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Bardoxolone Ameliorates Cerebral Ischemia/ Reperfusion Injury in Male Rats

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Abstract

Background: Ischemic cerebrovascular disease causes oxygen deprivation to the brain tissue. Reperfusion, which can occur spontaneously through dissolution of the thrombus or by therapeutic recanalization, leads to the generation of reactive oxygen species (ROS)and induces inflammation which further damages the brain tissue. **Methods:** Animal model of rats were subjected to general anesthesia during which an occlusion of bilateral common carotid arteries was done for half hour then reperfusion for one hour with and without prior treatment with Bardoxolone 3 mg/kg intraperitoneal (i.p) 24 hr. Brain tissues were sent for histopathological scoring assessment as well as for estimation of Nrf2, NF-κB and MDA levels. **Results:** The level of nuclear Nrf2 was increased significantly (P<0.05) in bardoxolone group. Brain tissue damage scores and the levels of NF-κB and MDA were decreased significantly (P<0.05) in rats treated with bardoxolone. Histological results in bardoxolone group showed reduced structural lesions. **Conclusions:** Neuroprotective effect of bardoxolone against cerebral I/R injury via up-regulation of nuclear Nrf2 protein and down-regulation of inflammation and oxidative stress.

Key word: Cerebral I/R; bardoxolone; Nrf2; MDA; NF-κB; oxidative stress.

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Introduction

Ischemia-reperfusion injury described as inadequate oxygen and nutrients supply to tissues due to impaired blood perfusion followed by restoration of blood supply that can lead to inflammatory conditions and generation of free radicals that may both aggravate local injury and impairedtarget organ function (1).Cerebral tissues are needed for a high and fixed oxygen supplywhile any vascular obstruction, temporary or permanent, affects this availability and lead to impairmentin neurological functions and eventually causes cerebral cell death (2). Reactive oxygen species induce the recruitment and activation of neutrophils through the release of cytokines such as TNF-α, IL-1, IL-6, IL-8, and triggering the up-regulation of P-selectin and ICAM-1. The release of such oxidants causes direct injury to the endothelium (3).Nrf2 is a member of the CNC family called erythroid-cell-derived-protein with CNC-homology

(ECH) and the transcription-factor can be classified into a seven of domains called Nrf2–ECH-homology (Neh) domains Neh-1 to Neh-7 (4). In stressful condition, those cysteine residues can be modified leading to the release of Nrf2 from the DLG motif side and remains attached to ETGE motif side and make Nrf2 free to translocate to the nucleus (5). Within the nucleus, Nrf2 forms a dimerization complex with the masculo-apo-neurotic-fibro sarcoma (Maf) protein that has facilitated the binding of Nrf2 to anti-response elements (AREs) within the regulatory regions to induce a wide variety of enzymes (6). These enzymes include NQO-1, SOD1, and HO-1 (7) through reactions of oxidation-reduction, nucleophile attack processes, efflux transporter of toxic metabolites, and maintenance of reduced conditions by thiol-containing molecules(8).Bardoxolone is one of the synthetic triterpenoid compounds (9).It has anti-oxidative activities (10) through activation of Keap1/Nrf2/ARE pathway (11) which makes it a potent agent used in the presence of excessive ROS.The molecular mechanism of action of the bardoxolone is activation of Nrf2 and blocking the activity of NF-κB thereby plays an important role in suppress of inflammation(12). In the absence of bardoxolone, Nrf2 binds to Keap1 which makes it subjected to ubiquitination. Bardoxolone disrupts Keap1-Nrf2 complex at its cysteine residues, causes release of Nrf2 and translocation of Nrf2 to the nucleus (13).In the current study, we evaluate the role of bardoxolone in ameliorating damage caused by cerebral I/R injury.

Materials and Methods

Twenty-four Albino male rats were obtained from the animal house at Faculty of Science, University of Zakho. The animals were kept in the animal house at Faculty of Medicine, University of Kufa. The experiment was approved by the University of Kufa-Animal Care and Research Committee, and the investigation according to the Laboratory Animals Guide Care.

