Methylene blue administration during cardio-pulmonary resuscitation and early reperfusion protects against cortical blood-brain barrier disruption

Miclescu Adriana, Sharma Hari Shanker, Cécile Martijn, Wiklund Lars

Department of Surgical Sciences/Anaesthesiology and Critical Care Medicine, Uppsala University Hospital, SE-751 85 Uppsala, Sweden

Objective: The present study was designed to study the effects of cardiac arrest and cardiopulmonary resuscitation (CPR) on blood-brain barrier (BBB) permeability and subsequent neurological injury. It also tests the cerebral effects of MB on the maintenance of BBB integrity, the production of nitric oxide (NO) and regulation of nitric oxide synthases (NOS) in cerebral cortex.

Intervention: The control group (CA, n=16) underwent 12 min cardiac arrest without subsequent CPR, after which the brain of the animals was removed immediately or after 15 and 30 min. The other two groups with 12 min cardiac arrest and subsequent 8 min CPR received either an infusion of saline (CA-MB group, n=10) or an infusion of saline with MB (CA+MB, n= 12) started one minute after the start of CPR and continued 50 min after return of spontaneous circulation (ROSC). In both the latter (CA-MB and CA+MB) groups the brains were removed for histological analysis at the following time points: 30, 60, 180 min after ROSC.

Main Results: In all the groups an increase of necrotic neurons and albumin immunoreactivity was demonstrated with increasing duration of ischemia and reperfusion time. The immunohistochemistry analysis indicated less blood brain barrier disruption in the animals receiving MB (CA+MB group) evidenced by decreased albumin leakage ($P<0.01$), water content ($P=0.02$), potassium ($P=0.04$), but also decreased neuronal injury ($P<0.001$) in this group in comparison with the group that was not treated with MB (CA-MB). Similarly, MB treatment reduced nitrite/nitrate ratio ($P=0.02$), iNOS expression ($P<0.01$), and nNOS expression ($P<0.01$).

Conclusion: Cerebral edema, increase BBB permeability and neurologic injury are observed early in ischemia induced by cardiac arrest. MB markedly reduced BBB disruption and subsequent neurologic injury. In addition with these cerebral morphologic effects, the exposure to MB is associated with a decrease of NO as measured by nitrate/nitrite content and partially inhibition of NOS activity.

Key words: Cardiopulmonary resuscitation (CPR); Blood-brain barrier (BBB); Methylene Blue (MB); Nitric oxide (NO); Nitrite/nitrate content; Nitric oxide synthases (NOS)

Introduction

The role of the blood-brain barrier (BBB) in inducing neuronal damage following cardiac arrest (CA) is still not well clarified. Suggested causes of disruption of the BBB in ischemia and reperfusion are thought to be multifactorial and to involve effects of glutamate, calcium, zinc and nitric oxide (NO) as well as other free radicals (1). Upregulation of nitric oxide synthase (NOS) is associated with increased production of NO that induces breakdown of the BBB (2). It has been suggested that pretreatment with pharmacological agents that reduce excess nitric oxide (3) or oxidative stress (4) might reduce disruption of BBB permeability (3, 4) caused by ischemia/reperfusion injury. Methylene blue (MB), a non-toxic dye and also a scavenger, recently proved to be a possible aid in resuscitation from cardiac arrest by attenuating oxidative, inflammatory, myocardial and neurologic injury (5, 6). The therapeutic effects of MB are ascribed to the presence of the nitric oxide/cyclic guanylyl monophosphate (NO/c-GMP) signaling system and to its action on iron-containing enzymes. Thus, MB has direct inhibitory effects on nitric oxide synthases (7) and also blocks synthesis of cyclic guanosine monophosphate (cGMP) by inhibiting the soluble guanylate cyclase enzyme (8). Due to xanthine oxidase inhibition (9), MB also protects against the toxic effects of some free oxygen radicals (10).

The present study was designed to test the possible protective effects of MB on maintenance of BBB integrity after cardiac arrest and CPR. Furthermore, the study tried to identify the potential regulation of nitric oxide synthases (NOS) and consequently quantify the nitric oxide metabolite levels after global brain ischemia. We previously demonstrated that the most serious effects on cerebral blood flow and oxygen metabolism occurred in cerebral cortex (11) that is most vulnerable to ischemic insult. Thus, this study focused on the changes in BBB function and neuronal injury in this particular cerebral area.

Material and Methods

The Uppsala Institutional Review Board for Animal Experimentation approved this prospective, randomized, laboratory animal study. The piglets were handled according to the guidelines of the Swedish National Board for Laboratory Animals and the European Convention of Animal Care. Anesthesia was used in all surgical interventions.

Animals

The following inclusion criteria were applied: No apparent pre-existing disease, $PaCO_2$ between 5-5.5 kPa, PaO_2 > 10 kPa (75 mm Hg) at baseline after stabilization. The animals included in this study (n=45) were 12-14 weeks old and of so-called triple breed. Piglets were obtained from a single provider source. All piglets were kept fasting but had free access to water during the night, and were taken to the laboratory on the morning of the experiment.

Anesthesia

Immediately upon arrival at the laboratory, the animals received an intramuscular injection of 6 $mg \cdot kg^{-1}$ Zoletil® (tiletamine and zolazepam, Reading, France) combined with 2.2

$mg \cdot kg^{-1}$ Rompum® (xylazine, Bayer, Germany) and atropine 0.04 $mg \cdot kg^{-1}$ (Atropin®, NM Pharma, Stockholm, Sweden). A peripheral venous catheter (18 Gauge) inserted in an ear vein was used for induction and maintenance of anesthesia, as well as for fluid administration. All the piglets received an intravenous injection of 20 mg morphine (Morfin Bioglan®). An injection of 100 mg ketamine (Ketaminol®,Veterinaria AG, Switzerland) was added as an intravenous bolus to achieve surgical anesthesia. The absence of motor response to painful stimuli was considered indicative of an adequate level of anesthesia. An intravenous infusion of 8 $mg \cdot kg^{-1} \cdot h^{-1}$ sodium pentobarbital (Apoteket, Sweden), morphine (Morphine®, Pharmacia, Uppsala, Sweden) 0.5 $mg \cdot kg^{-1} \cdot h^{-1}$ and 0.25 $mg \cdot kg^{-1} \cdot h^{-1}$ pancuronium bromide (Pavulon®, Organon, Netherlands) dissolved in 2.5% glucose solution was started and used for maintenance of anesthesia. The piglets were secured in the supine position and were tracheotomized, which was done to secure a free airway during the experiment. The animals were mechanically ventilated (Servo-i V3.1, Siemens Medical, Solna, Sweden) with 30% oxygen in air after preparation. Volume-controlled mechanical ventilation was instituted with an I: E ratio of 1:2, a fixed frequency of 25/min and a minute volume set to maintain arterial $PaCO_2$ at an average of 5-5.5 kPa. A positive end-expiratory pressure (PEEP) of 5 cm H_2O was applied to prevent atelectasis. The capnogram and saturation level were displayed continuously until extubation (CO_2SMO Plus-8100, Novametrix, Wallingford, CT, USA), as were leads II and V5 of the electrocardiogram (ECG).

Fluid administration

All animals received fluid replacement with acetated Ringer's solution (Ringer-acetat®, Frezenius Kabi, Stockholm, Sweden) by means of an infusion pump (Life Care®, Abbot Shaw, USA), as follows: 30 $mL \cdot kg^{-1}$ during the first hour of preparation in order to obtain a pulmonary artery wedge pressure of 7-10 mm Hg, and a continuous infusion of 10 $mL \cdot kg^{-1} \cdot h^{-1}$ thereafter.

