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Ruta graveolens water extract (RGWE) ameliorates ischemic damage and improves neurological deficits in a rat model of transient focal brain ischemia

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ABSTRACT

Introduction and aims: The limited therapeutic options for ischemic stroke treatment render necessary the identification of new strategies. In recent years, it has been shown that natural compounds may represent a valid therapeutic opportunity. Therefore, the present study aimed to evaluate the protective effect of Ruta graveolens water extract (RGWE) in an in vivo experimental model of brain ischemia.
Methods: RGWE effects on ischemic damage and neurological function were evaluated in adult rats subjected to transient occlusion of the Middle Cerebral Artery (tMCAO), receiving two intraperitoneal injections of RGWE, 100 and 300 min after the induction of ischemia. In addition, astroglial and microglial activation was measured as GFAP and IBA-1 expression by immunofluorescence and confocal microscopy analysis.
Results: Treatment with RGWE containing 10 mg/kg of Rutin, the major component, ameliorates the ischemic damage and improves neurological performances. Interestingly, the pro-inflammatory states of astrocytes and microglia, respectively detected by using C3 and iNOS markers, were significantly reduced in ipsilateral cortical and striatal areas in ischemic RGWE-treated rats.
Conclusions: RGWE shows a neuroprotective effect on brain infarct volume extent in a transient focal cerebral ischemia model and this effect was paralleled by the prevention of pro-inflammatory astroglial and microglial activation. Collectively, our findings support the idea that natural compounds may represent potential therapeutic opportunities against ischemic stroke.

1. Introduction

According to World Health Organization, stroke represents the second leading cause of death worldwide (World Health Organization). Regarding European countries, Wafa and colleagues predict an increase in incidence and prevalence largely dependent on the increase in the number of people who survive stroke [43].

The consequences are devastating: in most cases, stroke patients may be interested by contralateral hemiparesis or hemiplegia, and several cognitive and behavioral symptoms may be also present in dependence of the brain areas affected [41].

The only FDA-approved pharmacological treatment consists in thrombolytic therapy with recombinant tissue Plasminogen Activator (t-PA) administered up to 4,5 h after the onset of stroke. However, t-PA suffers of a narrow therapeutic window and hemorrhagic transformation can occur [26]. Furthermore, it has been proposed that toxicity

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phenomena may occur through NMDA receptor activation that can exacerbate brain damage [21,42].

In the light of these considerations, emerged the idea that therapeutic strategies directed toward single targets may be ineffective to resolve such a complex disorder. Therefore, a one-compound-multi-target strategy could represent a valid alternative. To this aim, natural compounds have been investigated in a series of studies based on preclinical models showing the ability to modulate different intracellular signaling pathways with protective effects both on ischemic damage and on the integrity of the blood-brain barrier [10].

In particular, flavonoids showed a marked effectiveness in limiting ischemic brain damage by interfering with different survival pathways [10].

Ruta graveolens, commonly known as rue, is a native plant of southern Europe that has spread all over the world. Rue is a medicinal plant used since ancient times, in fact, it was already known by the ancient Greeks and Romans as well as at the medical school of Salerno. In recent years it has been the subject of great attention in drug discovery field given its ability to cure various diseases due to the presence, in the extract, of numerous compounds, including some flavonoids, with known curative action. Indeed, the water extract of Ruta graveolens (RGWE) is rich in neuroactive compounds and it has proved effective in different experimental settings both in neuro-oncology and in neurodegeneration as well as to interfere with angiogenesis [14,23,25].

A biochemical characterization has been performed to identify some of the components of the aqueous extract which all belong to the class of flavonoids. The major component of RGWE is Rutin, quercetin-3-O-rutinoside, which is present with an indicative amount of 120 mg/g [25]. However, in these experiments, Rutin has been able to exert the effects shown by RGWE [23,25].

Interestingly, Rutin has been used to manage brain ischemia and ischemia/reperfusion (IR) injury in a number of rat models including brain ischemia and IR [32,36]. Nevertheless, the therapeutic effects of the whole extract have never been tested.

On these premises, the present work aims to analyze the effects of the administration of RGWE in a rat model of transient focal cerebral ischemia based on middle cerebral artery occlusion in a reasonable therapeutic time window.

2. Methods

2.1. Animals

46 Male Sprague–Dawley rats (Charles River Laboratories, Calco, Varese, Italy), weighing 200–250 g, were housed under diurnal lighting conditions (12 h darkness/light) and in a conditioned room (23 °C). Experiments were performed according to the international guidelines for animal research and approved by the Animal Care Committee of "Federico II", University of Naples, Italy. Animals, during any surgical or invasive procedure, were anesthetized using a mixture of oxygen and sevoflurane at 3.5% (Medical Oxygen Concentrator LFY-I-5A), and the rectal temperature was maintained at 37 ± 0.5 °C with a heat-controlled mat (Harvard Apparatus). All efforts were made to minimize animal suffering and to reduce the number of animals used. Among 46 animals used for the present study, 17 have been excluded from the statistical analysis. In particular, 3 died during the surgical procedures, 5 were excluded because the brains were damaged in the sampling and 9 were excluded because the surgical procedure was unsuccessful.

In vitro experiments were accrued out on primary culture from prenatal mice. Briefly, C57BL/6 J mice, obtained from the Charles River Laboratories Italia s.r.l. (Milan, Italy), were housed in the animal facility of the Institute of Genetics and Biophysics under a 12-h light-dark schedule at a constant temperature and with food and water ad libitum. Consistent with ethical responsibilities and 3 R principles, every effort has been made to minimize animal suffering and reduce the number of animals used (three for each experiment). Embryonic age (E) was established by considering the day of insemination (as confirmed by the vaginal plug) as day E0. Prenatal brains were quickly isolated and sited in PBS without calcium and magnesium and supplemented with 33 mmol/L glucose. The ventral midbrain was carefully dissected under a stereoscope in sterile conditions.

