Original Article

DOI: 10.5582/ddt.2013.v7.6.261

Cerebrolysin attenuates cerebral and hepatic injury due to lipopolysaccharide in rats

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ABSTRACT: This study aimed to investigate the effect of cerebrolysin on oxidative stress in the brain and liver during systemic inflammation. Rats were intraperitoneally challenged with a single subseptic dose of lipopolysaccharide (LPS; 300 µg/kg) without or with cerebrolysin at doses of 21.5, 43 or 86 mg/kg. After 4 h, rats were euthanized and the brain and liver tissues were subjected to biochemical and histopathological analyses. Cerebrolysin revealed inhibitory effects on the elevation of lipid peroxidation and nitric oxide induced by LPS. In contrast, the decrease in reduced glutathione level and paraoxonase activity induced by LPS was attenuated by an injection of cerebrolysin in a dose-dependent manner. Moreover, cerebrolysin reduced LPS-induced activation of brain NF-κB and reversed LPS-induced decline of brain butyrylcholinesterase and acetylcholinesterase activities in a dose-dependent manner. Histopathological analyses revealed that neuronal damage and liver necrosis induced by LPS were ameliorated by cerebrolysin dose-dependently. Cerebrolysin treatment dose-dependently attenuated LPS-induced expressions in cyclooxygenase 2, inducible nitric oxide synthase, and caspase-3 in the cortex or striatum as well as the liver. These results suggest that cerebrolysin treatment might have beneficial therapeutic effects in cerebral inflammation. Cerebrolysin might also prove of value in liver disease and this possibility requires further exploration.

Keywords: Cerebrolysin, cholinesterases, lipopolysaccharide, NF-κB, oxidative stress

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1. Introduction

Cerebrolysin is a brain derived peptide preparation produced by the biotechnologically standardized enzymatic breakdown of purified porcine brain proteins. It consists of a mixture of approximately 25% low molecular weight biologically active peptides (< 10 kDa) and 75% free amino acids, based on total nitrogen (*1*,*2*). The solution is free of proteins, lipids, and antigenic properties. Cerebrolysin has been shown to exert neurotrophic and neuroprotective actions, increasing the viability of cortical neurons and promoting their growth (*3*), increasing the tolerance of neurons to ischemic damage and slowing the execution of the cell death (*4*). The drug also has been shown to rescue the alterations in neurogenesis in amyloid precursor protein (APP) transgenic (tg) mice by protecting neural precursor cells and decreasing their rate of apoptosis (*5*) and to enhance neurogenesis in the ischemic brain (*6*). Cerebrolysin treatment resulted in significant improvements in the memory and global score and delayed progression in patients with Alzheimer's disease and vascular dementia (*1*,*7*) and improved the outcome after moderate and severe head injury (*2*) having only mild and transient adverse reactions. The efficacy of cerebrolysin persisted for up to several months after treatment, suggesting that cerebrolysin has not merely symptomatic benefits, but a disease-delaying potential (*7*).

Oxidative stress and increased inflammatory response have been implicated in the pathogenesis of neurological disorders such as stroke and ischemia/reperfusion injury (*8*) and in neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, and multiple sclerosis (*9*). The brain is highly susceptible to oxidative stress in view of the high rate of oxygen utilization, its high content of polyunsaturated fatty acids, the presence of redox-active transition metals such as Cu^{2+} and Fe^{2+} and the paucity of antioxidant enzymes (*10*). The presence of systemic

inflammation also has profound effects on brain functions (*9*). The systemic injection of lipopolysaccharide (LPS), a product of the gram negative bacterial cell wall causes increased brain oxidative stress and neuroinflammation and thus represents a useful model for studying the effect of systemic inflammation on brain function (*11,12*). The mammalian Toll-like receptor 4 (TLR4) on immune cells is the signal-transducing receptor that when activated by the bacterial LPS triggers the acute inflammatory cascade (*13*). When administered peripherally at subseptic doses, LPS results in impaired antioxidant mechanisms and mitochondrial redox activity, increased lipid peroxidation (*11*,*12*), induction of cyclooxygenase 2 (COX-2) mRNA in the rat brain (*14*) and an up-regulation of cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6 in brain as well as in peripheral tissues and plasma (*15*). Studies also indicated that short-term systemic inflammation produced *in vivo* by administration of LPS can result in neuronal damage by itself (*16*) or exacerbates damage in an existing cerebral pathological state (*17*).

The aim of this study was therefore to investigate the effect of cerebrolysin on oxidative stress in the brain during systemic inflammation caused by intraperitoneal injection of LPS in rats. We also examined the effect of cerebrolysin on brain acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities and on nuclear factor-kappaB (NF-κB), a multisubunit transcription factor that is critical for inducible expression of multiple genes involved in inflammatory responses (*18*). Moreover, the effect of cerebrolysin on oxidative stress and liver tissue damage caused by LPS was examined.

2. Materials and Methods

2.1. *Animals*

Sprague Dawley rats of either sex weighing (130 ± 10 g) were used. Animals were obtained from the Animal House Colony of the National Research Centre (Cairo, Egypt) and housed in stainless steel wire meshed suspended rodent cages under environmentally controlled conditions (25 ± 2°C and the light/dark cycle of 12/12 hours). Standard laboratory food and water were provided *ad libitum*. Animals received human care in compliance with guidelines of the Ethical Committee of National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. *Drugs and chemicals*

LPS derived from *Escherichia coli* (Serotype 055: B5, Sigma-Aldrich, St Louis, MO, USA) was used and dissolved in sterile saline, aliquoted, and frozen at −20°C. Cerebrolysin (EVER Neuro Pharma GmbH, Unterach, Austria) was used and dissolved in isotonic (0.9% NaCl) saline solution immediately before use. The doses of cerebrolysin in the study were based upon the human dose after conversion to that of rat according to Paget and Barnes (*19*) conversion tables.

