ABSTRACT

The aim of the present study was investigated the improvement of antioxidant enzymes activities and blood brain barrier by Immunox V drug in cerebral spinal fluid of meningitis induced rat model. Twenty four albino rats were selected and divided into three groups of eight; Control group (n=8), Infected group (n=8) and Immunox V treated group (n=8). Our results showed that there were significantly (p<0.001) decreased in all antioxidant enzymes activities along with increased the activities of adenylate kinase, xanthine oxidase enzymes (p<0.001) as well as malonalldialdehyde (MDA) levels in the CSF of infected group as compared to control group. The levels of total protein, calcium and phosphorus were also increased significantly (p<0.001) along with decreased (p<0.001) the glucose level in CSF of infected group as compared to control group. After administration of immunox V for one week treatment, all enzyme activities along with MDA levels as well as biochemical parameters were improved and come back to normal level in the infected plus treated group. These findings indicated that immunox V drug play a therapeutic role in the improvement of oxidant and antioxidant levels and prevent the blood brain barrier during bacterial meningitis infection.

Key words: Meningitis, blood brain barrier, antioxidant enzymes, MDA, ceftriaxone and vancomycin, fixed dose combination.

Introduction

Bacterial meningitis is a common disease with high morbidity and mortality rate in the world wide (22). Meningitis is an important clinical problem and it is characterized by an intense inflammatory response of sub arachnoid and ventricular space, breakdown of the blood brain barrier (BBB), subsequent brain edema and vasculitis of the blood vessels (23, 26). The blood brain barrier is a physical and metabolic barrier that separates the peripheral circulation from the central nervous system and serves to regulate and protect the micro environment of the brain. It protects the integrity and function of the brain by selectively regulating the entry and exit of biologically important substances (18). It is effectively blocks the vast majority of circulating bacteria from entering the brain, its defensive function fails in bacterial meningitis infections. Bacterial meningitis, a serious brain infection, can develop rapidly into a life-threatening infection even in healthy children or adults. Free radicals are also important factor for induction of bacterial meningitis (29). These radicals (superoxide anion, NO) are responsible for the development of intracranial complications, brain edema and brain damage etc. It also became evident that free radical, especially nitric oxide, are important mediators of meningitis-associated pathophysiological changes, at least during the early phase of the disease (11). During meningitis, the neurological and immunological responses, the blood brain barrier is compromised.
ratio of free radical generating and free radical scavenging enzymes may be disturbed which leads to causes several kinds of macromolecules such as lipid, protein and nucleic acids (12).

Antibiotics either alone or in combination therapy are an important approach to address the management of chronic and acute diseases. Immunox V is a fixed dose combination of ceftriaxone plus vancomycin antibiotic. Ceftraixon is a third generation cephalosporin class of antibiotic. It has excellent activity against the common bacteria causing meningitis in children (16). Vancomycin is a glycopeptide antibiotic used in the prophylaxis and treatment of infections caused by Gram-positive bacteria. Ceftriaxone has been shown to be additive or synergistic in combination with vancomycin, against several gram-negative pathogens (4, 5, 28). The aim of the present study was investigated the therapeutic role of Immunox V in the changes of blood brain barrier and antioxidant enzyme activity in cerebral spinal fluid of experimental induced meningitis rat model.

Materials and Methods
Chemicals & drug
All the biochemicals used in the present study were procured from Sigma, St. Louis, MO, USA. Other chemicals, purchased locally, were of analytical grade. Methicillin-resistant staphylococcus aureus (MRSA) bacterial organism was isolated from infected patients from PGIMS Chandigarh, India. An antibiotic such as Immunox V (a fixed dose combination of ceftriaxone plus vancomycin) was obtained from Venus Remedies Ltd. India. The ratio of ceftriaxone plus vancomycin was 2:1 respectively.

Bacterial strain
Methicillin-resistant Staphylococcus aureus (MRSA) bacterial strain maintained on nutrient agar slant were grown in septic culture in nutrient broth at 37 °C for 24 hours. Organisms were harvested by centrifuged at 2348 g for 15 min, washed three times, and suspended in phosphate buffer saline (0.2 M, pH 7.0) to the desired concentration.

Meningitis model
For the purpose of this investigation, it was necessary to developed a model of experimental meningitis in the rat. Total sixteen rats (infected) were anesthetized intramuscularly with 10mg/kg of ketamine (Ketolar; Parke-Davis, Prat de Llobregat, Spain). Control rat (n=8) was received 25µl of normal saline by intravenously. Meningitis infection was induced by direct intracisternal injection of 25 µl of saline containing log10 6 CFU / ml MRSA strain via a 24-gauge needle. Meningitis infection was induced in rat within 7 to 10 days.

