Clinical and Applied Thrombosis/Hemostasis
Volume 25: 1-8

DOI: 10.1177/1076029619871359
journals.sagepub.com/home/cat
SAGE

Fisetin Prolongs Therapy Window of Brain Ischemic Stroke Using Tissue Plasminogen Activator: A Double-Blind Randomized Placebo-Controlled Clinical Trial

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Abstract

Recombinant tissue plasminogen activator (rt-PA) can be utilized to treat ischemic stroke with safety and effectiveness but limited by a narrow therapeutic window. In the present clinical trial among patients with stroke, we sought to evaluate the potential of fisetin to extend the therapeutic window of rt-PA treatment. Patients with stroke were divided based on their onset-to-treatment time (OTT) and then randomly assigned to receive the rt-PA treatment combined with fisetin or placebo. Primary outcome was evaluated using the National Institutes of Health Stroke scale (NIHSS), and secondary outcome was assessed by serum levels of matrix metalloproteinase (MMP) 2, MMP 9, and C-reactive protein (CRP). Fisetin dramatically improved the treatment outcomes of the patients with stroke in the delayed OTT strata, as revealed by lower NIHSS scores. The beneficial effect of fisetin was likely attributable to reduced levels of MMP-2, MMP-9, and CRP in the serum, as evidenced by strong linear correlations between serum levels of such markers with the NIHSS scores in all enrolled patients. Fisetin may possess the potential to supplement traditional rt-PA treatments among patients with stroke, particularly for those with delayed OTT, and thereby extend the otherwise narrow therapeutic window and improve the treatment outcomes.

Keywords

ischemic stroke, recombinant tissue plasminogen activator (rt-PA), fisetin, C-reactive protein (CRP)

Date received: 24 May 2019; revised: 24 July 2019; accepted: 30 July 2019.

Introduction

Brain ischemic stroke, with an increasing incidence rate, is among the leading causes of motility and morbidity globally.[1] Brain damages after ischemic stroke often result in severe short- and/or long-term sensorimotor and neurological dysfunctions, along with pathological injuries such as disruption of the blood–brain barrier (BBB) and cerebral edema. Currently, treatment within 3 hours after the symptoms onset using the recombinant tissue plasminogen activator (rt-PA), a serine protease for the degradation of fibrin clots,[2] is widely applied clinically as an effective therapy for acute brain ischemia. Although rt-PA is indeed effective when administered in a timely fashion, delayed treatments beyond the initial 3 hours reportedly yielded less satisfactory outcomes and were more likely to cause adverse responses including intracerebral hemorrhage and hyperperfusion, thereby hindering a broader clinical application of the rt-PA treatment.[3,4]

During the past several decades, natural compounds derived from fruits, vegetables, or plants have attracted great interest for their therapeutic potential against various diseases. Fisetin is a bioactive flavonoid enriched in fruits and vegetables such as apple, strawberry, persimmon, cucumber, and onion,[5,6] and has been recently recognized for its beneficial effect on health and well-being. Fisetin is reported to exert neuroprotective actions.[7] For instance, using cultured PC12 cells as a model of permanent focal ischemia, fisetin significantly increased cell survival after hydrogen peroxide challenge.[8] In addition, in a

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mouse stroke models, fisetin attenuated postischemic immune cell infiltration, activation and infarct size,[9] and reduced the behavioral deficits following a stroke.[10]

To further assess the clinical efficacy of fisetin in expanding the therapeutic window of rt-PA treatment against acute brain ischemic stroke, we hereby designed the present clinical trial and treated patients with stroke who were receiving delayed rt-PA with fisetin. The treatment outcomes were examined using clinical scores as well as biochemical parameters and compared to those of the patients with stroke receiving rt-PA combined with placebo.

Methods

Ethical Statements

The current intent-to-treat clinical trial was performed during January 2015 and December 2018 in compliance with the guidelines laid down by the Declaration of Helsinki and received the approval of the Ethics Committee of Cangzhou Central Hospital. All patients (or their family) provided signed written informed consent before enrollment into this study.

