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ORIGINAL ARTICLE

Monoacylglycerol Lipase Inhibitor is Safe when Combined with Delayed r-tPA Administration in Treatment of Stroke

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Abstract— Administration of tissue plasminogen activator (tPA) during first 3–4.5 h after ischemic stroke is the main therapeutic strategy; however, its using after that, leads to reperfusion injury and neurotoxic effects. Additionally, inflammation has a critical role in secondary injury after late reperfusion therapy. Thus, this project was designed to explore the effects of JZL-184 (JZL), an agonist of type 1 cannabinoid receptor (CB1), on the side effects of recombinant tPA (r-tPA), which is administrated after 5 h of stroke onset in the mice middle cerebral artery occlusion (MCAO) model. After established the model of MCAO mouse, they were put to six groups, including intact, control, vehicle, JZL (4 mg/kg), r-tPA (9 mg/kg), and JZL plus r-tPA. Thereafter, brain levels of IL-10, TNF-α, and matrix metalloproteinase − 9 (MMP9), brain edema and infarction, and behavioral functions have been determined in the groups. JZL alone or in combination with r-tPA, but not r-tPA, reduced brain edema, infarct volume, brain levels of TNF-α, MMP9, and also improved behavioral tests. JZL and JZL plus r-tPA also increased brain levels of IL-10. According to the results, JZL can improve the effects of r-tPA to overcome stroke SSE, when used after 5 h of stroke onset. Based on the fact that there is limitation regarding using r-tPA after 3 h of stroke onset, using a combination of r-tPA/JZL can be considered for a future therapeutic strategy.

KEY WORDS: stroke; MCAO; JZL-184; r-tPA; secondary side effects.

INTRODUCTION

Brain stroke is a main human disorder, which is developing in both developed and developing countries [1]. Unexpected obstruction of brain blood vessels by clots or emboli is considered as a main cause for the induction of stroke [1]. Following brain stroke, the cells within the central region of brain stroke are damaged at once; however, cells within the penumbra region are alive without normal function [2, 3]. In the case of untreated stroke, the penumbra region cells will be died and may lead to the secondary side effects (SSE) of stroke [2, 3]. Accordingly, in medicine, tissue plasminogen activator (tPA) is administrated during the first 3 h after the onset of stroke to dissolve vessel clots and increase blood flow to the penumbra region cells. However, if tPA is used after 3 h, it causes deterioration of stroke *via* increasing the inflammation in the damaged region. It has been reported that inflammation is a critical factor, which is induced following induction of brain stroke [4]. Moreover, inflammation is a main factor, which is involved in the pathogenesis of SSE [3].

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Therefore, investigators are using various anti-inflammatory drugs in combination with tPA to reduce tPA SSE by down-regulation of inflammation to protect penumbra region cells [5–7]. Brain pro-inflammatory molecules, including cytokines and matrix metalloproteinase (MMP) are the main factors underlying inflammation, edema, and infarction. These phenomena are the critical risk factors promoting damage in penumbra region and deterioration of stroke SSE, including behaviors and neurological disorders [1, 8, 9]. Thus, the new therapeutic strategies focusing on down-regulation or upregulation of the pro-inflammatory agents, such as tumor necrosis factor-alpha (TNF-α) and MMP9, as well as the use of anti-inflammatory molecules, such as interleukin-10 (IL-10) [10], can be considered for treatment of brain stroke, especially in the case of the patients with a stroke with more than 3 h after onset of stroke.

Recently, JZL-184 (JZL) has been introduced as a potent irreversible inhibitor of monoacylglycerol lipase (MAGL). MAGL is the main enzyme involved in the catabolism of endocannabinoid 2-arachidonoylglycerol (2-AG) and production of arachidonic acid (AA). It appears that JZL is a main inhibitory factor for production of AA pro-inflammatory metabolites, including prostaglandins (PG), leukotriens (LK), and platelet activator factors (PAFs) [11, 12]. Additionally, JZL modulates brain inflammation *via* affecting the cannabinoid receptor type 1 (CB1) and upregulation of the receptor ligand, 2-AG [11, 13]. Furthermore, it has been documented that JZL reduces TNF-α, as the pro-inflammatory, and increases IL-10, as the anti-inflammatory cytokines [14].

