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Antioxidant and Blood Brain Barrier Status in Cerebrospinal Fluid of Bacterial Meningitis Rat Model after Vancoplus Treatment

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The aim of this study was to compare the efficacy of ceftriaxone, vancomycin and its fixed dose combination of ceftriaxone plus vancomycin (Vancoplus) for the treatment of meningitis induced by methicillin-resistant *Staphylococcus aureus* (MRSA) in rat model. The MRSA strain ATCC 43300 was used to induce meningitis in rat model. The rats were fed standard pelleted diet and water *ad libitum*. Thirty rats were divided into five groups containing six rats in each group. The vancomycin group received 14.2 mg kg⁻¹ b.wt./day, the ceftriaxone group received 28.57 mg kg⁻¹ b.wt./day, the vancoplus group received 42.8 mg kg⁻¹ b.wt./day, control and infected group received normal saline. Present findings showed that activities of antioxidant enzymes such as superoxide dismutase and catalase were significantly increased ($p < 0.001$) along with decreased ($p < 0.001$) in lipid peroxidation (malondialdehyde) level in vancoplus treated group as compared to ceftriaxone and vancomycin. The level of adenylate kinase and xanthine oxidase enzymes also become lowered in vancoplus treated group as compared to ceftriaxone and vancomycin. The levels of total protein, calcium and phosphorus were also increased significantly ($p < 0.001$) along with decreased ($p < 0.001$) in glucose level in cerebral spinal fluid of infected group as compared to control group. After treatment with vancoplus, levels of total protein, calcium and phosphorous become reduced along with raised in glucose level as compared to ceftriaxone and vancomycin group. These findings indicate that vancoplus is more effective than ceftriaxone and vancomycin alone for improvement of oxidant and antioxidant levels, it also crosses the blood brain barrier more faster than ceftriaxone and vancomycin alone and cure bacterial meningitis.

Key words: Meningitis, methicillin-resistant *Saphylococcus aureus*, ceftriaxone plus vancomycin, blood brain barrier, antioxidant

INTRODUCTION

Bacterial meningitis found worldwide which is an important infection of the Central Nervous System (CNS) that leads to serious neurological sequelae, including cognitive impairment (Koedel *et al.*, 2002; Beek *et al.*, 2002). According to a World Health Organization, about 171,000 people worldwide die from bacterial meningitis every year. Even with antimicrobial treatment, fatality rates are as high as 5 to 10% in the developed world. The incidence and mortality rates are much higher in third-world countries. Between 10 and 20% of those who do survive bacterial meningitis suffer permanent damage such as mental retardation, deafness, or epilepsy (Porter, 2004).

Bacterial meningitis can also cause seizures, hearing loss, mental changes and a wide variety of other complications. People of any age are susceptible to bacterial meningitis infections. The bacteria often live harmlessly in a person's mouth and throat. In rare instances, however, they can break through the body's immune defenses and travel to the fluid surrounding the brain and spinal cord. There they begin to multiply quickly. Soon, the thin membrane that covers the brain and spinal cord (meninges) becomes swollen and inflamed, leading to the classic symptoms of meningitis. Meningitis is usually caused by an infection with a virus or a bacterium. Bacterial meningitis can be a serious medical emergency. Many different types of bacteria can cause bacterial meningitis. In newborns, the most common causes are Group B. *streptococcus*, *Escherichia coli* and *Listeria monocytogenes*. But in older children, meningitis is caused by *Streptococcus pneumoniae* (pneumococcus) and *Neisseria meningitidis* (meningococcus).

Levels of antioxidant and oxidant become imbalanced during bacterial meningitis and produced free radical and oxidative stress (DeMenezes *et al.*, 2009; Aycicek *et al.*, 2006, 2007). Caksen (2003) reported comparative antioxidant status between acute bacterial meningitis and encephalitis. In childhood meningitis it has been reported that natural or synthetic antioxidants might prevent disease progression, tissue damage and distributed blood brain barrier (Ray *et al.*, 2000).

The blood-brain and blood-cerebrospinal fluid (CSF) barriers, situated at the cerebral capillaries and the choroid plexuses, form an impediment to the movement of solutes from blood to brain. These barriers are formed, respectively, by the capillary endothelial cells and the plexus epithelial cells. As well as acting as passive permeability barriers, both tissues have numerous transport systems. Such systems are necessary to circumvent the barriers to move nutrients like glucose into

the brain, to remove waste products from the brain and to provide a stable brain micro environment. The cerebrospinal fluid (CSF) must be examined for general appearance, consistency and tendency to clot. CSF analysis should include cell counts (including a WBC differential), glucose and protein analysis and Gram staining of the centrifuged sediment. The use of C-reactive protein levels has been shown to play an important role in differentiating among the various types of meningitis. Spiro and Spiro (2004) reported that polymerase chain reaction has a high sensitivity for detection of viral meningitis as well as bacterial meningitis.