Patient Selection

A total of 215 patients admitted into Cangzhou Central Hospital for acute ischemic stroke were initially recruited for this study. Inclusion criteria include (1) clearly defined onset time, (2) deficit(s) measurable on the National Institutes of Health Stroke scale (NIHSS),[11] (3) computed tomographic scan of the brain showing no signs of intracranial hemorrhage at the time of the recruitment.

Exclusion criteria include (1) platelet counts below 100 000 per mm^3 or glucose levels above 22.2 mmol/L or below 2.7 mmol/L; (2) diastolic blood pressure above 110 mm Hg or systolic blood pressure above 185 mm Hg; (3) seizure at the stroke onset; (4) international normalized ratio above 1.6; (5) minor or rapidly improving symptoms; (6) signs of intracranial, subarachnoid, gastrointestinal, or urinary tract hemorrhage within 21 days; (7) arterial puncture at a noncompressible site within 7 days; (8) received heparin or anticoagulants within 2 days prior to the onset of the stroke; (9) major head surgery or intracranial neurosurgical procedure within 3 months; (10) consumed any supplements containing fisetin within 2 days prior to the stroke; and (11) received aggressive treatments in order to reduce the blood pressure to the aforementioned limits.

Onset-to-Treatment Time Determination

The onset time of the stroke was first determined by interviewing patients and/or family members present when the symptoms were initially noticed and was later validated by corroborating evidence such as ambulance reports. The possibility of onset during sleep was also carefully examined. If the patient woke up with stroke symptoms, the onset time of the stroke was established as the last time when the individual was known to be awake and/or without any stroke symptoms. The patient would not be included if the stroke onset time could not be determined with certainty. The onset-to-treatment time (OTT) was then calculated as the interval between the stroke onset and the treatment initiation. The OTT between 0 and 3 hours is categorized as normal OTT, and OTT of 3 to 5 hours is categorized as delayed OTT.

Randomization and Treatment

A total of 192 eligible patients eventually participated in the current study and were randomly assigned to different groups using a permuted-block randomization with varying block sizes and stratified according to their OTT. Patients were then treated with rt-PA ("Actilyse"; Boehringer Ingelheim, Germany) in the dose of 0.9 mg/kg body weight, which was administered intravenously with the initial 10% as a bolus and the remaining 90% as a constant infusion over 1 hour; 100 mg of Wax Tree derived fisetin ("Novusetin", Bioriginal, Anaheim, California) or cellulose as the placebo was mixed with rt-PA and delivered at the same time. After the initial administration, patients received continuous treatment of either 100 mg placebo or 100 mg fisetin daily for a period of 7 days. A daily dose of 100 mg fisetin was chosen based on the Food and Drug Administration model of conversion from an in vivo dose to the safe human equivalent dosage, for this trial was the first intervention study in patients with stroke.[12] In the initial treatment as well as the following daily treatments, fisetin and placebo were packed to mask their contents to both the investigator and the patients.

Definition of Treatment Outcome

The NIHSS score was employed to evaluate the neurological deficits of the patients.[11] National Institutes of Health Stroke scale, a 0- to 42-point scale, quantifies neurologic deficits in 11 categories, with zero indicating normal function with no sign of neurologic deficit. Scores of 0 to 10 were regarded as favorable outcomes. All evaluations were conducted by an investigator blind to the group assignment.

Enzyme-Linked Immunosorbent Assay

Samples of patients' blood were harvested before the start of rt-PA treatment as the baseline, and at 1 day and 7 days posttreatment as end point. Samples were immediately centrifuged to isolate the serum, which was stored at $-80^{\circ}C$ within 1 hour after collection to avoid the degradation. Serum levels of matrix metalloproteinase (MMP) 2, MMP-9, C-reactive protein (CRP), cystatin C, and neuropeptide Y were assessed using Human ELISA kits (Sigma-Aldrich, St. Louis, MO) in accordance to the provided protocols.

