Rodent models of cardiopulmonary bypass: utility in improving perioperative outcomes Fellery de Lange

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# Proefdiermodellen voor hart-long machine gebruik: toepassingen ter verbetering van perioperatieve uitkomsten

(met een samenvatting in het Nederlands)

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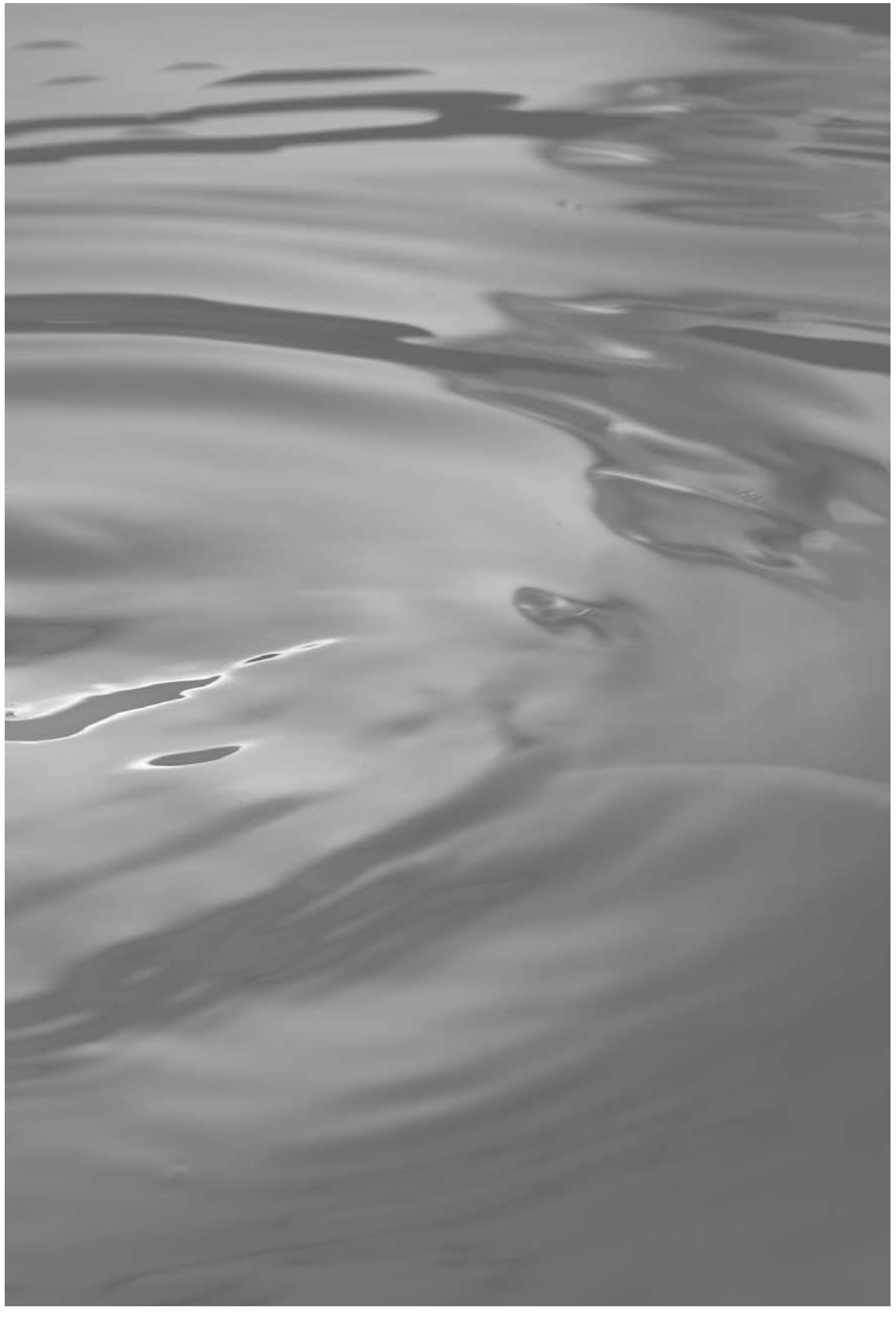
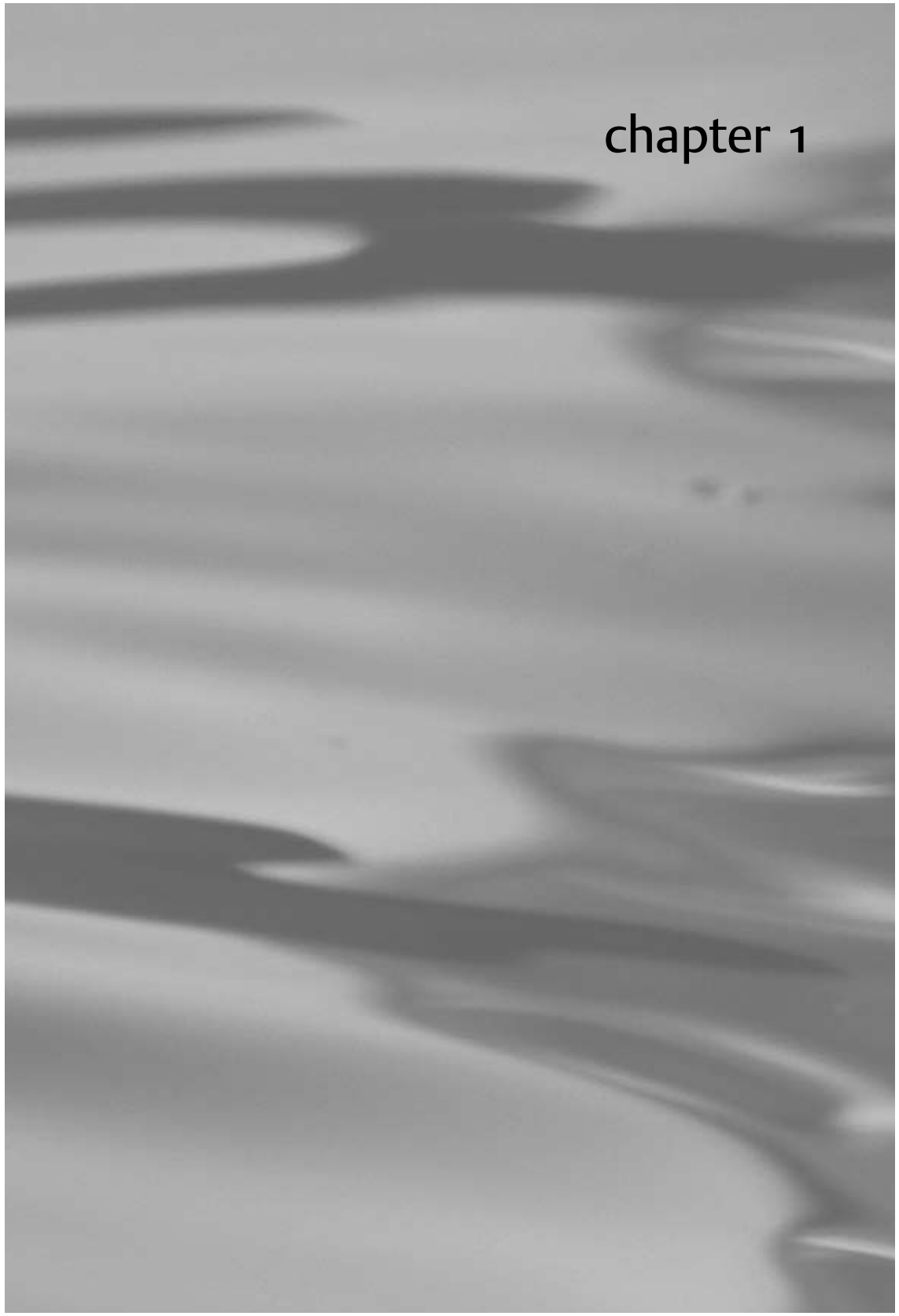


# Contents



chapter 1

# General introduction

## Introduction

On May 6, 1953, a Philadelphia surgeon named John H Gibbon Jr. performed the world's first successful open heart procedure in which total heart-lung bypass was employed. Using an extracorporeal circuit of his own design, he repaired an atrial septum defect in an 18-year-old girl. The efforts leading to that success had started twenty years earlier when he watched a patient die of a massive pulmonary embolus while trying to think of "a way to take the blue venous blood out and getting oxygen into it while carbon dioxide escaped." [1] Together with his wife, a research assistant, he composed a circuit consisting of glass and cork connections, a venous reservoir and a vertical revolving cylinder functioning as oxygenator, and tested this on animals. His laboratory used cats as their laboratory animals, mainly because their size fitted his technical resources. He describes: "I must confess that when our supply ran low, I could often be found prowling around Beacon Hill at night, tuna fish and sack in hand, looking for strays." [1] In 1935 he successfully used this prototype of a heart-lung machine to keep a cat alive for 26 min. However, foaming of blood and clotting in the bypass system were limiting its use. In 1939 heparin was "discovered" and patency of the sytems became less of a problem. In 1950 Gibbon teamed up with engineers from the International Business Machines Corporation (IBM) and developed a screen oxygenator in which blood was "filmed" over a vertical screen of stainless steel wire. When several of these screens were suspended in a clear plastic case through which oxygen was blown their mortality rates among test animals dropped from 80% to 12 %.[1] In later years several more advanced types of bubble oxygenators were developed and consistently used in open heart surgery in the 1970s and '80s.

In the early days of valvular replacement surgery (1960s) severe cerebral complications were frequently observed. Fatal ischemic brain injury was observed in 9 to 14% of patients.[2-4] However, variations in methodology were responsible for large differences in the incidence of central nervous system (CNS) complications observed.[5] Prospective studies reported postoperative CNS complications to be very common and detectable in over 60% of patients, whereas retrospective studies reported cerebral complications detectable in as little as 1-6% of patients.[6-9] Mechanisms contributing to these complications were thought to be mainly cerebral hypoperfusion and macro-and microembolizations of air, particulate matter or aggregated blood elements.[5] At the end of the 1980s evidence accumulated that bubble oxygenators - compared to membrane oxygenators- produce large quantities of microbubbles that can be detected in cerebral and carotid arteries when compared to membrane oxygenators.[10,11] As a result bubble oxygenators were slowly replaced by membrane oxygenators, and in modern cardiac surgery these are now standard practice.





Despite these advances in surgical and perfusion techniques, neurological complications after cardiac surgery remain a problem. These complications now mainly present as deficits in memory and learning. Newman *et al* reported these neurocognitive deficits occuring in up to 36% of patients after 6 weeks.[12] A systematic review by Van Dijk *et al* showed 22.5% of patients exhibiting cognitive decline.[13] To elucidate the pathophysiological mechanisms underlying these more subtle neurological complications, laboratory research in appropriate animals models is neccessary. An economical rodent model for CPB with extensive possibilities of cognitive testing was recently developed by Grocott and Mackensen.[14,15] This rat model was the basis for this thesis.

In *Chapter 2* we validated a CPB set-up for rats, based on the first description by Grocott and Mackensen. In *Chapter 3* we studied the effects of CPB on cognitive function in animals with an increased vulnerability to cerebral dysfunction due to diabetes or old age. In *Chapter 4* we investigated the effect of several perioperative CPB temperature strategies on cognitive performance.
A new method to deliver bilateral cerebral embolization - as might occur during cardiac surgery- is described in *Chapter 5* The use of MRI detectable microspheres enables quantification of the embolic load. *Chapter 6* describes a method based on the existing rat CPB model in which full cardioplegic arrest with good survivability can be achieved. *Chapter 7* focuses on anticoagulation. In this chapter heparin, the historical first choice for anticoagulation, is compared to a novel factor IX RNA molecule and its antidote. *Chapter 8* reports on the effects of a perfluorocarbon solution thought to minimalize gaseous embolization during CPB. C*hapter 9* contains the results of a pooled analysis of five separate rat CPB studies to facilitate detection of subtle differences in cognitive testing. Finally, *Chapter 10* discusses the different applications of the rat CPB model in a wider context and outlines its future utilizations.

## Reference List


1. Gibbon JH: History. Medical World News November 3, 1972;
2. Gilman S: Cerebral disorders after open-heart operations. New Eng J Med 1965; 272: 489-98
3. Javid H, Tufo HM, Najafi H, Dye WS, Hunter JA, Julian OC: Neurological abnormalities following open-heart surgery. J Thorac Cardiovasc Surg 1969; 58: 502-9
4. Tufo HM, Ostfeld AM, Shekelle R: Central nervous system dysfunction following open-heart surgery. JAMA 1970; 212: 1333-40
5. K.A.Sotaniemi: Prevalence and causes of cerebral complications in cardiac surgery, Cerebral damage before and after cardiac surgery. Edited by A.Willner. Kluwer Academic Publishers, 1993, pp 37-46
6. Sotaniemi KA, Mononen H, Hokkanen TE: Long-term cerebral outcome after open-heart surgery. A five-year neuropsychological follow-up study. Stroke 1986; 17: 410-6
7. Shaw PJ, Bates D, Cartlidge NE, French JM, Heaviside D, Julian DG, Shaw DA: Neurologic and neuropsychological morbidity following major surgery: comparison of coronary artery bypass and peripheral vascular surgery. Stroke 1987; 18: 700-7
8. Coffey CE, Massey EW, Roberts KB, Curtis S, Jones RH, Pryor DB: Natural history of cerebral complications of coronary artery bypass graft surgery. Neurology 1983; 33: 1416-21
9. Branthwaite MA: Prevention of neurological damage during open-heart surgery. Thorax 1975; 30: 258-61
10. Deverall PB, Padayachee TS, Parsons S, Theobold R, Battistessa SA: Ultrasound detection of micro-emboli in the middle cerebral artery during cardiopulmonary bypass surgery. Eur J Cardiothorac Surg 1988; 2: 256-60
11. Blauth C, Smith P, Newman S, Arnold J, Siddons F, Harrison MJ, Treasure T, Klinger L, Taylor KM: Retinal microembolism and neuropsychological deficit following clinical cardiopulmonary bypass: comparison of a membrane and a bubble oxygenator. A preliminary communication. Eur J Cardiothorac Surg 1989; 3: 135-8
12. Newman MF, Kirchner JL, Phillips-Bute B, Gaver V, Grocott H, Jones RH, Mark DB, Reves JG, Blumenthal JA: Longitudinal assessment of neurocognitive function after coronary-artery bypass surgery. New Eng J Med 2001; 344: 395-402
13. Van Dijk D, Keizer AM, Diephuis JC, Durand C, Vos LJ, Hijman R: Neurocognitive dysfunction after coronary artery bypass surgery: a systematic review. J Thorac Cardiovasc Surg 2000; 120: 632-9
14. Grocott HP, Mackensen GB, Newman MF, Warner DS: Neurological injury during cardiopulmonary bypass in the rat. Perfusion 2001; 16: 75-81
15. Mackensen GB, Sato Y, Nellgard B, Pineda J, Newman MF, Warner DS, Grocott HP: Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001; 95: 1485-91

chapter 2

# Cardiopulmonary bypass and long-term neurocognitive dysfunction in the rat

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## Abstract

Neurologic and neurocognitive complications after cardiac surgery with cardiopulmonary bypass (CPB) have been reported repeatedly. To better understand its etiology and design protective strategies, an appropriate animal model may prove useful. Although impaired short-term neurocognitive function has been recently demonstrated after CPB in rats, the demonstration of persistent long-term neurocognitive changes would be more relevant from a clinical perspective. We hypothesized that CPB results in long-term impairment of neurocognitive performance in rats. Male rats were exposed to either 60 min of normothermic non-pulsatile CPB, using a roller-pump and a neonatal membrane oxygenator, or to cannulation only (sham animals). Long-term neurocognitive function was assessed at 4 to 7 weeks after CPB (Can test), and again after 12 weeks (Morris water Maze) in both operated groups and in a non-operated control group, followed by histologic evaluation of the hippocampus. In separate groups of CPB and sham animals, we also performed plasma TNF-$\alpha$ and IL-6 measurements. There were no significant differences in long-term neurocognitive performance or histological outcome between the three groups. Cytokine patterns were also similar in both operated groups. We conclude that CPB did not appear to cause long-term neurocognitive dysfunction in this model of CPB in young healthy rats. The lack of long-term deficits may be due to the absence of clinically important etiologic factors such as atheromatous and gaseous embolization in this model. Similar cytokine patterns in both operated groups suggest that surgical trauma rather than exposure of blood to extra-corporeal circuit was probably responsible for the inflammatory response.






## Introduction

Neurological complications after cardiac surgery with cardiopulmonary bypass (CPB) have been reported in several clinical studies. Stroke occurs in 3% of patients, but the risk can increase to more than 6% in patients older than 75 years.[1] In contrast, neurocognitive deficits (NCD), which can be detected by repeated neuropsychological testing, occur in up to 36% of patients after 6 weeks.[1,2] These complications mainly present as deficits in memory and learning capacity, although personality changes or psychiatric pathology such as depression can also manifest. The current opinion about the causative mechanism of neurologic damage after CPB is that it results from cerebral embolization, hypoperfusion, generalized inflammation or a combination of these factors.[3,4] To elucidate mechanisms underlying this complication, and to support the development and pre-clinical testing of possible neuroprotective strategies, a reproducible and economical animal model for CPB is needed. A survival model for extra-corporeal circulation in the rat was recently developed by Grocott et al.[5] In this model, consistent neurocognitive deficits and immunological changes have been observed 3-12 days after CPB.[5-9]

From a clinical perspective, persistent long-term neurocognitive changes are more relevant than potentially reversible changes in the early postoperative period. Moreover, Newman et al. recently reported that the incidence of neurocognitive decline increased from 24% one year after undergoing heart surgery with CPB to 42% after 5 years.[2] Therefore, we investigated longer-term neurocognitive function in this rat model. We hypothesized that the animals would express impairment of neurocognitive performance at 12 weeks, compared with both sham-operated and non-operated control rats. In addition, we hypothesized that CPB animals would express higher levels of circulating inflammatory cytokines.

## Materials and methods

**Experimental groups**

Male rats (Wistar, 4 months old, 450-550 g; Harlan, Horst, The Netherlands) were used. Rats were housed in groups in Macrolon® cages with sawdust bedding. An 8 am on / 8 pm off lighting cycle was used. After a 1-week acclimatization period, all animals started habituation and training for neurocognitive testing, as described below. Rats that met training criteria were divided into 3 groups: a non-operated control group (n=7), a sham group undergoing only cannulation for CPB and an equal duration of anesthesia (n=9), and a group undergoing 60 minutes of normothermic CPB (n=12). In addition, a separate group of untrained rats was used for multiple blood sampling for tumor necrosis factor

alpha (TNF-$\alpha$) and interleukin-6 (IL-6) determination, using the same CPB protocol. For the latter experiment, rats were randomly divided into 2 groups: sham surgery (n=10) or 60 minutes of CPB (n=9). Sample size calculations were based on previous experiments with this model.[7,8] It was calculated that 12 animals per group would be needed for a desired power of 0.80, with an $\alpha = 0.05$. Using this group size, detection of a difference of 2.5 errors per test with a standard deviation of 2.0 in the Can test is possible.
The animals received humane care in compliance with the European Convention on Animal Care and the study protocol, including all procedures, was approved by the Utrecht University Animal Experimentation Committee.

**Anesthesia, surgical preparation and cardiopulmonary bypass**

The experimental protocol for the CPB was based on the model for extracorporeal circulation in the rat, as developed by Grocott et al.[5] Anesthesia was induced with 4% halothane in $O_2$/air (1:1). The trachea was intubated (16-gauge intravenous catheter, Abbott B.V., Hoofddorp, The Netherlands) and the lungs were ventilated (Amsterdam Infant Ventilator; HoekLoos, Amsterdam, The Netherlands). Tidal volume was set to achieve normocapnia (verified by capnography and arterial blood gas analysis), with $O_2$/air (0.5:1) at 40 $min^{-1}$ (0.5 s inspiration time). Anesthesia was maintained with 2.0-2.5% halothane. During CPB, rats were anesthetized with intravenous fentanyl (125 $\mu g.kg^{-1}$), fluanisone (4 $mg.kg^{-1}$) and midazolam (2 $mg.kg^{-1}$). Atropine (40 $\mu g.kg^{-1}$) was added to prevent respiratory problems due to excessive secretions in the post-surgical period. Rectal temperature was maintained at 37.5 ± 0.5˚C, using an electrical heating pad and aluminium foil covering.
The left femoral artery was cannulated (26-gauge catheter) for blood pressure monitoring. Thereafter, the mean arterial pressure was kept between 70-100 mm Hg by adjusting the halothane concentration as necessary, typically 2.0-2.5%. Immediately after insertion of the arterial line, 200 $IU.kg^{-1}$ heparin was administered. The tail artery was cannulated for arterial inflow using a 22-gauge catheter; a multi-orifice 4.5-French cannula (adapted from a Desilets-Hoffman catheter, Cook , Son, The Netherlands) was advanced into the right heart, using the right common jugular vein for access.
The extracorporeal circuit setup is shown schematically in Figure 1. The system consists of a glass venous reservoir, a peristaltic pump (Pericor® SF70, Verder, Haan, Germany), a neonatal membrane oxygenator (0140GM, Polystan, Værløse, Denmark) and a custom glass counter-flow heat exchanger with built-in bubble trap. All components were connected with polyethylene tubing. For each experiment, a new sterile oxygenator was used. The venous reservoir and heat exchanger were thoroughly cleaned and sterilized prior to use. The CPB circuit was primed with 10 mL of heparinized (90 IU) gelatin solution (Gelofusine®) and 40 mL of blood from two heparinized (200 $IU.kg^{-1}$) donor rats that were exsanguinated under deep halothane anesthesia.





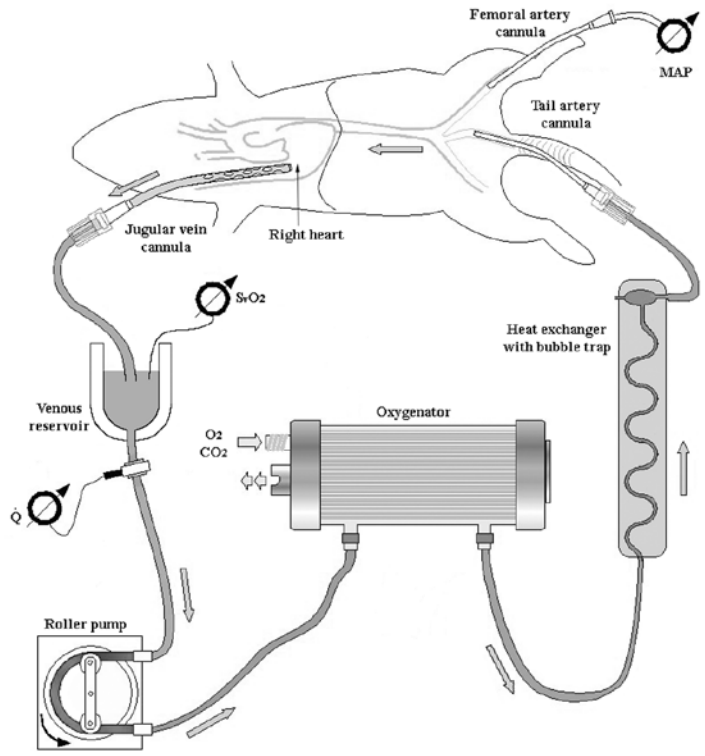


*Figure 1*
*Schematic impression of the experimental setup.*

During CPB, blood flow and venous saturation were continuously monitored. Targeted CPB flow was 100-110 $mL.kg^{-1}.min^{-1}$, corresponding to 60-70% of normal cardiac output. Samples for blood gas analysis and hemoximetry (ABL775 and OSM3, Radiometer Copenhagen, Denmark) were taken from the venous side of the CPB circuit at 15 and 60 minutes of CPB. After weaning from CPB and disconnection from the circuit, protamine (150 $IU.kg^{-1}$ i.v.) was administered to neutralize heparin. All cannulae were removed, the wounds sutured and halothane anesthesia was terminated. Nalbuphine (1 $mg.kg^{-1}$) was given intramuscularly for postoperative analgesia. Animals were allowed to recover in a warmed, oxygen-enriched box for 16 hours, after which they were returned to their cages.

**Neurocognitive testing**

Investigators performing the neurocognitive tests were blinded to treatment allocation. The protocol for neurocognitive testing is shown in Figure 2. For evaluation of memory and learning capacity in the first two months after CPB, the animals were trained in the Can-test, a novel non-aversive spatial-object discrimination task, recently developed by Popovic et al.[10] Briefly, 7 white soft drink cans were placed upside down on a pedestal in a square arena (100x100x45 cm). One of the cans was marked, baited (0.3 mL of water in the bottom indentation) and put in a fixed position among the others. Rats were water-deprived for 18 hours prior to training. Training consisted of 10 trials daily, with a maximum time of 180 s per trial. A first visit to a non-rewarded can was counted as a reference memory error (RME), every subsequent visit to that same can as a working memory error (WME). Failure to visit any can was scored as 'not active' (NAC); activity but failure to find the rewarded can was scored as 'not found' (NFO). Rats that did not meet inclusion criteria at the 5th day of pre-training (i.e. ≥ 80% of trials without errors or inactivity, and ≤ 4 cumulative errors) prior to the operation, were eliminated from the study. Rats were trained at postoperative days 4-10, 12, 14, 16, 18, 20, 24 and 28, with the baited can in the same position as during pre-training (longitudinal task, LT). In postoperative week 5, the baited can was colored and positioned randomly for every trial (simple visual task, SVT; 4 days). In the next week, this baited can was in a variable position among cans of 6 different colors (complex visual task, CVT; 4 days). Finally, in week 7, all cans were marked with a different surface texture for tactile discrimination and in a randomized position for every trial (complex tactile task, CTT; 4 days).

At week 11 after surgery, the rats were switched to an 8 am off / 8 pm on lighting cycle for Morris water maze testing of spatial learning capacity in week 12 as described previously.[11,12] Briefly, the Morris water maze consisted of a large circular black pool (210 cm diameter, 50 cm height, filled to a depth of 30 cm with water at 28 ± 1˚C), which was placed in a darkened room, illuminated by sparse red light. Within the pool a submerged platform (black, round, 8 cm diameter, 1 cm below surface) was hidden in a fixed location, 55 cm from the edge of the pool. The rat could climb on the platform to escape from the necessity of swimming. The animals were given 3 swimming trials per day on 5 consecutive days with a different starting position in each trial. The rat was given a maximum of 120 s to find the hidden

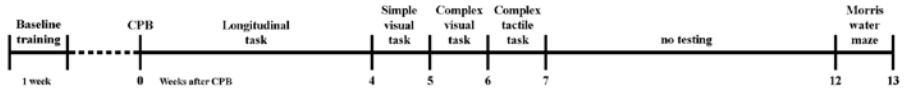


*Figure 2*

*Timeline, showing the sequence of neurocognitive testing.*





platform and was allowed to stay on it for 30 s. Rats that failed to locate the platform were put on to it by the experimenter for 30 s.

Morris maze performance was analyzed for latency to find the platform, distance swum and swimming speed, using a video-tracking system (Ethovision tracking software v1.90, Noldus Technologies, Wageningen, The Netherlands).

**TNF-α and IL-6 analysis**

In the two groups that underwent CPB or sham cannulation specifically for repeated TNF-α and IL-6 determination, the experimental protocol was essentially the same. The tail artery cannula was left *in situ* for sampling until 4 hours after the start of CPB; animals were decapitated 9 hours after start of CPB. Blood samples were taken from the venous reservoir during CPB at 15 and 60 minutes, from the tail artery 2 and 4 hours after start of CPB, and from the jugular vein following decapitation. A total of 0.5 mL of blood was taken for each sample; plasma was separated by centrifugation in EDTA-tubes and stored at -20˚C. The investigator performing the cytokine analysis was blinded to the treatment allocation.