2.2. Transient focal ischemia

Transient focal ischemia was induced in rats by middle cerebral artery occlusion through a minimally invasive surgical procedure as previously described [18]. Male rats were anaesthetized using a mixture of oxygen and sevoflurane at 3.5% (medical oxygen concentrator LFY-I-5A). Under an operating stereomicroscope (Nikon SMZ800, Nikon Instruments, Florence, Italy), a 5–0 surgical monofilament nylon suture (Doccol, Sharon, MA, USA) was introduced through the right external carotid artery into the circle of Willis for 19 mm to reach the origin of Middle Cerebral Artery (MCA) blocking blood flow through the artery. To confirm the achievement of ischemia cerebral blood flow (CBF) was monitored, as previously described [35], through a disposable microtip fiber optic probe (diameter 0.5 mm) connected through a master probe to a laser Doppler computerized main unit (PF5001; Perimed Järfälla, Sweden) and analyzed using PSW Perisoft 2.5. Animals were excluded from experimental groups if they did not show a CBF reduction of at least 70% or if they die after the surgical procedure. Rectal temperature was constantly monitored and maintained at 37 ± 0.5 °C through a heated mat and a lamp.

2.3. Drugs

Ruta graveolens is a perennial and spontaneous plant spread in the Mediterranean area. It is not a protected species. Leaves of Ruta graveolens were collected from plants conserved at the Experimental Section of Medicinal Plants at the Botanical Garden of Naples, Italy, with the permission of Prof. Paolo De Luca, director of the "Orto Botanico". The harvest period was spring-summer months. The water extract has been prepared as follows: 250 g of whole leaves were finely chopped with scissors and then put in a flask with 1 liter of distilled water and covered with aluminum foil. Leaves were boiled for 60 min. Boiled leaves were separated from the liquid phase, representing the water extract, using a 3 mm funnel filter paper. Such liquid phase was filtered with a 0.22 μm filter (MILLEX®GP, MILLIPORE, Bedford, MA), collected in a beaker covered with parafilm, and frozen under liquid nitrogen. The extract was then lyophilized (VirTis-SP Scientific). After lyophilization, the powder was stored at 4^0 C. We prepared a stock solution by resuspending the powder with distilled water to a concentration of 50 mg/ml [24].

Reverse-phase high-performance liquid chromatography (RP-HPLC) with photodiode array detection and liquid chromatography (LC) mass spectrometry (MS) using a quadrupole time of flight (ESI-Q/TOF) mass spectrometer equipped with an electrospray ionization (ESI) source was carried out on RGWE, leading to the identification of five molecules, all belonging to the flavonoid's family. The molecules identified were the followings: Rutin (quercetin-3-O-rutinside), quercetin 3-O-glucosyl rhamnosyl-galactoside, epigallocatechine 3-O-gallate, isorhamnetin, isorhamnetin 3-O-rutinoside. The most abundant component is Rutin. Rutin levels within the aqueous extract quantified by RP-HPLC by using the Rutin standard were estimated to be 120 mg/g of lyophilized extract [25].

Ischemic animals were divided into 5 experimental groups: 1) ischemic rats receiving intraperitoneal administration (i.p.) of RGWE vehicle; 2) ischemic rats receiving i.p. the low dose of RGWE containing 10 mg/kg of Rutin; 3) ischemic rats receiving i.p. the intermediate dose of RGWE containing 30 mg/kg of Rutin; 4) ischemic rats receiving i.p. the high dose of the extract containing 100 mg/kg of Rutin; 5) ischemic rats receiving 10 mg/kg of Rutin. The doses of extract administered were based on a previous quantization of the Rutin content [25].

Doses were based on previous studies [23] and on the basis of preliminary experiments, all animals were subjected to two i.p. administrations at two different time intervals, the first early after the starting of reperfusion and the second 300 min after cerebral ischemia induction.

2.4. Evaluation of the infarct volume and neurological deficits

Neurological scores were evaluated before euthanasia according to the following two scales: a general neurological scale and a focal neurological scale, as previously described [18]. For the general neurological score, the following 6 items were measured: (1) conditions of the hair (2) position of ears, (3) conditions of the eyes, (4) posture, (5) spontaneous activity on the bench, and (6) the presence of epileptic behavior. The scores obtained rating the 6 items were then summed to have a general neurological score ranging between 0 and 28 depending on the severity of signs. For the focal neurological score, 7 elements were considered: (1) symmetry of the body, (2) gait, (3) the ability to climb, (4) the presence of circling behavior, (5) front limb asymmetry, (6) compulsory circling, and (7) whisker response. A focal neurological score was obtained by summing the score of each item.

After neurological evaluation animals were transcardially perfused under deep (Sevoflurane) anesthesia with 0,9% saline solution, followed by 60 ml of 4% paraformaldehyde in phosphate-buffered saline. The brains were removed and postfixed overnight at + 4 C° and cryoprotected in 30% sucrose phosphate buffer saline. Brains were sectioned frozen on a sliding cryostat at 40 μm thickness. Nissl staining was performed as previously described [8]. Slide-mounted sections at 400 μm intervals were dipped for 7 min in a 0.5% solution of Cresyl Violet in distilled water supplemented with acetic acid (16 N solution, 60 drops/l). Slides were then rinsed in distilled water, dehydrated through graded ethanol baths (95%, 100%; 5 min each), delipidated for 8 min in xylene, and coverslipped with Eukitt Mounting Medium (Bio-Optica, Milan, Italy). Infarct volume was calculated by summing the infarction areas of all sections and by multiplying the total by slice thickness. To avoid that edema could affect the infarct volume value, infarct volume has been expressed as a percentage of the infarct volume, calculated by dividing the infarct volume by the total ipsilateral hemispheric volume.

