

Myricetin Ameliorates Brain Damage Induced by Cerebral Ischemia-Reperfusion Injury in Rats

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Abstract

Background: Interruption of blood flow may result in considerable tissue damage via ischemia/ reperfusion injury induced oxidative stress in brain tissues. The present study aimed to investigate the effects of myricetin treatment in rats cerebral I/R injury. **Material and Methods:** The study was carried out on 36 Wistar-albino rats, divided into four groups including Sham group, I/R group, I/R+(control-vehicle DMSO) and I/R+ myricetin 50 mg/kg intraperitoneally 1 hour before induction of ischemia. Measurement of brain tissue IL-1 β , ICAM-1, caspase-3, Notch 1 and Jagged 1 was done after one hour of reperfusion with assessment of the brain infarcted area and histopathological analysis. **Results:** Myricetin attenuates the cerebral I/R injury induced increase in inflammatory cytokine (IL-1 β), adhesion molecule (ICAM-1) and proapoptotic enzyme (caspase-3). Additionally, it reduces the size of infarcted area and histopathological damage. However, such protective effect was not found to be mediated by Notch 1 pathway because myricetin treatment didn't show changes in the increased levels of Notch 1 and Jagged 1 seen in brain with I/R injury. **Conclusions:** Myricetin has a neurocytoprotective effect against cerebral I/R injury which is manifested as anti-inflammatory anti-apoptotic preserving cell structure and viability, nevertheless this effect is not mediated through Notch 1 pathway.

Key words: Cerebral ischemia/reperfusion, interleukin-1 β , Jagged 1, myricetin, Notch 1

INTRODUCTION

Ischemic stroke is more common than hemorrhagic with over 80% of cardiovascular deaths occur in low- and middle-income countries.^[1] Limitation of blood flow through cerebral artery due to thrombosis can cause ischemic stroke which affects cellular homeostasis due to insufficient oxygen and nutrient supply.^[2] Reperfusion damage is defined as further corrosion of ischemic brain tissue by a series of biochemical events on reestablishing cerebral circulation.^[3] A perplexing sequence of events can lead to cerebral damage, starting with cellular acidosis that causes impairment in enzymatic activities and chromatin clumping inside the nucleus, the reestablishment of bloodstream to ischemic tissue delivers oxygen, leading to excessive increase in reactive oxygen species production.

In addition, many inflammatory cascades are triggered by oxidative stress, leading to massive cytokines production such as interleukin (IL)-1 β and intercellular adhesion molecule (ICAM)-1 which will eventually result in cell death due to destruction of numerous cellular structures.^[4] In the period of reperfusion, chemotactic agents cause the adherence of activated leukocytes to endothelial cells. Matrix metalloproteinases and neutrophil-derived oxidants are then produced which cause injury to the blood-brain barrier, the leukocytes then leave the capillary and penetrate the brain

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tissue to release inflammatory mediators which damage the ischemic penumbra.^[5] Studies have shown that reducing ICAM-1 expression due to decreasing IL-1 β production was associated with decreased infarction size.^[6] Apoptosis is activated in response to hypoxic-ischemic insult and in response to oxidative stress in reperfusion injury (RI); the ischemic penumbra is commonly the most affected area by apoptosis.^[7] Once caspase-3 is activated, it will cleave numerous protein substrates, this will either direct to the start of certain proteins that enhance cell killing or inactivate proteins that are important for cell survival and eventually enhance cell killing.^[8] Notch 1 signaling is very essential for nervous system development, it is expressed significantly in neural stem cells (NSCs) controlling proliferation, differentiation, and maintenance in normal or pathogenic state.^[9] Studies which have shown that Notch may have a significant part in activation of microglia and lymphocyte infiltration in cerebral ischemia (CI).^[10] Jagged 1 is a cell shell ligand that operates on the preserved Notch signaling pathway. It has been shown to be upregulated in CI as an adaptive response to enhance neurogenesis in response to stroke.^[11] The field of drug industry has been centered on the neuroprotective effects of natural compounds since traditional herbal medicine with antioxidative, anti-inflammatory, or antiapoptotic properties showed protective in many animal models of intellectual ischemia.^[12] Myricetin is a phenolic compound commonly institute in tea, vegetables, berries, and red wine, existing either as a free form or as a glycoside.^[13] It is poorly absorbed from the intestine with strong antioxidant effect.^[14] In rat model of intestinal ischemia/reperfusion (I/R) injury, myricetin was created to decline the production of inflammatory cytokines such as tumor necrosis factor- α , IL-1 β , and IL-6 in addition to malondialdehyde and increasing the levels of superoxide dismutase and glutathione in the intestinal tissues, leading to improvement in I/R-induced intestinal injury.^[15] The main objective of our study is to assess the prospective neuroprotective consequence of myricetin in a rat model of CI/RI.

