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Combined Neuroprotective Action of JWH-015 and AM251 in the CA1 Hippocampal Area of Rat Model of Transient Global Cerebral Ischemia

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Abstract

Objectives: Transient global cerebral ischemia (TGCI) is induced by occlusion of the bilateral common carotid artery occlusion (BCCAO), and it mediates neuronal cell death in the CA1 hippocampal area. AM251 is a cannabinoid receptor type 1 (CB1) blocker that has been known to be protective against transient focal cerebral ischemia. JWH-015 is a selective agonist of CB2 and activator of CB1 that is involved in the promotion of neuronal recovery and survival. The aim of this study was to investigate the role of combined application of JWH-015 and AM251 in the rat model of GCI.

Materials and Methods: Male Wistar rats underwent 20 minutes of ischemia followed by reperfusion. Then, 1 mg/kg JWH-015 and 2 mg/kg AM251 were administered through the caudal vein. The groups were control, sham, ischemia, vehicle, AM251, JWH-015 and AM251 + JWH-015. Animals were sacrificed 1 week after BCCAO.

Results: The AM251 + JWH-015 group indicated a significant increase in the protein expressions of AKT1, Bcl-XL, Bcl-2 and Bad 14-3-3, but it showed a considerable decrease in the protein expressions of Bad and JNK1/2 ($P \leq 0.05$ vs. AM251, and JWH-015 groups). The AM251 + JWH-015 group had a significantly higher number of alive cells and lower number of apoptotic cells in the CA1 hippocampal area and it also had a considerable improvement in spatial memory ($P \leq 0.05$ vs. AM251, and JWH-015 groups).

Conclusions: The results of this study indicated that combined application of AM251 and JWH-015 could be neuroprotective against detrimental effects of ischemia probably via suppression of neuronal apoptosis and maintenance of their survival.

Keywords: CA1, Survival, Apoptosis, Global cerebral ischemia

Introduction

Ischemic stroke is known as a brain damage results from reduced levels of glucose and oxygen supply caused by blockage of cerebral arteries (1, 2). The transient global cerebral ischemia (TGCI) is induced by bilateral common carotid artery occlusion (BCCAO) (3), which reduces brain blood flow (4). TGCI mediates neuronal cell death resulting in severe neurological and neurobehavioral dysfunction (5). Among various areas in the brain, neurons of the CA1 hippocampal area are highly vulnerable to ischemia (6) so the apoptosis of these neurons is an important phenomenon of brain ischemia/reperfusion injury (7).

A main action of endocannabinoids is to protect tissue from pathological lesion (8). Cannabinoids effects are attributed to the activation of cannabinoid receptors type 1 (CB1) and type 2 (CB2) (9). There is a rise in the levels of CB1 and CB2 in the brain tissue after BCCAO and reperfusion (BCCAO/R) (4). Both receptors are expressed in the brain (10). CB2 has been linked to neuroprotection (11) through reduction of ischemic-induced microcirculatory dysfunction (4). CB2 is involved in modulation of hippocampal CA1 synaptic plasticity (10). Blockage of the CB1 receptor by AM251 has been known to be protective against transient focal cerebral ischemia; however, CB1 receptor agonist could also be capable of reducing neuronal loss of hippocampus following TGCI and decreasing the infarct volume following focal cerebral ischemia induced by middle cerebral artery occlusion (12). JWH-015 is an indole that serves as a selective agonist of CB2 and activator of CB1 (13). Viscomi et al reported that application of JWH-015 against remote axotomy-induced apoptosis could decrease neuronal apoptosis through Phosphatidylinositol-3 kinases, protein kinase B or AKT(PI3-K)/AKT pathway and consequently, promote neuronal recovery and survival (14). The goal of

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the present study was to assess a possible role of combined exogenous application of JWH-015 with AM251 in the CA1 hippocampal area of TGCI in rats.

