

Effect of New Compound with Antioxidant Potential in the Energy Metabolism of Adults Rats after Pneumococcal Meningitis

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Abstract: Problem statement: In bacterial meningitis, the *Streptococcus pneumoniae* can modify the cerebro-spinal fluid (CSF) homeostasis and initiation of the host inflammatory response with presence cytokine and leukocyte migration into the subarachnoidal space. Despite the availability of highly effective antibiotics, the disease is often fatal or causes long-term neurological problems in affected patients. **Approach:** The aim of this study was to evaluate the effect of this new compound P0801 in the oxidative stress, mitochondrial respiratory chain and brain Creatine Kinase activity (CK) in adult rats after meningitis by *S.pneumoniae*. **Results:** In our study, the group of animals with pneumococcal meningitis that received P0801, with or without antibiotic therapy at 24 h after induction decreased protein carbonyls in cerebral cortex and in the hippocampus at 48 h after induction, being that the same time decreased lipid peroxidation. In the animals that received antibiotic therapy and or treatment with P0801 at 24 h after induction of meningitis, there were increased by the activity of complex I, II, IV and enzyme succinate dehydrogenase in cerebral cortex, being that at 48 h in the group that received antibiotic therapy and or P0801, there were increased by the activity of complex I, II and succinate dehydrogenase in the cerebral cortex and or hippocampus. In our study, the CK activity was increased in hippocampus and cerebral cortex at 24 h after induction meningitis in the group that received antioxidant treatment and only in cerebral cortex in the group that received antibiotic therapy with antioxidant treatment. **Conclusion/Recommendations:** The study with new compound is an attempt to coadjuvant treatment with the antibiotic to minimize the oxidative damage and energy metabolism during illness. New studies are needed to clarify the action of this new compound as coadjuvant, evaluating side effects and their formulation, since it might look promising in the development of novel therapeutic agents in further studies.

Key words: *Streptococcus pneumoniae*, meningitis, energy metabolism, oxidative damage, antioxidant treatment, cerebral cortex, hippocampus

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INTRODUCTION

Pneumococcal Meningitis (PM) are characterized by an intense inflammatory host reaction of the central nervous system that contributes to the development of cortical necrosis and hippocampal apoptosis (Bellac *et al.*, 2006). The highest rate of learning deficits in founding in meningitis caused by *Streptococcus pneumoniae* compared with other infecting agents as *Haemophilus influenza* or group B Streptococcus (Merkelbach *et al.*, 2000; Bedford *et al.*, 2001; Beek *et al.*, 2002). The *S. pneumoniae* is the cause of the most severe and most frequent form of adult bacterial meningitis. The microorganism can modify the cerebro-spinal fluid (CSF) homeostasis and initiation of the host inflammatory response with presence cytokine and leukocyte migration into the subarachnoidal space (Quagliarello *et al.*, 1991; Ostergaard *et al.*, 2000; Koedel *et al.*, 2002). Because of this may occur increase the release of metabolites proteolytic as reactive oxygen species (ROS), resulting in endothelial damage and increased permeability of blood brain barrier (BBB). The production of ROS can contribute neuroinflammatory process and has an important role in the pathophysiology of bacterial meningitis can cause inhibition of activity of complexes of the enzymes of mitochondrial respiratory chain, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of Na⁺, K⁺-ATPase and decreased in cellular energy metabolism (Cuzzocrea *et al.*, 2001). The inflammatory host response can to contribution altered brain physiology, BBB breakdown, brain edema, increasing intracranial pressure and brain injury (Brandt, 2010). Inflammation contributes to morbidity and mortality, but is itself unresponsive to antibiotics. Therapy with antibiotics is only partially effective in preventing mortality and development of neurological sequelae caused by pneumococcal meningitis. Despite the availability of highly effective antibiotics, the disease is often fatal or causes long-term neurological problems in affected patients (Baraff *et al.*, 1993; Grimwood *et al.*, 1995). Therefore, new adjunctive therapies that may modulate the inflammatory process are needed (Koedel *et al.*, 2002; Scheld *et al.*, 2002).

In this study, was to evaluate the effect of a new compound P801, containing fatty acids with poly-L-lysine, cysteine, retinoic acid, coenzyme Q10, ascorbic acid, taurine, methionine and alpha-tocopherol with potential antioxidant activity in the oxidative stress, mitochondrial respiratory chain and brain Creatine kinase activity in adult rats after meningitis by *S. pneumoniae*.

MATERIALS AND METHODS

Animals: Male Wistar rats (300 g of body weight) were obtained from our breeding colony. The animals were

housed five to a cage with food and water available *ad libitum* and were maintained on a normal 12-h light/dark cycle. All procedures were approved by the Animal Care and Experimentation Committee of the UNESC, Brazil and followed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

