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Combination of Bardoxolone and DMF Ameliorates Cerebral Ischemia/Reperfusion Injury in Male Rats

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Abstract

Background: Ischemic stroke has been ranked as the second cause of death in patients worldwide. Inflammation which is activated during cerebral Ischemia/Reperfusion (I/R) is an important mechanism leading to brain injury. The present study aimed to investigate the effect of Berberine on cerebral I/R injury and the role of inflammation in this process.

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+ Berberine 5 mg/kg injected intraperitoneally 1 hour before induction of ischemia. Measurement of brain tissue IL-1β, ICAM-1 (Intercellular Adhesion Molecule-1), caspase-3, Notch 1 and Jagged 1 was done after one hour of reperfusion in addition to assessment of the brain infracted area and histopathological analysis.

Results: Berberine attenuates cerebral I/R injury induced increase in inflammatory cytokine (IL-1β), adhesion molecule (ICAM-1) and proapoptotic enzyme (caspase-3). Additionally, it reduces the size of infracted area and histopathological damage; such protective effect could be mediated by Notch 1 signaling pathway since Berberine further unregulated the increased levels of Notch 1 and Jagged 1 seen in brain with I/R injury.

Conclusions: Berberine has a neurocytoprotective outcome against cerebral I/R injury which is manifested as anti-inflammatory anti-apoptotic effect that preserved cell structure and viability, in the meantime this effect could be mediated by Notch 1 signaling pathway.

Keywords: Cerebral I/R; Berberine; IL-1β; Notch 1; Jagged 1

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Introduction

Cerebral ischemia reperfusion injury (CI/ RI) is defined as deterioration of ischemic brain tissue by a series of biochemical events upon reestablishing cerebral circulation [1]. Ischemic stroke usually causes brain tissue damage where there is almost total cerebral blood flow arrest to undergo necrotic cell death within min, the less rigorously affected tissue that borders the necrotic core is called "ischemic penumbra" which is usually metabolically active but still nonfunctional since the cerebral blood flow is not completely ceased from this area due to collateral blood flow, the main mechanism of cell death in the ischemic penumbra is apoptosis [2]. Inflammation is the essential process in the pathophysiology of I/R injury, leukocytes infiltration is usually the starting event. In the period of reperfusion, chemotactic agents cause the adherence of activated leukocytes to endothelial cells. IL-1β is one of the isoforms of the proinflammatory cytokine IL-1, it has been found to have an important role in many diseases including stroke [3]. Its expression is enhanced within 30 min after I/R injury [4]. Intercellular adhesion molecule (ICAM-1) is a protein ligand that belongs to the immunoglobulin supergene family; it is present on microvascualr endothelium and glial cells, its expression increases during inflammation of brain. Apoptosis is activated in response to hypoxic ischemic insult and in response to oxidative stress in reperfusion injury; the ischemic penumbra is commonly the most affected area by apoptosis [5,6]. An increase in caspase-3 activity was recognized after an experimental model of cerebral ischemia in rats [7]. Notch signaling pathway is associated with numerous regulatory processes like cellular proliferation, programmed cell death and many developmental adaptations like epithelial- mesenchymal transition in superior eukaryotes [8]. Notch 1 signaling is very essential for nervous system development, it is expressed significantly in neural stem cells and neuroblasts in which it controls proliferation, differentiation and maintenance in normal or pathogenic state, and this makes it crucial for neurogenesis and neural specification [9,10]. Abnormal

Notch signaling is linked to many developmental disorders like neurodegenerative disease and cancer [11,12]. Notch signaling also has a role in cerebral I/R injury but there has been contradicting pieces of evidence about it. One study revealed that Notch signaling pathway activation contributes to the neuroprotective effects of isoflurane preconditioning in a mouse model of global cerebral ischemia [13]. Jagged 1 (JAG 1) is one of the 5 cell surface ligands that operate mainly on the preserved Notch signaling pathway. Jagged 1 expressing cells exist in the sub ventricular zone (SVZ) near and apposing to Notch 1 expressing cells [14]. Jagged 1 has been shown to be unregulated in cerebral ischemia in SVZ neural progenitor cells as an adaptive response to enhance neurogenesis in response to stroke [15]. Various therapeutic plans which meant to protect from the harmful effects of ischemia reperfusion have been suggested by experimental animal studies and clinical trials. Neuroprotective agents can target specific path physiological step in cerebral ischemia reperfusion injury like oxidative stress, apoptotic cell death and so many others [16]. Increasing consideration in the field of drug industry has been centered on the neuroprotective effects of natural compounds from traditional herbal medicine, herbal compounds with anti-oxidative, anti-inflammatory or anti-apoptotic properties showed protective or therapeutic effects in many animal models of cerebral ischemia [17]. Berberine is a natural alkaloid of the isoquinoline class. Various studies have been conducted on Berberine over the last two decades revealing many beneficial pharmacological and therapeutic effects for this alkaloid. The main objective of our study is to assess the potential neuroprotective effect of Berberine in a global model of cerebral ischemia reperfusion injury after bilateral common carotid artery occlusion (BCCAO) in rat, and to investigate the potential role of Notch signaling pathway in mediating these effects.

