Restorative Neurology and Neuroscience 21 (2003) 141–150
IOS Press

Release patterns of astrocytic and neuronal biochemical markers in serum during and after experimental settings of cardiac surgery

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Abstract. *Objective:* Brain injury and altered psychomotor development in infants, children and adults after cardiac surgery using cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest (DHCA) is still a matter of concern. Early diagnosis and identification of brain injury that has occurred or is ongoing by measurement of biochemical markers in serum may have diagnostic and prognostic value. The aim of the experimental studies in an animal model was therefore to investigate the release patterns of astroglial and neuronal markers in serum and to determine the morphological and immunohistochemical changes in the brain of animals undergoing similar perfusion conditions of CPB and a period of DHCA.
Methods: Fourteen New Zealand rabbits, (weight, 3.1 ± 0.25 kg) were anesthetized, intubated and mechanically ventilated. Four animals were sham operated and served as controls. After median sternotomy the animals were connected to CPB by cannulation of the aorta and right atrium. Full flow CPB (200–250 ml/kg/min) was initiated to achieve homogeneous systemic cooling. Circulatory arrest of 60 minutes was induced when rectal and nasopharyngeal temperature of 14°C was achieved. After rewarmed reperfusion and establishment of stable cardiac ejection the animals were weaned from CPB and monitored for 6 hours. Then the animals were killed, the brain was immediately removed and cut in standardized sections. These were fixated, embedded in paraffin and stained for further quantitative histological studies. In the brain astrocyte reactivity for S-100B was assessed immunocytochemically (DPC® Immustain Los Angeles, USA). Monoclonal mouse anti-human neurospecific enolase (NSE) antibody was used for the localization of NSE in the fixed and paraffin embedded brain (NSE-DAKO, H14). The concentrations of S-100B protein and neurospecific enolase (NSE) in the serum were analyzed using a commercially available immunoluminometric assay (LIA-mat®, Sangtec® 100, Byk-Sangtec). Immunospecific monoclonal anti-parvalbumin antibody was used for the detection of parvalbumin in the brain. Serum concentrations of parvalbumin were analyzed using a newly developed ELISA method.
Results: In all experimental animals a significant increase of the serum concentration of the astroglial protein S-100B was found immediately after reperfusion and the termination of CPB. In contrast the serum levels of the neuronal proteins parvalbumin and NSE were not increased, but rather decreased. Light microscopy and electron microscopy revealed perivascular astrocytic swelling and minor neuronal cell injury. In comparison to the sham operated animals, increased immunohistochemical staining of S-100B was found. This increased reactivity of S100B antibody was found in the astrocytic processes with immediate connection to the perivascular space and around the perivascular oedema. The immunocytochemical stainings for NSE and parvalbumin in the neuronal cells was not different from that of sham-operated animals and indicated well preserved neurons.

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Conclusion: The marked perivascular swelling of the astrocytic processes may support the assumption of re-perfusion injury of the blood-brain barrier and could be indicative of the source of measured S-100B in the serum. Injury to astrocytes and their increased immunocytochemical reaction for S-100B seem to precede damage of the neurons following CPB and 60 minutes of DHCA. Immunostaining of NSE and parvalbumin may indicate integrity of the neuronal cells.

Keywords: Protein S-100B, neuron-specific enolase (NSE), parvalbumin, cardiopulmonary bypass, deep hypothermic circulatory arrest, brain injury

1. Introduction

Brain injury and altered psychomotor development in infants and children after cardiac surgery using cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest (DHCA) is still a matter of concern [1–4]. In addition to neuromonitoring methods for the evaluation of brain perfusion and oxygenation [5–7], early diagnosis and identification of ongoing brain injury by measurement of biochemical markers in serum may have an additional diagnostic and prognostic value. The Ca^{2+}-binding protein S-100B is predominantly located in astroglial cells and has been identified in the brain as being related to trophic function in low concentrations and to toxic and neurodegenerative pathways in higher concentrations [8–10]. The astroglial cells make up 50–60% of the central nervous system and constitute, with the endothelial cells, a support tissue system that acts as a supply and protective barrier between the cerebral microvasculature and the neurons [11]. Neuron-specific enolase (NSE) is a glycolytic enzyme specifically expressed in neurons and has been used as a marker of neuronal cell damage in serum and cerebrospinal fluid [12–15]. Increased serum levels of the brain marker protein S-100B and NSE have been shown to identify adults with postoperative brain damage and psychomotor alterations after cardiac surgery [16–25].