Analysis of plasma IL-6 levels was performed in 9 animals of the CPB group and 10 animals of the sham group, TNF-α level was determined in 4 animals of both groups. Assays were performed using rat-specific ELISA kits (For IL-6: Quantikine® M IL-6 Immunoassay, minimum detection level of 10 pg.mL$^{-1}$ (R&D Systems, Minneapolis, MN, USA); for TNF-α: CT 072, minimum detection level of 6.3 pg.mL$^{-1}$ (U-CyTech, Utrecht, The Netherlands)).

**Histological examination**

When Morris maze testing was complete, the animals were anesthetized and the brain was fixed *in situ* by transcardial perfusion with 4% buffered formalin. The brain was removed, incubated with sucrose and cut into 10-μm-thick slices using a freezing microtome. From each brain, the the most dorsal slice with two cross sections of each lateral ventricle (-3.6 mm from the bregma) was stained with hematoxylin and eosin for light microscopic evaluation of the number of normal and abnormal cells in the CA1, CA2 and CA3 regions of the hippocampus. Cytoplasmatic eosinophilia, karyorrhexis or pyknosis were used as criteria for cellular abnormality.

**Statistical analysis**

Results of cognitive tests were calculated as means ± standard deviation per group, and analyzed by repeated measurements ANOVA, with Greenhouse-Geiser correction if appropriate. Because of a highly skewed distribution of the IL-6, the data were analyzed by the Mann-Whitney test. Physiological parameters and hippocampal cell count data were analyzed using the Student's *t*-test, with Bonferroni correction for the cell count analysis. $p$-values < 0.05 were considered significant.

## Results

During the surgical cannulation, physiological parameters were comparable for both groups (Table 1). However, during extracorporeal circulation, the mean arterial pressure (MAP) was higher in the CPB group and the hematocrit and temperature values were significantly higher in the sham group.

**Neurocognitive testing**

In all groups, there was a significant time effect during the neurocognitive tests (all $p < 0.001$). Can-test results are displayed in Figure 3. Error scores were corrected for activity level and calculated as cumulative number of errors per day, divided by the number of 'active trials': (number of errors)/(10-NAC). No differences could be detected in either reference memory errors (LT: $F(2,22)=.083$, $p=.92$; SVT: $F(2,25)=.180$, $p=.84$; CVT: $F(2,25)=1.35$, $p=.28$; CTT: $F(2,25)=2.75$, $p=.083$) or working memory errors (LT: $F(2,22)=1.45$, $p=.26$; CVT: $F(2,25)=2.20$, $p=.13$; CTT: $F(2,25)=.920$, $p=.41$), except for the simple visual task ($F(2,25)=3.85$, $p=.035$;).

*Table 1*

| | Sham (n=9) | CPB (n=13) |
|---|---|---|
| Weight (g) | 495 (1) | 500 (8) |
| Cannulation time (min) | 69 (4) | 67 (3) |
| **Before CPB (t = 0)** | | |
| MAP (mm Hg) | 69 (12) | 79 (19) |
| $pCO_2$ (mm Hg) | 48.0 (3.4) | 43.9 (1.7) |
| temperature (˚C) | 38.2 (0.7) | 37.6 (0.4) |
| CPB flow ($mL.min^{-1}.kg^{-1}$) | - | 89 (37) |
| **t = 30 min** | | |
| MAP (mm Hg) | 58 (11) | 79 (5)* |
| Hematocrit (%) | 37.9 (3.2) | 30.4 (1.7)* |
| temperature(˚C) | 37.7 (0.4) | 37.3 (0.3)† |
| CPB flow ($mL.min^{-1}.kg^{-1}$) | - | 102 (20) |
| **t = 60 min** | | |
| MAP (mm Hg) | 71 (13) | 85 (11)‡ |
| $pCO_2$ (mm Hg) | 41.3 (3.1) | 44.7 (1.2) |
| Hematocrit (%) | 38.3 (2.0) | 31.2 (2.1)* |
| temperature (˚C) | 37.4 (0.5) | 37.4 (0.3) |
| CPB flow ($mL.min^{-1}.kg^{-1}$) | - | 99 (18) |

*Physiologic parameters during surgery and CPB. Values are means (SD) per group (* p<0.001, † p=0.015, ‡ p=0.013). MAP = mean arterial pressure.*

Post-hoc analysis of the results of the latter task showed a significant difference only on day 1 of testing ($F(2,25)=3.73$, $p=.038$).

Morris maze latencies and distances are displayed in Figure 4. Swimming speed was similar in the three groups. Latency time and swimming distance in the CPB group were not significantly different from the sham and control groups on any of the 5 testing days ( $F(2,25)=1.09$, $p=.35$ for latency, $F(2,25)=.756$, $p=.48$ for distance).

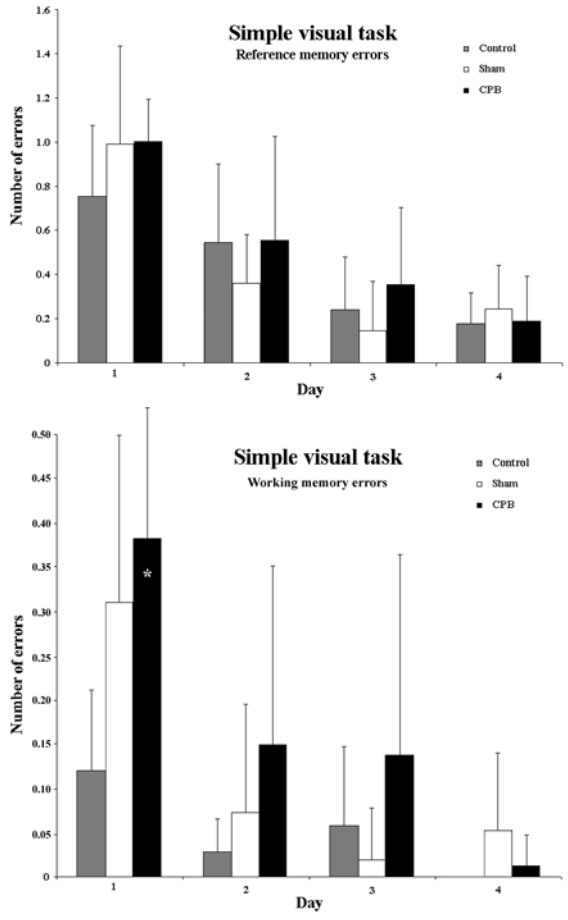


*Figure 3*

*Assessment of neurocognitive function with the Can-test. Upper panel: simple visual task reference memory errors. Lower panel: simple visual task working memory errors. All values are the number of errors per active trial ± SD. * p=0.04.*

**Immunologic analysis**

IL-6 levels were extremely variable between animals: a slight increase was observed at 2 h after CPB, but this increase was also present at the corresponding time point in sham animals. No differences could be detected between the sham-operated group and rats subjected to extra-corporeal circulation (Figure 5). TNF-α was not detected in any of the plasma samples, i.e., the concentration remained below the detection level of the ELISA kit (6.3 pg.mL$^{-1}$).

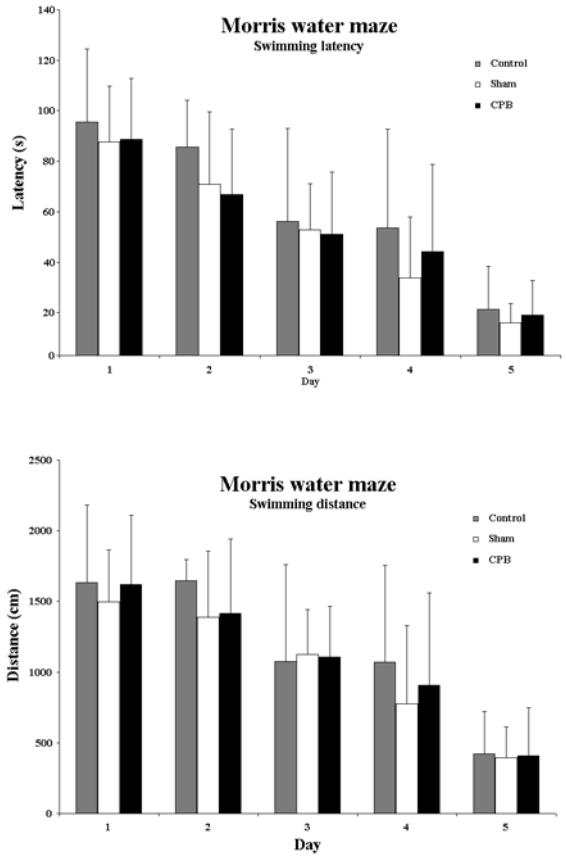


*Figure 4*

*Assessment of neurocognitive function with the Morris water maze at 12 weeks after CPB. Upper panel: latency to find the platform (in seconds ± SD). Lower panel: swimming distance to the platform (in centimeters ± SD).*

**Histology**

There was no evidence for necrosis or infarction in any of the sections from either the CPB, sham or control groups. The number of cells counted is shown for every group in Table 2. No significant differences in histological outcome were detected between the three groups.

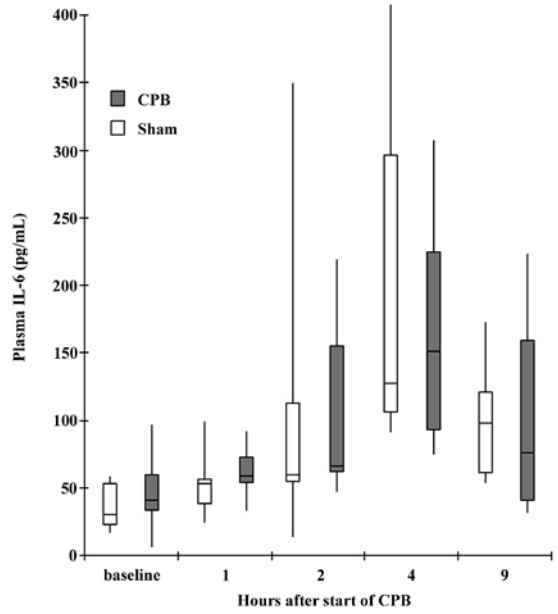


*Figure 5*

*Evaluation of the inflammatory response during and after CPB. Plasma IL-6 concentrations are shown as medians per group.*

*Table 2*

| | CA-1 + CA-2 | | CA-3 | |
|---|---|---|---|---|
| | *Normal* * | *Abnormal* ˚ | *Normal*† | *Abnormal*‡ |
| **Control** | 1155 (215) | 3 (2) | 131 (31) | 0 (0) |
| **Sham** | 957 (141) | 3 (3) | 122 (16) | 0 (0) |
| **CPB** | 1062 (146) | 4 (5) | 135 (46) | 0 (0) |

*Normal and abnormal neuronal cell counts by hippocampal area. Values are means (SD) per group. (* p=0.22, ˚ p=0.97, † p=0.83, ‡ p=0.40).*

## Discussion

The present study showed no impairment of long-term (i.e. 12 week) neurocognitive performance in rats that had undergone 60 min of normothermic CPB. This is in contrast to previous studies where neurocognitive decline has been demonstrated in a similar (albeit with several potentially important differences) model. Mackensen et al. found worse neurologic scores 1, 3 and 12 days after CPB and a significant difference in latency to find the platform in the Morris Water Maze in the same time period.[8] Ma et al. reported similar results with this model, and demonstrated that administration of Xenon during CPB could attenuate the neurologic and neurocognitive deficits.[7]

The apparent discrepancy between the results of the present study and previous studies using a similar model warrants discussion. There are several explanations for these differences. It is conceivable that CPB animals in our study had developed cerebral dysfunction at some earlier time point after CPB, but that it had become undetectable after 4-12 weeks. However, longitudinal assessment of a partial recovery from NCD with the repeated application of the Morris water maze (MWM) is technically impossible. During initial exposure to the maze it takes the animal several days to learn and memorize the location of the hidden platform, based on various environmental cues. Once a rat has learned this 'trick', a second test, even in a different spatial setup is comparatively easier to learn. This precludes detection of subtle differences in spatial learning capacity with repeated application of the MWM. Unlike the MWM, the Can-test allows longitudinal testing and introduction of progressively more difficult paradigms. In the current study, longitudinal testing was initiated on day 4 after CPB, initially assessing memory for the pre-training task (finding the marked can). Four weeks later, several novel tasks were introduced (changing the means of distinguishing the target can, and also its position). On the first day of these novel tasks, we found a significantly higher number of errors in the CPB group as compared the non-operated control group. However, since there were no differences in Can-test performance between control, sham and CPB animals at any further time point, this finding is most likely due to chance rather than being of importance for our conclusion that neurocognitive dysfunction – if present – had recovered by the end of week 4. Also, when these results are combined with those of previous studies using this model, it is most likely that CPB in young rats may not be a sufficiently noxious stimulus to cause long-lasting NCD.

Another possible explanation for the observed lack of difference in neurocognition between CPB and sham animals is that the tests used were not sensitive enough to pick up subtle deficits at 12 weeks. Although not yet widely used, the non-aversive setup of





the Can-test allows assessment of spatial/object learning capacity in both short and long-duration behavioral studies, including subtle neurocognitive impairment in diabetic rats.[10] Moreover, it permits correction of test results for behavioral parameters such as activity level and is thus regarded as being sensitive to subtle differences in cognitive function in rats. On the other hand, the MWM is a well-known and widely accepted test for evaluation of spatial learning capacity[12], but is often regarded as having the disadvantage of being aversive. The swimming induces major psychological stress, which could in turn cloud the validity of the results obtained. However, in previous studies with a comparable model the MWM has proven to be sensitive enough to detect differences in cognitive performance.

The observed differences in physiological parameters between the sham and CPB groups during the CPB period need consideration, as they could possibly have confounded the results. The lower mean arterial pressure in the sham group could have influenced the outcome if it had resulted in a different cerebral blood flow (CBF). However, the mean MAP in this group was still 58 mm Hg, which is well above the lower limits of cerebral circulatory autoregulation. It is thus not likely that CBF was decreased in this group. Also, the lower hematocrit in the CPB group could have confounded the results if it had resulted in a relevant decrease in cerebral oxygenation. Nonetheless, a hematocrit of 31% in combination with sufficient oxygenation is unlikely to have led to neuronal hypoxia in these animals. Finally, the difference in temperature halfway the CPB period was small but statistically significant. However, the known effects of body temperature on the outcome after cardiac surgery are mainly associated with either hypo- or hyperthermia [3], while both values here are considered normothermic. Therefore, it is unlikely that this difference has influenced the outcome of either group.

An additional and unexpected finding was the observed similarity in cytokine patterns in the sham and CPB groups. Because there are no oxygenators for use in small animals on the market, we used the smallest commercially available (neonatal) oxygenator. However, the membrane surface of this oxygenator remained approximately 25 times oversized compared with the clinical situation (0.66 $m^2.kg^{-1}$ vs. 0.02 - 0.04 $m^2.kg^{-1}$). As a result, there was a very large blood-membrane contact surface area in this system. Therefore, we expected to observe a considerable inflammatory response after CPB with this setup.[13] We did not detect a TNF-$\alpha$ acute phase response and we found a late rise in IL-6 levels, with a comparable pattern in both the sham and the CPB group. Taken together, these data suggest that the inflammatory response induced by the circuit was limited, and more likely caused by surgery or anesthesia alone than by the cardiopulmonary bypass per se.

Several reports have suggested that the inflammatory response during CPB is principally caused by myocardial and pulmonary hypoxia.[14-16] In the present model, cardiac output was maintained (albeit on a low level) and ventilation was continued during what was in effect 'partial-CPB', possibly preventing the 'necessary' hypoxia to cause an inflammatory reaction. Moreover, several clinical studies have failed to demonstrate a clear rise in TNF-$\alpha$ level during and after CPB.[17,18] Also, in previous studies conducted with this model by other groups, the oxygenator was cleaned, sterilized and reused because of its high cost ($350-400 per unit).[6] It is conceivable that, despite cleaning, the membranes were still partly coated with protein residues, which might augment the inflammatory response observed by these groups. Finally, the use of the steroid fluanisone (as a component of Hypnorm®) for maintenance of anesthesia during the CPB period could theoretically have influenced the inflammatory response. However, an anti-inflammatory effect of Hypnorm®, which has been used widely for anesthesia in laboratory animals, has never been reported. We therefore assume that this effect is likely small or negligible.

Because the animals in this study survived for three months after CPB, we did not expect to see a difference in the number of abnormal cells in the hippocampus, since both necrotic and apoptotic cells would have been removed by then. The only long-term histological marker suggesting previous cerebral damage would have been a lower absolute neuronal cell count, leading to shrinkage of cortex and/or hippocampus. Since histological results were identical in CPB and control rats, we conclude that CPB alone is unlikely to cause long-term morphological changes in HE-stained brain sections. This finding is in agreement with the absence of histological changes in previous studies with this model.[7,8] However, these results should be interpreted in the light of potential neuronal repopulation of previously injured cerebral structures, which could have masked a difference in cell count present at an earlier timepoint.

If cognitive dysfunction after CPB in the rat is only transient, and if a rise in cytokines can be prevented by maintaining myocardial and pulmonary oxygenation, the clinical relevance of using this partial-CPB preparation to test protective strategies for CPB may need re-evaluation. The current rodent model differs in several aspects from the clinical situation. First, the thoracic cavity remains closed, and access to arterial and venous circulation is by percutaneous routes. One might approximate the clinical situation more closely by using an open chest setup to achieve and maintain ventricular fibrillation. Such a model was recently outlined by Gourlay et al [19], but survival and long-term outcomes within this model have not yet been described. Second, several factors that have been suspected of being clinically important in causing neurological pathology are absent in this model including cerebral arterial embolization from atherosclerotic plaques and reinfused cardiotomy blood contaminated with fat, thrombi and other debris [20] and cerebral hypoperfusion. Finally, young animals were used in this model for a condition that mainly affects the eldery.[1] The aged brain is different from the younger brain in several important respects, including size, distribution and type of neurotransmitters, metabolic function, and capacity for plasticity, making it more vulnerable to the systemic inflammatory response. One thus might argue that the use of relatively young, healthy animals is not appropriate.

## Conclusion

This model of partial CPB presented herein is probably an insufficient stressor of the central nervous system to achieve persistent changes in neurocognition. Nonetheless, we believe that certain aspects of the present model remain relevant for studying the pathophysiology of cerebral damage and neurocognitive dysfunction after cardiac surgery with CPB, but potentially it needs to be extended to incorporate other relevant perturbations, such as the addition of cerebral embolization (gaseous and/or particulate) that can occur during clinical cardiopulmonary bypass. Further, the influence on outcome of this model of various known risk factors, such as advanced age or diabetes, could also be evaluated.

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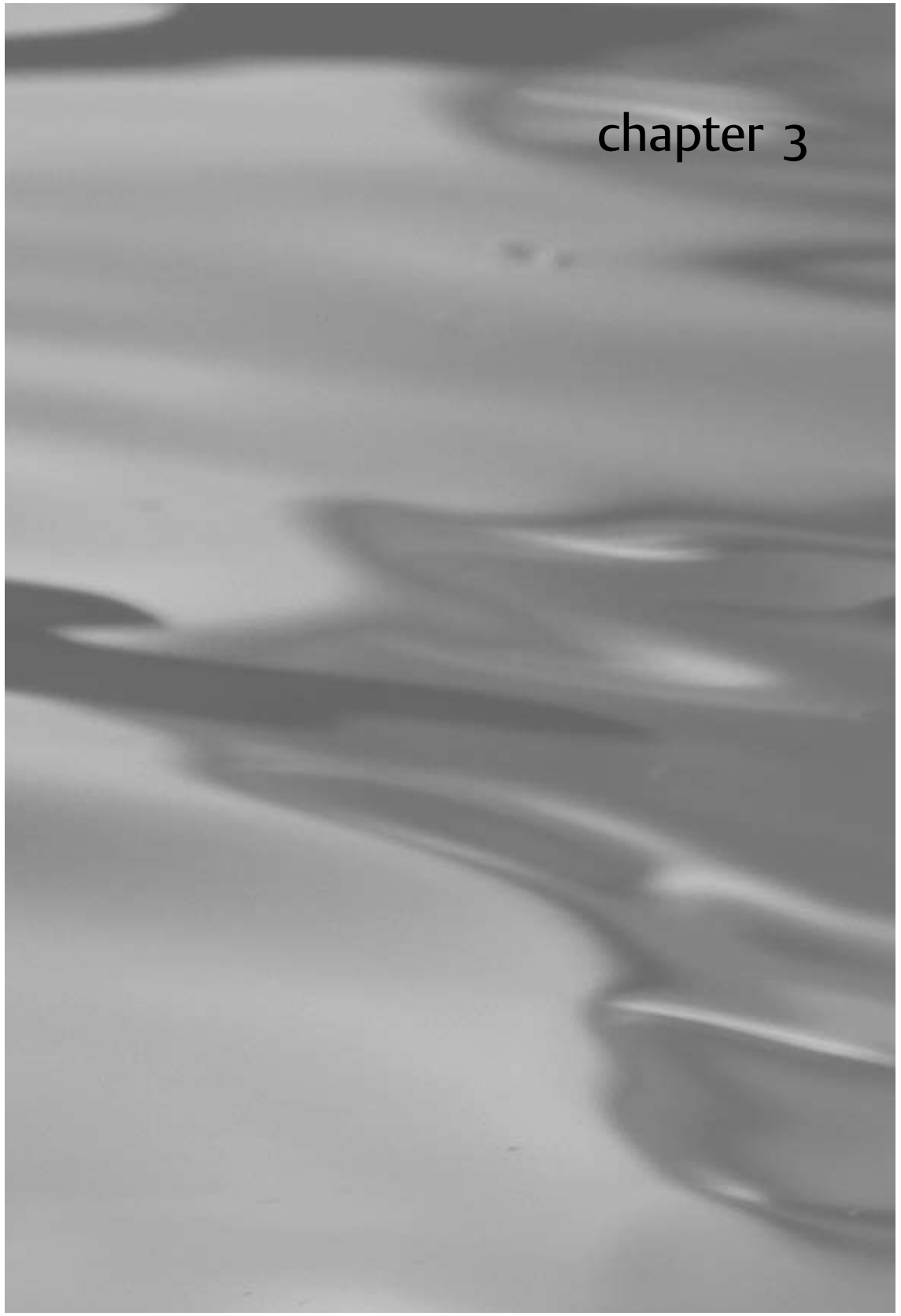
## Reference List


1. Roach GW, Kanchuger M, Mangano CM, Newman M, Nussmeier N, Wolman R, Aggarwal A, Marschall K, Graham SH, Ley C: Adverse cerebral outcomes after coronary bypass surgery. Multicenter Study of Perioperative Ischemia Research Group and the Ischemia Research and Education Foundation Investigators. New Eng J Med 1996; 335: 1857-63
2. Newman MF, Kirchner JL, Phillips-Bute B, Gaver V, Grocott H, Jones RH, Mark DB, Reves JG, Blumenthal JA: Longitudinal assessment of neurocognitive function after coronary-artery bypass surgery. New Eng J Med 2001; 344: 395-402
3. Grocott HP, Homi HM, Puskas F: Cognitive dysfunction after cardiac surgery: revisiting etiology. Semin Cardiothorac Vasc Anesth 2005; 9: 123-9
4. Selnes OA, Goldsborough MA, Borowicz LM, McKhann GM: Neurobehavioural sequelae of cardiopulmonary bypass. Lancet 1999; 353: 1601-6
5. Grocott HP, Mackensen GB, Newman MF, Warner DS: Neurological injury during cardiopulmonary bypass in the rat. Perfusion 2001; 16: 75-81
6. Hindman BJ, Moore SA, Cutkomp J, Smith T, Ross-Barta SE, Dexter F, Brian JE, Jr.: Brain expression of inducible cyclooxygenase 2 messenger RNA in rats undergoing cardiopulmonary bypass. Anesthesiology 2001; 95: 1380-8
7. Ma D, Yang H, Lynch J, Franks NP, Maze M, Grocott HP: Xenon attenuates cardiopulmonary bypass-induced neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2003; 98: 690-8
8. Mackensen GB, Sato Y, Nellgard B, Pineda J, Newman MF, Warner DS, Grocott HP: Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001; 95: 1485-91
9. Sato Y, Laskowitz DT, Bennett ER, Newman MF, Warner DS, Grocott HP: Differential cerebral gene expression during cardiopulmonary bypass in the rat: evidence for apoptosis? Anesth Analg 2002; 94: 1389-94
10. Popovic M, Biessels GJ, Isaacson RL, Gispen WH: Learning and memory in streptozotocin-induced diabetic rats in a novel spatial/object discrimination task. Behav Brain Res 2001; 122: 201-7
11. Biessels GJ, Kamal A, Urban IJ, Spruijt BM, Erkelens DW, Gispen WH: Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: effects of insulin treatment. Brain Res 1998; 800: 125-35
12. Morris RG, Garrud P, Rawlins JN, O'Keefe J: Place navigation impaired in rats with hippocampal lesions. Nature 1982; 297: 681-3
13. Gu YJ, Boonstra PW, Akkerman C, Mungroop H, Tigchelaar I, Van Oeveren W: Blood compatibility of two different types of membrane oxygenator during cardiopulmonary bypass in infants. Int J Artif Organs 1994; 17: 543-8
14. Diegeler A, Doll N, Rauch T, Haberer D, Walther T, Falk V, Gummert J, Autschbach R, Mohr FW: Humoral Immune Response During Coronary Artery Bypass Grafting :A Comparison of Limited Approach, "Off-Pump" Technique, and Conventional Cardiopulmonary Bypass. Circulation 2000; 102: 95-100
15. Wan S, DeSmet JM, Barvais L, Goldstein M, Vincent JL, LeClerc JL: Myocardium is a major source of proinflammatory cytokines in patients undergoing cardiopulmonary bypass. J Thorac Cardiovasc Surg 1996; 112: 806-11
16. Wan S, LeClerc JL, Vincent JL: Inflammatory response to cardiopulmonary bypass: mechanisms involved and possible therapeutic strategies. Chest 1997; 112: 676-92
17. Brix-Christensen V, Petersen TK, Ravn HB, Hjortdal VE, Andersen NT, Tonnesen E: Cardiopulmonary bypass elicits a pro- and anti-inflammatory cytokine response and impaired neutrophil chemotaxis in neonatal pigs. Acta Anaesthesiol Scand 2001; 45: 407-13
18. Hayashi Y, Sawa Y, Nishimura M, Tojo SJ, Ichikawa H, Satoh H, Yamaguchi T, Suhara H, Ohtake S, Matsuda H: P-selectin monoclonal antibody may attenuate the whole body inflammatory response induced by cardiopulmonary bypass. ASAIO 2000; 46: 334-7
19. Gourlay T, Ballaux PK, Draper ER, Taylor KM: Early experience with a new technique and technology designed for the study of pulsatile cardiopulmonary bypass in the rat. Perfusion 2002; 17: 191-8
20. Moody DM, Brown WR, Challa VR, Stump DA, Reboussin DM, Legault C: Brain microemboli associated with cardiopulmonary bypass: a histologic and magnetic resonance imaging study. Ann Thorac Surg 1995; 59: 1304-7




# Effects of cardiopulmonary bypass on neurocognitive performance and cytokine release in old and diabetic rats

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## Summary

**Background**

Age and diabetes mellitus have been identified as independent risk factors for cognitive decline after cardiac surgery with cardiopulmonary bypass (CPB). We tested the effects of CPB on cognitive function in aged and diabetic rats utilizing the Morris water maze (MWM).

