Rejuveinix Mitigates Sepsis-Associated Oxidative Stress in the Brain: Clinical Impact Potential in COVID-19 and Nervous System Disorders

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Research Article

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Abstract

Here, we demonstrate that our anti-sepsis drug candidate Rejuveinix (RJX), at a dose level that is >10-times lower than its maximum tolerated dose (MTD) for human subjects, improves the survival outcome in the LPS-GalN model of sepsis and multi-organ failure. One hundred (100) percent (%) of untreated control mice remained alive throughout the experiment. By comparison, 100% of LPS-GalN injected mice died at a median of 4.6 hours. In contrast to the invariably fatal treatment outcome of vehicle-treated control mice, 40% of mice treated with RJX (n=25) remained alive with a 2.4-fold longer median time survival time of 10.9 hours (Log-rank X^2=20.60, P<0.0001). Notably, RJX increased the tissue levels of antioxidant enzymes SOD, CAT, and GSH-Px, and it reduced oxidative stress in the brain. These findings demonstrate the clinical impact potential of RJX as a neuroprotective COVID-19 and sepsis drug candidate, which is currently being evaluated in a placebo-controlled, double-blind Phase II multi-institutional US study in hospitalized patients with COVID-19 associated viral sepsis.

Introduction

Severe viral sepsis caused by SARS-CoV-2, the causative agent of coronavirus disease 2019 (COVID-19), shows a rapid progression associated with a cytokine release syndrome (CRS) and a high case fatality rate due to the development of ARDS and multi-organ failure in high-risk COVID-19 patients [1, 2]. Our anti-sepsis drug candidate, Rejuveinix (RJX), is a rationally designed formulation of naturally occurring antioxidants and anti-inflammatory compounds with a significant clinical impact potential for COVID-19 associated viral sepsis [3, 4]. RJX showed a very favorable clinical safety profile in a recently completed double-blind, placebo-controlled, randomized, two-part, ascending dose-escalation Phase 1 study in healthy volunteers (Protocol No. RPI003; ClinicalTrials.gov Identifier: NCT03680105) [5]. It has now entered a randomized, double-blind, placebo-controlled Phase 2 study in hospitalized patients with critical COVID-19.

More than a third of patients with COVID-19, especially those with severe to critical COVID-19 who are treated on an intensive care unit (ICU), develop central nervous system (CNS) symptoms and signs (e.g., headache, dizziness, ataxia, seizure, delirium, confusion, impaired consciousness) consistent with CNS involvement and /or neurological complications [6–13]. Cerebrovascular complications of viral sepsis, including ischemic or hemorrhagic stroke, CNS involvement in CRS, hypoxia as well as the interplay of comorbidities, have been implicated as contributing factors [6–13]. Due to the neuro-invasive capability of SARS-CoV-2, acute disseminated encephalomyelitis (ADEM) and viral encephalitis have also been suspected in some patients [13].

Oxidative stress caused by the massive production of reactive oxygen species (ROS) is thought to play a major role in the pathogenesis of severe viral sepsis in COVID-19 [14]. Chaudry et al. recently proposed that reactive oxygen intermediates and oxidative stress may play an important role in the pathophysiology of COVID-19 associated CNS disease, reminiscent of their role in Parkinson's disease [15]. Notably, RJX exhibited potent antioxidant activity and mitigated lipid peroxidation in prophylactic

and therapeutic settings, as reflected by significantly decreased tissue MDA levels and normalization of the tissue levels of the antioxidant enzymes SOD, CAT, and GSH-Px as well as ascorbic acid [5]. The primary objective of the present study was to obtain experimental proof of concept that RJX can mitigate sepsis-associated oxidative stress in the brain.