In addition to the use of conventional brain markers S-100B and NSE, determination of other specific neuronal metabolites in serum may have also an additional diagnostic value after brain ischemia. Parvalbumin is a neuronal Ca^{2+}-binding protein with molecular weight of 12 kDa [26–29]. Parvalbumin is found in a subpopulation of gamma aminobutyric acid (GABA) neurons in most brain regions including the cortex, hippocampus and cerebellum [30]. It acts as a slow-onset calcium buffer and has an effect on short-term synaptic plasticity in the cerebellum [31] and in the hippocampus [32], for a review, see [33]. Several immunohistochemical studies have demonstrated its restricted distribution in a subpopulation of rapid firing neurons [27] and in neurons with high oxidative capacity [34]. Parvalbumin-immunoreactive cells represent a neuronal subset, which has been found to be resistant to degenerative processes in Alzheimer's disease [35]. Although immunocytochemical identification of parvalbumin in the brain has been well known since 1981 [28], determination of parvalbumin concentration in serum first became possible after isolation and characterization of monoclonal antibodies against parvalbumin [36].

The patterns of release into the blood stream of such neuronal and astrocytic markers and their related immunocytochemical distribution in the brain compartments in respect to hypothermic cardiopulmonary bypass have not been studied so far.

Thus, we report our results in an experimental study of the concentrations of astrocytic protein S-100B and the neuronal proteins NSE and parvalbumin in serum and the relationship to immunohistochemical changes in the brain in rabbits that underwent CPB with a 60-minute period of deep hypothermic circulatory arrest (DHCA) followed by reperfusion and rewarming.

2. Materials and methods

Fourteen New Zealand White rabbits (weight, 3.1 ± 0.25 kg BW) were used for the experiment with approval of the Institution of Animal Care for the state of Berlin (Reg.0146/98). All animals were premedicated with intramuscular Xylazin (Rompun, 5 mg kg^{-1} BW) and Ketamin (Ketanest, 50 mg kg^{-1} BW). After endotracheal intubation and the establishment of central intravenous lines, anesthesia was maintained by a continuous infusion of Fentanyl (100 μg $kg^{-1}h^{-1}$) and Midazolam (0.2 mg $kg^{-1}h^{-1}$) and a fractionated dosage of Pancoronium (0.2 mg $kg^{-1}h^{-1}$). Each animal was supported using a volume control ventilator (Servo 900, Siemens) at a peak respiratory pressure of 20–23 cm H_2O, at an inspired oxygen fraction of 21–30%, and at a rate of 20–25 breaths min^{-1} to achieve a normal pH and arterial carbon dioxide tension.

After median thoracotomy 4 animals were kept sedated and ventilated for the same period without connection to CPB and served as control group.

2.1. Cardiopulmonary bypass (CPB)

Nonpulsatile CPB was initiated by the use of a microporous polypropylene membrane oxygenator, which had been selected for the size and flow requirements of the animals (Safe-Micro, Polystan, Denmark), and an arterial filter (Dideco/Sorin, Italy), which used a nonpulsatile roller-pump (Stöckert, Munich, Germany). The priming volume of 300 ml consisted of fresh homologous blood collected from two sacrificed animals, which served as blood donors on the day of the study, and an electrolyte solution to obtain a target hemoglobin value of approximately 7–8 mg dl^{1}. To achieve a pH value of 7.4, $NaHCO_3$ 8.4% was added to the prime.