Materials and Methods

A total of 36 adult male Wistar Albino rats weighing (200-400gm) were purchased from College of Science, University of Zakho, they were housed in the animal house at University of Kufa. The experiment was approved by University of Kufa-Animal Care and Research Committee, and the investigation according to the Laboratory Animals Guide Care. After 2 weeks of adaptation, the rats were distributed randomly into 4 groups as follows:

- Group 1 (Sham group): In this group of rats, the anesthetic and surgical processes were performed without BCCAO.
- Group 2 (Control group): In this group of rats, BCCAO was performed for 30 min, and then reperfusion was allowed for 1 hr.
- Group 3 (Control-vehicle group): One hour before ischemia, the rats were injected intraperitoneally by dimethyl sulfoxide (DMSO) and then BCCAO was performed for 30 min, followed by reperfusion for 1 hr.
- Group 4 (Treatment group): in this group of rats, Berberine 5 mg/kg was injected intraperitoneally 1 hr. before BCCAO.

Drug preparation

Berberine chloride (CAS: 633-65-8) was purchased from Sigma Aldrich St Louis, the dose was prepared immediately before use by dissolving it in (5% DMSO) in a dose of 5 mg/kg [18,19] and was injected intraperitoneally 1 hr before induction of ischemia.

Induction of global brain ischemia

A global model of brain ischemia was induced by BCCAO, animal's temperature was kept at about 37℃ by the aid of a light bulb, and the rats were anesthetized by ketamine at a dose of 100 mg/kg and xylazine at a dose of 10 mg/kg intraperitoneally [20]. Then after being placed on the back and fixed firmly in the supine position, a small incision was performed in the middle of the neck by fine surgical tools, and the carotid arteries which exist underneath the trachea were isolated from the vagal nerves bilaterally and occluded by mini vascular clamps to induce ischemia, after 30 min of occlusion the clamps were removed and reperfusion was allowed for 1 hr.

Preparation of Samples

After one hour of reperfusion, the rats were decapitated, and the brains were isolated and washed in ice-cold phosphate buffer solution, they were kept on ice and weighed then sectioned into 3 main coronal slices, one slice was kept in 10% formalin for histopathological analysis. The other slice was kept in the freezer at-20 for 20 min to enable further sectioning to more uniform coronal slices for TTC (2,3,5-Triphenyl Tetrazolium Chloride) 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 1X cocktail protease inhibitor, and 0.2% triton X -100 then homogenized by ultrasonic liquid processor, the homogenates were then centrifuged at 15,000 g for 30 min at 4℃ and the supernatants were withdrawn and stored at -80℃ for measurement of other markers by ELISA technique [21].

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

They measured by Elabscience rat ELISA kits which are sandwich type enzyme–linked immunosorbent assay 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 2,3,5-triphenyltetrazolium chloride (TTC) staining technique [22], the tetrazolium salt is reduced by dehydrogenises which are present in the mitochondria to a red colored formazan product so viable tissue will be stained red while infracted tissue will be left unstained. TTC stain solution, 0.2 % (w/v), was freshly prepared by dissolving the TTC powder in PBS, the staining procedure (by immersion method) was as follows:

2 mm thick coronal slices was quickly sectioned after brain isolation, the slices were kept moistened in cold PBS during the slicing process, then the slices were transferred to the freshly prepared 0.2% TTC solution in a flat bottomed covered dish and incubated for 30 min at 37℃ in dark since the stain is light sensitive, the dish was 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 stainless areas were defined as infracted 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 embedded 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 [23].