**Methods**

Aged rats (26 mo) were randomized into a sham group (cannulation but no CPB, n=11) and a 90 min CPB group (n= 11). In addition, young rats (n=14) were made diabetic with streptozotocin 9 weeks prior, and randomized to a sham or 90 min CPB group. Cytokine release (IL-6) and short-term MWM performance (days 8 to 14 postoperatively) were assessed in all animals, long-term MWM performance (8 weeks postoperatively) only in aged rats.

**Results**

There were no differences between the aged groups in short-term (p=0.58) or long-term MWM performances (p=0.69). The diabetic animals also showed no differences between the sham and CPB groups in MWM performance (p=0.64). IL-6 assays showed an increased inflammatory response after CPB in the diabetic animals, but not in the elderly groups.

**Conclusions**

Ninety minutes of normothermic CPB had no deleterious effect on neurocognitive outcome in elderly or chronically diabetic animals, suggesting that CPB in itself is not a sufficient stressor of the CNS.






## Introduction

Neurological and cognitive impairments after cardiac surgery are serious complications with major impacts.[1,2] The use of cardiopulmonary bypass (CPB) is thought to be an important contributing factor. A reliable small animal CPB model would allow study of the relative importance of different etiologic factors and provide a tool for efficient preclinical testing of putative neuroprotective strategies and compounds. Grocott and Mackensen [3,4] were the first to decribe a survival model for CPB in healthy young rats.

Several human studies have identified age and diabetes as independent risk factors for neurocognitive decline after cardiac surgery.[2,5] Increased vulnerability of the aging brain to various insults has been repeatedly been demonstrated in experimental models of neurologic injury.[6-9] Also, experimentally induced diabetes in young rats causes irreversible cerebral microvascular changes, decreased cognitive performance and increased infarct volumes in experimental stroke models.[10]

The present study was designed to incorporate these clinically relevant risk factors into the rat model for cardiopulmonary bypass.[3,4] We exposed aged and diabetic rats to 90 min of CPB and evaluated neurocognitive outcome and the inflammatory response. We hypothesized that 90 min of CPB would lead to cognitive impairment in the Morris water maze in these at risk groups.

## Methods

The Utrecht University Animal Experimentation Committee approved all procedures and testing, according to the European Convention on Animal Care.

**Experimental groups**

*Old rats*

38 Male Wistar-Hannover rats (Harlan, Horst, The Netherlands), 25-27 months old from a population with a median lifespan of 26 months, were randomized into two groups: i) a group subjected to 90 min of CPB and ii) a sham-operated group

*Diabetic rats*

Male Wistar rats, weighing 400 - 450 g (n=14) were injected eight to nine weeks before the start of the experiment with 33 $mg \cdot kg^{-1}$ streptozotocin (STZ) i.v. (Serva, Heidelberg, Germany) to induce chronic diabetes mellitus[11] and randomized into CPB and sham animals.

**Anaesthesia, surgical preparation and cardiopulmonary bypass system**

Anaesthesia was induced using 5% isoflurane in oxygen-enriched air. The animals were intubated with a 16 G catheter (Abbott, Hoofddorp, The Netherlands) used as ETT and mechanically ventilated ($FiO_2$ 40%, frequency 30/min, peak pressure 15 cm $H_2O$) to achieve normocapnia (verified by mainstream capnography and arterial blood gas analysis). Atropine 0.05 mg was given subcutaneously to prevent excess pulmonary secretions. Anaesthesia was maintained with 2.0-2.5 % isoflurane. The surgical procedure [12] started with cannulation of the femoral artery with a 26 G catheter (Abbott, Hoofddorp, The Netherlands) for blood pressure monitoring. Immediately thereafter 150 IU of heparin and 5 μg of fentanyl were given. Glucose (Accu-Check Sensor 2, Roche, Almere, The Netherlands), blood gases (ABL 775 and OSM 3, Radiometer, Copenhagen, Denmark) and hematocrit (Hct) were analyzed. The experiment was discontinued if the starting Hct was below 0.34 to control for effects of health on behavioural performance.[13] The tail artery was cannulated with a 20G catheter for arterial inflow and a second dose of 150 IU of heparin was administered. A modified multi-orifice 4.5 French pediatric cannula (Desilets-Hoffman Catheter, Cook, Son, The Netherlands) was advanced through the right external jugular vein into the right atrium for venous outflow. The CPB circuit consisted of a newly designed 8 ml Plexiglas® venous reservoir, a roller pump (Verder, Haan, Germany) and a small-volume oxygenator (M.Humbs, Valley, Germany, Ing.Humbs@t-online.de). The oxygenator has two Plexiglas® shells (12.8 cm x 12.8 cm x 2.7 cm) that carry a sterile, disposable three layer artificial diffusion membrane, made with hollow polypropylene fibers (Jostra AG, Hirrlingen, Germany). The surface area available for gas exchange was 558 $cm^2$. To prevent excessive heat loss, the oxygenator had an integrated heat exchanger. A glass bubble trap was located between the arterial outflow of the oxygenator and the animal. All parts were connected through disposable silicone tubing. The circuit was primed with 8 ml of hydroxyethyl starch (HES) 6 % and 4.5 ml of homologous donor blood, obtained from sham-operated animals as described below. CPB with a flow rate of 100-150 $mL.kg^{-1}min^{-1}$, adjusted to maximize flow and to maintain a minimal venous reservoir blood level, was carried out for 90 min. During CPB, HES was added as needed. Mean arterial pressure was kept between 60-80 mmHg, if necessary with the use of phenylephrine 10 $\mu g \cdot mL^{-1}$, 0.1-1.0 $mL \cdot hr^{-1}$.

At the start of the CPB/sham period, pancuronium (0.2 $mg \cdot kg^{-1}$) and fentanyl 5 μg were administered. During CPB ventilation was stopped and anaesthesia maintained with isoflurane added to the oxygenator gas flow (600 $mL \cdot min^{-1}$ of $O_2$ and 18 $mL \cdot min^{-1}$ of $CO_2$). Temperature was monitored using a thermistor placed underneath the left temporal muscle adjacent to the skull, and maintained at 37.5 ±1.0˚C using an electrical heating pad and a heat-reflecting blanket.

After 90 minutes of normothermic bypass, ventilation was restarted ($FiO_2$ =1) and the extracorporeal circuit disconnected. The cannulas were removed and wounds closed. The animals remained ventilated for another 60 minutes, and recovered in an oxygen-enriched environment thereafter. Sham animals underwent the same cannulation procedures, except for connection to the extracorporeal circuit and discontinuation of ventilation. In addition, 9 mL of blood was replaced with HES 6 % at the start of the sham period, to reach a similar Hct as the CPB animals.

**Neurocognitive testing**

*Old animals*

The investigator performing the tests was blinded to treatment allocation. The animals underwent neurocognitive testing in our Morris water maze (MWM) setup[12], a 2 m diameter black pool with a fixed hidden platform submerged 1 cm below the water surface in one quadrant and various visual cues at the surrounding walls. The rat could climb on the platform to escape from the necessity of swimming. Testing was performed one hour after start of the darkening period in a red-lighted room and consisted of three trials daily. Each trial the animals were placed in the pool at a randomly assigned, different quadrant. Trials were limited to a 120 s search period, after which the animal was placed on the platform. A computerised video tracking system (Ethovision v1.9, Noldus, Wageningen, The Netherlands) measured latency (time to reach the platform), swimming speed and overall distance. Testing for short-term effects was started at the 7th postoperative day and continued for seven days to allow a latency floor value to be reached. Long-term effects were assessed in a reversed MWM setup during a four day period starting at 8 weeks postoperatively. In the reversed set-up, the platform was located in another quadrant than in the short-term set-up and cues were changed.

*Diabetic animals*

The diabetic animals started neurocognitive testing at the 4th post-operative day and continued testing for 5 days, at which a latency floor value was expected to be reached. No long-term testing was performed because of the poor physical condition of the animals due to the untreated diabetes.

**IL 6 analysis**

The experimental protocol for the IL-6 determinations was the same for the old and diabetic animals. Blood samples from each animal were taken just before the start of CPB, after 90 minutes of CPB, and 1 hr after the end of CPB. A total of 0.5 mL of blood was taken for each sample; plasma was separated in EDTA-tubes (Microtainer, BD, Alphen aan den Rijn, the Netherlands) and stored at -80˚C. Duplicate analyses of plasma IL-6 levels were performed by an investigator blinded to group allocation. Assays were performed using

rat-specific Elisa kits (Quantikine M IL-6 Immuno assay; R&D Systems, Minneapolis, MN, USA).

**Statistical Analysis**

Data from cognitive tests are presented as means ± standard deviation and analyzed by repeated measurements ANOVA, with Greenhouse-Geisser correction if appropriate. Data from cytokine assays are presented as medians with minimum/maximum. Differences between group cytokine levels were assessed using the Mann-Whitney U test. Physiologic values were analyzed using the Student's t-test. *P*-values < 0.05 were considered significant. An estimation of the number of animals per group was based on group sizes and differences found in earlier studies of Mackensen [3], Ma [14] and Dieleman [12]. Although in these studies an oversized oxygenator was used, this did not influence our a priori power analysis, since the exposure of blood to the extracorporeal system is thought to play a more important role than oxygenator surface per kg the animal is being exposed to.

## Results

*Old animals*

Of the initial 38 aged rats, only twenty-two (n=11 per group) were actually used in the experiment. The others had to be excluded because of: death during transportation and housing (n=1), visible masses (n=7) and low body weight or hematocrit < 0.34 after the first analysis (n=8). Two animals in the CPB group died within the first postoperative hour; one due to catheter-related problems (cardiac tamponade), the other of unknown causes.

Table 1 shows physiologic values during the experiment. During CPB, temperatures were lower in the CPB group compared to the sham group. At 1hr postoperatively, $PaO_2$ was also lower (but > 100 mmHg) in the CPB group.

*Diabetic animals*

The weight of the animals decreased from around 400 g at the time of the STZ injection to 370 g nine weeks thereafter, due to severe metabolic dysfunction. All animals survived surgery and completed the study (n=7 for each group). The perioperative physiologic values of the diabetic groups are listed in Table 2. At the start of the experiment all blood glucose levels were > 17 mmol·$L^{-1}$. Mean arterial pressure was somewhat lower in the CPB group, but still within in the normal range. At the end of CPB, temperatures were lower in the CPB group. In addition, in the postoperative period, $PaO_2$ was lower (but > 13.3 kPa) in the CPB animals.





*Table 1: Physiologic values Old animals. Means ± (SD)*

| | | CPB | | | |
|---|---|---|---|---|---|
| | **baseline** | **30 min** | **60 min** | **90 min** | **1 hr post** |
| **Weight (g)** | | | | | |
| sham | 659 (56) | | | | |
| CPB | 649 (64) | | | | |
| **Hematocrit (%)** | | | | | |
| sham | 40 (3) | 27 (3) | 28 (3) | 29 (2) | 28 (2) |
| CPB | 42 (3) | 27 (2) | 27 (3) | 28 (3) | 28 (5) |
| **Glucose (mmol/L)** | | | | | |
| sham | 5.6 (1.7) | | | | |
| CPB | 4.7 (1.8) | | | | |
| **Pericranial Temperature ( ºC)** | | | | | |
| sham | 37.5 (0.4) | 37.2 (0.4) | 37.5 (0.5) | 37.4 (0.5) | 37.4 (0.7) |
| CPB | 37.6 (0.5) | 36.8 (0.3)[a] | 36.8 (0.5)[a] | 36.9 (0.5)[a] | 36.9 (0.3)[a] |
| **CPB flow (ml/min)** | | | | | |
| sham | | – | – | – | |
| CPB | | 61 (8) | 58 (6) | 55 (4) | |
| **MAP (mmHg)** | | | | | |
| sham | 86 (17) | 74 (7) | 71 (12) | 66 (7) | 66 (10) |
| CPB | 78 (15) | 69 (8) | 69 (12) | 73 (9)[a] | 71 (9) |
| **pH** | | | | | |
| sham | 7.45 (0.03) | 7.40 (0.04) | 7.42 (0.05) | 7.40 (0.04) | 7.39 (0.04) |
| CPB | 7.45 (0.04) | 7.37 (0.05) | 7.36 (0.05) | 7.36 (0.06) | 7.37 (0.03) |
| **$PaCO_2$ (kPa)** | | | | | |
| sham | 5.5 (0.7) | 5.6 (0.7) | 5.3 (0.9) | 5.3 (0.8) | 5.5 (0.7) |
| CPB | 5.9 (0.9) | 5.7 (0.7) | 5.9 (0.8) | 6.1 (1.2) | 6.0 (0.8) |
| **$PaO_2$ (kPa)** | | | | | |
| sham | 25.9 (6.3) | 27.1 (5.7) | 27.1 (4.4) | 27.6 (4.4) | 28.3 (4.7) |
| CPB | 21.6 (7.3) | 22.9 (8.8) | 24.4 (10.5) | 26.5 (11.7) | 16.0 (4.9)[a] |
| **$HCO_3^-$ (mmol/L)** | | | | | |
| sham | 27.9 (1.8) | 25.5 (1.5) | 24.9 (1.5) | 25.0 (1.5) | 24.7 (1.9) |
| CPB | 29.3 (1.6) | 24.1 (1.7) | 23.9 (2.1) | 23.6 (2.2) | 25.0 (2.0) |

*CPB=cardiopulmonary bypass, MAP= mean arterial pressure*

*a= p<0.05 when compared to sham animals*

**Neurocognitive tests**

*Old animals*

Figure 1A shows MWM performance (latency) of old animals. In each group there was improvement over time ($p<0.001$) as evidence of learning. A latency floor was reached on day 13 postoperatively, implying no further learning. There was no difference between the study groups ($p=0.58$). The reverse MWM at 8 weeks also showed improvement over time ($p=0.024$), but again without differences between the groups ($p=0.69$). There were no significant differences in swimming speed, or in distance swum (data not shown).

*Table 2: Physiologic values Diabetic animals. Means ± (SD)*

| | | CPB | | | |
|---|---|---|---|---|---|
| | **baseline** | **30 min** | **60 min** | **90 min** | **1 hr post** |
| **Weight (g)** | | | | | |
| sham | 374 (49) | | | | |
| CPB | 367 (26) | | | | |
| **Hematocrit (%)** | | | | | |
| sham | 44 (3) | 25 (4) | 27 (1) | 26 (3) | 25 (3) |
| CPB | 46 (6) | 25 (2) | 25 (3) | 24 (3) | 26 (5) |
| **Glucose (mmol/L)** | | | | | |
| sham | 21.6 (4.3) | 20.1 (4.7) | 21.8 (4.0) | 20.4 (4.0) | 23.8 (8.2) |
| CPB | 23.8 (5.3) | 17.8 (2.6) | 19.8 (2.8) | 19.1 (3.1) | 20.8 (3.4) |
| **Pericranial Temperature ( ºC)** | | | | | |
| sham | 37.4 (0.7) | 36.8 (0.4) | 36.6 (0.5) | 36.7 (0.8) | 36.6 (0.4) |
| CPB | 37.4 (0.4) | 37.0 (0.6) | 37.1 (0.2)$^{\beta}$ | 37.3 (0.4) | 37.2 (0.8) |
| **CPB flow (ml/min)** | | | | | |
| sham | | – | – | – | – |
| CPB | | 65 (08) | 65.4 (12.4) | 65.2 (9.5) | – |
| **MAP (mmHg)** | | | | | |
| sham | 93 (23) | 80 (16) | 84 (10) | 81 (09) | 90 (15) |
| CPB | 85 (11) | 66 (07)$^{\beta}$ | 76 (04) | 78 (05) | 72 (10)$^{\beta}$ |
| **pH** | | | | | |
| sham | 7.39 (0.06) | 7.38 (0.06) | 7.36 (0.04) | 7.38 (0.07) | 7.37 (0.04) |
| CPB | 7.39 (0.04) | 7.38 (0.06) | 7.37 (0.08) | 7.33 (0.11) | 7.33 (0.05) |
| **$PaCO_2$ (kPa)** | | | | | |
| sham | 6.8 (1.5) | 5.2 (1.2) | 5.6 (0.7) | 5.2 (0.9) | 6.0 (0.9) |
| CPB | 6.9 (1.5) | 5.7 (1.1) | 5.9 (1.2) | 6.8 (2.5) | 5.9 (0.7) |
| **$PaO_2$ (kPa)** | | | | | |
| sham | 26.3 (3.1) | 28.3 (2.7) | 24.7 (7.1) | 27.3 (5.4) | 34.8 (14.0) |
| CPB | 21.6 (3.3)$^{\beta}$ | 31.5 (6.8) | 30.9 (9.5) | 32.4 (10.1) | 16.1 (8.9)$^{\beta}$ |
| **$HCO_3^-$ (mmol/L)** | | | | | |
| sham | 29.6 (2.4) | 24.1 (1.7) | 24.5 (1.9) | 25.2 (1.7) | 22.6 (2.2) |
| CPB | 29.4 (2.5) | 21.7 (2.9) | 23.0 (2.4) | 22.2 (1.8)$^{\beta}$ | 25.3 (2.3) |

*CPB=cardiopulmonary bypass, MAP=mean arterial pressure*

*β= p<0.05 when compared to sham animals*

*Diabetic animals*

In the diabetic animals, both the CPB and sham group showed significant learning over time ($p$<0.001), but there were no differences between the groups ($p$=0.64). Latencies did not improve further after four days of testing, reaching a floor at an average of 32 s at day 8 after surgery (Figure 1B).

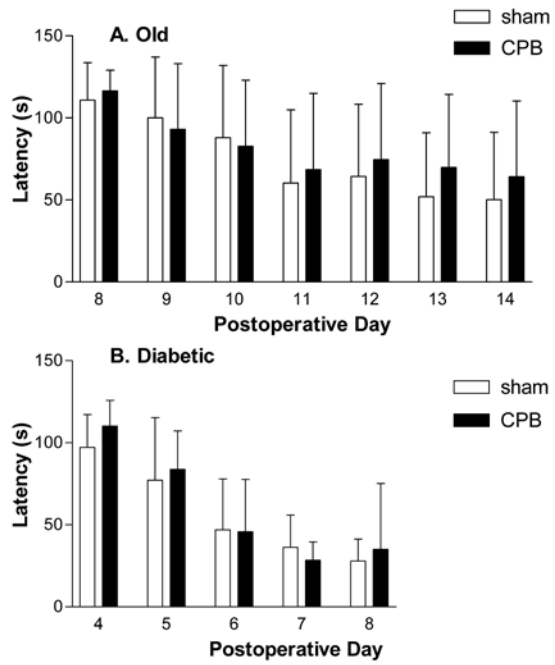


*Figure 1*

*A. Latency in Morris water maze for old animals, short-term testing. Means + SD (n=11 per group). No differences between the groups (p=0.58).*

*B. Latency in Morris water maze for diabetic animals, short-term testing. Means + SD (n=7 per group). No differences between the groups (p=0.64). CPB=cardiopulmonary bypass*

**IL-6 analysis**

IL-6 levels are shown separately for the old and diabetic groups (figure 2A and 2B).

In the elderly animals, IL-6 concentrations were not different between the two groups at any time point. In both sham and CPB animals, the values at 90 min CPB and 1 hr post CPB were higher than the baseline values ( $p$= 0.02 and $p$= 0.001 for the sham animals, $p$=0.01 and $p$=0.003 for the CPB animals). Baseline values in diabetic animals were not statistically different ($p$=0.45) between the sham and CPB group, but at 90 min CPB and 1 hr post CPB IL-6 levels in the CPB group were significantly higher than in the sham group (p=0.002 and $p$=0.005 respectively). In addition, a time effect was noted in the CPB group: the IL 6 levels were higher at 90 CPB and 1 hr post CPB timepoints when compared to baseline ($p$= 0.003 for both timepoints). In the sham group, only the 1 hr post CPB value was different from the baseline value ($p$= 0.03).

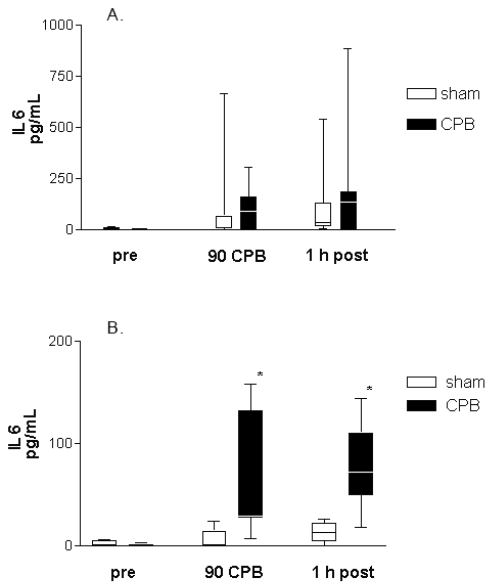


*Figure 2*
*A. Boxplot (medians and minimum/ maximum values) IL-6 levels for old animal groups. No differences between the groups at each timepoint.*
*B. Boxplot (medians and minimum/ maximum values) IL-6 levels for diabetic animal groups. IL-6 levels were significantly higher (*) in the CPB group compared to sham animals at timepoint 90 CPB (p=0.002) and 1 hr post (p=0.005). CPB=cardiopulmonary bypass*





## Discussion

In the present studies we subjected rats with increased vulnerability for CNS dysfunction (aged and diabetic animals) to 90 minutes of normothermic CPB and evaluated short- and long-term neurocognitive outcome in the Morris water maze. Compared to sham-operated aged or diabetic animals, there was no evidence of impaired visuo-spatial learning in animals exposed to 90 min of normothermic CPB. In the aged animals, plasma levels of the pro-inflammatory cytokine IL-6 increased during CPB and in the post CPB period, but were comparable between CPB and sham animals. However, in the diabetic animals, CPB did cause a significant increase in IL-6 levels when compared to sham operated animals.

The results of this study are in accordance with our previous study [12], in which we found that CPB in young healthy animals did not lead to cognitive impairment in the MWM. We stated that using young healthy animals in a model for a condition that mainly affects the elderly was not appropriate. We hypothesized that by using animals with increased susceptibility for neurcognitive deficits, CPB would induce cognitive impairments.
We opted to use old animals because of their well-known susceptibility to CNS injury. Not only do aged rats demonstrate increased infarct volumes in stroke models [8,9], they also have a reduced capacity for neurogenesis after ischemia [15] and increased apoptosis and impaired MWM performance after intermittent episodes of hypoxia.[6] Reduced capacity for regeneration and loss of neurons and synapses, especially in the hippocampus, leads to deficits in spatial learning and memory.[16,17]
Treating rats with streptozotocin produces an established animal model for diabetes.[11] In this model, it has been shown that 8 weeks after non-treated streptozotocin-induced diabetes, functional and cognitive deficits appear.[18,19] We therefore chose to use animals from this model for diabetes as a second group with increased vulnerability for neurocognitive deficits. As expected, all animals in our study suffered from severe, irreversible hyperglycemia after treatment with streptozotocin. Because their hyperglycemia was present more than 9 weeks, all the diabetic complications of this model would have been present in our animals.

As in our previous study with this model, we used the Morris water maze to assess cognitive function in our animals. The MWM is a well established and widely used spatial memory test for rats and mice, in which functional hippocampal integrity is essential for a normal performance.[20] Also, the MWM has repeatedly been shown to be sufficiently sensitive to detect deficits in spatial memory in aged rats in particular.[6,17,20] It is therefore unlikely that we would have missed any difference in cognitive performance because our test was too insensitive.

Since these results apparently contradict two previous studies using this model, further discussion is warranted. Mackensen [3] reported a significant difference in MWM latency between young rats subjected to 60 min CPB and sham groups at days 3 to 12 after surgery. Ma [14], using the same experimental set-up, reported that rats that were exposed to Xenon during CPB had lower MWM latencies the first two days of testing. Both experiments ended at day 12 postoperatively and no long-term neurocognitive outcomes were studied. An important difference between the present study and this previous work, including our own [12], is that we now used a much smaller oxygenator. Until very recently, no miniature oxygenators were commercially available, and therefore the smallest neonatal oxygenator had to be used. The membrane surface of that oxygenator was approximately 20 times overdimensioned compared to the clinical situation (0.66 $m^2 \cdot kg^{-1}$ vs. 0.02-0.04 $m^2 \cdot kg^{-1}$). For this study, we used a recently developed small animal oxygenator with a surface area of 0.09 $m^2 \cdot kg^{-1}$ in these rats. As a result, both priming volume and the blood-membrane contact could be reduced. Also, in the previous studies by other groups, the oxygenator was cleaned, sterilized and reused, whereas we now were able to use a new membrane for each animal. It is conceivable that, despite rinsing, the oxygenator fibres in those earlier studies were still partly coated with protein residues, which might have augmented the inflammatory response. In contrast, in our own study using a large non-reused oxygenator, we were unable to demonstrate neither cognitive deficits nor an increased inflammatory response in the animals subjected to CPB. It is possible that reuse of the oxygenator as done by the other groups may have contributed to both the observed impaired cognitive outcomes and the differences in activation of the immune system.

Despite the use of an appropriately sized oxygenator, we still needed to add 4.5 mL of donor blood to maintain Hct > 0.28. In the sham animals we diluted to the same hematocrit as the CPB animals. By combining these factors, analysis of inflammatory markers becomes more relevant. To investigate the effect of CPB on inflammation, we chose to measure the pro-inflammatory cytokine IL-6 because it is released relatively late in the inflammatory cascade. IL-6 levels are also consistently increased after cardiac surgery.[21] Indeed, in the diabetic rats the IL-6 levels were significantly higher in the CPB group than in the sham animals. The lack of a between-group difference in the old animals could be due to the larger variability in the data (fig. 3). As a result of inter-individual differences in the 'speed' of the aging process in rats, the variation in the elderly population is larger than in young animals. Also, many of these animals may still have had underlying conditions that influenced their cytokine response. In contrast, the diabetic animals were part of a more homogenous group, all with the same underlying illness for the same period of time. As expected, exposure to CPB increased IL-6 levels in these animals.
An important limitation of this study is the variance between old animals. Although we had anticipated on the increased variance and increased our group size, due to the very high prevalence of visible masses and low preoperative hematocrit values, the number of excluded animals exceeded our estimates and 11 animals per group were included. We decided not to add animals to the study at a later point (from a different batch), because this is methodologically incorrect. This is even more so in studies where neurocognitive performance is the primary outcome measure, since animals from a different population would inevitably have different previous cognitive "experiences", and therefore have uncomparable responses. A sample size of 12 still would have allowed an increase of 50% in MWM latency to be detected. For the diabetic animals a post-hoc power analysis on day 2 shows that to detect a 45 % increase in latency in this study with 80% power, 9 animals are needed. Because the noted differences between the animals are minimal, we believe that the group sizes as chosen would have allowed us to detect apparent cognitive decline in these animals. The risk of having encountered a type II error in this experiment is thus relatively low. Other limitations to our study include the relativey short timespan we used to measure IL 6. This was chosen deliberately so we did not influence our cognitive test results by stressful blood sampling in awake animals. As a result, only the trend is measured and we may have missed the peak levels. However, in this rat model peak IL 6 levels occur between 2 and 4 h after start of 60 min CPB (unpublished observation) and therefore the time point 1 h after 90 min of CPB would still be sufficient to detect different rates of cytokine release.