Thus, it appears that JZL could be considered as a candidate to be used in combination with tPA to reduce its SSE in the case of administration after 3 h. Additionally, it has been reported that more than 85% of the human stroke is the ischemia type [2]; thus, the present study was designed to evaluate the effects of JZL on the tPA SSE in the case of administration after 5 h in mice model of middle cerebral artery occlusion (MCAO). Accordingly, brain edema and infarction, brain levels of IL-10, TNF-α and MMP9, and also behavioral functions have been evaluated in the MCAO model, which have been administrated with JZL and recombinant tPA (r-tPA) alone and in combination with each other.

MATERIAL AND METHOD

Animals

Seventy-eight male mice weighing 25–35 g were introduced to this study. The animals were kept in the standard conditions, including 12:12-h light/dark cycles at 25 °C and free access to food and water. The experimental procedures were approved by Local Ethical Committee with Code: IR.RUMS.REC.1395.135. Accordingly, the animals were handled using the standard methods to minimize the pain and stress.

Experimental Groups

The mice were randomly divided into intact, control, vehicle, (JZL) 4 mg/kg [13], r-tPA 9 mg/kg, and JZL plus r-tPA groups [15]. In each group, five mice were used to evaluate TNF-α, IL-10, and MMP-9 brain levels, and eight mice were used for assessment of brain infarct volume, edema, and behavioral experiments. Accordingly, 13 MCAO mice without treatment with any drug and treated with 300 mg/kg dimethyl sulfoxide (DMSO 10%) were introduced to control and vehicle groups, respectively [16]. JZL group were 13 animals, which treated with JZL immediately after induction of stroke, while r-tPA group contains the ischemic animals, which were treated with r-tPA (9 mg/kg) 5 h after induction of stroke. JZL plus r-tPA group contains 13 animals, which were treated with both JZL plus r-tPA 5 h after induction of stroke.

Induction of Mice Model of Middle Cerebral Artery Occlusion (MCAO)

MCAO model has been created using a standard method, which has been described previously [17, 18]. Briefly, after anesthetizing using ketamine (80 mg/kg) and xylazine (4 mg/kg), a small incision between the right ear and the eye was made, and the temporalis muscle was retracted. Therefore, the skull was exposed to create a 1 mm^2 in diameter hole, just above the MCA. Then, the dura was removed, and carefully, the proximal MCA was cauterized.

Infarct Volume and Brain Edema Measurement

Zhang and Iadecola protocol [17] were used to evaluate the infarct volume. Briefly, 48 h after induction of MCAO, the mice were sacrificed and the brain was removed, and 1-mm-thick coronal slices have been created by using a cutting block. The slices were stained in a standard condition using 2, 3, 5-triphenyltetrazolium chloride 1% (Sigma Chemical Co., St. Louis, MO, USA). During the protocol, the infarcted and non-infarcted brain tissue were stained white (remained unstained) and red (stained), respectively. Then, the infarct zone was demarcated to analyze using the ImageJ software (NIH Image,

version1.61, Bethesda, Maryland, USA), and all the infarct areas were summed and multiplied in the thickness of the brain sections (1 mm) [17].

The following standard formula was used to correct infarct volume: measured infarct area × (1 − [(ipsilateral hemisphere area − contralateral hemisphere area)/contralateral hemisphere]) [19].

The following formula has also been used to calculate brain edema as the percentage, which was introduced by O'Donnell and colleagues [20]: (volume of left hemisphere − volume of right hemisphere)/volume of right hemisphere.

MMP-9, IL-10, and TNF-α Brain Levels

Brain levels of IL-10 (eBiosciences, USA), MMP-9 (R and D system, USA), and TNF-α (eBiosciences, USA) were examined using commercial ELISA kits. In brief, the homogenized brain samples were added to the anti-IL-10, anti-MMP-9, and anti-TNF-α capture antibody pre-coated ELISA plates, respectively. The plates were washed after a 2-h incubation in dark room temperature. Then, HRP-conjugated detection antibodies were added to the wells and incubated in dark room temperature for an hour. Then, the HRP substrate, 3, 3′, 5, 5'-tetramethylbenzidine (TMB), in addition to H_2O_2, was added and incubated in dark place for 15 min. 2N sulfuric acid was used to stop the reactions and the optical densities (OD) were obtained at 450 nm by an ELISA reader (Bio-Rad, USA).