Infarct volumes and neurological scores were evaluated in a blinded manner by individuals who did not perform the surgical procedures [19].

2.5. Tissue processing, immunostaining, and confocal immunofluorescence

Immunostaining and confocal immunofluorescence procedures were performed as previously described [1,2,5,6]. The primary antibodies used were the following: rabbit polyclonal anti-Iba1 (Wako, Cat# 019–19741); mouse anti-iNOS (Santa Cruz, Cat# sc7271); rabbit polyclonal anti-GFAP (Abcam, Cat# ab7260); rabbit monoclonal anti-TMEM119 [28−3] (Abcam, Cat# ab209064); mouse monoclonal anti-GFAP (Merck Millipore, Cat# MAB360) and mouse monoclonal anti-C3 (Santa Cruz, Cat# sc28294). Then, sections were incubated with fluorescence-labeled secondary antibodies (Alexa 488- and Alexa 594-conjugated anti-mouse or anti-rabbit immunoglobulins G). The fluorescent DNA-binding dye Hoechst-33258 was used to stain nuclei. Images were observed using a Zeiss LSM 700 laser (Carl Zeiss) scanning confocal microscope. Digital images were taken with an optical thickness of 0.7 μm and a resolution of 1024 × 1024.

2.6. Quantitative confocal studies

Quantitative studies were performed in single and double-labeling experiments. Fluorescence intensities of GFAP, Iba1 and TMEM119 were quantified in pixel intensity by the NIH image software [7]. Co-localization studies of GFAP/C3 and TMEM119/iNOS were performed by using the colocalization plugin of NIH image software.

For quantitative analysis, digital images were taken with 40 × objective. Identical exposure times and laser power settings were applied to all the photographs from each experimental set.

2.7. The intensity of GFAP and IBA-1 immunostaining

The fluorescence intensity of GFAP and IBA-1 in the cortex and striatum was evaluated on double-labeled tissue. Four consecutive and identical sections were selected to analyze the fluorescence intensity of GFAP and IBA-1. During the phase of image acquisition by a confocal microscope, standard conditions were regulated. Digital images were taken with × 20 objective. Image J software was used to measure the fluorescence intensity of GFAP and IBA-1 [1,37].

2.8. Cell cultures

The cells were dissociated from E11 mouse ventral midbrain and cultured as described. In brief, the tissues were dissected and dissociated using mechanical trituration with a fire polished Pasteur pipette in a culture medium (see below) and 0.01% pancreatic deoxyribonuclease (Sigma, Milan, Italy); cells were centrifuged 10 min at 500 g, suspended in neural basal medium (NBM, Invitrogen, Milan, Italy) counted and, plated in NBM at a density of 18.000/cm^2 in dishes coated with 15 μg/ml of poly-D-lysine (Sigma). NBM was complemented by B27 (Invitrogen), the basic fibroblast growth factor (bFGF, 20 ng/ml, Sigma), the N-terminal fragment of the SHH protein (50 ng/ml), and FGF8 (10 ng/ml, Sigma). Half of the medium was changed every 3 days. After 6 days the medium supplements were withdrawn, except for B27. Cultures were left for an additional 6 days. 1 mg/ml of RGWE was added to the incubation medium for 48 h. Experiments have always been carried out at least in triplicate sister samples for every experimental point analyzed.

2.9. RNA isolation and real-time PCR

Total RNA was isolated from midbrain primary cultures using TriReagent (Sigma) following the manufacturer's instructions. The analyses were performed in triplicate samples for each experimental point analyzed and were processed separately. RNA yield and integrity were measured by A260 spectrophotometry. Briefly, 2 μg of RNA was reverse transcribed, using random hexanucleotides (New England Biolabs 6 μM) and 200 U of Moloney-murine leukemia virus reverse transcriptase (New England Biolabs). SYBR Green qRT-PCR reactions were performed in 96-well plates using CFX96 Real-Time PCR (Biorad). Thermal cycling conditions comprised initial steps at 95 °C for 2 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Gene expression levels were quantified from real-time PCR data by the comparative threshold cycle (CT) method using hypoxanthine phosphoribosyl transferase (HPRT) as an internal control gene. The relative expression level of the gene of interest was then expressed as $2^{-\Delta CT}$ wherein ΔCT = CT gene of interest - CT HPRT.

2.10. Scavenging activity

Rutin and RGWE hydrogen peroxide scavenging activity was measured by spectrophotometric analysis. Briefly, PBS solution of Rutin or RGWE was added to 1.0% H_2O_2 (in PBS) to obtain the final concentration(s) to be tested and read at 230 nm in a microplate reader as previously described in [25]. The hydrogen peroxide scavenging activity (Hs) was measured according to the following equation: Hs= (Ac - Ar)/Ac × 100. Where Ac is the absorbance at 230 nm of H_2O_2 in PBS and Ar is the absorbance of H_2O_2 in presence of Rutin or RGWE. 2 mM ascorbic acid was used as positive control and 10% DMSO as negative control. All measures were repeated five times.

2.11. Statistical analysis

Values were expressed as means ± S.E.M. All experiments were performed and analyzed in a blinded manner. Statistically significant differences among means were determined by ANOVA followed by Student-Newman-Keuls post-hoc test for ischemic damage. Neurological deficits were analyzed through a nonparametric two-tailed Mann-Whitney test. The differences among means of fluorescence intensity of the two groups were determined by the Unpaired t-test. Statistical significance was accepted at the 95% confidence level ($P < 0.05$). Analyses were performed by using GraphPad Prism 5.0 (La Jolla, CA, USA).

3. Results

3.1. RGWE treatment decreases ischemic damage in rats subjected to tMCAO

In order to analyze the neuroprotective potential of RGWE in adult rats subjected to tMCAO and treated with RGWE, ischemic damage was evaluated through the Nissl staining technique [8] 24 h after the induction of the ischemia.