Preparation and procedures

An 18-G arterial catheter was advanced into the aortic arch via a branch of the right carotid artery for withdrawal of blood samples and measurement of blood pressure. A 14-G saline-filled double lumen catheter was placed into the right atrium via a cutdown of the right external jugular vein to measure right atrial pressure and for drug administration. This catheter was connected to pressure transducers, which were calibrated to ambient pressure at the level of the right atrium.

Measurements of hemodynamic variables

During CPR and after ROSC, hemodynamic parameters including leads II and V5 of the ECG, heart rate (HR), systemic arterial blood pressure, and right atrial pressure were continuously displayed (Solar 8000 monitor, Marquette Medical Systems, Milwaukee, WI, USA) and recorded (Workbench 3.0, Strawberry Tree Inc., Sunnyvale, CA, USA) until the end of the experiment.

Samples

Samples of arterial blood were taken for blood gas analysis and acid-base balance (ABL 300, Radiometer, Co-

penhagen, Denmark) at baseline, and at 14, 16 and 19 min during CPR, and after ROSC at 5, 15, 30, 60 and 120 min, and before 180 min. Oxygen saturation and hemoglobin were determined simultaneously on an OSM3 Hemoximeter (Radiometer, Copenhagen, Denmark) at the same time points.

Experimental protocol

After preparation, the piglets were ventilated with 30% oxygen in air, stabilized for one hour, after which baseline measurements were obtained before cardiac arrest. Thereafter, ventricular fibrillation was induced by a 50-Hz, 40-60-V alternating transthoracic current applied via two subcutaneous needle electrodes. Cardiopulmonary arrest was defined as a decrease in aortic blood pressure to below 25 mm Hg and the presence of ventricular fibrillation on the ECG. Mechanical ventilation was stopped at this point. After 12 min of untreated cardiac arrest, closed-chest CPR was performed with a pneumatically driven automatic piston device for CPR (Lucas®, Jolife AB, Lund, Sweden), and mechanical ventilation with 100% oxygen was resumed with the same ventilatory settings as before induction of cardiac arrest. One minute after commencement of CPR all animals received 0.4 $U \cdot kg^{-1}$ of vasopressin (Arg^8-vasopressin, Sigma Chemicals Co, St Louis, MO, USA) as a bolus administered via the right atrial catheter. After 8 min of external chest compressions, a monophasic countershock was delivered through defibrillation electrode pads (Medtronic Physio-Control Corp. Seattle, WA, USA) at an energy level of 200 J (Figure 1). If restoration of spontaneous circulation (ROSC) was not accomplished, another two defibrillatory shocks (200 J, 360 J) and a bolus injection of epinephrine 20 $\mu g \cdot kg^{-1}$ were administered. DC shocks were then applied at the same energy level of 360 J during a maximum period of 5 min. CPR was discontinued if ROSC was not achieved during this time. Restoration of spontaneous circulation was defined as return of co-ordinated electrical activity resulting in a systolic blood pressure greater than 60 mm Hg for at least 10 consecutive minutes. If the arterial pH was less than 7.20 or the base deficit more than 10 mmol/L at 5 min after ROSC, acidosis was corrected with 1 $mmol \cdot kg^{-1}$ of a tris buffer mixture (Tribonat®, Kabi Fresenius, Stockholm, Sweden) and by increasing minute ventilation, aiming at a $PaCO_2$ within the range of 5.0 to5.5 kPa. After the resuscitation phase and ROSC, dobutamine (Dobutrex®, Eli Lilly Sweden, Solna, Sweden) was administered in a solution of 12.5 mg/ml starting at $5\mu g \cdot kg^{-1} \cdot min^{-1}$ to keep systolic blood pressure above 70 mm Hg. After completion of the study, all animals received an injection of 10 mL potassium chloride 20 mmol/mL and were sacrificed. Sixteen animals served as controls and received no further interventions except anesthesia and placement of catheters. Within 5 min after death, the skull of all animals was opened with the animal in the prone position and the brain was rapidly removed for histopathological analysis.

To study disruptions of the BBB and the brain water and electrolyte content, 38 pigs were divided into three groups. The first group (CA, n=16)

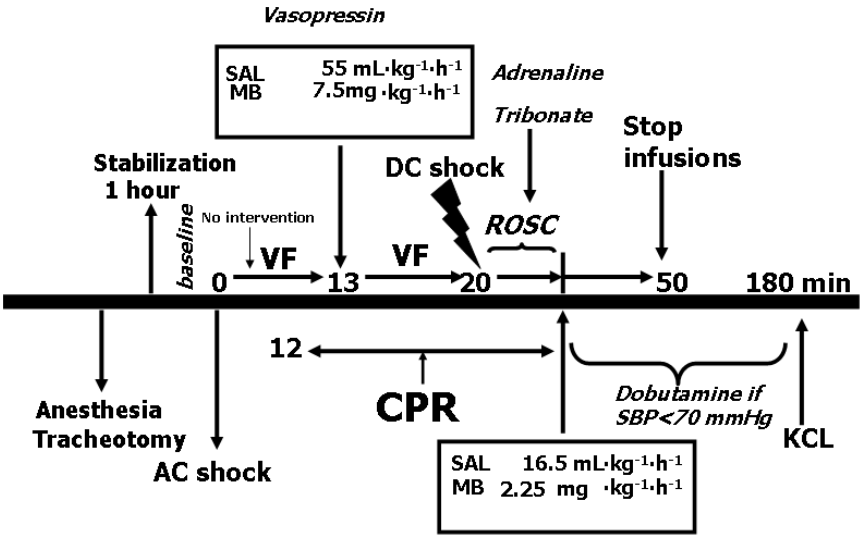

Figure 1 Experimental protocol*. After stabilization for 1 h, animals were subjected to 20 min of ventricular fibrillation (VF) including 12 min of cardiac arrest and 8 min of cardiopulmonary resuscitation (CPR). MB (methylene blue); SAL (saline); ROSC (restoration of spontaneous circulation); AC (alternating transthoracic shock); DC (defibrillatory shocks); KCL (potassium chloride)***.**

that served as control group, underwent cardiac arrest without subsequent CPR. In some animals the brain was removed immediately (n=5) after cardiac arrest, and in others it was removed at 15 min (n=5) or 30 min (n=6) after cardiac arrest. The other two groups underwent cardiac arrest and subsequent CPR and received either an infusion of saline (CA-MB group, n=10) or an infusion of saline with MB (CA+MB, n= 12). One minute after the start of CPR the animals in the last two groups were randomly assigned to receive an infusion of 55 $mL \cdot kg^{-1} \cdot h^{-1}$ normal saline (CA-MB group, n=10) or the same dose of saline infusion with 7.5 $mg \cdot kg^{-1} \cdot h^{-1}$ methylene blue (Metyltioninklorid 10 $mg \cdot mL^{-1}$ equivalent to 8.56 $mg \cdot mL^{-1}$ water free MB, Apoteket, Umeå, Sweden) (CA+MB group). After ROSC the normal saline infusion was reduced to 16.5 $mL \cdot kg^{-1} \cdot h^{-1}$ and the methylene blue infusion was reduced to 2.25 $mg \cdot kg^{-1} \cdot h^{-1}$. In these latter groups the brains were removed for histological analysis in three animals (CA-MB) and four animals (CA+MB), respectively, at each of the following time points: 30, 60, 180 min after ROSC.

