# The Effect of Rosa Damascena Extract on Expression of Neurotrophic Factors in the CA1 Neurons of Adult Rat Hippocampus Following Ischemia

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**Abstract-** Ischemic stroke is an important cause of death and disability in the world. Brain ischemia causes damage to brain cell, and among brain neurons, pyramidal neurons of the hippocampal CA1 region are more susceptive to ischemic injury. Recent findings suggest that neurotrophic factors protect against ischemic cell death. A dietary component of Rosa damascene extract possibly is associated with expression of neurotrophic factors mRNA following ischemia, so it can have therapeutic effect on cerebral ischemia. The present study attempts to evaluate the neuroprotective effect of Rosa damascene extract on adult rat hippocampal neurons following ischemic brain injury. Forty-eight adult male Wistar rats (weighing 250±20 gr and ages 10-12 weeks) used in this study, animals randomly were divided into 6 groups including Control, ischemia/ reperfusion (IR), vehicle and three treated groups (IR+0.5, 1, 2 mg/ml extract). Global ischemia was induced by bilateral common carotid arteries occlusion for 20 minutes. The treatment was done by different doses of *Rosa damascena* extract for 30 days. After 30 days cell death and gene expression in neurons of the CA1 region of the hippocampus were evaluated by Nissl staining and real time PCR assay. We found a significant decrease in NGF, BDNF and NT3 mRNA expression in neurons of CA1 region of the hippocampus in ischemia group compared to control group ($P$<0.0001). Our results also revealed that the number of dark neurons significantly increases in ischemia group compared to control group ($P$<0.0001). Following treatment with Rosa damascene extract reduced the number of dark neurons that was associated with NGF, NT3, and BDNF mRNA expression. All doses level had positive effects, but the most effective dose of *Rosa damascena* extract was 1 mg/ml. Our results suggest that neuroprotective activity of *Rosa damascena* can enhance hippocampal CA1 neuronal survival after global ischemia.



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**Keywords:** Brain ischemia; Cell death; Rosa damascene extract; Neurotrophic factor

## Introduction

Stroke, a brain attack, is the leading cause of serious, long-range disability with about 6,000,000 people suffering each year (1). Stroke is defined as an acute neurological dysfunction due to interruption or extreme reduction of blood supply to specific brain region (2). There are two main kinds of stroke, 87 percent of strokes are ischemic, and it occurs when a blood vessel supplying blood to part of the brain obstructs by the formation of a clot and 13 percent are hemorrhagic, and it occurs when a weakened blood vessel in the brain ruptures (3).

Depriving brain tissue of oxygen and nutrients leads to the certain type of cellular damage (4). All the neurons of the ischemic area damage and cell death occur among the different type of brain cells, but the pyramidal neurons of the hippocampal CA1 region are particularly susceptive to ischemic injury (5).

CA1 pyramidal neurons are found in the CA1 area of the hippocampus, a major part of the human's brain which is located in the medial region of the temporal lobe, and include the cornu ammonis (CA1-4). Hippocampus plays a critical role in the formation, organization, and retrieval of new memories. The principal cell type in this area is

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pyramidal neuron, which integrates spatial, contextual, and emotional information (5).

Under pathologic conditions such as ischemia NTFs such as BDNF, NGF, and GDNF express in brain cells. The production reaches a maximum level at 3 hours with a gradual decrease by day 1 (6).

These neurotrophic factors are a family of biomolecules that include NGF, BDNF, NT3, NT4 (7).

The protein level of BDNF, the regional enhancement of BDNF mRNA expression in hippocampus approved to be predominantly enhanced by anthocyanin (8). Anthocyanidin is a class of flavonoid. Bioflavonoids are a group of natural antioxidant that can be found in a great deal of fruits and vegetables (9).