Materials and Methods

Animals and Surgical Procedure

Male Wistar rats weighing (200-250 g) were obtained from Medicine Faculty of Tehran University of Medical Sciences (TUMS). The standard circumstance of illumination (12 hours light-dark cycle), humidity (55%-65%) and constant temperature (22°C) were applied to all animals. Food and water ad libitum are easily provided for rats, and they were also let to be acclimatized to the new environment for 1 week without doing any procedures. Experiments were carried out on rats by adhering to the guidelines of Ethical Committee of Tehran University of Medical Sciences and the national and institutional guidelines for animal care.

Animals were kept fast overnight but were allowed free access to water before the surgery. Using 60 mg/kg ketamine (Sigma, USA) and 10 mg/kg xylazine (Serva Feinbiochemica, New York), the rats were anesthetized. Anterior midline incision of the cervical area was performed to isolate CCAs sheets. Left and right common carotid arteries were isolated from carotid sheets and then the vagus nerves were freed from carotid arteries carefully. Both common carotid arteries were occluded using Yashargil Aneurism microclips for 20 minutes (15) and then the clasps were removed. CCAs were visually inspected for blood flow recovery and no formation of blood clots within the vessels. After reperfusion, all animals were allowed to survive for 1 week. A rectal thermistor was used to monitor body temperature which maintained at 37 ± 5°C during ischemia. One rat died during ischemia, and 2 rats died post-ischemia.

Experimental Procedure

Seventy-seven rats were divided into 7 groups as follows: control, sham, ischemia, vehicle (DMSO), JWH-015, AM251 and JWH-015 + AM251. After one week for adaptation, the rats in control group received no surgery or treatment, and sham group received surgery with no ischemia. Ischemia group received 20-minute BCCAO, Vehicle groups underwent ischemia induction and received intracaudal administration of DMSO 1 hour after BCCAO. JWH-015 group received intracaudal injection of 1 mg/kg JWH-015 (Sigma, USA) and AM251 group received intracaudal administration of 2 mg/kg AM251 (Sigma, USA) 1 hour after BCCAO. These drugs were prepared with dimethyl sulfoxide (DMSO, Sigma, USA) and stored at 4°C. JWH-015 + AM251 group received intracaudal administration of 1 mg/kg JWH-015 + 2 mg/kg AM251 1 hour after BCCAO. In this study, samples were collected under 2 different conditions. First, 3 samples were collected for Western blot analysis 1 week after BCCAO. Next, spatial working was assessed by Morris water maze (MWM) (n = 8), 1 week after BCCAO and continued until 5 days. Then, animals were sacrificed after MWM test and samples were collected for Cresyl violet (Nissl) staining and TUNEL assay so that MWM training and testing may not affect the results of WB.

Western Blot Analysis

Extraction of total protein from hippocampus was performed using RIPA buffer containing phosphatase inhibitor and protease cocktails (Sigma, USA). Protein concentration was evaluated using Bradford protocol (Bio-Rad, MI, USA), and proteins were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Transferring of proteins onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) incubated for 1 hour with 5% bovine serum albumin (BSA) (Sigma, USA) in 100 mL TBST was then performed. PVDF membranes were then probed with primary antibodies against Bad 14-3-3 (1:500 dilution, Santa Cruz Biotechnology, CA, USA), AKT1 (1:2000 dilution; Abcam, Cambridge, MA, USA), Bad (1:1000 dilution, Cell Signaling, USA), Bcl-XL (1:3000 dilution; Abcam, Cambridge, MA, USA), Bcl-2 (1:1000 dilution; Abcam, Cambridge, MA, USA), c-Jun N-terminal kinase (JNK) 1/2 (1:1000 dilution, Santa Cruz Biotechnology, CA, USA) and β-actin (1:500, Abcam, Cambridge, MA, USA). Membranes were washed 3 times in TBST buffer and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 1 hour at room temperature. Immunoreactive visualization of proteins was investigated by enhanced chemiluminescence system (ECL-plus, Lumigen, Inc. Southfield, MI, USA) and the band densities were normalized to β-actin and quantified using ImageJ analyzer (NIH, Bethesda, MD, USA). For each experiment, 3 trials were performed.

