brain by reducing cytosolic hydrogen peroxides and catalyzing the dismutation of $O_2^{\bullet-}$, respectively [35] whereas GSH is one of the primary endogenous antioxidant, which removes H_2O_2 and lipid peroxide [36]. As observed in our study, transient ischemia/reperfusion causes a marked increase of MDA in brain homogenate. This may be caused by the decrease of SOD and GSHPx activity as in such a condition $O_2^{\bullet-}$ and H_2O_2 formed during ischemia/reperfusion cannot be efficiently scavenged. Consequently, GSH content was significantly reduced by its consumption due to scavenging of the fast generating ROS. BM-21 administration significantly elevated SOD and GSH-Px activities and obviously $O_2^{\bullet-}$ and H_2O_2 should be scavenged more efficiently to further decreasing lipid peroxidation and therefore neuronal damage. In agreement with these facts a decrease of MDA and an increment of GSH concentration were observed in treated animals. These findings suggest that the neuroprotection exerted by BM-21 appear to correlate well with the increment of brain antioxidant defenses. A decrease of susceptibility of brain homogenates to oxidative stress *in vitro* was also observed by treatment of normal gerbils with BM-21 at a dosing protocol that effectively reduces death, neurological score and infarct volume. Thus, administration of BM-21 renders the brain tissue less vulnerable to free radical action that might have important implications in the preventive effects exerted on ischemic/reperfusion *in vivo*.

Transient global ischemia in gerbils can induce neuronal death in hippocampal CA1 region. This is regularly observed from 3 to 7 days after reperfusion [23]. It was shown that a reduction of enzymatic antioxidant enzymes, a decrease of GSH content and the increment of $O_2^{\bullet-}$ have been observed in hippocampal tissues and CA1 region after forebrain ischemia insult [37-42]. Indeed, it has been suggested that the lowered antioxidant capacity of this brain region is likely to be associated to their greater vulnerability toward ischemia.Therefore, our findings support the notion that restoration of brain oxidative status may be an important factor involved in the hippocampal CA1 region preservation observed in our work.

Brain edema is a crucial feature of ischemic injury and is classified as cytotoxic or vasogenic[43]. Inflammation an oxidative stress in the brain impairs cerebral microcirculation and blood barrier [29, 44-46] inducing edema. A previous report has demonstrated the inhibition *in vitro* of COX-1 and FLA2 by BM-21. Thus, the dual effect exerted by BM-21 on the arachidonic acid pathway together with the antioxidant action may account for the decrease of brain edema. This limits the extent of ischemia-induced secondary damage and may have a role in the neuroprotection against ischemia.

The mechanisms by which BM-21 decreased the damage caused by ischemia-reperfusion in the brain have not yet completely explained. We have previously found multi-radical effects of BM-21 and halassiolin B on free radical and several ROS [13, 14]. Moreover, an early *in vitro* study [47] showed that BM-21 protect cultured hepatocytes against *t*-butilhydroperoxide by increasing GSH concentration and decreasing MDA level suggesting an antioxidant effects. These results are in agreement with those [13] showed that BM-21 and halassiolin B applied topically prevented UVB mouse skin injury against UVB-acute damage. This view was subsequently supported by the fact that the lipophilic fractions obtained from BM-21 (F1) inhibited the LPS-stimulated nitrosative stress in RAW 264.7 cells and exhibited ROS scavenger capacity [48]. In accordance, topically applied F1 also prevented skin damage by UVB radiation and significantly attenuated MDA concentration and increased GSH and SOD activity in mouse skin within the first 48 h after acute exposure to UVB irradiation [48]. A recent *in vivo* study showed that BM-21 given by oral route elicited significant neuroprotection in the model of acrylamide-induced neurotoxicity by preventing the decrease in antioxidant enzymes GSHPx and SOD in sciatic nerve, cerebellum and brain caused by ACR. The increase in enzyme activities was accompanied by a significant increase in GSH concentration and decrease in