## Materials and Methods

### Plant extract

Flowers of *Rosa damascena* were collected in May 2015 from Urmia located in North West of Iran. Voucher specimen was authenticated and then deposited in the laboratory of the department of Pharmacognosy in the school of pharmacy, Tehran University of Medical Sciences. The air dried petal part of flowers was powdered by crushing. The resulting powder was soaked with 1500 ml alcohol 70% and was remanded for 7 days. one, two and four days after starting, they were given a shake and added in alcohol 70%. The extract was given a shake, filtered and evaporated in a rotating evaporator on the reduced pressure until dryness. The resulting extract kept at -4$^{o}$ c until being used.

### Animals

Forty-eight adult male Wistar rats (weighing 250±20 gr and age 10-12 weeks) were used in this study. The animals had free access to water and food and were maintained (on a 12/12 hours light/dark cycle). All procedures used in this study were based on The animal's protocol of Animal Research and experiments approved by the Tehran University of Medical Sciences.

### Rats model of global ischemia

Under 100 mg/kg ketamine and 10 mg/kg xylazine anesthesia, the cervical vessels were exposed, then Vagus nerves were carefully separated, and global ischemia was induced by bilateral common carotid arteries ligation for 20 minutes (10).

### Groups and drug administration

The animals were randomly divided into 6 groups (n=8); control, ischemia/reperfusion (IR), vehicle (IR+1 ml DMSO 10%), treatment I (IR+0.5 mg/ml *Rosa damascena* extract), treatment II (IR+ 1 mg/ml *Rosa damascena* extract), treatment III (IR+ 2 mg/ml *Rosa damascena* extract). *Rosa damascena* extract was dissolved in dimethyl sulfoxide 10% (DMSO10%) and injected intraperitoneally after ischemia-reperfusion for 30 days.The injection was done at the same time of biological hours for all animals.

### Nissl staining

Under anesthesia (intraperitoneal injection of 100 mg/kg Ketamin and 10 mg/kg Xylasin ) transcardial perfusion and fixation were done by 4% buffer paraformaldehyde. Animals brains were removed and stored in 4% paraformaldehyde in PBS for 24 hours. Tissue processing and paraffin embedding were done routinely. By using Rotary Microtome coronal section of 5-µm-thickness prepared on the bases of Paxinos Atlas of Rat Brain. The sections mounted and exposed to Nissl staining (11).

### Cell counting

The cell counting was done in the dorsal part of the hippocampal CA1 region. Five sections were selected from each animal (a total of 15 from each group). Sections were observed under an Olympus microscope (CX31, Tokyo, Japan) with a 40× objective lens; image was captured by using a digital camera (Olympus, Japan) and displayed on a computer monitor. The cell with massive shrinkage and abnormal basophilia were detected as dark neurons (12).

### Real time assay

Real-time PCR assay was accomplished using ABI 7300 Real Time PCR System with the Power SYBR Green PCR Master Mix (Applied Biosystems). GAPDH was used as the housekeeping gene for normalizing data. Selected primer sequences included NGF forward 5´-CATCGCTCTCCTTCACAGAGTT-3´, NGF reverse 5´-TGTACGGTTCTGCCTGTACG -3´; NT3 forward5´-ATGGCTCCTGCAAAGCTGAT-3´, NT3 reverse 5´-ATGCTCCATGTGTCCCCTTG-3; BDNF forward 5´-CAATCGAAGCTCAACCGAAGA-3´, BDNF reverse 5´-GGGAACCCGGTCTCATCAAA-3´; GAPDH forward 5´-CCAGCTACTCGCGGCTTTAC-3´, reverse 5´-GTTCACACCGACCTTCACCA-3´. Reaction conditions: 95° C for 10 minutes, followed by 40 cycles of 95° C for 15 seconds, and then 60° C for 1 minute. Finally, the $2^{-\Delta\Delta Ct}$ technique was used for comparative quantification of data and further normalization to GAPDH and fold change compared to control.

