Cerebro-Protective effect of bosentan in brain ischemia reperfusion injury

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Abstract:

Background: Ischemia reperfusion injury following acute ischemic insult is responsible for extension of injury. Bosentan is an endothelin receptor antagonist, which is currently used as a strong vasoconstrictor. This study aims to investigate the effects of bosentan on ischemia reperfusion injury after brain ischemic stroke in rat model.

Methods: Forty male Wistar rats were randomly allocated into four study groups: Sham group: Rats underwent the anesthesia & surgery for an identical period to the other 3 study groups without intervention. Control group: Rats underwent anesthesia & surgery including bilateral common carotid artery ligation (BCCAL) for 30 minutes and then reperfusion for 1 hour. Vehicle group: Four days before ischemia, rats were administered with a vehicle (5% gummi arabicum) and then anesthesia &BCCAL surgery were performed. Bosentan treat group: Four days before
ischemia, rats were administered with bosentan (100mg/kg/day) and then anesthesia & BCCAL surgery were performed.

**Results:** Bosentan treated group exhibited lower concentration of interleukin 6 (IL-6) in the brain (2168.0±30.67 pg/mL) than in the control group (2571.37±96.58 pg/mL) \((P\leq0.05)\). In addition, interleukin 10 (IL-10) levels were significantly high in the bosentan group (275.7±15.97 pg/mL) when compared to the control group (244.05±12.23 pg/mL) \((P\leq0.05)\). This was associated with a non-significant reduction in the brain levels of endothelia nitric oxide synthase (eNOS) in the bosentan treated groups (90.23±1.14 ng/mL) when compared to the control group (90.94±2.48), \((P>0.05)\).

**Conclusion:** Bosentan treatment have protective effects against the inflammatory damage following ischemia reperfusion injury following acute myocardial infarction.

1. **Introduction:**

Brain ischemia reperfusion injury may follow acute myocardial infarction, which may increase the degree of myocardial damage [1]. After complete stroke, recanalization may be implemented to restore blood flow, which may predispose to reperfusion injury[1]. Studies showed that inflammatory mediators, like cytokines, have important role in initiating inflammatory reaction and compound reperfusion injury[2]. Recanalization of the occluded cerebral vessels using thrombolysis is not currently considered as the therapeutic option because of the high risk of cerebral hemorrhage [3]. Moreover, the reperfusion may cause further damage. Therefore, other pharmacological options are recommended against the molecules that initiate ischemic damage [4].

One of the most promising therapies is that targets inflammatory response [5]. Inflammatory response may arise early within 30 minutes after ischemic insult and it may have important role in progression into cell necrosis [6].Histological examination after cerebral ischemic insult reveals infiltration of polymorphonuclear leucocytes and macrophages within 6-12 hours after ischemia which becomes more intense after 2-3 days [7]. Cytokines are small glycoproteins that mediate inflammatory and immune response [8]. During brain ischemia, they stimulate the production of adhesion molecules, attract leucocytes and induce thrombogenesis by increasing platelet activating factor, plasminogen-activator inhibitor-1 and tissue factor [9]. One of these molecules is interleukin 6 (IL-6). Serum level of IL-6 was higher in patients with acute stroke than in normal individual. Moreover, it correlates with the size
of brain infarction[10]. IL-6 also increases the expression of phospholipase A2, which mediate the synthesis of leukotrienes, prostaglandins and platelet activating factor[11].

On the other hand, interleukin 10 (IL-10) is considered as anti-inflammatory cytokine, which steps down the inflammatory response [12]. IL-10 expression is increased after brain insult to increase neuronal cells survival[13]. IL-10 deficiency results in endothelial damage, increased oxidative stress and inability of vasculature to respond to physiological demands [14]. In preclinical studies, IL-10 knockout mice show higher inflammatory response in the brain after occlusion of middle cerebral artery, whereas, systemic and intraventricular administration of IL-10 decrease infarction size [14].