Immunohistochemistry, brain water and electrolyte content

a. Fixation

One of the brain hemispheres was immersed in 4% buffered formalin and stored at 4º C for 1 week. Small tissue pieces (<3x5 mm) were cut from the cerebral cortex and processed for histology or immunohistochemistry. The tissue pieces were dehydrated in a graded series of alcohol, rinsed in xylene, and embedded in low-temperature paraffin (56-58º C) according to a standard protocol (12). Multiple 3-5- µm-thick sections (6 to 8) were cut from each tissue block and collected on glass slides. After deparaffinization, duplicate sections were stained with either Nissl (Cresyl violet) or hematoxylin and eosin using a commercial protocol (13). For each animal the number of distorted neurons in one whole section was counted at least three times in a blinded fashion and the median values were recorded for data analysis.

b. Albumin immunostaining

Immunostaining of the endogenous albumin to detect leakage across the BBB is a well-standardized method in neuropathological laboratories and has previously been used by several groups (14, 15, 16). Immunohistochemical analysis for albumin was performed on paraffin embedded (3-µm thick) sections using a rabbit polyclonal anti-human albumin antibody (DAKO, Glostrup, Denmark), and the streptavidin-HRP-biotin technique as described previously (17, 18). Briefly, the endogenous peroxidase activity was blocked with 3% H_2O_2 and 10% normal goat serum, followed by incubation with the primary antibodies (1: 400 for albumin). This was followed by incubation with biotinylated linking antibody and HRP Vector laboratories, Burlingame, Ca, USA), with brief rinses in PBS between incubations. The reaction was visualized using DAB 3,3'-Diaminobenzidine (Vector Laboratories, Burlingame, CA, USA). Reagent controls (omitting the primary antibody or substituting nonimmune serum for the primary antibody in the staining protocol) on tissue sections were used to confirm the specificity of the primary antibodies used (19, 20). The numbers of albumin-positive cells were counted three times, in a blinded fashion, in one identical area of the cortex from each animal. The median value was used for the final calculation. It is often difficult to differentiate glia from neurons, particularly when edema and neural damage alter the shape of cells. Although most albumin that is present inside cells appears to be in neurons, a few glial cells can also be positive. However, albumin leakage into the extracellular environment is also seen in these cases. Therefore, albumin-positive cells appear to represent both damaged neuron and glial cells.

c. Immunohistochemistry of NOS

Immunostaining was performed on 3-µm thick paraffin sections using a monoclonal NOS antiserum as described earlier (21, 22, 23). In brief, the antibodies of nNOS were diluted 1:5000 and applied for 48 h with continuous shaking at room temperature (10). The immune reaction was developed using a peroxidase-antiperoxidase technique and visualized in the light microscope. No

immunoreactivity was detected in controls where the primary antibody step was omitted. The number of nNOS positive cells in each group was counted in a blind fashion (10).

d. Western Blot

Protein homogenates of brain samples were prepared by rapid homogenization in Tissue Extraction Reagent II (Invitrogen Corporation) according to the manufacturer's instructions. The protein concentration was determined using the Bio-Rad RC DC kit. Protein extracts (50 μg) for each group were pooled from 3 animals, resolved by electrophoresis on 12% SDS-acrylamid gels and transferred to Hybond-P PVDF membrane (GE Healthcare). The membranes were blocked with Phosphate-buffered saline (PBS) containing 0.2% Tween-20 and 5% non-fat dry milk, prior to incubation, overnight at 4°C, with either anti NOS1 (1:10,000, Euro-Diagnostica AB), anti-NOS2 (1:10,000, abcam), anti-NOS3 (1:200, Santa Cruz Biotechnology) or anti–actin (1:200, Sigma) in PBS-Tween-20. After washing, membranes were incubated (1 h at RT) with anti-rabbit IgG-conjugated to horseradish peroxidase (1:10,000). After washing, immunoreactive bands were visualized by enhanced chemiluminescence using Lumi-Light^{plus}(Roche Diagnostics) and protein band densities were digitally quantified and normalized to the loading control (β actin) using Quantity One software (Biorad).

e. Cerebral cortex tissue injury (evidenced histologically)

Three-micrometer paraffin sections from identical tissue blocks from the cerebral cortex were cut and stained with Haematoxylin and eosin or Nissl for light microscopy to analyze cellular changes. For each animal, the numbers of distorted neurons in one whole section were counted at least three times, in a blinded fashion, and the median values were recorded for data analysis.

f. Brain water and electrolyte content

Small tissue pieces of pig cerebral cortex were used to measure water and electrolyte content according to a standard protocol (24). The water content was calculated from the difference between dry and wet weights of the sample (12), and ion content (Na^+, K^+ and Cl^-) was measured from the dry weight of the samples as described earlier (18, 25). These measurements were made in slices from the same hemisphere of the brain at the level of the parietal cerebral cortex. To measure water content, after obtaining the wet weight, the samples were placed in an oven, maintained at 90 °C, for 72 h or until three dry weight measurements were constant. Water content was calculated according to the formula: wet weight-dry weight/wet weight × 100 (26, 27). To measure ions, after obtaining the dry weight, the samples were heated at 550 °C for 24 h to produce ash. The ash was dissolved in 5 mL of 3 mmol nitric acid and diluted with deionized water (1: 10). Na^+, K^+ and Cl^- were determined at 330, 404 and 512 nm, respectively, with an Atomic Absorption Spectrophotometer (Packard Instruments, Downers, IL, USA) using an air-acetylene flame (28, 29).

Measurement of nitrite and nitrate in the cerebral cortex of pigs

a. Sample preparation:

Immediately after removal, the brain tissue was dissected out at 4° C, then frozen on dry ice and stored at -70 °C until assay.

b. Tissue

For sample preparation, the tissue was thawed and homogenized in 20 mM Tris buffer, 10 mM EDTA (pH 7.4), and centrifuged at 4000 x g for 15 min, before the total protein content in cytosol was determined using Lowry method (30).

c. Fluorometric Assay of nitrite and nitrate

Nitrite and nitrate levels were measured using fluorescence of 2, 3-diaminonapthotriazole as described earlier (31, 32). The cytosol was filtered to remove hemoglobin through a 10 kDa cutoff filter at 15 000 g for 90 min and then processed for nitrite or nitrate measurement (33). The NOx levels were measured following reduction of nitrate to nitrite with nitrate reductase and NADPH regenerating system (G-6-P/G-6-PDH). The complete reaction was developed using nitrate reductase 30mU: NADPS 3 µM; G-6-P 750 µM; G-6-PDH 48 mU in a total reaction volume of 100 µl. The samples were incubated at room temperature for 90 min. At the end of incubation, 30 µl DAN reagent (50 µg/ml, 0.62 N HCl) was added, and after 10 min, 30 µl of 1.4 N NaOH was added to the incubation mixture. The fluorescence was measured in an Aminco-Bowmann Spectrophotofluorometer Series 2 (Thermo Elecron Corp, Madison, WI, USA) at excitation wave lengths of 360 nm and 450 nm. The NOx levels were quantified using external standard solutions of sodium nitrite or sodium nitrate reduced with reductase. For nitrite measurements, the samples were not treated with reductase. The brain NO levels were expressed as pmoles/mg protein (33).

Statistical analysis

Water content, ion analysis, hemodynamic data were analyzed using ANOVA followed by Bonferroni's test for multiple group comparison. The histological and immunohistochemical results obtained for all groups were compared using Kruskal Wallis non-parametrical analysis of variance and if a statistical difference was detected post-hoc analysis of each group compared to the control group was performed by the Dunn`s multiple comparisons test.

Results

Out of 45 animals, seven were not resuscitated, thus leaving 38 to be studied after ROSC. There were no differences between groups at baseline, before cardiac arrest.

a.Hemodynamic parameters

During CPR, mean arterial pressure (MAP) increased significantly, but it stayed below baseline values at all points ($P<0.001$ versus baseline). Heart rate increased significantly after restoration of spontaneous circulation and then gradually returned to baseline values by the end of the first hour after ROSC. After ROSC, there was a period of 5-10 min of spontaneous overshoot in arterial

blood pressures, in all groups (P <0.0001). After that a hypotensive period gradually followed in all groups, with the lowest levels at 30 minutes after ROSC (P<0.001).The decreased blood pressure lasted approximately 1 h after ROSC, followed by a gradually increased to baseline levels. Differences between the two groups that underwent CPR were seen at 15 and 30 min after ROSC with increased MAP in CA+MB group in comparison with the group untreated with MB (P<0.01) (Fig.2).

b. Blood gases Compared to pre-arrest values, it was observed that during the 8 min of CPR the arterial blood pH gradually decreased, but acidemia was not profound in any of the three groups. In contrast acidemia was profound (<7.2) 5 min after ROSC and needed correction with tris buffer mixture and increased ventilation.