### Statistical analysis

Data were presented as mean±SEM. One-way ANOVA and Tukey multiple comparisons were used to analyze the data. Significance was established if $P<0.05$.

## Results

### Results for nissl staining

The number of dark and live neurons of the hippocampal CA1 region was counted in all groups. The mean number of dark neurons number in ischemia and vehicle group was significantly more than the control group ($P<0.0001$). Following the treatment, the mean number of dark neurons significantly was less than dose-dependently compared to ischemia and vehicle groups ($P<0.001$). All doses used in this study had positive effects on neuronal survival, but the most effective one with ($P<0.001$) was 1 mg/ ml.

In addition, our results showed that the number of live neurons in ischemia and vehicle groups was significantly lower than the control group ($P<0.0001$), and when live neurons of the hippocampal CA1 region of treated groups compared to ischemia and vehicle groups, a significant increase was observed ($P<0.001$).

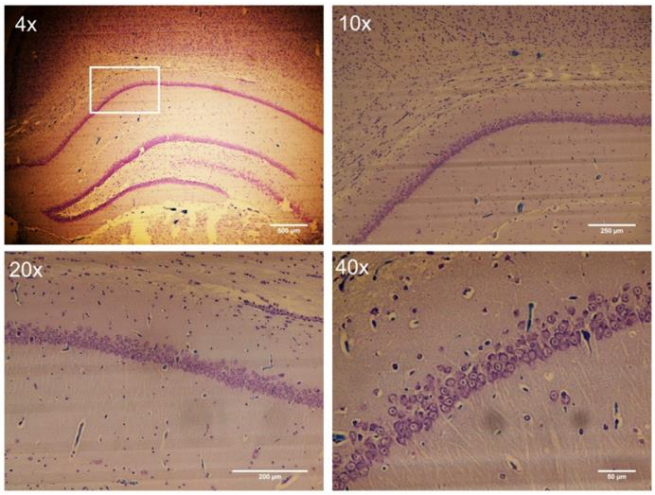


**Figure 1.** CA1 region of hippocampus at different magnification

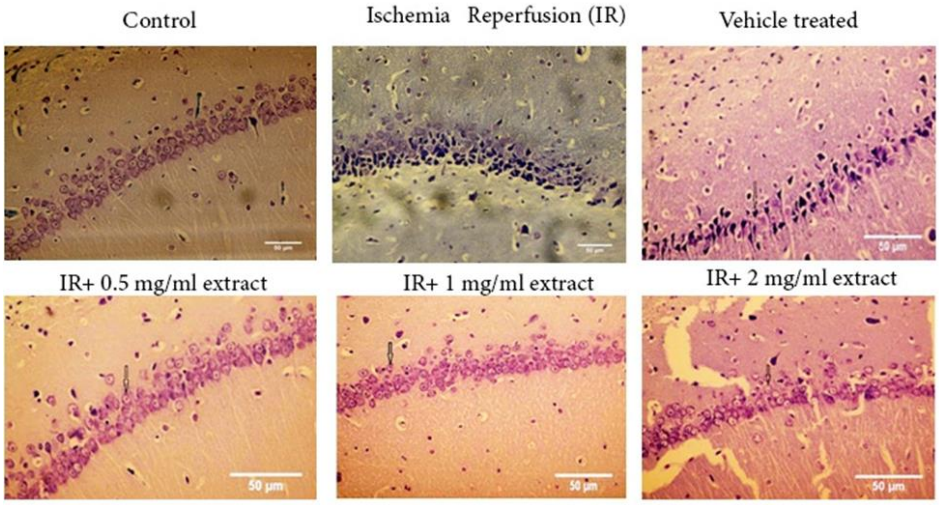


**Figure 2**. Photomicrographs showing the morphological features of the hippocampal CA1 region in different groups following Cresyl Violet Staining. Dark cells were assessed in the hippocampal CA1 region after 30 days in non-ischemic (control), vehicle-treated, ischemia-reperfusion (IR), IR+ 0.5, 1, and 2 mg/ml ,Rosa damascene extract treated. Degenerating dark cells showed oval or triangular nuclei. Magnification: 400×.