Behavioral Testing

The sensorimotor functions of evaluated animals were evaluated using a standard method, adhesive removal (glue tape) test [21]. Accordingly, the test was performed daily for 2 days before and at 24 and 48 h after MCAO insult, and the averaged latency to remove glue tape was recorded during three trials [22].

Neurological Disorder Assessment

Rating neurological disorders were recorded using Bederson grading system [9] at 24 and 48 h after MCAO induction. Rating neurological disorders were rated using the score scales. Accordingly, lack of disorder, bend the front limb, bend the front limb to add controlling pushing strength in side, turning to one side, and turning to one side plus loss of consciousness or death were considered as score 0 to 5.

Statistical Analysis

Brain levels of IL-10, MMP9 and TNF-α, brain infarct volume, paresis, and sensorimotor as well as neurological disorders were analyzed using ANOVA following with Tukey test. Differences among groups with $p < 0.05$ were considered significant and final data were presented as mean ± standard error. Data from Bederson test was also analyzed using Kruskal-Wallis test, as the non-parametric test, and reported as medians and percentiles 25 and 75. Differences with $p < 0.05$ were considered significant.

RESULTS

Brain Edema

Evaluation of the cerebral brain edema 48 h after MCAO showed that edema was significantly decreased in JZL ($p = 0.007$) and JZL plus r-tPA ($p = 0.045$) groups in comparison to vehicle group. Edema was also decreased in the JZL ($p = 0.003$) and JZL plus r-tPA ($p = 0.020$) groups when compared to r-tPA group. There were no significant differences between vehicle and control groups ($p < 0.1$), vehicle and r-tPA ($p = 0.998$), JZL and JZL plus r-tPA ($p = 0.870$), as well as JZL plus r-tPA groups ($p < 0.1$, Fig. 1).

Brain Infarction

Data analysis revealed that the infarct volumes significantly decreased in the JZL ($p < 0.001$) and JZL plus r-tPA ($p < 0.001$), but not r-tPA ($p = 0.162$) groups when compared to the vehicle group. There were significant differences between r-tPA and JZL plus r-tPA ($p = 0.010$) and also between r-tPA and JZL ($p = 0.007$). However, there were not significant references between control and vehicle ($p > 0.1$) and JZL and JZL plus r-tPA ($p = 0.993$) groups (Fig. 2).

TNF-a, IL-10, and MMP9 Levels

Data analysis regarding brain levels of IL-10, MMP9 and TNF-α 48 h after induction of MCAO demonstrated that, although r-tPA, JZL, and JZL plus r-tPA administration leads to upregulation of IL-10, it was significant only in JZL ($p = 0.044$) and JZL plus r-tPA ($p = 0.043$) groups. There were no significant differences between control and vehicle ($p > 0.1$) and also among r-tPA, JZL, and JZL plus r-tPA groups (Fig. 3). However, IL-10 serum levels were significantly upregulated in all groups when compared to intact group (Fig. 3).

The results also demonstrated that brain levels of MMP9 decreased significantly in JZL ($p = 0.010$) and

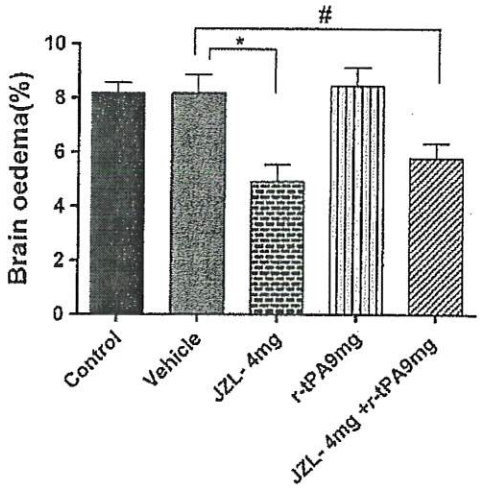

Fig. 1. The effects of r-tPA, JZL, and JZL-184 plus r-tPA on the brain edema in MCAO model. The figure shows that JZL (*) and JZL-184 plus r-tPA (#) significantly reduced brain edema when compared to vehicle group. The volume for intact group was zero; hence, it was not added to the figure.