Cresyl Violet (Nissl) Staining

Animals were perfused using paraformaldehyde 4% (Sigma, USA) in 0.1 M phosphate buffer (pH 7.2), and paraffin-embedded hippocampal tissue samples were sectioned (5 μm thicknesses with an interval of 120 μm). Then, slides were deparaffinized and stained with 0.5% cresyl violet. Olympus microscope (CX31, Japan) which was equipped with a digital camera (Leica, Germany) was used for observation of sections at 400 X magnification. A total number of 100 cells were counted per 3 high power fields. Purple-stained cells with intact morphology and light cytoplasm were chosen as viable cells, and blue-stained cells with triangular shapes were chosen as dead cells.

TUNEL Assay

Evaluation of DNA fragmentation was performed using an in situ cell death detection kit (Roche) based on the manufacturer's protocol, Deparaffinized sections were treated with 10 μg/mL of proteinase K for 30 minutes and incubated with 20 μL of terminal deoxynucleotidyl

transferase enzyme. Samples were then incubated with 450 µL of labeling solution for 1 hour, horseradish peroxidase conjugated anti-fluorescein antibody Fab fragments for 30 minutes, and 50-100 µL of DAB substrate for 10 minutes. After counterstaining with methyl green, the sections were assessed with a fluorescence microscope (IX2 ILL100; Olympus, Japan) at 560 nm.) A total number of 100 brown-stained apoptotic cells of CA1 hippocampal area were counted blindly in 6 randomly chosen areas per 3 high power fields using ImageJ analyzer (NIH, Bethesda, MD, USA). Positive control samples were incubated with 2000 U of DNase I recombinant in Tris-HCl (pH 7.5) at 25°C for 10 minutes prior to labeling. Negative control samples were coated only with the labeling solution.

Morris Water Maze

Learning and spatial memory were evaluated by MWM. A large circular black pool (diameter: 180 cc and height: 60 cm), filled with opaque water (25 ± 1°C) to a depth of 35 cm was used in this study. The pool was surrounded by non-motile external cues as a guide for rats in finding their routes. The tank was partitioned into 4 equal quadrants. A hidden platform was located 1 cm below the water level in the center of the fourth quadrant, Throughout the MWM test, the platform was fixed and its location did not change. The test lasted for 5 consecutive days between 10:00 AM and 12:00 PM. Animals were allowed to have 4 training trials (training test). Rats were tracked by a video camera (Nikon, Melville, NY, USA) which was installed above the pool. Visual test was evaluated on day 1 by placing a platform coated by a transparent covering on the water surface (visible test). During trial days 2, 3 and 4, the platform had no transparent covering at 0.5 cm below the water surface. During the training test, the rats were kindly placed in the water from different sides of the pool (North, South, East and west) randomly, and were allowed to find out the platform in 60 seconds. There was a 20-second interval between the 2 consecutive trials. During each trial, animals were manually placed on the platform where they could not find the platform by themselves within 60 seconds.

On the last day (day 5) or probe day, the platform was removed and long-term or reference memory was checked by allowing animals to swim for 60 seconds in the pool that was not equipped with the platform. All rats in this day were placed in the pool from one side (west). At the end of day 5, the period of time spent in the target quadrant (fourth quadrant where the platform was placed) and the speed of animals in recognizing the exact location of the platform were recorded.

Statistical Analysis

Homogeneity of variances was evaluated using Levene test and all variables had a normal distribution based on the results of Shapiro-Wilk test. Data were analyzed with one-way analysis of variance (ANOVA) using SPSS version 22.0 (SPSS Inc., Chicago, IL). Tukey test was used as a post-hoc for all equal variances. Data were presented as mean ± SD and the differences between the groups were considered statistically significant at a $P \leq 0.05$.