lipid peroxidation [16]. Therefore our results of *in vitro* and *in vivo* experiments support that the radical trapping action of the extract may have the capacity to directly quench free radical species in brain and this characteristic certainly contributes to its neuroprotective effects on global ischemia. This effect may preserve brain antioxidant status by protecting the inactivation of antioxidant enzymes from free radical attack [49] and GSH content. However, other mechanism that might account for the maintenance of redox balance is probably the modulatory effects exerted by some flavones identified as components of the extract on Nrf2/ARE pathway [50, 51]. Thus, BM-21 could prevent ischemic damage via either direct scavenging of ROS or the induction of cellular defense detoxifying/antioxidant enzymes. However, as there is no direct data addressing such effect under ischemic gerbil's brain, this hypothesis awaits further investigation. It is noteworthy that stroke is associated with acidification [52] that can induce neuronal death mostly caused by the activation of the acid sensitive ion channels (ASICs)[53]. Since BM-21 and halassiolin B inhibited ASICS and exhibited antinociceptive effects *invivo* given by oral route at the same dose [15] another mechanism of neuroprotection on global ischemia in Gerbil may be related to the inhibition of ASICS.

Overall, oral administration of BM-21 was neuroprotective in terms of reducing ischemic damage in experimental transient BCCAO in Mongolian gerbil. BM-21 given at doses that decreased brain infarct size and prevent the loss of hippocampal CA1region also improved the protective defenses against oxidative stress in brain. Thus, the neuroprotective activity of BM-21 in this model is at least in part the result of its antioxidant properties. Interestingly, the phytochemical analysis *T. testidinum*BM-21 extract indicated the presence of phytoconstituents including poliphenols, flavonoids and proannthocyanidins as major constituents [14] which partially explain the neuroprotective action due to their multiple effects as modulators of neuronal function [54] . The present result, together with the neuroprotective effect previously observed in

ACR model [16], suggests that the active components of BM-21 can cross the blood brain barrier and reach brain tissue at concentrations capable of developing protective activity against ischemic insult when given by oral route. Thus, BM-21 extract seems to possess potential as a supplement in health food products. However, the central neuroprotective effects of BM-21 observed in the present study need to be substantiated in other animal model more closely related to clinical ischemic events.

Acknowledgement

References

1. Warlow, C.; Sudlow, C.; Dennis, M.; Wardlaw, J.;Sandercock, P. Stroke, Lancet, 2003, 362(9391),1211-1224. DOI: 10.1016/S0140-6736(03)14544-8.
2. Piantadosi, CA.;Zhang, J. Mitochondrial generation of reactive oxygen species after brain ischemia in the rat, Stroke, 1996, 27(2),327-331.
3. Phillis, JW. A "radical" view of cerebral ischemic injury, Progress in Neurobiology, 1994, 42(4),441-448.
4. Gaudet, RJ.; Alam, I.;Levine, L. Accumulation of cyclooxygenase products of arachidonic acid metabolism in gerbil brain during reperfusion after bilateral common carotid artery occlusion, Journal of Neurochemistry, 1980, 35(3),653-658.
5. Garthwaite, J.; Garthwaite, G.; Palmer, RM.;Moncada, S. NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices, European Journal of Pharmacology, 1989, 172(4-5),413-416.
6. Voko, Z.; Hollander, M.; Hofman, A.; Koudstaal, PJ.;Breteler, MM. Dietary antioxidants and the risk of ischemic stroke: the Rotterdam Study, Neurology, 2003, 61(9),1273-1275.
7. Cassidy, A.; Rimm, EB.; O'Reilly, EJ.; Logroscino, G.; Kay, C.; Chiuve, SE.;Rexrode, KM. Dietary flavonoids and risk of stroke in

women, Stroke, 2012, 43(4),946-951. DOI: 10.1161/STROKEAHA.111.637835

8. Ahn, BR.; Moon, HE.; Kim, HR.; Jung, HA.;Choi, JS. Neuroprotective effect of edible brown alga Eiseniabicyclis on amyloid beta peptide-induced toxicity in PC12 cells, Archives of Pharmacal Research, 2012, 35(11),1989-1998. DOI: 10.1007/s12272-012-1116-5