In conclusion, we were unable to demonstrate that exposing old or diabetic rats to 90 min of normothermic CPB caused persistent changes in neurocognitive function as measured by Morris water maze performance. In the present model of CPB - using an appropriately sized, non-reused extracorporeal circuit – CPB by itself is not a sufficient stressor of the central nervous system, not even in animals with increased susceptibility for CNS injury. These results are in agreement with recent clinical observations suggesting that avoidance of CPB using off-pump coronary artery bypass techniques has only limited effects on improving cognitive outcomes in humans.[22-24] Nonetheless, the current rat CPB model may prove to be an excellent starting point to investigate the additive effects of CPB on perioperative (embolic) neurological injury during cardiac surgery.

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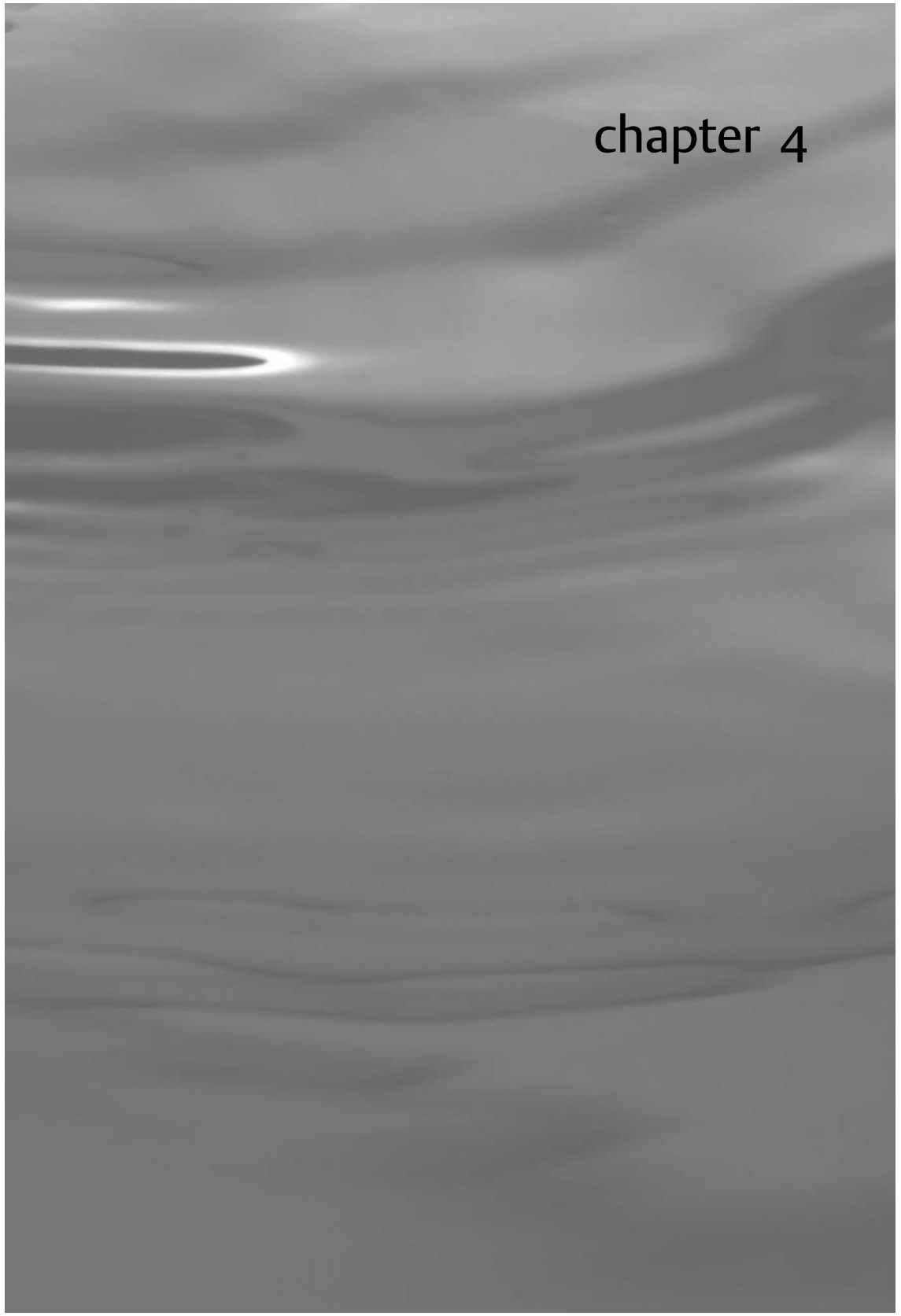
## Reference List


1. Newman MF, Kirchner JL, Phillips-Bute B, Gaver V, Grocott H, Jones RH, Mark DB, Reves JG, Blumenthal JA: Longitudinal assessment of neurocognitive function after coronary-artery bypass surgery. New Eng J Med 2001; 344: 395-402
2. Ridderstolpe L, Ahlgren E, Gill H, Rutberg H: Risk factor analysis of early and delayed cerebral complications after cardiac surgery. J Cardiothorac Vasc Anesth 2002; **16**: 278-85
3. Mackensen GB, Sato Y, Nellgard B, Pineda J, Newman MF, Warner DS, Grocott HP: Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001; 95: 1485-91
4. Grocott HP, Mackensen GB, Newman MF, Warner DS: Neurological injury during cardiopulmonary bypass in the rat. Perfusion 2001; 16: 75-81
5. Newman MF, Croughwell ND, Blumenthal JA, Lowry E, White WD, Spillane W, Davis RD, Jr., Glower DD, Smith LR, Mahanna EP, .: Predictors of cognitive decline after cardiac operation. Ann Thorac Surg 1995; 59: 1326-30
6. Gozal D, Row BW, Kheirandish L, Liu R, Guo SZ, Qiang F, Brittian KR: Increased susceptibility to intermittent hypoxia in aging rats: changes in proteasomal activity, neuronal apoptosis and spatial function. J Neurochem. 2003; 86: 1545-52
7. Siqueira IR, Cimarosti H, Fochesatto C, Salbego C, Netto CA: Age-related susceptibility to oxygen and glucose deprivation damage in rat hippocampal slices. Brain Res. 2004; 1025: 226-30
8. Kharlamov A, Kharlamov E, Armstrong DM: Age-Dependent Increase in Infarct Volume Following Photochemically Induced Cerebral Infarction: Putative Role of Astroglia. Gerontol A Biol Sci Med Sci. 2000; 55: B135-B141
9. Davis M, Mendelow AD, Perry RH, Chambers IR, James OFW: Experimental Stroke and Neuroprotection in the Aging Rat Brain. Stroke 1995; **26**: 1072-8
10. Duverger D, MacKenzie ET: The quantification of cerebral infarction following focal ischemia in the rat: influence of strain, arterial pressure, blood glucose concentration, and age. J.Cereb. Blood Flow Metab 1988; 8: 449-61
11. Biessels GJ, Gispen WH: The impact of diabetes on cognition: what can be learned from rodent models? Neurobiol.Aging 2005; **26 Suppl 1**: 36-41
12. Dieleman JM, de Lange F, Houston RJ, Biessels GJ, Bar PR, Mackensen GB, Grocott HP, Kalkman CJ: Cardiopulmonary bypass and long-term neurocognitive dysfunction in the rat. Life Sci 2006; 551-8
13. Spangler EL, Waggie KS, Rea W, Roberts D, Hengemihle J, Danon D, Ingram DK: Relationship of hematological variables to learning performance in aged Fischer-344 rats. Neurobiol.Aging 1995; 16: 85-9
14. Ma D, Yang H, Lynch J, Franks NP, Maze M, Grocott HP: Xenon attenuates cardiopulmonary bypass-induced neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2003; 98: 690-8
15. Jin K, Minami M, Xie L, Sun Y, Mao XO, Wang Y, Simon RP, Greenberg DA: Ischemia-induced neurogenesis is preserved but reduced in the aged rodent brain. Aging Cell 2004; **3**: 373-7
16. Gallagher M, Rapp PR: The use of animal models to study the effect of aging on cognition. Annu Rev Psychol. 1997; 48: 339-70
17. Wyss JM, Chambless BD, Kadish I, van Groen T: Age-related decline in water maze learning and memory in rats: strain differences. Neurobiol Aging 2000; 21: 671-81
18. Kamal A, Biessels G-J, Urban IJA, Gispen WH: Hippocampal synaptic plasticity in streptozotocin-diabetic rats: impairment of long-term potentiation and facilitation of long-term depression. Neuroscience 1999; 90: 737-45
19. Kamal A, Biessels GJ, Duis SE, Gispen WH: Learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: interaction of diabetes and ageing. Diabetologia 2000; 43: 500-6
20. D'Hooge R, De Deyn PP: Applications of the Morris water maze in the study of learning and memory. Brain Res Rev 2001; 36: 60-90
21. Hall RI, Smith MS, Rocker G: The systemic inflammatory response to cardiopulmonary bypass: pathophysiological, therapeutic, and pharmacological considerations. Anesth Analg 1997; 85: 766-82
22. Cheng DC, Bainbridge D, Martin JE, Novick RJ: Does off-pump coronary artery bypass reduce mortality, morbidity, and resource utilization when compared with conventional coronary artery bypass? A meta-analysis of randomized trials. Anesthesiology 2005; 102: 188-203
23. Van Dijk D, Jansen EW, Hijman R, Nierich AP, Diephuis JC, Moons KG, Lahpor JR, orst C, Keizer AM, Nathoe HM, Grobbee DE, De Jaegere PP, Kalkman CJ: Cognitive outcome after off-pump and on-pump coronary artery bypass graft surgery: a randomized trial. JAMA 2002; 287: 1405-12
24. Hammon JW, Stump DA, Butterworth JF, Moody DM, Rorie K, Deal DD, Kincaid EH, Oaks TE, Kon ND: Single crossclamp improves 6-month cognitive outcome in high-risk coronary bypass patients: The effect of reduced aortic manipulation. J Thorac and Cardiovasc Surg 2006; 131: 114-21




# Effect of limited rewarming and postoperative hypothermia on cognitive function in a rat CPB model

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## Abstract

**Background**

Clinical studies have failed to demonstrate significant benefits of hypothermia for the prevention of postoperative cognitive dysfunction (POCD) after cardiopulmonary bypass (CPB). One explanation for this might be that potentially injurious cerebral hyperthermia occurs during rewarming at the end of CPB off-setting the protective benefits of hypothermia. In this study, we investigated the relative influence of CPB temperature, rewarming strategies, and postoperative temperature in a model CPB in the rat.

**Methods**

Four groups of male Sprague-Dawley rats were surgically prepared and subjected to 90 min of CPB. Group A was normothermic (37.5°C) during and after CPB. Group B underwent hypothermic (32°C) CPB followed by rewarming to 37.5°C at the end of bypass. Group C had hypothermic (32°C) CPB followed by limited rewarming to 35°C. Group D had normothermic CPB with hypothermia (35°C) induced only postoperatively. Groups were compared for POCD determined by the performance in the Morris water maze on postoperative days 3-9. Histologic analysis of the brains (CA1 and CA3 hippocampal regions) was also undertaken.

**Results**

Hypothermia induced only during (Group B vs Group A) or after CPB (Group D vs Group A) conferred no significant POCD benefit. Hypothermia when induced during CPB and continued into the postoperative period, demonstrated a significant improvement in water maze performance vs all other temperature regimens. (Group C vs Group A, $p$ = 0.044; Group C vs Group B, $p$ = 0.011; Group C vs. Group D, $p$ = 0.012). No histological differences between groups were demonstrated.

**Conclusions**

The combination of hypothermic (32°C) CPB coupled with limited rewarming and prolonged postoperative hypothermia (35°C) decreased POCD after CPB in rats.






## Introduction

Cardiac surgery and the use of cardiopulmonary bypass (CPB) has been associated with a high incidence of postoperative cognitive dysfunction (POCD).[1,2] The etiology of this cognitive impairment after cardiac surgery is multifactorial and may include cerebral microembolization, global cerebral hypoperfusion, systemic and cerebral inflammation, cerebral temperature perturbations, cerebral edema, and possible blood-brain barrier dysfunction, all superimposed on genetic influences that may alter susceptibility to injury or alter repair from injury once it has occurred.[3] Many neuroprotective strategies, pharmaceutical as well as surgical, have been investigated; however clinical success has been limited.[3-5] Extensive laboratory studies have demonstrated that hypothermia is a potent therapeutic tool to attenuate the effects of experimental cerebral ischemia.[6-9] However, several large clinical studies in cardiac surgical patients have failed to demonstrate any benefits of hypothermia for the prevention of POCD.[10-12] One possible explanation for this lack of effect may be related to the rewarming that occurs prior to the end of CPB. Overly aggressive rewarming can result in the brain being exposed to dangerously elevated blood temperatures that may neutralize any neuroprotective effect afforded by hypothermia and might itself be deleterious to the brain.[13] One clinical trial did demonstrate a benefit of hypothermic CPB by integrating limited rewarming while maintaining prolonged postoperative hypothermia.[14] However, a clear distinction as to whether the patients benefited from the postoperative hypothermia per se or the avoidance of rewarming could not be made. The purpose of this study was to investigate the effect of different CPB temperatures, rewarming strategies, and postoperative temperature on POCD after CPB in a rat model.

## Methods

The study was approved by the Duke University Animal Care and Use Committee and all procedures met the National Institutes of Health (NIH) guidelines for animal care.[15]

**Anesthesia, surgical preparation and cardiopulmonary bypass**

Male 375- 400 g Sprague-Dawley rats (Harlan; Indianapolis, IN) were housed two per cage under a 12 hour light dark cycle conditions with free access to food and water. After a 12 hour fast, anesthesia was induced with 3% isoflurane in 50% $O_2$ in a plastic box. After orotracheal intubation with a 14G catheter (Insyte BD Medical, Sandy, UT), the animals were mechanically ventilated (Harvard Model 687, Harvard Apparatus, Holliston, MA) to maintain a normal arterial carbon dioxide tension. During the surgical preparation and

during recovery, anesthesia was maintained with 1.5-2% isoflurane.

Surgical preparation consisted of cannulation of the tail artery with a 20G catheter (Insyte BD Medical, Sandy, UT), which later served as arterial inflow for the CPB circuit. One hundred fifty IU of porcine heparin was given after placement of the first catheter. Arterial blood pressure was monitored via the superficial caudal epigastric artery, which was cannulated with polyethylene tubing (PE-10 Intramedic Tubing, Becton-Dickinson, Sparks, MD). A modified multi-orifice 4.5 French pediatric catheter (modified Desilets-Hoffman Catheter, Cook, Bloomington, IN) was advanced through the right external jugular vein into the right heart for venous outflow. The CPB circuit [16] consisted of a glass venous reservoir, a peristaltic pump (Masterflex@, Cole-Parmer Instrument Co., Vernon Hills, Il) and a membrane oxygenator (Micro @ neonatal oxygenator, Cobe Cardiovascular, Arvada, CO) , all of which were connected with 1.6 mm ID silicone tubing (Tygon@, Cole-Parmer Instrument Co., Vernon Hills, Il). An in-line flow probe (2N806 probe and T208 flowmeter, Transonics Systems Inc., Ithaca, NY) was used to continuously measure CPB flow.

The CPB circuit was primed with approximately 30 mL of fresh whole blood obtained from 2 heparinized (100 IU per animal) donor animals. As necessary, 6% hetastarch (Hextend, Hospira Inc, Lake Forest, IL) was added to the reservoir to maintain an adequate circulating volume. During CPB, ventilation was discontinued, and anesthesia was maintained with fentanyl (30 $\mu g.kg^{-1}$ i.v.), midazolam (0.4 $mg.kg^{-1}$ i.v.), and atracurium (0.5 $mg.kg^{-1}$ i.v.) as a bolus injection followed by a continuous infusion of 2.5 $\mu g.kg^{-1}.min^{-1}$ fentanyl, 0.03 $mg.kg^{-1}.min^{-1}$ midazolam, and 0.08 $mg.kg^{-1}.min^{-1}$ atracurium. The oxygenator fresh gas flow (4% CO2, 66% O2 and 30% Air) was approximately 1 L/min.

All rats underwent 90 min of CPB and were randomized into 4 groups based on different perioperative and postoperative temperature regimens (n=13 per group). Group A was subjected to 90 min of normothermic (37.5°C) CPB, followed by a 6 hr postoperative normothermic period of 37.5°C. Group B underwent hypothermic CPB (32°C) for 75 min with rewarming over the last 15 min of CPB to 37.5°C. This temperature was then maintained for the first 6 hrs after CPB. Group C was subjected to 75 min of hypothermic CPB at 32°C with limited rewarming at the end of CPB to a temperature of 35°C which was maintained for the first 6 postoperative hours. Group D was normothermic during CPB, cooled to 35°C at the end of CPB and then maintained at that temperature for the first 6 postoperative hours. After 6 hours, all animals were allowed to rewarm to a normothermic temperature.

During surgery, CPB, and the first 6 hr after CPB, rectal and pericranial temperature were measured and servo-regulated (YSI 400 series thermistor and 73ATA Indicating controller, YSI, Yellow Springs, OH) according to group designation. The heating/cooling system consisted of a heating blanket, a convective forced-air system, as well as a water-jacketed





venous reservoir and arterial flow inlet in addition to the operating platform that was connected to either a warm water bath (36-38°C) or a water bath with ice water. At different time points (Table 1), blood gas analysis (alpha stat) and hemoglobin levels were performed using an IL1306 blood gas analyzer (Instrumentation Laboratories Inc, Lexington, MA) and an OSM3 Hemoximeter@ (Radiometer Inc, Copenhagen, Denmark). The targeted flow during CPB was 160-180 $mL.kg^{-1}min^{-1}$ closely resembling the normal cardiac output in the rat.[17] The animals were weaned from CPB without the use of any medication and heparin anticoagulation was allowed to dissipate spontaneously. The venous cannula and the epigastric arterial catheter were then removed and the wounds closed. The animals were ventilated for 6 hrs after CPB to allow any remaining neuromuscular blockade to dissipate as well as to maintain temperature control. After the last blood sample, the tail cannula was removed, the wound closed, and anesthesia discontinued. After tracheal extubation, the animals were placed in an oxygen enriched environment.

**Cognitive testing**

Starting on the third postoperative day and continuing for 7 days, the animals underwent cognitive testing in the Morris water maze (MWM).[16] The MWM is a 1.5 m diameter black pool filled with water (26 ± 1°C) with a fixed submerged platform in one quadrant and various visual clues present on the surrounding walls. The time taken by the rat to locate the hidden platform after having been placed in the pool (defined as the latency) was measured. During the testing period, the animals underwent daily testing with four trials per day. At each trial, the animals were placed in the pool in randomly assigned, different quadrants. If the platform could not be found, the maximum time spent swimming in the pool was limited to 90 s, after which the animal was placed on the platform for 15 seconds. Their performance (including swimming speed) was recorded and analyzed using a computerized video tracking system (Ethovision, Noldus, Wageningen, the Netherlands).

**Histologic examination**

After completion of the MWM testing, the animals were anesthetized with 3% isoflurane and underwent in situ brain fixation using an intracardiac injection of heparinized saline followed by 10 % buffered formalin. Twenty-four hours later, the brains were removed and stored in 4% formalin. Paraffin-embedded sections were serially cut in 5 $\mu m$ slices and then stained with hematoxylin and eosin for light microscopic evaluation. From each brain, the most dorsal slice with two cross sections of each lateral ventricle (-3.6 mm from bregma) was examined, and the total number of necrotic neurons in bilateral hippocampal CA1 and CA3 regions were recorded. Cytoplasmatic eosinophilia, karyorrhexis or pyknosis were used as criteria for cellular necrosis.

*Table 1. Physiologic Values, per group at different timepoints. Mean (sd)*

| | | CPB | | | | After CPB | | | |
|---|---|---|---|---|---|---|---|---|---|
| | baseline | 5 min | 30 min | 60 min | 90 min | 1 hr | 2 hr | 4 hr | 6 hr |
| **Weight (g)** | | | | | | | | | |
| A | 364 (9) | | | | | | | | |
| B | 360 (11) | | | | | | | | |
| C | 365 (16) | | | | | | | | |
| D | 362 (9) | | | | | | | | |
| **MAP (mmHg)** | | | | | | | | | |
| A | 85 (6) | | 75 (16) | 76 (14) | 81 (15) | 78 (9) | 74 (8) | 70 (8) | 74 (11) |
| B | 82 (3) | | 75 (12) | 75 (13) | 74 (8) | 78 (5) | 74 (7) | 75 (5) | 78 (4) |
| C | 82 (4) | | 79 (12) | 78 (10) | 75 (11) | 76 (7) | 76 (5) | 77 (6) | 79 (4) |
| D | 83 (5) | | 78 (16) | 77 (11) | 76 (16) | 76 (4) | 76 (5) | 73 (6) | 72 (7) |
| **CPB flow (ml/min)** | | | | | | | | | |
| A | | 50 (2) | 50 (2) | 51 (2) | 50 (3) | | | | |
| B | | 48 (3) | 48 (3) | 49 (4) | 51 (4) | | | | |
| C | | 49 (3) | 48 (3) | 49 (2) | 50 (3) | | | | |
| D | | 49 (2) | 50 (3) | 50 (2) | 49 (2) | | | | |
| **Hemoglobin (mg/dL)** | | | | | | | | | |
| A | 17.8 (0.7) | | 10.9 (1.0) | | 10.3 (0.8) | | 11.3 (0.8) | | 11.3 (1.0) |
| B | 16.9 (1.0) | | 10.5 (0.7) | | 10.7 (0.9) | | 11.6 (0.6) | | 11.7 (0.9) |
| C | 17.0 (0.9) | | 10.5 (0.9) | | 10.3 (0.8) | | 11.6 (0.8) | | 11.3 (0.9) |
| D | 17.4 (0.9) | | 10.6 (0.9) | | 10.9 (0.7) | | 11.7 (0.5) | | 11.5 (0.7) |
| **Glucose (mg/dL)** | | | | | | | | | |
| A | 78 (11) | | | 119 (12) | 126 (16) | | | | 130 (21) |
| B | 85 (6) | | | 130 (13) | 133 (18) | | | | 135 (15) |
| C | 81 (9) | | | 134 (22) | 144 (19) | | | | 136 (26) |
| D | 81 (6) | | | 119 (14) | 127 (20) | | | | 137 (13) |
| **pHa** | | | | | | | | | |
| A | 7.39 (0.03) | | 7.36 (0.04) | 7.40 (0.04) | 7.36 (0.03) | 7.38 (0.04) | 7.40 (0.05) | 7.40 (0.04) | 7.41 (0.03) |
| B | 7.42 (0.03) | | 7.40 (0.03) | 7.40 (0.02) | 7.39 (0.03) | 7.41 (0.03) | 7.40 (0.03) | 7.40 (0.04) | 7.41 (0.04) |
| C | 7.42 (0.03) | | 7.37 (0.06) | 7.35 (0.06) | 7.37 (0.08) | 7.42 (0.04)[d] | 7.41 (0.04) | 7.42 (0.04) | 7.41 (0.04) |
| D | 7.39 (0.03) | | 7.37 (0.09) | 7.33 (0.11) | 7.29 (0.16) | 7.37 (0.04) | 7.40 (0.03) | 7.43 (0.04) | 7.42 (0.03) |
| **$PaCO_2$ (mmHg)** | | | | | | | | | |
| A | 38 (3) | | 41 (4) | 38 (4) | 40 (3) | 38 (4) | 36 (5) | 37 (5) | 37 (4) |
| B | 36 (3) | | 38 (4) | 39 (5) | 40 (3) | 37 (3) | 36 (4) | 37 (4) | 37 (4) |
| C | 37 (4) | | 42 (7) | 46 (9) | 46 (11) | 37 (5) | 37 (5) | 36 (3) | 38 (4) |
| D | 39 (4) | | 42 (19) | 49 (19) | 58 (33) | 39 (3) | 36 (2) | 36 (3) | 36 (3) |
| **$PaO_2$ (mmHg)** | | | | | | | | | |
| A | 273 (73) | | 253 (120) | 295 (109) | 269 (80) | 258 (109) | 267 (65) | 262 (37) | 278 (44) |
| B | 259 (71) | | 410 (60)[a d] | 433 (58)[a d] | 337 (70) | 264 (80) | 258 (87) | 243 (82) | 260 (78) |
| C | 285 (90) | | 353 (85) | 351 (104) | 268 (122) | 265 (92) | 285 (102) | 271 (84) | 279 (82) |
| D | 214 (91) | | 294 (83) | 288 (106) | 272 (111) | 245 (78) | 269 (75) | 284 (68) | 276 (49) |
| **$HCO_3^-$ (mEq)** | | | | | | | | | |
| A | 23.3 (1.5) | | 23.4 (1.1) | 23.7 (1.1) | 23.0 (1.5) | 22.5 (1.5) | 22.8 (1.6) | 22.6 (1.7) | 23.7 (1.2) |
| B | 23.5 (0.6) | | 23.0 (1.0) | 24.2 (2.7) | 24.8 (1.8) | 23.4 (0.7) | 22.7 (1.3) | 23.4 (1.2) | 23.3 (1.0) |
| C | 24.1 (1.1) | | 23.4 (1.9) | 25.5 (2.3) | 24.4 (3.7) | 23.4 (2.0) | 23.4 (1.4) | 23.6 (0.9) | 23.8 (1.1) |
| D | 24.1 (0.9) | | 22.7 (1.3) | 25.4 (1.7) | 25.4 (2.4) | 22.8 (1.1) | 22.7 (1.4) | 23.7 (1.1) | 23.3 (1.3) |
| **Rectal temperature (C°)** | | | | | | | | | |
| A | 37.2 (0.3) | 37.3 (0.3) | 37.3 (0.3) | 37.4 (0.2) | 37.5 (0.1) | 37.5 (0.1) | 37.5 (0.1) | 37.5 (0.1) | 37.5 (0.1) |
| B | 37.4 (0.1) | 32.4 (0.8) | 32.0 (0.3) | 32.1 (0.2) | 35.6 (1.4) | 37.0 (0.6) | 37.4 (0.2) | 37.5 (0.1) | 37.5 (0.1) |
| C | 37.4 (0.3) | 32.3 (0.8) | 32.0 (0.3) | 32.0 (0.3) | 34.1 (1.3) | 34.9 (0.2) | 35.1 (0.2) | 35.1 (0.2) | 35.0 (0.1) |
| D | 37.4 (0.2) | 37.3 (0.3) | 37.4 (0.2) | 37.5 (0.1) | 37.5 (0.1) | 35.2 (0.4) | 35.1 (0.1) | 35.0 (0.1) | 35.1 (0.1) |
| **Pericranial temperature (C°)** | | | | | | | | | |
| A | 37.2 (0.4) | 37.2 (0.3) | 37.3 (0.3) | 37.4 (0.2) | 37.4 (0.1) | 37.5 (0.1) | 37.5 (0.1) | 37.4 (0.1) | 37.5 (0.1) |
| B | 37.4 (0.1) | 32.9 (0.7) | 32.0 (0.5) | 32.0 (0.2) | 34.7 (1.2) | 37.0 (1.0) | 37.3 (0.2) | 37.5 (0.1) | 37.5 (0.1) |
| C | 37.4 (0.3) | 33.2 (0.8) | 32.1 (0.2) | 32.0 (0.1) | 33.3 (1.0) | 34.9 (0.5) | 35.0 (0.1) | 35.0 (0.2) | 35.0 (0.1) |
| D | 37.4 (0.2) | 37.3 (0.2) | 37.4 (0.2) | 37.5 (0.1) | 37.5 (0.1) | 35.6 (0.4) | 35.2 (0.2) | 35.0 (0.1) | 35.1 (0.1) |

*[a]p < 0.05 vs group A*

*[d]p < 0.05 vs group D*

**Statistical analysis**

Morris water maze latencies were analyzed using repeated measurements analysis of variance (ANOVA) with Bonferroni post-hoc testing. Physiologic values and histological outcomes were compared using one-way analysis of variance with Bonferroni post-hoc testing where appropriate. Statistical significance was assumed when $p < 0.05$.