Results

Western Blot Analysis

Protein expressions of AKT1, Bad 14-3-3, Bad, JNK1/2, Bcl-2 and Bcl-XL were assessed by western blot. The AM251 + JWH-015 group showed a 2-fold increase in the protein expressions of AKT1 and Bad 14-3-3 with 1.84 ± 0.07 and 1.45 ± 0.11, respectively (for both $P < 0.001$ vs. ischemia). The AM251 + JWH-015 group also showed over 2-fold rise in the expression of Bcl-2 with 1.51±0.1 and it had over 5.5-fold rise in the mRNA expression of Bcl-XL with 1.84±0.19 ($P < 0.001$ vs. ischemia). This group showed over 6- and 2-fold fall in the mRNA expressions of Bad and JNK1/2 with 0.45 ± 0.06 and 0.58 ± 0.07, respectively ($P < 0.001$ vs. ischemia). The levels of protein expressions were significant in the AM251 + JWH-015 group compared with the AM251 and JWH-015 groups (for both $P < 0.001$) (Figure 1).

Cresyl Violet (Nissl) Staining

Nissl staining was performed to evaluate survival rate of CA1 hippocampal neurons. The number of alive cells showed a significant fall in the ischemic group with 7.38 ± 2.63 ($P < 0.001$ vs. control). The AM251 + JWH-015 group had a high rate of neuronal viability by about 12–fold with 91.7 ± 4.62 ($P < 0.001$ vs. ischemia). The rate of survival in the AM251 + JWH-015 group was significant compared with either AM251 group ($P <0.001$) or JWH-015 group ($P<0.02$) (Figure 2).

TUNEL Assay

The number of apoptotic cells rose significantly in the ischemic rats with 70.56 ± 0.62 ($P<0.001$ vs. control). Conversely, the AM251 + JWH-015 group had a notable fall in the number of TUNEL-positive cells with 12 ± 1.06 ($P<0.001$ vs. ischemia). The result of the AM251 + JWH-015 group was significant compared with the AM251 group ($P<0.001$) and JWH-015 group ($P<0.05$) (Figure 3).

Morris Water Maze

Spatial memory was investigated by evaluation of MWM in the probe stage. Rats in the AM251 + JWH-015 group had lower speed with 13.12 ± 3.20 ($P < 0.05$ vs. ischemia) and spent longer time with 27.11 ± 5.9 in the target quadrant ($P < 0.001$ vs. ischemia). The value for speed was significant in the AM251 + JWH-015 group compared with the AM251 group and JWH-015 group (for both $P<0.05$). Similarly, the value for time spent in the target quadrant was notable in the AM251 + JWH-015 group compared with the AM251 and JWH-015 groups (for both $P<0.001$) (Table 1).