Histopathological analysis and scoring of cerebral injury: The histopathological analysis and the scoring of brain damage were determined as follows [24]:

- 0 (normal) no morphological signs of damage
- 1 (slight) edema or eosinophilic or dark (pyknotic) neurons or dark shrunk cerebral purkenje cells
- 2 (moderate) at least two small hemorrhages
- 3 (severe) clearly infarctive foci (local necrosis)

Statistical analysis

Data were analyzed by the means of SPSS software (statistical package for social sciences) version 24, mean with standard error and standard deviation were considered as descriptive measures while One-Way ANOVA was considered to test significant differences between more than 2 groups, in which Post Hoc. Tukey test was used for multiple comparisons and Mann-Whitney U-test was used as nonparametric test to compare histopathological scores between 2 groups. Graph Pad 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 value ≤ 0.05.

Results

In order to evaluate the neuroprotective effects of berberine, a number of inflammatory and apoptotic parameters were examined after induction of global cerebral ischemia with and without pretreatment 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 cerebral ischemia and in mediating the proposed neuroprotective effects. Cerebral ischemia/ reperfusion injury is known to induce an inflammatory process, so that we were interested to evaluate the inflammatory status of the brain tissue in term of a proinflammatory cytokine; IL-1β, and an intercellular adhesion molecule; ICAM- 1 and the proposed protective effect with pre-treatment by berberine.

Effect on cerebral cytokine IL-1β level: The cerebral concentration of IL-1β was significantly (*p<0.05) elevated in control group in comparison to sham group (201.67±2.73 *vs.* 93.89±0.97 pg/ml), meanwhile control and control-vehicle groups showed insignificant differences between them. Berberine treatment group IL-1β cerebral concentration was significantly (*p<0.05) lesser than control-vehicle group (118.47±2.25 *vs.* 206.84±3.23 pg/ml). The changes in IL-1β 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 control group in comparison to sham group (23.67±0.32 *vs.* 8.89±0.34 ng/ml), while control and control-vehicle groups showed insignificant differences between them. Berberine treatment group ICAM-1 cerebral concentration was significantly (*p<0.05) lesser than control-vehicle group (17.15±0.25 *vs.* 22.78±0.26 ng/ml). The changes in ICAM-1 cerebral concentration are summarized in table 2.

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

Group	IL-1β concentration in pg/ml	
	Mean±SEM	Std.
Sham	93.89±0.97	2.37
Control	201.67±2.73	6.69
Control-Vehicle	206.84±3.23	7.91
Berberine	118.47±2.25	5.53

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

Group	ICAM-1 concentration in ng/ml	
	Mean ±SEM	Std.
Sham	8.89 ± 0.34	0.83
Control	23.67 ± 0.32	0.80
Control-Vehicle	22.78 ± 0.26	0.64
Berberine	17.15 ± 0.25	0.63

Berberine attenuates caspase-3 induced by CI/ RI: Caspase-3 activation is a marker for cell death and cerebral ischemia/ reperfusion injury is supposed to cause an elevation in the caspase-3 levels. Our results confirmed that cerebral concentration of caspase-3 was significantly (*p<0.05) elevated in control group in comparison to sham group (6.91±0.21 *vs.* 0.82±0.09 ng/ml), meanwhile control and control-vehicle groups showed insignificant differences between them. Berberine treatment group caspase-3 cerebral concentration was significantly (*p<0.05) lesser than control- vehicle group (2.42±0.17 *vs.* 6.71±0.20 ng/ml). The changes in caspase-3 cerebral concentration are summarized in table 3.

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

Group	Caspase-3 concentration in ng/ml	
	Mean±SEM	Std
Sham	0.82±0.09	0.22
Control	6.91±0.21	0.51
Control-Vehicle	6.71±0.20	0.49
Berberine	2.42±0.17	0.41

Notch signaling pathway is activated during CI/ RI: The Notch pathway is a highly conserved signaling system that controls cellular self-renewal and survival during the development of various tissues. Therefore, during cerebral ischemia/reperfusion 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 control group in comparison to sham group, meanwhile control and control-vehicle groups showed insignificant differences between them. Berberine treatment showed significant further elevation in Notch 1 cerebral level in comparison with control-vehicle group. The changes in Notch 1 receptor cerebral concentrations are summarized in figure 1.