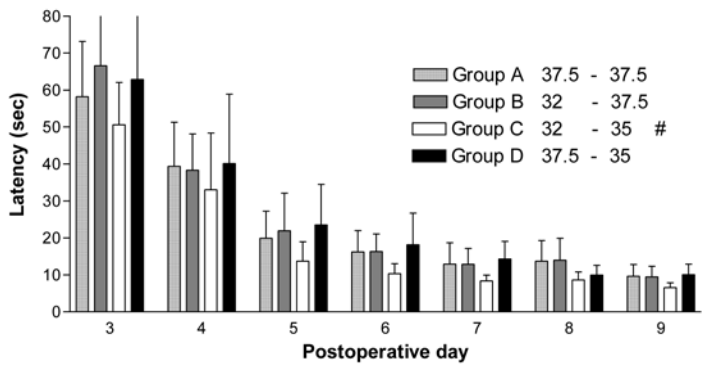



*Figure 1*

*Results of the cognitive testing performed in the Morris Water Maze in animals exposed to various combinations of normothermia and hypothermia during both cardiopulmonary bypass (CPB) as well as postoperatively. Group A was maintained at normothermia (37.5°C) both during and for 6 hours after CPB. Group B underwent hypothermic (32°C) bypass followed by a normothermic postoperative period. Group C was hypothermic during CPB (32°C) and kept hypothermic (35°C) for the first 6 postoperative hours. Group D was normothermic during CPB but was cooled (35°C) postoperatively. # Indicates lower latencies for Group C, thus better performance ($p < 0.05$). Data represent mean + SD.*

*Table 2. Histologic Results: Dead cells in the hippocampal CA1 and CA3 areas.*

| | Dead Cells | |
|---|---|---|
| **Group** | **CA 1** | **CA 3** |
| A | 15 ± 9 | 15 ± 6 |
| B | 15 ± 7 | 16 ± 6 |
| C | 12 ± 3 | 13 ± 3 |
| D | 11 ± 4 | 14 ± 5 |
| *p-value* | *0.25* | *0.46* |



## Results

There was one death in group A, three in group B, and three in group D, all related to surgical/technical difficulties including excessive blood loss during cannulation or mechanical failure of the pump connections during the procedure. Thus, there were a total of 45 rats in the experiment available for analysis (group A n=12, group B n=10, group C n=13 and group D n=10 rats).

Physiologic values at different time points during the experiment are presented in Table 1. The temperature differences at several time points were in accordance with the *a priori* group designations. There was a transiently higher arterial oxygen tension in group C, the group with hypothermic CPB and postoperative period. All other parameters were not significantly different between the groups.

Figure 1 represents the average latency for the animals to find the platform. In all groups there was daily improvement in the MWM latency providing evidence of learning over time ($p < 0.001$) and a plateau was reached, implying no further larning. There were no differences between Groups A vs. B, A vs. D, and B vs. D ($p = 1.00$ for all comparisons). However, Group C (hypothermic bypass with limited rewarming and prolonged postoperative hypothermia) performed better than all other groups (Group C vs. Group A, $p = 0.044$; Group C vs. Group B, $p = 0.011$; Group C vs. Group D, $p = 0.012$). The swimming speeds averaged between 39 ± 23 and 44 ± 25 $cm \cdot s^{-1}$ throughout the postoperative days with no significant differences among the four groups ($p = 0.206$) indicating no differences in motor capabilities between groups.

Histologic results are listed in Table 2. There were no differences between the four groups in the number of a dead hippocampal CA1 ($p = 0.25$), or for CA3 neurons ($p = 0.46$).

## Discussion

In the present study, we subjected rats to 90 min of CPB with different intraoperative and postoperative temperature regimens. The animals were subjected to either a normo- or hypothermic (32°C) CPB period in combination with a 6 hr hypothermic (35°C) or normothermic (37.5°C) postoperative period. In comparison to the other groups, the hypothermic CPB group with a period of postoperative hypothermia performed significantly better on the MWM test indicating better cognitive performance. This suggests that isolated hypothermia (during or after CPB) offers no significant cognitive protection in comparison to normothermic temperature strategies, but a combined strategy (hypothermic CPB with limited rewarming and a prolonged hypothermic period) decreases CPB-related POCD in rats. We speculate that this was most likely due to the

benefits of limited rewarming, as the group that only had postoperative hypothermia, demonstrated no neuroprotective benefit.

This study was designed to answer a pertinent clinical question. Although hypothermia itself is found to be neuroprotective in different types of studies [6-9], in cardiac surgery its neuroprotective properties remain unclear.[10-12,18] Hypothermic bypass has been examined in a number of trials focusing on neurocognitive outcome. McLean et al [10] were unable to detect any neuroprotective effect from moderate hypothermia in a study of 201 patients when compared to normothermia. Similarly, Mora et al [18] randomly assigned patients to hypothermic (< 28°C ) or normothermic (> 35°C ) temperature regimens and also found no influence of CPB temperature strategy on neurocognitive performance. In that study, neurocognitive performance deteriorated in more than half of each treatment group, and at the 6 week follow-up assessment, 15% in each group continued to have cognitive impairments. Importantly, they also reported a 10% incidence of new neurologic dysfunction (i.e., stroke) in the normothermic group vs. none in the hypothermic group. In comparison, the Warm Heart Investigators trial, a study with 1732 patients undergoing bypass surgery [12], showed no difference in stroke rate between two similar temperature groups.

A clinical study from our own investigative group compared normothermic (35.5-36.5°C; n=136) CPB with hypothermic (28-30°C; n=134) CPB for change in cognitive function 6 weeks postoperatively and found no difference between the two temperature groups.[11] One possible explanation for the lack of difference in the various hypothermia vs normothermia studies is that all of the hypothermic patients eventually had to undergo rewarming. This rewarming itself may have negated any benefit conferred by hypothermia by causing an overshoot in cerebral temperature.[19] Another limitation of these studies was the definition of normothermia. Some allowed temperature drift such that the patients were actually mildly hypothermic. Others actively rewarmed the patients such that potential harmful cerebral hyperthermia occurred.

To address the potential confounding effect of rewarming on neurocognitive outcome after hypothermic bypass, a second clinical trial was performed in which 165 patients undergoing hypothermic CPB (28-32°C) were assigned to a conventional or slow rewarming rate.[13] In the slow rewarming group in that trial, a 2°C difference between nasopharyngeal-perfusate temperatures was maintained during rewarming, while in the conventional group temperature gradients of 4-6°C were allowed. A significant association between change in cognitive function and rate of rewarming was found, with the slow rewarming group performing significantly better with respect to cognitive outcome.

The results of the present experiment corroborate this finding of a benefit to hypothermic CPB when coupled with limited rewarming and prolonged postoperative hypothermia. A slow rewarming rate appears to be essential for a beneficial effect on cognitive outcome. In our study, the bypass inflow temperature was never allowed to increase beyond a normothermic (37.5°C) level. This prevented any overshoot in cerebral temperature to be responsible for the poorer outcome in the group that was rewarmed prior to the end of CPB. Even in the absence of cerebral hyperthermia, the known uncoupling of cerebral blood flow (CBF) and metabolism during rewarming [20] may have still have led to ischemic injury in the brain.

The experimental design in this rat CPB model allowed for the investigation of different temperature strategies, especially those that cannot be performed in the clinical setting. For example, group D (normothermic CPB and hypothermic postoperative period) is relatively easy to perform in an experimental setup and allowed the assessment of the influence of a single postoperative hypothermic period after a normothermic CPB period. In the clinical setting, this isolated postoperative hypothermia would be difficult to justify.

Nathan et al [14] completed a study where 223 patients undergoing CABG were all cooled to 32°C at the start of CPB and randomly assigned to rewarming protocols with targets of either 34°C or 37°C. Surface rewarming was applied in the intensive care unit with strict avoidance of hyperthermia. One week after surgery, the hypothermic group had a lower incidence and severity of postoperative cognitive impairment, indicating possible beneficial effects of mild hypothermia. In a recent investigation the same authors[21] addressed the safety issues raised by mild postoperative hypothermia. They concluded that postoperative hypothermia is likely safe (with respect to bleeding outcomes, inotropic use, and times to tracheal extubation), and that complete rewarming after hypothermic cardiopulmonary bypass is not always necessary. In contemporary cardiac surgery, patients are not usually intentionally cooled without subsequent rewarming, but the Nathan et al results as well as the present animal study suggest that this avoidance of rewarming may have some merit if combined with mild intraoperative hypothermia.

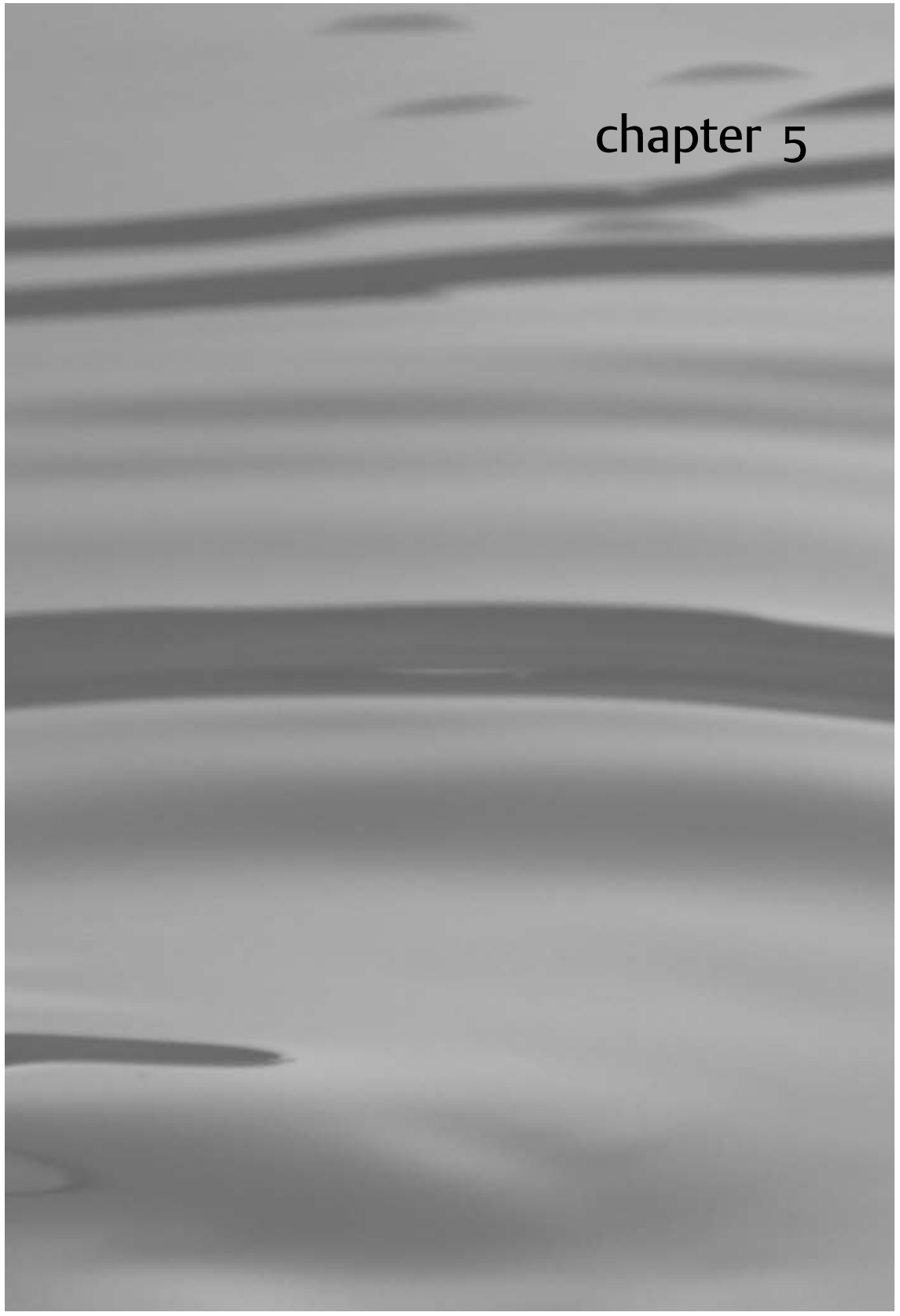
There were several limitations in this study. Unlike the clinical setting, we did not measure perfusate-nasopharyngeal temperature gradients, as has been used as a means of regulating rewarming rate.[11] However, we did measure pericranial temperature, a close correlate of brain temperature, by inserting a temperature probe between the temporal muscle and the cranium. In this way, we achieved cooling and rewarming within 15 min without any overshoot in pericranial temperatures. A second limitation was the lack of any discernible histological correlate injury to the cognitive differences. This absence of neurotic injury is not completely unexpected as brain dysfunction is frequently seen without any gross histological injury. This absence of necrotic brain injury in the presence of a functional deficit has previously been seen in models of CPB.[16,22] A final limitation was our relatively short-term outcome. We chose to terminate the animals after cognitive

testing on day 9. This was done in an effort to balance the logistics of accommodating a reasonable postoperative period of neurocognitive testing with a window within which gross histopathological changes (thought not seen here) might possibly be discernable.

In summary, we have demonstrated a neuroprotective effect of limited rewarming and prolonged postoperative hypothermia after hypothermic CPB in rats. This finding is consistent with clinical studies of various hypothermia and rewarming strategies and provides further evidence for limiting the rewarming rate and accepting some postoperative hypothermia.

## Reference List


1. Newman MF, Kirchner JL, Phillips-Bute B, Gaverm V, Grocott HP, Jones RH, Mark DB, Reves JG, Blumenthal JA. Longitudinal assessment of neurocognitive function after coronary artery bypass surgery. N Engl J Med 2001;344:395-402.
2. Roach GW, Kanchuger M, Mangano CM, Newman M, Nussmeier N, Wolman R, Aggarwal A, Marschall K, Graham SH, Ley C. Adverse cerebral outcomes after coronary bypass surgery. Multicenter Study of Perioperative Ischemia Research Group and the Ischemia Research and Education Foundation Investigators. N Engl J Med 1996;335:1857-63.
3. Grocott HP, Homi HM, Puskas F. Cognitive dysfunction after cardiac surgery: revisiting etiology. Semin Cardiothorac Vasc Anesth 2005;9:123-9.
4. Arrowsmith JE, Harrison MJG, Newman SP, Stygall J, Timberlake N, Pugsley WB. Neuroprotection of the brain during cardioulmonary bypass. A randomized trial of remacemide during coronary artery bypass in 171 patients. Stroke 1998;29:2357-62.
5. Mitchell SJ, Pellett O, Gorman DF. Cerebral protection by lidocaine during cardiac operations. Ann Thorac Surg 1999;67:1117-24.
6. Busto R, Dietrich WD, Globus MY, Valdes I, Scheinberg P, Ginsberg MD. Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. J Cereb Blood Flow Metab 1987;7:729-38.
7. Minamisawa H, Smith ML, Siesjo BK. The effect of mild hyperthermia and hypothermia on brain damage following 5, 10, and 15 minutes of forebrain ischemia. Ann Neurol 1990;28:26-33.
8. Minamisawa H, Nordstrom CH, Smith ML, Siesjo BK. The influence of mild body and brain hypothermia on ischemic brain damage. J Cereb Blood Flow Metab 1990;10:365-74.
9. Barone FC, Feuerstein GZ, White RF. Brain cooling during transient focal ischemia provides complete neuroprotection. Neurosci Biobehav Rev 1997;21:31-44.
10. McLean RF, Wong BI, Naylor CD, Snow WG, Harrington EM, Gawel M, Fremes SE. Cardiopulmonary bypass, temperature, and central nervous system dysfunction. Circulation 1994;90:II250-5.
11. Grigore AM, Mathew J, Grocott HP, Reves JG, Blumenthal JA, White WD, Smith PK, Jones RH, Kirchner JL, Mark DB, Newman MF. Prospective randomized trial of normothermic versus hypothermic cardiopulmonary bypass on cognitive function after coronary artery bypass graft surgery. Anesthesiology 2001;95:1110-9.
12. Randomized trial of normothermic versus hypothermic coronary bypass surgery. The Warm Heart Investigators. Lancet 1994;343:559-63.
13. Grigore AM, Grocott HP, Mathew JP, Phillips-Bute B, Stanley TO, Butler A, Landolfo KP, Reves JG, Blumenthal JA, Newman MF. The rewarming rate and increased peak temperature alter neurocognitive outcome after cardiac surgery. Anesth Analg 2002;94:4-10.
14. Nathan HJ, Wells GA, Munson JL, Wozny D. Neuroprotective effect of mild hypothermia in patients undergoing coronary artery surgery with cardiopulmonary bypass: a randomized trial. Circulation 2001;104:I85-191.
15. Guide for the Care and Use of Laboratory Animals. Revised 1996; National Institutes of Health publication No. 86-23.
16. Mackensen GB, Sato Y, Nellgard B, Pineda J, Newman MF, Warner DS, Grocott HP. Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001;95:1485-91.
17. Li SG, Randall DC, Brown DR. Roles of cardiac output and peripheral resistance in mediating blood pressure response to stress in rats. Am J Physiol 1998;274:R1065-9.
18. Mora CT, Henson MB, Weintraub WS, Murjin JM, Martin TD. The effect of temperature management during cardiopulmonary bypass on neurologic and neuropsychologic outcomes in patients undergoing coronary revascularization. J Thorac Cardiovasc Surg 1996;112:514-22.
19. Grocott HP, Mackensen GB, Grigore AM, Mathew J, Reves JG, Phillips-Bute B, Smith PK, Newman MF. Postoperative hyperthermia is associated with cognitive dysfunction after coronary artery bypass graft surgery. Stroke 2002;33:537-41.
20. Enomoto S, Hindman B, Dexter F, Smith T, Cutkomp J. Rapid rewarming causes an increase in the cerebral metabolic rate for oxygen that is temporarily unmatched by cerebral blood flow: A study during cardiopulmonary bypass in rabbits. Anesthesiology 1996;84:1392-400.
21. Nathan HJ, Parlea L, Dupuis JY, Hendry P, Williams KA, Rubens FD, Wells GA. Safety of deliberate intraoperative and postoperative hypothermia for patients undergoing coronary artery surgery: a randomized trial. J Thorac Cardiovasc Surg 2004;127:1270-5.
22. Ma D, Yang H, Lynch J, Franks NP, Maze M, Grocott HP. Xenon attenuates cardiopulmonary bypass-induced neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2003;98:690-8.



chapter 5

# Unilateral intracarotid injection of holmium microspheres to induce bilateral cerebral embolization in rats; validation obtained with MRI

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## Abstract

**Background**
Cerebral embolization models have been hindered by the fact that delivery is one-sided and cannot be quantified easily. We developed a model for bilateral cerebral micro-embolization.

**Methods**
To validate the quantification of holmium microspheres a phantom study was performed in which concentration of microspheres in solution was compared with the number of holmium-induced artifacts on MRI. After that identical microspheres were administered by unilateral injection in the carotid artery, while the opposite carotid artery was clamped. On post-injection MRI-scans, intracerebral delivery and right/left distribution of the microspheres was determined.

**Results**
In the phantom study it was shown that quantification by MRI is possible and that MRI artifacts represent single microspheres. In the rat brain, about one third of the injected dose was consistently located on the contralateral side. The administration resulted in a reproducible administration regarding distribution and number of microspheres.

**Conclusions**
The use of holmium microspheres enables quantification of delivered dose as single microspheres induce artifacts on MRI. By clamping the comtralateral carotid artery, one third of the dose is located in the contralateral hemisphere.






## Introduction

In the last few decades, many animal models of cerebral micro-embolization have been employed to study pathophysiological mechanisms of diffuse cerebral ischemia. In these microsphere animal models [1-4] an attempt is made to mimic the dislodging of particles as occurs during cardiac, orthopedic or cerebrovascular surgery and in stroke. However, all of these models have been hindered by the fact that injection in the carotid artery results only in ipsilateral hemispheric insults with unpredictable delivery which cannot be quantified directly.[2,3] We therefore developed a method of unilateral intracarotid injection for bilateral distribution of holmium-containing micro-embolic spheres. Because holmium is highly paramagnetic it can be used for quantitative detection by Magnetic Resonance Imaging (MRI).[5] By using phantoms with different concentrations of microspheres, we investigated whether individual microspheres can cause detectable distortions on MRI imaging.

## Methods

**Microspheres**
The microspheres used in the studies were poly(L-lactic-acid) microspheres containing 17.1% holmium acetylacetonate.[6,7] Because of the paramagnetic properties of holmium, the microspheres will cause pixel distortion on MRI images and can thus be quantified.[5] The microspheres had a diameter of 30μm (outer limits 20-50 μm).

**Phantom study**
To validate the quantitative MRI measurements in the rat brain, phantom experiments were performed. Therefore, two microsphere solutions in agar were produced with a calculated amount of 1000 and 125 microspheres per ml. For preparation of a 1% agar gel matrix, 0,6 g of dry agarose powder was mixed in 60 g of microsphere suspension in water. The microsphere agar suspensions were heated to 100°C for 3 minutes, resulting in transparent fluids. To prevent trapping of air bubbles, the mixed fluids were sonicated during cooling. The formed gels at room temperature were optically transparent. Syringes with 2 ml of agarose gel were used for the MRI measurements.
To measure the accuracy in the amount of microspheres in the suspensions, we determined the microsphere count per mL. Particle size distribution was determined at room temperature by using a Coulter Counter Multisizer 3 (Beckman Coulter Nederland, Mijdrecht, the Netherlands) with a 100 μm orifice. The calibration of the instrument was performed by using 20 μm latex beads (Coulter CC size standard L20; Beckman

Coulter Nederland). The amount of particles between 20 and 50 μm (microspheres) was determined in 2 ml of the diluted microsphere suspensions.

**Animal experiments**

The experiments were conducted in agreement with the applicable Dutch law, "Wet op de dierproeven" (art. 9) (1977), and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986), and approved by the ethical committee for animal experimentation of the University Medical Center Utrecht, Utrecht, The Netherlands.

*Animals*

Ten fasted male Wistar rats (HsdCpb:wu, Harlan, Horst, The Netherlands) weighing 475 – 525 g were used in this study. Wistar rats were chosen because this strain of rats has a complete Circle of Willis.[8]

*Anesthesia and surgical preparation*

Anesthesia was induced using halothane 4% in oxygen enriched air in a plastic induction box. The animals were orotracheally intubated using a 18G cannula (Abbott, Hoofddorp, The Netherlands) as tracheal tube and mechanically ventilated. Normocapnia (35-45 mmHg) was maintained using an infant ventilator with a respiratory frequency of 45 $min^{-1}$, a $FiO_2$ of 35% and maximum respiratory pressures of 1.5-2 kPa. Mainstream capnography was used for continuous respiratory monitoring. Rectal temperature was measured and maintained at 37°C with the use of a warming blanket and heated water pad. During subsequent surgical preparation, anesthesia was maintained with halothane 1.5-2%. Atropine 0.05 mg was given subcutaneously to prevent excess pulmonary secretions. All surgical wounds were locally infiltrated with lidocaine 1%.

The surgical procedure started with cannulation of the right femoral artery with a 26 G cannula (Abbott, Hoofddorp, The Netherlands) for continuous arterial pressure monitoring.

Through one midline cervical incision the common (CCA), the external (ECA) and internal (ICA) carotid arteries were bilaterally exposed. The right pterygopalatine artery was temporarily occluded during the cannulation and injection period. The right CCA was clamped and a PE 50 cannula was introduced retrogradely into the ECA with its tip ending near the bifurcation, then the CCA clamp was released. Thereafter, the contralateral CCA and ECA were clamped. A three minute time-period was allowed for flow adjustment. Injection of the microspheres into the right ICA was performed with a constant flow of 0.15 $ml.min^{-1}$. After injection the cannula was removed and the right ECA ligated. Four minutes after injection the clamps on the contralateral ECA and CCA were released. Skin and subcutaneous tissue were closed in two layers. The animals were ventilated for a further 60 min, after which the femoral artery catheter was removed and the skin closed. The animals recovered in a warm and oxygen enriched environment. Nalbuphine 0.5 mg i.m. was administered for postoperative analgesia. Forty-five days after the operation (as this was a pilot for long-term survival), the animals were anesthetized with halothane and decapitated. The brains were immediately removed and stored in 4% buffered formalin.

*Microsphere suspension*

The microspheres were homogeneously suspended in a 0.3 ml gelatin 8% with Tween 0.05% in saline solution for the animal study. The used solutions were sequentially prepared. To guarantee the homogeneousity, each solution was quickly cooled in ice in a syringe after preparation on a vortex to prevent any downward movement of the microspheres. At the moment of injection, the solution, soft-solid at room temperature, was directed through a warmed metal housing (37°C) after it had left the syringe, thus preventing microsphere trapping in the syringe or conus while a homogeneous (not sagged) solution was injected.

*Group designation*

The injected doses were calculated (by weight) approximately 5.000, 4.000, 3.000, 2.000, 1.500 microspheres (n=2 at each dose).

*Histology*

To determine whether microspheres were lodged individually or in clusters in the brain tissue, two rat brains obtained from rats injected with a calculated dose of 3000 microspheres, were embedded in paraffin and 5 μm paraffin sections were stained with hematoxylin and eosin. A total of thirty slices was evaluated for presence of microspheres.

***MRI protocol (phantom and animal study)***

All experiments were performed using a 4.7 T horizontal bore NMR spectrometer (Varian, Palo Alto, California, USA), equipped with a high-performance gradient insert (12cm inner diameter, maximum gradient strength 500 mT/m). For ex-vivo scanning of formalin fixed brains a homebuilt solenoid-coil (4 windings; Æ 35mm) was used for radio frequency transmission and signal reception. Fifty-one contiguous coronal T2-weighted spin echo images of 0.5 mm were collected with the following characteristics: repetition time=5000 ms; echo time = 17.5 ms; field of view = 25x25mm; matrix = 128 x 128; zero-filled = 256 x 256; two transitions. For scanning of the agar samples with different holmium concentrations a birdcage coil was used (Æ 65mm). An identical MR protocol (MRI sequence, repetition

time, echo time, resolution of images and number of transitions) was used as for the ex-vivo scanning of formalin fixed brains.

***MRI analysis***

The MRI scans of the rat brains were independently analyzed by two investigators (FL, JMD). They were blinded to number of microspheres injected in each rat. For each rat, all pixel distortions in the left and right side of the brain were counted in all slices.
The MRI scans of the phantoms were analyzed by one investigator (FL), who was unaware of the microsphere concentration of the phantoms.

***Statistical analysis***

For each hemisphere, the number of pixel distortions counted by the two experimenters was averaged. Using linear regression analysis, a regression coefficient and $R^2$ were estimated for the association between the number of microspheres injected and the distortions counted on MRI. *p*-values < 0.05 were considered significant.

## Results

**Phantom study**

The results of the validation experiments using the phantoms of highly diluted microsphere suspension are depicted in Table 1. The calculated microsphere concentration per mL is tabulated. The total number of MRI artifacts counted per phantom was 630 and 75 microspheres per ml agar gel, respectively. The Coulter counter results (number of particles per ml) are 671 and 79 microspheres per ml. The number of artifacts on MRI nearly equals the number of spheres in solution as counted by the Coulter counter, thus suggesting that single microspheres are counted in each MRI slice. A MRI image of the syringe with clearly identifiable pixel distortions caused by the holmium microspheres can be seen in Fig 1.