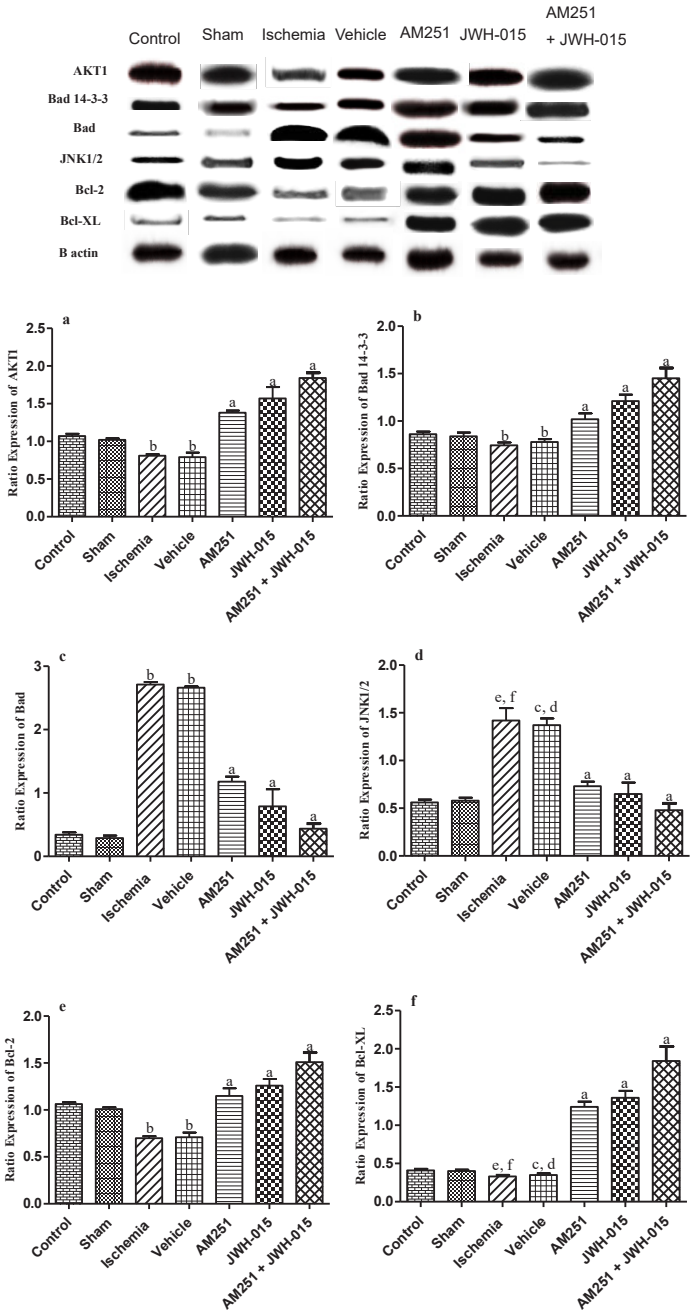

Figure 1. Western blot analysis of the role of combined application of AM251 and JWH-015 in protein expression of survival and apoptosis factors in the CA1 hippocampal area of rats underwent transient global cerebral ischemia (GCI) induction by bilateral common carotid artery occlusion and reperfusion (BCCAO/R). a, AKT1; b, Bad 14-3-3; c, Bad; d, JNK1/2; e, Bcl-2; and f, Bcl-XL. a, $P < 0.001$ vs all; b, $P < 0.001$ vs control and sham; c, $P < 0.02$ vs sham; (d, $P < 0.008$ vs control; e, $P < 0.01$ vs sham; and f, $P < 0.004$ vs control.

Discussion

The combined additive neuroprotective action of AM251 and JWH-015 in the TGCI model induced by 20-min BCCAO has been shown in this study. Hippocampus is supplied by the posterior circulation in human, but because of the variation in hippocampus supplier arteries in mammalian, the bilateral carotid arteries ligation was used in the animal model in this paper (16). Expression rates of factors related to the apoptosis or survival in the CA1 hippocampal area was assessed by western blot, and our results revealed high levels of protein expressions of AKT1, Bcl-2, Bad 14-3-3 and Bcl-XL but low levels of protein expressions of JNK and Bad in the AM251 + JWH-015 group compared with each of the AM251 and JWH-015 groups. Bad is a pro-apoptotic protein with a key role in the determination of cell death/survival. Bad inhibits Bcl-XL and Bcl-2 through which it promotes apoptotic cell death. Phosphatidylinositol 3-kinase (PI3-K)/AKT and JNK pathways are thought to be responsible for Bad regulation. An essential mediator of neuronal survival is AKT (17) while JNK is a mediator of cell death. PI3-K/AKT pathway is responsible for Bad inactivation while JNK

