Aortic Cannulation and Cardiopulmonary Bypass Independently Produce Selective Brain Lesions in Pig

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Abstract: Whether cardiopulmonary bypass alone or together with the manipulation of the aorta produce neurological complication remains controversial. Using a domestic pig model of cardiopulmonary bypass, we investigated the immediate effects of aortic cannulation and cardiopulmonary bypass on neuronal injury in different brain regions. We compared the presence of neuronal injury in three experimental groups: non-operated controls (n=3); operated controls with aortic cannulation without cardiopulmonary bypass (n=5); operated animals undergoing cardiopulmonary bypass (n=5). Pyknotic cells were counted in the prefrontal cortex, cerebellum and in the hippocampal formation. Calretinin immunohistochemistry was used to show possible ischemic damages in the hippocampus which is known to be one of the most sensitive brain regions to ischemia. Decreased calretinin immunoreaction and reduced number of calretinin-positive neurons were observed in the hippocampal dentate gyrus following aortic cannulation or cardiopulmonary bypass compared to the non-operated control group. Changes were more severe following cardiopulmonary bypass than after cannulation of the aorta alone. The frequency of pyknotic cell nuclei was not different in the control and experimental groups. Our experimental study suggests that both cannulation of the aorta alone and cardiopulmonary bypass affect a selected population of neurons.

Abbreviations: CA1-3 – Subregions of Ammon's horn (Cornu Ammonis), CPB – Cardiopulmonary bypass, DAB – 3,3'-diaminobenzidine, g – granule cell layer of the dentate gyrus, h – hilus of the dentate gyrus, l-m – stratum lacunosum-moleculare of Ammon's horn, m – molecular layer of the dentate gyrus, p – layer of somata of the pyramidal cells of Ammon's horn, r – stratum radiatum of the pyramidal layer of Ammon's horn, TB – Tris buffer

Introduction

Neurological injury can be a devastating complication of cardiac surgery that results in longer duration of hospitalization, increased costs, and increased probability of death. The manifestations of such injury are broad, ranging from neurocognitive dysfunction to stroke [1]. Fortunately, the incidence rate of stroke is relatively low [1, 2], but cognitive defects are relatively frequent and occur shortly after operation [1]. Approximately 90% of the cases in the clinical spectrum of cerebral complications are cognitive deficits and not major neurological complications [1]. Strategies aimed to reduce neurological injury during cardiac surgery have focused, for the most part, on the technical aspects of cardiopulmonary bypass (CPB) [2]. Potential mechanisms of neuronal damages include macroembolization of air or particulate matter; microembolization of gas, fat, aggregates of blood cells, platelets or fibrin, and particles of silicone or polyvinylchloride tubing; and inadequate cerebral perfusion pressure [2, 3]. Embolism causes transient ischemia, but depending on the length of vascular

obstruction it may also cause infarction. Neurological disturbances seen in CPB are associated with an ischemic period of hyperexcitability resulted by glutamate accumulation following ischemia as well as the production of the possibly neurotoxic nitrogen oxide [4, 5]. The resultant neuronal hyperactivity induces a cascade of cellular events causing necrosis and apoptosis, leading to acute or delayed cell death in selected areas of the brain, including the hippocampus, basal ganglia, and cerebellum [4, 5, 6]. However, a previous study showed that avoiding CPB in off-pump surgery reduced the inflammatory response as well as the perioperative release of markers of neuronal damage [7]. Therefore, off-pump operations reduce adverse neurological outcomes compared with on-pump procedures [7–14].

It has recently been reported, that aortic manipulation (cross or partial clamp) does not influence significantly the neurological outcome in off-pump patients [11]. In contrast, the conclusions of a recent retrospective study of large numbers of patients suggest that not CPB but the aortic manipulation was found to be an independent risk factor for cerebrovascular accident [15].

