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ORIGINAL ARTICLE

Ruta graveolens Linn. (RGLE) Reduces Autophagy and Improves Memory in the Rat Model of Dementia through mTOR Signaling Pathway Regulation

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ABSTRACT

Objectives: The present study evaluated *Ruta graveolens* Linn. extract (RGLE) for its possible role in the treatment of vascular dementia (VD) in the rat model and inhibition of autophagy in hippocampus tissues. **Materials and Methods:** The spatial working memory of the rats was assessed using the established Tmaze tests. A video camera was used for recording and water maze software (HVS Image 2020; HVS Image Software Ltd.) was used for analysis of the digital images to measure escape latency and swimming distances for each rat. **Results:** RGLE treatment of the VD rats significantly ($p < 0.05$) alleviated the impairment in spontaneously altered behaviors and significantly ($p < 0.05$) reduced escape latency. The VD-mediated decrease in distance travelled, swim time, and count of platform crossings was significantly ($p < 0.02$) alleviated by RGLE treatment of the rats. In RGLE-treated rats, the VD-mediated increase in Beclin-1 and Microtubule-associated protein light chain 3II (LC3II) expression in the hippocampus tissues was significantly ($p < 0.05$) alleviated. RGLE treatment prevented the suppression of the mammalian target of rapamycin (mTOR) phosphorylation in VD rat hippocampus tissues. The rapamycin-mediated suppression of p-mTOR and elevation of Beclin 1 and LC3II expression in the rat hippocampus could not be alleviated by RGLE treatment. **Conclusion:** In summary, RGLE effectively prevents VD-mediated cognitive impairment and neuronal damage in the rats. Moreover, autophagy was inhibited and mTOR pathway was activated in rats with VD by RGLE treatment. Therefore, RGLE may be studied further as a therapeutic agent for treatment of dementia.

Key words: Anti-inflammatory, autophagy, Beclin, dementia, memory loss

SUMMARY

- The study demonstrates that RGLE treatment improves VD-induced cognitive impairment in rats and inhibits neuronal damage. Moreover, the mTOR signaling pathway is activated and autophagy activation is inhibited in VD rats on treatment with RGLE. Therefore, RGLE may be developed as an effective treatment strategy for the treatment of VD.

Abbreviations used: VD = Vascular dementia, RGLE = *Ruta graveolens* Linn. extract, LC3II = Microtubule-associated protein light chain 3II, mTOR: mammalian target of rapamycin, DMSO = Dimethyl sulfoxide, MWM = Morris water maze, RIPA = Radioimmunoprecipitation assay buffer.

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INTRODUCTION

Vascular dementia (VD) alone accounts for 15% of the dementia cases and is the second most common type of dementia following Alzheimer's disease.[1] It is characterized by gradual loss of memory and learning ability of the patients.[2] Researchers believe that vascular risk factors play a significant role in the development and progression of VD disorder.[3] Presently licensed treatments are not available for VD, and the molecular mechanism involved in the pathogenesis of the disorder is yet to be discovered.

Autophagy is a cellular process involved in the maintenance of stability in the body through self-degradation of various damaged components such as mitochondria.[4,5] Excessive activation of autophagy causes an increase in self-digestion leading to self-degradation of vital organelles and proteins followed by cell death.[6] It has been demonstrated that brain neuronal damage induced by transient ischemia is associated with the over-activation of autophagy.[7,8] Thus, autophagy is involved in disorders of the central nervous system, and its down-regulation may be of therapeutic significance for these diseases.

Natural products have shown significant therapeutic properties against several diseases/disorders of the central nervous system such as VD,[9] amyotrophic lateral sclerosis,[10] Alzheimer's disease,[11] and Parkinson's disorder[12] in the animal models. Diversity of mechanisms including antiinflammatory effects, inhibition of oxidative stress, prevention of platelet aggregation, and down-regulation of apoptosis have been demonstrated to be associated with neuro-protective properties of these natural products.[13-16]

Ruta graveolens Linn., an odoriferous herbaceous plant belonging to the family *Rutaceae* is native to the Mediterranean region.[17] The