Treatment with the low dose of RGWE containing 10 mg/kg of Rutin induced a significant reduction of 60% ischemic volume (tMCAO + Vehicle: 49,86 ± 6.886 vs tMCAO + RGWE low dose containing 10 mg/kg of Rutin: 20,11 ± 3.548) ($p < 0.05$, one-way ANOVA) (Fig. 1).

3.2. RGWE treatment improves neurological deficits in rats subjected to tMCAO

The effects of RGWE treatment were also evaluated on the neurological function of rats subjected to tMCAO (Fig. 2). Ischemic rats treated with the low (10 mg/kg of Rutin) and intermediate (30 mg/kg of Rutin) doses of RGWE showed a significant reduction in general neurological deficits (Fig. 2a). Differently, only the low dose of the extract resulted in a significant reduction in focal neurological deficits while animals treated with the intermediate and the high dose of the extract did not show any significant improvement (Fig. 2b) ($p < 0.05$). (Fig. 3).

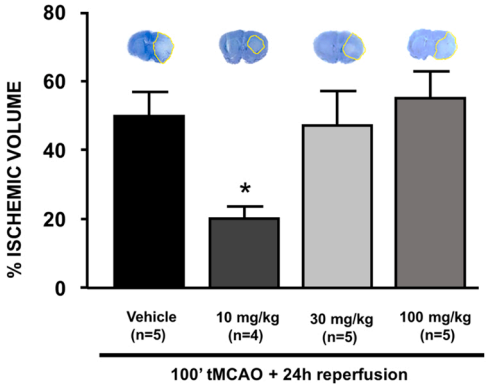

Fig. 1. Effect of RGWE administration on ischemic volume. Ischemic damage was assessed on rats 24 h after the induction of 100 min middle cerebral artery occlusion. On the top, representative pictures of Nissl-stained brain sections comprising the cortex and striatum are included. Brain damage is in white. Each column represents the mean ± S.E.M. n = 5 rats per tMCAO + Vehicle; n = 4 rats per tMCAO + RGWE containing 10 mg/kg of Rutin; n = 5 per tMCAO + RGWE containing 30 mg/kg of Rutin; n = 5 per tMCAO + RGWE containing 100 mg/kg of Rutin. * : $p < 0,05$ vs tMCAO + Vehicle. In the representative brain slice pictures, dashed line indicates infarct volume.

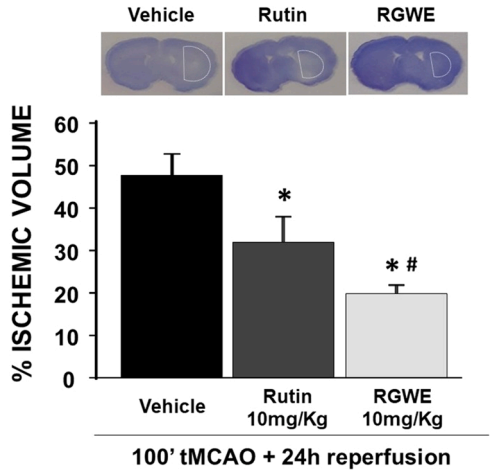

Fig. 2. RGWE induces a better neuroprotective effect than Rutin. Ischemic damage was assessed on rats 24 h after the induction of 100 min middle cerebral artery occlusion. On the top, representative pictures of Nissl-stained brain sections comprising the cortex and striatum are included. Brain damage is in white. Each column represents the mean ± S.E.M. n = 5 rats per tMCAO + Vehicle; n = 5 rats per tMCAO + 10 mg/kg of Rutin; n = 5 per tMCAO + RGWE containing 10 mg/kg of Rutin. * $p < 0,05$ vs tMCAO + Vehicle. *# $p < 0,05$ vs tMCAO + Vehicle and vs tMCAO + Rutin 10 mg/kg. Dashed line indicates infarct volume.

3.3. RGWE treatment reduced the ischemia-induced increase of the pro-inflammatory markers C3 and iNOS respectively in GFAP-positive astrocytes and in TMEM119-positive microglial cells

To investigate the effect of the extract on glia activation, immunohistochemical and confocal microscopy analysis were performed. Brain slices of the same height from ischemic animals treated with the vehicle or the RGWE low dose were compared. Low-dose RGWE treatment significantly prevented glial cells activation (Figs. 4 and 5). Interestingly, RGWE treatment (10 mg/Kg) strongly prevented the ischemia-induced expression of the astrocytic GFAP marker as well as the increase of the microglial TMEM119 marker both in the cortex and in the striatum of ischemic animals (Figs. 4 and 5).

More interestingly, RGWE treatment caused a significant reduction in the expression of the pro-inflammatory markers C3 and iNOS respectively in GFAP-positive astrocytes (Fig. 4) and in TMEM119-positive microglia (Fig. 5) in the cortex and in the striatum of ischemic animals.

3.4. RGWE determines BDNF overexpression in E11 mouse ventral midbrain primary culture

To verify whether Bdnf gene expression changes following RGWE treatment, we performed a Real-time PCR experiment on E11 ventral midbrain primary cultures treated for 48 h with 1 mg/ml of RGWE. As shown in Fig. 6, RGWE is able to induce a significantly increased BDNF mRNA compared to control (CTRL) cultures treated with vehicle.