2.2. Protein levels of S-100B, NSE and parvalbumin in the serum

Blood samples were collected preoperatively and at the following time points of the experiment: 15 min after the start of CPB, before the induction of DHCA, 15 minutes after reperfusion, and at the end of CPB. After centrifugation for 5 min, the serum samples were frozen at −80°C for further analysis. The S-100B protein and NSE serum concentrations were analyzed using a commercially available immunoluminometric assay (LIA-mat®, Sangtec® 100, Byk-Sangtec, Dietzenbach, Germany). Serum concentrations of parvalbumin were analyzed using an ELISA method described recently [37].

3. Histological and ultrastructural studies

The skull was carefully opened and the brain was immediately removed, cut in standardized sections and fixed for 48 hours in SOMOGYI solution consisting of paraformaldehyde, glutaraldehyde 25%, and picric acid with neutral pH (7.4) for subsequent light and electron microscopy [38]. The specimens for light microscopy were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. For electron microscopy the tissue was dehydrated and embedded in araldite.

4. Immunohistochemical staining

S-100B: S-100B is a small Ca^{2+}-binding protein, which is mainly localized in astroglial cells in the central and in Schwann cells in the peripheral nervous system [39]. S-100B is also present in the interstitial cells of the pineal gland, in fat cells, in myoepithelial cells, in melanocytes and in Langerhans' cells of the skin, and in reticulum cells of lymphoid organs [9]. Astrocytes diffuse S-100B staining in the brain were visualized by S-100B protein primary antibody, which employs a monoclonal antibody against bovine S-100 protein (DPC® Immustain, code CKS1S, DPC, Los Angeles, USA) using alkaline phosphate anti-alkaline phosphatase (APAAP) technique.

NSE: Monoclonal mouse anti-human NSE antibody was used for the localization of NSE in the fixed and paraffin embedded brain sections (DAKO-NSE, H14, Code No.M 873 Lot No.120). Enolases are homo- or heterodimers of three subunits: alpha, beta, and gamma [40]. The gamma subunits are expressed primarily in neurons. Gamma-gamma subunits may also be expressed in extended platelets, megakaryocytes, T-cells and smooth muscle cells [40]. The antibody used recognized the gamma-gamma subunit of NSE in the neurons.

Parvalbumin: Monoclonal anti-parvalbumin antibodies are immunospecific for the calcium-binding protein parvalbumin in fixed and paraffin-embedded brain sections (SWant, Swiss antibodies, Bellinzona, Switzerland). Monoclonal anti-parvalbumin antibody is a mouse IgG1 produced by fusion of mouse myeloma cells with spleen cells from mice immunized with parvalbumin which was purified from carp muscles [36]. The product reacts specifically and stains selectively parvalbumin by immunobinding [36] as determined by indirect immunoperoxidase staining and immunoblotting.

Statistics: Statistical analysis and presentation of the graph were performed using Stat-View software. Differences in serum levels of S-100B and other neuronal marker between different stages of the experiment were evaluated using the Wilcoxon test.

5. Results

Ten animals were in stable haemodynamic conditions before and during CPB. Perfusion pressure was kept at 40–50 mm Hg. In all experimental animals a significant increase of the serum concentration of the