Animals groups

After one week of acclimatization the rats were divided into four groups: sham group: rats were subjected to general anesthesia without occlusion of the bilateral common carotid arteries, control group (ischemic-reperfused): rats were subjected to general anesthesia followed by occlusion of the common carotid artery for half an hour then reperfusion for one hour but not receive any drug(14), vehicle group: rats were subjected to the same surgical procedure as in control group but received the vehicle of drugs, DSMO, intraperitoneal (i.p) 24 hr. before ischemia, bardoxolone group: rats were subjected to the surgical procedure as in control group plus bardoxolone at 3 mg/kg i.p 24 hr. before ischemia(15).

Drug preparation

Bardoxolone Cat No. SMB00376 was purchased from Sigma Aldrich, (Germany), was dissolved in 0.1% DMSO plus enough distilled water.

Induction of global brain ischemia

Rats were subjected to general anesthesia via injection of ketamine and xylazine i.p at 100 mg/kg and 10 mg/kg respectively. A neck incision was done and the carotid arteries were blocked by using vascular clamps on both sides at same time as possible for 30 min, then the clamps were removed from the arteries to begin reperfusion for 1 hr to induce global cerebral ischemia/reperfusion injury. After 1 hr of reperfusion, rats were culled via decapitation, brains were removed quickly and cold in pre-cold PBS solution, then transfer to freezer for 10 min, after that each

brain was cut to three slices, one for histopathological and immune-histopathological studies, second for TTC staining, and third for ELISA study.

Assessment of cerebral infarction using TTC stain

Brain tissues were stained by 2, 3, 5 Triphenyl tetrazolium chloride stain (TTC) which was purchased from Direvo Industrial Biochemical and was immediately prepared by dissolving it in PBS at 0.2 % (w/ v) concentration before slicing of the brains. After brains cuts into coronal slices, it immersed in TTC stain at 37°C for 30 min in a glass petri dish with aluminum foil covering to prevent the effect of light on TTC stain followed by immersion in PBS-10% PFA (16).Infarct volume was measured by using digital imaging (digital camera) and image analysis software (image J system). The infarct area was measured by Swanson's method (17).

Histopathological assessment

Brain tissues wereput in formalin and immersed in a paraffinwax, cut at 6 µm thick sections, then stained by hematoxylin-eosin (H and E). The pathological scoring scale used in this study as following (18): Normal (0): there are no edema, RBC, and eosinophilic neurons. Slight (1): there is edema or eosinophilic neurons. Moderate (2): there are edema, eosinophilic neurons, and a little number of RBC. Severe (3): there are edema, eosinophilic neurons, RBC, and necrosis.

Tissue preparation for MDA and NF-κB measurement

The brain tissues were cut into very small pieces under cold condition followed by homogenized with homogenization solution which contains PBS, cocktail inhibitor and Triton X100 for 20 min by using a ultrasonic liquid processor under cold condition followed by centrifuging and stored at −80°C for future analysis.

Cytoplasmic and Nuclear extraction of Nrf2

The extraction and isolation of Nrf2 tissue samples were performed according to nuclear and cytoplasmic extraction kit (Beyotime, Jiangsu, China).

Measurement levels of Nrf2, MDA, and NF-κB through ELISA technique

The collected supernatants obtained from groups were used for measuring of Nrf2 at cytoplasmic and nucleus level, in addition to MDA, and NF-κB levels by ELISA (Nanjing Pars Biochem CO., Ltd, China) according to manufacturer protocol.

Measurement levels ofTLR2, and TLR4 through immunohistochemistry

At end of reperfusion, brain tissues cut at 6 µm thick sections, dewaxing, and immersed in Xyleneand to be followed by dehydration using different concentrations of alcohol. Break down the crosslink between formalin and antigen, we used retravel buffer in water bath and then blocked the peroxidase effect by using blocking agent. Incubation the brain tissues with primary antibody purchased from Cusabio Company USA followed by secondary antibody and finally stained by using DAB and hematoxylin solution and see under microscope.

Results

Bardoxolone reduced infarction area percentage

Cerebral I/R injury cause an infarcted area which appears as a white color when stained by TTC stain while the valid area appeared as red color. A photographic analysis by using software image J showedsignificant increase ($p<0.05$) in percentages of infarcted areas compared to sham group (43.15 ±7.72% vs. 0.39 ±0.09%) while bardoxolone

treatment reduced infarction area percentage significantly ($p<0.05$) compared to control group (9.69±1.02% vs. 43.15±7.72%) as seen in table (1).