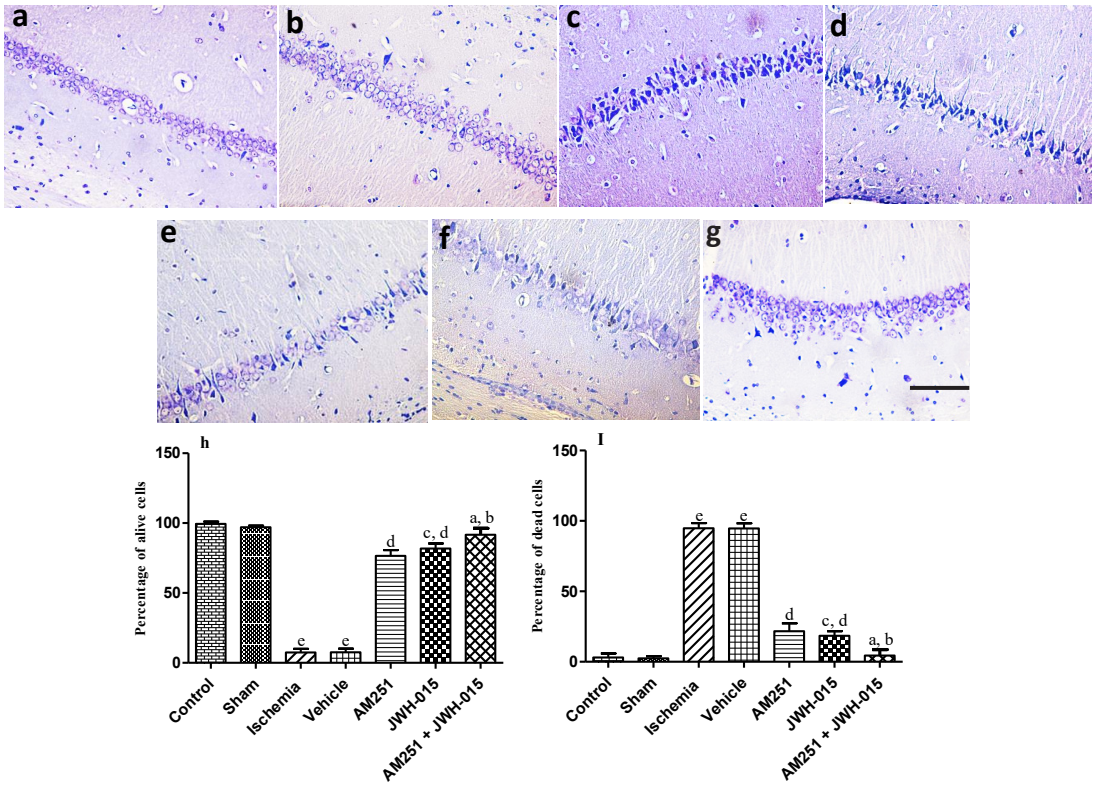

Figure 2. Cresyl violet (Nissl) staining to investigate a possible effect of combined application of AM251 and JWH-015 on survival rate of the CA1 hippocampal area in the model of transient global cerebral ischemia (GCI) induced by bilateral common carotid artery occlusion and reperfusion (BCCAO/R). a, control; b, sham; c, ischemia; d, vehicle; e, AM251; f, JWH-015; and g, AM251 + JWH-015. Viable neurons of CA1 hippocampal area are purple-stained cells with light cytoplasm and intact morphology, while dead neurons of CA1 hippocampal area are blue-stained cells with triangular shape (light microscope. Scale bar = 100 μm). h and I, Comparison of the percentage of alive and dead CA1 hippocampal neurons. a, $P < 0.02$ vs. JWH-015; b, $P < 0.001$ vs. AM251, Ischemia and Vehicle; c, $P < 0/01$ vs. JWH-015; d, $P < 0.001$ vs. Ischemia, Vehicle, Control and Sham; and e, $P < 0.001$ vs. Control and Sham.

pathway is responsible for Bad activation. Phosphorylation of Bad is mediated by AKT; this phosphorylated form has an affinity to bind with 14-3-3 to form Bad 14-3-3, which in turn decreases further attachment of Bad to Bcl-XL (18). JNK activation may control the transcription of Bcl-2 family members indirectly, including Bcl-2 and Bcl-XL downregulation and Bax upregulation (8).

It has also been reported that PI3-K/AKT pathway could be active by CB2 receptor stimulation (19). Murataeva et al reported that JWH-015 is not only a CB2 selective agonist but also a potent activator of CB1 receptors in neurons (13). Viscomi et al used JWH-015 in targeting remote axotomy-induced apoptosis, and they found that it is capable of decreasing neuronal apoptosis and promoting neuronal recovery and survival through PI3-K/AKT pathway (14).