Statistical Analysis

Data were expressed as mean (standard deviation). The normality of data distribution was determined using the Kolmogorov-Smirnov goodness-of-fit test. Then 2-tailed Student t test was utilized to examine normally distributed data, and the

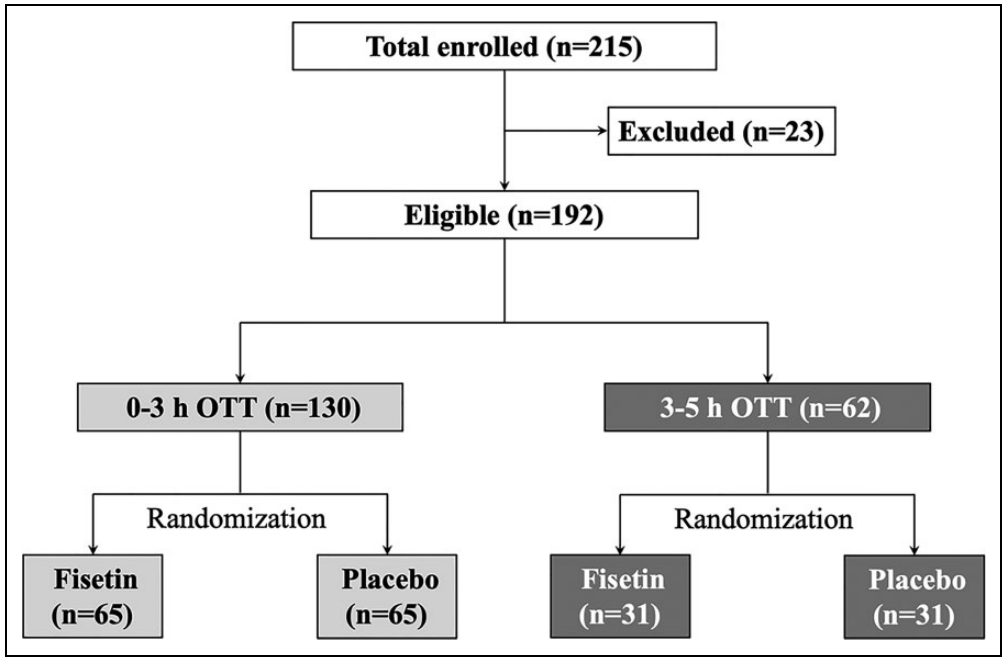

Figure 1. Flow chart of study design.

Mann-Whitney test was utilized to examine non-normally distributed data; *P* values less than .05 were regarded as indicative of statistical significance. Sample size of treatment groups was determined using Cohen d method.[13] Briefly, the mean values of MMP-2, MMP-9, and CRP from all groups were divided by their respective standard deviation to give a series of standardized effect size numbers. The largest number in all calculations was then used for reference to Cohen's d power table, compared to which our group size was still able to yield sufficient statistical significance and power.

Results

Patient Characteristics

During January 2015 and December 2018, a total of 215 patients were initially enrolled into this trial, among which 23 patients were excluded based on the inclusion and exclusion criteria. The remaining 192 eligible patients, with 130 patients in the 0- to 3-hour OTT strata and 62 in the 3- to 5-hour OTT strata, were assigned into 2 treatment groups in a random manner (Figure 1). Baseline characteristics and medical histories of all patients were listed in Tables 1 and 2. The baseline characteristics as well as preexisting medical conditions prior to the stroke onset were comparable among the 4 OTT strata and treatment groups. Importantly, as listed in Table 2, we did not observe any significant differences in the baseline NIHSS scores or the stoke deficits among 4 OTT strata and treatment groups.

National Institutes of Health Stroke Scale Score of All Eligible Patients 1 Day After Initial Treatment

Treatment outcome was assessed using the NIHSS scores as determined by an investigator blind to the group assignment.

Table 1. Medical History of All Eligible Patients in the Study.

	0- to 3-hour OTT (n = 130)		3- to 5-hour OTT (n = 62)	
Medical History	Fisetin (n = 65)	Placebo (n = 65)	Fisetin (n = 31)	Placebo (n = 31)
Hypertension	46	47	17	19
Transient ischemic attack	18	17	11	9
Stroke	11	12	10	8
Myocardial infarction	18	17	9	10
Atrial fibrillation	19	21	8	10
Angina pectoris	12	14	7	9
Preexisting disability	7	5	3	4
Valvular heart disease	8	6	4	4
Congestive heart failure	9	11	5	6
Smoking in previous year	29	26	15	13
Drinking in previous year	31	28	13	14

Abbreviation: OTT, onset-to-treatment time.