Effect of bardoxolone on cytoplasm and nuclear brain tissuelevelsofNrf2

Cerebral I/R injury caused translocation of Nrf2 from the cytoplasm to the nucleus. Our results showed significant ($p<0.05$) reduction in the level of cytoplasmic Nrf2 in control group comparing to sham group (49.23±1.30 vs. 73.41±2.11ng/ml) while bardoxolone treatment further reduced the level of Nrf2 in cytoplasm significantly ($p<0.05$) compared to control group (37.01±0.51 vs. 49.23±1.30 ng/ml) as seen in table (2).Consequently, bardoxolone treatment elevated the nuclear level of Nrf2 significantly ($p<0.05$) compared to control group (40.45±1.99vs. 31.49±0.43ng/ml) as seen in table (3).

Bardoxolone reduced MDA level in brain tissue

Our results showed that cerebral I/R injury caused elevation in MDA level compared to sham group (1.22±0.05 vs. 0.44 ±0.03µmole/ml) while MDA level of bardoxolone group was reduced significantly ($p<0.05$)as compared to control group(0.51±0.01vs. 1.22±0.05 µmole/ml) as shown in table (4).

Bardoxolone reduced NF-κB level in brain tissue

We showed that bardoxolone reduced level of NF-κB significantly ($p<0.05$) compared to control group (113.52±4.32vs. 166.18±7.73 ng/ml) as shown in table (5).

Bardoxolone inhibits expression of TLR2 and TLR4

Cerebral I/R injury caused increase in the expression of TLR2 and TLR4. We analyzed the TLRs level in brain tissue for all groups via the immunohistochemistry technique (IHC). We found that control and vehicle groups expressed TLR4 and TLR2 with weak-moderate intensity and few positive cells while bardoxolone inhibited their expression in brain tissues as shown in figure (1).

Bardoxolone reduced histopathological damage

Brain tissue appeared under the microscope as normal tissue, mild, moderate or severe damage depend on the present of edema, dark neurons, hemorrhagic area, or necrosis. The control group showed elevated damage score when compared to sham group with a significant reduction in brain tissue damage score in bardoxolone group compared to control group as shown in figure (2).

Discussion

Nrf2 has a central regulatory role as cellular anti-oxidative systems (7), therefore, drugs have Nrf2 activator properties are believed to have a protective effect against cerebral ischemic damage. In our study, we suggested that rats pretreated with bardoxolone ameliorates cerebral ischemia-reperfusion injury by increasing Nrf2 nuclear level, reduced infarct volume, decreased MDA content, modulation inflammation response by reducing level of NF-κB, TLR2, and TLR4.Free radicals are mediators caused cell injury and cell death (19) and can be detoxified by endogenous antioxidants induced by Nrf2 but overproduction of free radical can depleted store of these anti-oxidants (20). Pharmacological Nrf2 inducer can increase the nuclear level of Nrf2 and protect cells from free radical-induced cell death (21). We found that cerebral I/ R injury lead to increase of necrotic cell, oxidative stress and inflammation, while pre-treatment with bardoxolone reduced this percent significantly probably due to translocation of the Nrf2 to nucleus and induction of anti-oxidant genes. Many experiments have studied the effect of Nrf2 activator on the cerebral ischemia-reperfusion injury. Ren J et al. found that infarction area was increased in the