*Table 1. Comparison of the calculated concentrations (microspheres per ml), total number of MRI artifacts, its volume, the number of artifacts per ml and the Coulter counter results are tabulated per phantom. See text for details.*

| | calculated | MRI artifacts | Volume ml | artifacts per ml | Coulter |
|---|---|---|---|---|---|
| ph 1 | 1000 | 914 | 1.45 | 630 | 671 |
| ph 2 | 125 | 119 | 1.58 | 75 | 79 |

*ph 1= phantom 1  ph 2= phantom 2*

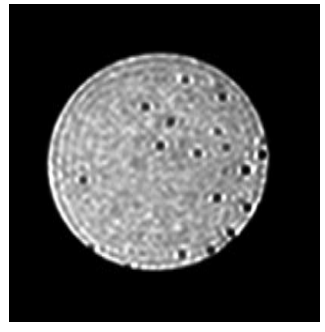

*Figure 1*
*MRI image of phantom containing holmium microspheres. The individual artifacts are clearly distinguishable.*

**Animal study**

The animals receiving the estimated dose of 5.000 and 4.000 microspheres were found dead within 36 h after injection with gross neurologic symptoms. The remaining animals lived throughout the experiment. The perioperatively measured blood pressures, $etCO_2$, and temperatures were all within their physiological range (data not shown).

*MRI of rat brains*

All brains of the surviving rats were scanned. Each brain was represented in approximately 34 slices. The “black hole” artifacts, caused by the holmium microspheres, were easily identifiable (see Figure 2). In Table 2, the number of counted artifacts and their right/left distribution is depicted. As expected, the number of artifacts increased with the injected dose. The regression coefficient was 0.48 (95% CI 0.35-0.61), $R^2$ was 0.98, ($p< 0.01$). On average, 66.4% (± 6.9) of the artifacts (and thus the microspheres) were located on the ipsilateral right hemispheres. Occlusion of the contralateral carotid artery and resulting redistribution of flow enhanced contralateral delivery of microspheres (33.7 ± 6.9 %). The mean difference in counted black hole artifacts between the two experimenters was 17 % (± 5%).

*Histology*

In all slices, only individual microspheres lodged in blood vessels were seen. See Figure 3.

*Table 2. Number of injected microspheres and number of artifacts counted on MRI and their distribution.*

| calculated | artifacts brain | % right | % left |
|---|---|---|---|
| 3000 | 1704 | 67.3 | 32.7 |
| | | | |
| 3000 | 1695 | 63.0 | 37.0 |
| 2000 | 1320 | 77.1 | 22.9 |
| 2000 | 1285 | 70.9 | 29.1 |
| 1500 | 1012 | 58.5 | 41.5 |
| 1500 | 930 | 61.3 | 38.7 |
| | mean | 66.4 | 33.7 |
| | sd | 6.9 | 6.9 |

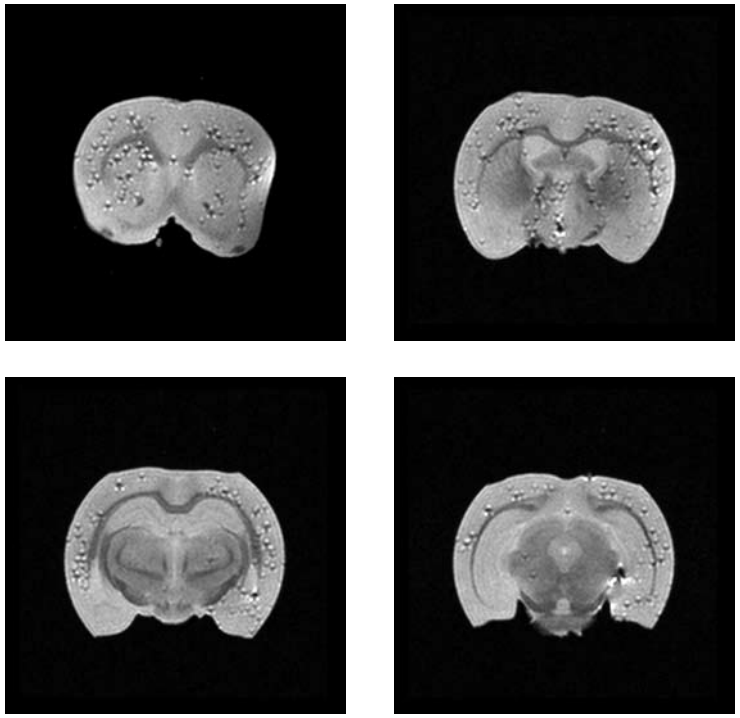

*Figure 2*

*T2 weighted spin echo images of the brain of a rat with a calculated dose of 3000 microspheres. Distinct black hole artifacts caused by the holmium microspheres are clearly visible. Right side on MRI corresponds to anatomical right side.*





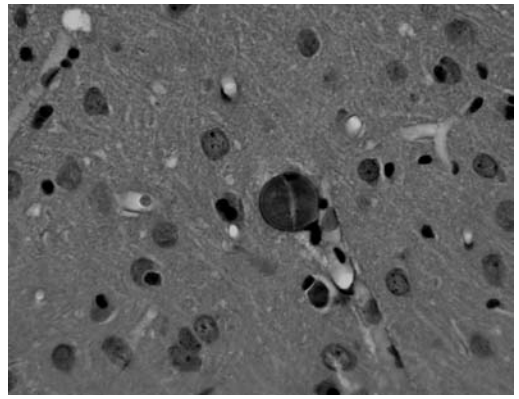

*Figure 3*

*H&E stained slice of rat brain tissue. In the middle a microsphere surrounded by endothelial cells is visible.*

## Discussion

In the present experiment, we subjected rats to a cerebral embolic load of MRI detectable holmium microspheres. Three minutes prior to injection, the contralateral internal and external carotid arteries were clamped. This maneuver resulted in a consistent bilateral distribution of the microspheres; one third of the embolic load was located in the contralateral hemisphere. Moreover, phantom studies showed that the number of detectable MRI artifacts correlated with the number of microspheres injected, thus suggesting that each artifact represents a single microsphere.

In the field of stroke research, numerous animal models for cerebral ischemia have been employed. The middle cerebral artery occlusion (MCAO) model, also known as the filament occlusion model, is widely used but infarct sizes are depending on many factors and not easily reproducible.[9,10] Existing micro-embolization models are also heterogeneous in nature due to variation in vehiculum used and injected dose and size of the microspheres. However, microsphere models better resemble the clinical situation in time-course of the lesions.[11,12] In analogy with the transition from TIA to stroke in humans, ischemic lesions in microsphere models develop slowly (effect increasing up to 24 hrs).[12] In addition, a large area “at risk” with varying degrees of injury available as possible targets of therapy is available. Micro-embolization models are very well suited for simulation of multiple cerebral embolizations during cardiac, cerebrovascular or orthopedic surgery. However, one major drawback of the currently available models is that delivery could not be quantified and was always unilateral. To better mimic the clinical situation, delivery of micro-emboli should preferably be bilateral.
De Ley *et al*[13] showed that in the anesthetized, normocapnic rat unilateral carotid occlusion will lower total cerebral blood flow to 75% of the normal value in a comparable degree on the occluded and non-occluded side. This persistence of a symmetrical hemispheric blood flow during occlusion can be used to reach a high degree of bilateral distribution in a rat model of microsphere embolization. In the experimental protocol, we allowed for a three minute period of flow adjustment after clamping of the left CCA and ECA. This period was based on an observation by Morita et al[14], stating that pial vessel diameter was stable after 60 s of unilateral carotid occlusion. To ensure complete flow adjustments, we decided to extend this period to three minutes.

In the current experiment, MRI detectable holmium microspheres, originally used for research on treatment of liver malignancies, were used to actually quantify delivery.[5]
During preparation and injection of microspheres the choice of the right vehiculum is essential. When stored in fluid, microspheres will settle down on the bottom by gravity and get trapped in the conus of the syringe, thereby importantly reducing cerebral delivery and reproducibility. We therefore used a gelatin formulation (gelatin 8% with Tween), liquid at body temperature but soft-solid at room temperature. The solution was warmed to 37 degrees Celsius just before reaching the tip of the cannula, thus ensuring delivery into the carotid artery. In many experiments, dextran was used as vehiculum of choice.[15,16] However, dextran injections in rats are a model for inflammation and shock [17,18] and we therefore think that direct injection into the cerebral arteries should be avoided. In our pilot experiments with dextran as vehiculum for the microspheres, several rats exhibited severe paw edema.
The use of MRI allows evaluation of delivery of the holmium-containing emboli. Distribution can thus be quantified and delivery failures can be recognized rapidly. Although we used the isolated fixed rat brain to study with MRI, anesthetized animals can also be evaluated without influencing outcome measures. By using phantom models with a known concentration of microspheres per ml (Coulter counter) we showed that the number of artifacts on MRI as counted in the models equals the number of microspheres in the solution. This implies that each microsphere causes a single artifact on MRI and thus exact number of delivery of microspheres, along with localisation, can be quantified quickly. The strength of the method described in this paper is that the delivered dose can be determined by MRI.

In conclusion, this paper describes a reproducible method for bilateral cerebral delivery of microspheres. The use of holmium microspheres makes it possible to quantify the exact amount and the distribution of these embolic particles by MRI, as each small MRI artifact represents a single microsphere. This quantification is important when cerebral embolic load is correlated to other outcome measures.

## Acknowledgement

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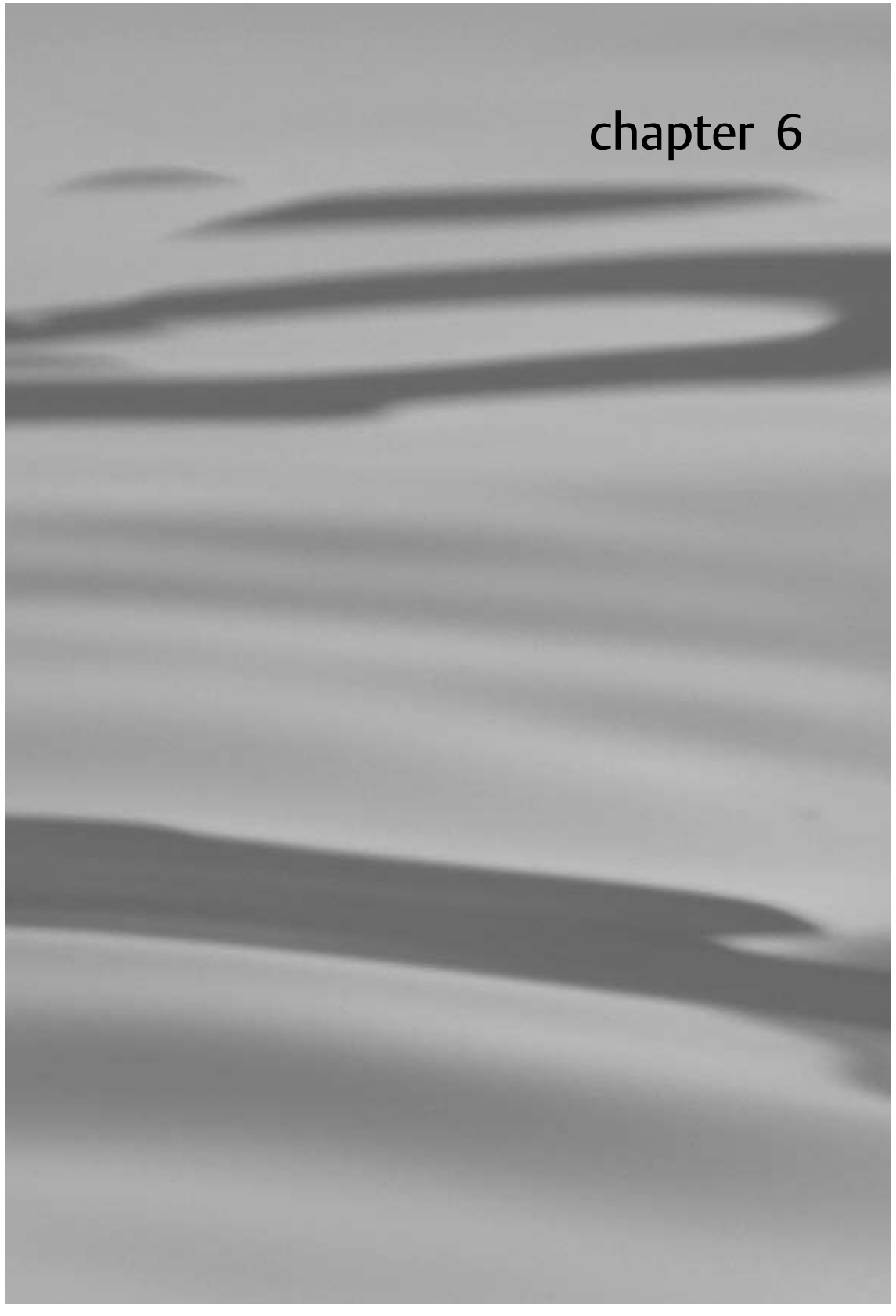
## Reference List


1. Bralet J, Beley P, Bralet AM, Beley A: Influence of various agents on the development of brain edema in the rat following microembolism. Protective effect of gamma-butyrolactone. Stroke 1979; 10: 653-6
2. Omae T, Mayzel-Oreg O, Li F, Sotak CH, Fisher M: Inapparent hemodynamic insufficiency exacerbates ischemic damage in a rat microembolic stroke model. Stroke 2000; 31: 2494-9
3. Takeo S, Taguchi T, Tanonaka K, Miyake K, Horiguchi T, Takagi N, Fujimori K: Sustained damage to energy metabolism of brain regions after microsphere embolism in rats. Stroke 1992; 23: 62-8
4. Zivin JA, DeGirolami U, Kochhar A, Lyden PD, Mazzarella V, Hemenway CC, Henry ME: A model for quantitative evaluation of embolic stroke therapy. Brain Res. 1987; 435: 305-9
5. Nijsen JFW, Seppenwoolde JH, Havenith T, Bos C, Bakker CJG, het Schip AD: Liver Tumors: MR Imaging of Radioactive Holmium Microspheres--Phantom and Rabbit Study. Radiology 2004; 231: 491-9
6. Nijsen JF, van Steenbergen MJ, Kooijman H, Talsma H, Kroon-Batenburg LM, van De WM, van Rijk PP, De Witte A, Van Schip AD, Hennink WE: Characterization of poly(L-lactic acid) microspheres loaded with holmium acetylacetonate. Biomaterials 2001; 22: 3073-81
7. Zielhuis SW, Nijsen JF, de Roos R, Krijger GC, van Rijk PP, Hennink WE, het Schip AD: Production of GMP-grade radioactive holmium loaded poly(L-lactic acid) microspheres for clinical application. Int.J Pharm. 2006; 311: 69-74
8. Lee RMKW: Morphology of cerebral arteries. Pharmacology & Therapeutics 1995; 66: 149-73
9. Aspey BS, Taylor FL, Terruli M, Harrison MJG: Temporary middle cerebral artery occlusion in the rat: consistent protocol for a model of stroke and reperfusion. Neuropathology and Applied Neurobiology 2000; 26: 232-42
10. Gerriets T, Stolz E, Walberer M, Muller C, Rottger C, Kluge A, Kaps M, Fisher M, Bachmann G: Complications and Pitfalls in Rat Stroke Models for Middle Cerebral Artery Occlusion: A Comparison Between the Suture and the Macrosphere Model Using Magnetic Resonance Angiography. Stroke 2004; 35: 2372-7
11. Beauchamp NJ, Jr., Barker PB, Wang PY, vanZijl PCM: Imaging of Acute Cerebral Ischemia. Radiology 1999; 212: 307-24
12. Mayzel-Oreg O, Omae T, Kazemi M, Li F, Fisher M, Cohen Y, Sotak CH: Microsphere-induced embolic stroke: an MRI study. Magn Reson.Med 2004; 51: 1232-8
13. de Ley G, Nshimyumuremyi JB, Leusen I: Hemispheric blood flow in the rat after unilateral common carotid occlusion: evolution with time. Stroke 1985; 16: 69-73
14. Morita Y, Fukuuchi Y, Koto A, Suzuki N, Isozumi K, Gotoh J, Shimizu T, Takao M, Aoyama M: Rapid changes in pial arterial diameter and cerebral blood flow caused by ipsilateral carotid artery occlusion in rats. Keio J Med 1997; 46: 120-7
15. Takagi N, Miyake K, Taguchi T, Tamada H, Takagi K, Sugita N, Takeo S: Failure in learning task and loss of cortical cholingergic fibers in microsphere-embolized rats. Exp.Brain Res. 1997; 114: 279-87
16. Miyake K, Takeo S, Kaijihara H: Sustained decrease in brain regional blood flow after microsphere embolism in rats. Stroke 1993; 24: 415-20
17. Kogure K, Ishizaki M, Nemoto M, Nakamura T, Suzuki M: Antishock effects of corticosterone on dextran-induced shock in rats. AJP - Endocrinology and Metabolism 1986; 251: E569-E575
18. Gulati OP, Sharma SC, Hammersen F: Hemodynamic, biochemical and morphological changes in the dextran and D-galactosamine-induced edemas in rats. Arch.Int.Pharmacodyn.Ther 1983; 263: 272-87



chapter 6

# A novel survival model of cardioplegic arrest and cardiopulmonary bypass in rats: a methodology paper

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Submitted for publication

## Abstract

**Background**

Given the growing population of cardiac surgery patients with impaired preoperative cardiac function and rapidly expanding surgical techniques, continued efforts to improve myocardial protection strategies are warranted. Prior research is limited to either large animal models or *ex vivo* preparations. We developed a new *in vivo* survival model that combines administration of anterograde cardioplegia with endoaortic crossclamping during cardiopulmonary bypass (CPB) in the rat.

**Methods**

Sprague-Dawley rats were cannulated for CPB (n=10). With ultrasound guidance, a 3.5 mm balloon angioplasty catheter was positioned via the right common carotid artery with its tip proximal to the aortic valve. To initiate cardioplegic arrest, the balloon was inflated and cardioplegia solution injected. After 30 min of cardioplegic arrest, the balloon was deflated, ventilation resumed, and rats were weaned from CPB and recovered. To rule out any evidence of cerebral ischemia due to right carotid artery ligation, animals were neurologically tested on postoperative day 14, and their brains histologically assessed.

**Results**

Thirty minutes of cardioplegic arrest was successfully established in all animals. Functional assessment revealed no neurologic deficits, and histology demonstrated no gross neuronal damage.

**Conclusion**

This novel small animal CPB model with cardioplegic arrest allows for both the study of myocardial ischemia-reperfusion injury as well as new cardioprotective strategies. Major advantages of this model include its overall feasibility and cost effectiveness. In future experiments long-term echocardiographic outcomes as well as enzymatic, genetic, and histologic characterization of myocardial injury can be assessed. In the field of myocardial protection, rodent models will be an important avenue of research.






## Introduction

Although considerable progress has been made in surgical techniques and other perioperative management to allow for the majority of patients to undergo cardiac surgery without significant mortality, more than 25% of this surgical population may still experience substantial morbidity related to adverse cardiovascular events. These include prolonged contractile dysfunction (stunning), myocardial infarction, low-output syndromes, and overt ventricular failure, all resulting in prolonged intensive care unit stay and reduced functional capacity at discharge, and ultimately contribute to overall mortality.[1, 2] Mortality after perioperative myocardial infarction (PMI) is 40-50%.[3] The etiology of myocardial dysfunction following cardiac surgery is multifactorial but frequently involves perioperative myocardial ischemia reperfusion injury.[4]

Since the advent of cardiopulmonary bypass (CPB), cardioplegic arrest has been an essential component of cardiac surgery but remains associated with ischemia-reperfusion injury to the myocardium.[5-7] As the population ages and percutaneous coronary interventions have become a standard therapy, patients referred for cardiac surgery generally present with a higher risk for perioperative cardiovascular complications.[8, 9] These complications are primarily due to increased co morbidities and more complex surgical interventions resulting in the need for more prolonged aortic crossclamp and CPB times, all making optimized myocardial protection strategies an essential component of cardiac surgery procedures. Experimental efforts to better understand the underlying mechanism associated with postoperative myocardial reperfusion injury and to improve established myocardial protection protocols have been limited to either costly large animal models or *ex vivo* heart preparations.[10-12] The use of normothermic cardioplegia solutions has shown beneficial results, but research is limited and principally relies on isolated heart models.[13-15] However, these models do not facilitate research on long-term effects of myocardial reperfusion injury or novel therapeutic interventions. To advance the field, additional research in a suitable rodent model with good survivability appears to be warranted. Such a model would not only allow to further elucidate mechanisms of adverse myocardial outcomes following cardioplegic arrest but also permits the characterization of genetic, proteomic, and histologic changes as well as long-term functional outcomes in response to injury and therapy. It might also facilitate further research aiming to optimize current cardioprotective strategies *in vivo* and facilitate myocardial gene delivery studies.[16, 17]

Based on an existing beating-heart model of CPB in the rat[18], we developed a novel *in vivo* survival model that allows administration of anterograde cardioplegia and endoaortic crossclamping. To rule out any gross neurological damage due to cannulation of the right carotid artery, a functional assessment and histological evaluation of the brains was performed.

## Methods

The study was approved by the Duke University Animal Care and Use Committee, and all procedures met the National Institutes of Health (NIH) guidelines for animal care.[19]
Male 400-425g Sprague-Dawley rats (Charles River Labs, Wilmington, MA, USA) were housed two per cage under a 12-hour light-dark cycle with food and water available ad libitum.

**Anesthesia, surgical preparation, cardiopulmonary bypass, and neurological assessment**

Fasted rats (n=10) were anesthetized with 5% isoflurane in 50% $O_2$ in a plastic induction box. After orotracheal intubation with a 14G cannula (Insyte BD Medical, Sandy, UT), the animals were mechanically ventilated (Harvard Model 687, Harvard Apparatus, Holliston, MA) 60 breaths·min$^{-1}$ with $FiO_2$ 0.6 while maintaining a normal arterial carbon dioxide tension. The maximal airway pressure did not exceed 20 mm$H_2O$. During subsequent surgical preparation, anesthesia was maintained with 1.5-2.0% isoflurane. A needle thermistor was inserted in the left temporal muscle adjacent to the skull to measure pericranial temperature. With both forced-air and surface heating systems, temperature during surgery was controlled at 36°C when not on CPB, during CPB, and cardioplegic arrest at 34°C. Towards the end of, and after CPB, temperature was controlled at 37°C. Electrocardiogram ECG) electrodes were placed on both front paws and left hind paw.
Surgical preparation consisted of cannulation of the tail artery with a 20 G catheter (Insyte BD Medical, Sandy, UT), which served as the arterial inflow cannula for the CPB circuit. 150 IU porcine heparin and 5 μg fentanyl were administered after placement of this first cannula. Mean arterial blood pressure was monitored via the superficial caudal epigastric artery, which was cannulated with polyethylene tubing (PE-10 Intramedic Tubing, Becton-Dickinson, Sparks, MD). A 3.5 mm angioplasty balloon catheter (Sprinter® OTW, Medtronic Inc, Minneapolis, MN) was retrogradely inserted into the ascending aorta via the right common carotid artery and positioned, under ultrasound guidance (SONOS 7500, Philips Medical Systems, Andover, MA), with its tip just above the aortic valve (Figure 1). This catheter served as an endoaortic crossclamp during the experiment, comparable with PortAccess™ surgery as performed in humans.[20] A multi-orificed 4.5 Fr catheter (modified Desilets-Hoffman Catheter, Cook, Bloomington, IN) was advanced through the right external jugular vein into the right atrium and served as a conduit for venous outflow. Repeat injections of 150 IU porcine heparin and 5 μg fentanyl were administered, and CPB was initiated. In addition, 0.2 mg pancuronium was administered prior to CPB. Muscle relaxation was used to prevent spontaneous ventilation that often interferes with venous return due to movement of the mediastinal structures relative to the venous outflow cannula.

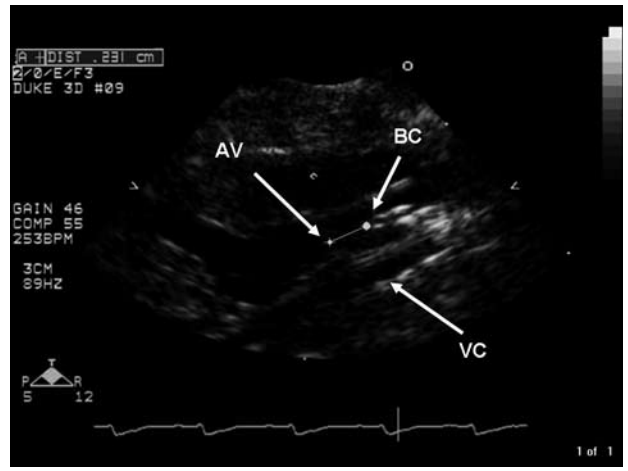


*Figure 1*

*Echocardiographic image of angioplasty balloon catheter positioned near aortic valve. Distance measure indicating a distance of 0.23 cm from tip of balloon catheter to aortic valve. AV = aortic valve, BC = tip of balloon catheter, VC = multi-orificed venous cannula advanced into right atrium.*

The CPB circuit (Figure 2) consisted of a 4 ml Plexiglas venous reservoir, a roller pump (Masterflex; Cole-Parmer Instrument Co., Vernon Hills, IL), and a custom-designed small-volume oxygenator (M. Humbs, Valley, Germany). The 4 ml priming volume oxygenator was built of two Plexiglas shells (12.8 cm x 12.8 cm x 2.7 cm) that carry a sterile, disposable three layer hollow fiber membrane providing a surface area for gas exchange of 558 cm$^2$.[18, 21] To prevent excessive heat loss, one of the shells had an integrated heat exchanger. An in-line flow probe (2N806 probe and T208 flowmeter, Transonics Systems Inc., Ithaca, NY) was used to continuously measure CPB flow. The entire circuit was primed with 10 mL of 6% hetastarch (Hextend, Hospira Inc, Lake Forest, IL). All parts were connected through single use silicone tubing. During CPB, a flow rate of 150 mL·kg$^{-1}$·min$^{-1}$ was maintained, and an average of 3 mL of hetastarch was added to compensate losses and extravasation. At the start of CPB, frequency of ventilation was lowered to 30 min$^{-1}$, and 0.5-1% isoflurane was administered through the oxygenator with 70% oxygen and additional $CO_2$ as needed (α stat blood gas management). After 15 min of CPB, the endoaortic clamp was quickly inflated while concomitantly 0.5 ml cardioplegia was infused via an infusion pump at a rate of 200 mL·hr$^{-1}$ through the central lumen of the angioplasty catheter. Cardioplegia solution consisted of 4 parts of standard undiluted adult cardioplegia induction solution (5% dextrose in 0.225% NaCl (655mL·L$^{-1}$) potassium

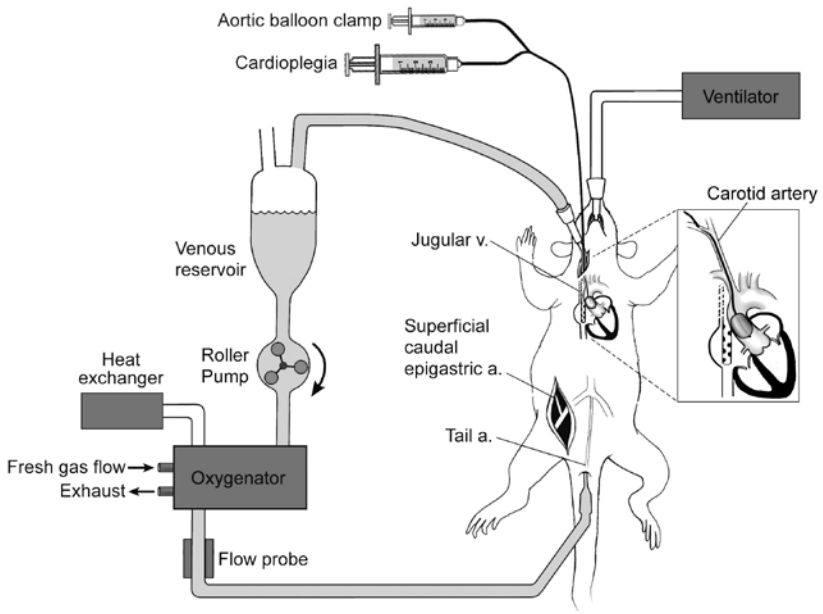


*Figure 2*

*Schematic diagram of rat CPB apparatus and surgical preparation highlighting the aortic balloon catheter serving as endoaortic crossclamp.*

chloride (95mEq·L$^{-1}$), tromethamine (238mL·L$^{-1}$), and citrate-phosphate-dextrose (CPD) solutions (60mL·L$^{-1}$) and 1 part esmolol 10 mg·ml$^{-1}$. Ventilation was discontinued. Cardioplegic arrest was confirmed both by ultrasound and electrocardiographically. The CPB flow rate was adjusted as needed to maintain a constant venous reservoir blood level. At 15 min of arrest, a second dose of 0.4 mL of cardioplegia was administered to uphold the arrest. After 30 min of cardioplegic arrest, the balloon was deflated and removed and ventilation restarted at a slower rate of 35 min$^{-1}$, and $FiO_2$ of 0.7. CPB was maintained for another 30 min to allow for rewarming of the animals. After discontinuation of CPB, the ventilatory rate was raised to 60 min$^{-1}$. The venous cannula was removed and the incision closed. During the entire experiment, mean arterial pressure was maintained above 45 mmHg, with the use of small doses of phenylephrine if necessary. After cessation of CPB, the remaining blood left in the CPB circuit was centrifuged for 5 min at 3000 rpm, and the supernatant discarded. Two mL of the remaining red blood cell concentrate was then re-infused. The animals were ventilated for another 60 min, after which the remaining cannulae were removed and the wounds closed. The rats recovered in a warmed and oxygen-enriched environment for at least 12 hr prior to return to their home cages. Blood gas analysis was performed before the start of CPB, at 15 min of CPB, at 15 min of arrest, at the end of 30 min arrest, at the end of the total of 75 min of CPB, and at 1 hr post CPB, using an IL-GEM Premier 3000 blood gas analyzer (Global Medical Instrumentation, Ramsey, MI).