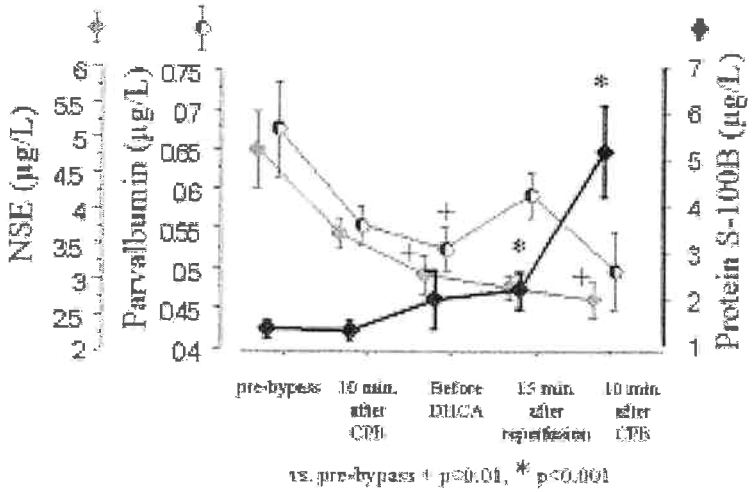

Fig. 1. Kinetic pattern of serum levels of astrocytic protein S-100B, the neuronal proteins parvalbumin and the neuronspecific enolase (NSE) before, during and after deep hypothermic circulatory arrest. In contrast to the significant elevation of the serum levels of S-100B from the base line values before the start of cardiopulmonary bypass (CPB), the neuronal biochemical markers were rather decreased, which may indicate haemodilution of the preexisting concentrations in the serum without further release after the connection to CPB.

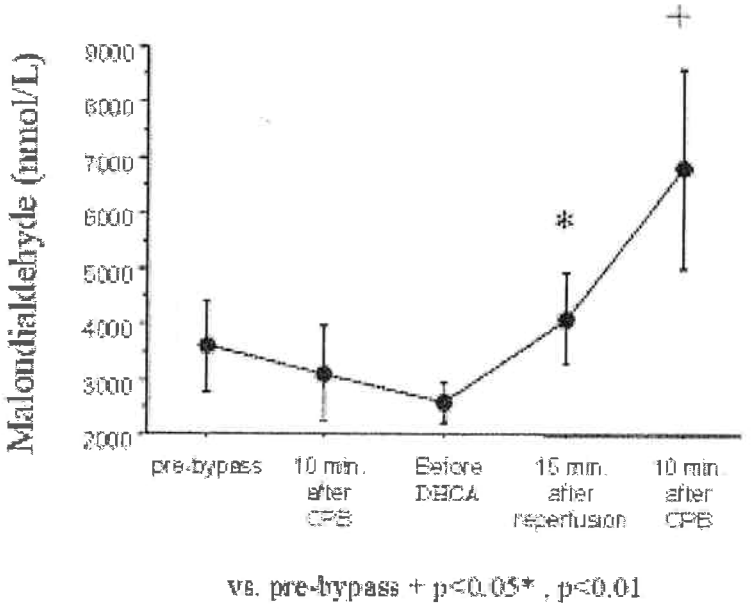

Fig. 2. Change in the concentration of malondialdehyde (MDA), a metabolite of lipid peroxidation in the serum before and after deep hypothermic circulatory arrest. Significant increase of the serum levels of MDA were found after reperfusion and rewarming.

protein S-100B was found immediately after reperfusion and the termination of CPB. In contrast the serum levels of the neuronal markers parvalbumin and NSE were not increased, but rather significantly decreased due to the haemodilution by CPB (Fig. 1). The concentration of parvalbumin increased slightly after reperfusion but remained below the preoperative values. The concentration of malondialdehyde (MDA) in serum after deep hypothermic circulatory arrest may indicate general lipid peroxidation in different organs and possible reperfusion injury at the cerebral level as well. A significant increase of the serum levels of MDA was found after reperfusion and rewarming (Fig. 2).