Although, more and more new powerful antibiotics have been used, mortality and neurologic deficits still occur frequently following bacterial meningitis (Pelkonen *et al.*, 2009; Coimbra *et al.*, 2007). Though vancomycin hydrochloride, a glycopeptide antibiotic and the drug of choice in the treatment of methicillin-resistant *S. aureus* infections, is highly efficacious, patients presenting themselves with mixed infections require concomitant therapy with a second antibacterial agent proven to be reliably effective against gram-negative bacteria (Toyoguchi *et al.*, 1997). Friedland (1993) recommended that combination of third-generation cephalosporins and vancomycin is used for the treatment of bacterial meningitis. Kaplan and Mason (1998) also reported that combination of ceftriaxone or cefotaxime with vancomycin is recommended by many experts for the treatment of meningitis caused by resistant strains. Investigators have also noted that combinations of glycopeptides and β -lactams can demonstrate additive or synergistic activity against MRSA isolates in *in vitro* tests, since they act as inhibitors at different stages of cell wall synthesis (Barr *et al.*, 1990; Sieradzki and Tomasz, 1997a, b). Sieradzki and Tomasz (1997a, b) reported that synergistic combination of vancomycin and β -lactams was used against GISA strains. The aim of this study was to assess the status antioxidant, oxidant and blood barrier parameters in the cerebrospinal fluid after the treatment of vancoplus.

MATERIALS AND METHODS

Study conduct: The study was carried out from 12th November 2008 to 20th February 2009 in Pre-clinical Unit of Venus Medicine Research Centre, Venus Remedies Ltd. Baddi (India).

Study chemicals: All the biochemicals used in the present study were procured from Sigma, St. Louis, MO, USA. Other chemicals purchased locally were of analytical

grade. The antibiotics ceftriaxone, vancomycin and its fixed dose combination vancoplus were obtained from Venus Remedies Ltd., India. The study was conducted in Pre-chemical Lab of Venus Medicine Research Centre, Venus Remedies Ltd., Baddi (India). The ratio of fixed dose combination of ceftriaxone+vancomycin in vancoplus were 2:1.

Meningitis model: Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 bacterial organism was purchased from IMTECH Chandigarh, India. Meningitis infection was induced by direct intracisternal injection of 25 μ L of saline containing $\log 10^6$ cfu mL^{-1} MRSA strain via a 24-gauge needle. Meningitis infection was induced in rat after ten days.

Experimental animals and treatments: Thirty albino rats (weighing 60 ± 5 g) were used in the experiment. The rats were fed standard pelleted diet and water *ad libitum*. The test room was air conditioned with temperature $23 \pm 2^\circ\text{C}$, humidity $65 \pm 5\%$ and with artificial fluorescent light (10-14 h of light and dark, respectively). This study was approved by Institutional Animal ethics committee. The rats were divided into five groups of six rats each as given below:

- **Group-I (n = 6):** Control (Normal Saline)
- **Group-II (n = 6):** Infected group treated with normal saline
- **Group-III (n = 6):** Ceftriaxone (28.57 mg kg^{-1} b.wt./day)
- **Group-IV (n = 6):** Vancomycin (14.2 mg kg^{-1} b.wt./day)
- **Group-V (n = 6):** Ceftriaxone-vancomycin (42.8 mg kg^{-1} b.wt./day)

The doses of above antibiotics was given as 10 mg mL^{-1} . The respective drugs were administered intravenously for 7 days according to body weight after 8 h interval in twice a day. At the end of treatment, the CSF was collected with a heparinized microtube (MICROPET. Becton, Dickson and Company) through a small hole made by a 23 g syringe needle. Thus, 100-120 μ L of CSF per rat could be readily obtained without mixture of the blood (Nirogi *et al.*, 2009).

Enzyme assays

Superoxide dismutase (SOD) assay: The reaction mixture composed of 1.0 mL carbonate buffer (0.2 M, pH 10.2), 0.8 mL KCl (0.015 M), 100 μ L of blood and 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 sec interval for 1 min 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 (Misra and Fridovich, 1972).