untreated group as compared to sham while reduced in pre-treatment rat with Nrf2 activator (resveratrol) (22). Ya et al. showed nuclear Nrf2 level increased significantly in control group after global ischemia as compared to sham group, while this level increased three folds in 5-hydroxymethyl-2-furfural (5-HMF) treatment group as compared tocontrol group (23). Imai et al. who studied the effect of bardoxolone in reducing hemorrhage induced by focal cerebral ischemia by an increased level of Nrf2 (24). Jun Zeng et al. found that liquiritigenin (Nrf2 activator) reduced the cytoplasmic level of Nrf2 and elevated its nuclear level (25). Cerebral I/ R injury is associated with oxidative stress, which plays a role in the impaired function and leads to neuronal death. MDA is a marker of oxidative stress due to its effect on the product of lipid peroxidation(26). We found that low MDA level was shown in sham group which increased significantly in control group after induction of BCCAO that indicated MDA generation due to increased stress. Administration of bardoxolone 24 hr. reduced MDA level significantly when compared with control group because of the buildup of Nrf2 protein in nucleus which reduces brain damage. Yao et al. showed that control mice have high levels of MDA in the brain tissue as compare to treated group (27).Zhang et al. shown that down-regulation of MDA in treated group (Nrf2 activator) when compared to the control group(28).These findings support our results in that I/R-induced oxidative stress and activating Nrf2 protects brain cells membrane from peroxidation. Many studies have involved cross-talk between both NF-κB and TLRs with Nrf2 (29-31). ROSis considered a major stimulatory factor of inflammation through activation of transcription factor NF-κB(32). We found that NF-κB level in sham group is very low and TLR2 and TLR4 do not express in both sham and bardoxolone groups.NF-κB level and TLRs increased significantly in control and vehicle groups that indicated I/R induced inflammatory response. We also noticed pretreatment by bardoxolone reduced NF-κB level and TLRs expression as compared to control group that indicated that as Nrf2 protein increased in nucleus reduce inflammation. Stephenson et al. showed that NF-κB increases three folds in the ischemic brain when compared to control group (33). Li et al. found NF-κB increases in control group while it decreased in ursolic acid (Nrf2 activator) treated group (34).Ying Wang et al shoedn that cerebral I/R caused elevation of TLR2 and TLR4 when measured by immunohistochemistry (35).Yanzhe Wang et al. who observed that ursolic acid treatment decrease the number of TLR-positive cells compared to control (36). Ischemia caused free radical generation which induced neuronal damage and mitochondrial impairment(37). We found that sham group has low damage score while damage scores increased significantly in control group after induction of ischemia when compared to sham group indicated I/R induces damage in brain tissue. Administration of bardoxolone reduced brain damage scores lower than untreated groups that indicated Nrf2 protein decreased brain damages. Beibei Chen et al. has shown that I/R caused marked neuronal damage and pyknotic nuclei in I/R group, while Nrf2 activator (rifampicin) group showed normal neurons (38). BH Clausen et al. who used monomethyl-fumarate as Nrf2 activator in middle cerebral artery occlusion model, they found elevation of Nrf2 reduced edema significantly in the treated group when compared to control group(39).Our results clarified the neuroprotective effect of bardoxolone in ameliorating the cerebral I/R injury through its ability to increase nuclear Nrf2 translocation and thereby down-regulation of inflammatory and oxidative stress markers which eventually reduced brain tissue damage.

References

1.Dorweiler B, Pruefer D, Andrasi B, et al. Ischemia-Reperfusion Injury: Pathophysiology and Clinical Implications. Eur J Trauma Emerg Surg. 2007;33(6):600-12.