2.3. *Study design*

Rats were randomly divided into 5 groups of 6 animals each. Group I (normal control) received saline intraperitoneally (0.1 mL). Groups 2-5 were intraperitoneally (*i.p.*) injected with LPS in a dose of 300 μg/kg for induction of endotoxemia. Following LPS injection, group 2 was given *i.p.* saline and kept as positive control, while groups 3-5 were administered *i.p.* cerebrolysin at doses of 21.5, 43, and 86 mg/kg, respectively. Rats were euthanized 4 h after LPS injection by decapitation under ether anaesthesia, brains and livers were then removed, washed with ice-cold saline solution (0.9% NaCl), weighed and stored at –80ºC for the biochemical analyses. The tissues were homogenized with 0.1 M phosphate buffered saline at pH 7.4, to give a final concentration of 10% (w/v) for the biochemical assays. The time selected for tissue sampling (4 h after *i.p.* administration of LPS) was based on previous studies that indicated the rise in plasma and tissue cytokines and inflammatory mediators after LPS administration (*20*).

2.4. *Biochemical analyses*

2.4.1. *Determination of lipid peroxidation*

Lipid peroxidation was assayed by measuring the level of malondialdehyde (MDA). Malondialdehyde forms a 1:2 adduct with thiobarbituric acid which can be measured by spectrophotometry. Malondialdehyde was determined by measuring thiobarbituric reactive species using the method of Ruiz-Larrea *et al.* (*21*), in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex having peak absorbance at 532 nm.

2.4.2. *Determination of reduced glutathione (GSH)*

GSH was determined by Ellman's method (*22*). The procedure is based on the reduction of Ellman's reagent by –SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid. The nitromercaptobenzoic acid anion has an intense yellow color which can be determined spectrophotometrically.

2.4.3. *Determination of nitric oxide*

Nitric oxide measured as nitrite was determined by using Griess reagent, according to the method of Moshage *et al.* (*23*) where nitrite, stable end product of nitric oxide radical, is mostly used as indicator for the production of nitric oxide.

2.4.4. *Determination of paraoxonase activity*

Arylesterase activity of paraoxonase (PON1) was measured spectrophotometrically in supernatants using phenyl acetate as a substrate (*24*). In this assay, arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate resulting in phenol formation. The rate of formation of phenol was measured by monitoring the increase in absorbance at 270 nm at 25ºC. The working reagent consisted of 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM calcium chloride and 4 mM phenyl acetate as the substrate. Samples diluted 1:3 in buffer were added and the change in absorbance was recorded following a 20 sec lag time. Absorbance at 270 nm was taken every 15 sec for 120 sec using a UV-VI8 Recording Spectrophotometer (Shimadzu, Kyoto, Japan). One unit of arylesterase activity is equal to 1 µM of phenol formed per minute. The activity was expressed in kU/L, based on the extinction coefficient of phenol of 1,310 M/cm at 270 nm, pH 8.0, and 25ºC. Blank samples containing water were used to correct for the spontaneous hydrolysis of phenylacetate.

2.4.5. *Determination of brain NF-κB*

NF-κB was measured in supernatants using commercially available ELISA kit (Glory Science Co., Ltd, Del Rio, TX, USA) using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of NF-κB.

2.4.6. *Determination of brain AChE activity*

AChE activity in the cortex was determined according to Gorun *et al*. (*25*). The principle of the method is the measurement of the thiocholine produced as acetylthiocholine is hydrolyzed. The color was read immediately at 412 nm.

2.4.7. *Determination of brain BChE activity*

BChE activity was measured spectrophotometrically in supernatants using commercially available kit (Ben S.r.l., Milano, Italy). In this assay cholinesterase catalyzes the hydrolysis of butyrilthiocholine, forming butyrate and thiocholine. The thiocholine reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) forming a colored compound. The increase in absorbance in the unit time at 405 nm is proportional at the activity of the cholinesterase in the sample.

2.5. *Histopathological examination*

The brain and liver tissue from different groups were collected and fixed in 10% formalin, dehydrated in graduated ethanol 50-100%, cleared in xylene, and embedded in paraffin. Sections 4-5 µm thick were prepared, stained with haematoxylin and eosin (H & E) stain, and examined for histopathological changes under light microscope.

2.6. *Immunohistochemical analysis*

For immunohistochemistry, 4 µm thick deparaffinized brain and liver tissue sections were used. Briefly, deparaffinized slices were incubated overnight with the antibodies against COX-2 diluted 1:100, inducible nitric oxide synthase (iNOS) diluted 1:100, and cleaved caspase-3 diluted 1:1,000. Endogenous peroxidase activity was blocked by incubation in 0.075% hydrogen peroxide in PBS. For antibody detection DAKO EnVision+ System, Peroxidase/DAB kit was employed. The sections were then counterstained with haematoxylin, dehydrated using graded alcohols and xylene, and mounted with Entelan. The immunostaining intensity and cellular localization of, COX-2, iNOS, and cleaved caspase-3 were analyzed by light microscopy.

2.7. *Statistical analysis*

Data were expressed as mean ± SEM. The data were analyzed by one-way ANOVA followed by Duncan's multiple range test, using SPSS software (SAS Institute Inc., Cary, NC, USA). A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. *Biochemical results*

3.1.1. *Lipid peroxidation*

The administration of LPS resulted in a significant increase in the level of MDA in brain and liver by 40.1% (36.48 ± 1.58 *vs*. 26.04 ± 1.92 nmol/g) and 73.8% (48.33 ± 1.3 *vs*. 27.8 ± 0.65 nmol/g), respectively, compared with the saline control group. Brain MDA significantly decreased by 19.8% following treatment with cerebrolysin at 86 mg/kg, compared with the LPS control group (29.24 ± 2.0 *vs*. 36.48 ± 1.58 nmol/g). Meanwhile, in the liver, MDA significantly decreased by 38.5, 44.5, and 45.6% after cerebrolysin doses of 24.5, 43, and 86 mg/kg, respectively (Table 1).

3.1.2. *GSH*

Following LPS challenge, GSH decreased by 42.8% (3.49 ± 0.08 *vs*. 6.10 ± 0.58 µmol/g) and 36.6% (5.20 ± 0.39 *vs*. 8.20 ± 0.33 µmol/g) in brain and liver, respectively, compared with the saline control group. A significant increase by 17.5% was observed in brain GSH after the highest dose of cerebrolysin (4.10 ± 0.14 *vs*. 3.49 ± 0.08 µmol/g). Meanwhile, the level of GSH in liver was not significantly altered by treatment with cerebrolysin (Table 1).