Materials And Methods

Rejuveinix (RJX)

RJX is a proprietary composition of naturally occurring antioxidants and anti-inflammatory agents, which, in combination, provide potent and immediate tissue protection. Its ingredients include ascorbic acid, magnesium sulfate heptahydrate, cyanocobalamin, thiamine hydrochloride, riboflavin 5' phosphate, niacinamide, pyridoxine hydrochloride, and calcium D-pantothenate. RJX is a two-vial system, and A and B are each of the two vials. Vial A contains the active ingredients and minerals, whereas Vial B contains the buffer, sodium bicarbonate, as the Vial A content is acidic [5]. RJX is being developed as an anti-inflammatory and antioxidant treatment platform for patients with sepsis, including COVID-19 patients with viral sepsis and ARDS [5].

LPS-GalN Model of Fatal Cytokine Storm, Sepsis, and Multi-organ Failure

The ability of RJX, DEX, and RJX plus DEX to prevent fatal shock, ARDS, and multi-organ failure was examined in the well-established LPS-GalN model [5]. In this model, LPS is combined with GalN, which further sensitizes mice to LPS-induced systemic inflammatory syndrome and multi-organ failure. Male BALB/c mice (6–8 weeks old) were obtained from the Firat University Experimental Animal Center. Mice had ad libitum access to standard rodent chow and water throughout the study. The care and treatment of the animals were in accordance with the *Guide for the Care and Use of Laboratory Animals*. The research protocol was approved by the Animal Care and Use Committee of Firat University. BALB/c mice were randomly divided into different treatment groups. All mice were genetically identical, of the same age, and the LPS-GalN challenged mice were injected with the same amount of LPS-GalN. This statistical equivalency of mice allowed using a pseudo-randomization convenience allocation to assign mice to identified cages. For random treatment allocation, cages were randomly selected to receive one of the specified treatments. We applied the concealment of treatment allocation and blind outcome assessment to reduce the risk of bias in our conclusions. Health care assessments were performed by animal care technicians not involved in treatment assignments or treatments. Investigators did not participate in individual health status or outcome assessments. Untreated normal control mice did not receive any treatments.

All mice except for the untreated normal control mice were challenged with an otherwise lethal dose of LPS mixed with GalN. Specifically, all mice were challenged with an *i.p.* injection of LPS plus D-galactosamine (Sigma, St. Louis, MO). D-Galactosamine (Sigma Chemicals), which was dissolved at a 32

mg/ml final concentration in phosphate-buffered saline (PBS), was mixed with an equal volume of diluted, sonicated LPS immediately before dosing. This freshly prepared LPS-galactosamine mixture (LPS-GalN) was used immediately after preparation. Each mouse received a 500 µL i.p. injection of LPS-GalN (consisting of 100 ng of LPS plus 8 mg of D-galactosamine). In Part A of the study, vehicle control mice were treated with 0.5 mL normal saline (NS), i.e., an aqueous solution of 0.9% NaCl instead of RJX. NS was administered intraperitoneally (*i.p*) 2 hours before and 2 hours after the *i.p* injection of LPS-GalN. Test mice received 0.7 mL/kg RJX dose (2 hours before and 2 hours after LPS-GalN. Mice were monitored for mortality for 24 h. The Kaplan-Meier method, log-rank chi-square test, was used to analyze the 24 h survival outcomes of mice in the different treatment groups. At the time of death, lungs and liver were harvested, fixed in 10% buffered formalin, and processed for histopathologic examination. 3 µm sections were cut, deparaffinized, dehydrated, and stained with hematoxylin and eosin (H & E) and examined with light microscopy.