An animal model of meningitis: All surgical procedures and bacteria inoculations were performed under anesthesia, consisting of an intraperitoneal administration of ketamine (6.6 mg kg⁻¹), xylazine (0.3 mg kg⁻¹) and acepromazine (0.16 mg kg⁻¹) (Hoogman *et al.*, 2007; Bellac *et al.*, 2007; Grandgirard *et al.*, 2007). *S. pneumoniae* (ATCC 6303) was cultured overnight in Todd Hewitt broth and grown to logarithmic phase. In the morning of the experiment the culture was centrifuged for 10 min at (5000×g) and resuspended in sterile saline to the concentration 5×10⁹ CFU/mL (Grandgirard *et al.*, 2007; Irazuzta *et al.*, 2008). Rats underwent a cisterna magna tap with a 23-gauge needle. The position of the needle was verified by the free flow of clear cerebro-spinal fluid. CSF was withdrawn and the animals received either 10 µL of sterile saline as a placebo (sham) or an equivalent volume of the *S. pneumoniae*. At the time of inoculation, the animals received fluid replacement (10 mL of saline subcutaneously) and were returned to their cages (Irazuzta *et al.*, 2008). Following their recovery from anesthesia, the animals were supplied with food and water *ad libitum*. Meningitis was documented 16 h after induction of the meningitis by a quantitative culture of 5 µL of CSF obtained by puncture of the cisterna magna and cultured quantitatively on sheep blood agar plates to document that they had meningitis (Bellac *et al.*, 2007; Grandgirard *et al.*, 2007). The animals received antibiotic therapy beginning at 16 h after induction (ceftriaxone, 100 mg kg⁻¹ body weight twice a day intraperitoneally) and antioxidant treatment with a new compound P0801 (6,87 mg kg⁻¹ body weight twice a day intraperitoneally). The animals were killed by decapitation at 24 h and 48 h after inoculation (Bellac *et al.*, 2007; Grandgirard *et al.*, 2007). The brain was removed and hippocampus and cerebral cortex were isolated and stored at -80°C.

Determination of lipid peroxidation and carbonyl groups:

As an index of lipid peroxidation, we used the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction (Draper and Hadley, 1990). Briefly, the samples were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 30 min. TBARS were determined based on absorbance at 532 nm. The oxidative damage to

proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH), as previously described (Levine *et al.*, 1990). Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in DNPH and the absorbance was monitored at 370 nm.

Activities of mitochondrial respiratory chain enzymes: Hippocampus and cerebral cortex were homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250mM sucrose, 2mM EDTA, 10mM Trizma base, 50 IU mL⁻¹ heparin). The homogenates were centrifuged at 800×g for 10 min and the supernatants kept on-80°C until used for the enzyme activity determination. The maximal period between homogeneous preparation and enzyme analysis was always less than 5 days. Activities of mitochondrial respiratory chain enzymes: NADH dehydrogenase (complex I) was evaluated according to the method described by Cassina and Radi (1996) by the rate of NADH-dependent ferricyanide reduction at 420 nm. The activities of succinate-2,6-Dichloroindophenol (DCIP)- oxidoreductase (complex II) and Succinate Dehydrogenase (SDH) was determined by the method described by Fischer *et al.* (1985). Complex II activity was measured by following the decrease in absorbance due to the reduction of 2,6-DCIP at 600nm. SDH activity was measured by following the decrease in absorbance due to the reduction of 2,6-DCIP at 600nm in the presence of phenazine methasulphate. The activity of succinate: cytochrome c oxidoreductase (complex III) was determined according to the method of Fischer *et al.* (1985). Complex III activity was measured by cytochrome c reduction using succinate as substrate at 550nm. The activity of cytochrome c oxidase (complex IV) was assayed according to the method described by Rustin *et al.* (1994), measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm. The activities of the mitochondrial respiratory chain complexes were expressed as nmol. Min⁻¹. mg protein⁻¹.

Creatine Kinase (CK) activity assay: Creatine kinase activity was measured in brain homogenates pre-treated with 0.625 mM lauryl maltoside. The reaction mixture consisted of 60 mM Tris-HCl, pH containing 7.5, mM phosphocreatine, 9 mM MgSO₄ and approximately 0.4-1.2 µg protein in a final volume of 100 µL. After 15 min of pre-incubation at 37°C, the reaction was started by the addition of 3.2 µmol of ADP plus 0.8 µmol of reduced glutathione. The reaction was stopped after 10 min by the addition of 1 µmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 100 µL 2% µ-naphtol and 100 µL 0.05% diacetyl in a final volume of 1 mL and read

spectrophotometrically after 20 min at 540 nm. Results were expressed as nmol. min⁻¹. mg protein⁻¹.

Protein content: The protein content was determined by the method described by Lowry *et al.* (1951) using bovine serum albumin as standard.

Statistical analysis: The data oxidative stress were Analyzed Using Analysis of Variance (ANOVA) followed by post hoc Tukey and the data mitochondrial respiratory chain enzymes and Creatine Kinase (CK) activity were analyzed followed by Student's t test. All analyses were expressed as mean ± S.D. and were performed using the Statistical Package for the Social Science (SPSS) software version 17.0.

RESULTS

In the cerebral cortex of animals with pneumococcal meningitis decreased protein carbonyls in the group that received antioxidant treatment with new compound P0801, with or without antibiotic therapy at 24 h after induction. In the hippocampus there was not altered (Fig 1a). In the group that received only antibiotic therapy increased protein carbonyls in the hippocampus, however, in the group that received antioxidant treatment there was decreased protein carbonyls at 48 h after induction meningitis, being that in the cerebral cortex there was not altered (Fig. 1b).

In the group that received only antioxidant treatment increased lipid peroxidation in the cerebral cortex at 24 h after induction meningitis, being that in the hippocampus there was not altered (Fig. 2a). In the Fig 2b shows decreased lipid peroxidation in the group that received only antioxidant treatment in hippocampus at 48 h after induction meningitis, being that in the cerebral cortex there was not altered.

In the animals with pneumococcal meningitis that received antibiotic therapy or antioxidant treatment with new compound P0801 at 24 h after induction of meningitis, the activity of complex I in cerebral cortex was increased in the group that received antibiotic therapy and antioxidant treatment (Fig. 3a), being that in 48 h was increased in the group that received only antioxidant treatment (Fig. 4a). The activity of complex II in cerebral cortex was increased in the group that received antioxidant treatment at 24 h (Fig. 3b) and at 48 h in the hippocampus also (Fig. 4b). The activity of complex III there was not altered at 24 and 48 h after induction meningitis (Fig. 3c and 4c). The activity of complex IV was increased in the cerebral cortex at 24 h after induction meningitis in the group that received antioxidant treatment and in the group that received antioxidant treatment and antibiotic therapy (Fig. 3d). At 48 h there was not altered (Fig. 4d).

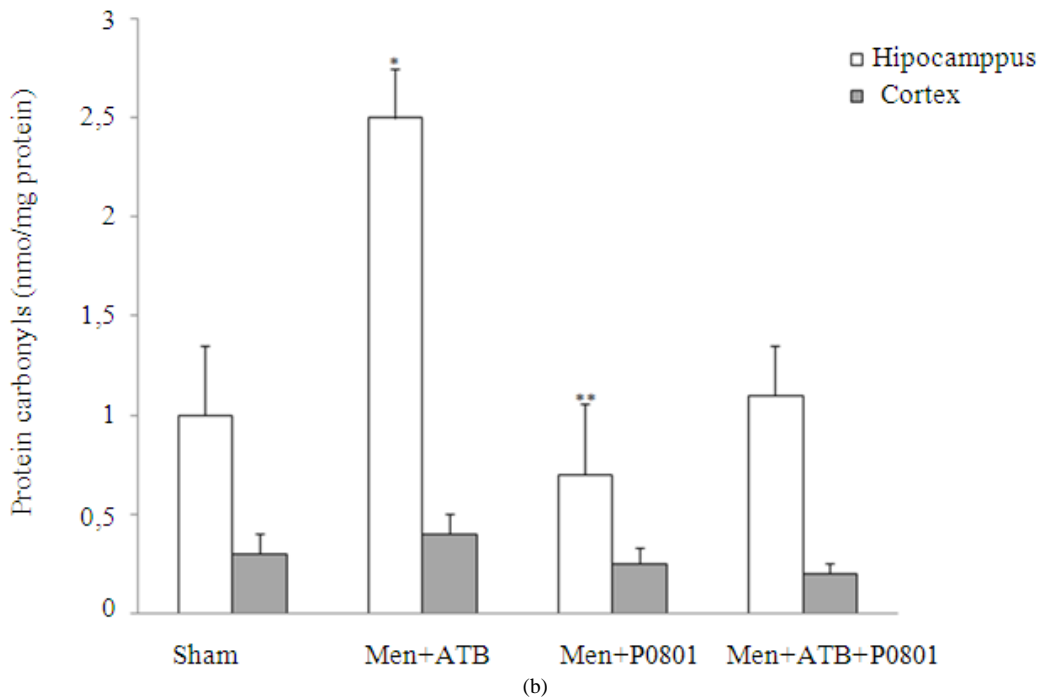
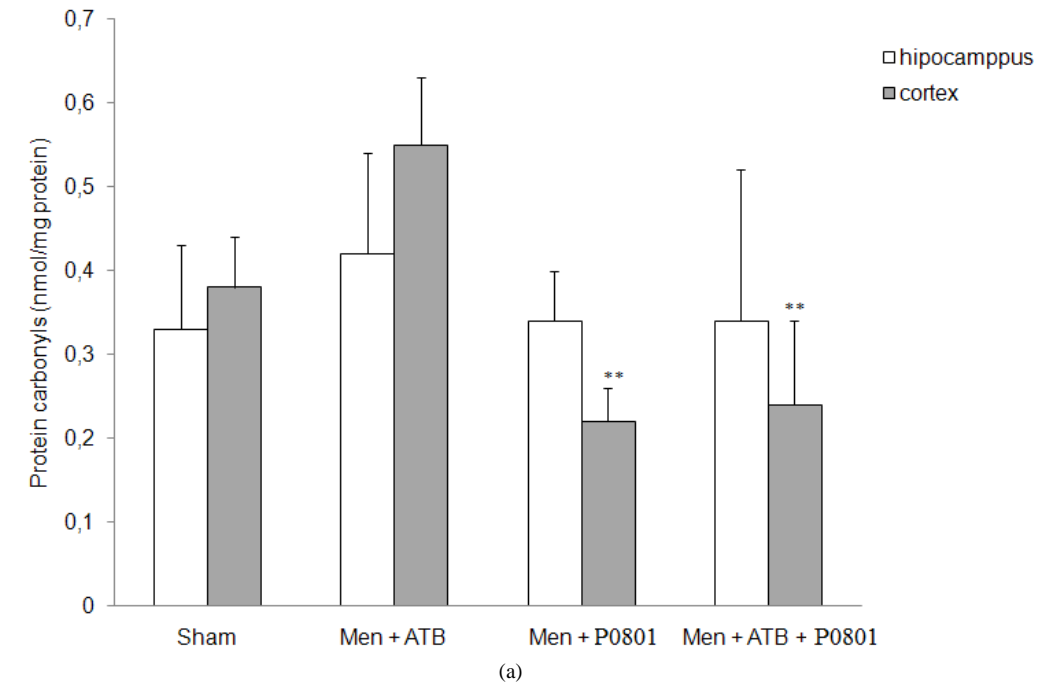


Fig. 1: Protein carbonyls in the hippocampus and cerebral cortex of rats after pneumococcal meningitis that received antibiotic therapy or antioxidant treatment with P0801 at 24 h (A) and at 48 h (B) after induction of meningitis. Results are expressed as mean \pm S.D. of 5 animals in each group. Symbols indicate statistically significant when compared with sham group * $p < 0.05$, ** $p < 0.05$ when compared with meningitis group that receive antibiotic therapy and *** $p < 0.05$ when compared with meningitis group that received antibiotic therapy and antioxidant treatment

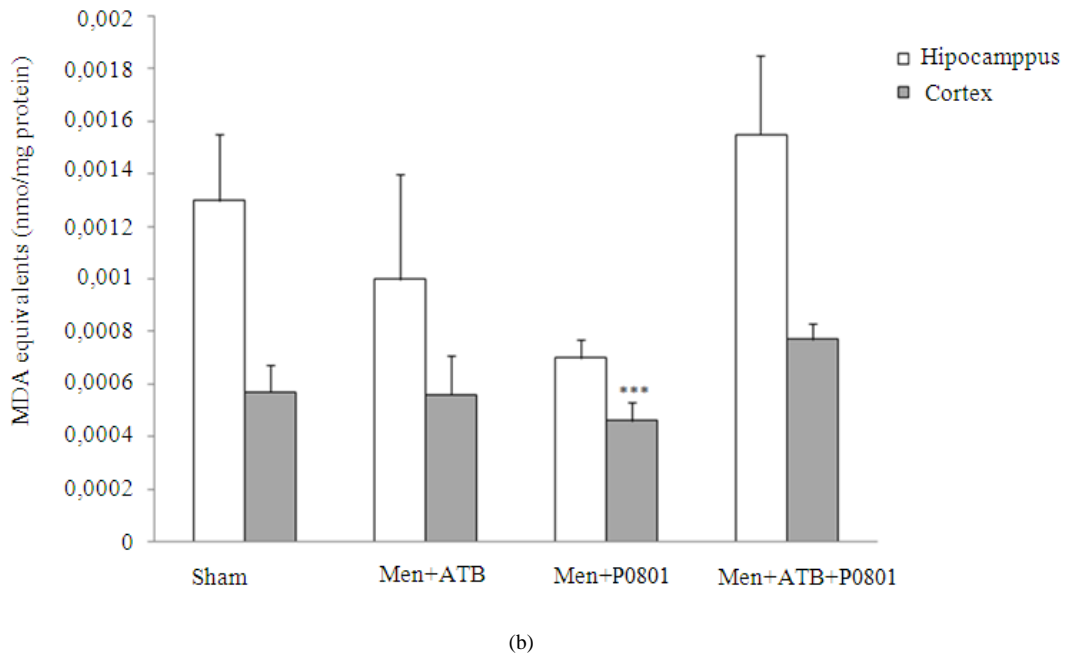
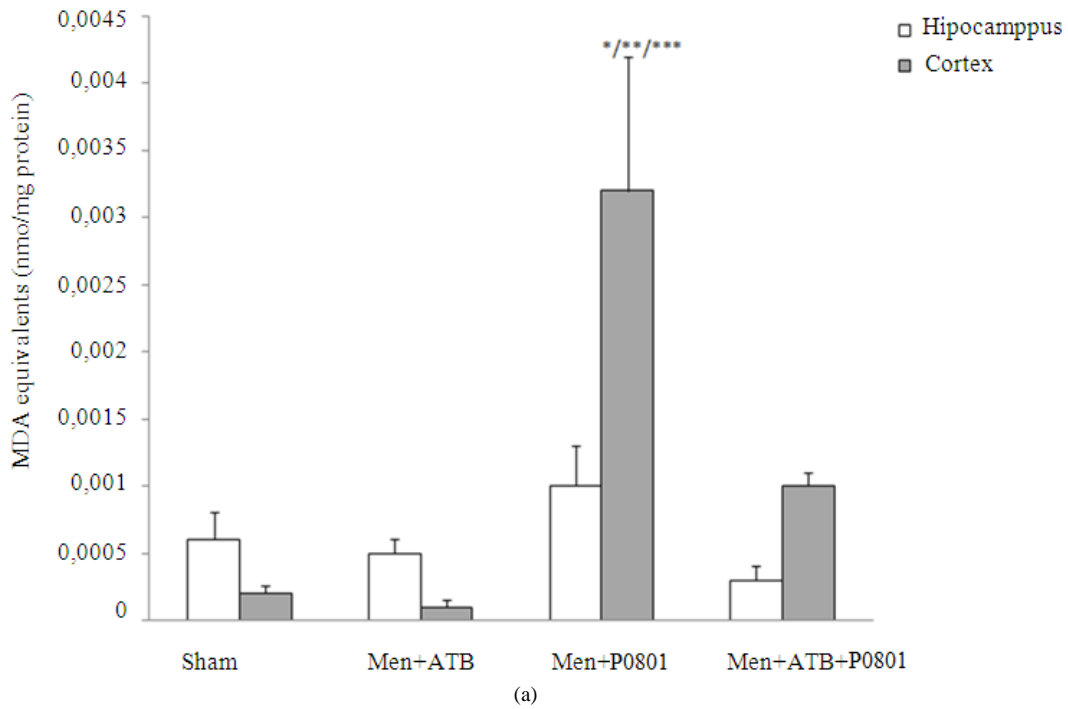
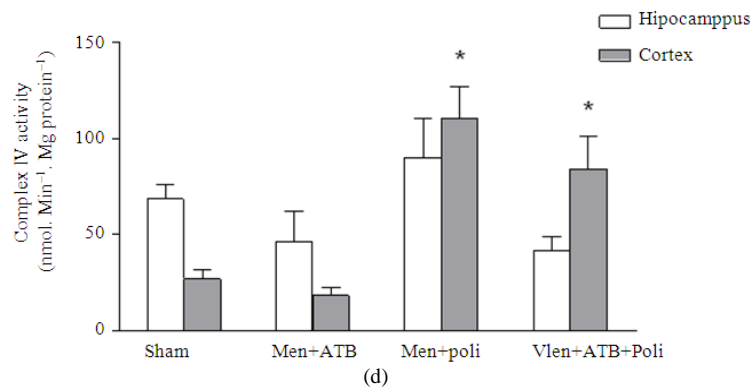
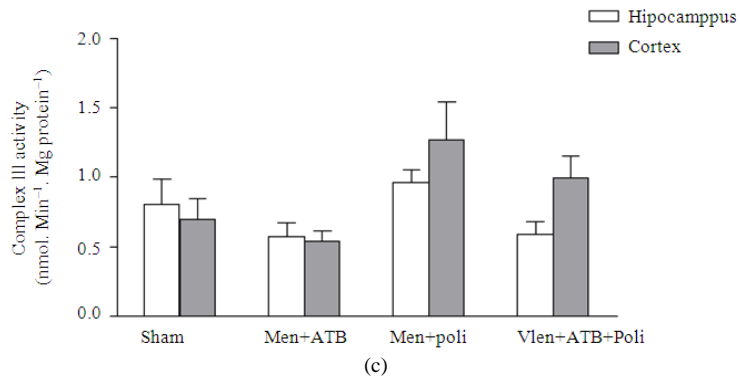
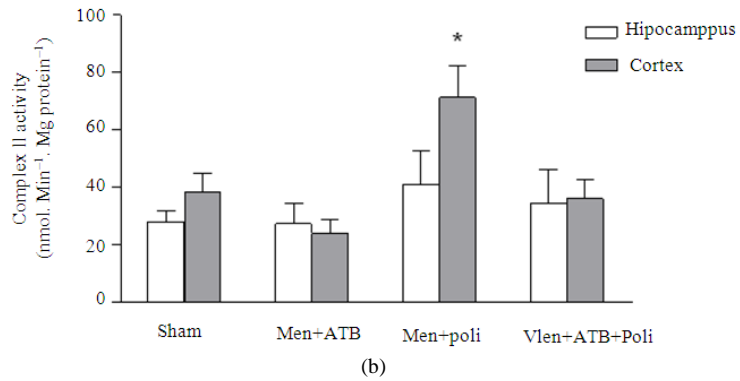
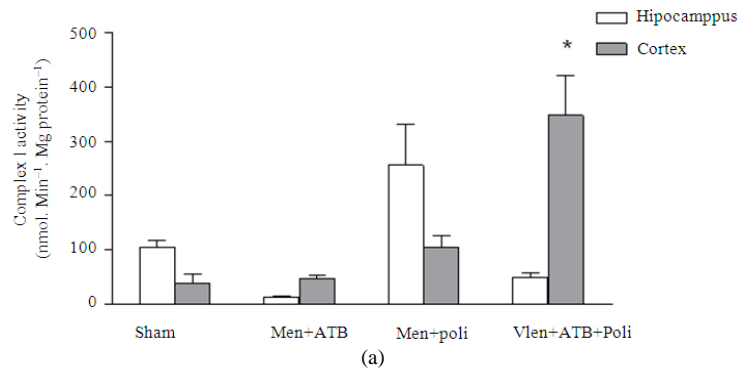


Fig. 2: Lipid peroxidation in the hippocampus and cerebral cortex of rats after pneumococcal meningitis that received antibiotic therapy or antioxidant treatment with P0801 at 24 h (A) and at 48 h (B) after induction of meningitis. Results are expressed as mean \pm S.D. of 5 animals in each group. Symbols indicate statistically significant when compared with sham group * $p < 0.05$, ** $p < 0.05$ when compared with meningitis group that receive antibiotic therapy and *** $p < 0.05$ when compared with meningitis group that received antibiotic therapy and antioxidant treatment



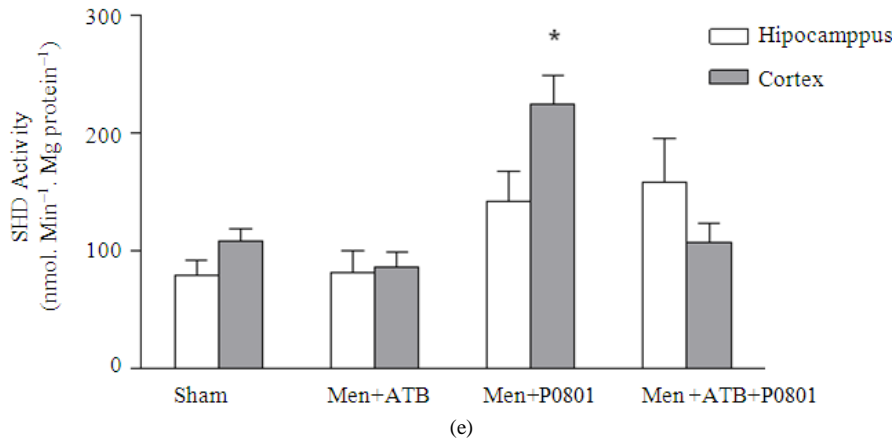
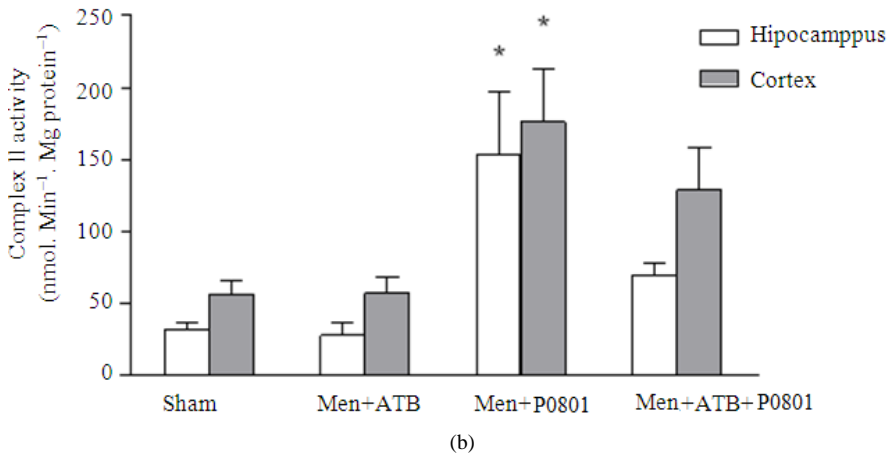
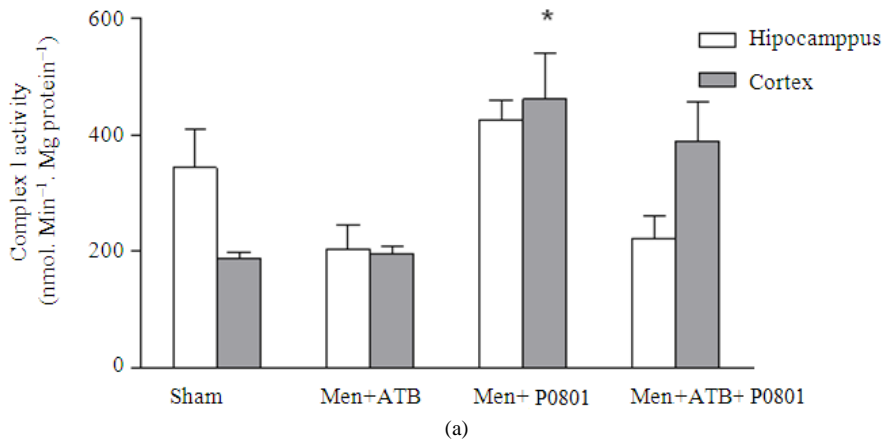


Fig. 3: Activity of complexes I (A), II (B), III (C), IV (D) and SDH (E) in the hippocampus and cerebral cortex of rats after pneumococcal meningitis that received antibiotic therapy or antioxidant treatment with P0801 at 24 h after induction of meningitis. Results are expressed as mean \pm S.D. of 5-7 animals in each group. Symbols indicate statistically significant when compared with sham group * $p < 0.05$



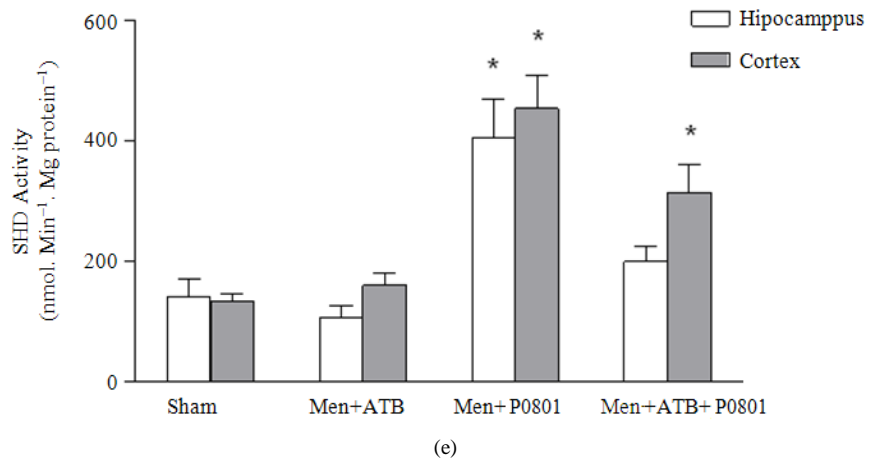
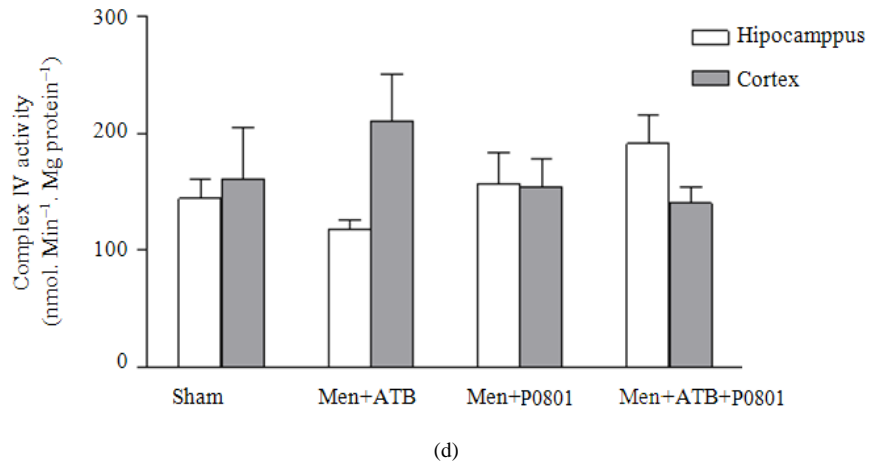
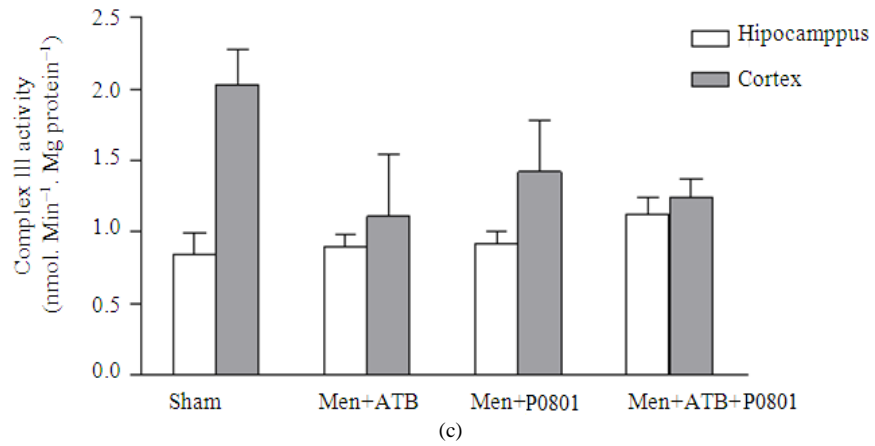