3.1.3. *Nitric oxide*

A significant increase in the level of nitric oxide by 56.7 and 102.6% was observed in brain (29.52 ± 0.72 *vs*. 18.84 ± 0.52 µmol/g) and liver (30.75 ± 1.22 *vs*. 15.18 ± 1.62 µmol/g), respectively. Cerebrolysin inhibited the rise in brain nitric oxide in a dose dependent manner by 22.4, 27.2, and 42.7% in brain. Similarly, cerebrolysin treatment resulted in decreased nitric oxide levels in the liver by 28.3, 32.0, and 34.5% (Table 1).

3.1.4. *Paraoxonase*

The activity of the enzyme was significantly decreased in both the brain and liver by LPS injection. In the brain PON1 activity decreased by 42.5% (7.46 ± 0.44 *vs*. 12.97 ± 0.77 kU/L). In the liver PON1 activity decreased by 36.9% (24.42 ± 0.80 *vs*. 38.67 ± 2.1 kU/L). Cerebrolysin inhibited the decline in PON1 activity in both brain and liver tissue in a dose-dependent manner. In brain PON1 activity increased by 59.7, 92.1, and 104.6% by cerebrolysin doses of 24.5, 43, and 86 mg/kg as compared to LPS control. In the liver, PON1 activity increased by 24.5, 34.6 and 38.2% by cerebrolysin as compared to LPS control (Table 1).

3.1.5. *NF-κB*

NF-κB in the brain was markedly increased following endotoxin administration (16.90 ± 0.12 *vs*. 2.20 ± 0.08 U/L). Treatment with cerebrolysin resulted in a significant and dose-dependent reduction in brain NF-κB by 15.7, 39.1, and 59.3% (14.25 ± 0.62, 10.30 ± 0.28, 6.88 ± 0.26 U/L) by cerebrolysin given at 24.5, 43 and 86 mg/kg as compared to LPS control (16.90 ± 0.12 U/L) (Table 2).

3.1.6. *AChE*

The administration of LPS was followed by a significant decrease in brain AChE activity by 26.5% (5.68 ± 0.24 *vs*. 7.73 ± 0.32 µmol SH/g/min). The decline in AChE was reversed by cerebrolysin given at 43 and 86 mg/kg which increased AChE activity by 23.6 and 29.2%, respectively, as compared to the LPS control (7.02 ± 0.54 and 7.34 ± 0.56 *vs*. 5.68 ± 0.24 µmol SH/g/min) (Table 2).

3.1.7. *BChE*

Following LPS challenge, a significant decrease in brain BChE activity by 23.9% was observed as compared to saline control (217.6 ± 8.9 *vs*. 285.8 ± 15.6 U/L). Cerebrolysin (24.5, 43 and 86 mg/kg) given to LPS-treated rats resulted in a significant and dose-dependent elevation in brain BChE activity by 26.7, 128.9, and 239.5% (275.7 ± 14.9, 498.1 ± 17.1, 738.8 ± 25.6 U/L) as compared to LPS control (217.6 ± 8.9 U/L). BChE activity increased by 74.3 and 158.5% by 43 and 86 mg/kg cerebrolysin as compared to the saline control (Table 2).

Table 1. Effect of cerebrolysin on malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (NO) levels and paraoxonase (PON1) activity in brain and liver of rats treated with lipopolysaccharide

	Saline	LPS	LPS + cerebrolysin 24.5 mg/kg	LPS + cerebrolysin 43 mg/kg	LPS + cerebrolysin 86 mg/kg
Brain					
MDA (nmol/g.tissue)	26.04 ± 1.92	36.48 ± 1.58*	32.00 ± 1.08*	31.92 ± 1.13*	29.24 ± 2.0+
GSH (µmol/g.tissue)	6.10 ± 0.58	3.49 ± 0.08*	3.39 ± 0.12*	3.83 ± 0.06*	4.10 ± 0.14*
NO (µmol/g.tissue)	18.84 ± 0.52	29.52 ± 0.72*	22.90 ± 0.66*,+	21.48 ± 1.42+	16.92 ± 0.84+
PON1 (kU/L)	12.97 ± 0.77	7.46 ± 0.44*	11.91 ± 0.86+	14.33 ± 0.65+	15.26 ± 0.72+
Liver					
MDA (nmol/g.tissue)	27.80 ± 0.65	48.33 ± 1.30*	29.74 ± 1.70+	26.80 ± 1.42+	26.30 ± 1.27+
GSH (µmol/g.tissue)	8.20 ± 0.33	5.20 ± 0.39*	5.66 ± 0.18+	5.54 ± 0.21*	5.40 ± 0.36*
NO (µmol/g.tissue)	15.18 ± 1.62	30.75 ± 1.22*	22.05 ± 1.79*,+	20.90 ± 1.36*,+	20.14 ± 1.30*,+
PON1 (kU/L)	38.67 ± 2.10	24.42 ± 0.80*	30.40 ± 2.10*,+	32.86 ± 1.51+	33.74 ± 1.98+

Results are mean ± S.E. Six rats were used per each group. Data were analyzed by one-way ANOVA and means of different groups were compared by Duncan's multiple range test. $p < 0.05$ was considered statistically significant. * $p < 0.05$ *vs.* saline group. + $p < 0.05$ *vs*. LPS control group.

Table 2. Effect of cerebrolysin on NF-κB, acetylcholinesterase (AChE) activity and butyrylcholinesterase (BChE) activity in rat brain tissues after lipopolysaccharide (LPS) in rats

	Saline	LPS	LPS + cerebrolysin 24.5 mg/kg	LPS + cerebrolysin 43 mg/kg	LPS + cerebrolysin 86 mg/kg
NF-κB (U/L)	2.20 ± 0.08	16.90 ± 0.12*	14.25 ± 0.62*,+,#	10.30 ± 0.28*,+	6.88 ± 0.26*,+,#
AChE (µmol SH/g/min)	7.73 ± 0.32	5.68 ± 0.24*	6.15 ± 0.61*	7.02 ± 0.54+	7.34 ± 0.56+
BChE (U/L)	285.8 ± 15.6	217.6 ± 8.9*	275.7 ± 14.9+,#	498.1 ± 17.1*,+	738.8 ± 25.6*,+,#

Results are mean ± S.E. Six rats were used per each group. Data were analyzed by one-way ANOVA and means of different groups were compared by Duncan's multiple range test. $p < 0.05$ was considered statistically significant. * $p < 0.05$ *vs*. saline group. + $p < 0.05$ *vs*. LPS control group. # $p < 0.05$ *vs*. LPS + cerebrolysin at 43 mg/kg-treated group.