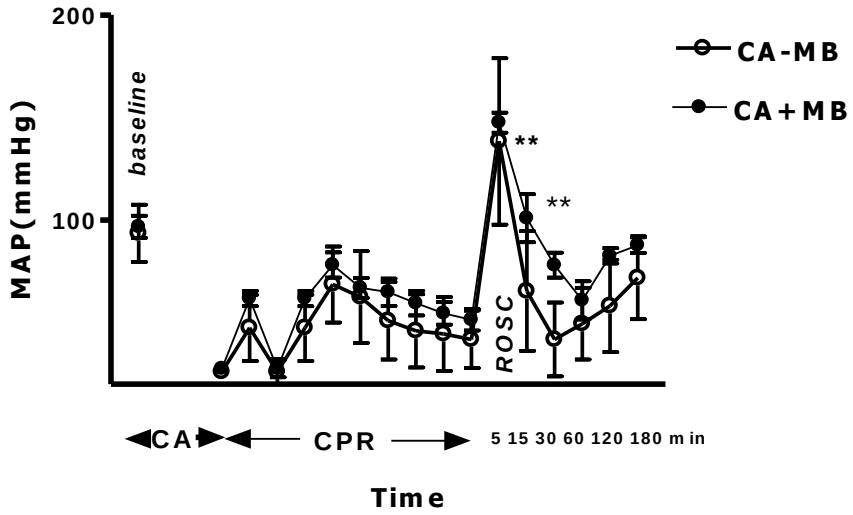

Figure 2 ***Mean arterial pressure.*** *The group treated with methylene blue (CA+MB) represented with black circles and the group without MB (CA-MB) denoted with white circles. Significant differences (P**<0.01) between groups were seen at 15 and 30 minutes after return of spontaneous circulation (ROSC). CA-cardiac arrest; CPR cardiopulmonary resuscitation.*

Albumin immunoreactivity, water, electrolyte content and neuronal injury

During the experiment the brain water content increased progressively, with a maximum in all the groups at the end of the experiment in comparison with controls (P<0.001). This was accompanied by an increase in brain sodium content in all groups (P<0.05) and an increase in potassium content in the CA group (P<0.001), as well as a decrease in cerebral potassium content in the CA+MB group (P<0.01) at 180 min. The chloride ion simultaneously accumulated in the CA group. In all the groups an increase in necrotic neurons and albumin

immunoreactivity was demonstrated with increasing duration of ischemia and reperfusion time (P<0.001). The immunohistochemical analysis indicated less BBB disruption in the animals receiving MB (CA+MB group), as evidenced by a decreased albumin leakage (*P*=0.004), tissue content of water (*P*=0.02), sodium (P=0.06) and potassium (*P*=0.04), as well as decreased neuronal injury (*P*=0.008) in comparison with the group that was not treated with MB (CA-MB) (Fig.3). The cerebral contents of albumin, water, sodium and potassium, as well as neuronal damage, were decreased at every time point (30, 60, 180 min) in the CA+MB group compared to the CA-MB group. A significant difference in chloride content between the CA+MB and CA-MB groups was observed only at 30 min (*P*<0.01). The changes in immunohistochemical staining were assessed using semi-quantitative analysis, and the positive albumin cells were counted in the same region of the cortex (Fig. 4).

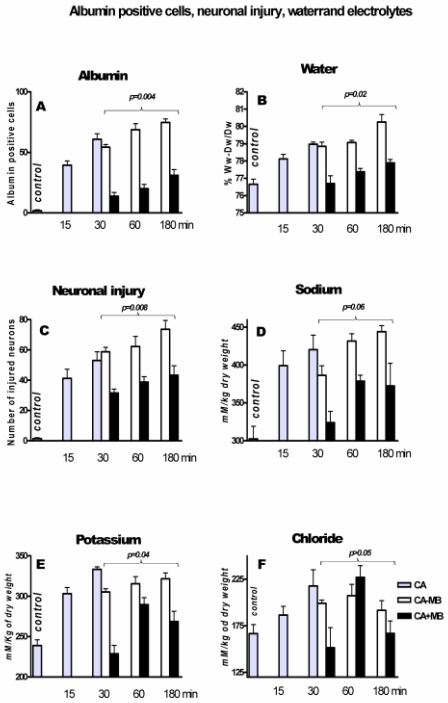

Figure 3. ***Graphic representations of alterations in blood-brain barrier (BBB) immunoreactivity for albumin (A), brain edema (B), neuronal injury (C) and tissue electrolytes content [(sodium (D), potassium (E), chlor(F)] in cerebral cortex.*** *Untreated animals (CA-MB group) exhibited profound increase in the permeability of BBB for albumin (p=0.004), increase in brain water (p=0.02), and potassium (p=0.04). Semiquantitative data in the same group evidenced significant increase in the number of distorted neurons as a measure of neuronal injury (p=0.008). The groups described were: CA group (cardiac arrest without CPR); CA-MB group (cardiac arrest with CPR without administration of MB) and CA+MB (cardiac arrest with MB administration).*

The CA group was sacrificed immediately after cardiac arrest. There was a mild to moderate degree of nerve cell damage in the cortical regions that increased over time, but that was mitigated by the administration of MB (P=0.008). Moderate to severe nerve cell damage was also present in the subcortical regions, such as the mid-thalamic nuclei and the brain stem reticular formation. The magnitude of nerve cell damage was most pronounced in brain stem (13).

b. Nitrate, nitrite and nitrite/nitrate content

In general, there was a rapid increase in the values of nitrate, nitrite and the nitrite/nitrate content throughout the experiment.The increase over time in nitrite levels was more pronounced than that for nitrate levels. However, the increase was mitigated in the group that was treated with MB (CA+MB) as compared to the group that did not receive MB (CA-MB). Thus, methylene blue administration reduced both nitrate and nitrite content, with a more important reduction in nitrite resulting in a lower nitrite/nitrate ratio in the CA+MB group (P=0.02) in comparison with the CA-MB group.