One day after the initiation of rt-PA treatment, in the 0- to 3-hour OTT strata, distribution of NIHSS scores of patients receiving simultaneous fisetin treatment was statistically indistinguishable from that of patients receiving placebo (Figure 2; 0- to 3-hour OTT/fisetin vs 0- to 3-hour OTT/placebo). In the 3- to 5-hour OTT strata, treatment outcome of patients given placebo was noticeably less satisfactory (Figure 2; 3- to 5-hour OTT/placebo), in comparison with patients from the 0- to 3-hour OTT strata regardless of treatment (Figure 2; 0- to 3-hour OTT/fisetin and 0- to 3-hour OTT/placebo). This finding was in agreement with prior reports on the therapeutic window of rt-PA treatment as ideally within the first 3 hours of stroke onset. However, within this 3- to 5-hour OTT strata, patients receiving fisetin exhibited great improvement as

Table 2. Baseline Characteristics and Deficits of All Eligible Patients in the Study.[a]

	0- to 3-hour OTT (n = 130)		3- to 5-hour OTT (n = 62)	
Baseline Characteristics	Fisetin (n = 65)	Placebo (n = 65)	Fisetin (n = 31)	Placebo (n = 31)
Age, year	61.9 ± 6.2	62.4 ± 7.3	62.6 ± 6.1	63.1 ± 7.6
Gender (male/female)	32/33	34/31	16/15	14/17
Weight, kg	65.7 ± 8.2	66.3 ± 7.5	67.1 ± 8.4	65.4 ± 7.9
Blood pressure, mm Hg				
Systolic	151 ± 19	153 ± 21	152 ± 24	156 ± 23
Diastolic	83 ± 11	81 ± 12	80 ± 13	82 ± 11
Deficits				
Level of consciousness	15	16	15	14
Best visual	29	31	30	28
Best gaze	28	29	31	29
Sensory	41	38	40	42
Best language	39	42	40	37
Extinction and inattention	28	26	30	29
Ataxia	4	5	6	4
Dysarthria	46	51	49	52
Average motor	39	40	37	42
Median NIHSS (range)	14 (1-34)	13 (2-32)	13 (2-33)	15 (2-35)

Abbreviations: NIHSS, National Institutes of Health Stroke scale; OTT, onset-to-treatment time.
[a]All intergroup differences were statistically insignificant (P > .05).

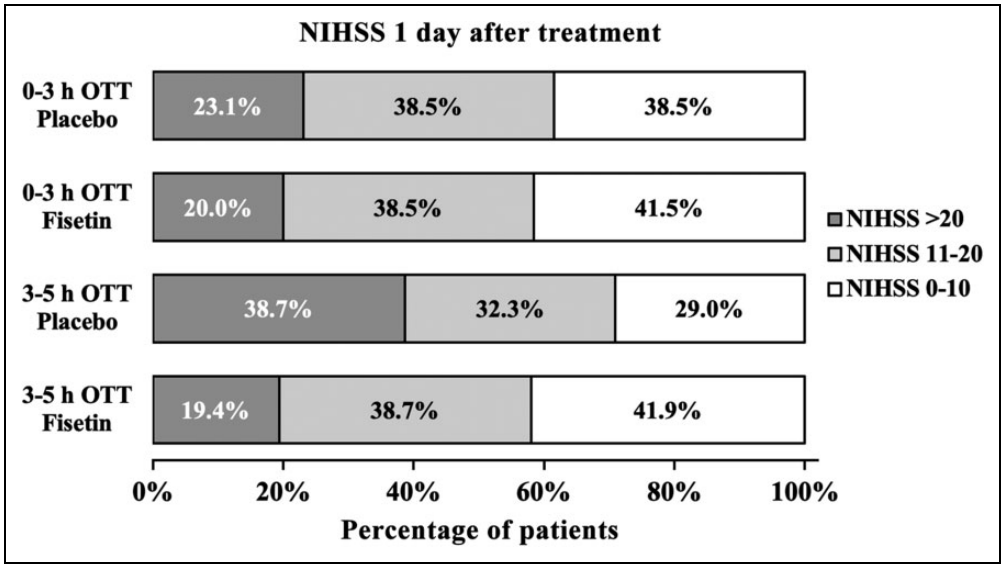

Figure 2. NIHSS score of all eligible patients 1 day after initial treatment. Scores of 0 to 10 was considered to indicate a favorable outcome. NIHSS indicates National Institutes of Health Stroke scale; OTT, onset-to-treatment time.