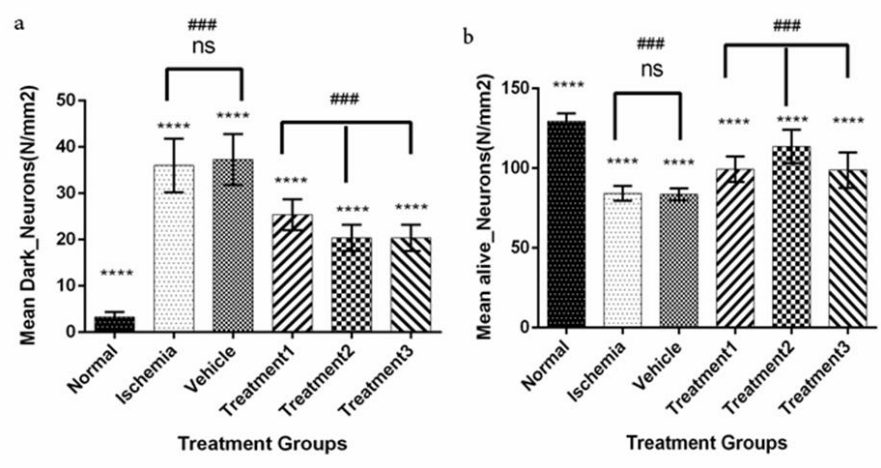


**Figure 3**. The mean of dark neurons (a) and live neurons (b) in the hippocampal CA1 area in different groups. Control (non-ischemic), Ischemia (Ischemia Reperfusion), Vehicle (Vehicle treated), treatment 1 (Ischemia-reperfusion + 0.5 mg/ml extract), treatment 2 (Ischemia-reperfusion + 1 mg/ml extract), treatment 3 (Ischemia-reperfusion + 2 mg/ml extract). **** indicate $P<0.0001$, ### $P<0.001$

**Real-time assay**

30 days after ischemia expression of NGF, NT3 and BDNF mRNA in pyramidal neurons of the hippocampal CA1 region were assayed.

The decreased expression of NGF, NT3 and BDNF mRNA in pyramidal cells of animals was observed in ischemia and vehicle groups compared to control group ($P<0.0001$).

In CA1 pyramidal neurons, NGF, NT3 and BDNF mRNA expression significantly increased in treated animal by *Rosa damascena* extract in comparison with ischemia and vehicle groups ($P<0.0001$), and animals treated with 1 mg/ml dose of the extract showed higher level of NGF,NT3 and BDNF mRNA expression in their pyramidal neurons than other treated group ($P<0.001$).