9. Heo, SJ.; Cha, SH.; Kim, KN.; Lee, SH.; Ahn, G.; Kang, DH et al. Neuroprotective effect of phlorotannin isolated from Ishigeokamurae against H(2)O(2) -induced oxidative stress in murine hippocampal neuronal cells, HT22, Applied Biochemistry and Biotechnology, 2012, 166(6),1520-1532. DOI: 10.1007/s12010-012-9545-7

10. Yende, SR.; Harle, UN.;Chaugule, BB. Therapeutic potential and health benefits of Sargassum species, Pharmacog Rev, 2014, 8(15),1-7. DOI: 10.4103/0973-7847.125514

11. Kang, SM.; Cha, SH.; Ko, JY.; Kang, MC.; Kim, D.; Heo, SJ et al. Neuroprotective effects of phlorotannins isolated from a brown alga, Ecklonia cava, against H2O2-induced oxidative stress in murine hippocampal HT22 cells, Environmental Toxicology and Pharmacology, 2012, 34(1),96-105. DOI: 10.1016/j.etap.2012.03.006

12. Kim, JH.; Lee, NS.; Jeong, YG.; Lee, JH.; Kim, EJ.;Han, SY. Protective efficacy of an Ecklonia cava extract used to treat transient focal ischemia of the rat brain, Anatomy and Cell Biology, 2012, 45(2),103-113. DOI: 10.5115/acb.2012.45.2.103

13. Regalado, EL.; Rodriguez, M.; Menendez, R.; Concepcion, AA.; Nogueiras, C.; Laguna, A et al. Repair of UVB-damaged skin by the antioxidant sulphated flavone glycoside thalassiolin B isolated from the marine plant Thalassia testudinum Banks ex Konig, Mar Biotechnol (NY), 2009, 11(1),74-80. DOI: 10.1007/s10126-008-9123-8

14. Regalado, EL.; Menendez, R.; Valdes, O.; Morales, RA.; Laguna, A.; Thomas, OP.; Hernandez, Y.; Nogueiras, C.;Kijjoa, A. Phytochemical analysis and antioxidant capacity of BM-21, a bioactive extract rich in polyphenolic metabolites from the sea Grass Thalassia testudinum, Natural Product Communications, 2012, 7(1),47-50.

15. Garateix, A.; Salceda, E.; Menendez, R.; Regalado, EL.; Lopez, O.; Garcia, T et al. Antinociception produced by Thalassia testudinum extract BM-21 is mediated by the inhibition of acid sensing ionic channels by the phenolic compound thalassiolin B, Molecular Pain, 2011, 7,10. DOI: 10.1186/1744-8069-7-10

16. Menendez, R.; Garcia, T.; Garateix, A.; Morales, RA.; Regalado, EL.; Laguna, A.; Valdes, O.;Fernandez, MD. Neuroprotective and antioxidant effects of Thalassia testudinum extract BM-21, against acrylamide-induced neurotoxicity in mice, Journal of Pharmacy and Pharmacognosy Research, 2014, 2(3),53-62.

17. McGraw, CP. Experimental cerebral infarction effects of pentobarbital in Mongolian gerbils, Archives of Neurology, 1977, 34(6),334-336.

18. Molina, V.; Arruzazabala, ML.; Carbajal, D.; Valdes, S.; Noa, M.; Mas, R.; Fraga, V.;Menendez, R. Effect of policosanol on cerebral ischemia in Mongolian gerbils, Brazilian Journal of Medical and Biological Research, 1999, 32(10),1269-1276.

19. Markwell, MA.; Haas, SM.; Bieber, LL.;Tolbert, NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Analytical Biochemistry, 1978, 87(1),206-210.

20. Ohkawa, H.; Ohishi, N.;Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Analytical Biochemistry, 1979, 95(2),351-358.

21. Ellman, GL. Tissue sulfhydryl groups, Archives of Biochemistry and Biophysics, 1959, 82(1),70-77.

22. Cini, M.; Fariello, RG.; Bianchetti, A.;Moretti, A. Studies on lipid peroxidation in the rat brain, Neurochemical Research, 1994, 19(3),283-288.