Effect on cerebral Jagged 1 ligand

The cerebral concentrations of Jagged 1 ligand was significantly (*p<0.05) elevated in control group in comparison to sham group, meanwhile control and control-vehicle groups showed insignificant differences between them. Berberine treatment showed significant (*p<0.05) further elevation in Jagged 1 cerebral level in comparison with control-vehicle. The changes in jagged 1 ligand cerebral concentrations are summarized in figure 2.

Berberine attenuates cerebral infarction size induced by CI/ RI

Cerebral ischemia/reperfusion injury causes infarction in the

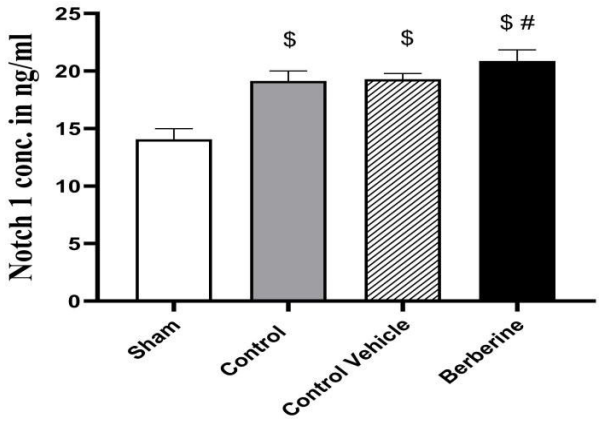

Figure 1: Cerebral Notch 1 concentration (ng/ml) in the experimental groups. Values expressed as mean ±SEM ($ *vs.* Sham, # *vs.* Control vehicle).

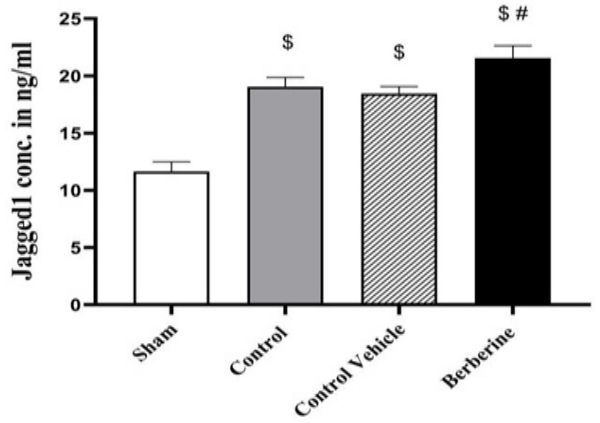

Figure 2: Cerebral Jagged 1 concentration (ng/ml) in the experimental groups. Values expressed as mean ±SEM ($*vs.* Sham, # *vs.* Control vehicle).

affected area of the brain which appears as white color when stained by TTC stain while the valid viable area appeared as red color. Figure 3 shows the brain tissues stained with TTC stain with different experimental groups. The infarction percentage was significantly (*p<0.05) elevated in control group in comparison to sham group (52.98±2.69% compare to 0%), while each of control and control–vehicle groups showed insignificant differences between them.

Berberine treatment group showed significant (*p<0.05) decrease in infarction percentages in comparison to control-vehicle group (19.08±0.53 *vs.* 51.51±2.84%). The changes in the infarction percentage are summarized in table 4.

Berberine 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 presence of edema, dark neurons, hemorrhagic area, or necrosis as shown in figure 4.

Regarding histopathological scores, there was statistically significant (*p<0.05) difference between control group and sham group, while the difference between control and control-vehicle group was insignificant, Berberine treatment significantly (*p<0.05) improved cerebral injury in comparison to control vehicle group as shown in table 5.

Discussion

In our study, the inflammatory mediators IL-1β and ICAM-1 were

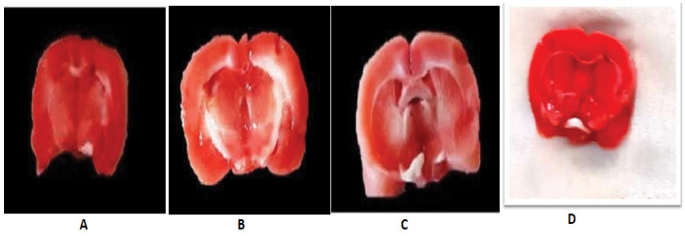

Figure 3: TTC stained coronal brain slice showing normal brain of sham group with no infarction (A), significant increase in cerebral infarction percentage in both control group (B) and control-vehicle group (C), significant decrease in cerebral infarction percentage for about 14% in berberine treated group (D).