Fig. 4: Activity of complexes I (A), II (B), III (C), IV (D) and SDH (E) in the hippocampus and cerebral cortex of rats after pneumococcal meningitis that received antibiotic therapy or antioxidant treatment with P0801 at 48 h after induction of meningitis. Results are expressed as mean \pm S.D. of 5-7 animals in each group. Symbols indicate statistically significant when compared with sham group *p<0.05

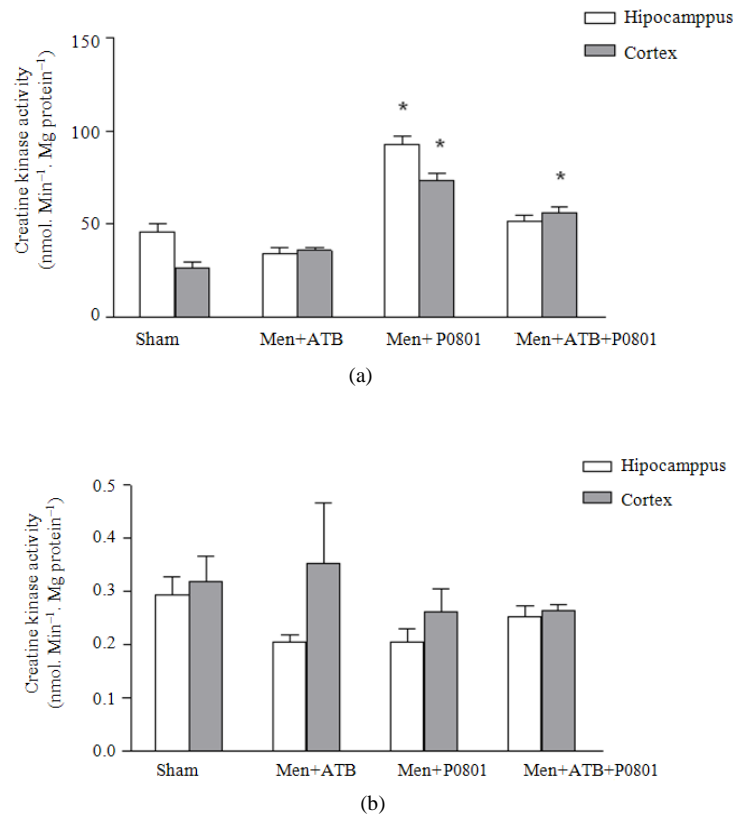


Fig. 5: Creatine Kinase (CK) activity in hippocampus and cerebral cortex of rats after pneumococcal meningitis that received antibiotic therapy or antioxidant treatment with P0801 at 24 h (A) and 48 h (B) after induction of meningitis. Results are expressed as mean \pm S.D. of 5-7 animals in each group. Symbols indicate statistically significant when compared with sham group * $p < 0.05$

The activity of enzyme succinate dehydrogenase (SHD) was increased in cerebral cortex in the group that received antioxidant treatment at 24 h after induction meningitis (Fig. 3e), being that in 48 h was increased in the hippocampus also, being in this time increased in cerebral cortex in the group that received antibiotic therapy and antioxidant treatment (Fig 4e).

In the CK activity was increased in hippocampus and cerebral cortex at 24 h after induction meningitis in the group that received antioxidant treatment and only in cerebral cortex in the group that received antibiotic therapy with antioxidant treatment (Fig. 5a). At 48 h there was not altered in the CK activity (Fig. 5b).

