Anti Viral And Anti Inflammatory Effect of Rosmarinic Acid

In An Experimental Murine Model of Japanese Encephalitis

Vivek Swarup, Joydeep Ghosh, Soumya Ghosh, Amit Saxena and Anirban Basu

National Brain Research Centre, Manesar, Haryana, India

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To whom correspondence should be addressed:
Anirban Basu
National Brain Research Centre,
Manesar, Haryana-122050, India
Tele: 91-124-2338921
Email: anirban@nbrc.res.in
**Abstract:**

Rosmarinic acid (RA) reduced the mortality of mice infected with Japanese Encephalitis virus (JEV). A significant decrease in viral load (p<0.001) and proinflammatory cytokine levels (p<0.001) was observed in JEV infected animals treated with RA as compared to infected without treatment, at 8-9 days post infection.
Flaviviruses are important human pathogens causing a variety of diseases ranging from mild febrile illnesses to severe encephalitis. Among them, Japanese encephalitis virus (JEV) targets the central nervous system (CNS) and is a major cause of acute encephalopathy in children (1). Clinically, infection with JEV results in increased levels of inflammatory mediators like TNF-α, IL-6, IL-8 and RANTES in the serum and cerebrospinal fluid (CSF) (3, 9, 12) which bears a direct correlation with mortality rate in JE patients (14). As increased microglial activation and subsequent induction of proinflammatory mediators like TNF-α, IL-6 and MCP-1 following JEV infection influences the outcome of viral pathogenesis (2), it is possible that the increased microglial activation triggers bystander damage since infected animals eventually succumb to infection.

Rosmarinic acid (RA) a phenolic compound found in various Labiatae herbs (6, 11), possess several anti-inflammatory properties (7, 16). Besides, the anti-oxidative property of RA has been demonstrated by its ability to reduce liver injury induced by D-galactosamine (15) and LPS (8), through scavenging of superoxide molecules (13) and inhibition of cyclo-oxygenase-2 (Cox-2).

In the present study, we investigated the efficacy of RA as a therapy against murine JE. Four to five weeks old BALB/c mice of either sex were infected intravenously (through tail vein) with lethal dose of $3 \times 10^5$ p.f.u of JEV virus (GP78 strain). From fifth day post infection, animals started to show symptoms of JE including limb paralysis, poor pain response, restriction of movements, pilo-erection, body stiffening and whole body tremor. Within ninth day of post-infection all animals succumbed to infection. RA (Tocris Bioscience) was dissolved in 1X phosphate buffered saline (PBS) at a
concentration of 2.0 mg/ml and stored at −20°C. After one day following virus inoculation animals started receiving RA intra-peritoneally, twice daily (25 mg/kg of body weight) until the first animal died from the group of infected animals, which did not receive any RA treatment. All experiments were performed according to the protocol approved by the Institutional Animal Ethics Committee.

Animals from control (PBS Injected), JEV infected, JEV infected and RA treated were perfused with PBS containing 7 U/ml heparin, and followed by a fixative containing 2.5% PFA in PBS and their brains were processed for cryostat sectioning. The sections were stained with Iba-1 (Wako Chemical, Japan) a marker for activated microglia (4). Activated microglia were counted from five different fields of cortex by using the software IM50 (Leica) and images were captured under 20X magnification (2). The average numbers of activated microglia was plotted as a graph.

Western blot analysis was performed with protein isolated from brain tissues of all the four groups of animals, at 8-9 days post infection (depending upon the mortality of the infected animals) (2). Briefly, ten microgram of each sample was electrophoresed and transferred onto a nitrocellulose membrane. The membranes were then blocked and probed with several primary antibodies that include: JEV Nakayama strain and Cox-2 (1:1000, Chemicon), Phospho NF-κB and IκB-α (1:1000, Cell Signaling Tech.), and β-tubulin (1:1000, Santa Cruz Biotechnology). Appropriate HRP conjugated secondary antibodies were used for all of them. Using chemiluminescence reagent blots were developed and images were captured and analyzed using Chemigenius Bioimaging System (Syngene). We have also performed western blot analysis with protein isolated from JEV infected and RA treated animals at death.
The mouse cytokine bead array (CBA) kit (BD Biosciences) was used to quantitatively measure cytokine levels in the brain tissue lysates isolated from all four groups of animal, and analysis was performed as described (2). CBA was also performed with protein isolated from JEV infected and RA treated animals at death. BV-2, a mouse microglial cell line was either mock infected or adsorbed with JEV (multiplicity of infection=5) for 1h. After adsorption, unbound viruses were removed by washing with PBS and cells were incubated in fresh serum free medium either in the presence or absence of 25μM RA for additional 18hrs. Following incubation the cell lysate was collected and CBA was performed. Western blot analysis and CBA was also performed on the protein isolated from JEV infected animals that succumbed, even after RA treatment.

All comparisons between groups were performed using One-way ANOVA; with Bonferroni method for post hoc pairwise multiple comparisons to detect p values of <0.05 between individual group means.

RA treatment following JEV infection reduced the mortality rate down to 20% (12 out of 15 animals survived following RA treatment in JEV infected group) (Fig 1A). While all infected animals that did not receive any RA treatment succumbed to infection, treatment with RA alone had no effect on the behavioral outcome of animals (Data not shown). Immunohistochemistry revealed both qualitative and quantitative differences in microglial activation in infected animals treated with RA as compared to infected without treatment. In the infected brain, the number of star-shaped ‘activated’ microglia (Fig. 1C) appeared to be more frequent (more than 30 fold) than in control (Fig. 1B) or JEV and RA treated group (Fig. 1D). A significant induction in JEV specific proteins (17 kDa and
84 kDa) was observed in infected group (Fig. 2). RA treatment completely abolished the expression of viral proteins and significantly reduced viral mRNA transcripts (data not shown). Interestingly, viral proteins were also absent in JEV infected mice which succumbed even after RA treatment.