During the first 14 postoperative days, the animals were checked daily for their overall well-being and wound healing. To determine if any neurologic injury occurred (because of intraoperative usage and postoperative ligation of the right common carotid artery), neurological function was assessed at day 14, using a previously established neurological scoring system.[22] In brief, it was derived by evaluating four different functions (general status, simple motor deficit, complex motor deficit, and sensory deficit). The score given to each animal was the sum of all four individual scores: 0 was the best and 48 the worst score possible.[22]

**TTC staining**

On postoperative day 14, the animals were sacrificed by inhalation of 5% isoflurane followed by decapitation. The brains were serially sliced into 2 mm coronal sections with the use of a brain matrix. Brain sections were immediately incubated in TTC (2,3,5-Triphenyl-tetra-zolium-chlorid) at 37°C for 20 minutes and subsequently stored in 10% phosphate-buffered formalin to detect any focal cerebral injury.

## Results

Full cardioplegic arrest over 30 min was successfully achieved in all animals. In two out of the ten animals, the initial arrest after the induction bolus of cardioplegia was incomplete as seen on the ECG. However, with a subsequent dose of 0.2 mL of cardioplegia, full arrest was achieved. None of the animals received more than 0.9 mL of cardioplegia in total. Following 30 min of cardioplegic arrest, when the endoaortic balloon was deflated, spontaneous cardiac rhythm with frequencies identical as pre-CPB resumed within seconds. Separation from CPB without the use of inotropic agents was achieved in all animals.

Table 1 displays the physiologic parameters of all animals. Because of the nature of CPB, rats demonstrated lower MAP, higher $PaO_2$ values, and lower hemoglobin concentrations during CPB as compared to baseline values. Retransfusion of 2 ml of concentrated blood from the CPB circuitry allowed the hemoglobin level to rise again. All animals survived the postoperative period and underwent neurological testing at day 14. Out of the 48 point maximum (worst) score, the animals scored 2.5 ± 1.4 points on average, consistent with no neurological deficits. Histological assessment of the brain showed no infarcted areas in any of the animals.

*Table 1. Physiologic parameters*

| | | CPB | | | | |
|---|---|---|---|---|---|---|
| | **Baseline** | **15 CPB** | **15 arrest** | **30 arrest** | **75 CPB** | **1 h post** |
| **Weight (g)** | 419 (7.7) | | | | | |
| **Hematocrit (%)** | 41 (1) | 27 (2) | 28 (2.7) | 29 (2.1) | 29 (3) | 33 (2) |
| **Glucose (mg/dl)** | 100 (14) | | | | 136 (12) | |
| **Temperature (˚C)** | 35.6 (0.6) | 34.8 (0.6) | 34.4 (0.5) | 34.2 (0.4) | 35.6 (0.7) | 36.9 (0.5) |
| **CPB flow (ml/min)** | | 51 (5) | 65 (9) | 62 (11) | | |
| **MAP (mmHg)** | 62 (7) | 61 (5) | 48 (5) | 46 (5) | 68 (15) | 75 (8) |
| **Arterial pH** | 7.42 (0.02) | 7.42 (0.06) | 7.46 (0.06) | 7.43 (0.08) | 7.38 (0.08) | 7.38 (0.06) |
| **$PaCo_2$ (mmHg)** | 40 (2) | 35 (5) | 34 (5) | 36 (5) | 42 (7) | 41 (5) |
| **$Pao_2$ (mmHg)** | 208 (33) | 366 (52) | 348 (69) | 325 (107) | 308 (68) | 285 (63) |
| **$HCO_3$ (mmHg)** | 26.1 (1.6) | 22.5 (1.4) | 23.9 (2.1) | 24.2 (1.8) | 25.6 (2.0) | 24.4 (3.0) |

*CPB= cardiopulmonary bypass*
*MAP= mean arterial pressure*
*PaCo2 = Partial pressure of carbon dioxide*
*Pao2 = Partial pressure of oxygen*
*HCo3 = Standard bicarbonate*

## Discussion

Research focusing on cardioprotective strategies during cardiac surgery has been hindered by the lack of a suitable small animal model that would allow for complete cardioplegic arrest with good survivability. Most previous research was performed in isolated heart models.[10, 14, 15] While these models allow investigating the immediate effects of therapeutic interventions or different cardioplegia solutions, they preclude the assessment of long-term histological, biochemical, or functional outcomes. Survival studies using dogs[13, 23, 24] or pigs[25] have been performed but are limited due to sample size and costs. To our knowledge, no small animal cardioplegic arrest model has been described so far. Although a number of rat CPB models have been described over the years[26-30], all of





them were resembling beating heart CPB, and none of them included any form of aortic crossclamping and anterograde cardioplegia administration. Therefore, this is a novel *in vivo* survival CPB model that allows minimal invasive administration of anterograde cardioplegia with endoaortic crossclamping with resulting cardioplegic arrest in rats.

Due to the excellent survivability and ease of postoperative cardiac recovery, this model lends itself to the investigation of genomic and proteomic changes as well as histological alterations that can be assessed at any time point and new therapeutic interventions aiming to optimize cardioprotection. Over the last several years, substantial preclinical advances have been made in gene- or cell-based therapies for myocardial protection and in rescue strategies for myocardial ischemia-reperfusion injury, all aimed to employ different types of genes, vectors, and delivery routes.[31, 32] Among these, cardiac gene delivery methods with the use of CPB, in which the adenoviral vector is administered following cardioplegic arrest, allow prolonged myocardial exposure time to the adenoviral vector and improved gene transfer.[16, 17] We speculate that the model described here will therefore facilitate direct intracoronary administration of medications, gene vectors, or cells and might even allow for ultrasound-mediated gene transfer. Because exposure time and coronary flow are major determinants of efficient intracoronary delivery, complete cardioplegic arrest with negligible coronary flow and long wash-in periods will likely optimize delivery and limit extracardiac expression.[16, 17]

In the search for a suitable model to study novel cardioprotective strategies, we adapted a rodent CPB model that we had previously described and utilized.[33, 34] In the original description by Grocott et al.[29], the smallest available human oxygenator was used, which was largely oversized for rodents. The model was later adapted, and a small, appropriately sized rat oxygenator was developed and inserted, thereby abolishing the need of donor blood to prime the circuit and allowing effects of appropriately sized CPB to be determined.[18, 35] Careful positioning of the venous outflow catheter allowed for optimal venous drainage, and CPB flows consistent with a normal cardiac output in rats could be achieved. The natural cardiac rhythm, however, was unaltered and pilot studies indicated that when venous drainage was not optimal, the heart continued to variably eject. Therefore, we adapted this model by inserting the smallest commercially available angioplasty balloon catheter with a central lumen retrogradely into the common carotid artery with the tip carefully positioned just proximal to the aortic valve. Inflation of the balloon resulted in effective aortic crossclamping, and concomitant administration of cardioplegia solutions through the lumen allowed for immediate cardioplegic arrest confirmed by ultrasound and ECG.

The right common carotid artery provided direct access to the ascending aorta, combining the correct vessel size with a relatively short distance to the aortic valve. A full sternotomy and direct cannulation of the heart is very invasive and would likely

cause increased mortality. At the end of the experiment when the catheter is removed, the right common carotid artery is permanently ligated. Although it has been described that ligation of one carotid artery in young healthy rats is without consequences[36], the exclusion of gross neurological or histological abnormalities appeared to be justified before utilizing or developing this model further. The absence of neurological deficits was screened with a 48-point neurologic scoring system[22], and the absence of any signs of cerebral infarction following TTC staining of the brains 14 days after surgery supported this prior work. Because the scope of the current work was purely to develop the experimental technique and demonstrate the technical feasibility of achieving 30 min of full cardioplegic arrest in rats with good survivability, we did not quantify cardiac function postoperatively. However, the model described here permits comprehensive perioperative echocardiographic evaluation of cardiac function in future studies. Another major advantage of this model is its minimal invasiveness (closed chest), and that it can be performed by one operator only, thus facilitating higher experimental throughput at lower costs. As an indication, the disposable membrane for the oxygenator costs $65, the Plexiglas parts of the CPB system (venous reservoir, oxygenator shell) can be re-used after vigorous cleaning and sterilizing while the CPB tubing was discarded after each bypass run. After extensive training, a perioperative mortality less than 10-15% can be achieved.

The model has several potential pitfalls. The endoaortic catheter requires exact and precise positioning close to the aortic valve. Ultrasonographic imaging is essential for positioning of the angioplasty catheter as well as assessing the cardioplegic arrest. When the catheter is not positioned optimally, incomplete arrest will be the result, and the balloon might even damage the brachiocephalic trunk (too high) or the left ventricle (too deep). If the injection of cardioplegia is executed too forcefully, dilatation of the left ventricle will occur. Measuring the pressure at the tip of the balloon catheter combined with the use of an infusion pump solves this issue. Use of the manufacturer's inflation device prevents overinflation of the endoaortic balloon. After failed cardioplegic arrest on the first attempt, repeated small doses of cardioplegia will result in complete arrest. Although we did not measure potassium in these animals, earlier pilot experiments revealed that hyperkalemia does not occur with the dosing regimen used in this protocol. Immediate return to regular sinus rhythm at preoperative rates following deflation of the endo-aortic balloon and restoration of preoperative hemodynamics present further indirect evidence that the cardioplegic regimen did not result in hyperkalemia.

As this work was entirely focused on developing the minimal invasive technique to accomplish CPB with cardioplegic arrest, we have not yet systematically investigated possible definite outcomes. However, study endpoints such as technical feasibility, effective cardioplegia and survival were successfully demonstrated. The model described will not only facilitate further research to elucidate mechanisms of myocardial reperfusion injury following cardioplegic arrest and CPB but also to evaluate novel approaches to improved myocardial protection. Due to its minimal invasiveness and ease of recoverability, short- and long-term effects of constituents of cardioplegia, duration of cardioplegic arrest, CPB time, and potentially also direct gene transfer on myocardial function and histological outcomes can be assessed better than in isolated heart models. In addition, the model allows for the investigation of unique animal strains with varying susceptibility to myocardial injury depending on either their genetic background (consomic) or preexisting disease (e.g., diabetes, old age, hypertension).

## Conclusion

This novel small animal CPB model with cardioplegic arrest allows for both the study of myocardial ischemia-reperfusion injury as well as the evaluation of new cardioprotective strategies. Major advantages of this model include its overall feasibility and cost effectiveness. In the field of myocardial protection, rodent models will remain an important avenue of research. Models such as the one described here will likely be utilized to not only assess longer-term functional outcomes but also characterize the enzymatic, genetic, and histologic response to of myocardial injury and protective strategies.

## Reference List


1. Steuer J, Granath F, de Faire U, Ekbom A, Stahle E. Increased risk of heart failure as a consequence of perioperative myocardial injury after coronary artery bypass grafting. Heart 2005 Jun;91(6):754-8.
2. Steuer J, Horte LG, Lindahl B, Stahle E. Impact of perioperative myocardial injury on early and long-term outcome after coronary artery bypass grafting. Eur Heart J 2002 Aug;23(15):1219-27.
3. Mangano DT. Perioperative cardiac morbidity. Anesthesiology 1990 Jan;72(1):153-84.
4. Shernan SK. Perioperative myocardial ischemia reperfusion injury. Anesthesiol Clin North America 2003 Sep;21(3):465-85.
5. Kloner RA, Jennings RB. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 1. Circulation 2001 Dec 11;104(24):2981-9.
6. Kloner RA, Jennings RB. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 2. Circulation 2001 Dec 18;104(25):3158-67.
7. Park JL, Lucchesi BR. Mechanisms of myocardial reperfusion injury. Ann Thorac Surg 1999 Nov;68(5):1905-12.
8. Alexander KP, Anstrom KJ, Muhlbaier LH, Grosswald RD, Smith PK, Jones RH, et al. Outcomes of cardiac surgery in patients > or = 80 years: results from the National Cardiovascular Network. J Am Coll Cardiol 2000 Mar 1;35(3):731-8.
9. Pretre R, Turina MI. Cardiac valve surgery in the octogenarian. Heart 2000 Jan;83(1):116-21.
10. Bopassa JC, Vandroux D, Ovize M, Ferrera R. Controlled reperfusion after hypothermic heart preservation inhibits mitochondrial permeability transition-pore opening and enhances functional recovery. Am J Physiol Heart Circ Physiol 2006 Nov;291(5):H2265-71.
11. Khan TA, Bianchi C, Araujo E, Voisine P, Xu SH, Feng J, et al. Aprotinin preserves cellular junctions and reduces myocardial edema after regional ischemia and cardioplegic arrest. Circulation 2005 Aug 30;112(9 Suppl):I196-201.
12. Knott EM, Sun J, Lei Y, Ryou MG, Olivencia-Yurvati AH, Mallet RT. Pyruvate mitigates oxidative stress during reperfusion of cardioplegia-arrested myocardium. Ann Thorac Surg 2006 Mar;81(3):928-34.
13. Corvera JS, Kin H, Dobson GP, Kerendi F, Halkos ME, Katzmark S, et al. Polarized arrest with warm or cold adenosine/lidocaine blood cardioplegia is equivalent to hypothermic potassium blood cardioplegia. J Thorac Cardiovasc Surg 2005 Mar;129(3):599-606.
14. Dobson GP, Jones MW. Adenosine and lidocaine: a new concept in nondepolarizing surgical myocardial arrest, protection, and preservation. J Thorac Cardiovasc Surg 2004 Mar;127(3):794-805.
15. Torracca L, Pasini E, Curello S, Ceconi C, Coletti G, Alfieri O, et al. Continuous versus intermittent warm blood cardioplegia: functional and energetics changes. Ann Thorac Surg 1996 Oct;62(4):1172-8; discussion 8-9.
16. Davidson MJ, Jones JM, Emani SM, Wilson KH, Jaggers J, Koch WJ, et al. Cardiac gene delivery with cardiopulmonary bypass. Circulation 2001 Jul 10;104(2):131-3.
17. Jones JM, Wilson KH, Koch WJ, Milano CA. Adenoviral gene transfer to the heart during cardiopulmonary bypass: effect of myocardial protection technique on transgene expression. Eur J Cardiothorac Surg 2002 May;21(5):847-52.
18. de Lange F, Dieleman JM, Jungwirth B, Kalkman CJ. Effects of cardiopulmonary bypass on neurocognitive performance and cytokine release in old and diabetic rats. Br J Anaesth 2007 Aug;99(2):177-83.
19. Guide for the care and use of laboratory animals, Commission on Life Sciences, National Research Council, National Institutes of Health, Revised 1996. Washington: National Academy Press; 2005.
20. Stevens JH, Burdon TA, Peters WS, Siegel LC, Pompili MF, Vierra MA, et al. Port-access coronary artery bypass grafting: a proposed surgical method. J Thorac Cardiovasc Surg 1996 Mar;111(3):567-73.
21. Jungwirth B, Mackensen GB, Blobner M, Neff F, Reichart B, Kochs EF, et al. Neurologic outcome after cardiopulmonary bypass with deep hypothermic circulatory arrest in rats: description of a new model. J Thorac Cardiovasc Surg 2006 Apr;131(4):805-12.
22. Yokoo N, Sheng H, Mixco J, Homi HM, Pearlstein RD, Warner DS. Intraischemic nitrous oxide alters neither neurologic nor histologic outcome: a comparison with dizocilpine. Anesth Analg 2004 Sep;99(3):896-903
23. Murphy CO, Pan C, Gott JP, Guyton RA. Microvascular reactivity after crystalloid, cold blood, and warm blood cardioplegic arrest. Ann Thorac Surg 1995 Oct;60(4):1021-7.
24. Schmidt FE, Jr., MacDonald MJ, Murphy CO, Brown WM, 3rd, Gott JP, Guyton RA. Leukocyte depletion of blood cardioplegia attenuates reperfusion injury. Ann Thorac Surg 1996 Dec;62(6):1691-6; discussion 6-7.
25. Fischer UM, Klass O, Stock U, Easo J, Geissler HJ, Fischer JH, et al. Cardioplegic arrest induces apoptosis signal-pathway in myocardial endothelial cells and cardiac myocytes. Eur J Cardiothorac Surg 2003 Jun;23(6):984-90.
26. Dong GH, Xu B, Wang CT, Qian JJ, Liu H, Huang G, et al. A rat model of cardiopulmonary bypass with excellent survival. J Surg Res 2005 Feb;123(2):171-5.
27. Fabre O, Zegdi R, Vincentelli A, Cambillaud M, Prat A, Carpentier A, et al. A recovery model of partial cardiopulmonary bypass in the rat. Perfusion 2001 May;16(3):215-20.
28. Gourlay T, Ballaux PK, Draper ER, Taylor KM. Early experience with a new technique and technology designed for the study of pulsatile cardiopulmonary bypass in the rat. Perfusion 2002 May;17(3):191-8.
29. Grocott HP, Mackensen GB, Newman MF, Warner DS: Neurological injury during cardiopulmonary bypass in the rat. Perfusion 2001; 16: 75-81
30. Hayashi Y, Sawa Y, Nishimura M, Satoh H, Ohtake S, Matsuda H. Avoidance of full-sternotomy:

effect on inflammatory cytokine production during cardiopulmonary bypass in rats. J Card Surg 2003 Sep-Oct;18(5):390-5.

31. Melo LG, Pachori AS, Kong D, Gnecchi M, Wang K, Pratt RE, et al. Molecular and cell-based therapies for protection, rescue, and repair of ischemic myocardium: reasons for cautious optimism. Circulation 2004 May 25;109(20):2386-93.
32. Suzuki K, Murtuza B, Fukushima S, Smolenski RT, Varela-Carver A, Coppen SR, et al. Targeted cell delivery into infarcted rat hearts by retrograde intracoronary infusion: distribution, dynamics, and influence on cardiac function. Circulation 2004 Sep 14;110(11 Suppl 1):II225-30.
33. Dieleman JM, de Lange F, Houston RJ, Biessels GJ, Bar PR, Mackensen GB, et al. Cardiopulmonary bypass and long-term neurocognitive dysfunction in the rat. Life Sci 2006 Jul 4;79(6):551-8.
34. Mackensen GB, Sato Y, Nellgard B, Pineda J, Newman MF, Warner DS, et al. Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001 Dec;95(6):1485-91.
35. Welsby IJ, Jones WL, Arepally G, De Lange F, Yoshitani K, Phillips-Bute B, et al. Effect of combined anticoagulation using heparin and bivalirudin on the hemostatic and inflammatory responses to cardiopulmonary bypass in the rat. Anesthesiology 2007 Feb;106(2):295-301.
36. Omae T, Mayzel-Oreg O, Li F, Sotak CH, Fisher M. Inapparent hemodynamic insufficiency exacerbates ischemic damage in a rat microembolic stroke model. Stroke 2000 Oct;31(10):2494-9.

chapter 7

# Factor IXa aptamer and matched antidote replace heparin and protamine and improve cardiac function after cardiopulmonary bypass in the rat

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## Abstract

**Background**

Heparin is the anticoagulant of choice for use in cardiac surgery employing cardiopulmonary bypass (CPB) because of its unique combination of pharmacologic properties including rapid onset of action, ease of monitoring and reversal with protamine. However, the clinical benefits of heparin and protamine are offset by a number of serious side-effects such as heparin-induced thrombocytopenia or allergic reactions, all leading to increased morbidity and mortality.

Searching for alternative anticoagulants to use during CPB, this study compares a novel single stranded RNA molecule (aptamer) that inhibits factor IXa and its specific antidote with heparin/protamine in rats subjected to CPB.

**Methods**

Using a model of normothermic CPB in rats, markers of anticoagulation (aPTT and ACT), inflammatory markers (TNF-$\alpha$, IL-1$\beta$, IL-6 and IL-10) and platelet counts were determined at different time points in two groups: heparin/protamine or factor IXa aptamer/antidote (n=12 per group). At 3 hrs post-CPB, myocardial shortening fraction was determined using ultrasound. Thereafter, the hearts were formalin fixed and analyzed histologically.

**Results**

Factor IXa aptamer replaced heparin as the sole anticoagulant during CPB and all animals had patent CPB circuits. Both antidotes showed immediate, long lasting reversal. The aptamer-antidote group showed similar inflammatory profiles but less decrease in platelet count and an improved cardiac performance 3hrs postoperative.

**Conclusions**

Aptamer to FIXa and its matched antidote provide excellent anticoagulation and reversal with improved cardiac performance when compared to heparin/protamine. This novel anticoagulant pair may lead to equivalent CPB conditions with an advantageous side effects profile as compared to heparin and protamine.






## Introduction

Although low-molecular weight heparin is gradually replacing heparin for the treatment of most patients with venous thromboembolism and acute coronary syndromes, unfractionated heparin remains the anticoagulant of choice in most cardiology and cardiac surgery settings. It is only recently that clinicians are reconsidering their "historical" first choice and novel anticoagulants targeting specific steps in the coagulation cascade are at various stages of development. Considerations for this change of view are the significant side-effects of heparin and its antidote protamine. Heparin indirectly inhibits thrombin but has no effect on fibrin-bound thrombin. Despite high-dose heparin anticoagulation, considerable thrombin-mediated hemostatic activation still occurs during cardiopulmonary bypass (CPB).[1] This hemostatic activation results in clinically relevant consumption of natural circulating anticoagulants and has been independently associated with thrombotic complications after cardiac surgery.[2] In addition, heparin is associated with heparin-induced thrombocytopenia (HIT), a life-threatening complication of heparin therapy affecting up to 3% of all cardiac surgery patients.[3,4] HIT is caused by antibodies directed to complexes containing heparin, and an endogenous platelet protein, platelet factor 4 (PF4). The diagnostic hallmark of HIT is the development of thrombocytopenia, which is mild (usually 50-100,000 $10^{-9}$/L) and rarely causes bleeding. Thrombocytopenia in HIT, however, serves as clinical harbinger of thrombotic complications that can develop in approximately 20-50% of patients.[3] Mortality from thrombotic complications in unrecognized HIT is exceptionally high (6-27%).[3,5] Even without causing thrombocytopenia, a positive PF4 antibody status is an independent predictor for adverse outcome in cardiac surgery patients.[6,7] Outside the field of cardiac surgery, the presence of these anti-heparin-PF4 antibodies has also been associated with increased death or vascular events.[8,9]

To date, unfractionated heparin is the only antidote-reversible anticoagulant available. However, protamine sulfate, a positively-charged molecule which reverses heparin's activity has its own limitations. Besides having endogenous anticoagulant properties at high doses, it can induce severe hypersensitivity reactions and it has been associated with increased mortality after cardiac surgery.[10]

We recently reported on a new class of drugs, aptamers, as reversible antagonists of coagulation factor IXa (FIXa).[11-13] Aptamers are single stranded RNA oligonucleotides that fold into a specific structure that enables them to directly bind to and inhibit a protein target.[14] After administration of the antidote, also an RNA molecule, the action of the aptamer is reversed. Advantageous properties of this class of drugs include high affinity and specificity for their targets with low immunogenicity and modifiable bioavailability. It now is possible to rationally design aptamer-antidote pairs to desired protein targets.[14,15]

In this study we compared the effects of FIXa aptamer-antidote pair on clinically relevant clotting parameters, platelet count, cytokine release, short-term histopathological impact, physiologic parameters and cardiac performance with heparin-protamine in an established rodent CPB model.

## Methods

The study was approved by the Duke University Animal Care and Use Committee and all procedures met the National Institutes of Health (NIH) guidelines for animal care.[16]

**Animals and groups**

Twenty-four male 425-450 g Sprague-Dawley rats (Charles River Labs, Wilmington, MA, USA) were used for this study. They were housed three per cage under a 12 hour light-dark cycle conditions with food and water available *ad libitum*. Rats were randomly assigned to one of two treatment groups (n=12 per group); the first group received heparin 600 IU/kg before CPB and protamine 1.25 mg/100IU of porcine heparin at the end of CPB and the other group received 10 mg/kg of FIXa aptamer before the start of CPB and 50 mg/kg antidote oligonucleotide at the cessation of CPB. Both drugs were diluted in saline to a volume of 0.3 mL. The experimenters performing the surgery, CPB, echocardiography, organ harvest and histopathology were blinded to treatment allocation.

**Anesthesia, surgical preparation and cardiopulmonary bypass**

After a 12 hour fast, anesthesia was induced with 5% isoflurane in 50% $O_2$ in a plastic induction box. After orotracheal intubation with a 14G catheter (Insyte BD Medical, Sandy, UT), the animals were mechanically ventilated to a maximum airway pressure of 18 mm$H_2O$ (Harvard Model 687, Harvard Apparatus, Holliston, MA) and a normal arterial carbon dioxide tension was maintained. During the surgical preparation, anesthesia was maintained with 2-2.5% isoflurane. A needle thermistor was inserted in the temporal muscle adjacent to the skull to measure pericranial temperature. With both forced-air and surface heating systems temperature was maintained at a target temperature of 36.5-37.0 $^{\circ}$C

The model used in this experiment was based on a model developed by Mackensen et al.[17] Briefly, arterial blood pressure was monitored via the superficial caudal epigastric artery, which was canulated with polyethylene tubing (PE-10 Intramedic Tubing, Becton-Dickinson, Sparks, MD). The superficial epigastric vein was cannulated with a 24 G catheter (Insyte BD Medical, Sandy, UT) and used solely for administration of drug and antidote. Further preparation consisted of cannulation of the tail artery with a 20 G catheter (Insyte BD Medical, Sandy, UT), which later served as arterial inflow for the CPB circuit. The full dose of porcine heparin (600 IU/kg) or aptamer 10 mg/kg and 5 μg of fentanyl was given. A modified multi-orifice 4.5 French pediatric catheter (modified Desilets-Hoffman Catheter, Cook, Bloomington, IN) was advanced through the right external jugular vein into the right heart for venous outflow.