Domestic pig is an animal model that is closer to the human than the common laboratory animals. The acute effects of the cannulation of the aorta alone and CPB was investigated on possible neuronal injury in the prefrontal neocortex, in the cortical layers of the cerebellum and in the hippocampal formation. Prefrontal cortex was chosen, because previous studies suggested that neuronal damage of this region may be responsible for the cognitive deficits of patients after CPB [1, 4]. The cerebellum and the hippocampal formation belong to the most sensitive brain regions to ischemia and damages of both brain regions cause deficits in memory formation [16, 17]. The Purkinje cells of the cerebellum and the pyramidal cells of the CA1 area of the hippocampus are shown to be selectively vulnerable to the decreased cerebral blood flow, both in human pathology and in experimental animals. In addition to the cell death of the CA1 pyramidal cells, acute degeneration of calretinin-immunoreactive neurons of the hilus of the hippocampal dentate gyrus was observed in the rodent model of global ischemia [18].

The aims of the present study were the followings: 1) to investigate whether acute neuronal injury can be induced by the cannulation of the aorta alone or by its combination with CPB. 2) to determine whether neuronal injury or acute cell death were among the consequences of the operation itself either aorta cannulation or CPB. 3) to describe the distribution of possible vulnerable neurons in the layers and subregions of the neo- and archicortex that can be affected by the operation.

Material and Methods

Animal preparation for cardiopulmonary bypass

All animals received humane care in compliance with the guidelines ‘Principles of Laboratory Animal Care’ formulated by the National Society for Medical

Research and the 'Guide for the Care and Use of Laboratory Animals' published by the National Institute of Health (NIH Publication No. 88–23, revised 1985).

Animal CPB model was used as previously described [19]. Briefly, 13 male and female neurologically mature young domestic pigs (commercial farm, 20 to 30 kg, 89–100 days old) were used. Preanaesthesia was induced with xylazine (2.2 mg/kg), ketamine (20 mg/kg), and atropine (0.03 mg/kg) intramuscularly. After endotracheal intubation, the animals were ventilated mechanically with 60% oxygen and 40% nitrogen. The ventilator rate and tidal volume were adjusted to maintain the arterial carbon dioxide level. Anaesthesia was maintained with 1.0% to 2.0% isoflurane. The temperature probe was placed in the oesophagus to monitor the core temperature. Catheters were placed in the left femoral artery and vein for withdrawal of blood samples and measurement of blood pressure. Ear vein was used for infusion and drug support. Preanaesthesia and anaesthesia were performed similarly in control and experimental animals, because in control animals the chest was opened for transcardial perfusion.

The chest was opened via median sternotomy. Heparin at 300 IU/kg was given intravenously. A cannula (size 18) was inserted into the ascending aorta and used for arterial blood return to the body during CPB. Venous cannulas were placed into both the superior (size 26) and inferior (size 28) venae cavae. The CPB circuit consisted of Pemco roller pumps (Pemco Inc. Cleveland, Ohio), cardiotomy reservoir (Minimax 1316, filtered hard-shell reservoir), arterial filter (Capiox), water bath (Hemotherm), and a membrane oxygenator (Minimax Plus 3381, hollow fiber oxygenator with plasma resistant fiber) with integral heat exchanger. The circuit was primed with 500 ml lactated Ringer's solution, 1000 ml homologous blood, and 5,000 IU heparin. Sodium bicarbonate was given to maintain the pH around 7.40. Blood electrolytes and osmolality were monitored and measured with a NOVA analyzer (Biomedica). It was maintained within the normal range. Similar to that, hematocrit was maintained from 23% to 30% (the normal pig hematocrit is about 30%) during CPB.

Experimental groups

The overall strategy was to compare the presence of neuronal injury in three experimental groups: non-operated controls (n=3), operated controls with the cannulation of the large vessels without CPB (n=5) and CPB, performed for 30 minutes (n=5).

Perfusion

At the end of the experiment, animals under deep anaesthesia were perfused using a J shaped cannula inserted into the aorta. The animals were perfused first with approximately 2–3 litres of phosphate buffered saline at room temperature (20–24°C) followed by 5 litres of solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The flow was non-pulsative and the above

described extracorporal pump system was used with a volume rate of half a litre/min. Only the head of the animal was perfused, therefore the descending aorta was clamped. Animals with cannulation alone were perfused 1 hour after the start of cannulation, whereas animals with CPB were perfused 30 minutes after the end of 30 minutes extracorporal circulation.