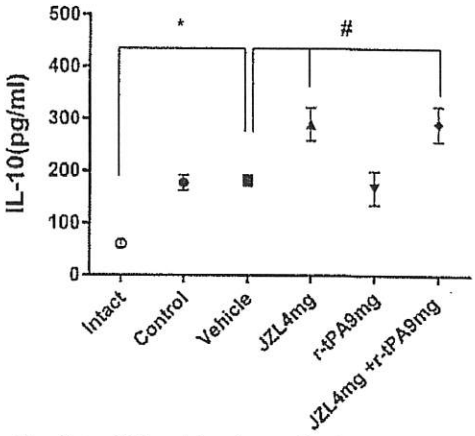

Fig. 3. The effects of JZL, r-tPA, and a combination of JZL plus r-tPA on the brain levels of IL-10 in MCAO mice. Administration of JZL (#) and JZL plus r-tPA (#) led to upregulation of IL-10 in the brain of MCAO model. IL-10 was significantly increased in all groups when compared to intact group (*).

JZL plus r-tPA ($p = 0.006$), but not in r-tPA ($p > 0.1$) groups, when compared to vehicle group. Accordingly, there were significant differences between r-tPA and JZL ($p = 0.0027$) and JZL plus r-tPA ($p = 0.019$) groups (Fig. 4). The statistical analysis revealed that JZL ($p = 0.783$) and JZL plus r-tPA ($p = 0.749$) reduced brain levels of MMP9 down to intact group (Fig. 4).

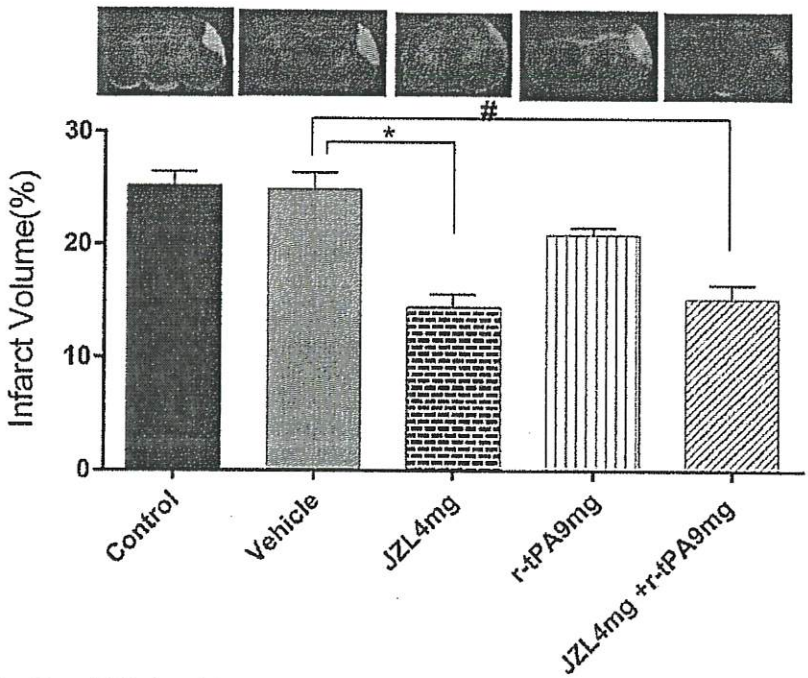

Fig. 2. The effects of JZL, r-tPA, and JZL plus r-tPA on the infarct volumes in MCAO model. Infarct volumes are presented as a percentage of affected ipsilateral hemispheres. Data are expressed as a mean ± SEM. JZL (*) and JZL plus r-tPA (#) significantly reduced the infarct volumes when compared to vehicle group. The volume for intact group was zero; hence, it was not added to the figure.