In comparison with the sham-operated animals

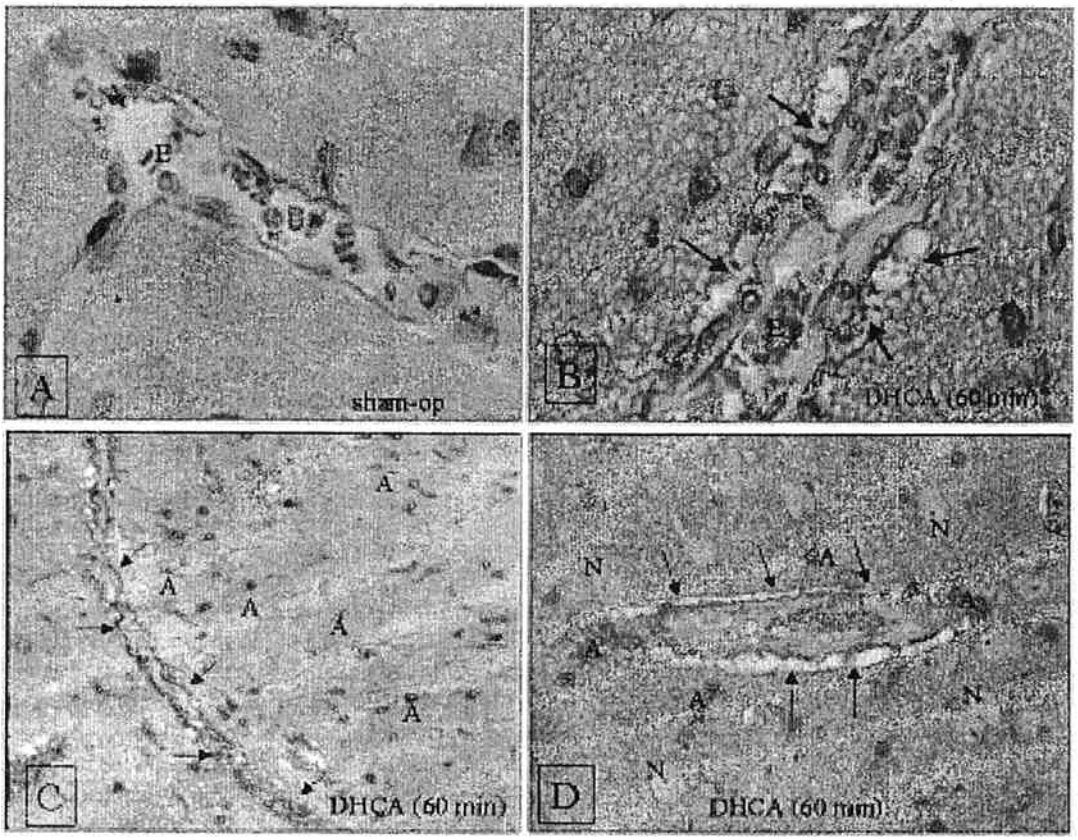

Fig. 3. Marked swelling of the astrocytic endfeet around small capillaries in the hippocampus [arrows]. In comparison with the control animals (panel A), increased diffuse immunoreactivity of S-100B (red staining) was found in the experimental animals (panel B-D). The protein S-100B was present in higher concentrations in the cytoplasm of astrocytes [C,D] as well as in the processes in perivascular regions of small capillaries [red staining, arrows].

(Fig. 3(a)) diffuse increased immunostaining of S-100B was found. This increased reactivity to S100B antibody was found in astrocytic cell bodies, in astrocytic processes extending into the perivascular region and around perivascular oedema (Fig. 3). In contrast to the perivascular hydropic astroglial cell injury, no significant neuronal injury was found in the investigated brain regions (Fig. 3(d)).

The mode of morphological changes was predominantly manifested as marked swelling of the perivascular astrocytic processes around small capillaries. These morphological changes were confirmed by electron microscopy (Fig. 4).

In contrast to astrocytes, neuronal cells positively stained for NSE and parvalbumin were found well preserved without any morphological sign of degeneration or swelling (Figs 5 and 6). Washout of NSE staining in the intercellular space was found sporadically in some hypoxic neurons.