Histological methods

For all histological methods, control and experimental animals were processed without knowing their identity. Brains were removed and post-fixed for a week in the same fixative used for perfusion. The following regions were chosen for histological experiments: the cerebellum, prefrontal cortex and the archicortical hippocampal formation. From the cerebellum, tissue slices were cut at the middle parasagittal level of the right hemisphere at the primary fissure. From the hippocampal formation a complete cross-section was cut at the mid-septo-temporal level, therefore the dentate gyrus, subregions of Ammon's horn, subiculum and parasubiculum were in the section. From the prefrontal cortex, coronal tissue slices were cut from the right hemisphere across the superior arcuate sulcus, from a region approximately equals with the superior frontal gyrus in humans. The tissue slices were approximately 10 mm thick.

The tissue blocks were sectioned with a Vibratome (Technical Products Incorporation, St Luis, MO, U.S.A) at 60 mm for calretinin immunohistochemistry and after paraffin embedding with a conventional sliding microtome at 10 mm for Nissl staining.

Immunohistochemistry: Following cutting, sections were processed immediately. After washing three times for 15 minutes each in 0.05 M Tris buffer (TB, pH 7.6), the free-floating sections were incubated in normal horse serum (1%) in TB for 1 hour at room temperature. The primary monoclonal antibody against calretinin (1:2500) containing 0.4% Triton X-100 (Swant, Bellinzona, Switzerland,) was than added. After incubation with the primary antibody at 4°C on a shaker for three days, sections were washed three times for 15 min each with TB and then incubated with a biotinylated pan-specific universal secondary antiserum (Vector Laboratories, Burlingame, CA) diluted 1:50 in TB, for 2 hours at room temperature. Then sections were washed with TB three times 15 min each and incubated with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, 1:50) for 2 hours at room temperature. After further washing in TB the reaction product was visualized with 3,3'-diaminobenzidine (DAB) as the chromogen (10 mg DAB in 25 ml TB (pH 7.4) with 25 ml 3% H_2O_2 added just before use). Progress of the reaction was monitored using a microscope and the reaction stopped in TB (pH 7.6). After a final wash in TB, sections were mounted on slides and air dried overnight. One third of the sections were counterstained with 1% Cresyl Violet according to the Nissl method to reveal the general cytoarchitecture. All sections were

dehydrated in alcohol, cleared in xylene, and covered with DPX (Mountant for histology, Fluka, Switzerland). Specificity of the antiserum was controlled by the producer. In addition, in sections processed with the omission of the primary antiserum no staining was detected.

In the Nissl stained sections the number of pyknotic cells were counted in the neocortex, cerebellum and in hippocampal formation. In each animal and each region 1000 cells, including the pyknotic cells, were counted.

Results

Aortic cannulation alone and CPB do not increase the rate of immediate cell death

In all histological preparations the numbers of pyknotic cell nuclei were counted in the examined brain regions. There were very few pyknotic cells (<0,1%) both in the neo– and archicortex in the experimental and control groups (Table I.). Most pyknotic cells were found in the hilus of the hippocampal dentate gyrus and in the granular layer of cerebellum. It has to be noted that hilus is relatively sparse in cells, whereas the granule cell layer of the cerebellum is fully packed with granule cells. In both areas the ratio was around 0.1%, whereas in other layers there were less pyknotic cells (Table 1).

Calretinin immunoreactivity in the dentate gyrus

In the non-operated control group the distribution and the number of calretinin-immunoreactive (ir) cells and fibers in the dentate gyrus (Figures 1A and B) were similar to that described in a previous study [20]. In the outer one-third of the molecular layer of the dentate gyrus, and in the stratum lacunosum-moleculare of Ammon's horn large numbers of bipolar calretinin-ir neurons were identified as Cajal-Retzius cells [20]. The inner half of the molecular layer and the hilus of the dentate gyrus contain calretinin-positive local circuit neurons, while in the supragranular layer a dense axonal plexus was observed (Figures 1A and B). Similarly to other species, the sources of origin of these axons are probably the neurons of the supramammillary nucleus [21, 22].

