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Neuroprotective effects of *Thalassia testudinum* leaf extract BM-21 on focal ischemia in rats

[Efectos neuroprotectores del BM-21, extracto obtenido de las hojas de *Thalassia testudinum* sobre la isquemia focal en ratas]

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Abstract

Context: The extract from the marine plant *Thalassia testudinum* BM-21, standardized to thalassiolin B content (5.8 ± 0.3%), possesses antioxidant, anti-inflammatory and neuroprotective effects on acrylamide-induced neurotoxicity in mice and global ischemia in Mongolian gerbils.

Aims: To determine whether or not BM-21 possesses neuroprotective effects against cerebral ischemia induced by permanent middle cerebral artery occlusion (pMCAo), a clinically relevant model of stroke.

Methods: BM-21 was administered orally (400 mg/kg, once-a–day/10 days) prior to ischemia. Twenty-four hours after occlusion, we studied neurological signs, infarct volume, cerebral edema, histological damage and oxidative stress in cortex and striatum. In addition, brain susceptibility to *in vitro* lipid peroxidation induced by kainic acid and 2,2'-azobis(2-amidinopropane) dihydrochloride was studied after the BM-21 administration.

Results: BM-21 prevented behavioral deficit; reduced infarct volume and cerebral edema; markedly decreased neuronal damage in striatum and cortex region. After occlusion, there was a significant increase of oxidative stress in cortex and striatum. Treatment of ischemic rats with BM-21 (400 mg/kg) prevented lipid peroxidation and protein damage and increased the antioxidant enzymatic activities and glutathione. BM-21 also inhibited the *in vitro* lipid peroxidation in total brain homogenates.

Conclusions: Oral pre-treatment of BM-21 protects rats against pMCAo ischemia-induced damage in the striatum and cortex. Results suggest that the protection of BM-21 involve at least partially, the increase resistance to oxidative stress.

Keywords: neuroprotection; oxidative stress; permanent middle cerebral artery occlusion; *Thalassia testudium*.

Resumen

Contexto: El extracto obtenido de las planta marina *Thalassia testudinum* (BM-21), estandarizado acorde al contenido de thalassiolin B (5.8 ± 0.3%), posee efectos antioxidantes, antiinflamatorios y neuroprotectores sobre la neurotoxicidad inducida por acrilamida y la isquemia global en gerbos de Mongolia.

Objetivos: Evaluar si el BM-21 ejerce efectos neuroprotectores sobre la isquemia inducida por la oclusión permanente de la arteria cerebral media en ratas (pMCAo), un modelo relevante desde el punto de vista clínico.

Métodos: El BM-21 fue administrado previamente por vía oral (400 mg/kg, 10 días/1 administración/día). Tras la oclusión (24 h) se investigaron; síntomas de daño neurológico, volumen de infarto, edema cerebral y daño histológico y estrés oxidativo en cuerpo estriado y corteza, así como el efecto del BM-21 sobre la peroxidación lipídica *in vitro* inducida por ácido kaínico y dihidrocloruro de 2,2'-azobis(2-amidinopropano).

Resultados: BM-21 previno la disfunción conductual, disminuyó el volumen de infarto, el edema cerebral, y el daño neuronal en cuerpo estriado y corteza. La oclusión indujo el incremento del estrés oxidativo en cuerpo estriado y corteza. El BM-21 disminuyó la peroxidación lipídica y el daño a proteínas e incrementó las actividades de las enzimas antioxidantes y el glutatión. También el BM-21 inhibió la peroxidación lipídica inducida *in vitro*.

Conclusiones: El BM-21 previno el daño neuronal inducido por la isquemia en corteza cerebral y cuerpo estriado en ratas, efecto que involucra al menos parcialmente, el incremento de la resistencia frente al estrés oxidativo.

Palabras Clave: estrés oxidativo; neuroprotección; oclusión permanente de la arteria cerebral media; *Thalassia testudium*.

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INTRODUCTION

Brain infarction from ischemia is a major cause of disability and death from the new born to the elderly. Development of an effective therapeutic strategy for ischemia has been a priority and challenge of neuroscientists for decades as brain stroke lack effective treatment. There is a general agreement that one of the major mechanisms involved in neuronal cell death during ischemia is oxidative stress (Hara et al., 1993; Hayashi et al., 1999; Lee et al., 2000). Therefore, consumption of antioxidant may prevent incidence and consequences of brain ischemia. Polyphenols are natural antioxidants that are present in vegetables, grains, fruits bark, roots, tea, and wine. Epidemiological studies have supported that the consumption of polyphenols can decrease the incidence of stroke (Cassidy et al., 2012; Bhullar and Rupasinghe, 2013). In this context, the neuroprotective effect of plant flavonoids has been thoroughly documented (Panickar et al., 2010; Gopinath et al., 2011; Tang et al., 2016).

Marine organisms have developed mechanisms of adaptation to adverse environmental factors (Sanders, 1993; Tomanek et al., 2010). Production of bioactive metabolites is one of such mechanisms (Hay and Fenical 1998; Engel et al., 2002; Pallela et al., 2010). However, marine organisms have not received all the attention they merit and thus the pharmacological properties of marine phytochemicals are not as well-known as are those of land plants. Nevertheless, it is true that over the past several years marine organisms have attracted the attention as a source of novel antioxidants. Among them, seaweeds are considered to be a rich source of polyphenolic antioxidants (Khairy and El-Sheikh, 2015) that exhibit in some cases neuroprotective actions (Pangestuti and Kim, 2011; Kang et al., 2012; Kim et al., 2012; Yende et al., 2014).