Lipid peroxidation as a biomarker of oxidative stress was determined and expressed as the amount of malondialdehyde (MDA, nmol/g tissue) in the brain, as previously described [5]. The enzymatic activities of SOD, CAT, and GSH-Px in the brain specimens were determined using the commercially available kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's procedures. In MDA assays, tissue samples (0.3 g) were analyzed for MDA using high-performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) [5]. Specifically, an HPLC system equipped with the LC solution Software (Shimadzu, Kyoto, Japan), a pump (LC-20AD), a UV Detector (SPD-20A), a column oven (CTO-10ASVP), an autosampler (SIL-20 A), a degasser unit (DGU-20A5), and a column (Inertsil ODS-3, 250x 46 mm, 5 mm) was used. Tissue samples were homogenized on ice in a glass–glass homogenizer in a mixture of 0.5 ml of HClO4 (0.5 M), 2.5 ml distilled water, and 2[6]-di-tert-butyl-p-cresol (BHT). Then, the samples were centrifuged at 4500 rpm for 5 min, and supernatants were injected into the HPLC system. The addition of acid was necessary to precipitate proteins and release the MDA bound to the amino groups of proteins and other amino compounds. The mobile phase was 30 mM KH2PO4–methanol (82.5 + 17.5, v/v %, pH 3.6), and the flow rate was 1 ml min^{-1}. The injection volume was 30µL, and chromatograms were scanned at 250 nm.

Statistical Analyses

Statistical analyses employed standard methods, including analysis of variance (ANOVA) and/or, nonparametric analysis of variance (Kruskal-Wallis) using the SPSS statistical program (IBM, SPPS Version 21), as reported [5]. Furthermore, the Kaplan–Meier method, log-rank chi-square test, was used to investigate survival and fatality in each group. P-values < 0.05 were considered significant.

Results

RJX improves survival outcomes after LPS-GalN induced sepsis.

One hundred (100) percent (%) of untreated control mice remained alive throughout the experiment. By comparison, 100% of LPS-GalN injected mice died at a median of 4.6 hours (Fig. 1). RJX was examined

for its protective activity at a dose level, which is > 10-fold lower than its maximum tolerated dose (MTD) of 0.759 mL/kg for human subjects (viz.; 4.2 mL/kg of a 6-fold diluted solution). RJX-treated mice had an improved survival outcome after being injected with LPS-GalN. In contrast to the invariably fatal treatment outcome of vehicle-treated control mice, 40% of mice treated with RJX (n = 25) remained alive with a 2.4-fold longer median time survival time of 10.9 hours (Log-rank $X^2 = 20.60$, $P < 0.0001$) (Fig. 1).

RJX reduces the oxidative stress in the brain after LPS-GalN induced sepsis.

No histopathologic brain lesions were observed in any of the mice challenged with LPS-GalN. However, the brain MDA levels measuring lipid peroxidation were markedly elevated, and the levels of the antioxidant enzymes SOD, CAT, and GSH-Px in the brain were dramatically reduced in LPS-GalN treated mice consistent with severe oxidative stress (Fig. 2). RJX decreased the brain MDA levels and normalized in a dose-dependent manner the reduced levels of the antioxidant enzymes SOD, CAT, and GSH-Px.

Discussion

COVID-19 has caused a marked increase in all-cause deaths in the US and has become the third leading cause of death for persons aged 45 through 84 years and the second leading cause of death for older persons. Patients with high-risk COVID-19 are in urgent need of treatment platforms capable of preventing the disease progression and/or reducing the case mortality rate by stopping or reversing the pulmonary as well as the systemic inflammatory process that causes the ARDS and culminates in multi-organ failure [1, 2, 16]. Here we extend our previous study and provide experimental evidence that RJX can improve the survival outcome in the LPS-GalN mouse model of fatal sepsis.

SOD, CAT, and GSH-Px are three pivotal antioxidant defense enzymes, and their levels are altered by the level of oxidative stress that is a hallmark of severe inflammation of sepsis [14]. Oxidative stress caused by the massive production of reactive oxygen species (ROS) is thought to play a major role in the pathogenesis of severe viral sepsis in COVID-19 as well [14]. The MDA levels were markedly elevated in the brain specimens of the LPS-GalN challenged mice which is consistent with increased lipid peroxidation. In parallel, the levels of the antioxidant enzymes SOD, CAT, and GSH-Px were profoundly suppressed due to severe oxidative stress. RJX exhibited potent antioxidant activity and mitigated lipid peroxidation, as reflected by significantly decreased tissue MDA levels and normalization of the tissue levels of the antioxidant enzymes SOD, CAT, and GSH-Px as well as ascorbic acid. We hypothesize that RJX will shorten the time to resolution of ARDS and viral sepsis in COVID-19 patients by preventing the development of a fulminant cytokine storm as well as reversing the cytokine-mediated multi-system inflammatory process and oxidative stress, thereby mitigating the inflammatory organ injury.