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plant has been used traditionally for anti-septic, anti-spasmodic, stimulant, abortifacient, expectorant, and anti-rheumatic properties. Phytochemical analysis has led to isolation of many phytochemicals including rutin, quercitin, rutacridone, rutacridone epoxide, graveoline, and gravacridonodiol from the plant.[17] The plant extract has been demonstrated to possess anti-inflammatory,[18,19] anti-diabetic,[20] and anti-hyperlipidemic[21] properties. Moreover, studies have also reported anti-rheumatic properties, analgesics,[22] and anti-androgenic[23,24] properties of the plant. The present study investigated the effect of *Ruta graveolens* Linn. extract (RGLE) on VD-induced learning and memory loss in a rat model and explored the underlying signaling pathways.

MATERIALS AND METHODS

Rats and grouping

A total of 60 male Sprague–Dawley rats (body weight 250–280 g) were obtained from the Animal Center of Xi'an Jiaotong University (Xi'an, China). All rats were individually housed under 12 hr light and dark cycles at a 23 ± 2°C temperature and given free access to standard rat chow and water. Experimental protocols were conducted on rats in accordance with the guidelines issued by the Ethics Committee for Animals, National Institutes of Health, and USA. The study was approved by the Committee for Care and Use of experimental animals of Xi'an Jiaotong University. The rats were separated randomly into five groups of 12 each: Normal, VD, RGLE, rapamycin (R), and RGLE and rapamycin (RGLE + R) groups. Rats were intra-gastrically given 10 mg/kg RGLE as a single dose 1 day before the surgery. Rapamycin (1 mmol/ml; 50 µl) was injected into rats either alone or in combination with RGLE into the lateral ventricle at 2 µl/min rate. After 24 hr of RGLE and/or rapamycin administration, the rats were subjected to a twovessel occlusion procedure to induce VD. Rats in the normal group were subjected to same procedure except ligation of the two vessels.

Tmaze for assessment of behavioral changes

Spatial working memory of the rats was assessed using the established Tmaze tests.[25] After 1 month of the RGLE treatment, the rats were subjected to Tmaze tests in which each trial involved a sample and a choice run. The sample run consisted of forcing the rats through the right or left arm for getting sugar, whereas the second arm was closed using a sliding door. During the choice run, both the arms were kept open and animals were allowed to enter the arm of their choice. Initially, the interval time between the sample and choice runs was fixed at 10 sec, which was later on delayed by 90 and 180 sec. Five trials were conducted daily, and the time gap between two trials for each rat was 10 min. A number of corrections indicated visit of the rat to the arm of the Tmaze not visited earlier.

Morris water maze (MWM)

Opaque water was taken in a circular pool having a 180 cm diameter, a 60 cm height, and a 35 cm depth maintained at a 25 ± 1°C temperature. The white platform was placed in one of the four quadrants of the pool, and the rat was placed gently in water. The rats were placed in such a way that they faced the quadrant not having a platform. The time taken by each rat to locate the hidden platform and the path traveled to reach the same were taken as the escape latency and distance traveled, respectively. Each rat was given 15 sec to rest on the platform prior to closing the trial. Rats that failed to locate the platform in 90 sec were guided toward it and give 15 sec to on for rest. Training was continued for 5 days, twice daily with an interval of 3 hr, and each trial was started from a different quadrant. A video camera was used for recording and water maze software (HVS Image 2020; HVS Image Software Ltd.) was used for analysis of the digital images to measure escape latency and swimming distances for each rat. On day 6 of the MWM test, trials were held without the hidden platform.

Nissl staining

Rats were sacrificed by cervical dislocation after euthanasia with sodium pentobarbital (200 mg/kg) through intra-peritoneal route. Brains of the rats were extracted, dissected, and subsequently fixed for 3 days at 4°C with 4% paraformaldehyde prior to paraffin embedding. The tissues were sliced into 5µm thin sections, followed by staining for 45 min with 1% Toluidine Blue at 60°C. Survival of the neurons was assessed by examining the Nisslpositive cells in the rat hippocampus using a light microscope (model, BX53; Olympus Corporation) at x400 magnification.