BM-21 is an aqueous-ethanolic extract from the leaves of *Thalassia testudium*, a Caribbean flowering seagrass widely distributed along Cuban coast. Polyphenols represent 29.5 ± 1.2% of the components in this extract (Regalado et al., 2012). The extract is known to exert anti-inflammatory effects (Llanio et al., 2006) and antioxidant actions (Regalado et al., 2009; 2012). BM-21 also promoted the recovery of mouse skin after acute UVB damage (Regalado et al., 2009) and exhibited antinociceptive effects (Garateix et al., 2011). Thalasiolin B, (chrysoeriol 7-b-D-glucopyranosyl-2"-sulfate), the most abundant active component of the extract appears to be responsible for both effects (Regalado et al., 2009; Garateix et al., 2011). Also apigenin 7-O-β-D-glucopyranosyl-2″-sulphate (thalassiolin C), chrysoeriol 7-O-β-D-glucopyranoside, apigenin 7-O-β-D-glucopyranoside, 5,7-dihydroxy-3',4'-dimethoxyflavone 7-O-β-D-glucopyranoside, luteolin-3'-sulphate, chrysoeriol and apigenin has been identified as other phenolic phytochemicals of the extract (Regalado et al., 2012). The extract also showed a hepatoprotective effect in vitro in primary cultured rat hepatocytes mainly due to antioxidant action (Rodeiro et al., 2008). A recent *in vivo* study showed that the extract standardized to thalassiolin B content (5.8 ± 0.3 %) administered by oral route (200 and 400 mg/kg/10 days) produced significant neuroprotection in the model of ACR-induced neurotoxicity by preventing the decrease in antioxidant enzymes glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in sciatic nerve, cerebellum and brain (Menéndez et al., 2014). BM-21 also was neuroprotective in the model of global ischemia in Mongolian gerbil as it reduced ischemic damage, decreased brain infarct size and prevented the loss of hippocampal CA1 region (Menéndez et al., 2015). The extract concomitantly improved the protective defenses against oxidative stress in the brain. However, human cerebral ischemia often (50.8%) results from a transient or permanent occlusion of the middle cerebral artery (MCA) (Yee Sien et al., 2007). Thus, the neuroprotective effects of BM-21 observed on global ischemia in gerbils need to be validated in another animal model more closely related to the clinical situation. In consequence, the present study was conducted to assess whether or not BM-21 possesses neuroprotective effects on cerebral infarction induced by permanent focal middle cerebral artery occlusion (pMCAo) in rats, a clinically-relevant model of stroke (Engel et al., 2011).

MATERIAL AND METHODS

Plant material

Thalassia testudinum was collected in March 2008 from "La Concha" Beach (22° 05" 45"" N, 82° 27" 15"" W) and taxonomically identified by Dr. Areces J.A. from the Institute of Oceanology, Havana, Cuba. A specimen (No. IdO39) was retained in the herbarium of the Cuban National Aquarium from identification. BM-21 was obtained as previously described (Regalado et al., 2009). The extract used in the present study contains a percentage of flavonoids and thalassiolin B within the quality criteria established (Garateix et al., 2011).

Materials

All chemicals were from Aplychem (Darmstadtz, Germany), except 2,2-azo-bis-2-amidinopropane hydrochloride (AAPH), obtained from Polyscience (Warington, PA) and triphenyl tetrazolium chloride (TTC) that was from Sigma-Aldrich Co. (St Louis, MO). Kit for the determination of SOD activity was from Randox (Crumlin, U.K.). Ketamine and Xylacine were from (Norepley, S.A, Montevideo, Uruguay). All the other reagents were of the highest purity available. Spectrophotometer UV/visible-120-2 was from Shimadzu Corporation (Japan).

Animals and treatment

Experiments were carried out using male Wistar rats from National Center for Laboratory Animals (CENPALAB), Santiago de Las Vegas, Havana, Cuba and Institute Clemente Estable (Montevideo, Uruguay). Rats weighting 250-350 g had access to food and water *ad libitum*, and were housed in groups of six in a temperature-controlled environment on a 12 h light/dark cycle. A total of 70 animals were randomly assigned into three experimental groups; an ischemic group orally treated for 10 days prior to ischemia with vehicle (distilled water); ischemic treated group receiving by oral route BM-21 (400 mg/kg once a day for 10 days); and the sham operated group was administered with vehicle. The dosage was selected according to our previous results, which showed that BM-21 at 400 mg/kg was the optimal dose in preventing acrylamide induced neurotoxicity in mice (Menendez et al., 2014) and the neuronal damage in a gerbil model of ischemia (Menendez et al., 2015). Also at this dose BM-21 exerted antinociceptive effects (Garateix et al., 2011). BM-21 and vehicle were administered by gastric gavage at 0.01 mL/g body weight previously dissolved in distilled water. 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.

Permanent focal middle cerebral artery occlusion (pMCAo)

Prior to surgery procedure, animals were anaesthetized by intraperitoneal route by ketamine (80 mg/kg) and xylacine (5 mg/kg). During the operation, the body temperature of the animals was continuously kept at 37°C by means of a thermostat controlled plate. Permanent focal cerebral ischemia was induced as described by (Sydserff et al., 1996) with minor modifications. In brief, a surgical midline incision was made to expose the left common, internal and external carotid arteries. The external carotid (ECA) and the common carotid arteries (CCA) were closed by a ligature. The occipital artery was electrocauterized and the internal carotid artery (ICA) was temporarily occluded using a micro- aneurysm clip. A small incision was then made in the common carotid artery, and a 19 mm length of 4-0 monofilament nylon sutures, its tip rounded by heating, was introduced into the ICA. The pterygopalatine branch of the ICA was clipped to prevent incorrect insertion of the occluding filament that was carefully advanced and slowly pushed forward through ICA until a light resistance was felt, resulting in pMCAo. After surgery, animals were then returned to their cages and closely monitored until they recovered from anesthesia. In sham-operated group, the external carotid artery was surgically prepared for insertion of the filament, but the filament was not inserted.