DISCUSSION

Several experimental studies over the past few years have documented that bacterial meningitis cause cell damage in two areas of the brain: the cortex and the hippocampus (Leib *et al.*, 2000; Pfister *et al.*, 2000; Loeffler *et al.*, 2001). Other studies have shown that

apoptosis in the hippocampus and cortical necrosis due to pneumococcal meningitis also contribute to damage brain (Grandgirard *et al.*, 2007; Irazuzta *et al.*, 2008). In pneumococcal meningitis, the inflammation caused by the organism increases the production of polymorphonuclear leukocytes causing bacterial cell lysis and, simultaneously, the leukocytes enhance release of proteolytic metabolites such as ROS, resulting in endothelial damage and increased permeability of the BBB (Waggener, 1974; Kontos *et al.*, 1992). With the generation of ROS in the brain of rats with pneumococcal meningitis, brain energy metabolism may be compromised by the high dependence of ATP in the CNS. The formation of ROS can cause mitochondrial damage, impairing oxidative phosphorylation and limiting the production of ATP (Sen *et al.*, 2007). Ghielmetti *et al.* (2003) showed that the level of ATP and ADP were decreased in the cerebral cortex by approximately 25% in rats affected by pneumococcal meningitis.

Barichello *et al.* (2010a) reported that in the animals infected with meningitis and untreated with antibiotic therapy, the levels of lipid peroxidation and protein carbonyls in the hippocampus and cortex were increased at 24 and 48 h after induction of meningitis. In comparison, the meningitis animals that received antibiotic therapy responded differently, the lipid peroxidation and protein carbonyls were decreased in the hippocampus and cortex at 24 and 48 h after induction. In spite of effective antimicrobial therapy and intensive care, the outcome of pneumococcal meningitis remains poorly with a mortality rate of up to 30% and permanent sequel due to neuronal injury in up to 50% of the survivors (Beek *et al.*, 2004; Weisfelt *et al.*, 2006). Success in the treatment of patients with bacterial meningitis and the development of improved strategies for disease management rely on knowledge of key pharmacologic principles for use of antimicrobial agents that are efficacious in the unique environment of the CSF, including penetration of the drug across the BBB, the activity of the drug in purulent CSF and the intrinsic pharmacodynamic properties of the drug (Sinner and Tunkel, 2004).

In our study, the group of animals with pneumococcal meningitis that received antioxidant treatment with new compound P0801, with or without antibiotic therapy at 24 h after induction decreased protein carbonyls in cerebral cortex and in the hippocampus at 48 h after induction. In the group that received only antioxidant treatment increased lipid peroxidation in the cerebral cortex at 24 h after induction meningitis, however, in the hippocampus at 48 h after induction meningitis decreased lipid peroxidation. The antibiotic therapy with antioxidant treatment can be studied as a potential treatment for bacterial meningitis, since it appears to decrease the oxidative stress.

The formation of oxidative stress may participate in the neuroinflammatory process and cause mitochondrial abnormalities, inhibiting the action of respiratory chain enzymes and decreasing the energy metabolism (Mancuso *et al.*, 2006; Sayre *et al.*, 2008). In this context, Barichello *et al.* (2010b) evaluated the activities of mitochondrial respiratory chain complexes in the brain of rats submitted to meningitis by *S. pneumoniae*. The results demonstrated that the activity of complex I was inhibited only 24 h after induction of meningitis in the structures striatum, cerebellum and hippocampus. The activities of complex II, III e IV were increased in the brain structures at 24 and 48 h after induction meningitis. In fact, a number of devastating neurodegenerative disorders are associated

with complex I deficiency, resulting in a decline of energy production by the respiratory chain and in increased production of Reactive Oxygen Species (ROS) (Bailey *et al.*, 2005; DiMauro and Hirano, 2005; Petrosillo *et al.*, 2008). In our results, in the animals with pneumococcal meningitis that received antibiotic therapy and or antioxidant treatment with new compound P0801 at 24 h after induction of meningitis, there were increased of the activity of complex I, II, IV and enzyme succinate dehydrogenase in cerebral cortex, being that at 48 h in the group that received antibiotic therapy and or antioxidant treatment there were increased of the activity of complex I, II and enzyme succinate dehydrogenase in the cerebral cortex and or hippocampus.

Barichello *et al.* (2009) verified that 24 h after the induction of the meningitis were observed a decrease in CK activity, being that at 48 h after meningitis, the CK activity was not altered. In our study, the CK activity was increased in hippocampus and cerebral cortex at 24 h after induction meningitis in the group that received antioxidant treatment and only in cerebral cortex in the group that received antibiotic therapy with antioxidant treatment.

Animal studies have shown that treatment antioxidants may protect against brain damage resulting from bacterial meningitis (Christen *et al.*, 2001; Halliwell and Gutteridge, 2007). Klein *et al.* (2006) shows that the treatment antioxidant with NAC (n-acetylcysteine) to be one promising therapeutic agent because it reduced both cerebral and cochlear complications of experimental pneumococcal meningitis. The study with new compound is an attempt to coadjuvant treatment with the antibiotic to minimize the oxidative damage and energy metabolism during illness.

CONCLUSION

Antibiotic treatment of pneumococcal meningitis with new compound antioxidants may be promising as a coadjuvant treatment by minimizing the damage effects of disease.

In our studies, the new compound seems to minimize oxidative stress and energy metabolism caused by meningitis, which together with the antibiotic can contribute to an effective treatment, decreasing any risk of sequelae due to meningitis. However, New studies are needed to clarify the action of this new compound as coadjuvant, evaluating side effects and their formulation, since it might look promising in the development of novel therapeutic agents in further studies.

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