3.5. RGWE possess antioxidant effects higher than Rutin

To assess the antioxidant properties of Rutin and RGWE, we performed H_2O_2 scavenging test with increasing doses of the two compounds. The concentrations of Rutin tested are those inferred to be present in the RGWE doses by mass spectroscopy analysis [25] As shown in Fig. 7, Rutin and RGWE have a remarkable scavenging activity as compared to DMSO used as negative control. Interestingly, RGWE has higher antioxidant properties as compared to Rutin alone at all concentrations (***$p < 0.0001$ 1 mg/ml RGWE vs 300 μg/ml Rutin) and the scavenging activity of 1 mg/ml RGWE is comparable to that of ascorbic

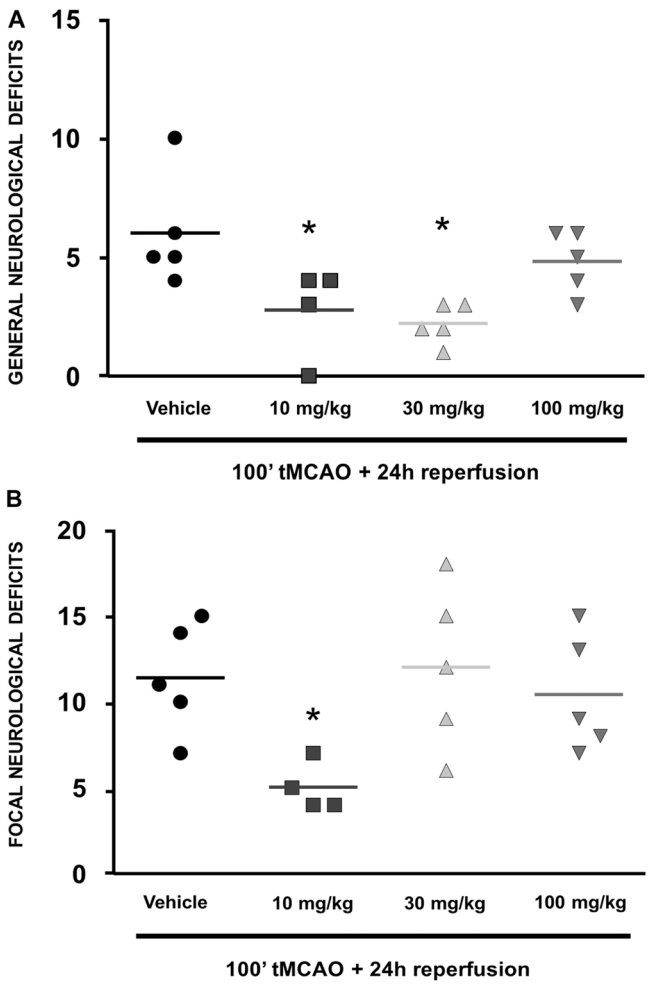

Fig. 3. Effects of RGWE administration on General (A) and Focal (B) Neurological Deficits. General and Focal Neurological scores were measured in ischemic rats 24 h after the induction of ischemia before sacrifice. Each column represents the mean ± S.E.M. n = 5 rats per tMCAO + Vehicle; n = 4 rats per tMCAO + RGWE containing 10 mg/kg of Rutin; n = 5 per tMCAO + RGWE containing 30 mg/kg of Rutin; n = 5 per tMCAO + RGWE containing 100 mg/kg of Rutin. * : $p < 0,05$ Vs tMCAO + Vehicle.

acid used as positive control.

4. Discussion

The present study demonstrated for the first time that: 1) treatment with Ruta graveolens water extract exerts a neuroprotective effect in a rat model of transient focal brain ischemia resulting in a significant reduction in ischemic damage; 2) it also resulted in a significant improvement in general and focal neurological deficits in ischemic animals; 3) the administration of the low dose of the extract caused a significant reduction in the fluorescence intensity of pro-inflammatory astroglial and microglial activation, in cortex and striatum of ischemic rats.

In recent years, the importance of natural compounds has become consolidated in various experimental settings based on preclinical models of cerebral ischemia. One of the most important features is represented by the ability of these compounds to modulate different targets simultaneously, given the complexity of their molecular structure capable of interacting with different pharmacological targets, thus modulating the function of the neurovascular unit, the integrity of the blood-brain barrier and counteracting the main pathological mechanisms of brain ischemia [10,46].

In our study, the effect of RGWE in inducing a reduction of the ischemic lesion was higher than Rutin alone, thus suggesting that RGWE components act synergistically in determining the neuroprotective effect. This aspect is further confirmed by in vitro experiments showing the scavenging ability of RGWE compared to Rutin alone.

The neuroprotective potential of RGWE should be considered an emerging property since it is likely that the pharmacological synergy between the components of the extract is responsible for a possible additive effect that determines the efficacy of cerebral ischemia.

In fact, experimental evidence on the neuroprotective effect of Rutin, the main component of the extract tested in this study was already present in the literature. In particular, Khan and co-authors showed significant neuroprotection when adult male Wistar rats were pre-treated with Rutin, orally (25 mg/kg), once a day for 21 days before the induction of middle cerebral artery occlusion [28]. However, this study brings an important limitation due to the pre-treatment that is difficult to pursue in humans. In our study, this limit is overcome since a post-treatment following the induction of ischemia was carried out in a therapeutic window up to 5 h from the induction of ischemia, to evaluate the neuroprotective potential of the extract in a translatable optic. In addition, we carried out experiments using the whole extract that contains different components.

Indeed, the aglyconic part of Rutin, i.e., quercetin, was shown to exert a protective effect on pial microcirculation in an animal model of incomplete global cerebral ischemia based on bilateral common carotid artery occlusion. Quercetin induced a dilation of all arterial orders through eNOS activation together with an activity of radical scavenger, an increase in the integrity of the blood-brain barrier and a decrease in vascular permeability. In addition, a significant reduction in ischemic damage has been described [30].

In accordance with the literature, we reported that our extract acts by reducing the formation of free radicals and by triggering the overexpression of the brain neurotrophic factor BDNF, a mechanism which could be at the basis of the reduction of ischemic damage.

Furthermore, one of the most important factors in the context of brain ischemia is given by the inflammatory reaction that occurs in the acute phase following the ischemic insult. There is a huge body of evidence showing the ability of natural compounds to modulate the production of proinflammatory cytokines and chemokines [11,46]. In the same vein, the results of the present study show that RGWE is able to significantly lower astroglial and microglial activation which represents one of the key factors in postischemic inflammation.

Although the understanding of the molecular mechanisms underlying the observed effects of RGWE on rats subjected to tMCAO is beyond the aim of our present study, our results let us hypothesize that several different mechanisms might have contributed to the beneficial effects of RGWE.

First of all, the antioxidants that are present in the RGWE could have exerted a detoxification of free radicals, the latter well known to play an important role in the pathophysiology of brain ischemia [44]. Indeed, we have previously shown that Rutin is highly expressed in RGWE and it is endowed with a potent antioxidant property [25]. Moreover, other compounds present in RGWE are endowed with antioxidant properties. In particular, RGWE contains a number of polyphenols such as quercetin and epigallocatechin gallate, isorhamnetin and isorhamnetin-3-O-rutinoside. These polyphenols might have played a role in ameliorating the outcome of rat ischemic brain in our experimental setting. For example, it has been shown that isorhamnetin protects primary human brain microvascular endothelial cells from toxicity induced by methylglyoxal and oxygen–glucose deprivation. Interestingly, such protection relies on the interference of different processes including inflammation, apoptosis, and oxidative stress [25].

Moreover, many polyphenols, including epigallocatechin-3-gallate (EGCG), possess the property to chelate metal, including iron, the latter being toxic and involved in ferroptosis, a type of cell death shown to occur also in ischemic brain [4].

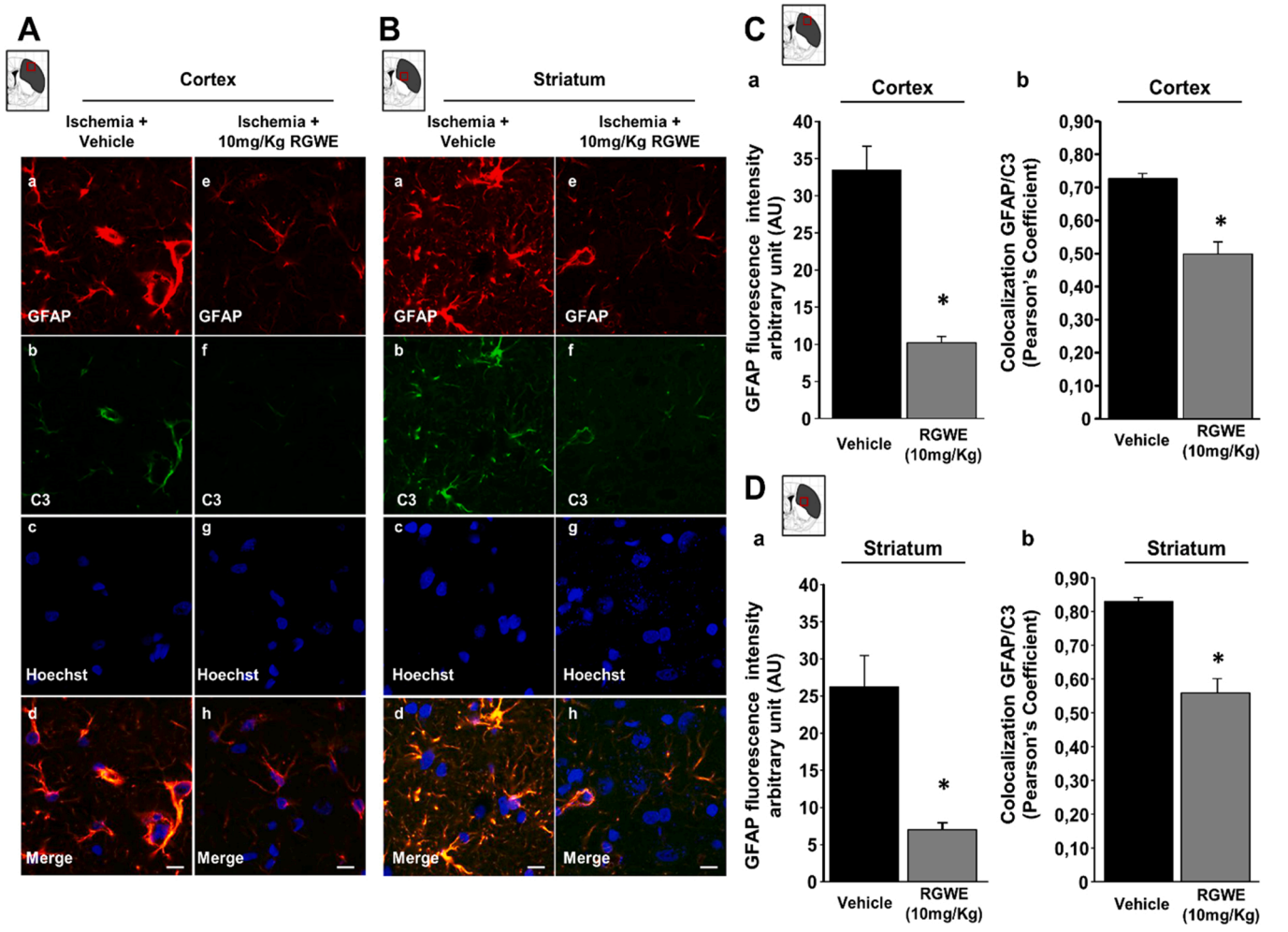

Fig. 4. Expression of the pro-inflammatory marker C3 in cortical and striatal GFAP(+)-positive cells Representative brain slice cartoons indicating the area of interest are on the top of the Figures. A-B. Confocal images displaying the expression of the astrocytic marker GFAP (red) and the pro-inflammatory marker C3 (green) in cortex and striatum of ischemic rat treated with Vehicle or RGWE (10 mg/Kg). Nuclei were stained by the nuclear dye Hoechst-33258. Scale bars: 10 μm. C-D. Quantification of GFAP fluorescence intensities (arbitrary unit, AU) (C,a;D,a) and quantitative analyses of GFAP/C3 colocalization values (C,b;D,b) (Pearson's Coefficient) in cortex and striatum of ischemic rat treated with Vehicle or RGWE (10 mg/Kg). The values represent the mean±SEM; (n = 3–4 indipendent experimental replicates); *p < 0.05 versus Ischemia + Vehicle.