compared to those given placebo (Figure 1; 3- to 5-hour OTT/fisetin vs 3- to 5-hour OTT/placebo).

National Institutes of Health Stroke Scale Score of All Eligible Patients 7 Days After Initial Treatment

Following the rt-PA treatment on the first day, patients who were initially administered simultaneous fisetin continuously received daily fisetin, while patients initially receiving placebo were continuously given daily placebo, both for 7 days. The NIHSS scores of all patients at the end of 7-day treatment are presented in Figure 3. The distribution of NIHSS scores in different OTT strata and treatment groups exhibited identical trend as on day 1 following the initial rt-PA treatment (Figure 3).

Serum Levels of MMP-2, MMP-9, and CRP

Next, we evaluated the serum levels of MMP-2, MMP-9, and CRP in patients from all groups at baseline, 1 day, and 7 days following the initial rt-PA treatment (Figure 4). We found that 1 day after the initial treatment, serum levels of both MMPs and

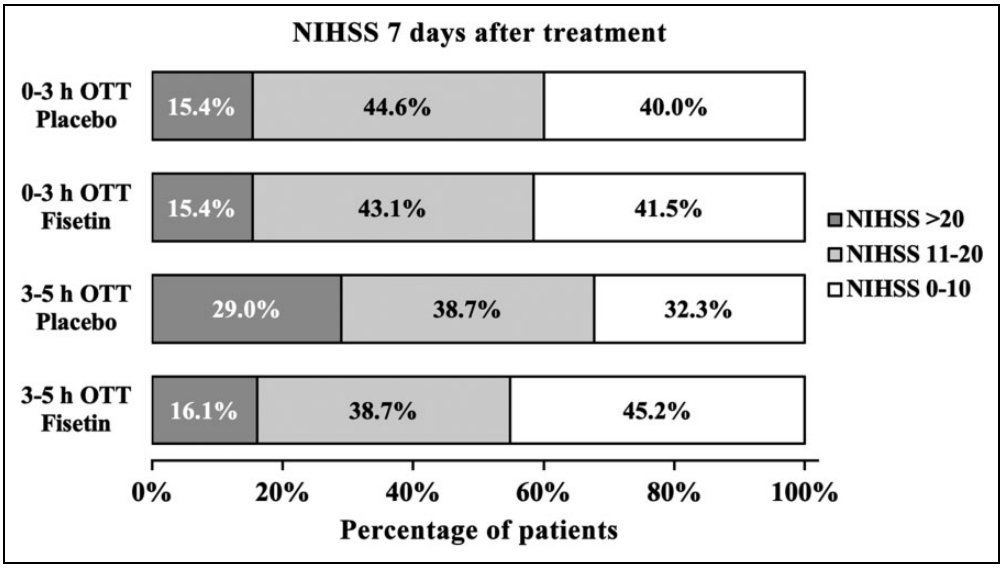

Figure 3. NIHSS score of all eligible patients 7 days after initial treatment. Scores of 0 to 10 was considered to indicate a favorable outcome. NIHSS indicates National Institutes of Health Stroke scale; OTT, onset-to-treatment time.

