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ORIGINAL STUDY

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Abstract

Background: With a high death rate, stroke is a very concerning condition. It continues to rank third in terms of both death and disability combined and as the second most common cause of death. The annual number of new stroke cases exceeds 12.2 million. RtPA is the only pharmaceutical agent that has been approved to treat stroke, which is unfortunate. It is recommended that rtPA be given within 4-5 hours of an appropriate diagnosis of stroke onset. During a stroke, the angiotensin II type 2 receptors (AT2R) serve to protect the brain. Compound 21 (C21), a selective non-peptide agonist of the angiotensin II type 2 receptor (AT2R), protects against impairments in neurological function and cerebral damage brought on by ischemic stroke.

Materials and Methods: Twenty-eight-year-old adult Randomly selected Sprague-Dawley rats were split into four equal groups. The sham group was anesthetized concurrently with the other groups. Control that had 30 minutes of ischemia induction followed by 60 minutes of reperfusion. The vehicle group received the identical therapy as the control group, but before 120 minutes of reperfusion, they received an intraperitoneal injection of the vehicle (1 milliliter per kilogram of 10% DMSO). Treatment groups received the same care as the control group, but they are injected intraperitoneal with Compound 21 (0.3 mg/kg) prior to 120 minutes.

Results: The induction of ischemia/reperfusion in rats (control group) significantly ($P \leq .05$) elevated the size of the infarction area in the brain compared to the treatment and sham groups. This work investigated the neuroprotective efficacy of C21 in the context of cerebral ischemia-reperfusion injury. Histological examination demonstrated considerable cerebral tissue damage in the ischemia/reperfusion gr. ($p<.05$), which was markedly diminished in the C21 group ($p<.05$). PCR findings indicated Rbfox3 expression was considerably decreased ($p <.05$) in the control gr. relative to sham gr., whereas it was significantly elevated in C21 gr. ($p <.05$). The results indicated C21 may alleviate cerebral dysfunction caused by ischemia-reperfusion damage in male rats via modulating the Rbfox3 signaling pathway, leading to a reduction in apoptotic events.

Conclusion: There was a decrease in the infarction and damage score in the group receiving C21 treatment, which is thought to be neuroprotective against cerebral ischemia/reperfusion in the male rat model. Additionally, a rise in fox3 gene expression all contributed to the antiapoptotic impact following ischemia-reperfusion damage.

Keywords: Apoptosis, Compound 21, Cerebral I/R, Fox3, Cerebral damage and infarction

1. Introduction

During an acquired brain injury (ABI), two critical distinct regions of the brain are identified: the ischemic core and the ischemic penumbra. The brain's ischemic center undergoes an abrupt decline in blood flow, occurring within minutes after an ischemia event, resulting in irreparable damage and eventual cellular death [1]. However, necrosis begins in the first few hours following the onset of ABI in the ischemic core, but apoptosis inside the ischemic penumbra may happen hours or days later.

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ABI is typified by important chemical processes, including as excitotoxicity, Ca2+ overload, and free radical overproduction, which cause apoptosis in many cells. DNA breakage, cytoskeletal and nuclear protein breakdown, protein cross-linking, apoptotic body formation, production of ligands for phagocytic cell receptors, and ultimately phagocytic cell uptake are the outcomes of apoptosis [2]. To identify the presence of apoptosis, distinctive ladder-like DNA bands are created during gel electrophoresis. The mRNA expression of genes linked to apoptosis is measured at various phases of apoptosis using the real-time quantitative polymerase chain reaction (RT-qPCR) [3]. In order to identify the biochemical characteristics of apoptotic cells, mitochondrial membrane potential analysis is also utilized.