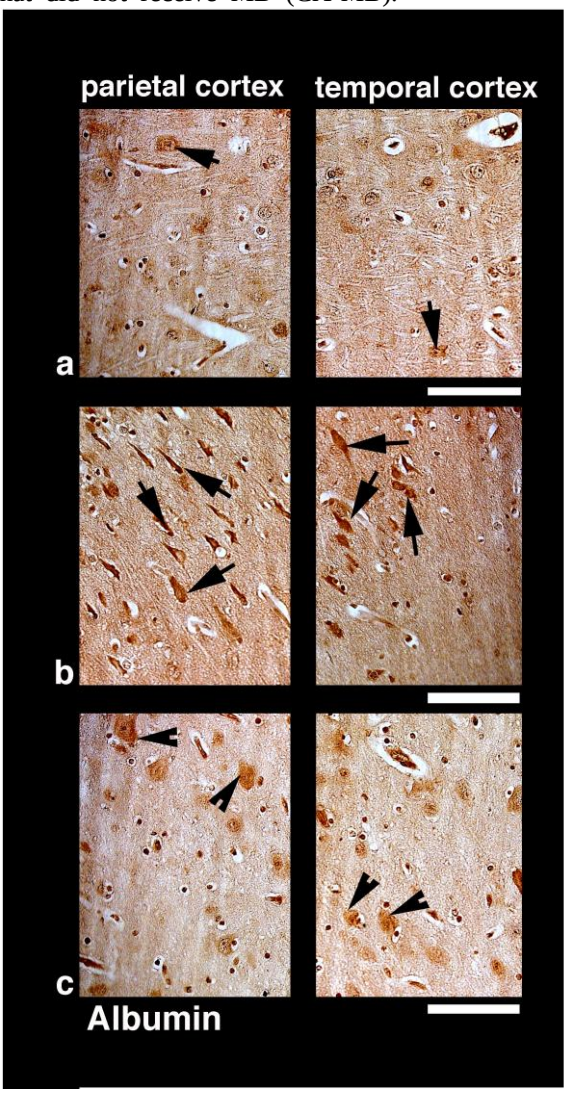

Figure 4. ***Representative examples of albumin immunostaining*** *in the parietal cortex (left panel) and temporal cortex (right panel) of pig brain from control (CAwithout CPR) (a), CA-MB group at 30 min (b) and methylene blue treated animals (CA+MB) (30 min, c). Control group occasionally exhibited a few albumin positive cells in the cortex (a). CA-MB 30 min resulted in a massive increase in numbers of albumin positive cells in the cortical areas (b). Many albumin-containing neurons are showing deformed shape and perineuronal edema can be clearly seen in this group (arrows). The neuropil is swollen and sponginess is evident around the damaged neurons in this group (b). CA+MB 30 min showed a marked reduction in albumin positive cells (arrow-heads) on the cerebral cortex. Moreover the shape of neurons is also very similar to that seen in the control group indicating quite good neuroprotection in MB treated group following CA 30 min (c). Sponginess and edema in the neuropil is much less apparent in MB treated CA group (c).*

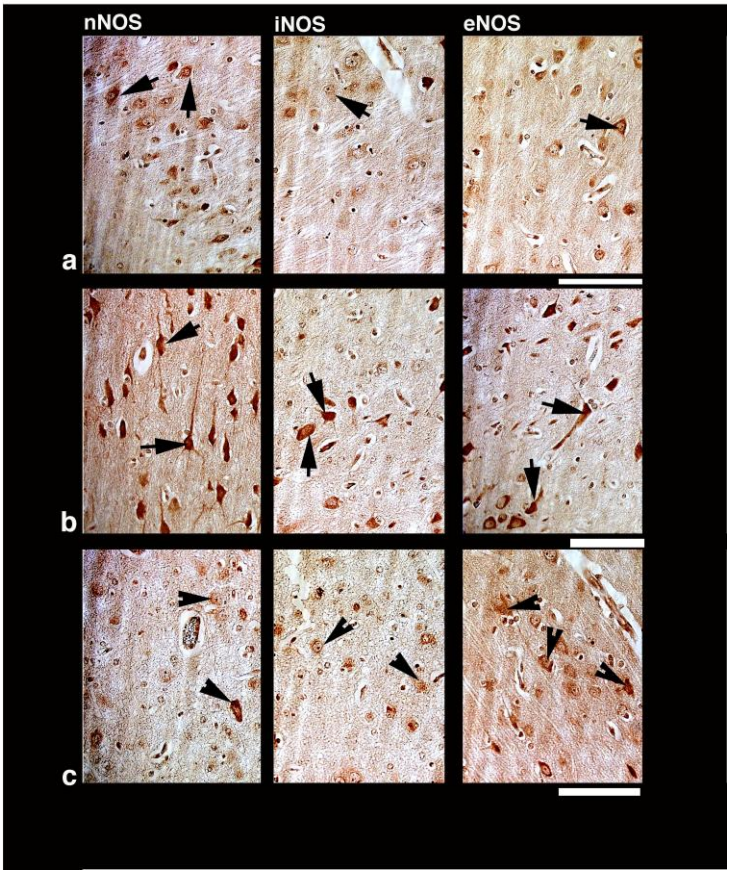

Figure 5. ***Representative example of neuronal (left panel), inducible (middle panel) and endothelial (right panel) nitric oxide synthase (NOS) expression*** *in the parietal cerebral cortex of pig brains in control (a), cardiac arrest (CA-MB) 30 min (b) and following methylene blue (MB) treated CA group 30 min (CA+MB) (c). Several neurons express constitutive neuronal NOS (nNOS) in the control cerebral cortex (a), whereas only a few cells show inducible NOS (iNOS) expression (arrows). Some neurons in control group exhibited endothelial NOS (eNOS) expression (arrows) together with endothelial cells. CA 30 min markedly upregulated nNOS expression in several neurons in the cortex (b). The nNOS expression is clearly seen in deformed neurons and axons distributed in the neuropil (arrows). Much intense nNOS expression is seen in deformed and damaged nerve cells showing marked perineuronal edema. These neurons are present in areas showing sponginess and edema in the cortex. On the other hand, a distinct increase in iNOS is seen in the similar cortical areas, however, the number of iNOS positive cells is much less compared to nNOS positive cells in the same animal. The pattern of iNOS expression in neurons is also markedly different than nNOS expression (middle panel, arrows). In CA*

group, pronounced increase in eNOS immunostaining is seen in several neurons located widespread in the cortical areas (b right panel) compared to control group (a). The eNOS containing cells are deformed and show intense immunoreaction product largely in the neuronal cytoplasm (arrows, right panel, b). MB-treatment profoundly reduced the number of nNOS and iNOS immunostaining in nerve cells in the cortical areas following CA 30 min (c, left and middle panel, respectively). Few neurons in this group show intense NOS immunoreaction product (arrows). The occurrence of perineuronal edema and sponginess of neuropil are much less apparent in MB-treated CA group (c). However, eNOS expression by MB treatment at 30 min is only slightly attenuated (c, right panel). Thus, eNOS expression is seen in many neurons although the intensities of immunoreaction product are much less intense compared to untreated CA group (b, right panel). Interestingly, perineuronal edema and sponginess is much less around the eNOS immunostained neurons in MB-treated CA group (c, right panel).

c.NOS activity/expression (Figs. 5,6,7,8)

In the control condition there was very little activity/expression of any of the NOS isoforms in the cerebral cortex indicated by immunohistochemistry. Experimental global ischemia from cardiac arrest rapidly led to the upregulation of the nNOS and iNOS isoforms. After only 5 min of untreated CA, significant nNOS expression was obvious. Thus, nNOS activation increased over time in all groups, resulting in an increased number of activated cells in comparison with iNOS ($p<0.001$). In contrast, there were no changes in eNOS expression over any of the time points studied. MB mitigated iNOS and nNOS upregulation at 30 min after ROSC, and thus less iNOS ($p<0.01$) and nNOS ($p<0.01$) activation was seen at this time point in the group that was treated with MB in comparison with the CA-MB group. However, MB appeared to attenuate nNOS and iNOS activity only during the first 30 min after ROSC, i.e. during the time of administration; no effects were observed later. In contrast to this, in the group with MB we observed by immunohistochemistry an activation of eNOS not only at 30 min ($p=0.02$), but also at all time points until the end of the experiment ($P<0.01$).

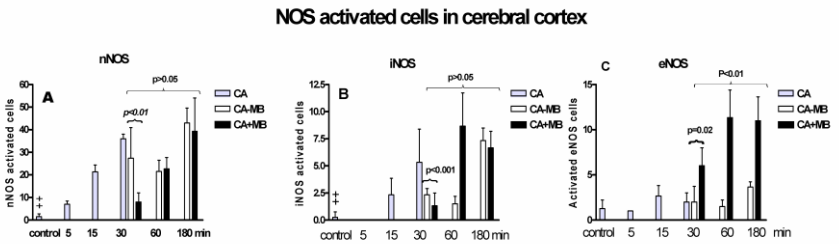

Figure 6. ***NOS activity in cerebral cortex.*** *Panel A shows iNOS activity; panel B represents nNOS activity; panel C shows eNOS expressions. Data are expressed as means± SD. The gray bars represent the control group, where NOSs activation was determined at baseline, and at 5, 15, and 30 min after cardiac arrest. The white bars represent the CA-MB group and the black bars the CA+MB group. In these groups NOSs expressions were obtained at 30, 60, and 180 min after ROSC.*

Ischemic neurons tend to pick up all kind of stainings. Accordingly, we have done a few of control experiments in order to fully appreciate the presented data. Therefore the immunohistochemical (IHC) stainings included a negative control where the primary antibody is omitted in order to exclude unspecific binding of the secondary antibody, and a positive control (Figure 7).