Animals and Treatments
The animals were quarantined for a period of three weeks to ensure stabilization before use. Twenty four rats (all males, weighing 60 to 70 g) were used throughout the experiment. The mice were fed standard pelleted diet and water ad libitum. The test room was air conditioned with temperature 23 ± 2°C, humidity 65 ± 5%, and with artificial fluorescent light (10 and 14 hours of light and dark, respectively). The study was approved by the institutional animal ethical committee. The rats were divided in to three groups of eight mice each.

Control group (n=8) : received normal saline (0.9% NaCl)
Infected group (n=8) : received intracisternal injection of MRSA strain (log10 6CFU / ml )
Infected + treated group (n=8) : received Immunox V drug (42.85 mg/kg/body weight)

Immunox-V drug was given to infected group according to their body weight by intravenously for seven days treatment and CSF
sample (0.02 to 0.08ml) was collected from lumber puncture.

Enzyme assays

**Superoxide dismutase (SOD) assay**

SOD activity was determined by the Method of Misra and Fridovich (15). The reaction mixture consisted of 1.0 ml carbonate buffer (0.2 M, pH 10.2), 0.8 ml KCl (0.015 M), 0.020 ml of CSF and distilled water to make the final volume to 3.0 ml. The reaction was started by adding 0.2 ml of epinephrine (0.025 M). The change in absorbance was recorded at 480 nm at 15 second interval for one minute at 25°C. Suitable control lacking enzyme preparation was run simultaneously. One unit of enzyme activity is defined as the amount of enzyme causing 50% inhibition of auto oxidation of epinephrine.

**Catalase assay**

Catalase activity was measured by the method of Luck (14). The reaction mixture consisted of 0.3 ml phosphate buffer, (0.2 M; pH 6.8), 0.1ml H₂O₂ (1 M) and distilled water to make the final volume to 3.0 ml. The reaction was started by adding the suitable aliquot of CSF. The change in the absorbance was recorded at 15 sec. interval for one minute at 240 nm at 25°C. Suitable control was run simultaneously. One Unit of enzyme activity was defined as the amount of enzyme that liberates half of the peroxide oxygen from H₂O₂ in 100 sec at 25°C.

**Glutathione reductase Assay**

GR activity to be measured by Carlberg & Mannervik (3). The reaction mixture consisted of 1.5 ml of potassium phosphate buffer (0.2 M; pH 7.0 containing 2 mM EDTA), 0.15 ml of 2 mM NADPH, 0.2 ml of oxidized glutathione and distilled water to make up the final volume 3.0 ml. The reaction to be started by adding the suitable aliquot of CSF preparation in the linearity range. The change in absorbance was measured at 340 nm wave length for one minute at 15 sec. interval. Control lacking enzyme to be also run simultaneously. One unit of enzyme activity to be defined as the formation of NADP in one minute by one ml of enzyme preparation using extinction coefficient of 6.22.

**Adenylate kinase assay**

Adenylate kinase (AK) activity was determined by method of Haslam & Mills with minor modification (8). The reaction mixture consisted of ADP 0.30 ml (4.0 mM), 0.55 ml of glucose (10 mM), 0.55 ml of MnCl₂ (10 mM), 0.30 ml of NADP (0.2 mM), 0.58 ml of Tris buffer (50 mM, pH 7.4), 10 µg of hexokinase (1 unit), 10 µg of glucose 6 phosphate dehydrogenase (1 unit), and added distilled water to make up 3.0 ml. The reaction was started by adding 20 µl of CSF sample. Change in absorbance was recorded at 340 nm at 15 seconds interval for one minute. Suitable control was run simultaneously. One unit of AK activity in the forward direction was defined as 1µmole of ADP removed /min at 37 °C. under experimental condition.

**Xanthine oxidase assay**

Assay of xanthine oxidase was carried out essentially according to the method described by Roussos (21). The assay mixture consisted of 0.30 ml Tris-HCl buffer, 50 mM (pH 7.4); 0.30 ml CuSO₄ (10 mM) ; 0.05 ml Xanthine, (2.58 mM per ml. in 0.05 M glycine buffer, pH 7.4); and added distilled water to make up 3.0 ml in volume. The reaction was started by adding 0.025 ml of CSF. Change in absorbance was recorded at 290 nm at 15 seconds interval for one minute. Suitable control was run simultaneously. One unit of activity has
been defined as change in absorbance at 290 nm in 1 minute by 1 ml of CSF.