According to the mitochondrial route, apoptosis can be detected early by the reduction in mitochondrial membrane potential [4]. Although the underlying processes are currently unclear, apoptosis may play a substantial role in the death of neurons after acute brain ischemia (ABI) [5]. Stroke, one of the primary causes of long-term morbidity and death in both industrialized and developing nations, can result from brain ischemia [6]. Thus, it is clinically crucial to prevent and treat stroke. Although AT2R is relatively weakly expressed in the majority of healthy tissues, it is significantly increased after tissue damage, including myocardial infarction (MI), vascular and neuronal injury, [7] and brain ischemia [8]. The targeted activation of the AT2R using the novel small-molecule ligand C21 has markedly improved comprehension of the fundamental molecular pathways linked to AT2R-mediated tissue protection, encompassing anti-inflammation, anti-fibrosis, and anti-apoptosis, and demonstrates substantial potential for pharmacological intervention in these conditions. [9] RbFox 3 is extensively utilized as a reliable indicator of neurons and neural stem cells and plays a part in the control of mRNA splicing. Thus, RbFox3 is a protein that is highly and selectively expressed in neuronal nuclei and functions in RNA splicing [10].

2. Materials and methods

Rats weighing 300–350 grams, numbering 28, obtained from the research center, were obtained from the National Center for Drug Control and Research in Baghdad, Iraq.

All animals treated under identical conditions and housed in the Kufa College of Science Animal House, which maintained a controlled temperature of 25° ± 1C and humidity levels of 60–65%. They received a regular chow diet, unlimited access to water, and experienced alternate 12-hour light and dark cycles [11].

Four groups are randomly assigned to the rats.with each group consisting of seven experimental rats:

Group-1: Sham: This group underwent the same surgical technique, including anesthesia and duration, but eliminated the Bilateral Common Carotid Artery Occlusion (BCCAO).

Group-2: Control: This group had anesthesia and (BCCAO) for thirty minutes, followed by one hour of reperfusion without intervention.

Group-3: Control-vehicle (C-vehicle): This group underwent the identical protocol as the control group, supplemented by an intraperitoneal injection of 1 ml/kg of the vehicle (10% DMSO) two hours before to the induction of ischemia/reperfusion (I/R).

Group 4: C 21 treated (0.3 mg/kg): This group underwent the same technique as the control group, supplemented by an intraperitoneal injection of 0.3 mg/kg of body weight of Compound 21 two hours prior to ischemia/reperfusion induction.

2.1. Preparation of samples for histopathology

After several steps of tissue dehydration using varying graded concentrations of alcohol, the isolated portion of the brain for H&E was fixed in 10% formalin for five to ten minutes. Finally, it was cleaned completely of alcohol using xylene before being submerged in paraffin wax. The automated tissue processor takes around 20 hours to complete this operation. Sections were obtained that were 5μ thick and stain [12].

No.	Tissue Type	Tissue Damage (Lesion)	Score
1	Brain	Necrosis and fibrosis	4
2	Brain	Inflammation (Microgiliosis)	3
3	Brain	Edema	2
4	Brain	Congestion	1
5	Brain	NO Pathological lesion	0

Histopathological scores.

2.2. PCR procedure

Place brain pieces in Trizol and freeze at −20°C.

RNA Extraction: isolate total RNA from the cells with an RNA extraction kit.

cDNA Synthesis: Transform the isolated RNA into complementary DNA (cDNA) via reverse transcription with a reverse transcriptase enzyme.

PCR Amplification:

- Develop primers for apoptosis-related genes (Rbfox3) and a housekeeping gene (e.g., GAPDH) for normalization purposes.