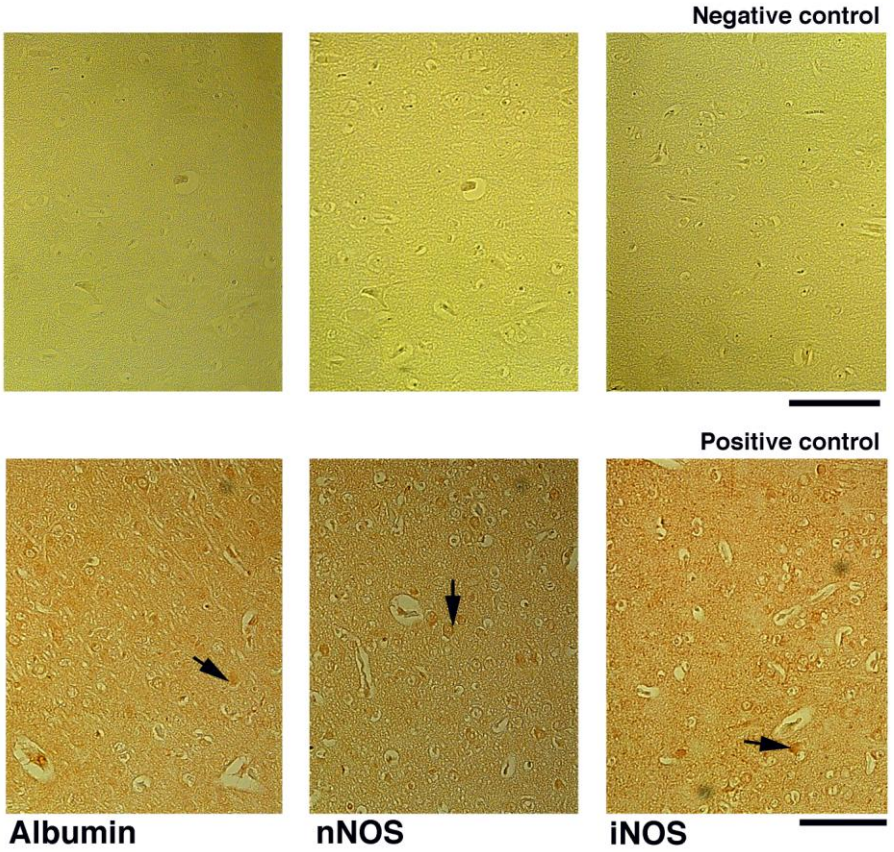

Figure 7. ***Negative and positive control images for albumin and NOS staining****. In negative control (antibodies are omitted) a very clean image represent specific staining The positive control represents 5 min controls (cardiac arrest without CPR) showing some mild immuno for NOS and albumin but this is much negligible than experimental groups.*

NOS activity was also investigated with Western blot analysis . In comparison with immunohistochemical analysis of NOS that were performed only from cerebral cortex, these Western blot analysis investigated NOS activity from different brain regions (Figure 8). Western blotting of superficial cerebral tissues pooled from all animals at respective time points revealed results which varied somewhat from the the immunohistochemical analysis for nNOS and iNOS but reasonably consistent with microscopical evaluation of immunostained sections of cerebral cortex in the

temporo-parietal region. No increase in eNOS protein expression could be shown using western blot technique.

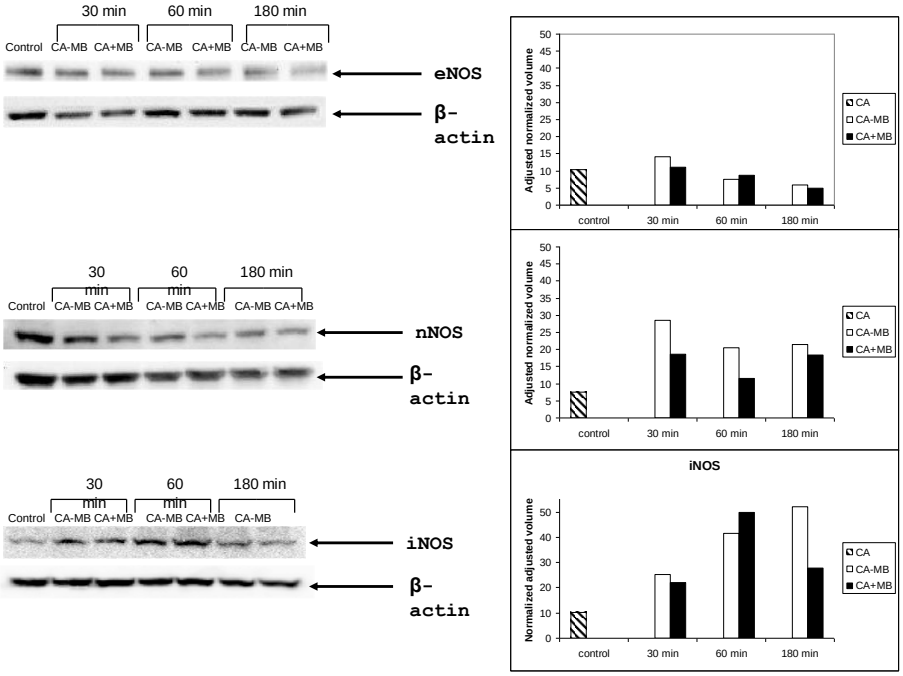

Figure 8. ***Expression of eNOS, nNOS and iNOS by western blot analysis.*** *Protein extracts of superficial cerebral tissue from 3 animals were pooled in control, CA-MB (white bars) and CA+MB (black bars) groups at 30, 60 and 180 min after ROSC respectively. The expression of ß-actin was determined as an internal control (A). Relative density analysis showed in the bar graphs for eNOS, nNOS and iNOS respectively, was expressed as a ratio to ß-actin after background subtraction (B)*

Discussion

It has long been accepted that global cerebral anoxia induced by circulatory arrest in humans can result in neurological damage and neurological dysfunction after only a 5-min cardiac arrest and subsequent restoration of spontaneous circulation (34). The present results provide experimental evidence for leakage of albumin, water and electrolytes into the brain parenchyma after cardiac arrest and ROSC. The BBB disruption began as early as during the initial phase of an untreated as well as a treated cardiac arrest and progressed throughout the 3 h observation period. MB administration during CPR and the initial phase after ROSC reduced these harmful effects considerably, but did not, on the other hand, reverse this ongoing detrimental process.

We have previously provided histopathological proof in a porcine model of BBB disruption as early as 5 min after untreated cardiac arrest (13). In the present investigation evidence of neurological injury was recorded 15

min after ROSC, and the magnitude of this injury increased with increasing time following the initial ischemic insult.

Evidence of widespread microvascular and neuronal damage 4 h after cardiac arrest and ROSC has previously been documented in infant piglets (35). In those studies it was considered that young animals might be more prone to a delayed increase in BBB permeability after cardiac arrest and CPR (36, 37). Maximal disruption of BBB has been reported at 3 hours after reperfusion, with normalization 24 h after ROSC (38). However, some studies have indicated that BBB postischemic disturbances are of a biphasic nature (39, 40) and that disruption of the BBB does not increase with extended periods of ischemia and recirculation (41). The discrepancy with the present results might be due to differences between various animal models, different methods for assessing BBB function and the different time points of investigation. In any case, there were no indications in the present study that leakage through the BBB was not a continuous process. This finding of an early leakage after cardiac arrest is also supported by studies using Evans blue (13). This dye has been shown to represent vascular protein leakage macroscopically (42).