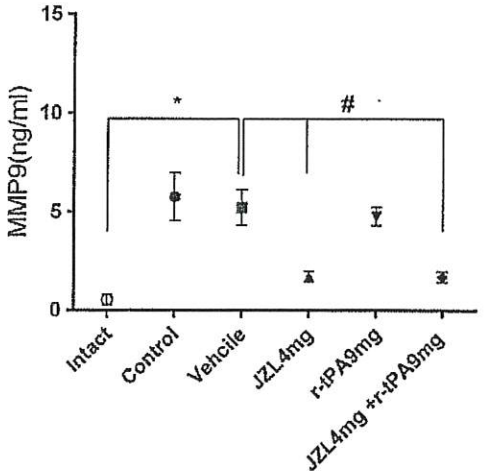

Fig. 4. The effects of JZL, r-tPA, and JZL plus r-tPA on the brain levels of MMP9 in the MCAO mice after 48 h. Data are expressed as mean ± SEM. Although JZL and JZL plus r-tPA significantly reduced brain levels of MMP9 in comparison to vehicle group (#), administration of r-tPA alone did not have these effects. MMP9 were not significantly different in JZL and JZL plus r-tPA groups in comparison to intact group (*).

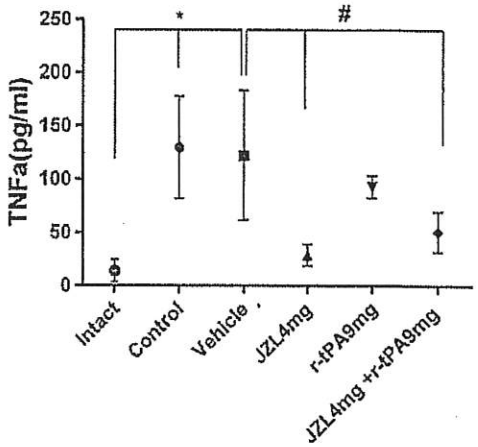

Fig. 5. The effects of JZL, r-tPA, and JZL-184 plus r-tPA on the brain levels of TNF-α in the MCAO mice. JZL and JZL plus r-tPA significantly decreased brain levels of TNF-α in the MCAO mice compared to vehicle group (#). TNF-α significantly increased in vehicle and control groups when compared to intact group (*).

However, brain levels of TNF-α significantly decreased in JZL ($p = 0.001$) and JZL plus r-tPA ($p = 0.014$) groups in comparison to vehicle group. The differences in r-tPA *versus* JZL ($p = 0.050$) were also significant. Brain levels of TNF-α were not significantly differences between vehicle *versus* r-tPA ($p = 0.722$), control *versus* vehicle ($p = 0.999$), and r-tPA *versus* JZL plus r-tPA ($p = 0.358$) groups. Figure 5 illustrates the brain levels of TNF-α in the evaluated groups. Interestingly, JZL ($p = 0.977$) and JZL plus r-tPA ($p > 0.1$) reduced brain levels of TNF-α to be as intact group.

Wire Suspension Test

Administration of JZL ($p = 0.027$ for 24 h and $p = 0.001$) and JZL plus r-tPA ($p = 0.028$ for 24 h and $p = 0.003$) improved wire suspension test times when compared to vehicle group (Fig. 6). However, r-tPA ($p = 0.973$ for 24 h and $p = 0.915$) was unable to improve wire suspension time in comparison to the vehicle group. Meanwhile, there were significant differences between intact group with all groups regarding wire suspension time (Fig. 6).

Glue Test

The results showed that there were no significant differences among groups regarding removing the label from the contralateral forepaw before administration of JZL and r-tPA ($p = 0.925$). Administration of JZL ($p > 0.001$) and JZL plus r-tPA ($p > 0.001$), but not and r-tPA ($p = 0.504$) alone, leads to improve the time of glue test in comparison to vehicle group after either 24 or 48 h. Figure 7 shows that there were significant differences between JZL/JZL plus r-tPA *versus* r-tPA in both 24 and 48 h after induction of MCAO.