Neurological evaluation

Before the surgery and 24 h after pMCAo animals, were subjected to a neurological evalua-

tion using a four-point scale as described (Menzies et al., 1992). Shams operated were used as control. The scale was as follow; 0: no apparent deficits behavioral; 1: contra lateral forelimb flexion when suspended by the tail; 2: decreased grip of the contra lateral forelimb while tail pulled; 3: spontaneous movement in all directions, contra lateral circling only if pulled by tail and 4: spontaneous contra lateral circling. Tests were conducted blinded in relation to treatment.

Morphometric evaluation

Infarct volume was calculated exactly as previously reported (Yang and Shua, 1998). Briefly, after completing neurological evaluation the animals were sacrificed under a light anesthesia with ether and brains were removed, frozen and coronally sectioned into five 2-mm thick slices (from rostral to caudal). Coronal sections of brain were stained with 2% TTC solution in saline for 10 min at 37°C. After fixing in 4% formal saline overnight, they were scanned using a flatbed scanner (Hewlett Packard HP Scanjet 3670). The area of infarction was measured in coronal brain sections by using image analysis software Image J, version 1.34. Measurements were made by manually outlining the margins of infarct areas. Infarct areas of all sections were cumulated to get total infarct area which was multiplied by thickness of brain sections to get the volume of infarction. To compensate for swelling, the following formula was applied: infarct size x contralateral hemisphere size/ipsilateral hemisphere size. The volumes of both hemispheres were calculated separately, and edema volume was calculated by subtracting the contralateral from ipsilateral volume (Thiyagarajan et al., 2004).

Histological evaluation and quantification of survival neurons

A total of 24 animals were used to examine ischemia-induced histological damage in treated and non-treated ischemic animals. Pseudo-operated animals served as controls. Twenty-four hours after pMCAo the animals were anaesthetized under a light anesthesia with ether and intracardially perfused with 100 mL of NaCl 0.9% and 150 mL of 4% paraformaldehyde. The brains were quickly removed and finally embedded in paraffin wax. The sections were selected from a brain region corresponding to levels +0.2 mm and +0.26 mm with respect to Bregma according to anatomical structures observed in Paxinos rat brain atlas (parietal cortex-striatum). Five-micrometer thick sections (20 µm) were cut on a microtome and stained with Nissl technique for light microscope examinations. In order to assess the effects of BM-21 treatment on the survival of the neurons in striatum and cortex in sections ipsilateral to the pMCAo (0.20 µm with respect to Bregma), photographs of 4 adjacent fields to the lateral ventricle were taken with a Olympus microscope (magnification 20X) and photographic camera (Microscope Digital Camera-DCM500, USB 2.0). On the photographs, the total population of survival neurons (those with echromatic nucleus and conserved cytoplasm) in the 3 experimental groups (n=6/group) were quantified.

Estimation of oxidative stress in homogenates of cortex and striatum after pMCAo

A total of 24 rats were assigned at random to three experimental groups as described previously and the same dosing protocol was used. After occlusion, rats were anesthetized under ether and euthanized to excise striatum and cortex of the left hemisphere. They were homogenized in 10 mM PBS, 7.4 pH (1:9, w:v) in an ice-cold bath, with a Potter–Elvehjem type homogenizer. Homogenates were centrifuged at 3000 *g* for 10 min and the resultant supernatants were used to determine biochemical parameters. Protein concentration was estimated with a modification of the Lowry procedure. Biochemical parameters were determined by spectrophotometric methods using and spectrophotometer UV-Vis (model 01205, Shimadzu Corporation). Estimation of malondialdeide (MDA) was assayed as thiobarbituric acid reactive substances (TBARS)(Ohkawa et al., 1979). Concentrations of MDA were expressed as µmol/mg protein. Protein carbonyl associated groups were determined after their derivatization with 2,4-dinitrophenylhydrazine (Dalle–Donne et al., 2003) and were expressed as nmol carbonyl groups/mg protein using molar coefficient absorption of 22,000 $M^{-1}cm^{-1}$. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity was measured according to commer-

cially available kit and expressed as IU/mg protein. The concentration of reduced glutathione (GSH) was measured by method of (Ellman, 1959) and expressed as nM by means of a molar absorption coefficient of 14,150 $M^{-1}cm^{-1}$.

Brain susceptibility to *in vitro* lipid peroxidation (LPO)

Sixteen male Wistar rats (250-350 g) were divided randomly into two groups (vehicle control and BM-21 treated group) and were similarly treated. At the end of the treatment period, rats were anesthetized with ether, euthanized and brains were quickly excised and homogenized as previously described. To measure the susceptibility to kainic-induced LPO (Yamamoto and Parayanthala, 2003) aliquots of tissue homogenate were incubated in presence of kainic acid 5.12 mg/mL (final concentration) at 37°C for 30 min. To assess brain susceptibility to AAPH-induced LPO (Yu-Jun et al., 2003) tissue samples were incubated with 50 mM AAPH (final concentration) under similar conditions. The extent of LPO was assessed by measuring TBARS concentration as previously described. In both oxidant system LPO status at 0 time were used as basal and were subtracted from the one obtained at 30 min. The TBARS levels were expressed in µmol/mg protein.