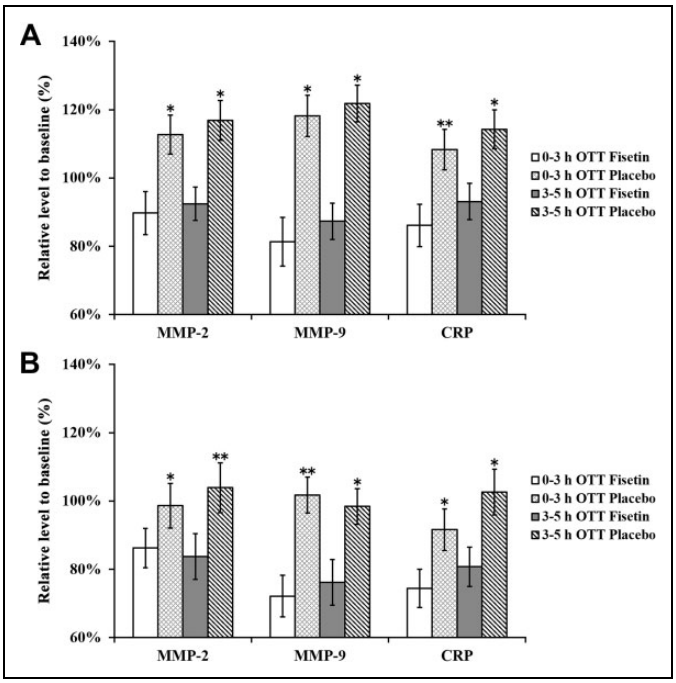

Figure 4. Serum levels of MMP-2, MMP-9, and CRP at (A) 1 day and (B) 7 days after initial treatment. Values were normalized as percentage to baseline values in respective treatment groups and expressed as mean (SD). *$P < .05$ fisetin versus placebo treatment at respective OTT groups. CRP indicates C-reactive protein; MMP, matrix metalloproteinase; NIHSS, National Institutes of Health Stroke scale; OTT, onset-to-treatment time; SD, standard deviation.

CRP in both 0- to 3-hour and 3- to 5-hour OTT strata were noticeably reduced in fisetin-treated patients than those treated with placebo (Figure 4A). After the 7-day continuous treatment, serum levels of all 3 markers were significantly lower than those at 1 day after treatment in all patients (Figure 4B).

Table 3. Strong Linear Correlations Between NIHSS Scores and Plasma Levels of MMP-2, MMP-9, and CRP in All Patients.

	NIHSS	*P* Value
MMP-2	$\gamma = 0.67$	.033
MMP-9	$\gamma = 0.71$	.028
CRP	$\gamma = 0.65$	.041

Abbreviations: CRP, C-reactive protein; MMP, matrix metalloproteinase; NIHSS, National Institutes of Health Stroke scale.

Nevertheless, in both OTT strata, fisetin treatment still markedly reduced the serum levels of MMP-2, MMP-9, and CRP.

Strong Linear Correlations Between NIHSS Scores and Plasma Levels of MMP-2, MMP-9, and CRP

Last but not least, we were curious about the correlations between the NIHSS score improvements and the reduction in MMP and CRP levels in the enrolled patients with stroke. To this end, we combined clinical and laboratory data for all 192 eligible patients throughout the entire study and performed correlation analysis (Table 3). Indeed, strong linear correlations between changes in the NIHSS scores and the serum levels of all 3 factors were observed, clearly indicating that the observed improvements in the NIHSS score were likely attributable to the reduced serum levels of MMP-2, MMP-9, and CRP.

Discussion

Despite the fact that rt-PA is effective in treating brain ischemic stroke, cautious considerations are needed during the clinical usage of rt-PA, for instance its relatively narrow OTT window of 3 hours.[2] There have been reports on serious side

effects of delayed rt-PA, including hyperperfusion leading to degradation of extracellular matrix and disruption of the BBB,[14-17] as well as hemorrhagic transformation leading to elevated incidence rate of symptomatic intracerebral hemorrhage.[18] Although in some parts of Europe, expanded treatment window of 4.5 hours is regarded safe and officially approved, percentage of patients exhibiting improvement when treated within 3 hours is twice as many as that when treated within the 3 to 4.5 hour extended window.[4] These aforementioned adverse effects have greatly limited the clinical efficacy and safety of rt-PA treatment. Therefore, alternative treatment such as stent retriever thrombectomy was occasionally employed to replace rt-PA treatment, on patients with prolonged OTT in particular. Stent retriever thrombectomy could ameliorate poststroke disability and improve the rate of functional independence, even when applied with OTT as long as 8 hours.[19] Nonetheless, as an invasive procedure, thrombectomy raises other complications and risks including anesthetic adverse effects or clot reforming that would necessitate subsequent procedures and is therefore poorly tolerated in older patients or individuals with chronic health problems such as bleeding or infection.