**Lipid peroxidation level**

Free radical mediated damage was assessed by the measurement of the extent of lipid peroxidation in the term of malonaldehyde (MDA) formed, essentially according to Ohkawa et al. (17). It was determined by thio barbituric reaction. The reaction mixture consisted of 25 µl of CSF preparation, 0.20 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of (20%, pH 3.5) acetic acid, 1.5 ml of 0.8% thio barbituric acid (TBA) and distilled water to make up the volume to 4.0 ml. The tubes were boiled in water bath at 95°C for one hour and cooled immediately under running tap water. Added 1.0 ml of water and 5.0 ml of mixture of n-butanol and pyridine (15:1 v/v) and vortexed. The tubes were centrifuged at 3500 rpm for 30 minutes. The upper layer was aspirated out and optical density was measured at 532 nm. The reference standard used was 1,1,3,3 tetra ethoxy propane.

**Biochemical analysis**

Glucose, total protein, phosphorus and calcium parameters were analyzed in CSF sample by using a commercially available standard kit (Bayer Diagnostics India Ltd., Baroda, Gujrat India).

**Histological analysis**

For histological analysis, animals were scarified and brain were excised from infected and infected plus treated group along with control group. Brain were immediately fixed in 10% formalin solution for 24 hours and then embedded in paraffin according to standard procedures. The brains were entirely sectioned along a coronal plane. Sections were stained with haematoxilin-eosin according to standard techniques. Morphological changes were assessed by using routine light microscopy.

**Statistical analysis**

The data obtained was analyzed statistically. All values are expressed as mean ± SD. One-way analysis of variance (ANOVA) with student-Newman-Keuls comparison test was used to determine statistical difference between control vs infected and treated groups. P values <0.05 were considered statistically significant.

**Figure. 1.** The simultaneous conversion of the enzyme xanthine dehydrogenase to its xanthine oxidase form and the break down of purine nucleotides to hypoxanthine create an ideal environment for the production of superoxide radical when O₂ is readmitted upon reperfusion.
Table 1: Status of oxidant and antioxidant enzymes parameters in CSF of meningitis induced rat

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameters</th>
<th>Control group (n=8)</th>
<th>Infected group (n=8)</th>
<th>Infected + treated group (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>SOD (mMole/min/ml)</td>
<td>425.52± 2.87</td>
<td>245.93 ± 2.76</td>
<td>404.3± 2.34</td>
</tr>
<tr>
<td>b</td>
<td>Catalase (mMole/min/ml)</td>
<td>119.45± 1.38</td>
<td>61.96 ± 1.12</td>
<td>97.36 ± 1.23</td>
</tr>
<tr>
<td>c</td>
<td>GR (mMole/min/ml)</td>
<td>0.344± 0.022</td>
<td>0.189± 0.002</td>
<td>0.290± 0.009</td>
</tr>
<tr>
<td>d</td>
<td>MDA (nmole/ml)</td>
<td>6.37± 0.08</td>
<td>16.49± 0.98</td>
<td>6.69± 0.23</td>
</tr>
<tr>
<td>e</td>
<td>XO (unit/min/ml)</td>
<td>0.312 ± 0.093</td>
<td>1.34± 0.27</td>
<td>0.945 ± 0.111</td>
</tr>
<tr>
<td>f</td>
<td>AK (mMole/min/ml)</td>
<td>149.21± 16.42</td>
<td>221.63 ± 29.87</td>
<td>185.06 ± 9.68</td>
</tr>
</tbody>
</table>

Value are expressed as Mean ± SD. Value in parenthesis represent % changes in control vs infected treated group and infected vs Immunox-V treated group( Infected plus treated group). Where SOD (superoxide dismutase); GR (Glutathione reductase); MDA (Malonaldehyde); XO (xanthine oxidase) and AK (adenylate kinase). a= *** p<0.001 (highly significant); b= ** p<0.01 (significant)

Figure 2. Histological analysis of the brain infected with MRSA strain and treatment with Immunox V drug in meningitis induced rat. Slide shows that A; Severe inflammation characterized by cellular exudates composed of PMNs entrapped in fibrin in the subarachnoid space. B; Inflammation reduced after administration of Immunox V drug for one week treatment. C; shows control normal cell.
Table 2: Status of biochemical parameters in CSF of meningitis induced rat modal