Bederson Test

Bederson test shows that JZL and JZL plus r-tPA significantly decreased Bederson results and consequently improved the neurologic damages ($p < 0.001$ for both 24 and 48 h) when compared to r-tPA, vehicle and control groups. Neurological deficits were not altered in r-tPA ($p > 0.05$ for both 24 and 48 h) when compared to vehicle and control groups (Table 1).

DISCUSSION

The obtained results showed that JZL, alone, reduced pro-inflammatory molecules and also improved the behavioral tests and also increased IL-10 brain levels, as anti-inflammatory molecules. Collectively, it appears that JZL alone can be considered as a key therapeutic factor for inhibition of stroke side effects and reduce damages to penumbra region cells. Additionally, the results were in parallel with previous investigations, which reported that r-tPA were unable to suppress stroke side effects when it is

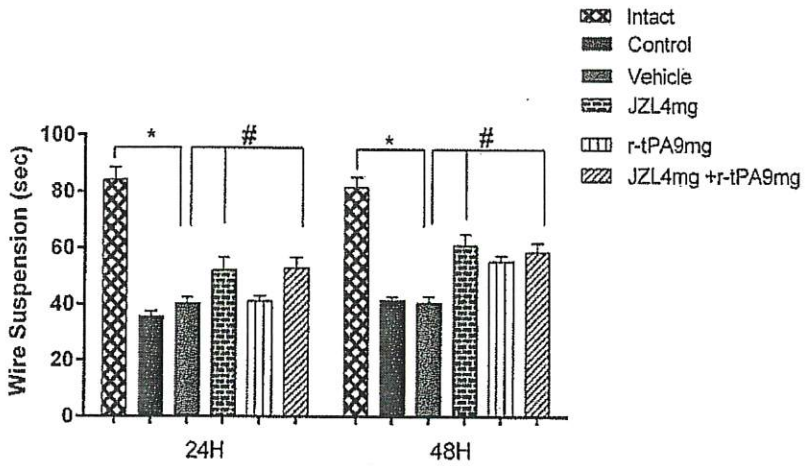

Fig. 6. The effects of JZL, r-tPA, and JZL plus r-tPA on the wire suspension (sec) test. JZL and JZL plus r-tPA (#) administration were associated with improved behavioral functions when compared to vehicle groups. Control and vehicle groups also had significant decreased wire suspension times in comparison to intact group (*).

administrated 3 to 4.5 h following induction of stroke [7]. Interestingly, JZL, when used with r-tPA, improved the anti-stroke activities of r-tPA and, hence, not only can be used alone to prevent stroke side effects, but also can be considered as a complementary drug to use in combination with r-tPA. Our previous investigation revealed that JZL can improve behavioral functions and also decrease brain inflammation as well as aspirin and MCAO animal model [14]. Therefore, the current results prove our previous investigation and demonstrated that JZL is a suitable component to reduce brain inflammation and damages to penumbra region cells. Moreover, another study by our research team demonstrated that AM251, an antagonist of CB1, can reduce regulatory effects of JZL on the inflammation and behavioral tests [23]. However, our previous study showed that there were no significant differences between AM251 administrated with JZL groups [23]. CB1 is the receptor responsible for activation of a pathway, which results in induction of brain anti-inflammatory conditions [24, 25]. Additionally, it has been reported that using JZL, in 4 mg/kg, led to 4–5-fold increased expression of 2-AG and consequently decreased AA in the brain tissues [26]. Therefore, it may be hypothesized that JZL reduces inflammation, alone and in combination with r-tPA, *via* both CB1 and AA dependent manner. Additionally, increased inflammation is another SSE of r-tPA, when it is administrated 5 h after the onset of stroke. The results demonstrated that r-tPA is unable to reduce brain levels of TNF-α and MMP9, as the main cause of inflammation in the brain; however, JZL plus r-tPA significantly reduced the pro-inflammatory markers. Again, the results indicated that JZL is able to reduce this side effect and can be considered as a main candidate to be used in combination with r-tPA.