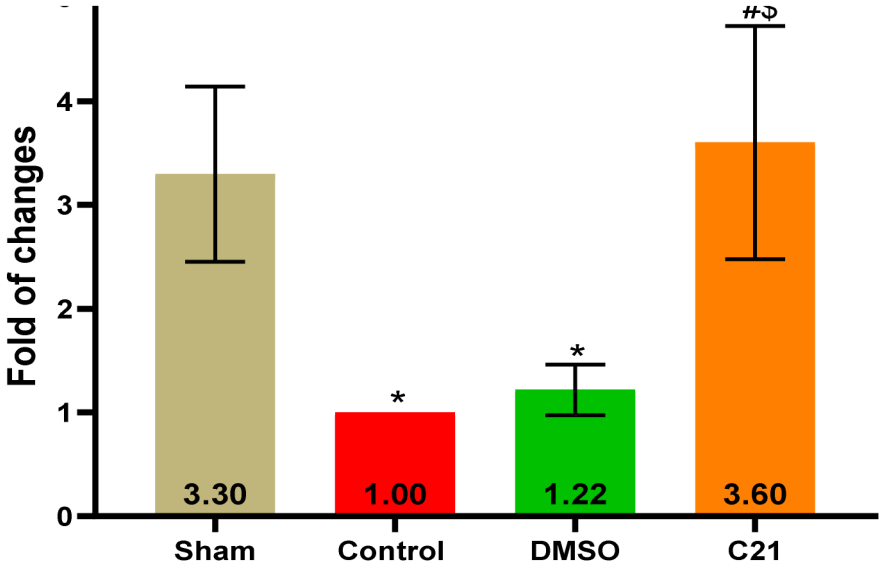

*Fig. 1. Gene expression of Rbfox3 among study groups. Results are expressed as Mean ± SD. * p < .05 Compared to the Sham group, # p < .05 Compared to the Control group, $ p < .05 Compared to the DMSO.*

Table 1. Descriptive Statistics of the Level of Rbfox3.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Sham	4	3.3111	.84488	.42244	1.9517	4.6405	2.23	4.04
Control	4	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
DMSO	4	1.2217	.24354	.12177	.8312	1.6063	1.02	1.56
C21	4	3.6994	1.12326	.56163	1.8121	5.3868	1.97	4.53

- Prepare the PCR reactions, incorporating cDNA, primers, dNTPs, and polymerase.

  Conduct an analysis of the PCR results by electrophoresis on an agarose gel and evaluate the expression levels of the apoptosis-associated genes. Assess the expression levels of pro-apoptotic and anti-apoptotic genes in treatment vs control groups. An elevated FOX3/GAPDH ratio might signify an anti-apoptotic effect [13].

2.3. Ethical approval

This study was approved by the Animal Care Committee at the University of Kufa, Iraq with reference number 20551 on (29/8/2024).

2.4. Statistical analysis

A statistical analysis of the experimental outcomes was performed using GraphPad Prism version 8, employing Tukey's multiple comparisons method. A one-way ANOVA was used to examine the significance of differences across groups. The data were presented as mean ± standard error of the mean (SEM), with a P value < 0.01 considered statistically significant.

3. Results

3.1. Effect of C21 on intracellular signaling protein after CIRI by PCR

When compared with the sham group, the Rbfox3 were significantly p0.05) lower in the control and vehicles groups, while the levels of RbFox3 expression in C21 group were higher compared with the control group. Fig. 1 and Table 1 presents a summary of the results of the PCR study:

3.2. Effect on cerebral infarcted area

The level of cerebral infarction size area has significantly (P < .05) increased in the control group in comparison to the sham. However, there is no significant difference between the control and C-vehicle groups. In contrast, there is a significant (P < .05) reduction in percentage of infarction in the treated group Compound21 (0.3 mg/kg) compared with the C-vehicle group. (Figs. 2 and 3) and (Table 2) present