Intact endothelium produces substances that protect against atherosclerosis and thromboembolism. Nitric oxide is one of these substances that are synthesized by enzyme nitric oxide synthase (NOS). Three types of this enzyme are found in our body. Neuronal NOS (nNOS), which is expressed in inhibitory neurons while inducible NOS (iNOS) is expressed by inflammatory cells in response for stress and inflammation [15]. Endothelial NOS (eNOS) is constitutively expressed by endothelial cells which plays important vascular protective role[16]. During ischemic stroke, nNOS and iNOS activity increase and result in greater injury and oxidative stress[16]. On the other hand, eNOS plays neuroprotective role during ischemic stroke[16]. Bosentan, an endothelin receptor blocker, has vasodilator activity and cardioprotective effect[17]. High levels of endothelin were shown in conditions associated with ischemic event suggesting that endothelin has important role in pathophysiology [18]. Moreover, use of endothelin antagonist in animal models of cardiac ischemia-reperfusion injury has shown reduction in infarction size and decrease inflammatory mediators involved in this type of injury[19]. Bosentan may have protective effects against global brain ischemia by improving cortical circulation through collateral cerebral vessels[19, 20]. In this study, we evaluated the effects of bosentan on inflammatory mediators and infarction size after ischemia reperfusion injury of the brain.

2. Materials & Methods

The study was approved by the Animal Research Ethics Committee, Faculty of Medicine, University of Kufa. Forty male Wistar rats were purchased from the Faculty of Veterinary Medicine, University of Kufa. The animals were
kept at the Animal House with an average temperature of 25°C and 60% humidity with 12 hours cycles of dark and light. They are allowed for free diet before starting the experiment.

2.1 Study design:

The animals were randomly allocated into four study groups:

Group 1 (Sham group): Animals were anesthetized and underwent the same operative procedure as the rest of the study groups without bilateral common carotid artery ligation (BCCAL).

Group 2 (Control group): Animals have the full operative procedure with BCCAL but without any treatment.

Group 3 (Control-Vehicle group): Animal had the same interference as the Vehicle group but they receive the vehicle (5% gummi arabicum) of the target treatment.

Group 4 (Bosentan treated group): Animals were treated with (bosentan 100mg/kg/day) orally for 4 days before the BCCAL operation, using Bosentas® CiplaMedCompany (62.5 mg dissolved in 5% gummi arabicum).

2.2 Induction of cerebral ischemia and reperfusion:

Rats were anaesthetized by ketamine plus xylazine (80mg/kg and 5mg/kg, respectively), injected intra-peritoneally [21]. Then the animals were placed on the surgical theater in supine position with arms and legs fixed by plaster. After small incision in the middle of the neck, both common carotid arteries were exposed and clamped with small vascular clamp for 30 minutes [22, 23]. Then, the clamp was removed after that for 1 hour and the animal was then sacrificed. The brain was isolated by decapitation and dissection.

2.3 Preparation of samples for biochemical analysis

The brain was washed with isotonic saline solution and cooled with ice. The brain was homogenized using high intensity ultrasonic liquid processor and phosphate buffered saline containing protease inhibitor cocktail. We take 10% (w/v) of the homogenate and centrifuged for 20 minutes at 14000×g. The supernatant kept in Eppendorf tubes and analyzed for measurement of IL-6, IL-10 and eNOS by ELIZA technique.

2.4 Histopathological test and scoring
Brain tissue was first isolated. After that, it was soaked in 10% formalin and then immersed in paraffin wax. Coronal slices were obtained and then longitudinal sections with 5µm thickness were done. These sections were stained with hematoxylin / eosin stain. Histopathological tests were done under supervision of pathologist[24]:

2.5 Measurement of Infarction Area

The area of cerebral infarction was measured by using 2, 3, 5-triphenyltetrazolium chloride (TTC) stain as soon as the brain removed and sliced. Then digitizer software was used for measurement of area of damage (infarction).

2.6 Statistical analysis

Data were analyzed using SPSS (IBM, version 20) program. One way ANOVA test was achieved to compare between the group means then Post-hoc LSD was followed for multiple comparisons. The significance level for statistical tests was 0.05 (P<0.05)

3. Results:

3.1: Effect of bosentan on cerebral IL-6:

The cerebral levels of IL-6 were significantly increased in the control group (2571.37±96.58 pg/mL), when compared to the sham group (1938.6±74.76 pg/mL), P value <0.05. In addition, Bosentan group showed significant reduction in concentration of IL-6 (2168.0±30.67 pg/ml) than control group, P<0.05. Table 1, Figure 1.

3.2: Effect of bosentan on cerebral IL-10

The concentration of IL-10 was significantly higher among the control group (244.05±12.23 pg/mL), as compared to the sham group (192.43±5.04 pg/mL), P<0.05. The Vehicle group showed no significant difference from the control group (240.90±9.75 pg/mL). Whereas, IL-10 was significantly higher in bosentan group (275.7±15.97 pg/mL), P<0.05, Table 1, Figure 1.