It is worth noting that some of the effects of polyphenols might be indirect, as in the case of EGCG and quercetin have shown to protect rodent brain cells from chemical toxicity or ischemia, respectively, via BDNF, a neurotrophic factor able to promote regeneration of neural cells and neurogenesis [17,20,45].

Secondly, the anti-inflammatory molecules may have also mitigated the inflammation that is initiated by cell damage occurring in the ischemic brain. A large body of in vitro and in vivo evidence exists, regarding the anti-inflammatory effects of polyphenols on brain cells [40]. For example, in a gerbil model of cerebral ischemia, green tea polyphenol, EGCG, is able to reduce brain edema, as well as the infarct volume [31].

Finally, RGWE might have interfered with signal transduction pathways, involved in neurodegeneration and/or neuroprotection. In this respect, previous experiments showed that RGWE is able to activate or inhibit extracellular signal-regulated kinases, (ERKs) according to the cell type [23,25]. Erk 1/2 may play a role in ischemia, including brain ischemia, although there are contrasting data on the biological effects of ERKs activation [29]. Some evidence points towards a protective role. For example, astragaloside VI, mitigates rat ischemic stroke by enhancing neurogenesis, via activation of ERKs signaling [12]. On the other side, ERKs-mediated toxicity has been observed in several experiments, as in the case of resveratrol whose protective effects are due to the inhibition of the ERK-STAT signaling pathway [9]. Indeed, it is well known that ERKs may play a pivotal role in ensuring neuroprotection as well as mediating detrimental effects on neurons, according to its kinetics and subcellular compartmentalization, although the molecular mechanisms are largely unknown [16,34]. Thus, we can speculate that in our experiments RGWE might have modulated ERKs signaling in several different brain cell types, leading to an amelioration of the clinical and histological outcome. It is worth noting that the therapeutic effect of RGWE is lost as the concentration of RGWE is increased. This does not come as a surprise since many compounds including flavonoids may exert different or even opposing effects at different concentrations. Indeed, a biphasic effect often referred to as hormesis, may be considered, to explain such an effect. Hormesis has been described as a process where exposure to a low dose of a compound that is toxic at a higher dose, induces a beneficial effect. Many compounds, including natural compounds such as polyphenols, have been shown to undergo hormesis, displaying a biphasic dose-effects [33]. As a matter of fact, recently Restov and colleagues showed that in C. elegans catechins in green tea, can be detrimental when administered at higher doses whereas at lower concentrations act as pro-oxidants being able to increase the life span of the cells [27]. Also, curcumin, the polyphenol agent extracted from the rhizome of Curcuma longa and often used as a spice, has been shown to play a hormetic effect in several experimental models of neuronal