2.Feigin VL, Krishnamurthi RV, Parmar P, Norrving B, Mensah GA, Bennett DA, et al. Update on the global burden of ischemic and hemorrhagic stroke in 1990-2013: the GBD 2013 study. Neuroepidemiology. 2015;45(3):161-76.
3.Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med. 2000;192(7):1001-14.
4.Itoh K, Igarashi K, Hayashi N, Nishizawa M, Yamamoto M. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. Molecular and cellular biology. 1995;15(8):4184-93.
5.McMahon M, Thomas N, Itoh K, Yamamoto M, Hayes JD. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. J Biol Chem. 2006;281(34):24756-68.
6.Nguyen T, Huang HC, Pickett CB. Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. J Biol Chem. 2000;275(20):15466-73.
7.Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol. 2007;47:89-116.
8.Suh N, Wang Y, Honda T, Gribble GW, Dmitrovsky E, Hickey WF, et al. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. Cancer Res. 1999;59(2):336-41.
9.Hyer ML, Shi R, Krajewska M, Meyer C, Lebedeva IV, Fisher PB, et al. Apoptotic activity and mechanism of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid and related synthetic triterpenoids in prostate cancer. Cancer Res. 2008;68(8):2927-33.
10.Yates MS, Tauchi M, Katsuoka F, Flanders KC, Liby KT, Honda T, et al. Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. Mol Cancer Ther. 2007;6(1):154-62.
11.Kawamoto Y, Nakamura Y, Naito Y, Torii Y, Kumagai T, Osawa T, et al. Cyclopentenone prostaglandins as potential inducers of phase II detoxification enzymes. 15-deoxy-delta(12,14)-prostaglandin j2-induced expression of glutathione S-transferases. J Biol Chem. 2000;275(15):11291-9.
12.Dinkova-Kostova AT, Liby KT, Stephenson KK, Holtzclaw WD, Gao X, Suh N, et al. Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. Proc Natl Acad Sci U S A. 2005;102(12):4584-9.
13.Ahmad R, Raina D, Meyer C, Kharbanda S, Kufe D. Triterpenoid CDDO-Me blocks the NF-kappaB pathway by direct inhibition of IKKbeta on Cys-179. J Biol Chem. 2006;281(47):35764-9.
14.Obadia N, Lessa MA, Daliry A, Silvares RR, Gomes F, Tibiriçá E, et al. Cerebral microvascular dysfunction in metabolic syndrome is exacerbated by ischemia-reperfusion injury. BMC neuroscience. 2017;18(1):67-.
15.Walsh J, Jenkins RE, Wong M, Olayanju A, Powell H, Copple I, et al. Identification and quantification of the basal and inducible Nrf2-dependent proteomes in mouse liver: biochemical, pharmacological and toxicological implications. J Proteomics. 2014;108:171-87.
16.Joshi CN, Jain SK, Murthy PSR. An optimized triphenyltetrazolium chloride method for identification of cerebral infarcts. Brain Research Protocols. 2004;13(1):11-7.
17.Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 1990;10(2):290-3.
18.Chandrashekhar VM, Ranpariya VL, Ganapaty S, Parashar A, Muchandi AA. Neuroprotective activity of Matricaria recutita Linn against global model of ischemia in rats. Journal of ethnopharmacology. 2010;127(3):645-51.
19.Singh U, Jialal I. Oxidative stress and atherosclerosis. Pathophysiology. 2006;13(3):129-42.
20.Ahmad S, Yousuf S, Ishrat T, Khan MB, Bhatia K, Fazli IS, et al. Effect of dietary sesame oil as antioxidant on brain hippocampus of rat in focal cerebral ischemia. Life Sci. 2006;79(20):1921-8.
21.Sinha K, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol. 2013;87(7):1157-80.
22.Ren J, Fan C, Chen N, Huang J, Yang Q. Resveratrol pretreatment attenuates cerebral ischemic injury by upregulating expression of transcription factor Nrf2 and HO-1 in rats. Neurochem Res. 2011;36(12):2352-62.
23.Ya B-L, Li H-F, Wang H-Y, Wu F, Xin Q, Cheng H-J, et al. 5-HMF attenuates striatum oxidative damage via Nrf2/ARE signaling pathway following transient global cerebral ischemia. Cell stress & chaperones. 2017;22(1):55-65.
24.Imai T, Takagi T, Kitashoji A, Yamauchi K, Shimazawa M, Hara H. Nrf2 activator ameliorates hemorrhagic transformation in focal cerebral ischemia under warfarin anticoagulation. Neurobiol Dis. 2016;89:136-46.
25.Zeng J, Chen Y, Ding R, Feng L, Fu Z, Yang S, et al. Isoliquiritigenin alleviates early brain injury after experimental intracerebral hemorrhage via suppressing ROS- and/or NF-κB-mediated NLRP3 inflammasome activation by promoting Nrf2 antioxidant pathway. Journal of neuroinflammation. 2017;14(1):119-.
26.Chen X, Zhang X, Wang Y, Lei H, Su H, Zeng J, et al. Inhibition of immunoproteasome reduces infarction volume and attenuates inflammatory reaction in a rat model of ischemic stroke. Cell death & disease. 2015;6(1):e1626-e.
27.Yao Y, Miao W, Liu Z, Han W, Shi K, Shen Y, et al. Dimethyl Fumarate and Monomethyl Fumarate Promote Post-Ischemic Recovery in Mice. Translational stroke research. 016;7(6):535-47.
28.Zhang D, Xiao Y, Lv P, Teng Z, Dong Y, Qi Q, et al. Edaravone attenuates oxidative stress induced by chronic cerebral hypoperfusion injury: role of ERK/Nrf2/HO-1 signaling pathway. Neurological research. 2018;40(1):1-10.