6. Discussion

Similar to the findings in clinical studies during cardiac surgery [18,22,23,41,42–44] the increased level of S-100B in serum in this experimental study was related to the non-pulsatile extracorporal circulation of cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest (DHCA). The histological and immunohistological investigation of the brain at the end of the experiment revealed characteristic morphological changes and an increased staining of S-100B in the astrocytes in different regions of the brain. In contrast to the preserved neurons, the initial morphological changes following reperfusion and rewarming after 60 minutes of total circulatory arrest in deep hypothermia were manifested predominantly in astrocytes. Cell injury consisted of swelling of the astrocytic endfeet around small vessels (Fig. 3). The diffuse distribution of the astrocytic Ca^{2+}-binding protein in the

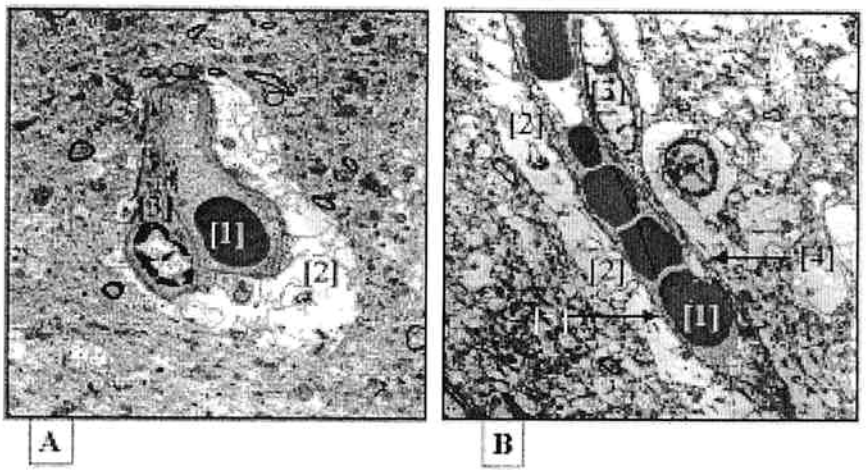

Fig. 4. Electron micrograph of cross-sectional (A) and longitudinal section (B) of hippocampal capillary with swollen astrocytic endfeet. The morphological constituents forming the blood brain barrier, such as the endothelial cells and the basement membrane appear well preserved. Red blood cell [1], swollen astrocytic endfeet [2], pericyte [3], basement membrane [4], endothelial cell [5].

brain was accompanied by increased accumulation of S-100B staining in perivascular regions (Fig. 3). These immediate histopathological and immunohistochemical changes and the related topographical localization adjacent to small capillaries may in part explain the mechanism underlying the release of S-100B from the brain into the blood stream. S-100B may easily pass through the injured blood brain barrier due to its low molecular weight and water solubility.

The most prominent morphological features after CPB and DHCA in our experiments were swollen astrocytes and perivascular oedema. The neurophysiological consequences of such morphological changes are unclear. The increased brain fluid accumulation however, may result in increased intracranial pressure and compromised brain perfusion and thus may be responsible for neurological alterations immediately after CPB [3,18,21,45].

The release patterns of the astroglial marker protein S-100B in to the serum without concomitant increased serum levels of the neuronal markers NSE and parvalbumin may confirm initial morphological alterations in the astrocytes rather than the neurons after deep hypothermic ischemia. The increased vulnerability of astrocytes in contrast to neurons with significant swelling of perivascular astrocytes may indicate a closer topographical relationship to the systemic circulation. During cardiac surgery, oxygen radical-induced membrane injury and inflammatory response in endothelial cells may occur [17,46,47]. Although the elevation of the lipid peroxidation product malondialdehyde in the serum may indicate general reperfusion injury in other organs, lipid peroxidation may occur in the brain as well and may in part explain the membrane injury and cell swelling of the astrocytic cell processes around small capillaries [17] (Figs 2 and 3). In our study the hydropic degeneration of the astrocytes was more visible than the morphological findings of Laursen in 1989, who demonstrated similar astrocytic injury following normothermic and hypothermic CPB [48,49].

Clinically, in neonates and infants with congenital heart disease after corrective surgery with CPB, we demonstrated postoperative characteristic release patterns of the astrocytic protein S-100B in serum that were associated with the presence or absence of immediate clinical neurological abnormalities [18,50,51]. Higher and persistently elevated post-bypass serum levels of protein S-100B have been shown to identify those patients with clinically detectable abnormal neurological findings such as seizures and brain lesions during their early postoperative course [16,18].