Following cannulation of the aorta alone, the number of the calretinin-ir interneurons in the hilus of the dentate gyrus decreased, and only a few cells per section could be found (Figures 1C and D). Similarly, the calretinin-ir axonal band

Table 1 – Percentage of pyknotic cells in the prefrontal cortex, hippocampal formation and in the cortical layers of cerebellum

Percentage of pyknotic cells (range of mean %)	Control	Aorta cannulation alone	CPB
Prefrontal cortex	0.01–0.05%	0.01–0.07%	0.02–0.06%
Hippocampus	0.03–0.08%	0.04–0.1%	0.02–0.07%
Cerebellum	0.04–0.1%	0.04–0.1%	0.03–0.09%

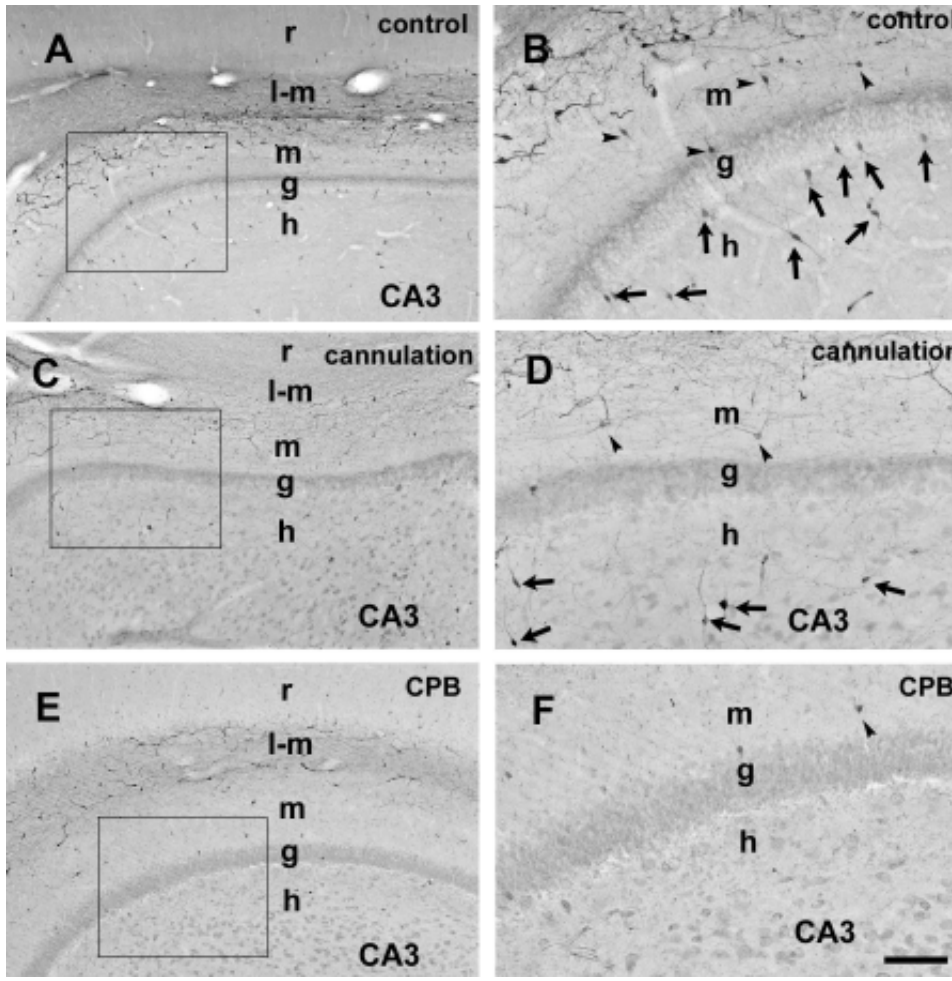

Figure 1 – A. Photomicrograph showing the distribution of calretinin-ir neurons and fibers in the hippocampal dentate gyrus of non-operated control animal. The area labelled by CA3 represents the border between the hilus of the dentate gyrus and the CA3 area of Ammon's horn. B. Boxed area in A is shown with higher magnification. Arrows point to calretinin-ir interneurons in the hilus (h). Arrowheads show calretinin-ir interneurons in the molecular layer (m) of the dentate gyrus. Large population of calretinin-positive bipolar Cajal-Retzius cells can be observed in the outer half of the molecular layer (m). A dense calretinin-ir axonal plexus delineate the border of the granule cell layer (g) and molecular layer (m). C. Photomicrograph showing calretinin-ir cells and fibers in the hippocampal formation of the pig following the cannulation of the aorta alone. D. Boxed area in C is shown with higher magnification. Arrows point to the calretinin-ir neurons in the hilus (h), whereas arrowheads show calretinin-positive neurons in the molecular layer (m) of the dentate gyrus. Note the pale staining of the calretinin-ir axonal plexus at the border between the granule cell (g) and molecular (m) layers. CA3 marks the border between the hilus and the CA3 area of Ammon's horn. E. Photomicrograph showing calretinin-ir cells in the hippocampal formation of the domestic pig following CBP. F. Boxed area in E is shown with higher magnification. The hilus (h) completely lacks of the calretinin-ir cells, while the arrowhead points to a single calretinin-ir interneuron in the molecular layer (m) of the dentate gyrus. The calretinin-positive fiber bundle that normally locates above the granule cell layer (g) is not visible. Bar = 250 μm for A, C, E and 80 μm for B, D, F.

from the supramammillary nucleus was much thinner and stained weaker (Figures 1C and D). In contrast, the calretinin-positive Cajal-Retzius cells were preserved in the outer molecular layer of the dentate gyrus and in the stratum lacunosum-moleculare of Ammon's horn (Figures 1C and D).

CBP resulted in a complete disappearance of the calretinin-ir hilar cells and the supragranular axonal plexus (Figures 1E and F). The calretinin-ir Cajal-Retzius cells in the outer molecular layer of the dentate gyrus and in the stratum lacunosum-moleculare of Ammon's horn were still preserved (Figure 1E).

Discussion