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. The global comparison was performed using One-Way ANOVA followed by Turkey's multiple comparison tests. Pair wise testing was done with the help of the unpaired Student t-test. Neurological deficits were presented as median ± 95% confidence interval and analyzed using nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison tests. $P<0.05$ was considered statistically significant.

RESULTS

Animals treated with BM-21 (400 mg/kg, showed no behavioral changes other than the neurological deficits induced by ischemia. Before pMCAo, neurological score was normal (score = 0) in all animals. pMCAo + Vehicle group exhibited the highest score (3.25 ± 1.23, $p<0.001$) corresponding to severe behavioral impairments 24 h after occlusion. Pre-treatment with BM-21 during 10 days resulted in a significant attenuation (0.87 ± 0.35, $p<0.05$) of rat neurobehavioral impairment when compared to pMCAo + Vehicle group (Fig. 1).

Twenty-four hours after pMCAo, rats developed infarcts affecting cortex and striatum. Fig. 2 represents the infarct volume in pMCAo rats treated either with vehicle or BM-21. As noted, treatment with BM-21 markedly reduced infarct areas at 24 h post pMCAo as evident from TTC-stained sections. When the total cerebral infarct volume was determined by computer-assisted imaging methods, a significant reduction ($p<0.001$) of the cerebral infarct volume (77.5%) was observed in the group pre-treated with BM-21 compared with the ischemic group (ischemic, 246.8 ± 31.25 mm^3; BM-21-treated, 55.39 ± 15.67 mm^3). No lesion was observed in Sham animals (data not shown).

Fig. 3 displays brain edema. In pMCAo caused a prominent increase in cerebral edema volume in the ipsilateral hemisphere as compared to contralateral hemisphere ($p<0.016$). However, pre-treatment with BM-21 significantly reduced by 71.2% edema volume ($p<0.001$) in relation to pMCAo + Vehicle group.

Fig. 4 shows representative histological sections of striatum and parietal cortex sections obtained at central coronal level. The figure shows that ischemia produced typical architecture loss in the cortex (E) and striatum (B) as dense network fibers of white substance and parenchyma of gray substance were not observed. In addition, necrotic neurons, proliferation of astrocyte and variable cavitation were observed as well as a decrease of cortex cells (E). In BM-21 treated rats, the histological appearance of brain sections (C and F) were similar to that of sham-operated animals (A and D). The typical architecture of the striatum and cortex was noted, which was especially observed in the striatum. In this section, it can be noted a clearly defined dense network fibers of white substance (indicated by arrows) in relationship to the parenchyma of gray substance. Additionally, the light coloration and the apparent decreased of cells, observed fundamentally in the cortex (E) suggested

the presence of an inflammatory process, which was not observed in the cortex sections of the animals treated with BM-21. A significant reduction ($p<0.001$) in the number of cells with normal structural features was observed in the pMCAo + Vehicle (striatum, 187 ± 6.5 cell/mm² *vs.* 60 ± 5 cell/mm²) and (cortex, 240 ± 10.5 cell/mm² *vs.* 90 ± 5.2 cell/mm²). In contrast, a significant recovery in the neuronal population was observed in the BM-21 pretreated group (striatum, 119.7 ± 10 cell/mm²; cortex, 200 ± 7.6 cell/mm²) (Fig. 5).

Table 1 describes results concerning oxidative stress in cortex and striatum. LPO determined as MDA concentration significantly increased ($p< 0.01$) in both areas when compared with the sham group. BM-21 treatment significantly attenuated the elevation of MDA levels induced by pMCAo in both brain regions ($p<0.05$ and 0.01, respectively). Occlusion also resulted in a significant ($p<0.05$) increase of protein carbonyl in both cortex (55.5%) and striatum (33.3%) in comparison to the pseudo operated group. BM-21 gave lower protein carbonyl content ($p<0.05$) in both brain regions that was almost similar than sham group. GSH content consistently declined ($p<0.001$) in pMCAo group treated with vehicle (cortex, 36.4%; striatum, 29.5%) while BM-21 treatment significantly increased GSH in cortex ($p<0.01$) and striatum ($p<0.001$) towards the normal level. Permanent occlusion significantly decreased SOD activity in cortex (34.7%, $p<0.01$) and striatum (43.7%, $p<0.05$) compared to vehicle sham group whereas the extract treatment significantly reduced the decreased SOD activity induced by pMCAo in both brain regions (cortex, $p<0.01$; striatum, $p<0.05$). Vehicle-pMCAo group showed decreased GSH-Px activity in cortex and striatum ($p<0.001$ and $p<0.05$, respectively) compared to control. Pretreatment with the extract significantly attenuated the decreased GSH-Px activity in cortex ($p<0.01$) and striatum ($p<0.05$), compared to vehicle plus pMCAo group.

Data referring to the *ex vivo* study (Fig. 6) convincingly showed that brain homogenate from BM-21 treated rats was less prone to LPO than those of control group as MDA concentration generated by kainic acid ($p<0.05$) and AAPH ($p<0.01$) were significantly reduced.