Catalase assay: The reaction mixture consisted of 0.3 mL phosphate buffer (0.2 M pH 6.8), 0.1 mL H_2O_2 (1 M) and water to make the final volume to 3.0 mL. The reaction was started by adding the suitable aliquot of enzyme preparation. The change in the absorbance was recorded at 15 sec interval for 1 min 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 H_2O_2 in 100 sec at 25°C (Luck *et al.*, 1965).

Measurement of lipid peroxidation: Free radical mediated damage was assessed by the measurement of the extent of lipid peroxidation in the term of malondialdehyde (MDA). It was determined by thiobarbituric reaction. The reaction mixture consisted of 100 μ L of diluted blood, 0.20 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% thio barbituric acid (TBA) and water to make up the volume to 4.0 mL. The tubes were boiled in water bath at 95°C for 1 h 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 min. The upper layer was aspirated out and optical density was measured at 532 nm. The reference standard used was 1,1, 3,3 tetraethoxypropane (Ohkawa *et al.*, 1979).

Glutathione reductase assay: GR activity to be measured by Carlberg and Mannervik (1985). 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 to measured at 340 nm wave length for 1 min 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 1 min by 1 mL of enzyme preparation using extinction coefficient of 6.22.

Adenylate kinase assay: Adenylate Kinase (AK) activity was determined by method of Haslam and Mills (1967) with minor modification. The reaction mixture consisted of

ADP 0.30 mL (4.0 mM), 0.55 mL of glucose (10 mM), 0.55 mL of $MnCl_2$ (10 mM), 0.30 mL of NADP (0.2 mM), 0.58 mL of Tris buffer (50 mM, pH 7.4), 10 μ g of hexokinase (10 unit), 10 μ g of glucose 6 phosphate dehydrogenase (1 unit) and added distilled water to make up 3.0 mL. The reaction was started by adding 20 μ L of CSF sample. Change in absorbance was recorded at 340 nm at 15 sec interval for 1 min. Suitable control was run simultaneously. One unit of AK activity in the forward direction was defined as 1 μ L 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 (1967). The assay mixture consisted of 0.30 mL Tris-HCl buffer, 50 mM (pH 7.4); 0.30 mL $CuSO_4$ (10 mM); 0.05 mL Xanthine, (2.58 mM mL^{-1} 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 sec interval for 1 min. Suitable control was run simultaneously. One unit of activity has been defined as change in absorbance at 290 nm in 1 min by 1 mL of CSF.

Total protein, glucose, calcium and phosphorous: These biochemical parameters were estimated by using commercially available standard kit (Bayer Diagnostics India Ltd., Baroda, Gujrat India).

Histology: Histology of meningeal cells was done by Conventional method of Hematoxylin and Eosin (H and E) staining protocol.

Statistical analysis: The resulting data was analyzed statistically. One-way analysis of variance (ANOVA) with student-Newman-Keuls comparison test was used to determine statistical difference between control and treated groups. The p-values <0.05 were considered statistically significant.

RESULTS

Animals of similar weight were placed in each treated group. Meningitis was occurred in infected group and its treatment with vancoplus has been shown in Fig. 1a-c. A significant ($p < 0.01$) decrease in superoxide dismutase and catalase activities in blood of rat were found in vancomycin and ceftriaxone treated groups as compared to control group. These activities were found to be increased significantly in vancoplus treated group ($p < 0.001$) as compared to ceftriaxone and vancomycin alone treated groups respectively (Table 1). Significant increase in MDA level in blood of mice were found in vancomycin ($p < 0.001$) and ceftriaxone ($p < 0.01$) alone treated groups as compared to control group. While in case of vancoplus, the MDA level was lowered significantly ($p < 0.001$) as comparison to ceftriaxone and vancomycin alone treated group and almost come near to normal level when compared to control group (Table 1).