Table 4: Cerebral infarction percentages in all experimental groups.

Group	Cerebral infarction size %	
	Mean ±SEM	Std.
Sham	0	0
Control	52.98 ± 2.69	6.61
Control-Vehicle	51.51 ± 2.84	6.97
Berberine	19.08 ± 0.53	1.31

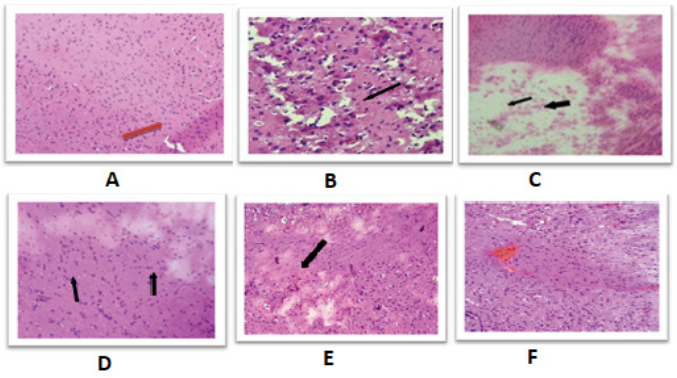

Figure 4: Photomicrograph of rat brain section stained with (H and E). (A) Sham group with score (0)(X10), (B) Control group with score (3), (Red arrow, edema) (Black arrow, necrosis) (40X), (C) Control-Vehicle group showing severe edema (arrows) (X10), (D) Control group with score (1), showing edema (arrows)(X10). (E) Control vehicle group with score (2), showing hemorrhage (arrows) (X10). (F) Berberine treatment group with score (1) showing moderate edema (arrows) (X10).

Table 5: Histopathological scores in all experimental groups.

Histological score	Groups							
	Sham		Control		Control-Vehicle		Berberine	
	n	%	n	%	n	%	n	%
Normal	6	100.0	0	0.0	0	0.0	2	33.3
Slight	0	0.0	0	0.0	0	0.0	3	50.0
Moderate	0	0.0	2	33.3	4	66.7	1	16.7
Severe	0	0.0	4	66.7	2	33.3	0	0.0
Total	6	100.0	6	100.0	6	100.0	6	100.0

significantly increased in control group. After permanent MCAO in mice, B H Clausen et al. have established that microglia-macrophages are the main cells that produce IL-1β [25]. Xinkang Wang et al. have shown that increased expression of IL-1β mRNA occurs within 1 hour after reperfusion in a rat model of tMCAO (Transient Middle Cerebral Artery Occlusion) [26], reaching maximum within 6 hours and persist for 2 days; Ye XH, et al. (2010) found early significant increase in IL- 1β level within the first hour of reperfusion after tMCAO in rats [27]. By interacting with integrins expressed on leukocytes surface, ICAM-1 is essential for the adhesion of leukocytes to activated endothelium surface [28]. Liesz A, et al. (2011) showed significantly decreased infiltration of