Previous research from our group has suggested that MB co-administered with hypertonic saline dextran may be an effective mechanism for attenuation of oxidative, inflammatory and neurological injuries (5, 6). MB may contribute to those effects by inhibiting NO production as well as reducing effects of excess NO, and/or by scavenging of oxygen-derived free radicals (5, 6). In the present study no HSD was administered. Therefore the neuroprotective effects must be ascribed to the MB, as the study protocols in these studies were otherwise identical. In our study, counting the distorted neurons in parietal-temporal cortex was used to compare the severity of post cardiac arrest induced brain damage. Findings by Radovsky et al (43), however, indicate that overall brain damage, rather than damage in any one specific region, is of most significance for the continued life of the experimental animal with cortical injury (43). Based on these and previous findings it seems that MB itself easily passes the BBB, since after systemic administration this dye crosses the BBB and selectively stains the brain tissues (45). Thus, in this situation MB has a much higher concentration in the brain tissue than in the circulation (44). This effect was demonstrated by measuring the distribution of MB with high-performance liquid chromatography (44), as well as by the obvious blue tint of the animal's brain when removed 5 min after it was sacrificed (34).

Systemic hypertension that occurs immediately after successful CPR increases cerebral blood flow as demonstrated previously by our group by means of the Laser Doppler technique (5, 6) and positron emission tomography (11). When BBB permeability is increased, it allows extravasation of albumin and other high and low molecular weight compounds (46) into the extracellular compartment of the brain, leading to vasogenic edema formation and subsequent cell injury . In contrast to this kind of reasoning the vasogenic edema that results after the systemic hypertensive response has been claimed to be a major contributor to

the BBB disruption (35, 47). In this context it may be noted that greater blood pressure in the group receiving MB in comparison with the group not receiving MB did not increase BBB permeability. On the contrary, lower albumin leakage, neuronal injury and water content was demonstrated in the group treated with MB. The lack of significant increase in water content and albumin in the MB treated group is consistent with the lack of an increase in BBB permeability. The rationale for our finding would be that the magnitude of the blood pressure increase after ROSC cannot be associated with the BBB disruption when MB had been administrated. The hyperemia that occurred after ROSC has been suggested to cause disruption of the BBB which ultimately may worsen neurological injury (3). These effects, and cerebral hyperemia observed after global ischemia have been suggested to be caused by high levels of NO produced during ischemia and early reperfusion (48). Thus, there is strong evidence that NO is involved in the mechanisms of neurotoxicity after cerebral ischemia (49). Hence, it seems logical to speculate that the mechanism behind less BBB disruption in the CA+MB group occurred as a result of NOS and guanylate cyclase inhibition by MB. It is noteworthy that this effect occurred in spite of the limited duration of the MB effect on nNOS and iNOS activation. Nitrite and nitrate are stable metabolites of NO, accessible to quantitative analysis. Therefore, measurement of nitrite and nitrate turned out to be the most suitable and practical method for assessing NO synthesis (50). MB decreased the nitrite/nitrate content, demonstrating an inhibitory effect on NO production and its subsequent oxidation.

All three NOS isoforms were expressed after global ischemia following cardiac arrest. However, both immunohistochemical and Western blot analysis confirmed previous studies demonstrating iNOS transcriptional induction occurs transcriptionally long after activation of post-transcriptionally activated eNOS and nNOS, suggesting that iNOS may not actively contribute to early cerebral injuries (51). Thus, it is generally agreed that action by nNOS is responsible for the formation of NO that initially is harmful to the brain (52). There are no previous data on the relative inhibition of the NOS isoforms by MB. Earlier studies suggest that the large NO tissue content produced a few hours after ROSC by activation of iNOS is detrimental, and that the considerably smaller tissue content of NO derived from eNOS is beneficial (53) due to its vasodilating effects after cerebral ischemia. Hence, it was also considered that the beneficial effects of non-selective inhibitors were limited, because they inhibit eNOS to a similar extent (51) and may therefore aggravate the effects of brain ischemia. MB was considered a non-selective inhibitor. However, the interpretation of this data requires caution in the light of other studies that describes dose-related neurotoxicity of methylene blue on central nervous system (54) for higher doses (between 5 mg/kg and 50 mg/kg), than the doses used in the present study and also possible interspecies differences (54). In this study the neuroprotection has been assessed in terms of BBB function and cerebral water content, together with histology. The limitation of the present

research is that the potential of neuroprotection concerning neurologic function in terms of electrophysiology or in behavior could not be fully assessed.

In conclusion:

The present results unequivocally demonstrate that MB markedly reduced BBB disruption and subsequent neurological injury after ischemia/reperfusion following cardiac arrest. Accordingly, this approach is worth testing in human cerebral ischemia and reperfusion after cardiac arrest.

Acknowledgements
The authors are grateful to Anders Nordgren, Monika Hall, Elisabeth Pettersson for excellent technical assistance.

References:

1. Kaur C, Ling EA: Blood brain barrier in hypoxic-ischemic conditions. *Curr Neurovasc Res* 2008;5:71-81
2. Kubes P, Granger DN: Nitric oxide modulates microvascular permeability. *Am J Physiol* 1992; 262: H611-H615
3. Schleien CL, Kuluz JW, Gelman B: Hemodynamic effects of nitric oxide synthase inhibition before and after cardiac arrest in infant piglets. *Am J Physiol Heart Circ Physiol* 1998;274:H1378-H1385
4. Schleien CL, Eberle B, Shaffner DH, et al: Reduced blood-brain barrier permeability after cardiac arrest by conjugated superoxide dismutase and catalase in piglets. *Stroke* 1994;25:1830-4
5. Miclescu A, Basu S, Wiklund L: Methylene blue added to a hypertonic hyperoncotic solution increases short-term survival in experimental cardiac arrest. *Critical Care Medicine* 2006;34:2806-13
6. Miclescu A, Basu S, Wiklund L: Cardio-cerebral and metabolic effects of methylene blue in hypertonic sodium lactate during experimental cardiopulmonary resuscitation.*Resuscitation* 2007; **75**:88-97
7. Mayer B, Brunner F, Schmidt K: Inhibition of nitric oxide synthesis by methylene blue. *Biochem Pharmacol* 1993;45:367-74
8. Mayer B, Brunner F, Schmidt K: Novel actions of methylene blue *Eur Heart J* 1993;14:22-6
9. Salaris SC, Babs CF, Voorhees III WD: Methylene blue as an inhibitor of superoxide generation by xanthine oxidase. A potential new drug for the attenuation of ischemia/reperfusion injury. *Biochem Pharmacol* 1991; 42:499-506
10. Kelner MJ, Bagnell R, Hale B, et al: Potential of methylene blue to block oxygen radical generation in reperfusion injury. *Basic Life Sci* 1988;49:895-8
11. Mörtberg E, Cumming P, Wiklund L: A PET study of regional blood flow after experimental cardiopulmonary resuscitation. *Resuscitation* 2007;75:98-104
12. Sharma HS, Cervos-Navarro J: Brain oedema and cellular changes induced by acute stress in young rats. *Acta Neurochir Suppl (Wien)* 1990;51:383-6
13. Wiklund L, Sharma HS, Basu S: Circulatory Arrest as a Model for Studies of Global Ischemic In-