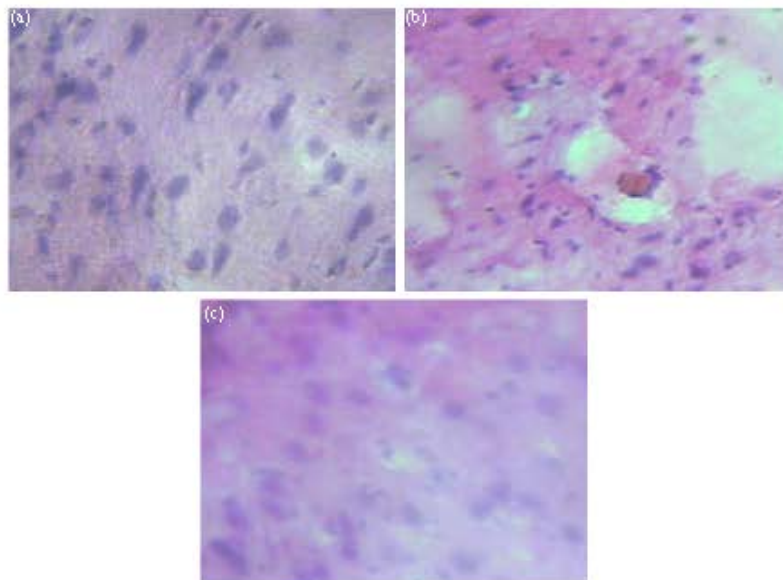


Fig. 1: Systematic presentation of meningitis and its treatment with vancoplus; (a) control (b) infected cell and (c) treated with vancoplus

Table 1: Comparison of oxidant and antioxidant enzymes of control, infected, ceftriaxone, vancomycin and the fixed dose combination of ceftriaxone plus vancomycin (vancoplus) treated groups in cerebrospinal fluid of rat meningitis model

Parameters	Control group	Infected group	Ceftriaxone treated group (n = 6)	Vancomycin treated group	Vancoplus treated group
SOD (mMole/min/mL)	425.520±1.57	240.280±2.49	255.930±2.76**	310.470±1.49**	419.860±2.41***
Catalase (mMole/min/mL)	194.150±1.48	86.460±1.48	117.450±1.22**	158.750±1.18**	188.450±1.57***
GR (mMole/min/mL)	0.547±0.027	0.179±0.080***	0.254±0.002**	0.328±0.016**	0.517±0.08***
MDA (nmole/mL)	6.480±0.12	19.650±1.28***	11.470±1.04*	8.490±0.17*	6.190±0.37ns
XO (unit/min/mL)	0.451±0.14	1.970±0.34***	1.240±0.37**	1.040±0.37**	0.428±0.18***
AK (mMole/min/mL)	165.280±2.89	264.150±2.49***	196.450±2.56**	167.590±3.47**	159.410±2.18***

SOD: Superoxide dismutase; GR: Glutathione reductase; MDA: Malonaldehyde; XO: Xanthine oxidase; AK: Adenylate kinase. Values are expressed in Mean±SD. ***p<0.001; **p<0.01; p<0.05; ns: Not significant

Table 2: Comparison of blood brain barrier of control, infected, ceftriaxone, vancomycin and the fixed dose combination of ceftriaxone plus vancomycin (vancoplus) treated groups in cerebrospinal fluid of rat meningitis model

Parameters	Control group	Infected group	Ceftriaxone treated group (n = 6)	Vancomycin treated group	Vancoplus treated group
Glucose (mg dL ⁻¹)	23.48±1.65	10.29±0.72	15.89±0.49**	19.26±0.67**	22.29±1.70***
Total protein (mg dL ⁻¹)	0.67±0.12	2.36±0.48	1.26±0.37**	0.98±0.37**	0.70±0.02***
Calcium (mg dL ⁻¹)	8.15±0.15	17.28±0.19	12.48±0.15**	10.39±0.28**	8.26±0.14***
Phosphorus (mg dL ⁻¹)	3.41±0.16	6.19±0.17	5.68±0.19**	4.67±0.27**	3.34±0.16***

Values are expressed in Mean±SD. ***p<0.001; **p<0.01

Similarly Glutathione Reductase (GR) activity in CSF was found to decreased significantly (p<0.001) in the infected groups as compared to the control group. Its activity slightly increased in ceftriaxone and vancomycin treated group (p<0.01) when compared to infected group. When infected group was compared to vancoplus treated group, the activity was increased significantly (p<0.001) and reached near to control level (Table 1).

Xanthine oxidase (XO) and adenylate kinase (AK) activities in CSF were found to be statistically significant increased (p<0.001) in the infected group when compared to the control group. It activity slightly decreased in ceftriaxone and vancomycin treated group (p<0.01) when compared to infected group. These enzyme activities were decreased significantly (p<0.001) in vancoplus treated group as compared to meningitis induced group and come back to control group (Table 1).

Glucose level was found to be lowered significantly (p<0.001) in the CSF of ceftriaxone and vancomycin treated group as compared to the control group. The glucose level in the CSF of vancoplus treated group was increased significantly (p<0.001) and reached almost near to control level. Calcium and phosphorus levels in the CSF of ceftriaxone and vancomycin treated group were increased significantly (p<0.001) when compared to the control group. After treatment of vancoplus for 7 days, the levels of calcium and phosphorus were decreased significantly (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 (Table 2).