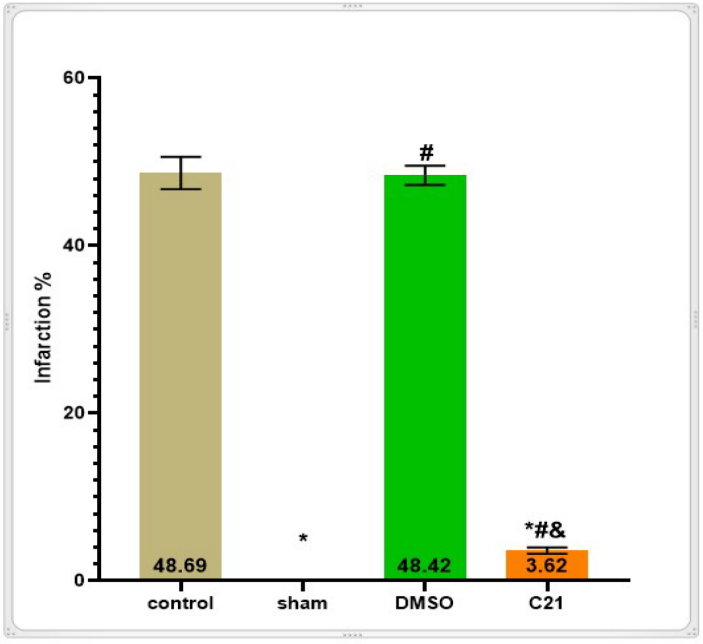

*Fig. 2. Error bar chart show difference in mean ± SEM values in percentage of infarction area in brain of all four groups. Results are expressed as Mean ± SD. * p < .05 in contrast to Control # p < .05 in contrast to Sham, and & p < .05 in contrast to DMSO gr.*

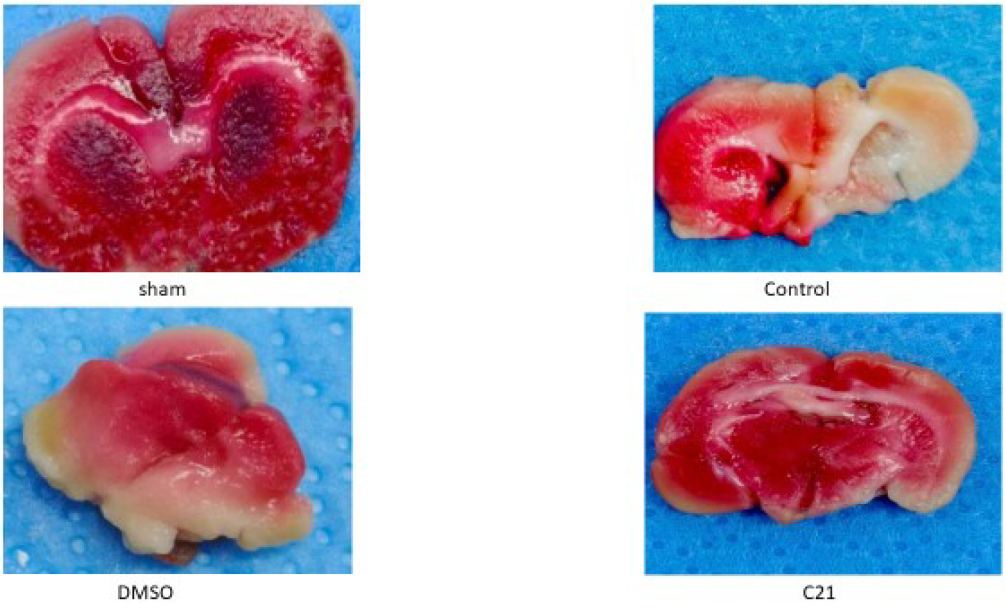

Fig. 3. Cross-section slices of rat brain stained with TTC stain. The white area represents an infarction region. The red color region represents a normal area.

a summary of the results of the infarction by TTC staining.

3.3. Histopathological finding

Occlusion of CCA for 30 min, followed by 60 min. of reperfusion-induced cerebral damage ranged from slight to severe, that characterized by edema, dark "eosinophilic" neurons, hemorrhagic areas, and necrosis. These changes have been examined by the pathologist under the microscope. The histological changes in each rat brain of the four study groups have been expressed in (Figs. 4 and 5) and (Table 3).

A: Normal morphology in sham group.
B: Control group The section shows sever break down in the brain parenchyma (necrotic lesion in

Table 2. The percentage of brain infarction size in the four study groups by using ANOVA test.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	48.6880	1.93376	0.86480	46.2869	51.0891	45.32	50.03
Sham	5	0.0000	0.00000	0.00000	.0000	.0000	.00	.00
DMS)	5	48.4200	1.15102	0.51475	46.9908	49.8492	46.87	50.09
C21	5	3.6160	0.38818	0.17360	3.1340	4.0980	2.95	3.91
Total	20	25.1810	24.03908	5.37530	13.9304	36.4316	.00	50.09