3.2. *Brain histopathology*

Sections from the cortex and striatum in saline-treated rats showed normal cytoplasm and nucleus in neurons (Figures 1A and E). Microscopic examination of sections from the cortex and striatum of LPS only treated rats revealed neuronal damage indicated by necrosis, gliosis, vacuolation of neuropil, and degenerative changes with shrunken, darkly stained pyknotic nuclei. Inflammatory cell infiltration was observed in cerebral cortex and striatum. In brain parenchyma vacuoles with hemorrhage were also noticed (Figures 1B and F). Examined brain sections of rats treated with LPS and cerebrolysin at 43 mg/kg showed no histopathological changes except for pyknosis of some nuclei (Figures 1C and G). Brain sections of rats treated with LPS and cerebrolysin at 86 mg/kg showed almost normal architecture and normal neuron cells similar to those of the control (Figures 1D and H).

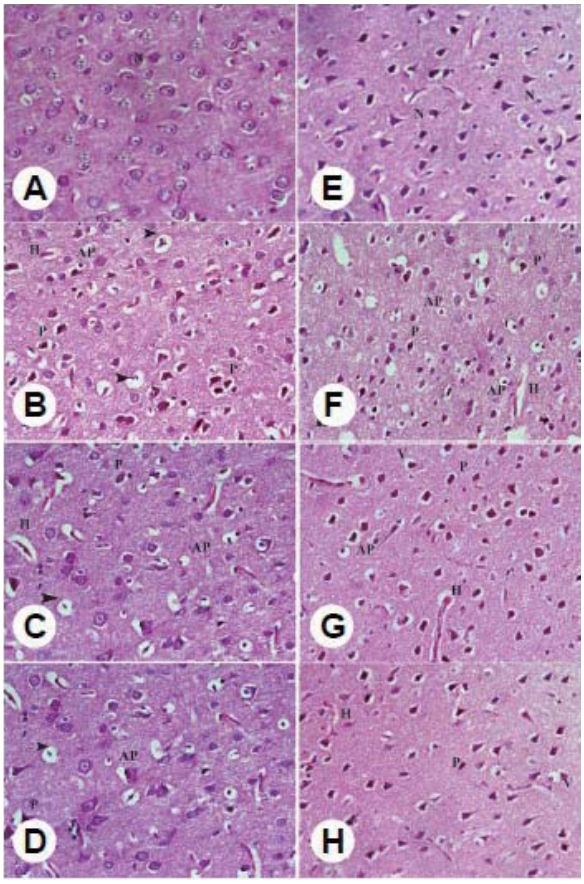

Figure 1. H & E stained sections from the rat cortex and striatum. (**A**) normal rat cortex showing the normal neurons (N); (**B**) cortex after LPS injection showing cytoplasmic vacuolation (arrow head), haemorrhage (H) and pyknotic darkly stained nuclei (P) with apoptotic cells (AP); (**C**) cortex after injection of LPS and cerebrolysin at 43 mg/kg showing moderate amelioration of damage with cytoplasmic vacuolations (arrow head), and pyknotic darkly stained nuclei (P) with apoptotic cells (AP); these changes were less as compared with LPS only treated group; (**D**) cortex after injection of LPS and cerebrolysin at 86 mg/kg marked amelioration of damage and recovery of the brain cells with few cytoplasmic vacuolations (arrow head) and pyknotic darkly stained nuclei (P) with apoptotic cells (AP); (**E**) normal rat striatum showing the neuron with the surrounding supporting cells with normal nuclei which showed dispersed chromatin and prominent nucleoli. The cytoplasm of these cells was basophilic; (**F**) striatum after LPS injection showing shrunken neurons with vacuolation of neuropil (V) and haemorrhage. Pyknotic (P) darkly stained and apoptotic nuclei (AP) were seen; (**G**) striatum after injection of LPS and cerebrolysin at 43 mg/kg showing amelioration of damage with less vacuolation of neuropil (V), hemorrhage, pyknotic (P) darkly stained and apoptotic nuclei (AP); (**H**) striatum after injection of LPS and cerebrolysin at 86 mg/kg: evidence of neuroprotection with markedly reduced number of damaged cells with few vacuolation of neuropil (V), hemorrhage, pyknotic (P) darkly stained and apoptotic nuclei (AP) (H & E, ×400).

3.3. *Brain immunohistochemistry*

3.3.1. *COX-2 expression*

Negligible COX-2-immunopositive neurons were seen in cortex and striatum of saline control group (Figures 2A and E, respectively). COX-2 expression in the cortex and

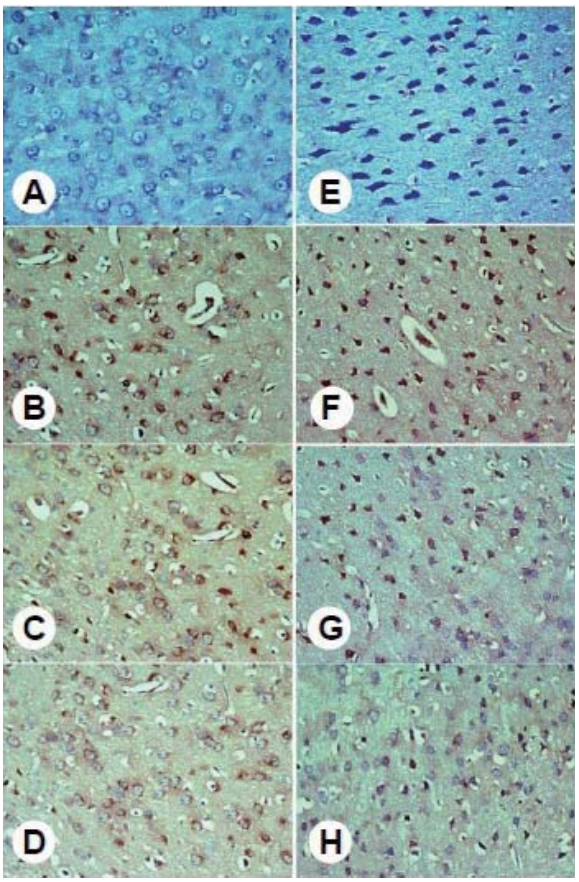

Figure 2. COX-2 immunohistochemistry of rat cortex and striatum. (**A**) negligible positive immunostaining in normal rat cortex; (**B**) strong COX-2 expression in cortex after LPS injection; (**C**) cortex after injection of LPS and cerebrolysin at 43 mg/kg: markedly decreased COX-2 expression; (**D**) cortex injection of LPS and cerebrolysin at 86 mg/kg: markedly decreased COX-2 expression. (**E**) striatum of control rat: negligible positive immunostaining; (**F**) strong COX-2 expression in striatum after LPS injection; (**G**) striatum after injection of LPS and cerebrolysin at 43 mg/kg: markedly reduced COX-2 expression; (**H**) striatum after injection of LPS and cerebrolysin at 86 mg/kg: almost normal COX-2 expression (COX-2 immunohistochemistry, haematoxylin counterstain, ×400). Brown color indicates positive.

striatum increased after LPS administration (Figures 2B and F, respectively). Sections from rats treated with LPS and cerebrolysin showed moderate to normal staining of COX-2 expression in the cortex (Figures 2C and D) and striatum (Figures 2G and H).

3.3.2. *iNOS immunoreactivity*

No iNOS immunoreactivity was observed in the cytoplasm of neurons in cortex and striatum of control rats (Figures 3A and E, respectively). The administration of LPS was followed by a significant increase in iNOS immunopositivity (Figures 3B and F). The administration of cerebrolysin resulted in a dose-dependent decrease in iNOS expression in neurons of cortex (Figures 3C and D) and striatum (Figures 3G and H).

3.3.3. *Cleaved caspase-3 immunoreactivity*

No caspase-3 immunoreactivity was observed in the cytoplasm of neurons in in cortex and striatum of control rats (Figures 4A and E). Caspase-3 immunostaining was observed in neurons distributed around the damage area and the number of immunopositive neurons increased after treatment with LPS (Figures 4B and F). On the other hand, caspase-3 immunostaining gradually decreased under treatment with cerebrolysin in a dose-dependent manner (Figures 4C, D, G, and H).

3.4. *Liver histopathology*

Figure 5A shows the normal hepatic architecture. In the liver sections from rats given only LPS injection,

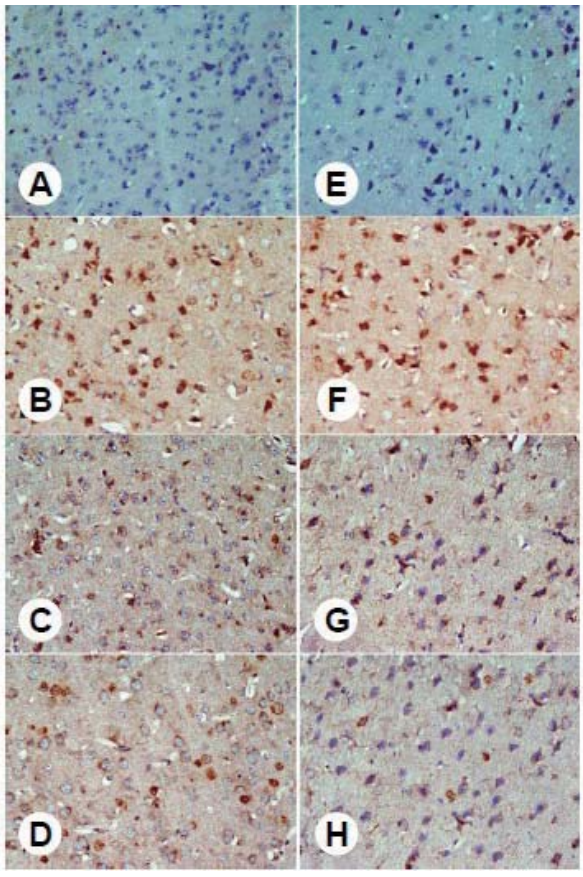

Figure 3. iNOS immunohistochemistry of rat cortex and striatum. (**A**) cortex of control rat: iNOS-immunopositive neurons; (**B**) significant increase in iNOS immunopositivity in cortical neurons after LPS injection; (**C**) cortex after injection of LPS and cerebrolysin at 43 mg/kg: markedly decreased iNOS expression compared to LPS control; (**D**) cortex after injection of LPS and cerebrolysin at 86 mg/kg: minimal iNOS expression. (**E**) striatum of control rat: no positive immunostaining; (**F**) striatum after LPS injection: significant increase in iNOS immunopositivity; (**G**) striatum after injection of LPS and cerebrolysin at 43 mg/kg: markedly decreased iNOS expression compared to LPS control; (**H**) striatum after injection of LPS and cerebrolysin at 86 mg/kg: minimal iNOS expression (iNOS immunohistochemistry, haematoxylin counterstain, ×400). Brown color indicates positive.

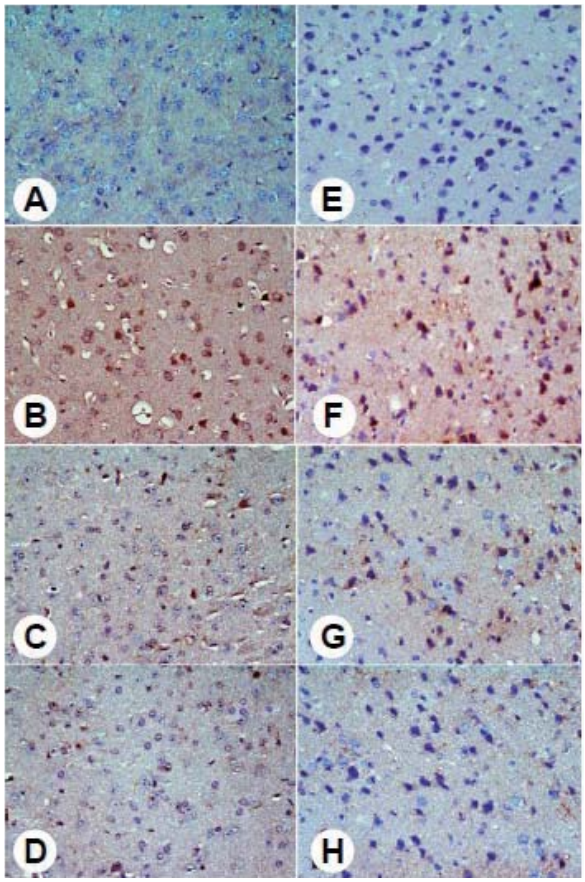

Figure 4. Expression of caspase-3 in cortical and striatal neurons. (**A**) normal rat cortex: caspase-3 immunohistochemistry not present; (**B**) cortex of LPS-treated rat: there is increased caspase-3 expression; (**C**) cortex after injection of LPS and cerebrolysin at 43 mg/kg; markedly decreased caspase-3 expression; (**D**) cortex after injection of LPS and cerebrolysin at 86 mg/kg: markedly decreased caspase-3 expression. (**E**) striatum of control rat: caspase-3 immunohistochemistry not present; (**F**) striatum of LPS-treated rat: there is increased caspase-3 expression; (**G**) striatum after injection of LPS and cerebrolysin at 43 mg/kg; markedly reduced caspase-3 expression to near normal; (**H**) striatum after injection of LPS and cerebrolysin at 86 mg/kg: caspase-3 expression nearly normal (Caspase-3 immunohistochemistry, haematoxylin counterstain, ×400). Brown color indicates positive.

large areas of pericentral necrosis with loss of hepatic architecture, vacuolar fatty change and inflammatory cell infiltration comprised predominantly of mononuclear cells and macrophages, have been found (Figures 5B and C). Treatment with cerebrolysin was associated with a dose-dependent improvement in the liver morphology (Figures 5D-F). When given at 43 mg/kg, the drug resulted in amelioration of liver damage with few necrotic areas being present (Figure 5E). The higher dose of 86 mg/kg almost completely prevented liver necrosis, showing minimal hepatic damage (Figure 5F).

3.5. *Liver immunohistochemistry*

3.5.1. *COX-2 expression*

In saline control group, negligible COX-2-immunopositive cells were seen in liver tissue (Figure 6A). However, after LPS injection increased expression of COX-2 immunopositivity was detected in the cytoplasm (Figure 6B). This was attenuated by cerebrolysin in a dose-dependent manner (Figures 6C and D).

3.5.2. *iNOS expression*

Negligible iNOS immunopositivity in the livers of control rats was noticed (Figure 7A). LPS intoxication increased iNOS immunoreactivity in necrotic areas (Figure 7B) which was slightly ameliorated by cerebrolysin at the dose of 21.5 mg/kg. Cerebrolysin given at 43 mg/kg markedly reduced cytoplasmic iNOS immunoreactivity as compared to the lower dose (Figure 7C), whereas in those treated with cerebrolysin at 86 mg/kg, iNOS expression was similar to controls (Figure 7D).

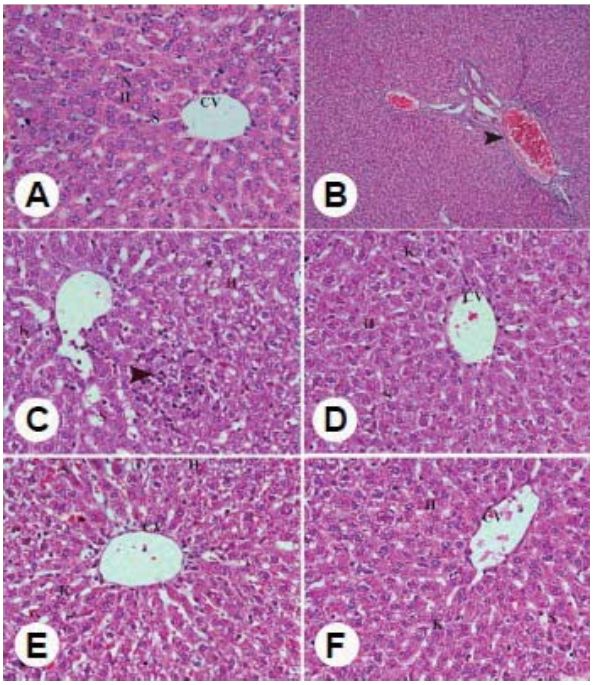

Figure 5. H & E stained sections from the rat liver. (**A**) normal liver with central vein (CV) and surrounding hepatocytes (H), sinusoids (S) and nucleus (N); (**B**) after LPS injection showing dilatation and congestion of portal tract with inflammatory cell infiltration around portal tract (arrow head); (**C**) after LPS injection showing pericentral necrosis with inflammatory cell infiltration (arrow head), degeneration of hepatocytes (H), dilatation and congestion of hepatic sinusoids (S) and activated Kupffer cells (K); (**D**) after injection of LPS and cerebrolysin at 21.5 mg/kg showing damage of hepatic cells with degeneration of some hepatocytes (H), dilatation of hepatic sinusoids (S) and activated Kupffer cells (K); (**E**) after injection of LPS and cerebrolysin at 43 mg/kg showing normal hepatic cells with less degeneration of some hepatocytes (H), dilatation of hepatic sinusoids (S) and activated Kupffer cells (K); (**F**) after injection of LPS and cerebrolysin at 86 mg/kg showing markedly reduced number of hepatocytes with normal hepatic cells (H), few dilatation of hepatic sinusoids (S) and activated Kupffer cells (K) (H & E, ×400).

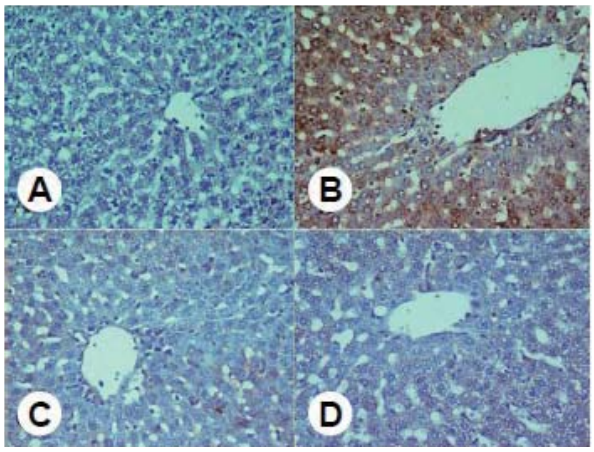

Figure 6. COX-2 immunohistochemistry of rat liver. (**A**) negligible positive immunostaining in normal rat; (**B**) strong COX-2 expression after LPS injection; (**C**) LPS and cerebrolysin at 43 mg/kg: nearly normal COX-2 expression; (**D**) LPS and cerebrolysin at 86 mg/kg: nearly normal COX-2 expression (COX-2 immunohistochemistry, haematoxylin counterstain, ×400). Brown color indicates positive.