29.Ding Y, Ren D, Xu H, Liu W, Liu T, Li L, et al. Antioxidant and pro-angiogenic effects of corilagin in rat cerebral ischemia via Nrf2 activation. Oncotarget. 2017;8(70):114816-28.
30.Wardyn JD, Ponsford AH, Sanderson CM. Dissecting molecular cross-talk between Nrf2 and NF-kappaB response pathways. Biochem Soc Trans. 2015;43(4):621-6.
31.Cuadrado A, Martin-Moldes Z, Ye J, Lastres-Becker I. Transcription factors NRF2 and NF-kappaB are coordinated effectors of the Rho family, GTP-binding protein RAC1 during inflammation. J Biol Chem. 2014;289(22):15244-58.
32.Pedruzzi LM, Stockler-Pinto MB, Leite M, Jr., Mafra D. Nrf2-keap1 system versus NF-kappaB: the good and the evil in chronic kidney disease? Biochimie. 2012;94(12):2461-6.
33.Stephenson D, Yin T, Smalstig EB, Hsu MA, Panetta J, Little S, et al. Transcription factor nuclear factor-kappa B is activated in neurons after focal cerebral ischemia. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2000;20(3):592-603.
34.Li L, Zhang X, Cui L, Wang L, Liu H, Ji H, et al. Ursolic acid promotes the neuroprotection by activating Nrf2 pathway after cerebral ischemia in mice. Brain Research. 2013;1497:32-9.
35.Wang Y, Ge P, Yang L, Wu C, Zha H, Luo T, et al. Protection of ischemic post conditioning against transient focal ischemia-induced brain damage is associated with inhibition of neuroinflammation via modulation of TLR2 and TLR4 pathways. Journal of Neuroinflammation. 2014;11(1):15.
36.Wang Y, Li L, Deng S, Liu F, He Z. Ursolic Acid Ameliorates Inflammation in Cerebral Ischemia and Reperfusion Injury Possibly via High Mobility Group Box 1/Toll-Like Receptor 4/NFκB Pathway. Front Neurol. 2018;9:253.
37.Su KG, Banker G, Bourdette D, Forte M. Axonal degeneration in multiple sclerosis: the mitochondrial hypothesis. Current neurology and neuroscience reports. 2009;9(5):411-7.
38.Chen B, Cao H, Chen L, Yang X, Tian X, Li R, et al. Rifampicin Attenuated Global Cerebral Ischemia Injury via Activating the Nuclear Factor Erythroid 2-Related Factor Pathway. Frontiers in cellular neuroscience. 2016;10:273-.
39.Clausen BH, Lundberg L, Yli-Karjanmaa M, Martin NA, Svensson M, Alfsen MZ, et al. Fumarate decreases edema volume and improves functional outcome after experimental stroke. Experimental neurology. 2017;295:144-54.

Group	Mean± SEM	95% CI		P value
		Lower	Upper	
Sham	0.39 ±0.09%	0.17%	0.61%	
Control	43.15 ±7.72%	23.30%	63.01%	#P<0.05
DMSO	42.18 ±2.11%	36.75%	47.62%	#P<0.05
Bardoxolone	9.69 ±1.02%	7.05%	12.32%	*P<0.05

Table 1: Percentages of infarcted areas for all experimental groups.
Data expressed by one-way ANOVA. # vs sham, * vs control.

Group	Mean±SEM	95% CI		P value
		Lower	Upper	
Sham	73.41±2.11	67.99	78.83	
Control	49.23±1.30	45.88	52.57	#P<0.05
DMSO	47.54±1.43	43.87	51.22	#P<0.05
Bardoxolone	37.01±0.51	35.69	38.32	*P<0.05

Table 2: Cytoplasm level of Nrf2 (ng/ml) in all experimental groups.
Data expressed by one-way ANOVA.* vs sham, # vs control

Group	Mean±SEM	95% CI		P value
		Lower	Upper	
Sham	22.67±0.72	20.82	24.52	
Control	31.49±0.43	30.40	32.59	#P<0.05
DMSO	31.66±0.64	30.01	33.30	#P<0.05
Bardoxolone	40.45±1.99	35.33	45.57	*P<0.05

Table 3: Nuclear level of Nrf2 (ng/ml) in all experimental groups.
Data expressed by one-way ANOVA. * vs sham, # vs control

Group	Mean± SEM	95% C I		Pvalue
		Lower	Upper	
Sham	0.44 ±0.03	0.35	0.52	
Control	1.22±0.05	1.09	1.36	#P<0.05
DMSO	1.23±0.05	1.11	1.35	#P<0.05
Bardoxolone	0.51±0.01	0.48	0.54	*P<0.05

Table 4: MDA levels (µmole/ml) for all experimental groups.
Data expressed by one way ANOVA.# vs sham, * vs control.