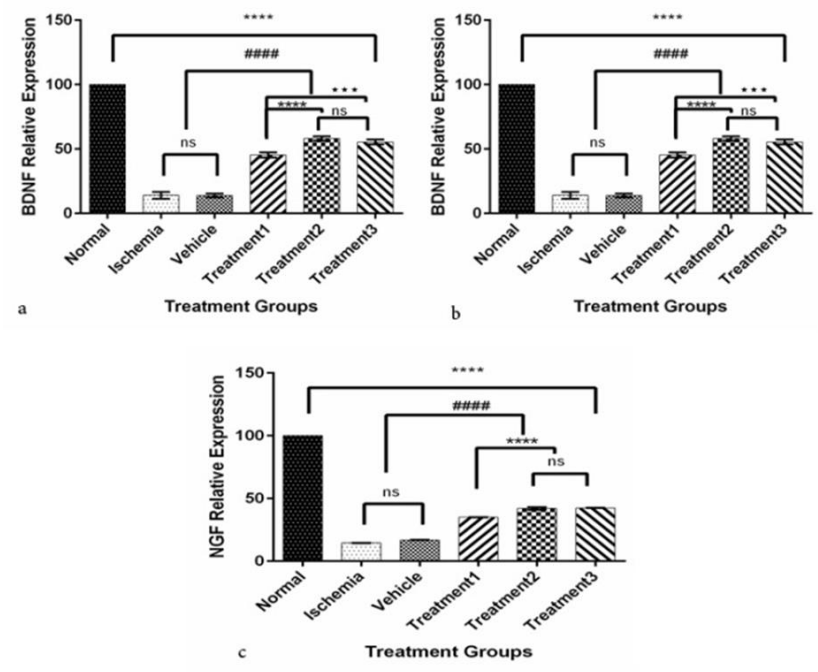


**Figure 4**. Expression of BDNF (a) , NT3 (b), NGF(c) genes expression in pyramidal cells of the hippocampal CA1 area. Control (non-ischemic), Ischemia (Ischemia Reperfusion), Vehicle (Vehicle treated), treatment 1 (Ischemia reperfusion + 0.5 mg/ml extract), treatment 2 (Ischemia reperfusion + 1 mg/ml extract), treatment 3 (Ischemia reperfusion + 2 mg/ml extract). **** indicate $P<0.0001$, *** $P<0.001$, #### $P<0.0001$

## Discussion

The cell damage and abnormal cell death have been reported following cerebral ischemia (13). Our results confirmed a significant increase in the number of dark neurons and decrease in the number of live neurons occur after global ischemia, the Volume density of pyramidal neurons in hippocampal CA1 region was significantly higher in treated group comparing to ischemia and vehicle groups. Many researches have mentioned the anti-inflammatory antibacterial and neurogenesis activity of this extract (14-17). So, it can be considered as a neuroprotective agent. Since the antioxidant potential of Rosa damascene extract has been determined, so it can reduce oxidative stress by ROS scavenging. ROS plays a critical role in ischemic neuronal cell death (18). Because the brain severely depends on the aerobic mechanism and contain high level of fatty acid which is more susceptible to peroxidation and in addition is not enriched in antioxidant defenses as compared to other tissues, so neural cells are more susceptive to oxidative damage in comparison with other tissues. The antioxidant compounds quench or inhibit free radical reaction by donating an electron to a rampaging free radical and delay or inhibit cellular damages (9). Our results suggest that the antioxidant activity of *Rosa damascena* extract can reduce toxicity of free radicals by ROS scavenging and enhance nerve cell survival, resulting in the protection of neurons during ischemic injury. In addition, in this study we attempted to investigate the changes in NGF, BDNF and NT3 mRNA expression in CA1 neurons cells following global ischemia. Neurotrophic factors are necessary for proliferation, differentiation, migration and survival of normally developing neurons and they also play effective roles in the protection of matures neurons under pathologic conditions (6). We hypothesized that cerebral ischemia changes expression of neurotrophic factors. Our results showed that global ischemia reduced expression of NGF, BDNF and NT3 mRNA and Rosa damascena extract plays an important role in enhancement of NGF, BDNF and NT3 mRNA expression in CA1 neurons cells, in treated groups. Since neuroprotective effect of neurotrophic factors has been determined (6), these results can suggest that neurotrophic factors enhance pyramidal neurons survival in CA1 area and reduce damages after ischemic insult. Our results showed that dosage of *Rosa damascene* extract should be controlled. In the present study, the most effective dose of the Rosa *da*mascena extract was 1mg/ml, All doses level had positive effects, though.

Our results indicated that 1mg/ml dose of *Rosa damascena* extract, administered i.p once daily for a 30-day span can increase NGF, BDNF and NT3 mRNA expression in CA1 cells and enhance the CA1 neurons survival following global ischemia. Many researches have mentioned that neurotrophic factors protect brain tissue from injury, that is why *Rosa damascena* extract-induced neuroprotection following cerebral ischemia may be related to these neurotrophic factors expression increases.

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