23. Kirino, T. Delayed neuronal death in the gerbil hippocampus following ischemia, Brain Research, 1982, 239(1), 57-69.

24. Hong, JT.; Ryu, SR.; Kim, HJ.; Lee, JK.; Lee, SH.; Yun, YP.; Lee, BM.;Kim, PY. Protective effect of green tea extract on ischemia/reperfusion-induced brain injury in Mongolian gerbils, Brain Research, 2001, 888(1),11-18.

25. Sharma, SS.;Gupta, S. Neuroprotective effect of MnTMPyP, a superoxide dismutase/catalase mimetic in global cerebral ischemia is mediated through reduction of oxidative stress and DNA fragmentation, European Journal of Pharmacology, 2007, 561(1-3),72-79. DOI: 10.1016/j.ejphar.2006.12.039

26. Harrison, MJ.;Brownbill, D.; Lewis, PD.;Russell, RW. Cerebral edema following carotid artery

ligation in the gerbil, Archives of Neurology, 1973, 28(6),389-391.

27. Payan, HM.;Conrad, JR. Carotid ligation in gerbils. Influence of age, sex, and gonads, Stroke, 1977, 8(2),194-196.

28. Myung, CS.; Shin, HC.; Bao, HY.; Yeo, SJ.; Lee, BH.;Kang, JS. Improvement of memory by dieckol and phlorofucofuroeckol in ethanol-treated mice: possible involvement of the inhibition of acetylcholinesterase, Archives of Pharmacal Research, 2005, 28(6),691-698.

29. Rao, AM.; Hatcher, JF.;Kindy, MS.;Dempsey, RJ. Arachidonic acid and leukotriene C4: role in transient cerebral ischemia of gerbils, Neurochemical Research, 1999, 24(10),1225-1232.

30. Brown, GC. Mechanisms of inflammatory neurodegeneration: iNOS and NADPH oxidase, Biochemical Society Transactions, 2007, 35(Pt 5),1119-1121. DOI: 10.1042/BST0351119

31. Thomas, NV.;Kim, SK. Potential pharmacological applications of polyphenolic derivatives from marine brown algae, Environmental Toxicology and Pharmacology, 2011, 32(3),325-335. DOI: 10.1016/j.etap.2011.09.004

32. Traystman, R.; Kirsch, J.;Koehler, R. Oxygen radical mechanisms of brain injury following ischemia and reperfusion, Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology, 1991, 71,1185-1195.

33. Sinet, PM.; Heikkila, RE.;Cohen, G. Hydrogen peroxide production by rat brain in vivo, Journal of Neurochemistry, 1980, 34(6),1421-1428.

34. Mizuno, Y.;Ohta, K. Regional distributions of thiobarbituric acid-reactive products, activities of enzymes regulating the metabolism of oxygen free radicals, and some of the related enzymes in adult and aged rat brains, Journal of Neurochemistry, 1986, 46(5),1344-1352.

35. Desagher, S.; Glowinski, J.;Premont, J. Astrocytes protect neurons from hydrogen peroxide toxicity, Journal of Neuroscience, 1996, 16(8),2553-2562.

36. Ballatori, N.; Krance, SM.; Notenboom, S.; Shi, S.; Tieu, K.;Hammond, CL. Glutathione dysregulation and the etiology and progression of human diseases, Biological Chemistry, 2009, 390(3),191-214. DOI: 10.1515/BC.2009.033

37. Homi, HM.; Freitas, JJ.; Curi, R.; Velasco, IT.;Junior, BA. Changes in superoxide dismutase and catalase activities of rat brain regions during early global transient ischemia/reperfusion, Neuroscience Letters, 2002, 333(1),37-40.

38. Saybasili, H.; Yuksel, M.; Haklar, G.;Yalcin, AS. Depolarization-induced superoxide radical formation in rat hippocampal slices, Neurochemical Research, 2002, 27(6),473-476.

39. Candelario-Jalil, E.; Mhadu, NH.; Al-Dalain, SM.; Martinez, G.;Leon, OS. Time course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils, Neuroscience Research, 2001, 41,233-241.

40. Haba, K.; Ogawa, N.; Mizukawa, K.;Mori, A. Time course of changes in lipid peroxidation, pre- and postsynaptic cholinergic indices, NMDA receptor binding and neuronal death in the gerbil hippocampus following transient ischemia, Brain Research, 1991, 540(1-2),116-122.