CB1 receptors are greatly expressed in neurons of various areas of CNS including the hippocampus (20). CB1 receptor antagonist AM251 has been known to be protective against transient focal cerebral ischemia (12). Recently, Dunbar et al reported attenuation of CA1 injury by AM251 (21). Interestingly, CB1 receptor agonist could also be capable of decreasing hippocampal neuronal loss following transient GCI and reducing the infarct volume after focal cerebral ischemia (12).

Since neuronal DNA fragmentation is mostly applied as an indicator of neuronal loss, the TUNEL assay was used to recognize apoptotic cells through labeling DNA strand breaks in CA1 neuron of the hippocampus (8). In addition, Nissl staining was performed to evaluate cell survival. The results indicated that ischemic rats had a significant higher number of TUNEL-positive cells and lower number of alive cells compared to the control. The same results were obtained with 20-min induction of ischemia by BCCAO in the works performed by Sharifi et al (15), Movassaghi et al (22) in rats and Mori et al (23) in mice. The results of TUNEL assay and Nissl staining in the ischemic rats were significantly counteracted by combined application of AM251 and JWH-015, as compared with each of the ischemia, AM251, and JWH-015 groups. The above findings of the present study indicate that combined administration of AM251 and JWH-015 has a potential to suppress apoptosis and maintain further survival of CA1 hippocampal neurons, which may involve in the stimulation of the AKT pathway and inhibition of the JNK pathway.

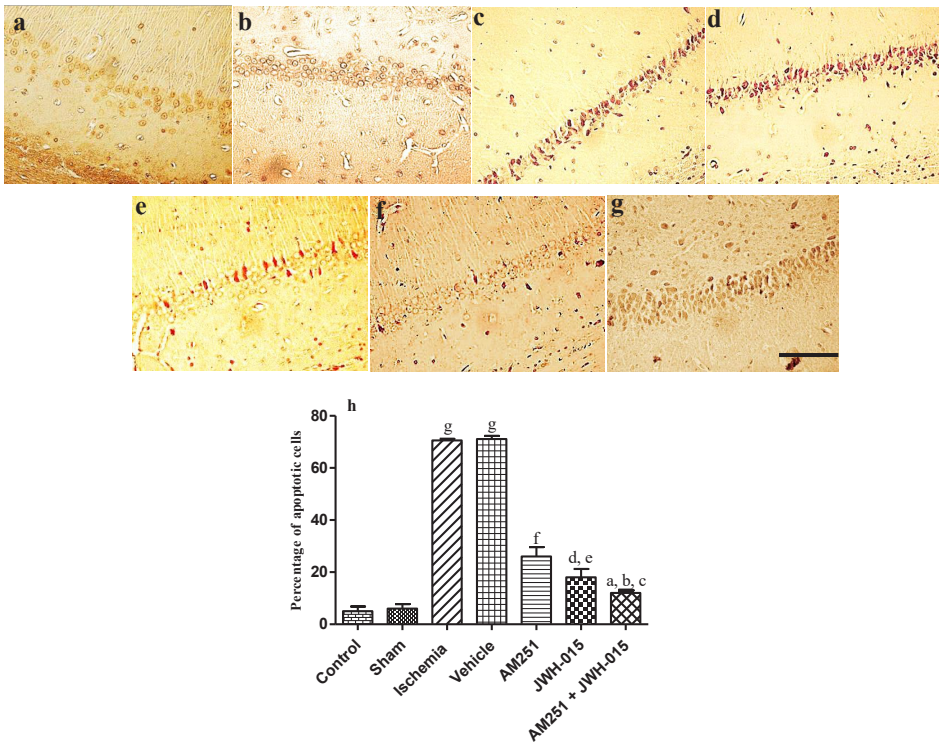

Figure 3. TUNEL assay to evaluate a possible role of combined administration of AM251 and JWH0-15 in apoptosis of neurons of CA1 hippocampal area in the rat model of transient global cerebral ischemia (GCI) induced by bilateral common carotid artery occlusion and reperfusion (BCCAO/R). a, control; b, sham; c, ischemia; d, vehicle; e, AM251; f, JWH-015; and g, AM251 + JWH-015. Dark brown-stained cells are TUNEL-positive neurons of the CA1 hippocampal area (fluorescence microscope. Scale bar = 100 μm). h, Percentage of TUNEL-positive cells. a, $P < 0.05$ vs. JWH-015; b, $P < 0.001$ vs. AM251, Ischemia and Vehicle; c, $P < 0.02$ vs. Control and Sham; d, $P < 0.01$ vs. AM251; e, $P < 0.001$ vs. Vehicle, Ischemia, Control and Sham; f, $P < 0.001$ vs. Ischemia and Vehicle; g , $P < 0.001$ vs. Control and Sham.