MATERIALS AND METHODS

Animals and experimental procedures

A total of 36 adult male Wistar albino rats weighing 200–400 g were purchased from College of Science – University of Zakho. They kept at University of Kufa with a temperature of the animal house maintained at about 25°C. After 2 weeks of adaptation, the rats were distributed randomly into four groups as follows: Group 1 (sham group): The anesthetic and surgical processes were performed on rats without bilateral common carotid artery occlusion (BCCAO). Group 2 (control group): BCCAO was performed for 30 min and then reperfusion was allowed for 1 h. Group 3 (control-vehicle group): Rats were injected intraperitoneally by DMSO 1 h before BCCAO was performed. Group 4 (treatment group):

Rats were injected with myricetin 50 mg/kg intraperitoneally 1 h previous to BCCAO.^[16] The experiment was approved by University of Kufa – Animal Care and Research Committee and the investigation according to the Laboratory Animals Guide Care.

Drug preparation: Myricetin (Chemical abstracts service CAS: 529-44-2) was purchased from Elabscience, the dose was prepared immediately by dissolving it in 5% DMSO in a dose of 50 mg/kg.^[16]

Induction of global brain ischemia

A global model of brain ischemia was induced by BCCAO,^[17] animals temperature was kept at about 37°C by the aid of a light bulb and was anesthetized by ketamine at a dosage of 100 mg/kg and xylazine at a dose of 10 mg/kg intraperitoneally.^[18] After being fixed firmly in the supine position, a small incision was performed in the middle of the neck and the carotid arteries were isolated from the vagal nerves bilaterally and occluded by mini vascular clamps to induce ischemia, after 30 min of occlusion, the clamp was detached and reperfusion was allowed for 1 h.

Preparation of samples

After 1 h of reperfusion, the rats were decapitated, and the brains were isolated and washed in ice-cold phosphate-buffered saline (PBS). While keeping on ice, they were sectioned into three main coronal slices; one was kept in 10% formalin for histopathological analysis. The second slice was kept in the freezer at –20°C for 20 min to enable further sectioning to more uniform coronal slices for 2,3,5-triphenyltetrazolium chloride (TTC) staining, while the last slice was mixed in 1:10 (w/v) ratio with ice-cold 0.1 M PBS (H 7.4) which contains $\times 1$ cocktail protease inhibitor and 0.2% triton $\times 100$ then homogenized by ultrasonic liquid processor; the homogenates were then centrifuged at 15,000 g intended for 30 min at 4°C and the supernatants were reserved and stored at –80°C for the measurement of other markers by enzyme-linked immunosorbent assay (ELISA) technique.^[19]

Measurement of study parameters

Measurement of tissue IL-1 β , ICAM-1, and caspase-3

They measured by Elabscience rat ELISA kits which are sandwich-type ELISA according to the manufacturer's protocol.

Measurement of tissue Notch 1

Notch 1 was measured by RayBio[®] rat Notch-1 ELISA kit which quantitatively measures Notch 1 receptor in plasma, serum, and tissue homogenates according to the manufacturer's protocol.

Measurement of tissue Jagged 1

Jagged 1 was measured by RayBio® rat Jagged 1 ELISA kit which quantitatively measures Jagged 1 protein in plasma, serum, and tissue homogenates according to the manufacturer's protocol.

TTC staining and measurement of infarction area

Estimation of the infarction area was accomplished by TTC staining technique.^[20] The tetrazolium salt is reduced by dehydrogenases which are present in the mitochondria to a red-colored formazan product so viable tissue will be stained red while infarcted tissue will be left unstained. TTC stain solution, 0.2% (w/v), was freshly prepared by dissolving the TTC powder in PBS, and brain slices were transferred to TTC solution in a flat-bottomed enclosed dish and incubated for 30 min at 37°C in dark, the dish needs to be shaken every 5 min. Then, the stain solution was removed and the slices were washed with PBS; finally, the slices were kept in 4% buffered formalin in a flat-bottomed transparent dish and photographed. The photos were analyzed using (ImageJ) software, the stained areas were defined as infarcted and calculated, and then, the infarction percentage was calculated and compared between different treatment groups and the control group.