Oxidative stress owing to mitochondrial dysfunction has been implicated in the pathophysiology of Alzheimer's disease (AD), the most common form of dementia, Parkinson’s disease (PD), the second most common progressive disorder of the central nervous system, and Huntington's disease (HD), a neurodegenerative disorder associated with cognitive decline and dementia. Notably, in a mouse CNS

model of severe oxidative stress, RJX rapidly and substantially increased the levels of the antioxidant enzymes SOD, CAT, GSH-Px that were reduced in the brains of LPS-GalN treated mice consistent with the severe oxidative stress. The results presented herein demonstrate for the first time that RJX could have therapeutic utility in the treatment of AD, PD, HD, and ALS. Furthermore, because of the well-established role of oxidative stress in the development and progression of ischemic stroke, one of the leading causes of mortality and morbidity, RJX could significantly improve the standard of care for stroke as well.

Declarations

Acknowledgments

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Authors' contributions

Each author has made significant and substantive contributions to the study, reviewed and revised the manuscript, provided final approval for submitting the final version. No medical writer was involved. F.M.U conceived the study, designed the evaluations reported in this paper, directed the data compilation and analysis, analyzed the data, and prepared the initial draft of the manuscript. Each author had access to the source data used in the analyses.

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Availability of data and material

Sets of data or summaries generated during the present study are available from the corresponding author upon reasonable request.

Ethics approval

The care and treatment of the animals were in accordance with the *Guide for the Care and Use of Laboratory Animals*. The research protocol was approved by the Animal Care and Use Committee of Firat University.

Consent to participate

Informed consent was obtained from all individual participants included in the current study.

Consent for publication

Consent of publication was obtained from all authors

Conflicts of interest

F.M.U. and M.V are employees of Reven Pharmaceuticals, the sponsor for the clinical development of RJX. M.T., M.G, and K.S declare no current competing financial interests