Hypoxic brain injury should not be expected when blood supply and oxygen availability at the cellular level is not altered during full flow CPB. However, alteration of microcirculation and cerebral oxygenation and additional other risk factors, such as inflammatory response and microembolization during non-pulsatile hypothermic perfusion, may contribute to mild to moderate global or focal brain injury [48,49,52].

Recent studies have demonstrated that the measured peak value of serum S-100B after CPB is predominantly contaminated with released S-100B from extracerebral sources, such as fat cells from the cardiotomy region [53,54]. However, the significant diffuse in-

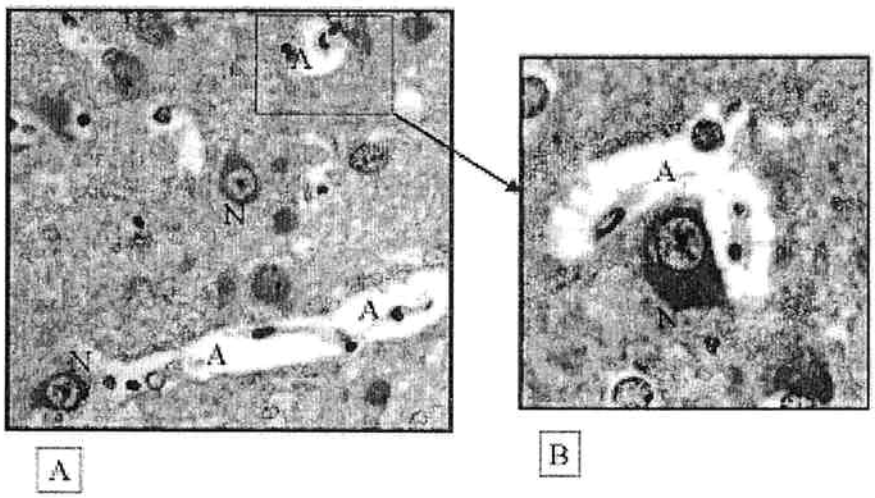

Fig. 5. The immunohistochemical staining of neurospecific enolase (NSE) (red staining) within the neurons [N] indicates intact cell membrane and cell structures in contrast to the edematous and swollen processes (arrow) of astrocytes [A].