Furthermore, our results showed that r-tPA did not alter the edema when compared to control and vehicle groups, while ZJL and JZL plus r-tPA reduced it signifi-

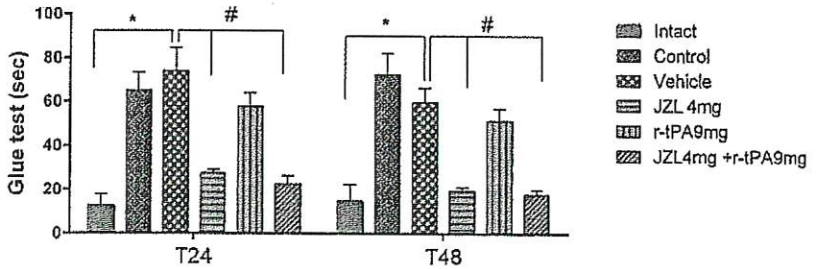

Fig. 7. The effects of JZL, r-tPA, and JZL plus r-tPA on the response remove in glue test. JZL and JZL plus r-tPA (#) administration were associated with improved response remove in adhesive test when compared to vehicle groups. There were significant differences between control/vehicle and intact groups regarding glue test time (*).

Table 1. The Results of Bederson Test in Different Groups at 24 and 48 h After Brain Ischemia

Group	Bederson 24 h	Bederson 48 h
Control	3 (3–3.25)	4 (3–4)
Vehicle	3 (3–3.25)*	3.5 (3–4)*
JZL 4 mg/kg	2 (1.75–2)**	1 (1–2)**
r-tPA 9 mg/kg	3(3–3)***	3(3–3)***
JZL 4 mg/kg plus r-tPA 9 mg/kg	2 (2–3)****	2 (2–2)****

Effect of JZl-184 and r-tPA on neurological deficits was measured by a 5-score scale at 24 and 48 h following stroke. Data are represented as median, 25th and 75th percentiles (percentiles in the parentheses)
*There was not a significant difference between control and vehicle groups
**JZL and JZL plus r-tPA groups have significantly decreased the results of Bederson test when compared to vehicle group
***There were no significant differences between r-tPA and vehicle group
****JZL plus r-tPA significantly improve the results obtained from Bederson test in comparison to vehicle group

cantly. There are some reports in parallel with our data and revealed that tPA did not change edema when it was applied out of its time window, and it might be related to its effects on the increased neuronal uptake of glucose [27], hypoxia tolerance [28], and activation of extracellular signal-regulated kinases1/2 (ERK 1/2)-cAMP response element binding protein (CREB)-activating transcription factor 3 (ATF3) signaling pathway [29]. However, our results revealed that using JZL and also JZL plus r-tPA reduced brain edema; hence, JZL can increase r-tPA neuroprotective effects.

Collectively, it may be concluded that JZL is a potential candidate, which can be used alone or in combination with r-tPA when more than 3 h past after the stroke onset. Accordingly, regarding the point that the experiments were performed on the animal model, it needs to be checked on human subjects to verify the effects of JZL on r-tPA SSE. The neuroprotective effects of JZL have also been investigated by Pihlaja et al., who reported that JZL, *via* suppression of inflammation, induces neuroprotection in an animal model of Alzheimer's disease [30]. Previous investigations have also demonstrated that JZL improves neuronal functions in animal models of Parkinson's disease and Down syndrome [31, 32]. The significant effect of JZL on neuronal functions *via* CB1 pathway and consequently decreased inflammation, have also been documented by some other investigators [33–35]. Based on the point that limitation time regarding using r-tPA in stroke is a main complication for treatment of the disorder, investigators are seeking for suitable components to reduce r-tPA SSE. Several investigations have been performed to test various components, such as aspirin [36, 37] and dipyridamole [38]. However, it appears that JZL can be considered as a suitable candidate for treatment of brain stroke.

Taken together, the presented results suggest that JZL may inhibit damage in penumbra region cells after administration of r-tPA after limitation times in the MCAO animal model *via* reducing the inflammation, brain infarction, and the subsequent improvement of neurological functions.

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