Total protein level in the CSF of ceftriaxone and vancomycin treated group was increased significantly (p<0.001) as compared to the control group. When

ceftriaxone and vancomycin treated group was compared to the vancoplus treated group, the protein level was found to be lowered (p<0.001) and come back to the normal level (Table 1).

DISCUSSION

Bacterial meningitis is inflammation of the tissue covering the brain and spinal cord called 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. Free radicals such as super oxide, nitric oxide are generated during meningitis infection (Van Furth *et al.*, 1996; Klein *et al.*, 2006). The blood brain barrier (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 malonaldehyde. There are various report suggested that meningitis infection changes the enzyme activities (Ray *et al.*, 2000) and blood brain barrier (Quagliarello *et al.*, 1986).

In the present study, all antioxidant enzymes activities were significantly decreased along with the increased XO and MDA levels in infected group as compared to control group. The increased levels of malondialdehyde also indicate 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 were 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.

Co-administration of ceftriaxone and vancomycin has been recommended as a standard choice for initial treatment of presumed pneumococcal meningitis since the mid-1990. Synergistic interaction of this combination was documented in the rabbit model (Friedland *et al.*, 1993) and in the CSF of the children with meningitis (Klugman *et al.*, 1995). It has been reported that fixed dose combination of ceftriaxone plus vancomycin reduces nephrotoxicity and acts as free radical scavengers (Soni *et al.*, 2009b). It has been also reported that the single individual administration of either vancomycin or ceftriaxone causes nephrotoxicity and hepatotoxicity due to induction of oxidative stress (Soni *et al.*, 2009a). Similar studies have also been reported with fixed dose combination of cephalosporin and aminoglycosides in presence of chemical vector that prevent oxidative stress against tissue injury (Chaudhary *et al.*, 2008).

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. The levels of total protein, calcium and phosphorus were significantly increased along with decreased glucose level in infected group as compared to control group. 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.

Ceftriaxone possesses a broad spectrum of antimicrobial activity including aerobic gram-positive, gram-negative bacteria and also a few anaerobic bacteria (Fritsche *et al.*, 2003; Rawls *et al.*, 2008; Chakraborty *et al.*, 2005). The drug is widely used in the treatment of infections caused by microorganisms resistant to conventional therapy or as an alternative to antibiotics with a low therapeutic index. It is also known to cause drug induced toxicities. It has also been reported that single ceftriaxone therapy induces lipid peroxidation level in liver of goat (Chakraborty *et al.*, 2005). Fekety (1990) reported that bleeding is probably the most serious side effect of cephalosporins. Ceftriaxone can also causes drug-induced gallstones. The potential of erythromycin and several other macrolides to cause hepatitis is well established (Roland *et al.*, 1991).

Vancomycin is reserved for the treatment of multi-drug resistant Staphylococcal infections that are life threatening. The typical indications are staphylococcal, enterococcal, or streptococcal infections: sepsis, endocarditis, joint infection. It is a toxic drug and its usage is best left to medical specialists. Unfortunately it

is also known that vancomycin cause nephrotoxicity in patients (Hodoshima *et al.*, 2004; Rybak *et al.*, 1990). Generation of free radicals and oxidative stress cause nephrotoxicity. There are several reports on toxic potential of antibiotics. Vancomycin is manifested by vestibular damage and/or cochlear damage, which leads to sensory hearing loss and tinnitus (Roland *et al.*, 1991). Duffull and Begg (1994) reported that nephrotoxicity is generally caused by the accumulation of the drug in renal cells. Creekmore (1998) also reported that vancomycin-induced nephrotoxicity were generally higher than the accepted therapeutic range. However, given drug's dependence on renal elimination, vancomycin concentrations would be expected to be elevated in patients with renal dysfunction.

Vancoplus 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 (Chaudhary *et al.*, 2008). 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 sulfhydryl group (Klein *et al.*, 2006). 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 (28). 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 (Quagliarello *et al.*, 1986).

The study concluded that by using chemical vector mediated technology, a fixed dose combination of ceftriaxone and vancomycin (vancoplus) having free radical scavenging properties which may be prevent form bacterial meningitis and improves the antioxidant enzymes activities. It may also be concluded that vancoplus crosses blood brain barrier more faster than ceftriaxone and vancomycin alone and effective for the treatment of bacterial meningitis.

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