jury and Neuroprotection. *Ann NY Acad Sci* 2005;1053:205–19
14. Salahuddin TS, Kalimo H, Johansson BB, et al: Observations on exudation of fibronectin, fibrinogen albumin in the brain after carotid infusion of hyperosmolar solutions. An immunohistochemical study in the rat in longlasting changes in the brain microenvironment and multifocal nerve cell injuries. *Acta Neuropathol (Berlin)* 1988;76:1–10
15. Pettersson CA, Salahuddin TS, Olsson Y: Presence of plasma proteins in spinal nerve roots. An immunohistochemical study in the rat. *Acta Neurol Scand* 1990; 82:21–7
16. Sharma HS: Pathophysiology of the blood–spinal cord barrier in traumatic injury. In: HS Sharma and J. Westman, Editors, *The blood–spinal cord and brain barriers in health and disease*, Elsevier, Academic Press, San Diego 2004; pp. 437–518
17. Sharma HS, Kretzschmar R, Cervos-Navarro J, et al: Age-related pathophysiology of the blood–brain barrier in heat stress. *Prog Brain Res* 1992;91: 189–196
18. Sharma HS: Pathophysiology of blood–spinal cord barrier in traumatic injury and repair. *Curr Pharmacol Des* 2005;11:1353-89
19. Gordh T, Chu H, Sharma HS: Spinal nerve lesion alters blood–spinal cord barrier function and activates astrocytes in the rat. *Pain* 2006; 124: 211–21
20. Sharma HS, Ali SF: Alterations in blood–brain barrier function by morphine and amphetamine. *Ann N Y Acad Sci* 2006;1074: 198–224
21. Sharma HS, Westman J, Alm P, et al: Involvement of nitric oxide in the pathophysiology of acute heat stress in the rat. Influence of a new antioxidant compound H-290/51. *Ann NY Accad Sci*1997; 813:581-90
22. Sharma HS, Alm P, Westman J: Upregulation of hemeoxygenase-II in the rat spinal cord following heat stress. In: Nielsen- Johanssen B, Nielsen R (eds) *Thermal physiology* 1997. The August Krogh Institute, Copenhagen 135-8
23. Sharma HS, Alm P, Westman J: Nitric oxide and carbon monoxide in the pathophysiology of brain functions in heat stress. In: Sharma HS, Westman J (eds) *Brain functions in heat environment* Prog Brain Res 1998;115: 297-333
24. Kiyatkin EA, Brown PL, Sharma HS: Brain edema and breakdown of the blood-brain barrier during methamphetamine intoxication: critical role of brain hyperthermia. *Eur J Neuroscience* 2007; 26 :1242–53
25. Joo F, Szucs A, Csanda E: Metiamide treatment of brain ødema in animals exposed to 90 yttrium irradiation. *J Pharm Pharmacol* 1976;28:162–3
26. Rapoport SI: *Blood–Brain Barrier in Physiology and Medicine*. 1976; Raven Press, New York
27. Sharma HS: Methods to produce brain hyperthermia. *Curr Prot Toxicol* 2005;14:1–24
28. Dux E, Temesvari P, Szerdahelyi P, et al: Protective effect of antihistamines on cerebral edema induced by experimental pneumothorax in newborn piglets. *Neuroscience* 1978;22:317–21

29. Sharma HS, Westman J: The Blood-Spinal Cord and Brain Barriers in Health and Disease. 2004 Academic Press, San Diego
30. Lowry OH, Rosebrough NJ, Farr AL, et al: Protein measurement with the folin phenol reagent. *J Bio Chem* 1951; 193:265-75
31. Sharma HS, Olsson Y, Dey PK: Early accumulation of serotonin in rat spinal cord subjected to traumatic injury. Relation to edema and blood flow changes. *Neuroscience* 1990;36:725-30
32. Misko TP, Schilling RJ, Salvemini D, et al: A fluorometric assay for the measurement of nitrite in biological samples. *Anal Biochem* 1993;214:11-6
33. Rao AM, Dogan A, Hatcher JF, Dempsey RJ. Fluorometric assay of nitrite and nitrate in brain tissue after traumatic brain injury and cerebral ischemia. *Brain Res* 793: 265-70
34. Wiklund L, Basu S, Miclescu A, et al: Neuro- and cardioprotective effects of blockade of nitric oxide action by administration of methylene blue. *Ann NY Acad Sci* 2007;1122:231-44
35. Caceres MJ, Schleien CL, Kuluz JW: Early endothelial damage and leukocyte accumulation in piglet brains following cardiac arrest. *Acta Neuropathol (Berl)* 1995;90:582-91
36. Schleien CL, Koehler RC, Shaffner DH: Blood-brain barrier integrity during cardiopulmonary resuscitation in dogs. *Stroke* 1990;21:1185-91
37. Schleien CL, Koehler RC, Shaffner DH, et al: Blood-brain barrier disruption following CPR in piglets. *Acta Neurochir Suppl (Wien)* 1990;51:213-5
38. Betz AL, Dietrich WD: Blood-brain dysfunction in cerebral ischemia. In Ginsberg MD, Bogousslavsky J: *Cerebrovascular disease: Pathophysiology, diagnosis and management*. Blackwell Science 1998;1:359
39. Mossakowski MJ, Lossinsky AS, Pluta R, et al: Abnormalities of the blood-brain barrier in global cerebral ischemia in rats due to experimental cardiac arrest. *Acta Neurochir Suppl (Wien)* 1994; 60:274-6
40. Preston E, Sutherland G, Finsten A: Three openings of the blood-brain barrier produced by forebrain ischemia in the rat. *Neurosci Lett* 1993;149:75-8
41. Pluta R, Lossinsky AS, Wisniewski HM, et al: Early blood-brain barrier changes in the rat following transient complete cerebral ischemia induced by cardiac arrest. *Brain Res* 1994; 633:41-52
42. Clasen RA, Pandolfi S, Hass GM: Vital staining, serum albumin and the blood-brain barrier. *J Neuropathol Exp Neurol* 1970; 29:266-84
43. Radovsky A, Safar P, Sterz F: Regional prevalence and distribution of ischemic neurons in dog brains 96 hours after cardiac arrest of 0 to 20 minutes. *Stroke* 1995;26:2127-34
44. Peter C, Hongwan D, Kupfer A, et al: Pharmacokinetiks and organ distribution of intravenous and oral methylene blue. *Eur J Clin Pharmacol* 2000;56:247-50
45. O`Leary JL, Petty J, Harris AB, et al: Supravital staining of mammalian brain with intra-arterial methylene blue followed by pressurized oxygen. *Stain Technol* 1968;43:197-20

46. Nelson CW, Wei EP, Povlishock JT, et al: Oxygen radicals in cerebral ischemia. *Am J Physiol* 1992; 263: H1356-H1362
47. Mayhan WG, Heistad DD: Role of veins and cerebral venous pressure in disruption of the blood-brain barrier. *Circ Res* 1986;59:216-20
48. Gally JA, Montague PR, Reeke Jr, et al: The NO hypothesis: possible effects of a short-lived, rapidly diffusible signal in the development and function of the nervous system. *Proc Nat Acad Sci USA* 1990;87:3547-51
49. Moro MA, Cárdenas A, Hurtado O, et al: Role of nitric oxide after brain ischaemia. *Cell Calcium* 2004;36:265–75
50. Tsikas D: Methods of quantitative analysis of the nitric oxide metabolites nitrite and nitrate in human biological fluids. *Free Radical Research* 2005;39:97-815
51. Murphy S, Gibson CI: Nitric oxide, ischaemia and brain inflammation. *Biochem Soc Transactions* 2007;35:1133-7
52. Won SJ, Kim DY, Gwag BJ: Cellular and molecular pathways of ischemic neuronal death. *J Biochem Mol Biol* 2002;35:67-86
53. Chan PH: Reactive oxygen radicals in signaling and damage in the ischemic brain. *J Cerebral blood flow metabolism* 2005;21:2-14
54. Vutskits L, Briner A, Klauser P, et al: Adverse effects of methylene blue on the central nervous system. *Anesthesiology* 2008;108:684-92