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameters</th>
<th>Control group (n=8)</th>
<th>Infected group (n=8)</th>
<th>Infected + treated group (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Glucose (mg/dl)</td>
<td>22.2 ± 1.89</td>
<td>9.07 ± 0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.2 ± 1.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>b</td>
<td>Total protein (mg/dl)</td>
<td>0.59 ± 0.03</td>
<td>1.51 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>c</td>
<td>Calcium (mg/dl)</td>
<td>7.28 ± 0.10</td>
<td>15.17 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.96 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>d</td>
<td>Phosphorus (mg/dl)</td>
<td>2.58 ± 0.07</td>
<td>4.42 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.96 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed in Mean ± SD. Value in parenthesis represent % changes in control vs infected treated group as well as infected vs Immunox-V treated group (Infected plus treated group). Where a= *** p<0.001 (highly significant); b= ** p<0.01 (significant)

Results

The results of present study showed that a significant decrease (42.2%, p<0.001; 48.1%, p<0.001) in super oxide dismutase (SOD) and catalase activities in the cerebral spinal fluid (CSF) of infected group as compared to the control group. These enzymes activities were found to be statistically significant increased (64.4%; 57.1%; p<0.001) in infected plus treated group as compared to the infected group after administration of Immunox V drug for seven days treatment. These enzymes activities were significantly increased (p<0.001) in the infected plus treated group when compared to the control group. The activities were reached almost near to the normal level after treatment of Immunox -V in the infected plus treated group.

Similarly glutathione reductase activity (GR) was found to decreased significantly (45.0%, p<0.001) in the infected groups as compared to the control group. When infected group was compared to infected plus treated group, the activity was increased significantly (53.4%, p<0.001) in infected plus treated group after treatments of Immunox V drug for 7 days treatment. The activity was increased in the infected plus treated group and reached near to control group after treatment of respective drug.

The levels of MDA (malonaldehyde) were observed statistically significant (158%, p<0.001) increased in the infected group as compared to the control group. These levels were found to be decreased (59.4%, p<0.001) in the infected plus treated group after treatment of respective drug (Immunox V) when compared to the infected group. MDA levels were decreased in the infected plus treated group and reached almost near to normal level as compared to control group.

Xanthine oxidase (XO) and adenylate kinase (AK) activities were found to be statistically significant increased (329.5%, p<0.001; 48.5%, p<0.001) in the infected group when compared to the control group. These enzyme activities were decreased significantly (29.4%, p<0.001;16.5%, p<0.01) in the infected plus treated group after treatment of fixed dose combination of ceftriaxone plus vancomycin drug (Immunox -V) as compared to meningitis induced group. These enzymes activities were lowered in the infected plus treated group and come back almost to the control group.

Glucose level was found to be lowered significantly (59%, p<0.001) in the CSF of infected group as compared to the control group. The level was elevated significantly (133.7%, p<0.001) in the CSF of infected plus treated group.
after treatment of immunox V drug and reached almost near to control level. Calcium and phosphorus levels were found to be increased significantly (108.4%, p<0.001; 71.3%, p<0.001) in the infected group as compared to the control group. After treatment of immunox-V for 7 days, the levels of calcium and phosphorus were decreased significantly (41%, p<0.001; 33.0%, p<0.001) in the CSF of infected plus treated group when compared to the infected group. These levels were decreased significantly and come back to near the control group.

Total protein level was increased significantly (155.9%, p<0.001) in the CSF of infected group as compared to the control group. When infected group was compared to the infected plus treated group, the protein level was found to be decreased (54.3%, p<0.001) and come back to the normal level after treatment of immunox-V drug for 7 days.

Discussion

Bacterial meningitis is the infection of the arachnoid membrane, subarachnoid space, and cerebrospinal fluid by bacteria. It is inflammation of the tissue covering the brain and spinal cord (the meninges). It is characterized by swelling of the meninges, increased pressure inside the skull blocks the flow of blood to the brain, starving the brain of nutrients and oxygen (27). Free radicals (super oxide, nitric oxide) are generated during meningitis infection (29,11). Under normal condition, xanthine oxidase is present as dehydrogenase which can not transfer electron to molecular oxygen. During ischemic condition, adenosine nucleotide pool is degraded to hypoxanthine and xanthine and simultaneously xanthine dehydrogenase is converted by selective proteolysis in to xanthine oxidase. Xanthine oxidase is free radical generating enzyme which acts on xanthine and hypoxanthine to produce uric acid and oxygen free radicals (Fig.1). Scheld et al reported that blood brain barrier altered during meningitis in the rat (27,10). Reese and Karnovsky investigated that cerebral capillary endothelium as the major site responsible for blood brain barrier (20). Increased permeability of the blood brain barrier (BBB) is a pathological hallmark in several neurological disorders (9). Oxidative stress causes the brain damage through disruption of BBB. The susceptibility of the BBB to oxidative damage may be due to the high influx of oxygen, relatively low antioxidant capacity, and the high membrane PUFA content (24). It has been suggested that loss of blood brain barrier integrity is free-radical mediated (1). Oxidation of the cell membrane is known to alter fluidity, enzyme activity, and transmembrane ion fluxes. It has been suggested that a free radical imbalance may influence the integrity of the BBB and causing increased permeability (24). This is supported by data from rat experiments showing that dietary enhancement with antioxidants protects against ischemia-mediated BBB disruption (25).