Tissue sampling for histopathology

The formalin-fixed slices underwent tissue processing to be fixed in paraffin wax and then were longitudinally cut into 5 µm sections; the sections were then stained with H and E stain for histopathological examination.^[21]

Histopathological analysis and scoring of cerebral injury

The histopathological examination for the scoring of brain damage was determined as follows:^[22]

- 0 – (normal) no morphological symbols of injury
- 1 – (slight) edema or eosinophilic or dark (pyknotic) neurons or dark shrunk cerebral Purkinje cells
- 2 – (moderate) as a minimum two tiny hemorrhages
- 3 – (severe) clearly infective foci (local necrosis).

Statistical analysis

Data were analyzed by the means of SPSS software (Statistical Package for the Social Sciences) version 24, mean with standard deviation was considered as descriptive measures. GraphPad Prism version 8 software was used to design the error bar charts for more clarification of data. Statistical significance in all the tests was considered when $P \leq 0.05$.

RESULTS

To evaluate the neuroprotective effect of myricetin, a number of inflammatory and apoptotic parameters were examined after the induction of global CI with and without pre-treatment with those agents in addition to infarction size assessment and histopathological analysis. Notch 1 receptor and Jagged 1 ligand expressions were also tested to examine the potential role of Notch signaling pathway in global intellectual ischemia and in mediating the proposed myricetin neuroprotective effects. Myricetin attenuates inflammatory parameters induced by CI/RI: Cerebral I/RI is known to induce an inflammatory process so that we were interested to evaluate the inflammatory status of the brain tissue in terms of a pro-inflammatory cytokine; IL-1 β , and ICAM; ICAM-1, and the proposed protective effect with pre-treatment by myricetin. Effect on cerebral cytokine (IL-1 β) level: The cerebral concentration of IL-1 β was significantly ($*P < 0.05$) elevated in the control group at the finish of the study in relationship with the sham group (201.67 ± 2.73 vs. 93.89 ± 0.97 pg/ml); meanwhile, the control and control-vehicle groups showed insignificant differences between them. Myricetin treatment group IL-1 β cerebral concentration was significantly ($*P < 0.05$) lesser than the control-vehicle group (124.62 ± 3.59 vs. 206.84 ± 3.23 pg/ml). The changes in IL-1 β cerebral concentration are summarized in Table 1. Effect on cerebral ICAM-1: The cerebral concentration of ICAM-1 was significantly ($*P < 0.05$) elevated in the control group at the finish of the study in comparison with the sham group (23.67 ± 0.32 vs. 8.89 ± 0.34 ng/ml), while the control and control-vehicle groups showed insignificant differences between them. Myricetin treatment group ICAM-1 cerebral concentration

Table 1: IL-1 β cerebral concentration in all experimental groups

Group	IL-1 β concentration in pg/ml	
	Mean \pm SEM	STD
Sham	93.89 \pm 0.97	2.37
Control	201.67 \pm 2.73	6.69
Control-vehicle	206.84 \pm 3.23	7.91
Myricetin	124.62 \pm 3.59	8.79

IL: Interleukin

Table 2: ICAM-1 cerebral concentration in all experimental groups

Group	ICAM-1 concentration in ng/ml	
	Mean \pm SEM	STD
Sham	8.89 \pm 0.34	0.83
Control	23.67 \pm 0.32	0.80
Control-vehicle	22.78 \pm 0.26	0.64
Myricetin	15.29 \pm 0.99	2.43