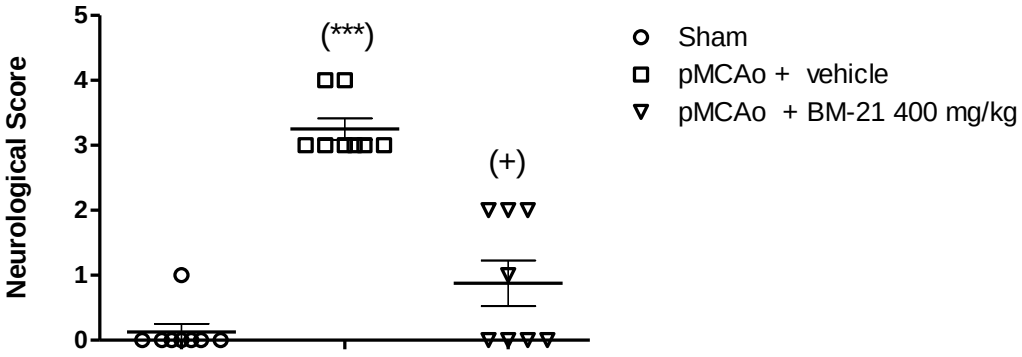

Figure 1. Neurological scores in rats after pMCAo (24 h) and effects of BM-21 administered 10 days before ischemia.

Values are means ± SEM; sham (n = 8); vehicle (n = 8) and BM-21 (n = 8). ***p≤0.01, Comparison vs. sham, ⁺p<0.05, comparison *vs.* pMCAo + Vehicle.

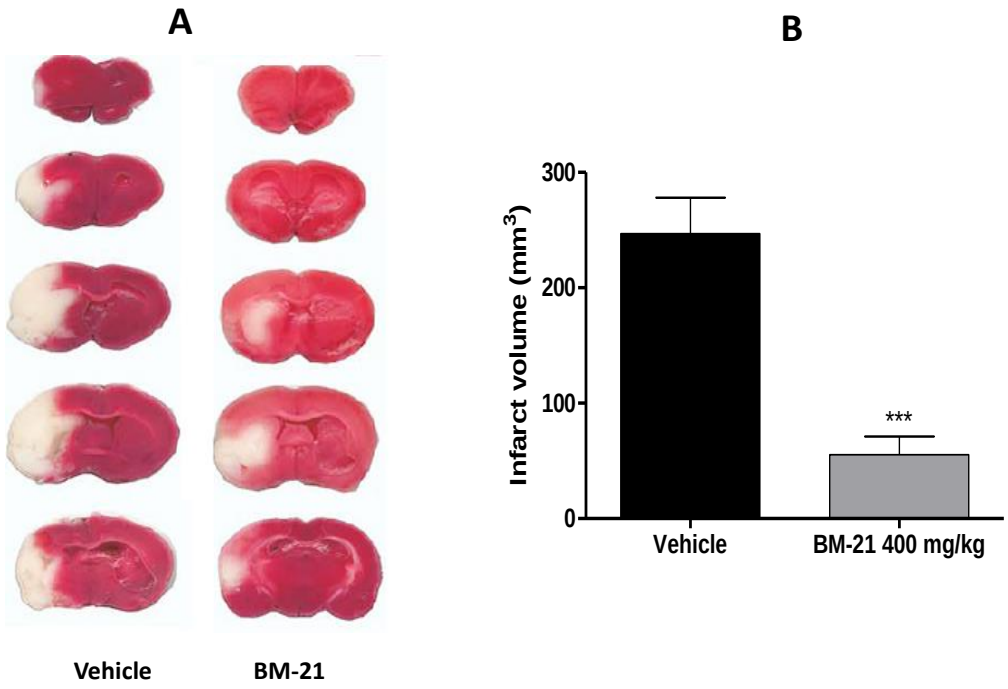

Figure 2. Representative photograph of coronal brain sections (A) and total infarct volume (B) 24 hours after pMCAo of vehicle and BM-21 treated rats.

Bars are means ± SEM for pMCAo+Vehicle (n=6) and pMCAo+BM-21 animals, (n=8). Significant decrease (***p<0.001) in total infarct volume in the group treated with BM-21 (Student's t-test).

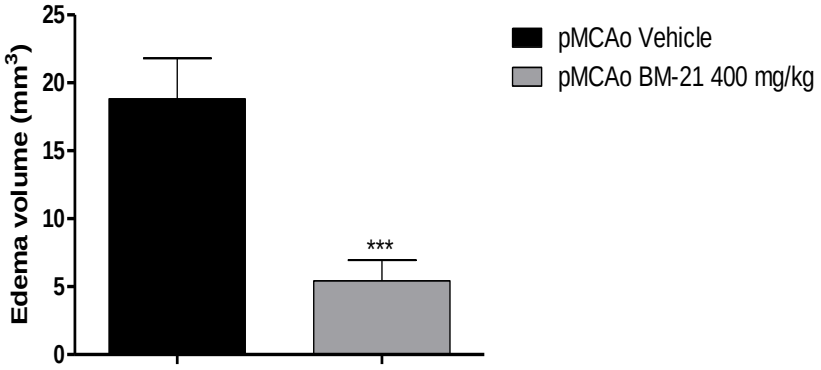

Figure 3. Edema volume after 24 h of pMCAo in vehicle and BM-21 treated group.

Bars are means ± SEM for pMCAo + Vehicle (n=6) and pMCAo + BM-21 (n=8) animals. ***p<0.001 *vs.* pMCAo + Vehicle (Student's t-test).

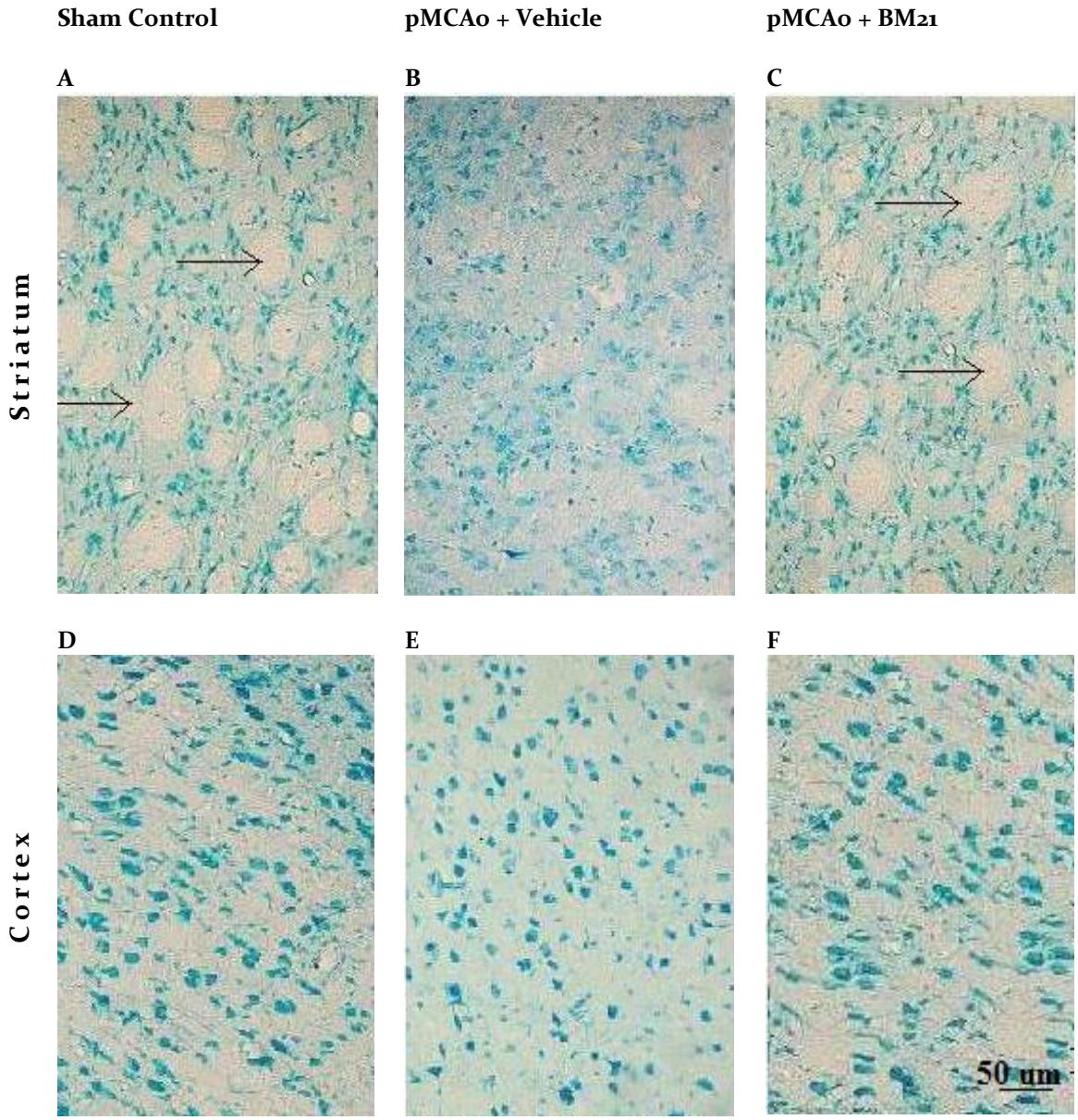

Figure 4. Histological examination of neuronal damage. Nissl staining of striatum and cortex areas at 24 hours after ischemic damage. The sections represent the striatum **(A, B, C)** and cortex **(D, E, F)** of a sham control rat **(A, D)** and ischemic rats; pretreated with vehicle **(B, E)** and BM-21 **(C, F)**.

Original magnification: 20 X, scale bar, 50 µm. This figure is representative of at least three experiments performed on different days. The arrows indicate the dense network fibers characteristic of the striatum.

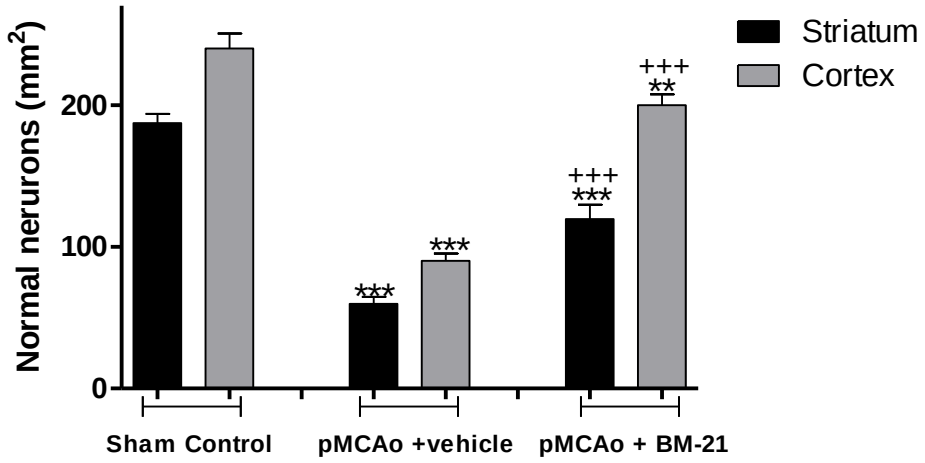

Figure 5. Number of normal neurons in striatum and cortex ipsilateral to the occluded artery counted in four fields adjacent to the lateral ventricles fields.

The number of survival neurons was assessed as the number of neurons in the same fields of sham operated animals. Each group represents the mean ± SEM, (n=6). **p≤0.01, ***p≤0.001; significant different *vs*. Sham control; (+++p ≤0.001) significant different *vs*. ischemic group (One way ANOVA followed by Tukey's multiple comparison test).

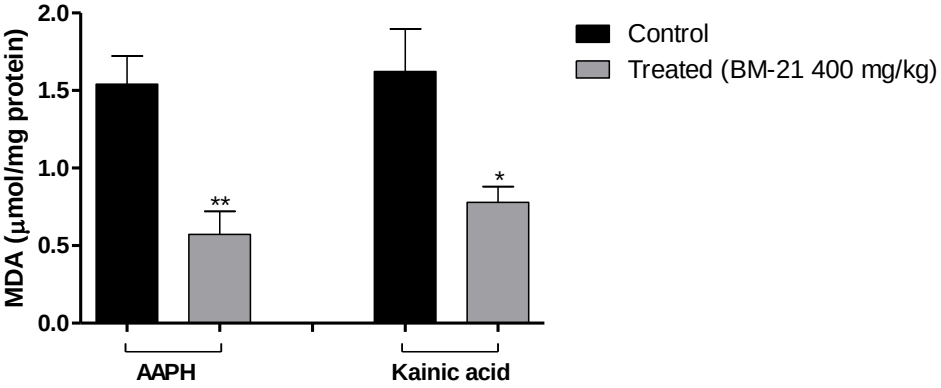

Figure 6. Effect of pre-treatment with BM-21 on the susceptibility of brain homogenates to *in vitro* lipid peroxidation.

Each bar represents the mean ± SEM of eight animals, *p≤0.05, **p≤0.01 *vs*. control (Student's t-test).

Table 1. Effect of BM-21 on oxidative stress in cortex and striatum of ischemic rats.

Group	GSH (nM)	SOD (IU/mg)	GSH-Px (IU/mg)	Protein carbonyls (µmol/mg)	MDA (µmol/mg)
Cortex					
Sham	67.60 ± 4.81	4.43 ± 0.40	1.23 ± 0.12	0.18 ± 0.02	2.95 ± 0.19
Vehicle	44.99± 2.75***	2.89 ± 0.25**	0.80 ± 0.14***	0.28 ± 0.03*	3.92 ± 0.23**
BM-21	62,96 ± 1.61++	4.05 ± 0.23+	1.07 ± 0.11++	0.20 ± 0.02+	2.98 ± 0.15+
Striatum					
Sham	59.78 ± 2.42	3.22 ± 0.49	1.05 ± 0.12	0.24 ± 0.04	2.45 ± 0.25
Vehicle	42.12 ± 2.96***	1.81 ± 0.25*	0.78 ± 0.08*	0.32 ± 0.03*	4.07 ± 0.25**
BM-21	65.60 ± 2.05***	3.28 ± 0.25+	1.12 ± 0.09+	0.19 ± 0.02+	2.57 ± 0.37++

Each group represents the mean ± SEM of eight animals. (*p<0.05, **p<0.01, ***p<0.001) significant different *vs*. sham; (+p<0.05, ++p<0.01) significant different *vs.* vehicle. One way ANOVA followed by Tukey's multiple comparison test.

DISCUSSION

In the present study, BM-21 produced significant neuroprotection in the pMCAo model after 10 days of treatment (400 mg/kg) prior to occlusion. This protective effect is evident through the amelioration of behavioral dysfunction 24 h after pMCAo, the reduction of infarct volume, brain swelling and neuron damage (cortex and striatum) in the ischemic hemisphere. Rats subjected to pMCAo are one of the most pertinent experimental models that resemble the clinical situation since most cases of human ischemic stroke are caused by permanent occlusion (Iadecola and Gorelick, 2004). Thus, the beneficial effect of BM-21 as a preventive agent in this model might have clinical relevance.

Among various brain regions, cortex and striatum are highly susceptible to brain ischemia. The striatum is one of most sensitive regions in focal ischemia because of the greater acidification and the abundance of dopaminergic neuronal endings and dopamine; both factors can increase the production of radical oxygen species (ROS) (Yang et al., 2011). Cortex is another sensitive area that contributes to the formation of the lesion after ischemia, mainly due to its irrigation by the MCA. In the present study, morphometric results revealed infarct area of coronal slices mainly located in striatum and cortex. This agrees with the common patterns of neural damage in focal ischemia in rats found by others authors (Kirisattayakul et al., 2013). Further, our results stated a significant reduction in infarct volume indicated that survival cells in striatum and cortex significantly increased by BM-21 treatment. Our histological evaluation by cresyl violet staining and total population of survival neurons agrees with the morphometric assessment of infarct volume.

The brain injury induced by pMCAo leads to neurobehavioral impairment. pMCAo damages core in the caudoputamen (García et al., 1995) a critical area for motor control (Tang et al., 2006). In addition, striatum and cortex areas are involved in supporting motor activity and motor coordination (Janac et al., 2006). Consistent with our histopathological results, all untreated rats with pMCAo showed neurologic deficits. Since BM-21 extract improved brain infarction and increased the number of cells with normal structural features in cortex and striatum, it is reasonable to expect the improvement of motor function.