In the present study, all antioxidant enzymes activities were significantly decreased along with the increased adenylyate kinase and xanthine oxidase as well as MDA levels in infected group as compared to control group (Table 1). The levels of total protein and phosphorus were significantly increased along with decreased glucose level in infected group as compared to control group (Table 2). It means meningitis infection caused by bacteria, altered the antioxidant enzyme activities as well as blood brain barrier (glucose, total protein, calcium and phosphorus) and causes oxidative stress. The increased levels of malondialdehyde indicate that the destruction in the tight junctions of the endothelial monolayer of BBB and increases its permeability. The level of total protein increased in
the CSF was caused due to change in the permeability of blood brain barrier. Changes in the biochemical events, such as energy failure, membrane depolarization, brain edema, production of oxygen-free radicals, and lipid per-oxidation, may lead to brain dysfunction. The remarkable decrease in the level of glucose may be due to physiological functioning of the choroid epithelium as well as from consumption by bacterial infection. Calcium is tightly regulated within the extracellular and intracellular compartments of the central nervous system, involving processes that include transport mechanisms across the blood brain barrier (BBB) and cellular membranes, extensive binding by proteins and other macromolecules, and sequestration within a variety of intracellular organelles. Calcium level was also increased in the infected group as compared to control group. This data was supported that disruption of the blood brain barrier caused due to bacterial meningitis.

After administration of a fixed dose combination of ceftriaxone plus vancomycin (Immunox V) for one week treatment, the levels of XO and MDA along with antioxidant enzymes activities were improved and came back to normal level in infected plus treated as compared to infected group. It mean that Immunox V drug act as an antioxidant. Similarly the levels of total protein, calcium and phosphorus along with glucose were also improved in treated group when compared to infected group.

The BBB is highly susceptible to oxidative stress, and hydrogen peroxide is an important mediator of oxidative cell injury. Hypoxia/reoxygenation cause opening of the BBB and endothelial release of hydrogen peroxide, in turn, increases lipid peroxidation and accumulation of malondialdehyde. There are various report suggested that meningitis infection changes the enzyme activities (19) and blood brain barrier (30).

Immunox V drug is a fixed dose combination of ceftriaxone plus vancomycin antibiotic. Since ceftriaxone has been combined with vancomycin and this combination was shown to be very effective as first line antibiotic combination therapy in the treatment of several severe infection including those observed in meningitis patient's (6). Co-administration of ceftriaxone and vancomycin has been recommended as a standard choice for initial treatment of presumed pneumococcal meningitis. Ceftriaxone is cephalosporin class antibiotic. It is thioether group containing antibiotics which are very effective in preventing the free radical-mediated oxidation of sulphydryl group (2). It has been also reported that cephalosporins protects against HOCl-driven oxidative injury, this defense is a consequence of a direct drug scavenging capacity towards HOCl (13). Vancomycin is glycopeptide which contain phenolic ‘OH group. Like other antibiotic (amoxicillin and cefadroxil), it also has powerful antioxidant which plays a significant role in the inhibition of bacterial expression of counter agents such as beta-lactamases (7).

Chemical vector mediated technology is used to provides compatibility of cephalosporins and glycopeptide without interfering in the pharmacokinetic property of drug component and prevents the oxidation of methionine group and thiazolidine and dihydrothiazine present in antibiotics (5). The component(s) of chemical vector having free radical scavenging potential that leads to reduction in oxidative stress.

Conclusions
The study concluded that a fixed dose combination of ceftriaxone and vancomycin (Immunox V) having free radical scavenging properties which is
helpful in the prevention of bacterial meningitis, reduces oxidative stress by improving the antioxidant enzymes activities and prevent in the alteration of blood brain barrier which is caused due to bacterial infection.

Acknowledgement
Authors are thankful to Dr. Vijay Naithani for valuable suggestions and also financial department of Venus Medicine Research Centre, Baddi, H.P for financial support.

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Article History:---------------------------
Date of Submission: 22-01-10
Date of Acceptance: 11-03-10
Conflict of Interest: NIL
Source of support: NONE