ICAM-1: Intercellular adhesion molecule

was significantly ($*P < 0.05$) lesser than the control-vehicle group (15.29 ± 0.99 vs. 22.78 ± 0.26 ng/ml). The changes in ICAM-1 cerebral concentration are summarized in Table 2. Myricetin attenuates caspase-3 induced by CI/RI: Caspase-3 activation is a marker for cell death and CI/RI injury is supposed to cause an elevation in the caspase-3 levels. Our results confirmed that the cerebral concentration of caspase-3 was significantly ($*P < 0.05$) elevated in the control group in comparison with the sham group (6.91 ± 0.21 vs. 0.82 ± 0.09 ng/ml); meanwhile, the control and control-vehicle groups showed insignificant differences between them. Myricetin treatment group caspase-3 cerebral concentration was significantly ($*P < 0.05$) lesser than the control-vehicle group (2.87 ± 0.24 vs. 6.71 ± 0.20 ng/ml). The changes in caspase-3 cerebral concentration are summarized in Table 3. Notch signaling path is activated during CI/RI: The Notch pathway is an extremely preserved signaling system that controls cellular self-renewal and survival during the development of various tissues. Therefore, during cerebral I/R injury, we proposed that this pathway is involved in the process of brain tissue damage. Effect on cerebral Notch 1 receptor: The cerebral concentration of Notch 1 receptor was significantly ($*P < 0.05$) elevated in the control group at the finish of the study in comparison with the sham group (19.14 ± 0.35 vs. 14.07 ± 0.37 ng/ml); meanwhile, the control and control-vehicle groups showed insignificant differences between them. On the other hand, myricetin treatment group was insignificantly different from control-vehicle group (19.07 ± 0.35 vs. 19.28 ± 0.20 ng/ml). The changes in Notch 1 receptor cerebral concentration are summarized in Table 4. Effect on cerebral Jagged 1 ligand: The cerebral concentration of Jagged 1 ligand was significantly ($*P < 0.05$) elevated in the control group in comparison with the sham group (19.04 ± 0.33 vs. 11.65 ± 0.34 ng/ml); meanwhile, the control and control-vehicle groups showed insignificant differences

Table 3: Caspase-3 cerebral concentration in all experimental groups

Group	Caspase-3 concentration in ng/ml	
	Mean \pm SEM	STD
Sham	0.82 \pm 0.09	0.22
Control	6.91 \pm 0.21	0.51
Control-vehicle	6.71 \pm 0.20	0.49
Myricetin	2.87 \pm 0.24	0.59

Table 4: Notch 1 cerebral concentration in all experimental groups

Group	Notch 1 concentration in ng/ml	
	Mean \pm SEM	STD
Sham	14.07 \pm 0.37	0.91
Control	19.14 \pm 0.35	0.87
Control-vehicle	19.28 \pm 0.20	0.49
Myricetin	19.07 \pm 0.35	0.87

between them. On the other hand, myricetin treatment group was insignificantly different from control-vehicle group (18.86 ± 0.22 vs. 18.45 ± 0.24 ng/ml). The changes in Jagged 1 ligand cerebral concentration are summarized in Table 5. Myricetin attenuates cerebral infarction size induced by CI/RI: CI/RI causes infarction in the affected area of the brain which appears as white color when stained by TTC stain while the valid area appeared as red color. Figure 1 shows the brain tissues stained with TTC stain with different experimental groups. The infarction percentage was significantly ($*P < 0.05$) elevated in the control group in comparison with the sham group ($52.98 \pm 2.69\%$ compare to 0%), while each of the control and control-vehicle group showed insignificant differences between them. Myricetin management group shows major reduce in infarction percentages in comparison with the control-vehicle group ($14.56 \pm 1.08\%$ vs. $51.51 \pm 2.84\%$). The changes in the infarction percentage are summarized in Table 6.

Myricetin attenuates brain tissue histopathological damage induced by CI/RI: Damage to the brain tissue appeared under the microscope as normal, mild, moderate, or severe tissue damage depend on the present of edema, dark neurons, hemorrhagic area, or necrosis as shown in

Table 5: Jagged 1 cerebral concentration in all experimental groups

Group	Jagged 1 concentration in ng/ml	
	Mean \pm SEM	STD
Sham	11.65 \pm 0.34	0.84
Control	19.04 \pm 0.33	0.82
Control-vehicle	18.45 \pm 0.24	0.61
Myricetin	18.86 \pm 0.22	0.55