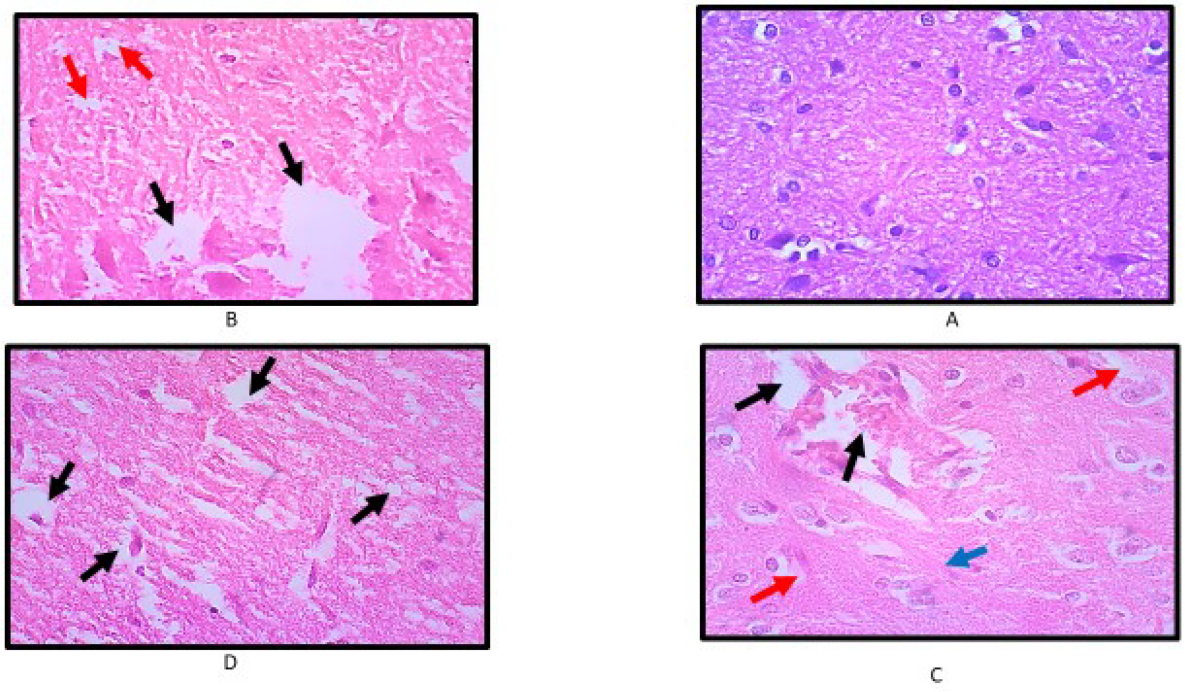

Fig. 4. Comparable H&E staining of cerebral tissue slices (magnification 40X).

the brain, Black arrows) with surrounding edematous lesion (brain edema, Red arrows).

C: Vehicle group shows severe breakdown in the brain parenchyma (necrotic lesion in the brain surrounding blood vessels with congestion Black arrow) with surrounding edematous lesion (brain edema, Red arrows) and moderate fibrous connective tissue can be seen in the section (blue arrow).

D: D: The C21 (0.3mg/kg) group shows a mild edematous lesion in the brain parenchyma (Black arrows) without any significant occupied lesion.

4. Discussion

Stroke tests have demonstrated that NeuN-positive cells decline more than in normal regions in disease-related centers. This result has been linked to neuronal death or destruction [14]. Neuroprotection, which may assist in stopping neuronal cells from dying, can be facilitated by AT2R activation. This may be especially important in cases of neurodegenerative disorders or ischemia [15].

Our study revealed that the C21-treated groups exhibited considerably greater levels of Rbfox3 gene expression in comparison to control and vehicle groups. Higher levels of NeuN expression may indicate a healthier neuronal population as a result of increased neuron survival in response to AT2R activation [16]. This support our study.