To see if the combined application of AM251 + JWH-015 could affect spatial memory, we evaluated MWM in the studying groups, and we found impairment of spatial memory in the ischemic group. CA1 hippocampal pyramidal neurons are particularly sensitive to ischemic injury, and their apoptosis is an outcome of transient GCI (15). Therefore, memory deficit is one of the outcomes of transient GCI (5), as in the study carried out by Movassaghi et al who found that exposure of rats to BCCAO could impair spatial memory (22). To assess learning and spatial memory MWM is used. There are several advantages of this test over other models including lack of motivational stimuli, for instance, loss of water and food, buzzer sounds and electrical stimulations. Memory deficit after BCCAO could be contributed to neurodegeneration in the hippocampus (23). Our results showed that BCCAO impairs reference memory. We noticed that rats in the AM251 + JWH-015 group had lower speed and spent a longer time in target quadrant which suggests low emotional state levels in these animals. To the best of our knowledge, these are the first findings denoting a protective role of combined application of AM251 and JWH-015 against spatial memory injury following GCI. CB1 and CB2 receptors modulate a vast variety of physiological functions including memory (10). CB1 receptor expresses at high levels in the hippocampus (24). Kim et al found that CB2 receptor is capable of modulating hippocampal CA1 synaptic plasticity (10). Data from MWM shed new on the beneficial role of combined administration of AM251 and JWH-015 in protecting neurons of CA1 hippocampal area.

In the light of this observation, combined administration of AM251 and JWH-015 may be one of the potential therapeutic aims for TGCI treatment.

Table 1. Spatial Memory Assessment by MWM to Evaluate the Role of AM251 Combined With JWH-015 in Rat Model of Cerebral Ischemia Induced by Transient BCCAO/R

Groups	Mean ± SD	
	Speed Q4 (cm/s)	Time Q4 (s)
Control	19.78 ± 4.75	26.43 ± 5.04
Sham	19.12 ± 4.91	28.12 ± 5.12
Ischemia	36.91 ± 6.3 [e]	5.54 ± 4.22 [e]
Vehicle	35.17 ± 6.71 [e]	5.23 ± 3.13 [e]
AM251	29.76 ± 4.68 [d]	13.11 ± 2.41 [d]
JWH-015	25.14 ± 3.50 [c, d]	18 ± 2.21 [d]
AM251+ JWH-015	13.12 ± 3.20 [a]	27.11 ± 5.9 [b]

Abbreviations: BCCAO/R, bilateral common carotid artery occlusion and reperfusion; MWM, Morris water maze
a, $P < 0.05$ vs. all groups; b, $P < 0.0001$ vs. JWH-015, AM251,Vehicle, and Ischemia; c, $P < 0.02$ vs. AM251; d, $P < 0,0001$ vs. Ischemia, Vehicle, Control and Sham; e, $P < 0.0001$ vs. Control and Sham

Conclusions

The outcomes got in this study demonstrated the neuroprotective function of combined AM251 and JWH-015 on neurons of CA1 hippocampal area against destructive effects of GCI probably via suppression of apoptosis and maintenance of cell survival. This study also suggests that combined administration of AM251 and JWH-015 has a protective role in AKT activation, which may involve JNK pathway inhibition. We suggest that these results be confirmed using AKT and JNK inhibitors in the next study.

Conflict of Interests

None to be declared.

Ethical Issues

Working with laboratory animals in all stages of research was in accordance with the standards and regulations of Ethical Committee of Tehran University of Medical Sciences.

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