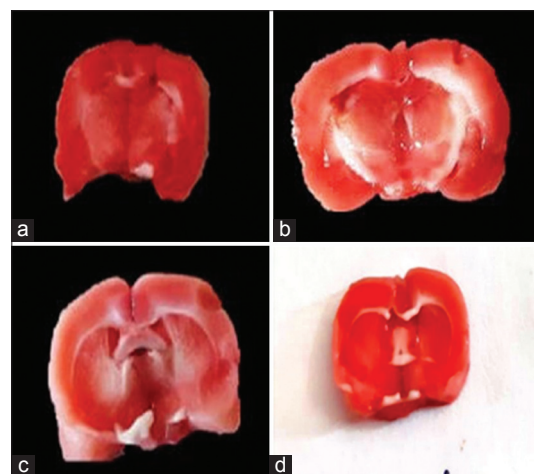


Figure 1: 2,3,5-triphenyltetrazolium chloride stained coronal brain slice showing normal brain of the sham group with no infarction (a), significant increase in cerebral infarction percentage in both the control group (b) and control-vehicle group (c), significant decrease in cerebral infarction percentage in myricetin-treated group (d)

Table 6: Cerebral infarction percentage in all experimental groups

Groups	Cerebral infarction percentage	
	Mean±SEM	STD
Sham	0	0
Control	52.98±2.69	6.61
Control-vehicle	51.51±2.84	6.97
Myricetin	14.56±1.08	2.65

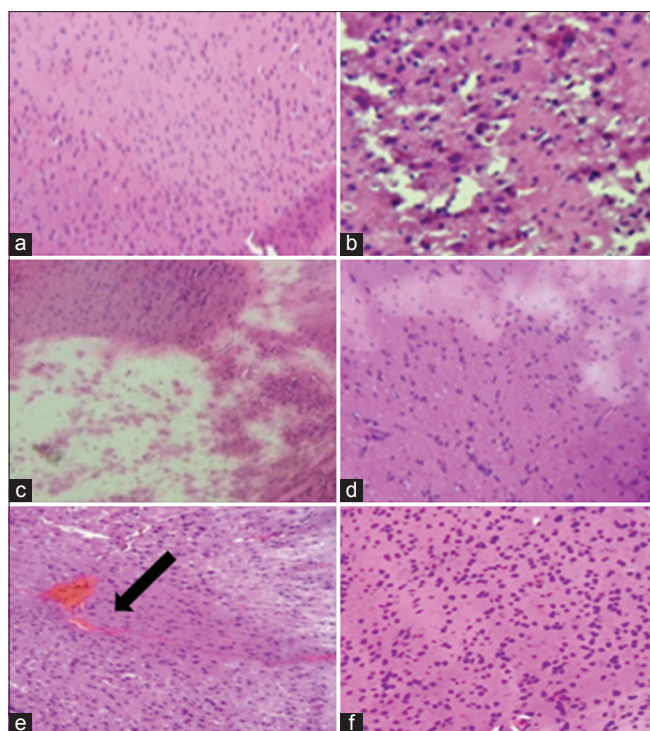


Figure 2: Photomicrograph of rat brain section stained with (H and E). (a) The sham group with score 0 ($\times 10$), (b) the control group with score 3, (red arrow, edema) (black arrow, necrosis) ($\times 40$), (c) the control-vehicle group showing severe edema (arrows) ($\times 10$), (d) the control group with score 1 ($\times 10$), showing edema (arrows). (e) The control-vehicle group with score 2, showing hemorrhage (arrows) ($\times 10$). (f) Myricetin treatment group with score 0 ($\times 10$)

Figure 2. Regarding histopathological scores, there was statistically significant ($*P < 0.05$) difference between the control group and sham group, whereas the difference between the control and control-vehicle groups was insignificant, myricetin treatment significantly ($*P < 0.05$) improved cerebral injury in comparison with the control-vehicle group (data not shown).