3.3: Effect of bosentan on cerebral eNOS

Cerebral concentration of eNOS did not show significant difference in bosentan group in comparison to control group (P>0.05), Table 1, Figure 1.
3.4: **Effect of bosentan on cerebral infarction size after ischemia reperfusion injury**

Cerebral infarction size after bosentan treatment was 20.72±1.95%, which is significantly less than that of control group 38.8 ±2.64 % as seen in Table 1.

**3.5 Results of Histopathological test**

By using histopathological scoring system mentioned above, bosentan treated group showed only grade 1 to grade 2 changes while control group showed grade 3 to about 70% as seen in Figure 2& 3.

**4. Discussion:**

Inflammatory response may extend the damage due to ischemia and reperfusion injury as the latter may trigger release of inflammatory cytokines[25]. IL-6 may be one of the important mediators of inflammatory response during cerebral ischemia and reperfusion injury [10, 26]. Endothelin increases level of IL-6 during cerebral ischemia – reperfusion injury [10, 26, 27]. Therefore, attenuation of IL-6 response may have beneficial effects on reduction of tissue damage after cerebral I/R injury [28].

Bosentan is a well-known blocker of both endothelia (ET\textsubscript{A}) and (ET\textsubscript{B}) receptors[29]. In our study we found that bosentan causes significant reduction in level of cerebral IL-6 following a four days treatment with bosentan. On the other hand, cerebral IL-10 concentration increased in bosentan-treated group. IL-10 has protective role in inhibition of the inflammatory response after cerebral I/R injury. Its concentration is increased during ischemic stroke [13, 29].

In our experiment, IL-10 cerebral concentration was increased in bosentan treated group. Our findings are in consistence with previous studies, which showed that bosentan reduces the concentration of inflammatory mediators like IL-6, IL-2, IL-8 in patients with systemic sclerosis[30]. Endothelial NOS concentration showed no significant change between different study groups. Nitric oxide, an endothelium-derived vasodilator, is synthesized by endothelial nitric oxide synthase. Nitric oxide produces peroxynitrite which may increase inflammatory response and oxidative stress. Furthermore, high concentration of nitric oxide after reperfusion may cause deleterious damage[31]. However, previous studies showed that bosentan therapy may lower eNOS expression after 28 days in animal model[32].

**5. Conclusion:**
Bosentan can be useful therapeutic agent to reduce the inflammatory response and decreases infarction size after cerebral I/R injury. Further studies are needed to explore other anti-inflammatory effects of bosentan during cerebral I/R injury.

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Table 1: The levels of the inflammatory markers among the four study groups, values are expressed as Mean± SEM, Significant P at or below 0.05.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>Control</th>
<th>Vehicle</th>
<th>Bosentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1938.6±74.76</td>
<td>2571.37±96.58</td>
<td>2487.12±90.19</td>
<td>2168.0±30.67^7S</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>192.43±5.04</td>
<td>244.05±12.23</td>
<td>240.90±9.75</td>
<td>275.7±15.97^3S</td>
</tr>
<tr>
<td>eNOS (pg/ml)</td>
<td>70.93±4.43</td>
<td>90.94±2.48</td>
<td>92.48±2.62</td>
<td>90.23±1.14^*</td>
</tr>
<tr>
<td>Cerebral infarction size (%)</td>
<td>0</td>
<td>38.8 ±2.64</td>
<td>36.6 ± 2.3</td>
<td>20.72±1.95^*</td>
</tr>
</tbody>
</table>

IL-6: Interleukin 6; IL-10: interleukin 10; eNOS: Endothelial nitrous oxide synthase.
*significant VS sham, # significant VS control, $significant VS vehicle
Figure 1: The levels of the anti-inflammatory markers among the four study groups. A: Interleukin 6 (pg/ml); B: Interleukin 10 (pg/ml); C: Endothelial nitric oxide synthase (pg/ml).

Figure 2: Percentage of the grade of the histopathological damage following cerebral I/R injury among the four study groups.
Figure 3: Histopathological image of rat brain (x20) from (A): Control group showing inflammatory cell infiltration with area of necrosis. (B) Bosentan group showing less areas of necrosis and inflammatory cell infiltration.

References:


