Chapter-II

Material & Method
Material and reagents
Oxidized glutathione (GSSG), reduced glutathione (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2,3,5-triphenyltetrazolium chloride (TTC), ethylene diamine tetra-acetic acid (EDTA), (-)-epinephrine, paraformaldehyde, glycine, dianinobenzidine (DAB), butylated hydroxytoluene (BHT), xylenol orange, ammonium sulphate, glucose-6 phosphate, glucose, methanol and sulphuric acid, cresyl violet, hematoxylin, eosin, hesperidin, silymarin, rutin, ATP kit, Monoclonal p53, Apaf-1, caspase-9, SOD, NF-kβ, Hsp70, iNOS, COX-2 antibodies were purchased from Sigma–Aldrich Chemicals Pvt. Ltd., India. Monoclonal GFAP antibody was purchased from Chemichon International, Temecula, CA and anti-rabbit IgG and anti-mouse IgG were purchased from Jackson Immuno Research Laboratories Inc., West Groove, PA, USA. TNF-α and IL-1β Immuno Assay Kits and CasPASE™ assay kit were purchased from eBioscience & BD Bioscience, USA. 4-0 silicone suture was purchased from Doccol co-operation, USA.

Animals and treatments
Male Wistar rats obtained from Central Animal House of Jamia Hamdard (Hamdard University), weighing 250 ± 10 g and age 16 weeks (approx.) at the start of the experiment, were used. Rats were housed in groups of four animals per cage at an ambient temperature of 25 ± 2 °C and relative humidity of 45–55% with 12 h light/dark cycles and had free access to standard rodent pellet diet and water ad libitum. The food was withdrawn 12 h before the surgical procedure. Experiments were conducted in accordance with the Animal Ethics Committee of the University, approved by the Government of India, New Delhi, India.

Methods
Experimental design
Experiments were designed to check the efficacy of Hesperidin (H), Silymarin (Sil), Naringenin (Nar), and Ellagic Acid (EA) in MCAO rat model of cerebral ischemia. In order to evaluate the mechanism of action of Hesperidin, Silymarin, Naringenin and Ellagic acid pre-treatment in cerebral ischemia a number of neurochemicals and functional biomarkers were selected.
Middle cerebral artery occlusion (MCAO)

The right MCAO was performed using an intraluminal filament model (Longa et al., 1989) as described by us (Salim et al., 2003). In brief, the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), a silicone rubber (4 0-3033REPK10, DOCCOL, USA) coated monofilament was introduced into the external carotid artery and advanced into the MCA via the internal carotid artery (17–20 mm) until a slight resistance was felt. Such resistance indicated that the filament had passed beyond the proximal segment of the anterior cerebral artery (Fig. 2.1). At this point, the intraluminal suture blocks the origin of MCA and occludes all sources of blood flow from internal carotid artery, anterior cerebral artery and the posterior cerebral artery. Two hours after the induction of ischemia, the filament was slowly withdrawn and the animals were then returned to their cages. Sham group rats, went to all surgical procedures except the MCAO. Thereafter, the animals were returned to their cages and given free access to food and water.

Post-operative care

Recovery of anaesthesia took approximately 4–5 h. The rats were kept in a well-ventilated room at 25 ± 3°C in individual cages till they gained full consciousness and then were housed together in a
group of four animals per cage. Food and water was kept inside the cages for 22h so that animals could easily access it without any physical trauma due to overhead surgery.

Neurological deficits
After 22 h of reperfusion, the neurological status of the animals was evaluated using two different methods:

**Flexion Test**
This method was first proposed by Bederson et al. (1986). Accordingly, four categories of neurological findings were scored: 0 = no observed neurological deficit; 1 = contralateral forelimb flexion with wrist flexion and shoulder adduction; 2 = reduced resistance to lateral push; 3 = circling movements towards the paretic side.

**Spontaneous motor activity (SMA)**
Spontaneous motor activity was evaluated for 5 min by placing the animals in their normal environment (cage). Neurological scoring was given as: 0 = rats moved around in the cage and explored the environment; 1 = rats moved in the cage but did not approach to all the sides and hesitated to move; 2 = rats barely moved in the cage and showed postural abnormalities (curved towards the paretic side); 3 = rats unable to move at all with their posture curved towards the paretic side.

**Behavioral tests:**
All of the behavioral studies were performed at room temperature in a calm room without any outside interference. All experiments were performed between 9:00 am to 5:00 p.m. in the laboratory at standard optimal conditions. All the tests were performed and analyzed by the subject blind to the experiment.

**Rota rod (muscular coordination)**
Omni Rotor (Omnitech Electronics, Inc., Columbus, OH, USA) was used to evaluate the muscular coordination (Kelly et al., 1998). The Rota rod unit consists of a rotating rod, 75 mm diameter, which was divided into four parts by compartmentalization to permit the testing of four rats at a time. After twice daily training for 2 successive days (speed 8 rpm) the rotational speed of the test
was increased to 10 rpm on the third day in a test session. The time each rat remained on the rotating bar was recorded for three trials of each rat at a 5 min interval and a maximum trial length of 180 sec per trial. The apparatus automatically records the time in 0.1 s when the rats fall off the rotating shaft. The speed was set at 10 r.p.m. and cut off time was 180 s, and the drug-native animals were trained on the rod, so that they could stay on it at least for the cut-off time. Data were presented as mean time on the rotating bar over the three test trials.

Grip test
Grip test was performed by the method of Moran et al (1985). The apparatus with a string of 50 cm length, pulled tightly between two vertical supports and elevated 40 cm from a flat surface was used. The rat was placed on the string at a point midway between supports and evaluated according to the following scale: 0 ~ fall off, 1 ~ hangs onto string by two forepaws, 2 ~ as for 1 but attempts to climb on string, 3 ~ hangs onto string by two forepaws plus one or both hind paws, 4 ~ hangs onto string by all fore paws plus tail wrapped around string, 5 ~ escape.

Adhesive-removal test
Adhesive removal test was performed by the method of Schallert et al (1982). Somatosensory deficit was measured both before and after 24 h of MCAO (Bouet et al., 2007). All rats were familiarized with the testing environment. In the initial test, 2 small pieces of adhesive-backed paper dots (of equal size, 113.1 mm²) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The rat was then returned to its cage. The time to remove each stimulus from forelimbs was recorded on 5 trials per day. Individual trials were separated by at least 5 minutes. Before surgery, the animals were trained for 3 days. Once the rats were able to remove the dots within 10 seconds, they were subjected to MCAO. The animals were given a maximum of 180 seconds to sense the tapes and remove them and were scored as 180 seconds if they did not succeed.

Evaluation of ischemic damage
Infarct volume analysis
The animals were sacrificed after 2 h of occlusion followed by 22 h of reperfusion. The brains were dissected out and kept in a brain matrix. A 1.5 mm coronal sections of the brains were cut down with the help of sharp blades and stained with 0.1% triphenyltetrazolium chloride (TTC) prepared into
normal saline at 37 °C for 15 min. TTC acts as a proton acceptor for many pyridine nucleotide-linked dehydrogenase along with the cytochromes, form an integral part of the inner mitochondrial membrane and make up the electron transport chain (Joshi et al., 2004). The tetrazolium salt is reduced by the enzymes into a red lipid soluble formazan. Viable tissues stain deep red while the infarcts remain unstained. For imaging, the sections were scanned by a high-resolution scanner (Hewlett Packard Scanjet automatic document feeder, IDHAO, U.S.A). The total mean infarct area of each section was observed by the change in colouration.

Biochemical studies

Tissue preparation for antioxidant enzymes and glutathione assays

After behavioral study, the animals were sacrificed and their brains were taken out to dissect hippocampus and frontal cortex to give 5% (w/v) homogenate (10 mM Tris HCl, pH 7.4 having 10 μl/ml protease inhibitors: 5 mM leupeptin, 1.5 mM aprotinin, 2 mM phenylethylsulfonylfluoride (PMSF), 3 mM pepstatatin A, 0.1 mM EGTA, 1 mM benzamidine and 0.04% butylated hydroxytoluene) and centrifuged at 800g for 5 min at 4 °C. This supernatant (S1) was used for the assay of TBARS and Na⁺ K⁺ ATPase and remaining S1 was recentrifuged at 10,500 × g for 15 min at 4 °C (S2) to separate post mitochondrial supernatant (PMS) which was used for the estimation of antioxidant enzymes and GSH.

Assay for TBARS, a marker of lipid peroxidation (LPO)

LPO was estimated by measuring the TBARS in accordance with the method of Utley et al.(1967) with some modification. Briefly, 0.2 ml S1 was pipetted in 2.0 ml flat bottom eppendorf tube and incubated at 37 °C in a metabolic water bath shaker at 120 strokes up and down; another 0.2 ml of the same S1 was pipetted in an eppendorf tube and placed at 0 °C incubation. After 1 h of incubation, 0.4 ml of 5% TCA and 0.4 ml of 0.67% TBA was added in both samples (i.e. 0 °C and 37 °C). The reaction mixture was centrifuged at 3,000 g for 15 min. The supernatant was transferred to another test tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the colour was read at 535 nm. The rate of LPO was expressed as nmol of TBARS formed/h/ mg protein using extinction coefficient of 1.56 × 10⁵ M⁻¹ cm⁻¹.
Assay for GSH content

GSH was determined as described by Jollow et al. (1967). PMS 0.1 ml was precipitated with 0.1 ml of sulfosalicylic acid (4 %). The samples were kept at 4 °C for 1 h and subjected to centrifugation at 1,200 g for 15 min at 4 °C. The assay mixture contained 0.1 ml of filtered aliquot (10 % w/v), 1.7 ml PB (0.1 M, pH 7.4) and 0.2 ml DTNB (4 mg/1 ml of PB, 0.1 M, pH 7.4) in a total volume of 2.0 ml. The yellow colour developed was read immediately at 412 nm. The GSH content was calculated as nmol GSH mg⁻¹ protein, using molar extinction coefficient of 13.6 x 10⁴ M⁻¹ cm⁻¹.

Determination of glutathione reductase (GR) activity

GR activity was assayed by the method of Carlberg et al. (1975), with some modification (Mohandas et al., 1984). The assay system consisted of 0.1 M PB pH 7.6, 0.1 mM NADPH, 0.5 mM EDTA, 1.0 mM GSSG and 0.1 ml PMS in a total volume of 2.0 ml. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using extinction coefficient of 6.22 x 10⁴ M⁻¹ cm⁻¹.

Determination of glutathione peroxidase (GPx) activity

GPx activity was measured according to the procedure of Mohandas et al. (1984). The reaction mixture consisted of 0.05 M PB pH 7.0, 1.0 mM EDTA, 1.0 mM sodium azide, 1 EU of 0.1 ml GR, 1.0 mM glutathione, 0.2 mM NADPH, 0.25 mM hydrogen peroxide and 0.1 ml of PMS in a final volume of 2.0 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22 x 10⁴ M⁻¹ cm⁻¹.

Glucose-6-phosphate dehydrogenase activity

The activity of glucose-6-phosphate dehydrogenase will be assayed by the method of Zaheer et al. (1965). The reaction mixture in a total volume of 3.0 ml will consist of 0.3 ml Tris HCl buffer (0.05 M, pH 7.6), 0.1 ml NADP (0.1 mM), 0.1 ml glucose-6-phosphate (0.8 mM), 0.1 ml MgCl₂ (8 mM), 0.1 ml cytosolic fraction and 2.3 ml distilled water. The change in absorbance will be recorded at 340 nm and the enzyme activity will be calculated as nmol NADP reduced/min/mg protein using molar extinction coefficient of 6.22 x 10⁴ M⁻¹ cm⁻¹.
**Determination of superoxide dismutase (SOD) activity**

SOD activity was measured spectrophotometrically, according to the method of Stevens et al. (2000), by monitoring the auto oxidation of (−)-epinephrine at pH 10.4 for 3 min at 480 nm. The reaction mixture contained glycine buffer (50 mM, pH 10.4) and 0.2 ml of PMS. The reaction was initiated by the addition of (−)-epinephrine. The enzyme activity was calculated in terms of nmol (−)-epinephrine protected from oxidation/min/mg protein using molar extinction coefficient of 4.02 x 10^3 M^-1 cm^-1.

**Determination of catalase activity**

Catalase activity was assayed by the method of Claiborne et al. (1984). Briefly, the assay mixture consisted of 0.05 M PB pH 7.0, 0.019 M hydrogen peroxide, and 0.05 ml PMS in a total volume of 3.0 ml. The changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

**Assay for Na^+ K^+-ATPase activity**

Na^+ K^+-ATPase activity was measured by the method of Sovoboda and Massinger (1981) with slight modification. The Na^+ K^+-ATPase activity was determined in two reaction media, A and B. The reaction mixture A consisted of 0.2 M KCl, 1.0 M NaCl, 0.1 M MgCl_2, 0.2 M Tris-HCl buffer (pH 7.4), 0.1 ml of homogenate in a total volume of 2.0 ml. The reaction mixture B consisted of 0.1 M MgCl_2, 10 mM ouabain, 1.0 M NaCl, 0.2 M Tris-HCl buffer (pH 7.4), 0.1 ml of homogenate in a total volume of 2.0 ml. The reaction was started by adding 0.12 ml of 25.0 mM ATP at 37°C and terminated after 15 min by adding of 0.1 ml chilled 10% TCA. The mixture was centrifuged and supernatant (0.5 ml) was used for the estimation of inorganic phosphorous by the method of Fiske and Subbarow (1925).

**Adenosine 5'-triphosphate (ATP) quantitation in mitochondria**

ATP content was determined in mitochondria by using ATP Sigma-Aldrich bioluminescent commercial kit as described by the manufacturers. The brain mitochondria were prepared according to the method described by Yousuf et al. (2007).
Genomic DNA extraction and gel electrophoresis

Genomic DNA was extracted according to the modified method of Simantov et al. (1996). Briefly, tissue samples (50 mg) were homogenized in 0.5 ml of a digestion buffer (10 mM Tris-HCl, 10 mM NaCl, 25 mM EDTA, 1 % SDS, 1 mg /ml proteinase K, pH 7.4). The samples were incubated at 50°C for 2 h and, if necessary, a second step of incubation was performed at 37°C for overnight. At the end of incubation, 50 μl of NaCl was added to samples. DNA was extracted twice with a mixture of phenol: chloroform: isoamyl alcohol (25: 24:1, v/v) and centrifuged at 3,000 x g for 10 min. DNA was precipitated with 1 ml of EtOH (100 %) and centrifuged at 3,000 x g for 4 min. After extensive washing with EtOH (70 %), DNA was re-centrifuged at 3,000 x g for 4 min and resuspended in buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Samples were incubated with RNase (0.5 mg/ml) for 30 min. Thereafter, DNA (10 μl) from each sample was loaded onto 1.2 % agarose gel, containing 0.5 μg/ml ethidium bromide and electrophoresed for the analysis of DNA fragmentation which was detected with UV transilluminator.

Assay for ADP-ribosyltransferase (polymerase)

The hippocampus and frontal cortex will be homogenized (10%) in 0.32 M sucrose, 10 mM Tris-HCl (pH7.4) in Taflon glass homogenizer and centrifuged at 1100 x g to get crude nuclear fraction (P1). The pellets will be resuspended in homogenizing buffer. Poly (ADP- ribosyl) polymerase activity will be estimated by method of Masmoudi et al. (1988) using 0.1 mM (3H)-NAD as a substrate in reaction mixture containing 10 mM Tris/HCl (pH 8), 0.4 mM dithiothritol, 4 mM MgCl₂ ans 25 μl of PMS in a total volume 125 μl. the reaction will be carried out at 37°C for 10 min. and terminated by the edition of 10 % trichloro acetic acid containing 0.02 M sodium pyrophosphate. After 30 min. at 4°C the precipitate will be collected on whatman GF/B glass fiber paper. Fiber paper dried for 15 min. at 100°C and radioactivity will be determined by counting at WALLAC-1410 Liquid Sanitation Counter.

Caspase activity

Caspase 3 activity was measured with kit (CasPASE™ assay kit supplied with Ac-DEVD-AFC substrate) according to the manufacturer's instructions. Tissue was weighed and homogenized in cooled lysis buffer and centrifuged at 12000 g for 3 min at 4 °C. The supernatant was taken from each sample, substrate (DEVD-AFC) was added and the solutions were incubated for one hour at 37 °C to allow a dissociation of 7-amino-4-trifluromethyl coumarin (AFC) from the conjugate
DEVD-AFC produces an optical change and activity was measured spectrophotometrically at 400 nm. Dilute 80 mM AFC solution in Cell Lysis Buffer to make 0, 10, 20, 40, and 80 mM stock solutions. Caspase-3 activity was calculated as nmol/h/mg protein.

Protein content
Protein content was determined by the method of Lowry et al. (1951). In brief, 20 µl sample and 980 µl H2O were added in the 10 ml of test tube. After mixing, 5 ml of alkaline copper reagent (2% Na2CO3 in 0.4% NaOH; 10% CuSO4; 0.0268% Na/K tartrate) was added to each tube, mix well and wait for 10-15 min. Add 0.5 ml of folin reagents (1:1 with H2O) was added to the each tube, vertex then wait for 30 min at room temperature. Read it against reagent blank at 660 nm in a spectrophotometer (Shimadzu – 1601, Japan). Standard was prepared by using different concentration BSA (0.03%) as a standard.

Histopathological evaluation
Assessment of neuronal damage by Cresyl violet
After 24 h of MCAO, animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 0.9% sodium chloride at 4 °C, followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brain was removed, kept in the same fixative for overnight at 4 °C and immersed in 0.1 M PBS containing 20%, 30% sucrose overnight at 4 °C, respectively. Sections were incubated in 0.5% cresyl violet solution for 3-5 min. The sections were rinsed in distilled water and differentiated in 95% alcohol, followed by dehydration in 100% alcohol. The sections were cleared in xylene and mounted in DPX mounting medium. Images were acquired using the light microscopy (BX51, Olympus, Tokyo, Japan).

Haematoxylin and Eosin staining
After 24 h of MCAO, animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 0.9% sodium chloride at 4 °C, followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brain was removed, kept in the same fixative for overnight at 4 °C and immersed in 0.1 M PBS containing 20%, 30% sucrose overnight at 4 °C, respectively. The brains were cut into 10-µm-thick coronal sections on cryostat (Leica, Germany). Every 10 sections of the cortex region, was mounted on glass slides, and processed for hematoxylin and eosin staining. In brief sections were incubated in haematoxylin for 2-4 mints, and rinsed in tap water followed by two
quick dips in 0.3% acid alcohols. Finally stained with eosin for 1-2 mints. The sections were rinsed in distilled water and differentiated in 95% alcohol, followed by dehydration in 100% alcohol.

**Immunofluorescence**

After 22 h of reperfusion the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 0.9% sodium chloride at 4 °C, followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brain was removed, kept in the same fixative for overnight at 4 °C and immersed in 0.1 M PBS containing 20%, 30% sucrose overnight at 4 °C, respectively. The tissues were kept in final sucrose solution till sectioning. The fixed tissues were embedded in OCT compound (polyvinyl glycol, polyvinyl alcohol, and water) and frozen at −20 °C. Coronal sections of 12 μm thicknesses were cut on a cryostat (Leica, Germany) and collected on gelatin-coated slides and immersed in wash buffer (sodium phosphate 100 mM, sodium chloride 0.5 M, Triton X-100, sodium azide) pH 7.4 for 20 min. After a pre-incubation for 1 h in blocking solution (10% normal goat serum, 0.3% Triton X-100 in PBS), sections were incubated overnight at 4 °C with primary antibody rabbit anti-GFAP (anti-glial fibrillary acidic protein; 1:200 dilution; Chemicon International, Temecula, CA) diluted in a solution of 0.3% Triton X-100 in PBS. The slides were washed with PBS to remove the unbound antibodies and sections were incubated with 1:500 dilution of Alexa Fluor 488 conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. Finally the sections were mounted on gelatin-coated slides, air dried and cover slipped. Omission of primary or secondary antibody served as controls.

**Immunohistochemistry**

After 22 h of reperfusion the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with 0.9% sodium chloride at 4 °C, followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brain was removed, kept in the same fixative for overnight at 4 °C and immersed in 0.1 M PBS containing 20%, 30% sucrose overnight at 4 °C, respectively. The tissues were kept in final sucrose solution till sectioning. The fixed tissues were embedded in OCT compound (polyvinyl glycol, polyvinyl alcohol, and water) and frozen at −20 °C. Coronal sections of 12 μm thicknesses were cut on a cryostat (Leica, Germany) and collected on gelatin-coated slides and immersed in wash buffer (sodium phosphate 0.1M, sodium chloride 0.5 M, Triton X-100, sodium azide) pH 7.4 for 20 min. After a pre-incubation for 1 h in blocking solution (10% normal goat serum, 0.3% Triton X-100 in PBS), sections were incubated overnight at 4 °C with primary
antibodies diluted (1:100 and 1:200 respectively) in a solution of 0.3% Triton X-100 in PBS. The slides were washed with PBS to remove the unbound antibodies and sections were incubated with a 1:2000 dilution of biotinylated anti-mouse/rabbit IgG secondary antibody for 1 h at room temperature. Finally the sections were mounted on gelatin-coated slides, air dried and cover slipped. Omission of primary or secondary antibody served as controls.

Western Blot
Western blot expression in the frontal cortex of the rats was carried out after 22 h of ischemia. Ipsilateral side of forebrain was dissected and homogenized at 4°C in a Tris-HCL buffer (50 mM, pH 7.6) containing: 150 mM KCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonylfluoride (PMSF), 1% Triton X-100, 0.5 μg/mL leupeptin and 1 μg/mL pepstatin (Ren et al., 2004). The homogenate was centrifuged at 15,000 g for 20 min and the supernatant used as the cytosolic fraction. Total cellular proteins (20 μg/lane cytosolic fractions) were separated on a 10% SDS-polyacrylamide gel and electro-transferred onto PVDF membranes (Hybond ECL, Amersham International, Bucks, U.K.). Equal loading and transfer of proteins was confirmed by temporally staining the membranes with Ponceau S solution. Nonspecific binding of proteins was prevented by treating the membranes with 5% nonfat dry milk in Tris-buffered saline/0.1% Tween 20 for 2 h at room temperature. The membranes were incubated overnight at 4°C with primary antibodies (1:500). After incubation with secondary antibody conjugated to horseradish peroxidase (anti-mouse, 1:10000), immunoreactive proteins were detected by the enhanced chemiluminescence’s system (ECL, Sigma) and serial exposures were made to radiographic film (Hyper film ECL, Amersham International).

Measurement of cytokines
Brains from MCAO, hesperidin treated MCAO and sham-control animals were removed without fixation after cervical dislocation 24 h following surgery. A 3-mm coronal section was taken from the injured area over the parietal cortex, snap-frozen in liquid nitrogen, and stored at -70°C until use. Brain samples were homogenized in a buffer consisted of 20 mM tris–HCl with pH 7.6, 100 mM KCl, 5 mM NaCl, 2 mM EDTA, 1 mM EGTA, 0.25 M sucrose, 2 mM DTT, 2 mM PMSF; pH 7.2. Homogenates were centrifuged for 12,000 g at 4°C for 15 min. Supernatants were removed and assayed in duplicate using TNF-α and IL-1β assay kits (eBioscience and BD Bioscience, USA)
according to the manufacturer's guidelines. Tissue cytokine concentrations were expressed as picograms of antigen per milligram of protein.

Statistics
Results are expressed as mean ± S.E.M. of eight animals. Differences between the means of experimental and control groups were analyzed by one-way ANOVA followed by Tukey-Kramer post-hoc test. The p-values less than 0.05 were considered statistically significant.