DISCUSSION

Various therapeutic plans used to protect from the harmful effects of I/R have been suggested by investigational studies and clinical trials. Neuroprotective agents can target

specific pathophysiological step in CI/RI such as oxidative tension and apoptotic cell death.^[23] The inhalation of gases like hydrogen gas was found to be neuroprotective by decreasing oxidative stress and free radicals formation;^[24] other inhalation anesthetics like isoflurane could discuss neuroprotection in animal models of CI/RI.^[25,26] Iadecola and Anrather have established that inflammation acting a key role in the propagation of ischemic cerebral injury.^[27] Resident microglial cells are considered the main source of IL-1 β in early brain injury after an ischemic insult.^[28] Haqqani *et al.* have illustrated a biphasic expression of IL-1 β mRNA, the early elevation occurs within 1 h of reperfusion, while late elevation occurs within 6–24 h of reperfusion after 20 min of temporary global intellectual ischemia.^[29] ICAM-1 is essential for the adhesion of leukocytes to activated endothelium surface.^[30] The enhanced expression of ICAM-1 found in cerebral endothelial cells is involved in promoting the infiltration of leukocytes to the brain after an ischemic insult as evidenced by Liesz *et al.* who showed significantly decrease penetration of immune cells to the brain after anti-ICAM-1 antibodies administration in an experimental model of stroke.^[31] We found that myricetin treatment significantly decreased pro-inflammatory cytokine IL-1 β and ICAM-1 cerebral levels; Sun *et al.* studied the defensive result of myricetin in intestinal I/RI in rats induced by clamping the superior mesenteric artery and found that myricetin treatment intragastrically caused significant reduction in pro-inflammatory cytokines.^[15] A study conducted on human umbilical vein endothelial cell to test the antioxidant, antiangiogenic, and cell adhesion effects of flavonoids demonstrated that myricetin significantly decreased IL-1 β induce appearance of vascular cell adhesion protein -1, ICAM-1, and E-selectin.^[32] Caspase-3 is the most plentiful effector caspase in the developing brain and was recognized in animal models of intellectual ischemia as a main mediator of apoptosis. Asahi *et al.* showed that caspase-3 mRNA was significantly elevated in rats brain after permanent middle cerebral artery occlusion,^[33] Liu *et al.* also confirmed that CI can trigger neurological destruction and front to neuronal apoptosis that may be connected with caspase-3.^[34] Comparable findings in caspase-3 upregulation after ischemia have been expanded to ischemic human being brain tissue.^[35] In our study, myricetin significantly decreased caspase-3 cerebral level. Scarabelli *et al.* demonstrated antiapoptotic effect of myricetin in rats myocardial I/RI through reducing death receptor Fas cell surface death receptor (FAS) expression and caspase-3 activation by inhibition of signal transducer and activator of transcription 1 phosphorylation.^[36] Similar results were found in rat intestinal I/R injury model, in which myricetin reduced caspase-3 expression while increasing B-cell lymphoma associate X protein (BAX) expression.^[15] Notch signaling trail acting a key role in preserving NSCs pool and regulating neurogenesis in embryonic and adult brain by inhibiting differentiation of neurons to permit consecutive waves of neurogenesis.^[37] Wang *et al.* demonstrated that Notch 1 receptor was expressed chiefly in neuronal precursor cells and immature neurons, while Jagged 1 ligand was predominant

in astrocytic cells in the subventricular zone (SVZ), they, in addition, showed to facilitate Notch 1 pathway which was activated as early as 4 h of reperfusion after transient middle cerebral artery occlusion in the SVZ of rat brain which was associated with enhanced neurogenesis in the SVZ.^[38] In our study, both Notch 1 receptor and Jagged 1 ligand were elevated in the control group, but myricetin treatment did not have significant effect on both Notch 1 and Jagged 1 proteins cerebral levels. Hence, we propose that myricetin neuroprotective effects are not mediated by Notch pathway regulation. A study showed that myricetin neither activated nor repressed Notch 1 signaling in human mammary epithelial cells-1 culture.^[39] Chandrashekhar *et al.* showed that global cerebral CI/RI significant increases in infarction size.^[21] CI/RI-induced severe and moderate cerebral injury revealed by histopathological scores while myricetin treatment showed normal brain histology and mild injury. This indicates that myricetin treatment significantly ameliorated cerebral injury. Shah *et al.* demonstrated that global CI for 30 min in rats produced congestion of blood vessels while necrosis was evident after 1 h of reperfusion.^[17] Sun *et al.* demonstrated that myricetin treatment in intestinal I/RI model in rat significantly reduced the damage score in histopathological examination in a dose reliant way.^[15]

CONCLUSION

Myricetin has a neurocytoprotective effect against cerebral I/R injury which is manifested as anti-inflammatory anti-apoptotic preserving cell structure and viability, nevertheless this effect is not mediated through Notch 1 pathway.

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