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.


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 impaired target organ function (1). Cerebral tissues are needed for a high and fixed oxygen supply while any vascular obstruction, temporary or permanent, affects this availability and lead to impairment in 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, nucelophile 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, DMSO, 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 were put in formalin and immersed in a paraffin wax, 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 of TLR2, and TLR4 through immunohistochemistry**

At end of reperfusion, brain tissues cut at 6 μm thick sections, dewaxing, and immersed in Xylene and 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 showed significant 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 tissue level of Nrf2**

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±1.99 vs. 31.49±0.43 ng/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±0.03 µmole/ml) while MDA level of bardoxolone group was reduced significantly (p<0.05) as compared to control group (0.51±0.01 vs. 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±4.32 vs. 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 to control 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). ROS is considered a major stimulatory factor of inflammation through activation of transcription factor NF-κB(22). 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. showed 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


<table>
<thead>
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<th>Group</th>
<th>Mean±SEM</th>
<th>95% CI</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>0.39 ±0.09%</td>
<td>0.17%</td>
<td>0.61%</td>
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<tr>
<td>Control</td>
<td>43.15 ±7.72%</td>
<td>23.30%</td>
<td>63.01%</td>
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<tr>
<td>DMSO</td>
<td>42.18 ±2.11%</td>
<td>36.75%</td>
<td>47.62%</td>
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<tr>
<td>Bardoxolone</td>
<td>9.69 ±1.02%</td>
<td>7.05%</td>
<td>12.32%</td>
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Table 1: Percentages of infarcted areas for all experimental groups. Data expressed by one-way ANOVA. # vs sham, * vs control.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean±SEM</th>
<th>95% CI</th>
<th>P value</th>
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<tr>
<td>Sham</td>
<td>73.41±2.11</td>
<td>67.99%</td>
<td>78.83%</td>
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<td>Control</td>
<td>49.23±1.30</td>
<td>45.88%</td>
<td>52.57%</td>
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<td>47.54±1.43</td>
<td>43.87%</td>
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<td>Bardoxolone</td>
<td>37.01±0.51</td>
<td>35.69%</td>
<td>38.32%</td>
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</table>

Table 2: Cytoplasm level of Nrf2 (ng/ml) in all experimental groups. Data expressed by one-way ANOVA. * vs sham, # vs control.
Table 3: Nuclear level of Nrf2 (ng/ml) in all experimental groups.
Data expressed by one-way ANOVA. * vs sham, # vs control

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean± SEM</th>
<th>Lower</th>
<th>Upper</th>
<th>Pvalue</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>0.44 ±0.03</td>
<td>0.35</td>
<td>0.52</td>
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<tr>
<td>Control</td>
<td>1.22±0.05</td>
<td>1.09</td>
<td>1.36</td>
<td>#P&lt;0.05</td>
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<tr>
<td>DMSO</td>
<td>1.23±0.05</td>
<td>1.11</td>
<td>1.35</td>
<td>#P&lt;0.05</td>
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<tr>
<td>Bardoxolone</td>
<td>0.51±0.01</td>
<td>0.48</td>
<td>0.54</td>
<td>*P&lt;0.05</td>
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Table 4: MDA levels (µmole/ml) for all experimental groups.
Data expressed by one way ANOVA.# vs sham, * vs control.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean± SEM</th>
<th>Lower</th>
<th>Upper</th>
<th>Pvalue</th>
</tr>
</thead>
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<tr>
<td>Sham</td>
<td>108.62±32.17</td>
<td>74.86</td>
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<tr>
<td>Control</td>
<td>166.18±7.73</td>
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<td>174.28</td>
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<tr>
<td>DMSO</td>
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<td>136.01</td>
<td>183.60</td>
<td># P&lt; 0.05</td>
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<tr>
<td>Bardoxolone</td>
<td>113.52±4.32</td>
<td>108.98</td>
<td>118.05</td>
<td>* P&lt; 0.05</td>
</tr>
</tbody>
</table>

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

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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 10x40).

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).