In the present study, the level of the cerebral damage score of histopathology has significantly increased ($P < .05$) in both control and C-vehicle groups compared with the sham group. While there is an insignificant difference between the control and C-vehicle groups. Based on the results of the present study, there is an agreement with many studies [17, 18]. where they showed a marked worsening in the brain and how the ischemia/reperfusion had a role in the brain damage as expressed in congestion of blood vessels, neutrophil infiltration, and neuronal necrosis.

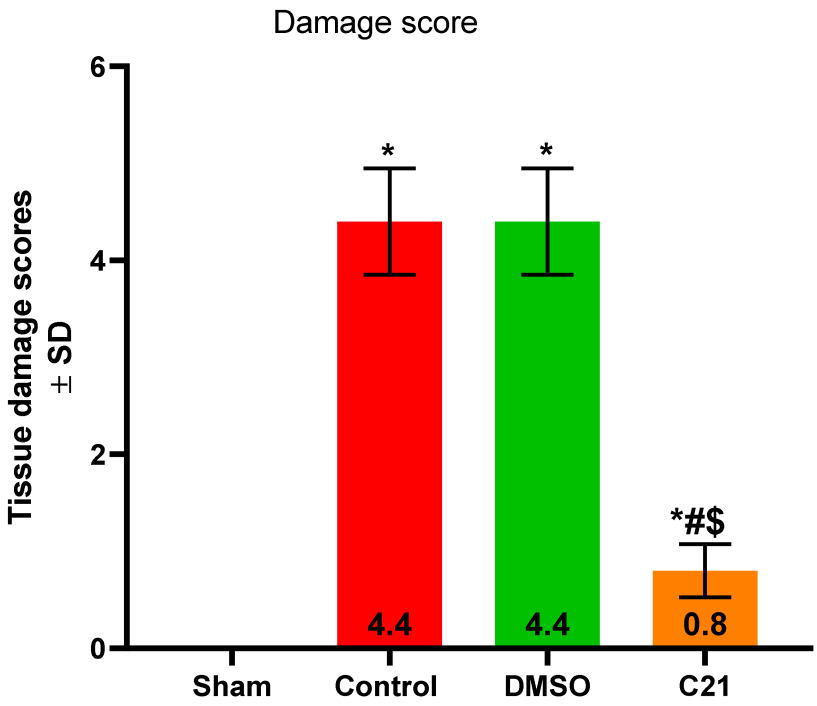

Fig. 5. Histopathological score percentage among the four study groups.

Table 3. Damage score in the four study groups by using ANOVA test.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
Sham	5	0.0000	.00000	.00000	.0000	.0000	.00	.00
Control	5	4.4000	.54772	.24495	3.7199	5.0801	4.00	5.00
DMSO	5	4.4000	.54772	.24495	3.7199	5.0801	4.00	5.00
C21	5	.8000	.27386	.12247	.4600	1.1400	.50	1.00

The pretreated groups of compound 21 attenuated the histopathological damaging score significantly ($P < .05$) as compared to the C-vehicle group. This study stated the role of AT2 agonist drug to ameliorates cerebral impairment after ischemia/reperfusion injury in the rat, as supported by a previous study that evaluated the beneficial role of C21 as cerebroprotective as appeared in the sections of histopathology [19]. This result supported by a prior research shown that C21 reduced necrotic lesions and maintained tubular shape in renal damage [20, 21]. So that, depending on the studies above on the beneficial protective role of C21 on the different body's organs and with the same context the present study also confirms the possibility of the C21 drug being considered as a neuroprotective due to its role in attenuating the histopathology damaging score.

In our study, the percentage of cerebral infarction after induction of BCCAO for 30 min. followed by 1hour of reperfusion has been determined using (TTC) staining method. It showed a significant increase in the infarction area in the control when compared with the sham group. The results of this study correspond with other studies [18–22].

5. Conclusion

Administration of Compound C21 led to an elevation in Rbfox3 gene expression and a decrease in damage score and infarction size in a male rat model of brain ischemia/reperfusion, demonstrating its antiapoptotic and neuroprotective effects.

Ethical Approval

The ethical Approval statement has been provided at the end of the "Materials and Methods" section (Page 3).

Funding Statement

This research received no external funding.

Conflict of Interest

The author declares no confict of interest.

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