immune cells to the brain after anti ICAM-1 antibodies administration in an experimental model of stroke [29]. Xing et al. have shown a significant increase in ICAM-1 after 60 min of ischemia in the course of their study on the neuroprotective effects of ischemic post conditioning after tMCAO in rats [30]. Cao et al. showed a significant early increase in ICAM-1 mRNA after 30 min of global cerebral ischemia while studying the effect of brain cooling in a rat model of global cerebral ischemia reperfusion injury [31]. We found that berberine decreased proinflammatory cytokine IL-1β and intercellular adhesion molecule (ICAM-1) cerebral levels. Zhang Y, et al. (2014) found that berberine significantly reduced IL-1β and ICAM-1 expression thereby reducing inflammation [32]. Zhang X, et al. (2012) revealed the neuroprotective effect of berberine after induction of permanent ischemia in rat by down-regulating NF-κB (Nuclear Factor Kappa-light-chain-enhancer of activated B cells) expression resulting in reduction in neurological deficits and infarct size [33], additionally berberine was found to down regulated IL- 1β and TNF-α (Tumor Necrosis Factor- α) after tMCAO in rats [34]. Our results come into agreement with D. P. Singh who demonstrated that pretreatment with berberine conferred neuroprotection after 15 min of transient ischemia and decreased the expression of inflammatory cytokines IL-1β and TNF-α [35]. In our study, caspase-3 cerebral level was significantly increased in control group. Xing et al. showed a significant elevation of caspase-3 activity in the cortex of rat brain after tMCAO in rats [30], Liang et al. showed significant elevation in caspase-3 activity after transient global cerebral ischemia in the course of their study on the neuroprotective effects of genistein in a rat [36]. In an in vitro study conducted by Liang et al. on PC12 cell line showed that apoptosis was inhibited by berberine pretreatment under hypoxic conditions by decreasing caspase-3 [37]. Our results come into agreement with Qichun Zhang Q, et al. (2016) who demonstrated that pretreatment with berberine conferred neuroprotection after tMCAO in rats [38]. Notch pathway plays a vital role in preserving NSCs pool and regulating neurogenesis in embryonic and adult brain by inhibiting differentiation of neurons to permit consecutive waves of neurogenesis [39]. Besides its participation in neural development, Notch pathway also plays a role in cerebral ischemia. Wang X, et al. (2009) demonstrated that In our study, both Notch1 receptor and Jagged 1 ligand cerebral levels were significantly elevated in control group in comparison with sham group, L. Chen et al. showed significant up-regulation of Notch 1 and Jagged 1 proteins in addition to NICD and Hes1 proteins after tMCAO for 90 min while studying the neuroprotective effects of ischemic preconditioning in focal CI/ RI suggesting the activation of Notch signaling pathway after cerebral ischemia reperfusion injury [40]. We found that berberine significantly elevated both Notch 1 and Jagged 1 proteins cerebral levels. Zhuang et al. studied the proliferation– enhancing activity on neural stem/progenitor cells of berberine which was the most potent compound to induce proliferation of neuronal stem cells [41]. Yu et al. showed that berberine pretreatment unregulated Notch 1 in the myocardium of rats subjected to MI/RI (Myocardial Ischemia/ Reperfusion Injury), similar effects were found in vitro by treating cultured cardiomyocytes subjected to ischemia with berberine [42]. The histopathological examination showed that berberine significantly ameliorated cerebral injury. Shah et al. demonstrated that both local and global cerebral ischemia for 30 min in rats produced congestion of blood vessels while necrosis was evident after 1 hour of reperfusion [43]. Chandrashekhar et al. also confirmed that blood vessel congestion, Neutrophil infiltration and necrosis were observed after global ischemia for in rats [23]. Our findings are in line with Sheng et al. who studied the antioxidant and ant apoptotic effect of berberine preconditioning on hepatic I/ RI in rats and revealed that berberine remarkably reduced the histopathological damage and improved liver function [44]. T. Zhang evaluated the effect of berberine pretreatment on myocardial ischemia triggered by isoproterenol which showed normal, well preserved cardiac muscle cell histology [45]. Measurement of infarction size, after TTC staining, revealed significant elevation in infarction size after CI/RI meanwhile, berberine significantly reduced infarction size. Chandrashekhar et al. confirmed that global cerebral CI/RI caused significant increase in infarction size [23]. Zhou et al. assessed the effect of berberine in decreasing cortical and sub cortical infarction size in mice with CI/RI [46-48]. Zhang et al. showed that berberine significantly reduced infarction size when given immediately after induction of permanent ischemia in rats [33].

Conclusion

From the findings of our study we can conclude the following Berberine has a neurocytoprotective outcome against cerebral I/R injury which is manifested as anti-inflammatory anti-apoptotic effect that preserved cell structure and viability, which could be mediated by Notch 1 signaling pathway, confirming the role of inflammatory mediators (IL-1β and ICAM-1) and apoptotic enzyme (caspase-3) in the pathogenesis of cerebral I/ RI, involvement of Notch signaling pathway in cerebral I/ RI. Berberine decreases the cerebral level of inflammatory mediators (IL-1β and ICAM-1) which might offer mechanistic approach for their protective effects. Berberine exhibits ant apoptotic effect in cerebral I/RI as evidenced by decreasing apoptotic enzyme (caspase-3) cerebral level. Berberine further up-regulated Notch signaling pathway which could have a role in berberine neuroprotective effects. Berberine decreases infarction size estimated by TTC stain and improve cerebral histopathological damage in cerebral I/ RI.

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