Western blotting

Rats were sacrificed by cervical dislocation after euthanasia with sodium pentobarbital (200 mg/kg) through the intra-peritoneal route. The hippocampi of the rats were extracted and subsequently homogenized on treatment with RIPA lysis buffer (Beyotime Institute of Biotechnology) mixed with phenylmethylsulfonyl fluoride. Lysate obtained was centrifuged at 4°C for 20 min at 12,000 × g to isolate the supernatant in which protein content was estimated by bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). Protein samples (30 µg) were resolved on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently transferred onto PVDF membranes. Non-specific sites in the membranes were blocked by incubation with 3% bovine serum albumin (SigmaAldrich) and Trisbuffered saline for 1 hr. Membrane incubation with primary antibodies was performed for overnight at 4°C. The primary antibodies used were anti-mTOR (cat. no. 2972; dilution 1:400), pmTOR (Ser2448; cat. no. 2971; dilution 1:250), LC3B (cat. no. 2775; dilution 1:1,000), βactin (cat. no. 4967; dilution 1:1,000, Cell Signaling Technology, Inc.), and Beclin 1 (cat. no. sc48341; dilution 1:1,000; Santa Cruz Biotechnology, Inc.). Then, membranes were subjected to incubation for 1 hr with horseradishperoxidase-conjugated antirabbit IgG secondary antibodies (Cell Signaling Technology, Inc). An enhanced chemiluminescence system (EMD Millipore) was used for detection of protein signals, and QuantityOne software (BioRad Laboratories, Inc.) was used for quantification of the signals.

Statistical analysis

The data expressed are the mean ± standard deviation of triplicate readings. All the data were analyzed using SPSS 16.0 statistical software (SPSS, Inc.). Differences between the groups were determined statistically using oneway ANOVA, followed by Tukey's multiple comparison test. At $P < 0.05$, differences were taken statistically significant.

RESULTS

RGLE alleviates VD-induced learning and memory loss in rats

Induction of VD caused significant ($p < 0.05$) changes in the behavior of rats in comparison to that of the normal group [Figure 1]. VD-induced behavioral changes in rats were effectively reversed by RGLE treatment in a dose-dependent manner when the interval time was fixed 10 sec between the sample and choice runs. RGLE treatment also prevented VD-induced behavioral changes in rats when the interval time between the sample and choice runs was increased to 90 and 80 sec. Treatment of rats with RGLE and rapamycin was ineffective in preventing VD-induced behavior changes when the interval time between the sample and choice runs was 10, 90, and 180 sec.

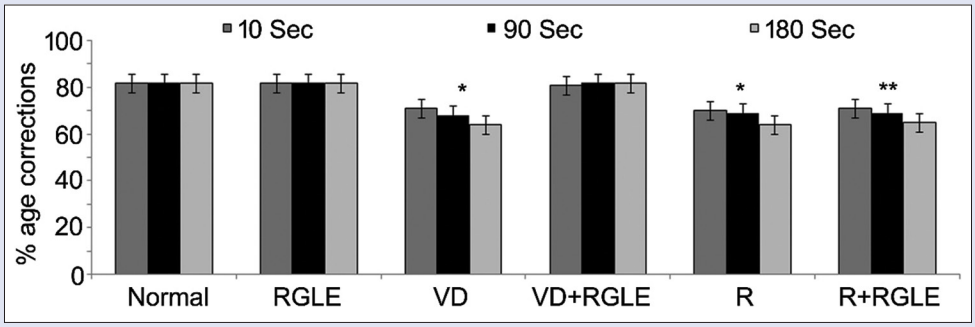

Figure 1: Effect of RGLE on VD induced changes in spatial working memory of the rats. T-maze test was performed to measure the corrections in rats when the interval time between the sample and choice runs was 10, 90, and 180 sec. The rats with VD were treated with 10 mg/kg doses of RGLE or RGLE and rapamycin, and then, the T-maze test was conducted to measure spatial working memory. *$p < 0.05$, **$p < 0.01$ vs. normal group