The pathophysiological mechanisms leading to neuronal injury following ischemia are complex and multifactorial although oxidative stress is one of the most important factors that exacerbate brain damage induced by cerebral ischemia (Piantadosi and Zhang, 1996). The harmful effect of overproduction of ROS during ischemia can be diminished by several defense mechanisms including catalase (CAT), SOD, GSH-Px and thiol-specific antioxidants. SOD and GSH-Px are considered the major endogenous antioxidant enzymes of the brain. SOD converts superoxide radical into hydrogen peroxide and molecular oxygen, while GSH-Px converts hydrogen peroxide into water. Therefore, the removal of hydrogen peroxide is critical to the efficacy of SOD in reducing oxidative stress. On the other hand, GSH is considered the most important intracellular non-protein thiol that has a crucial role scavenging hydrogen peroxide and lipid peroxides. The current work showed that there was a significant decline in the activity of the endogenous antioxidant enzymes SOD and GSH-Px in cortex and striatum in the ipsilateral hemisphere in ischemic rats. This may result in loss of protective activity exerted by these enzymes. In addition, GSH content significantly reduced because of the scavenging of the fast generating ROS during ischemia. Dysfunction of the antioxidant system in cortex and stratum may gradually contribute to an overall oxidative because free radicals are capable of inactivating antioxidant enzymes (Vessey and Lee, 1993) and damages GSH synthesis (Aoyama et al., 2008). Therefore, the resulting impairment of the endogenous antioxidant defense system during ischemia can cause oxidative stress, free radicals and damage to the neuronal components (cellular membranes, proteins, and DNA). The above is in agreement with the increment of LPO indicated by the increased MDA and the level of oxidatively modified protein in both brain areas. Our data clearly demonstrated that the administration of BM-21 decreased LPO and protein carbonyl and improved the antioxidant balance in cortex and striatum by increasing SOD and GSH-Px activity and GSH content. As the BM-21 has the ability to scavenge superoxide and hydroxyl radical (Regalado

et al., 2009; 2012) this may result in decreased inactivation of SOD and GSH-Px and enhancement of GSH concentration. These effects in turn may result in decreased LPO and protein oxidation.

Our results are consistent with the neuroprotective and the antioxidant properties previously observed on acrylamide-induced neurotoxicity in mice (Menéndez et al., 2014) and global ischemia in Mongolian gerbils (Menéndez et al., 2015). Therefore, the current results suggest again that the active antioxidant compounds of BM-21 can cross the blood–brain barrier and certainly function as antioxidants in cortex and striatum by directly scavenging pathological concentration of reactive oxygen formed during ischemia. However, another possibility is that BM-21 may act as an indirect antioxidant by increasing the activity of the cellular enzymatic antioxidants GSH-Px and SOD and GSH concentrations. In this regard, it has been shown that polyphenolic compounds can act as neuroprotective agents by modulating antioxidant defenses through its interaction with Nrf2 pathway. In line with these findings, pre-treatment of non-ischemic rats with BM-21 at the same dosage regimen significantly decreased the susceptibility of brain homogenate to kainic acid and AAPH-dependent LPO. This result suggests that the pre-treatment with BM-21 might improve the brain antioxidant status protecting rats against the oxidative stress increased by ischemia.

In ischemic stroke brain edema induced by oxidative stress and inflammation, is a crucial feature of ischemic injury. Brain edema formation has been described as cytotoxic and vasogenic (Klatzo, 1967). Free radicals exert their deleterious actions during both cytotoxic and vasogenic edema either by directly contributing to blood-brain-barrier (BBB) disruption or indirectly by triggering inflammatory pathway. Inflammatory mediators quickly released from injured tissue eventually lead to disruption of the BBB and brain edema. Previous results have shown superoxide scavenging action of BM-21 (Regalado et al., 2012) and the consistent elevation of SOD activity detected *in vivo* in several experimental models after BM-21 administration (Regalado et al., 2011; Menéndez et al., 2014). Besides, we also observed *in vivo* anti-inflammatory effects of BM-21 in acute models of inflammation in mice probably related to the inhibitory effect on phosphorlipase A_2 and cyclooxygenase 2 activity (Llanio et al., 2006) and antinociceptive effects in inflammatory models of pain (Garateix et al., 2011). Therefore, it is reasonable to suggest that the anti-inflammatory effects of BM-21 together with the antioxidant action may account for the decrease of brain edema.

It is noteworthy to note that BM-21 and thalassiolin B inhibited acid-sensing ion channels (ASICs) and exhibited antinociceptive effects *in vivo* given by oral route at the same dose that exhibited neuroprotective action (Garateix et al., 2011). During severe ischemia tissue pH falls below six that may cause neuronal death due to activation of ASICs (Xiong et al., 2004). Hence, another mechanism that contributes to the neuroprotective effects of BM-21 might be related to its effects on ASICs. In this regard, research shows that blockers of these channels can act as neuroprotective agents in models of ischemia (Xiong et al., 2004).

CONCLUSIONS

The treatment with BM-21 exhibited neuroprotective effects in the model of pMCAo. BM-21 extract significantly improved the neurological outcome 24 h after pMCAo and reduced total infarct and edema volume. Also, the extract also reduced neuron damage in cortex and striatum. The present results support the contribution of the antioxidant properties of BM-21 in its neuro-protective effects. Hence, our results show that BM-21 is a promising agent for the treatment of pathologies implicating neurodegeneration such as cerebral ischemia. However, further studies are needed to confirm this possibility.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	García TE	Menéndez R	Rivera F	Garateix A	Morales RA	Regalado E	Rodríguez JC	Dajas F
Concepts or ideas	X	X	X	X				X
Design	X	X		X		X		
Definition of intellectual content	X	X	X	X				X
Literature search	X	X						
Experimental studies	X	X			X		X	
Data acquisition	X	X			X		X	
Data analysis	X	X						
Statistical analysis	X	X						
Manuscript preparation	X	X			X			
Manuscript editing	X	X						
Manuscript review	X	X	X	X	X	X	X	X

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