Our findings in the present clinical study were promising. In patients in the 3- to 5-hour OTT strata, overall NIHSS scores were significantly improved compared to the placebo-treated controls in just 1 day after the initial rt-PA and fisetin treatment. More importantly, this treatment outcome was nearly indistinguishable from that of patients with 0- to 3-hour OTT, indicating that supplement of fisetin in rt-PA reperfusion could significantly broaden the effective OTT to at least 5 hours. In addition to the initial rt-PA/fisetin treatment, patients also received daily fisetin treatment for 7 consecutive days. Treatment outcome of all patients exhibited gradual improvement, as they recover from the acute stroke attack. However, detailed analysis of their NIHSS scores revealed the prolonged therapeutic effects of fisetin. We observed that fisetin treatment for 7 days could further promote the recovery of patients of the 3- to 5-hour OTT strata, to levels similar to the patients of the 0- to 3-hour OTT strata. This finding indicates that besides the role as a supplement in the initial rt-PA treatment, fisetin could also benefit the functional recovery after stroke, in agreement with the reported neuroprotective activities of fisetin in several prior studies.[9,10]

Another interesting finding in our current study is the reduced serum levels of MMP-2 and MMP-9 in patients receiving fisetin treatments. These results are also in line with previous reports, where fisetin suppressed the expression of both MMPs in human cancer cells[20,21] and experimental mice.[22] The intriguing observation is the relation between levels of MMPs with treatment outcome of rt-PA, for we found strong linear correlation between the reduction in serum levels of MMPs and improved NIHSS scores indicating more ideal treatment outcomes. This correlation strongly supported that the positive effect on stroke recovery was likely mediated by MMPs, which was inhibited by fisetin in both the initial rt-PA and the 7-day follow-up administrations. Consistent with our findings, both MMP-2 and MMP-9 were implicated in ischemic attacks, via degradation of collagen IV and laminin in the basement membrane to cause consequent disruption of the BBB[23,24] and cerebral hemorrhage.[25] In clinical usage of rt-PA, patients with upregulated MMP-9 levels face higher risk of parenchymal hemorrhage.[26] Additionally, symptoms as a result of the ischemic attack could be attenuated by excessive t-PA, which increases MMP-9.[27] In the present study, we found that treatment with fisetin greatly downregulated serum levels of both MMP-2 and MMP-9, which showed strong correlation with, and likely contributed to, the improved treatment outcomes in all patients regardless of their OTT strata.

On the other hand, we also observed significantly reduced serum level of CRP following fisetin treatment. Increase in the serum CRP level is reportedly associated with the development of ischemic attack and hemorrhagic stroke, as well as disease outcome.[28,29] However, not all studies confirmed that elevated routine serum CRP affects the outcome in patients receiving intravenous rt-PA for acute stroke.[30] Importantly, CRP is one of the most widely used predictive biomarker of stroke outcome in clinical practice.[31-33] Studies reporting that fisetin could reduce CRP have been previously documented. For example, in diabetic mouse model, fisetin was able to lower methylglyoxal-dependent protein glycation and inhibit expression of serum CRP.[34] Moreover, in patients with colorectal cancer, fisetin supplementation could significantly reduce plasma levels of interleukin 8 and CRP.[35] Our current study serves yet another instance, first of its kind among patients with stroke, demonstrating the activity of fisetin in antagonizing serum CRP levels. One limitation of the study is that possible coexistence of infection or inhospital infectious complications was not analyzed, which could potentially affect serum CRP level and should be considered in similar future studies.

In conclusion, the present randomized double-blind and placebo-controlled trial has provided the first clinical evidence on the role of fisetin as a supplement in rt-PA reperfusion therapy against acute brain ischemic stroke. Fisetin, when administered simultaneously with rt-PA, extended the otherwise narrow effective window of rt-PA treatment and dramatically improved the treatment outcome of this widely used stroke therapy.

Authors' Note

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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