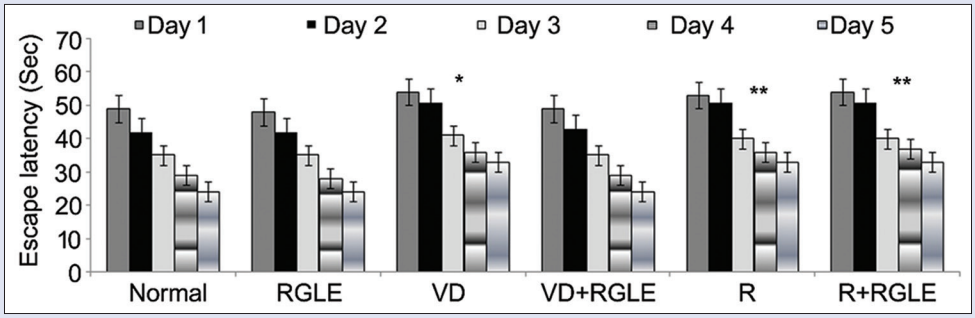

Figure 2: Effect of RGLE on VD induced changes in behavior of the rats. Morris water maze test was performed to determine escape latency of the rats. The rats with VD were treated with a 10 mg/kg dose of RGLE or RGLE and rapamycin, and then, Morris water maze test was conducted to measure changes in behavior. *$p < 0.05$, **$p < 0.01$ vs. normal group

RGLE alleviates VD-induced learning and memory loss in rats

The escape latency of the rats showed a significant ($p < 0.05$) increase after VD compared to the normal group [Figure 2]. However, RGLE treatment led to a significant ($p < 0.05$) reduction in VD-induced escape latency in rats compared to the VD group. Moreover, VD caused a significant ($p < 0.05$) reduction in distance travelled in the target quadrant and count of platforms crossed in rats compared to the normal group [Figure 3]. Moreover, VD caused a significant ($p < 0.05$) reduction in distance traveled in the target quadrant and time spent in swimming in rats compared to the normal group [Figure 4]. Treatment of the rats with RGLE significantly ($p < 0.05$) reversed VD-mediated reduction in distance traveled in the target quadrant and the time spent in swimming and lowering in the count of platforms crossed compared to the VD group. No significant ($p < 0.05$) changes were observed in VD rats on treatment with RGLE and rapamycin in the distance traveled in the target quadrant, the time spent in swimming, and the count of platforms crossed compared to the VD group.

RGLE targets Beclin 1 and LC3II expression and promotes the p-mTOR level in VD rats

In VD rats, the expression of Beclin 1 and LC3II proteins showed a prominent increase compared to the normal group [Figure 5]. However, RGLE treatment of the VD rats led to a remarkable suppression in the expression of Beclin 1 and LC3II proteins. Additionally, VD induction led to a significant (i < 0.05) lowering in p-mTOR (Ser2448) expression in rats compared to the normal group. On the other hand, treatment of the VD rats with a 10 mg/kg dose of RGLE caused a significant ($p < 0.05$) elevation in p-mTOR (Ser2448) expression.

Inhibitor of mTOR reverses the effect of RGLE on p-mTOR expression in VD rats

Rapamycin administration to VD rats caused a significant ($p < 0.05$) lowering in p-mTOR expression compared to the normal group [Figure 5]. Moreover, in rapamycin-administered VD rats, the expression of Beclin 1 and LC3II proteins showed a prominent increase compared to the normal group. Treatment of the rapamycin-administered VD rats with a 10 mg/kg dose of RGLE did not lead to any change in p-mTOR, Beclin 1, and LC3II protein expression.

RGLE reverses VD-induced neuronal death in rats

A number of neurons showed a significant ($p < 0.05$) reduction in rats after VD compared to the control group [Figure 6]. VD induction suppressed the viability of neurons in the CA1 region of the rat to 22% compared to 100% in the normal group. Treatment of the VD rats with a 10 mg/kg dose of RGLE significantly ($p < 0.05$) promoted the viability of neurons in the CA1 region compared to the VD group. VD-mediated reduction in viability of neurons in rat CA1 could not be reversed by RGLE treatment in the rapamycin-administered group.