The main findings of this study are that: 1) Both the cannulation of aorta alone and CPB cause injury of selected neuronal populations in the hippocampus.
2) Ischemia-induced changes of calretinin containing neurons and axons were more severe following CPB than after the cannulation of the aorta alone.
3) The operation itself did not result in an immediate increase in neuronal cell death in the examined regions.

Our findings about the fast disappearance of calretinin-immunoreactivity following CPB correlates with other results. The vulnerability of the hilar calretinin-containing interneurons to hypoxia and/or ischemia is known [18]. In addition, calretinin immunoreactivity rapidly disappears in the hilus following the termination of cerebral blood circulation caused by either the death of the animal or by the occlusion of the blood vessels supplying the hippocampal formation [23]. Calretinin staining of hippocampal neurons of the domestic pig brains, harvested from slaughter house, shows that hypoxia and post-mortem delay markedly decreases the immunoreactivity of calretinin-positive hilar neurons and that of the supragranular axonal plexus [20]. However, the quality of calretinin-staining of the Cajal-Retzius-type cells along the hippocampal fissure was not changed, showing that not all calretinin-positive neurons are sensitive for ischemia/hypoxia [20]. The markedly decreased calretinin-immunostaining following cannulation of the aorta alone may be caused by transient ischemia. Transient ischemia might also be caused by debris-emboli released from the wall of the aorta, but the aorta of our experimental animals lacked macroscopically visible sclerotic plaques [1, 8, 9].

In our experiments, CBP resulted in a stronger effect on the calretinin-containing hippocampal neurons and axons, but did not increase the number of pyknotic neurons. This may support previously published data that avoiding CPB and using off-pump surgery reduces the inflammatory response as well as the perioperative release of markers characteristic for neuronal damage [7].

Implications for human cardiac surgery

Strategies targeting the reduction of emboli floating in the cerebral circulation, adequacy of cerebral oxygenation, and minimization of the whole body

inflammatory response to the bypass circuit are one way to reduce the unwanted effects. Recent human studies have conflicting results whether avoiding CPB and applying the "aorta no-touch" technique may improve the neurological complications of patients undergoing cardiac surgery [8, 9, 12, 13, 15]. Cannulation of the aorta alone produces similar although more moderate effects on hippocampal neurons as the CPB.

In the clinical practice, neuropsychological deficits occur in 60 to 80% at the first week, and in 20 to 40% several weeks after operation [1]. Our recent results support the notion that pathological changes are very moderate in a few hours after the operation. Therefore, further experimental studies are needed to describe the long-lasting pathological effects that may occur after cardiac operations.

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