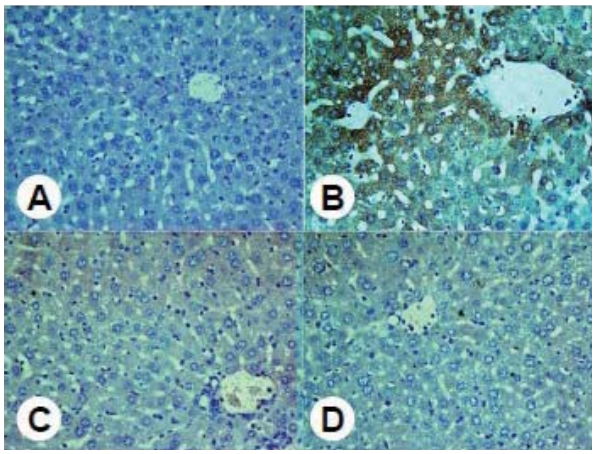

Figure 7. iNOS immunohistochemistry of liver. (**A**) control rat: negligible iNOS immunopositivity; (**B**) after LPS injection: markedly increased iNOS immunoreactivity in necrotic areas; (**C**) LPS and cerebrolysin at 43 mg/kg: more decrease in cytoplasmic iNOS immunoreactivity compared with the previous section; (**D**) LPS and cerebrolysin at 86 mg/kg: iNOS expression nearly normal (iNOS immunohistochemistry, haematoxylin counterstain, ×400). Brown color indicates positive.

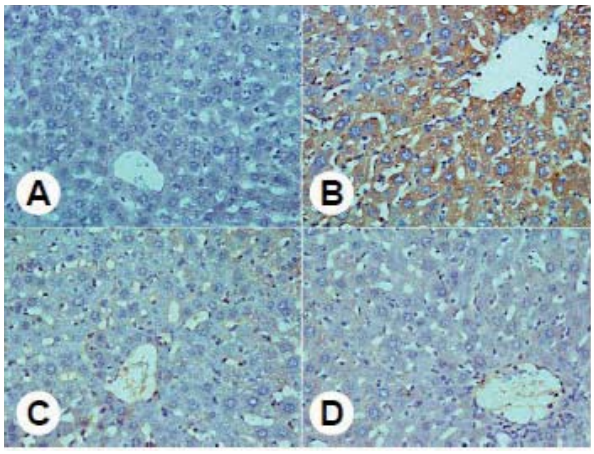

Figure 8. Caspase-3 immunohistochemistry in liver. (**A**) control rat: negligible caspase-3 immunopositivity; (**B**) LPS-treated rat: increased caspase-3 expression; (**C**) LPS and cerebrolysin at 43 mg/kg: markedly reduced caspase-3 expression; (**D**) LPS and cerebrolysin at 86 mg/kg: caspase-3 immunopositivity nearly normal (Caspase-3 immunohistochemistry, haematoxylin counterstain, ×400). Brown color indicates positive.

3.5.3.*Caspase-3 immunoreactivity*

Negligible caspase-3 immunopositivity was observed in the livers of control rats (Figure 8A). However, after LPS treatment a significant increase in caspase-3 immunoreactivity in the cytoplasm of hepatocytes was found (Figure 8B). This was subsequently reduced under treatment with different doses of cerebrolysin (Figures 8C and D).

4. Conclusion

This study examined the effect of cerebrolysin, a mixture of neurotrophic factors, on the development of oxidative stress and brain injury induced in rats by LPS endotoxin. The study demonstrates for the first time that cerebrolysin decreased the oxidative stress and the neuroinflammatory response and neuronal damage induced by peripherally injected LPS. The transcription factor NFκB, a central mediator of the immune response, which is critical for inducible expression of multiple genes involved in inflammatory responses is also reduced by the drug. These data suggest the usefulness of cerebrolysin in systemic inflammatory conditions involving the brain. We also showed that treatment with cerebrolysin decreased the LPS-induced liver injury.

The administration of LPS was associated with an increase in lipid peroxidation (measured as increased malondialdehyde) and a drop in GSH level, which indicates the development of oxidative stress and consumption of GSH by the increased generation of free radicals. Other researchers have reported decreased brain GSH and glutathione reductase activity in rats after the administration of LPS (1 mg/kg, *i.p.*) (*12*). Similarly, a single intraperitoneal dose of LPS (250 µg/mouse) was associated with GSH depletion, and lipid peroxidation and impairment in mitochondrial redox activity (*11*). Cerebrolysin at the highest dose examined caused a mild yet a significant decrease in brain MDA which was associated with an increase in GSH. Glutathione, a tripeptide of glycine, glutamic acid, and cysteine, is the most abundant nonprotein thiol in almost all aerobic species and which participates non-enzymatically and enzymatically in supporting cellular redox balance and in protecting against oxidative damage by reactive oxygen species (*26*). Studies have shown decreased glutathione content in brain of patients suffering from a number of neurological diseases (*27*), thereby implicating GSH consumption by free radicals in the pathogenesis of these disorders.

NF-κB is a ubiquitously expressed transcription factor that activates transcription of various inflammatory cytokines, adhesion molecules, and chemokines involved in the generation of acute inflammation (*18*). NF-κB is thus a critical intracellular mediator of the inflammatory cascade. Stimulation of cells by bacterial endotoxin or cytokines *e.g.*, TNF-α, and IL-1β leads to a dissociation of NF-κB from its inhibitory subunit IκBα and a rapid translocation of free NF-κB to the nucleus (*18*). NF-κB can also be activated by reactive oxygen species (*28*). In the present experiments, cerebrolysin decreased NF-κB levels in the brain after LPS challenge in a dose-dependent manner. This ability of cerebrolysin to decrease the induction of NF-κB activity might be involved in its neuroprotective effects by decreasing brain inflammation under pathological circumstances.

Reduced PON1 activity has been observed in the serum from patients with neurodegenerative diseases *e.g.*, Alzheimer disease and mixed dementia (*29*). This enzyme which possesses an organophosphatase, arylesterase, and lactonase activity and hydrolyzes many different substrates (*30*) is largely though to protect cellular membranes against oxidative stress (*31*). In the present study, markedly decreased PON1 activity was observed in the brain and liver after endotoxin challenge. Cerebrolysin was able to reverse the decline in PON1 activity after LPS injection, suggesting a stimulatory effect for cerebrolysin on the enzyme or reduction of oxidative stress by cerebrolysin with consequent sparing of PON1.