DISCUSSION

VD is the most common factor responsible for dementia caused mainly by cerebrovascular diseases.[26] Some of the common reasons responsible for VD include persistent hypertension, diabetes, and excessive smoking.[26] During the past few years, several studies have been performed to understand the molecular mechanism and pathogenesis of VD, but its treatment continues to be a challenge for clinicians.

Therefore, more studies are required to investigate the pathogenesis of the disease for the development of effective and successful treatment for VD.

Autophagy plays an important role in enabling the cells to adopt the stress associated with changes in internal and external environments.[27] Excessive activation of this dynamic catabolic process leads to the death of cells and plays a major role in ischemiamediated neuronal damage.[28,29] Down-regulation of autophagy has been reported to exhibit beneficial effects in the regulation of neurological deficits induced by cerebral ischemia.[30,31] Autophagy is commonly induced in cells by kinase mTOR, and it has been demonstrated that activation of mTOR inhibits cell death, whereas its down-regulation promotes cell death.[32] The mTOR pathway is directly inhibited by rapamycin, leading to induction of autophagy activation.[33] It is reported that activation of the mTOR pathway effectively protects neurons of hippocampi from injury induced by hypoxia and plays an important role in their recovery.[34] The present study used a reported protocol[35] to establish the VD rat model for investigation of RGLE in improving learning and memory potential. The study demonstrated that VD induction led to significant ($p < 0.05$) changes in the behavior of rats compared to the normal group. However, VD-induced behavioral changes in rats were effectively prevented by RGLE treatment. It was also found that treatment of the rats with RGLE and rapamycin could not prevent VD-induced behavior changes. RGLE treatment led to a significant ($p < 0.05$) reduction in VD-induced escape latency in rats compared to the VD group. Moreover, treatment of the rats with RGLE significantly ($p < 0.05$) reversed VD-mediated reduction in the distance traveled in the target quadrant, the time spent in swimming, and lowering in the count of platforms crossed compared to the VD group. These findings clearly demonstrated that RGLE treatment of the VD rats improved cognitive damage in the absence of the autophagy inhibitor, rapamycin.

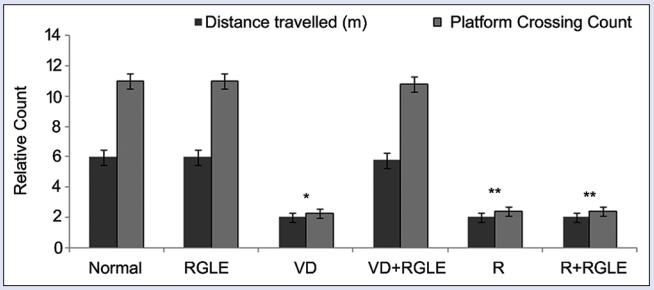

Figure 3: Effect of RGLE on VD induced changes in behavior of the rats. Morris water maze test was performed to determine the distance traveled in the target quadrant and the count of platforms crossed. The rats with VD were treated with a 10 mg/kg dose of RGLE or RGLE and rapamycin, and then, Morris water maze test was conducted to measure changes in behavior. *$p < 0.05$, **$p < 0.01$ vs. normal group

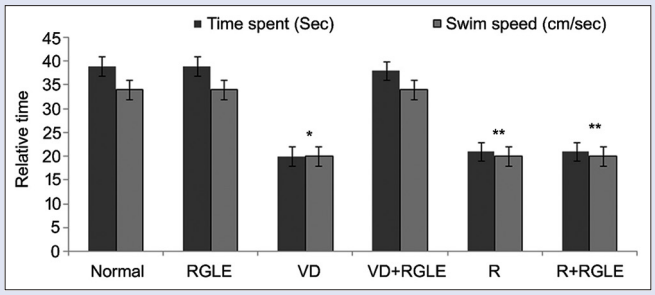

Figure 4: Effect of RGLE on VD induced changes in behavior of the rats. *Morris water maze* test was performed to determine the time spent in swimming and the swimming speed of the rats. The rats with VD were treated with a 10 mg/kg dose of RGLE or RGLE and rapamycin, and then, Morris water maze test was conducted to measure changes in behavior. *$p < 0.05$, **$p < 0.01$ vs. normal group