Group	Mean± SEM	95% C I		P value
		Lower	Upper	
Sham	108.62±32.17	74.86	142.39	
Control	166.18±7.73	158.07	174.28	# P< 0.05
DMSO	159.80±22.68	136.01	183.60	# P< 0.05
Bardoxolone	113.52±4.32	108.98	118.05	* P< 0.05

Table 5: NF-κB levels (ng/ml) for all experimental groups.
Data expressed by one-way ANOVA.# vs sham, * vs control.

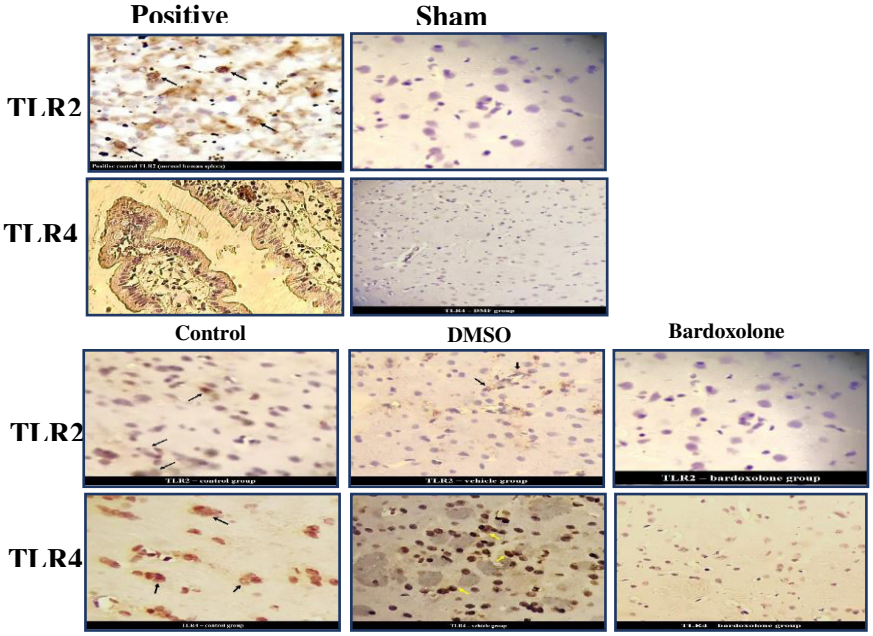
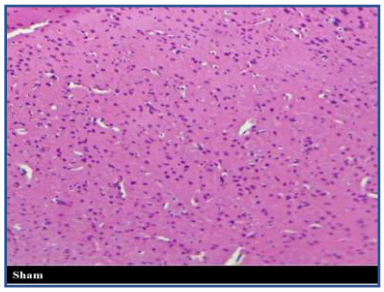
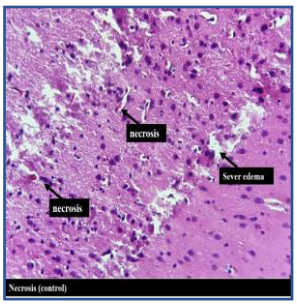


Figure 1: TLR2 and TLR4 expression in all experimental groups. Immunohistochemistry Staining showed that control and vehicle groups with 10-15 positive cells while for bardoxolone group <10% positive cells with moderate intensity. A positive control, using normal spleen tissue was used to test the validity of the antibody. (X 10×40).

A)



B)



C)

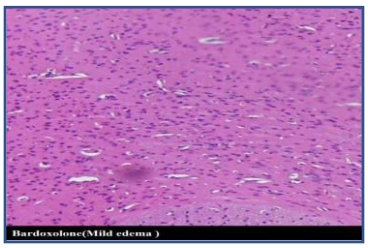


Figure 2: Histological scores of brain tissue damage. Sham (**A**), control (**B**), and bardoxolone (**C**) groups. The section stained with Hematoxylin and Eosin (X 100).