Cerebrolysin demonstrated a strong inhibitory effect on nitric oxide levels in brain after LPS challenge. The drug also attenuated the increased expression of iNOS in brain tissue. Increased generation of nitric oxide occurs during inflammatory conditions due to the action of the inducible form of nitric oxide synthase (iNOS). Glial cells (astrocytes and microglia) synthesize nitric oxide after the transcriptional expression of a Ca^{2+}-independent iNOS (*32*). Overproduction of reactive nitrogen species or nitrosative stress results in nitrosylation reactions that can alter the structure

of proteins and so inhibit their normal function (*33*). Synthesis of nitric oxide by both the inducible and constitutive NOS isoforms also contribute to the activation of apoptotic pathways in the brain during systemic inflammation induced by LPS (*34*). Moreover, LPS-activated microglia has been shown to mediate oligodendrocyte progenitor cell death involving nitric oxide-dependent oxidative pathway (*35*). In this way systemic inflammatory responses can affect neurogenesis, a process by which new neurons are added in the adult mammalian brain with the consequently the repair/replacement of the lost neuronal systems (*36*). Cerebrolysin thus by decreasing brain inflammation is likely to protect neurogenesis from oxidative and nitrosative mediated cell damage. In this context, there are data to suggest a beneficial effect for cerebrolysin on the process of neurogenesis. Thus in APP tg mice cerebrolysin might rescue the alterations in neurogenesis in APP tg mice by protecting neural precursor cells and decreasing the rate of apoptosis (*5*). Moreover, cerebrolysin treatment initiated 24 and 48 h after experimental stroke enhanced neurogenesis in the ischemic brain and improved functional outcome (*6*).

The inducible form of the enzyme cyclooxygenase, the rate limiting step in prostaglandin synthesis (COX-2) is undetectable in most tissues, but its expression can be induced by a variety of stimuli including LPS related to inflammatory response. Peripherally administered LPS results in increased expression of COX-2 in brain (*14*) and prostaglandin E2 (PGE2) generated in brain *via* COX-2 have been implicated in endotoxin-induced fever. LPS-induced COX-2 expression and PGE2 production appear to be mediated through NF-κB *via* activation of TLR4 (*37*). COX-2 has also been implicated in age-related neurodegenerative diseases *e.g.*, Parkinson's disease (*38*). The present study shows the presence of COX-2-immunopositive neurons in the cortex and striatum after LPS injection; this COX-2 expression being markedly reduced by cerebrolysin.

The activation of caspase proteases, especially caspase-3 is crucial for the execution of programmed cell death (apoptosis) (*39*). Caspase activation is an integral process of programmed cell death during various brain injuries (*40*). This study which measured caspase-3 expression by immunohistochemistry indicated significant increase of caspase-3 positive cells in cortex and striatum after LPS challenge. Cerebrolysin was found to inhibit the appearance of cells positive for caspase-3, suggesting that the drug protect cortical and striatal neurons at least partly by interfering with the activation of capsase-3.

Two different types of cholinesterases hydrolyze the neurotransmitter acetylcholine. AChE (EC 3.1.1.7) terminates the action of acetylcholine at the post-synaptic membrane in the neuromuscular junction. The other enzyme is BChE (EC 3.1.1.8) which hydrolyses acetylcholine as well as many other esters (*41*). The role of AChE in cholinergic neurotransmission is well established and the use of AChE inhibitors has been associated with improved cognition, behaviour, activities of daily living, and global functioning in mild-to-moderate Alzheimer's disease (*42*). The physiological functions of BChE are however, still unresolved. Severely demented Alzheimer's disease patients had significantly lower AChE and BChE activities in CSF than the controls had (*43*). In the present study, systemic endotoxin injection was associated with a decreased activity of both cholinesterases within the brain, suggesting a deleterious effect for systemic inflammation on brain function. This decline in the activity of both AChE and BChE was reversed by cerebrolysin, thereby, suggesting a neuroprotective effect for the drug. Markedly raised BChE activity above normal values after cerebrolysin treatment was however observed. The distribution of BChE in brain suggested that this enzyme may play a unique role in neuronal function (*44*). Studies also suggest a key role of BChE during neurogenesis (*45*). This might provide a possible explanation for the rise in BChE by cerebrolysin observed in the present study since the drug has been shown to promote neuronal growth (*3*) and enhances neurogenesis (*6*).

Although cerebrolysin is essentially a neuroprotective agent used in various cerebral pathologies, the present study also investigated its effects on the liver tissue integrity during endotoxaemia. It has been shown that in liver, TLR4 is expressed by all parenchymal and non-parenchymal cell types, and contributes to tissue damage due to different etiologies (*46*). Interestingly, lipid peroxidation induced by LPS in liver was markedly decreased by the drug despite no effect on GSH level. LPS causes iNOS expression in Kupffer cells and hepatocytes of the liver and consequent increased generation of nitric oxide (*47*) which can result in oxidant stress in the liver and consequent cell injury. In this respect, cerebrolysin, displayed marked inhibitory effect on nitric oxide level and on iNOS expression in hepatic tissue. The drug also reversed the LPS-induced depression of PON1 activity in the liver. Serum levels of PON1 decreases in chronic hepatitis and liver cirrhosis (*48*) and might be a potential test for the evaluation of liver function (*49*). Moreover, caspase-3 activation and COX-2 expression were inhibited by the agent in a dose-dependent manner. Histologically, cerebrolysin dose-dependently attenuated liver necrosis, inflammatory cell infiltration and the loss of hepatic architecture induced by LPS. These observations suggest a potential utility for this peptide mixture in reducing liver injury during endotoxaemia.

In summary, the present study showed that cerebrolysin significantly attenuated oxidative stress and nitric oxide levels in brain and liver after LPS challenge in rats. The drug also reduced the activation

of NF-κB, increased PON1, AChE, and BChE activities in brain. Moreover, cerebrolysin decreased caspase-3 activity, COX-2 and iNOS expression in cortex, striatum and liver and attenuated the brain and liver damage induced by LPS endotoxin, thus suggesting a neuroprotective and a hepatoprotective effect of the drug in endotoxaemia.

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(*Received March 18, 2013; Revised March 28, 2013; Re-revised December 9, 2013; Accepted December 10, 2013****)***