Multiple studies have demonstrated that excessive autophagy activation associated with ischemia or hypoxia leads to the death of the neurons.[36-39] In the present study, expression of Beclin 1 and LC3II proteins showed a prominent increase in VD rats compared to the normal group. However, RGLE treatment of the VD rats led to a remarkable suppression in the expression of Beclin 1 and LC3II protein expression. The VD induction also led to a significant ($p < 0.05$) lowering in p-mTOR (Ser2448) expression in rats compared to the normal group. On the other hand, treatment of the VD rats with RGLE caused a significant ($p < 0.05$) elevation in p-mTOR (Ser2448) expression. Administration of the p-mTOR inhibitor, rapamycin, to VD rats caused a significant ($p < 0.05$) lowering in p-mTOR expression compared to the normal group. Moreover, in rapamycin-administered VD rats, the expression of Beclin 1 and LC3II proteins showed a prominent increase compared to the normal group. Treatment of the rapamycin-administered VD rats with RGLE did not lead to any change in p-mTOR, Beclin 1, and LC3II protein expression.

Findings from the present study are in consistence with the previous reports that excessive activation of autophagy during VD promotes neuronal damage.[29,30] In the present study, the number of neurons showed a significant ($p < 0.05$) reduction in rats after VD compared to the control group. Treatment of the VD rats with RGLE significantly ($p < 0.05$) promoted the viability of neurons in the CA1 region compared to the VD group. VD-mediated reduction in viability of neurons in rat CA1 could not be reversed by RGLE treatment in the rapamycin-administered group.

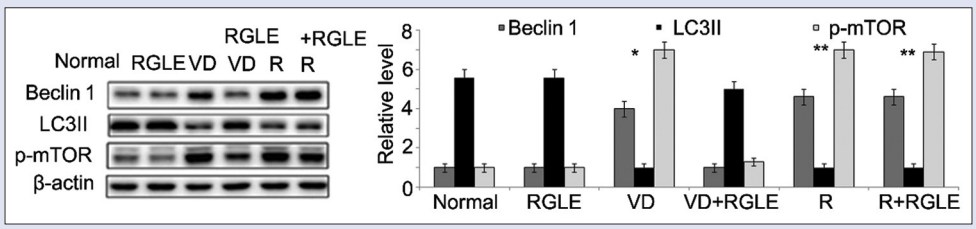

Figure 5: Effect of RGLE on Beclin 1, LC3II expression, and p-mTOR expression. The rats with VD were treated with a 10 mg/kg dose of RGLE, and then, expression of Beclin 1, LC3II, and p-mTOR in hippocampi was measured by western blotting. *$p < 0.05$, **$p < 0.01$ vs. normal group

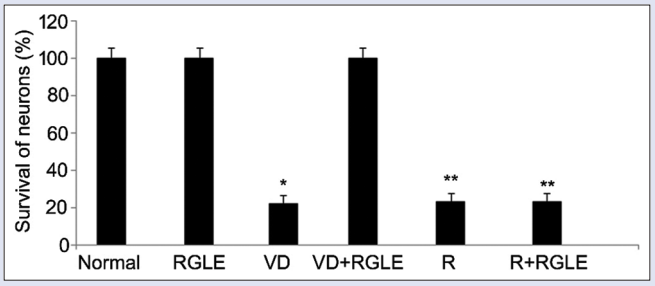

Figure 6: Effect of RGLE on viability of neurons in the CA1 region of the VD rats. The rats with VD were administered rapamycin and treated with a 10 mg/kg dose of RGLE. Loss of neurons in the CA1 region of the VD rats was detected by light microscopy using Nissl staining (magnification, x400) *$p < 0.05$, **$p < 0.01$ vs. normal group

CONCLUSION

In summary, RGLE treatment improves VD-induced cognitive impairment in rats and inhibits neuronal damage. Moreover, the mTOR signaling pathway is activated and autophagy activation is inhibited in VD rats on treatment with RGLE. Therefore, RGLE may be developed as an effective treatment strategy for the treatment of VD.

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Conflicts of interest

There are no conflicts of interest.

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