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Figures

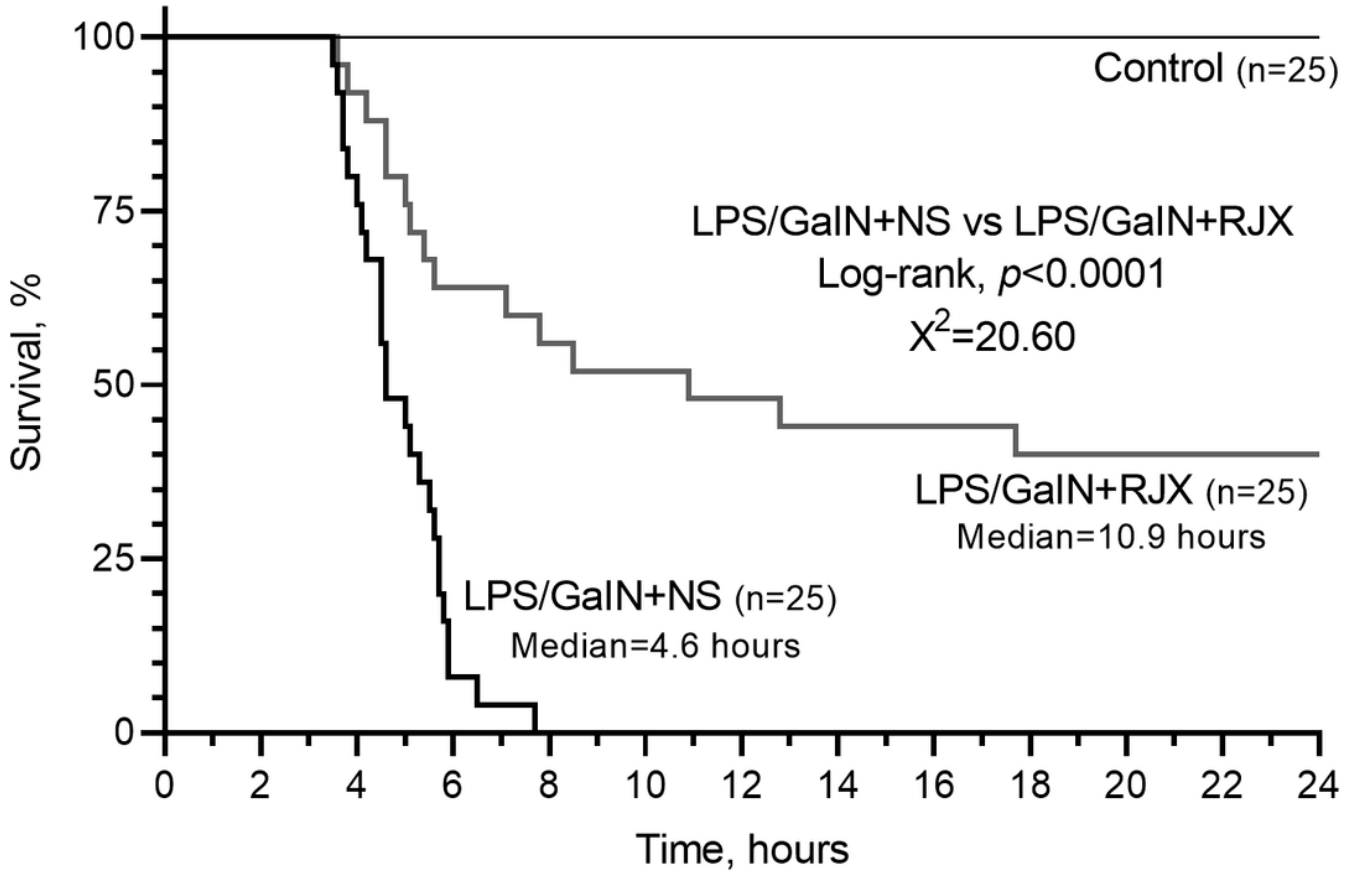

Figure 1

In Vivo Protective Activity of RJX in the LPS-GALN challenged mice. Groups of 25 BALB/C mice were treated with i.p injections of 6-fold diluted RJX (4.2 mL/kg, 0.5 ml/mouse) or vehicle (NS) 2 hours before or post-injection of LPS-GalN. Except for untreated mice (Control), each mouse received 0.5 ml of LPS-GalN (consisting of 100 ng of LPS plus 8 mg of D-galactosamine) i.p. Survival is shown as a function of time after the LPS-GalN challenge. Depicted are the survival curves for each group along with the median survival times and the log-rank P-value for the comparison of the LPS-GalN+RJX group versus the LPS/GalN+NS group.