As shown in Fig 3A, RA dramatically reduced the level of proinflammatory cytokines and chemokine. A significant (p<0.001) 5, 18, 6, 100, and 9 fold decrease in the level of IL-12, TNF-α, IFN-γ, MCP-1 and IL-6 respectively, was observed in infected animals treated with RA as compared to infected without treatment. In contrast, infected mice that succumbed even after RA treatment also had significantly high levels of proinflammatory cytokines as compared to uninfected one (Fig. 3A1). In line with studies that RA acts as a downstream inhibitor of IKK-β activity (5), western blot analysis revealed a significant (p<0.05) increase in IκB-α and considerable decrease in both pNF-κB and Cox-2 levels respectively, in infected animals treated with RA as compared to infected alone (Fig. 3B). JEV infected and RA treated group had reduced levels of pNF-κB and Cox-2 relative to infected and untreated group. Interestingly in infected animals that died even after RA treatment, had increased levels of NF-κB and Cox-2 and decreased levels of IκB-α than control group. High levels of proinflammatory mediators in infected animals that succumbed even after RA treatment despite decreased viral load, suggests that besides reduction in viral load proinflammatory mediator levels are important in determining the final outcome of the disease.

To confirm the in-vivo findings that RA modulate the release of proinflammatory cytokines and chemokines following JE, we determined the levels of proinflammatory mediators in mouse microglial cell line BV-2 infected with JEV in vitro. While JEV
infection of BV-2 increased the release of various proinflammatory cytokines, treatment of infected BV-2 cells with RA significantly (p<0.001) reduced the level of IL-12, TNF-α, IFN-γ, MCP-1 and IL-6 by 13, 14, 12, 25, and 12.5 fold respectively as compared to RA untreated infected animals (Fig. 4). No significant changes with RA treatment alone were observed.

In conclusion, our studies suggest that RA acts as a potent antiviral agent against Japanese encephalitis. Results from our in vivo experiments clearly indicate that RA reduces the 1) viral replication within the brain, and 2) secondary inflammation resulting from microglial activation, thereby suggesting its potential for treating JE. Both the antiviral and anti-inflammatory effects of RA are essential for reducing the severity of diseases induced by JEV. The studies presented here recommend RA as a strong candidate for further consideration as a therapeutic measure to reduce the neurological complications observed in JE.

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References:


**Figure Legends:**

**Figure 1:** *RA treatment significantly increases the survival of JEV infected mice.* 

A. Survival of mice infected with $3 \times 10^5$ p.f.u of JEV was significantly increased in groups that received RA treatment (n=15 for each group). Treatment with RA alone has no significant effect on the survival. Observation of animal survival experiments was performed in a masked manner to avoid bias toward any one group of animals. 

B-E. Cryostat sections from control (B), JEV infected (C) and JEV infected and RA treated (D) mouse brains were processed for Iba-1. While the control sections exhibited only resting microglia (B; arrow), the infected brains showed the presence of activated state (C; arrowhead). JEV infected and RA treated mice had mostly resting microglia (D; arrow). Scale bar is 25 µm. Iba1 positive activated microglia were counted and plotted as a graph (E). Values represent the means ± SEM from 5 random fields in 3 animals in each group (p<0.001)

**Figure 2:** *Antiviral efficacy of RA.* 

C – Control, I – JEV infected and RA – JEV infected and RA treated, and RA* – JEV infected and RA treated (dead) 

A. RA treatment in JEV infected mice completely reduced the levels of viral proteins. Protein isolated from control, JEV infected, JEV infected and RA treated BALB/c mice were analyzed by immunoblot. A significant decrease in the levels of JEV specific proteins (84kDa and 17kDa) in RA treated samples than infected ones. Data shown is a representative of four individual animals from each group.

**Figure 3:** *RA abrogates the increased expression of proinflammatory mediators.* 

A. Expression of IL-12, TNF-α, IFN-γ, MCP-1 and IL-6 was observed by CBA in Control,
JEV infected, JEV infected and RA treated animals, treated with RA alone and JEV infected and RA treated animals at death. Levels of IL-12, TNF-α, IFN-γ, MCP-1 and IL-6 were significantly reduced in RA treated samples as compared to the infected ones. p<0.001 (mean ± SEM). **A**. JEV infected mice that succumbed even after RA treatment had significantly high levels of proinflammatory cytokines when compared to uninfected one. p< 0.001 (mean ± SEM), n=4 for each group. **B**. Protein levels of control, JEV infected, JEV infected and RA treated animals, RA treated alone and JEV infected and RA treated animals at death were analyzed by immunoblot. A significant reduction in the levels of pNF-κB and Cox-2 in RA treated samples was observed as compared to infected ones (p<0.05). RA treatment reversed the level of IkB-α (p<0.05). Mice that died even after RA treatment, had increased levels of pNF-κB and Cox-2 and decreased levels of IkB-α than control group. Data shown is of two individual animals from total of four animals in each group.

**Figure 4:** RA decreases the proinflammatory mediators in vitro. Mouse microglial cell line BV2 was used to study the *in vitro* induction of proinflammatory cytokines and chemokine following JEV infection. There was a significant reduction in the levels of IL-6, MCP-1, IL-12, IFN-γ and TNF-α in JEV infected and RA treated samples than JEV infected only. Values represent the mean ± SEM from 3 independent experiments performed in duplicates (p<0.05).
A

![Graph showing cytokine levels](image)

A1

![Graph showing cytokine levels](image)

B

![Western blot analysis](image)