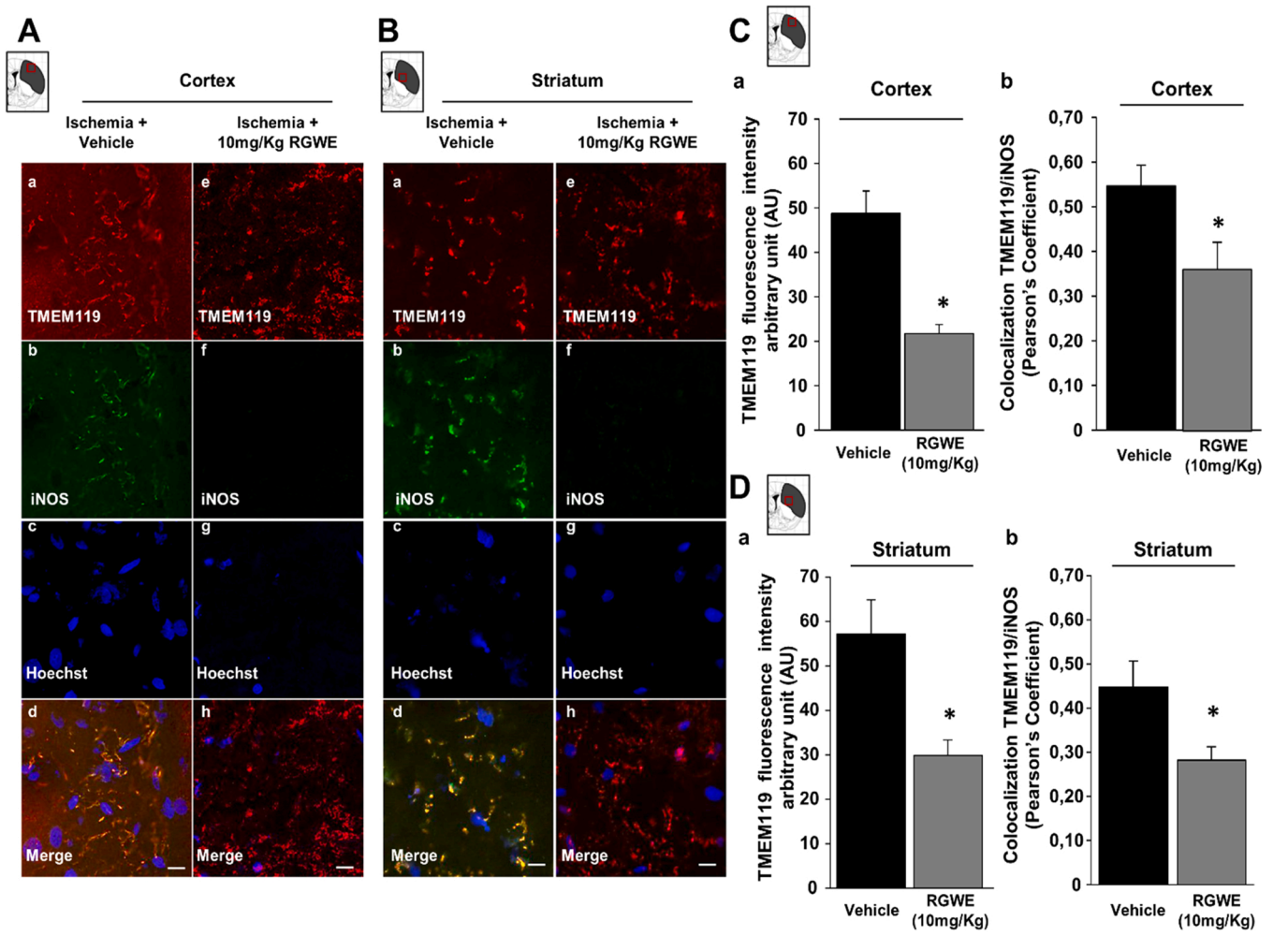

Fig. 5. Expression of the pro-inflammatory marker iNOS in cortical and striatal TMEM119(+)-positive cells. Representative brain slice cartoons indicating the area of interest are on the top of the Figures. A-B. Confocal images displaying the expression of the microglial marker TMEM119 (red) and the pro-inflammatory marker iNOS (green) in cortex and striatum of ischemic rat treated with Vehicle or RGWE (10 mg/Kg). Nuclei were stained by the nuclear dye Hoechst-33258. Scale bars: 10 μm. C-D. Quantification of TMEM119 fluorescence intensities (arbitrary unit, AU) (C,a;D,a) and quantitative analyses of TMEM119/iNOS colocalization values (C, b;D,b) (Pearson's Coefficient) in cortex and striatum of ischemic rat treated with Vehicle or RGWE (10 mg/Kg). The values represent the mean±SEM; (n = 3–4 indipendent experimental replicates); *p < 0.05 versus Ischemia + Vehicle.

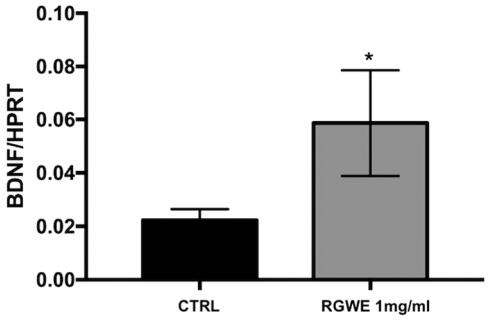

Fig. 6. RGWE is able to modulate BDNF gene expression. Expression levels of BDNF mRNAs, determined by Real-time RT-PCR in E11 mouse ventral midbrain primary cultures. The diagram shows the gene expression level of BDNF normalized to the housekeeping gene HPRT ($2^{-\Delta CT}$ method). Data are expressed as mean ± S.E.M, (n = 3). *Significantly different from control (CTRL) by Student's t-test (p < 0.05).

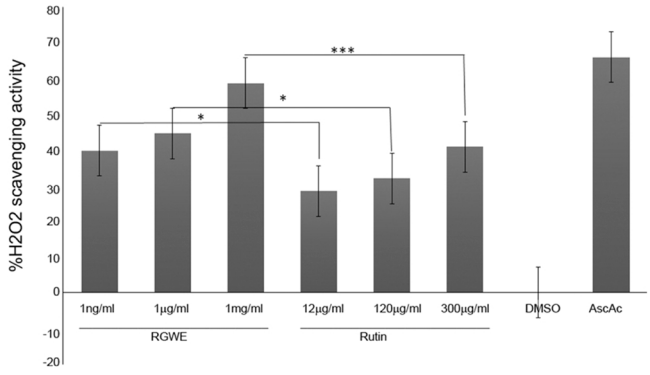

Fig. 7. RGWE shows higher scavenging activity than Rutin. The graph represents hydrogen peroxide scavenging activity of increasing doses of RGWE (1 ng; 1 μg and 1 mg/ml) and Rutin (12 μg, 120 μg and 300 μg/ml). The Rutin concentrations tested are those inferred to be present in the corresponding doses of RGWE. Data are expressed as ± SEM (n = 5); each concentration of RGWE has been compared with the corresponding of Rutin by Student T-test, *p < 0.01 and ***p < 0.0001. 10% DMSO was used as negative control, 2 mM Ascorbic acid as positive control.

damage including ischemia/reperfusion injury in rats [38]. In our experiments, several compounds acting as neurohormetics may have made rats brain better able to cope with ischemia. Moreover, as in other cases,

advanced "omic" approaches including genomic, proteomic, and epigenetic techniques, possibly combined with high-throughput screening and machine learning might help unveiling intracellular molecular targets as well as compounds present in the RGWE responsible for the biological effects [3,13,15,22,39]. In conclusion, taken together, our findings show that RGWE might be a potential source to search for compounds able to ameliorate the outcome of ischemic damage.

Contribution to authorship

MC, GP, LCD, FV, and OP contribute to the 1) conception and design of the study; MC, OC, AV and PB contribute to 2) acquisition and analysis of data; MC, OC, AV, and AC contribute to surgery experiments to induce stroke and brain damage evaluation in rats; GP, SA, LA, LCD and MC contribute to 3) drafting a significant portion of the manuscript and figures.

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Conflict of interest statement

All authors agree that there are no conflicts to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113587.

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