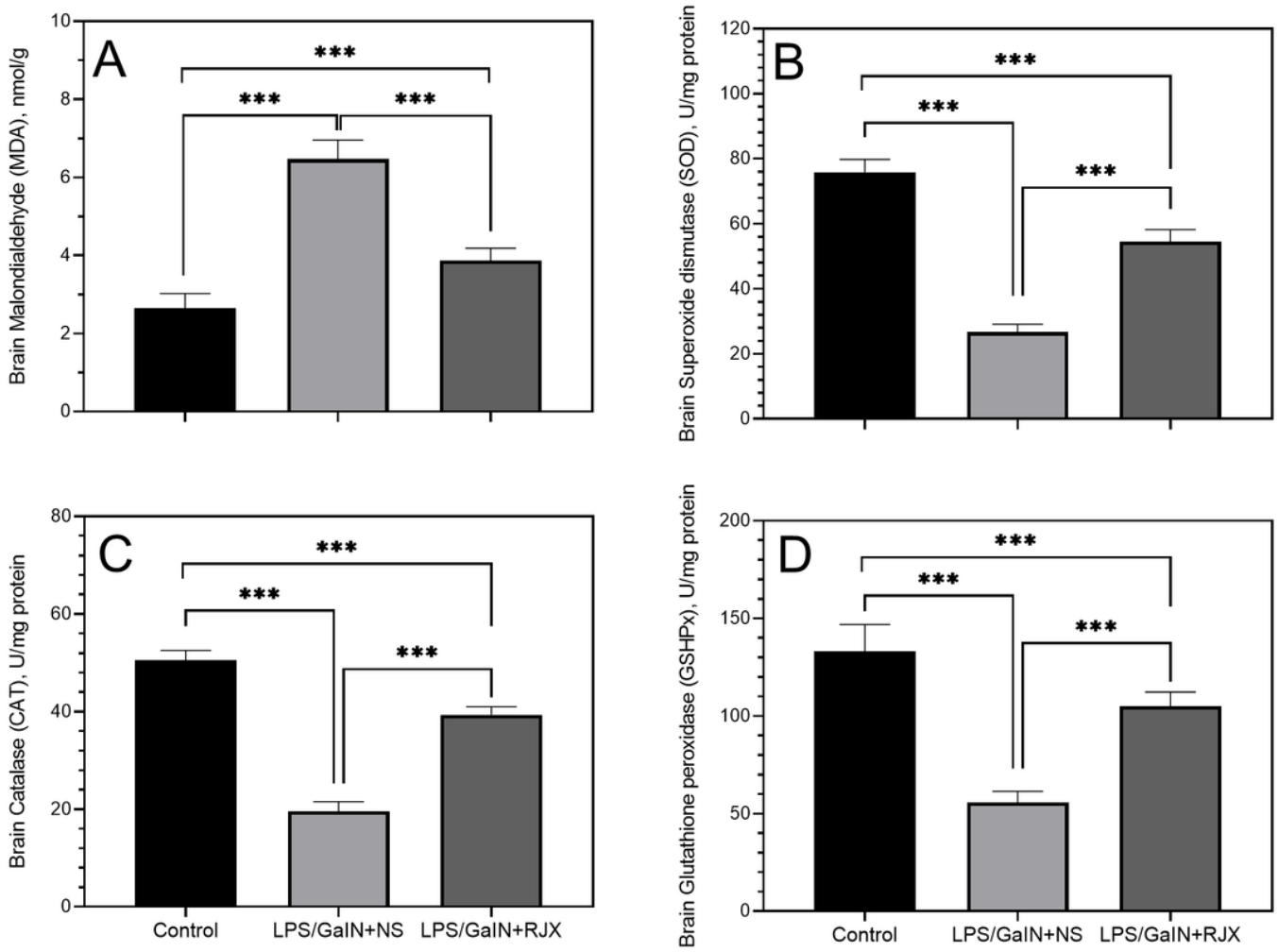

Figure 2

Effect of Rejuveinix (RJX) on brain malondialdehyde (MDA; Panel A), superoxide dismutase (SOD; Panel B), catalase (CAT; Panel C), and Glutathione peroxidase (GSHPx; Panel D) in the lipopolysaccharide-galactosamine (LPS-GalN) challenged mice. Each bar represents the mean and standard deviation for the measured parameter of mice from each specific treatment group. BALB/C mice were treated with i.p injections of 6-fold diluted RJX (4.2 mL/kg, 0.5 ml/mouse) or vehicle (NS) 2 hours before or post-injection

of LPS-GalN. Except for untreated mice (Control), each mouse received 0.5 ml of LPS-GalN (consisting of 100 ng of LPS plus 8 mg of D-galactosamine) i.p. (ANOVA and Tukey’s post-hoc test. Statistical significance between groups is shown by: *** $P < 0.001$).