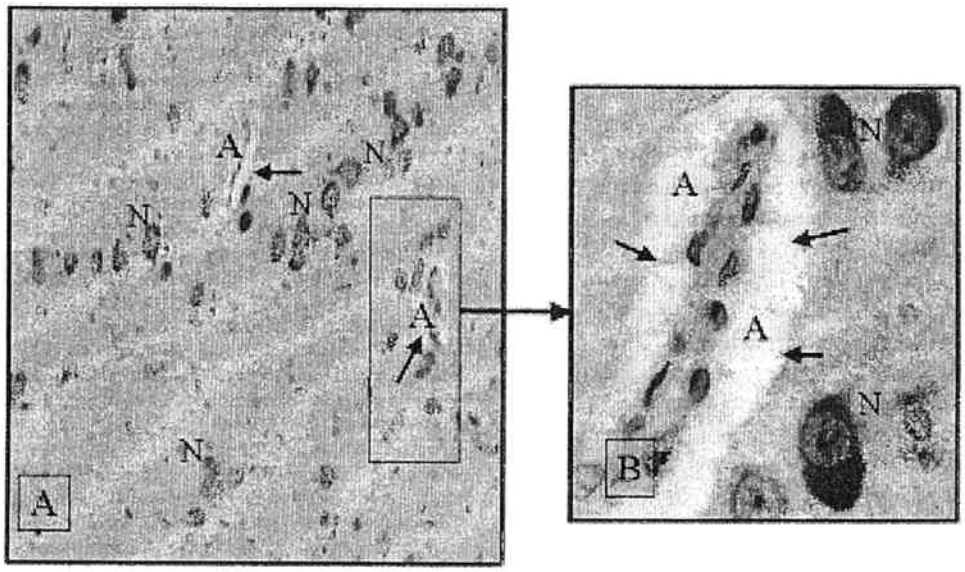

Fig. 6. Parvalbumin positive neurons [N] were predominantly found in restricted sub-populations of brain cells (panel A). Additional view with preserved parvalbumin positive neurons in contrast to adjacent swollen astrocytic cell processes [A]. Similar to NSE, the parvalbumin positive neurons were well preserved in contrast to the adjacent oedematous astrocytes (A, arrow).

creased immunohistochemical staining and cellular detection of S-100B in the brain and in cerebrospinal fluid indicates a cerebral contribution of the measured S-100B values in serum as well [11]. Nevertheless, until defined cut-off of abnormal serum levels for different age groups and different clinical settings have been established, the transiently increased serum levels immediately after uncomplicated cardiac surgery should be interpreted with caution.

In contrast to the gradual increase of astrocytic protein S-100B the serum, levels of the neuronal markers NSE and parvalbumin were not increased; rather, decreased serum levels were found (Fig. 1). The decreased serum levels may be explained by haemodilution of the extant prebypass serum levels without further significant release following the connection to the bypass circulation with additional 300 ml of donor prime blood.

The neuronal cells were found to be immunohistochemically positive for NSE in both, control and experimental animals (Figs 5 and 6). Immunohistochemical washout of NSE has been found to be indicative for neuronal degeneration after ischemia. Quantitative histological assessment revealed only sporadic neuronal cell death with washout of NSE in the hippocampal region after 60 minutes of deep hypothermic circulatory arrest. We assume that neuronal cell death with washout of the immunohistochemical staining of NSE occurs also in other brain regions. NSE with larger relative molecular mass than S100B (67 kDa vs. 22 kDa) is more unlikely to pass through the blood brain barrier. Thus, measurement of a neuronal marker in the serum does not necessarily reflect the actual concentration of NSE at cerebral levels. In addition, NSE may have an extracerebral source such as erythrocytes and peripheral neurons and may be released from hemolytic erythrocytes after CPB 55.

In the nervous system the Ca^{2+}-binding protein parvalbumin has been reported to be exclusively specific to neurons [28,34,56–58]. Additionally, parvalbumin is expressed at high levels in fast-twitch muscles, in particular, of small rodents [59], while expression levels in larger species including humans, are very low [60]. In the kidney, parvalbumin is also found in cells in the loop of Henle and the distal convoluted tubule of the kidney [61]. Parvalbumin is present in neurons within all layers of the cortex except layer I [27]. Parvalbumin-immunoreactive axons occur in different lamellae of the white matter containing axons belonging to association or projection neurons [56]. By acting as a slow-onset calcium buffer, parvalbumin affects the cytosolic calcium homeostasis, most notably the temporal aspects of calcium transients (for a review, see [33]). The fact that no elevations of parvalbumin, a molecule that is even smaller than S-100B was found in the serum after CPB strongly indicates that parvalbumin-containing neurons are not negatively affected at the time points analyzed in this study. On the other hand, the larger size of the neuronal marker NSE in comparison to S-100B and thus its reduced probability to pass the blood/brain barrier [35] may be an explanation for its absence in the serum after CPB.

In conclusion, the significant diffuse immunohistochemical staining of S-100B in the brain may indicate astroglial activation and possible astroglial cell injury rather than neuronal cell injury after 60 minutes of DHCA. Thus, serial measurement of astrocytic rather than neuronal markers is recommended for early diagnosis of brain injury after cardiac surgery. The protein S-100B is present in all brain regions and is smaller in size than the ubiquitous neuronal marker NSE that may favour passage through the blood brain barrier. Further clinical and experimental studies, however, are necessary to evaluate the diagnostic value and the precise molecular release mechanism of S-100B from brain cells into the serum.

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