41. Urabe, T.; Yamasaki, Y.; Hattori, N.; Yoshikawa, M.; Uchida, K.;Mizuno, Y. Accumulation of 4-hydroxynonenal-modified proteins in hippocampal CA1 pyramidal neurons precedes delayed neuronal damage in the gerbil brain, Neuroscience, 2000, 100(2),241-250.

42. Yamaguchi, S.; Ogata, H.; Hamaguchi, S.;Kitajima, T. Superoxide radical generation and histopathological changes in hippocampal CA1 after ischaemia/reperfusion in gerbils, Canadian Journal of Anaesthesia, 1998, 45(3),226-232. DOI: 10.1007/BF03012907

43. Klatzo, I. Presidental address. Neuropathological aspects of brain edema, Journal of Neuropathology and Experimental Neurology, 1967, 26(1), 1-14.

44. Bhakoo, KK.; Crockard, HA.; Lascelles, PC.;Avery, SF. Prostaglandin synthesis and oedema formation during reperfusion following experimented brain ischemia in the gerbil, Stroke, 1984, 15,891- 895.

45. Gaudet, RJ.; Alam, I.;Levine, L. Accumulation of cyclooxygenase products of arachidonic acid metabolism in gerbil brain during reperfusion after bilateral common carotid artery occlusion, Journal fo Neurochemistry, 1980, 35,653-658.

46. Xu, XS.; Ma, ZZ.; Wang, F.; Hu, BH.; Wang, CS.; Liu, YY et al. The antioxidant Cerebralcare Granule attenuates cerebral microcirculatory disturbance during ischemia-reperfusion injury, Shock, 2009, 32(2),201-209. DOI: 10.1097/SHK.0b013e3181996d61

47. Rodeiro, I.; Donato, MT.; Martinez, I.; Hernandez, I.; Garrido, G.; Gonzalez-Lavaut, JA et al. Potential hepatoprotective effects of new Cuban natural products in rat hepatocytes culture,

Toxicology In Vitro, 2008, 22(5),1242-1249. DOI: 10.1016/j.tiv.2008.04.006

48. Regalado, EL.; Rodriguez, M.; Menendez, R.; Fernandez, X.; Hernandez, I.; Morales, RA et al. Photoprotecting action and phytochemical analysis of a multiple radical scavenger lipophilic fraction obtained from the leaf of the seagrassThalassiatestudinum, Photochemistry and Photobiology, 2011, 87(5),1058-1066. DOI: 10.1111/j.1751-1097.2011.00945.x

49. Pigeolet, E.; Corbisier, P.; Houbion, A.; Lambert, D.; Michiels, C.; Raes, M.; Zachary, MD.;Remacle, J. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals, Mechanisms of Ageing and Development, 1990, 51(3),283-297.

50. Wu, PS.; Yen, JH.; Kou, MC.;Wu, MJ. Luteolin and Apigenin Attenuate 4-Hydroxy-2-Nonenal-Mediated Cell Death through Modulation of UPR, Nrf2-ARE and MAPK Pathways in PC12 Cells, PLoS One, 2015, 10(6),e0130599. DOI: 10.1371/journal.pone.0130599

51. Paredes-Gonzalez, X.; Fuentes, F.; Jeffery, S.; Saw, CL.; Shu, L.; Su, ZY.;Kong, AT. Induction of NRF2-mediated gene expression by dietary phytochemical flavones apigenin and luteolin, Biopharmaceutics and Drug Disposition, 2015.DOI: 10.1002/bdd.1956

52. Tombaugh, GC.;Sapolsky, RM. Evolving concepts about the role of acidosis in ischemic neuropathology, Journal of Neurochemistry, 1993, 61(3),793-803.

53. Xiong, ZG.; Zhu, XM.; Chu, XP.; Minami, M.; Hey, J.; Wei, WL et al. Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels, Cell, 2004, 118(6),687-698. DOI: 10.1016/j.cell.2004.08.026

54. Spencer, JPE. Flavonoids: modulators of brain function?,British Journal of Nutrition, 2008, 99(E-Suppl.1),ES60-ES77. DOI:10.1017/S0007114508965776