The CPB circuit consisted of a specifically designed 8 mL Plexiglas® venous reservoir, a roller pump (Masterflex®; Cole-Parmer Instrument Co., Vernon Hills, IL, USA) and a custom-designed small-volume oxygenator.[18] The 4 mL priming volume oxygenator was built of two Plexiglas® shells (12.8 cm x 12.8 cm x 2.7 cm) that carry a sterile, disposable three layer artificial diffusion membrane, made with hollow polypropylene fibers (Jostra AG, Hirrlingen, Germany) glued together in a crosswise fashion. The surface area available for gas exchange was 558 $cm^2$. To prevent excessive heat loss, one of the shells had an integrated heat exchanger. An in-line flow probe (2N806 probe and T208 flowmeter, Transonics Systems Inc., Ithaca, NY) was used to continuously measure CPB flow. The whole circuit was primed with 10 mL of 6% hetastarch (Hextend, Hospira Inc, Lake Forest, IL) and 0.2 mg of pancuronium. The dilutional effect of the prime caused Hct in the 0.22-0.24 ranges during CPB.

All parts were connected through silicone tubing, which was disposed after every bypass run. During CPB, ventilation was discontinued and isoflurane 0.2-1% in 100% $O_2$ was administered through the oxygenator. CPB with a flow rate of 150 $mL.kg^{-1}min^{-1}$, adjusted to maximize flow and to maintain a minimal venous reservoir blood level, was carried out for 60 minutes. Mean arterial pressure was kept between 50-60 mmHg. At 30 minutes of CPB, a repeated dose of 0.2 mg of pancuronium and 5μg of fentanyl was added to the circuit. After 60 min of CPB, ventilation was reinitiated and CPB was discontinued and the jugular venous canula removed. Immediately thereafter, protamine or antidote was administered. The animals remained ventilated for another 3 hours. After the last blood sample was obtained at the 3 hour time point, the heart was harvested from each study animal and preserved in 4% formaldehyde.

**Activated clotting time (act) and activated partial thromboplastin time (aptt)**

Coagulation assays were performed at the following six time points in three different phases of the experiment: i) Pre-CPB: at the beginning of the experiment and just after the start of CPB, ii) During CPB: at the 30 and 60 minute timepoints, and iii) Post-CPB: at the 20 and 60 minute time points after drug reversal. A Hemochron Jr. Signature Whole Blood Microcoagulation system (International Technidyne Corporation, Edison, New Jersey) was used for aPTT/ACT measurements. The results were represented as means of duplicated measurement. After 500s, the aPTT reached its out of range cutoff point.

**Blood gas analysis, interleukins and thrombocytes**

Blood gas analysis was performed before the start of CPB, at 30 minutes and 60 minutes of CPB, and at 1, 2 and 3 hour post-CPB, using a IL GEM Premier 3000 blood gas analyzer (Global Medical Instrumentation, Ramsey, MI, USA). Blood samples for TNF-$\alpha$ , IL-1$\beta$, IL-6 and IL-10 analysis were collected from the tail artery before the start of the experiment, during CPB at intervals of 30 minutes and post-CPB at 1, 2 and 3 hours. Samples were immediately centrifuged at 4°C and the serum frozen and stored at -80°C for analysis. Cytokine levels were analyzed by multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) microtitre plate, following manufacturer's instructions (Endogen® - Search Light™, Inc., Woburn, MA). Results were expressed as pg/ml and sensitivity for detection was 6.2 pg/ml for TNF-$\alpha$, 12.6 pg/ml for IL-1$\beta$, 12.6 pg/ml for IL-6, and 3.1 pg/ml for IL-10.

**Thrombocyte count**

Blood samples for thrombocyte counts were collected before start of CPB and 3 hours post CPB. Counts were performed using the Cell-Dyn 3700 (Abbott Diagnostics, Abbott Park, Il).

**Echocardiography**

Three hours after the cessation of CPB an echographic estimate of the left ventricular ejection fraction was obtained (Sonosite 180 with 10-5 MHz broadband linear array transducer, Bothell, WA) under 1.5% isoflurane anesthesia in all rats. A transthoracic cross-sectional view of the left ventricle was obtained at the midpapillary level. The end-diastolic diameter (EDD) and end-systolic diameter (ESD) were measured and the shortening fraction calculated (EDD-ESD/EDD*100). All measurements were executed using three different loops and averaged. All loops were reviewed off-line by a single, independent, board-certified (National Board of Echocardiography, Raleigh, NC) investigator (GBM) who was blinded to treatment designation.

**Pathology analysis**

Following echocardiographic assessment of ventricular function, the animals were decapitated under deep general anesthesia (isoflurane 5%) and cross-sections of the heart were obtained by sectioning through the right and left ventricles approximately midway from the apex to the base of the heart. After fixation in 4% phosphate-buffered formaldehyde, myocardial tissue samples were processed as follows. Samples were dehydrated with serial alcohols, embedded in paraffin, and 5$\mu$m paraffin sections were stained with hematoxylin and eosin. Samples were coded such that the pathologist performing the analysis (ADP) was blinded to treatment allocation.

Myocardial contraction band necrosis (MCBN), a marker of cell death[19] was quantified





in the subendocardial and myocardial layers of the right ventricle (RV), interventricular septum (IVS) and left ventricle (LV). The long and short axes of areas that exhibited hypereosinophilia and contraction bands were measured using a calibrated ocular micrometer and an estimated cross-sectional area calculated by multiplying the two diameters.

**Statistical analysis**

Physiological parameters, echo data, and APTT and ACT results were analyzed using the Mann-Whitney U test. Interleukin levels and thrombocyte counts were analyzed using repeated measure analysis of variance (ANOVA). P-values < 0.05 were considered significant.

For the analysis of the histology data, the Wilcoxon Rank-Sum Procedure was applied to compare lesion size within cardiac region, and across cardiac region, by treatment. In comparing the number of lesions within cardiac region by treatment, the Pearson Chi-Squared procedure was applied. In comparing the number of lesions across cardiac region, by treatment, the Cochran-Mantel-Haenszel (CMH) statistics were calculated. This procedure controls for the association between the lesion types for a given rat.

## Results

**Animals and circuit patency**

Out of each group, one animal died due to surgical difficulties during the procedure. All remaining animals (n=11 per group) completed the experiment.

In both, the heparin-treated and aptamer-treated animals, the CPB circuit remained patent throughout the entire CPB period. Gross examination of the reservoir, tubing and oxygenator revealed no evidence of thrombi and no clots were observed in either the arterial or venous cannulae.

**Physiologic parameters**

The physiological parameters measured during the course of the experiments included weight, hematocrit, pericranial temperature, cardiopulmonary bypass (CPB) flow, mean arterial pressure (MAP), pH, arterial partial pressures of carbon dioxide ($PaCO_2$), arterial partial pressure of oxygen ($PaO_2$) and bicarbonate ion. These results and their standard deviations are shown in Table 1.

There was a statistical difference between the two groups in CPB flow at 30 min CPB (heparin group 54 $mLmin^{-1}$ *vs*. aptamer group 58 $mLmin^{-1}$ , $p$= 0.037) and in MAP at 3 hours postoperative ( heparin group 51 mmHg *vs* aptamer group 59mmHg, $p$= 0.021).

*Table 1. Physiological parameters in the two groups at different timepoints. Means ± SD.*

| | | CPB | | | | |
|---|---|---|---|---|---|---|
| | **baseline** | **30 min** | **60 min** | **1 hr post** | **2 hr post** | **3 hr post** |
| **Weight (g)** | | | | | | |
| Heparin | 446 (17) | | | | | |
| Aptamer | 444 (22) | | | | | |
| **Hematocrit (%)** | | | | | | |
| Heparin | 36.3 (2.2) | 24.3 (1.8) | 24.2 (1.7) | 22.7 (1.4) | 21.0 (1.7) | 20.3 (1.4) |
| Aptamer | 36.4 (1.4) | 24.2 (1.4) | 23.9 (1.3) | 22.8 (1.5) | 21.3 (1.6) | 19.6 (2.0) |
| **Pericranial Temperature (˚C)** | | | | | | |
| Heparin | 36.3 (0.5) | 35.3 (0.3) | 35.4 (0.3) | 36.2 (0.5) | 36.1 (0.9) | 36.3 (0.5) |
| Aptamer | 36.4 (0.3) | 35.2 (0.2) | 35.2 (0.2) | 36.2 (0.7) | 36.6 (0.2) | 36.5 (0.3) |
| **CPB flow (ml/min)** | | | | | | |
| Heparin | | 54 (3) | 54 (5) | | | |
| Aptamer | | 58 (6)* | 58 (4) | | | |
| **MAP (mmHg)** | | | | | | |
| Heparin | 59 (7) | 56 (12) | 68 (15) | 74 (10) | 62 (14) | 51 (5) |
| Aptamer | 56 (5) | 52 (14) | 67 (9) | 64 (15) | 65 (10) | 59 (8)* |
| **pH** | | | | | | |
| Heparin | 7.49 (0.06) | 7.45 (0.04) | 7.45 (0.04) | 7.52 (0.05) | 7.49 (0.05) | 7.52 (0.07) |
| Aptamer | 7.48 (0.03) | 7.44 (0.06) | 7.44 (0.03) | 7.45 (0.12) | 7.50 (0.03) | 7.49 (0.09) |
| **$PaCo_2$ (mmHg)** | | | | | | |
| Heparin | 39 (4) | 42 (4) | 42 (4) | 37 (6) | 36 (3) | 29 (6) |
| Aptamer | 39 (3) | 43 (7) | 44 (4) | 39 (4) | 36 (3) | 31 (6) |
| **$Pao_2$ (mmHg)** | | | | | | |
| Heparin | 128 (32) | 496 (77) | 507 (90) | 356 (80) | 368 (101) | 388 (105) |
| Aptamer | 148 (40) | 522 (53) | 534 (60) | 363 (96) | 390 (117) | 405 (139) |
| **$HCO_3^-$ (mmHg)** | | | | | | |
| Heparin | 29.3 (1.2) | 28.6 (1.4) | 29.3 (2.0) | 30.2 (2.3) | 27.5 (3.4) | 24.5 (3.8) |
| Aptamer | 28.7 (1.2) | 29.1 (1.1) | 29.8 (1.1) | 28.8 (2.1) | 27.7 (3.3) | 24.6 (3.9) |

**= p< 0.05 compared to heparin group*

*CPB=cardiopulmonary bypass; post= post CPB, after cessation of CPB*

**Clotting parameters**

The activated clotting time (ACT) and activated partial thromboplastin time (aPTT) are represented in Figure 1A.In both groups the ACT was normal before anticoagulation therapy (99 ± 8 s in the heparin group and 96 ± 9 s in the aptamer group). After anticoagulation, a significant rise in ACT was observed in both groups. In the heparin group, at the start of CPB the ACT was 389 ± 32 s, in the aptamer group 335 ± 48 s, (p<0.05). Neither group received a subsequent dose of anticoagulant during the course of the experiment. At 30 and 60 minutes CPB, the ACT remained stable in the heparin and aptamer-treated animals (349 ± 55 s *vs* 292 ± 39 s, p= 0.03 and 320 ± 54 *vs*. 254 ± 51, *p*= 0.01 respectively). After administration of the antidote, both levels decreased to 130 s, which remained stable to the end of experiment.





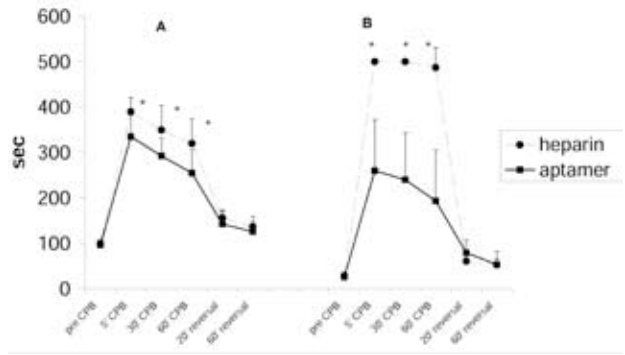



*Figure 1*

*ACT (1A) and APTT (1B) measurements at different time points during CPB and after drug reversal with the antidote. Means ± SD. CPB= cardiopulmonary bypass. * P< 0.05*

The aPTT results are represented in Figure 1B. In both groups the aPTT before anticoagulation measured less than 30 s. After anticoagulation, the aPTT measured 500 s in the heparin group (out of range - high) during most of the CPB period. In the aptamer group, the aPTT rose to 260 ± 112 s at the start of CPB, 240 ± 103 s at 30 CPB and 193 ± 112 s at the end of CPB (*p*< 0.001 compared to heparin group at all time points). After administration of the respective antidote, the levels returned to near-baseline in both, the heparin and aptamer group without statistical difference, 60 ± 13 s and 79 ± 28 s respectively (*p* = 0.2). There was no rebound effect during the remainder of the experiment. The thrombocyte count in the heparin group was 729 ± 93 (before CPB) and 325 ± 85 $x10^9$/L (post CPB) respectively. In the aptamer group 530 ± 124 and 247 ± 121 $x10^9$/L platelets were counted (see Figure 2) Both groups had a lower post CPB thrombocyte counts then pre CPB (p<0.001), but the decrease in the heparin group was more pronounced than in the aptamer group (interaction effect, *p*=0.007).

**Interleukin results**

Ten complete data sets were obtained and analyzed per group. The results are depicted in Figure 3a-d**.** The levels of all four markers were below detection at the start of the experiment in both groups.

The TNF-$\alpha$ levels for both the heparin- and aptamer-treated groups peaked 60 minutes after the end of CPB (Figure 3a). Repeated measure ANOVA reflected no difference between the two groups (*p*=0.18). In both sets of animals, TNF-$\alpha$ levels returned to near pre-CPB levels (*p*=0.97).

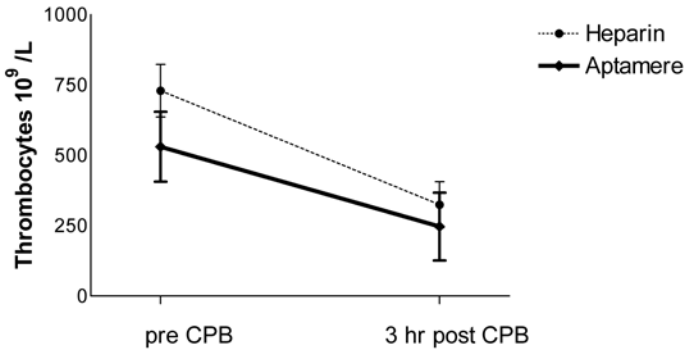
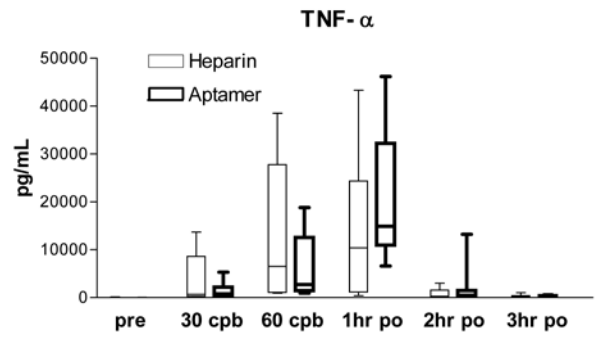


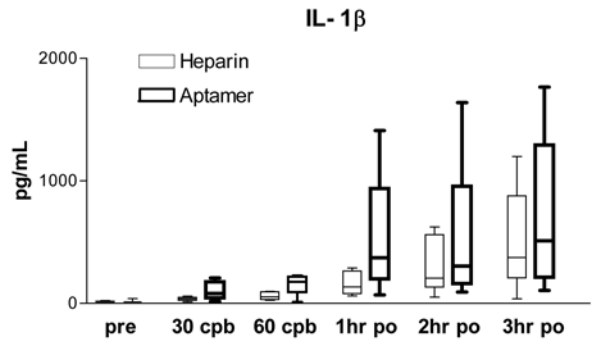

*Figure 2*

*Thrombocyte counts (109/L) at two perioperative time points (pre CPB and 3 hours post CPB). Means ± SD. Repeated measurement ANOVA showed less decrease in the aptamer group when compared to heparin (p=0.007); CPB= cardiopulmonary bypass.*

*3 A*



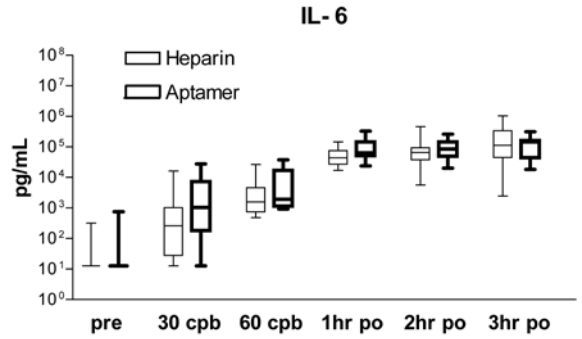

*3 B*








*3 C*




*3 D*

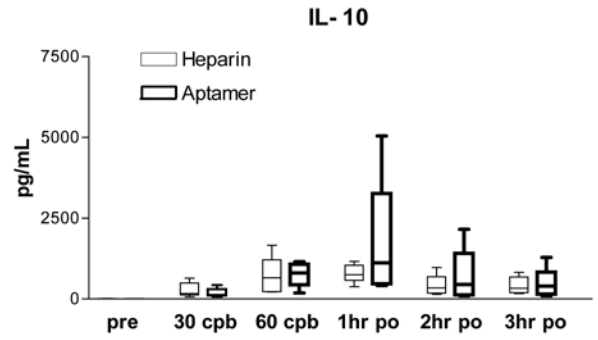



*Figure 3 A-D*

*Boxplots (median and minimum/maximum values) displaying plasma levels of four different cytokines (TNF-α, IL-1β, IL-6 and IL-10) at different time points during the experiment. Time during CPB in minutes and post CPB in hours (hrs). CPB=cardiopulmonary bypass. PO=post CPB. There were no differences between the groups.*

There was no significant difference between the IL-1β levels between the aptamer- and heparin-treated animals over the course of the experiment ($p$=0.34; Figure 3b). In both groups, levels rose significantly 60 minutes after CPB and continued to rise to the last measured time point 3 hours after cessation of CPB.

The IL-6 levels followed a similar pattern to the IL-1β data (Figures 3b, 3c). There was an increase in IL-6 levels post CPB in both groups, however there were no differences between the groups ($p$=0.47). Figure 3d shows the levels for IL-10. Despite a trend towards higher peak levels in the aptamer group (1,782 ± 1,675 pg/ml) compared to the heparin-treated animals (782 ± 253 pg/ml), no significant differences were detected using repeated measures ANOVA .

**Pathology**

All hearts of the studied animals were analyzed for signs of myocardial cell death as reflected in the cross-sectional areas. The number and size of lesions in the right ventricle (RV), interventricular septum (IVS) and left ventricle (LV) are summarized in Table 2. There were no significant differences between groups in number or size of lesions in the RV, IVS or LV.

**Echocardiography**

At the three hour time point, the aptamer-treated group showed a better global cardiac function compared to the heparin-treated animals with a left ventricular shortening fraction of 60 ± 16 % versus 42 ± 8 % ($p$ = 0.01).

*Table 2: Histopathological data: number and size of areas of myocardial contraction band necrosis in the two groups. Means ± SD.*

| | | RV | IVS | LV |
|---|---|---|---|---|
| Number of MCBN | Heparin | 1.3 (0.9) | 0.6 (0.5) | 2.1 (1.0) |
| | Aptamer | 1.2 (1.1) | 0.5 (0.5) | 2.9 (0.9) |
| Area size (mm2) | Heparin | 1.6 (1.6) | 0.6 (0.8) | 3.6 (3.0) |
| | Aptamer | 1.1 (1.4) | 0.4 (0.9) | 5.2 (2.5) |

*MCBN: myocardial contraction band necrosis, RV= right ventricle, IVS= interventricular septum, LV= left ventricle*

## Discussion

In the present study, we subjected rats to 60 min of CPB with two different anticoagulant-antidote strategies. Rats, fully anticoagulated with heparin during CPB and reversed with protamine, were compared with rats anticoagulated with FIXa aptamer and its specific antidote. Our results show that the aptamer-antidote oligonucleotide pair targeting FIXa successfully replaces heparin-protamine in this model. Inhibition of FIXa with the anticoagulant aptamer resulted in a similar inflammatory response to CPB but alleviated the decline in platelet count when compared to heparin anticoagulation. In addition, treatment with factor IXa appeared to improve echocardiographically assessed cardiac performance at three hours postoperatively. We speculate that use of RNA technology





as in the FIXa aptamer and its matched antidote may lead to alternative reversible anticoagulant regimes that ensure equivalent anticoagulation during CPB with decreased long-term side effects as described for heparin and protamine.

Unfractionated heparin has long been established as the anticoagulant of choice for use in cardiac surgery. This is largely due to its rapid onset of action, short half-life, ease of monitoring and reversibility with protamine. The clinical benefits of heparin for CPB, however, are compromised by its immunogenicity. Heparin sensitization and complications of Heparin-Induced Thrombocytopenia (HIT) are increasingly recognized as a serious adverse outcome of cardiac surgery and CPB.[3-5] In addition, platelet factor 4 (PF4)/heparin antibodies form in 30-50% of patients undergoing cardiac surgery and are independent risk factors for postoperative adverse events after cardiac surgery.[4-7]

Heparin's antidote, protamine sulfate also has limitations. It has its own anticoagulant effect and if given in excess doses can result in uncontrollable bleeding.[20] In addition to dosing difficulties and rare severe anaphylaxis associated with its use, protamine sulfate also results in severe unwanted side effects including profound hypotension, bradycardia, complement activation and pulmonary hypertension.[10] The most widely studied alternative drug to heparin is the direct thrombin inhibitor bivalirudin. A recent report described the safe use of bivalirudin during CPB in patients undergoing a wide variety of cardiac surgery procedures.[21] Cardiologists have already noted a superior safety profile for bivalirudin in acute coronary syndromes in comparison with heparin.[22] However, the action of bivalirudin can not be reversed by an antidote and its use requires drastic changes in surgical technique.[23]

In the search for other alternatives to heparin, factor IXa has been identified as a promising target. Factor IXa plays a central role in initiation and amplification of the coagulation cascade.[24] It has already been shown that inactivated factor IXa can maintain a patent CPB circuit [25], implying that specific inhibitions of factor IXa may be clinically useful. Aptamers represent a recently developed class of drugs consisting of a folded single-stranded chain of oligonucleotides (RNA) that can inhibit a specific protein target by binding to it. Aptamers are generated by *in vitro* screening of complex nucleic-acid based combinatorial shape libraries (>$10^{14}$ shapes per library) employing a process termed SELEX (Systematic Evolution of Ligands by EXponential Enrichment).[26] Recently, a specific aptamer against factor IXa and its complementary oligonucleotide antidote that reverses its activity by binding to it, was developed.[11-15] Our results employing this specific factor IXa aptamer-antidote pair corroborate the earlier findings by Spanier et al.[25] Anticoagulation with the factor IXa aptamer maintained the patency of the rodent CPB circuit for the entire duration of CPB. As expected, both the changes in ACT and aPTT levels in the aptamer-treated group were less pronounced than those in the heparin-treated group. This by no means implies insufficient anticoagulation. We decided to use the clinically used ACT and

aPTT as measures of anticoagulation, though when a factor IXa specific anticoagulant is used, different levels in ACT and aPTT imply anticoagulation due to the nature of the test and the type of anticoagulant used. As a reference, patients with hemophilia B (complete factor IX deficiency) will have aPTTs as high as 130 s (more than twice baseline) and be completely anticoagulated. In cardiac surgery following full-dose heparinization for CPB, the aPTT will be out of range; both examples exhibit complete anticoagulation at different aPTT values due to different anticoagulants used. Other factors making interpretation of our results difficult include species-specific responses to anticoagulation. In the rat, the highest attainable levels for ACT are around 480 s for which a heparin dose of 1200 IU/kg is required.[27] Based on our experience, the heparin dose used in this study (600 IU/kg) provides sufficient anticoagulation for up to 90 min CPB with ACTs around 350-400 s. In humans during cardiac surgery an ACT of 480 s is considered a safe margin for CPB, but this is not feasible in rats. This explains why the anticoagulation profile in aptamer-treated rats was different from the heparin-treated animals, while both profiles reflect sufficient anticoagulation.

Because the coagulation system exhibits an extensive cross-talk with cellular and humoral aspects of the inflammatory response[28] and since inflammatory cytokines increase during cardiac surgery and are associated with an increased incidence of postoperative complications, we measured inflammatory markers TNF-$\alpha$, IL-1$\beta$, IL-6 and IL-10 that are known to be elevated in CPB surgery.[29] All cytokines showed similar patterns in the aptamer-antidote treated groups compared to the heparin-treated animals. Because FIXa aptamer interacts upstream of thrombin in the coagulation cascade, we assumed that thrombin formation would be reduced, thus leading to less inflammation. To our surprise, the inflammatory profile was similar between groups which means that we could not reproduce earlier findings in a porcine model of CPB.[11] The reason for this remains unclear. Species-specific differences (neonatal swine versus adolescent rats) and discrepancies in the experimental protocols (absence of cardioplegic arrest, aortic cross-clamp and myocardial ischemia-reperfusion in the current model) may all have played a role. Further, the avoidance of sternotomy and cardiotomy suction, potent sources of tissue factor and thrombin [30], may have resulted in relative lower cytokine levels hence making detection of significant differences between groups more difficult.

Analysis of the thrombocyte counts showed that in both groups platelet numbers declined three hours postoperatively when compared to pre-CPB. This is consistent with the clinical scenario. Interestingly, the decline in platelets was slightly attenuated in the aptamer group. However, a lower baseline level (reason unclear) resulted in around 300x $10^9$ platelets/L in both groups at the end of the experiment. As platelet counts are known to fall up to two days after cardiac surgery [31], the slower decline in platelet numbers in the aptamer group may have continued into the postoperative phase.





Assessment of early postoperative cardiac function demonstrated that anticoagulation with the factor IXa aptamer and its specific antidote resulted in preserved cardiac function when compared to the heparin/protamine-treated animals. This is in support of recent data from a porcine model of CPB where aptamer/antidote treated animals demonstrated preserved cardiac output measurements as opposed to heparin/protamine treated animals.[11] In part, this effect might be explained by the avoidance of protamine which has been linked to a transient reduction of epicardial blood flow and other hemodynamic side effects such as systemic hypotension and pulmonary hypertension.[10,32] Of interest, the significant difference in cardiac function in the present work did not correlate with the histological findings demonstrating no changes in myocardial contraction band necrosis (MCBN). MCBN is a marker of cell death and can be identified shortly after irreversible myocyte injury (e.q 20 min of reduced nutritive flow).[19] It is histologically characterized by irreversible hypercontraction of the myocyte with markedly thickened Z-lines and short sarcomeres.[19] Overall, the degree of myocardial injury represented by MCBN was mild in both groups and again, the functional differences may best be explained by the transient effects of protamine that besides its effects on epicardial flow also has been shown to directly inhibit the contractility of myocytes and reduce the response to adrenergic stimulation in isolated porcine myocytes.[33,34]

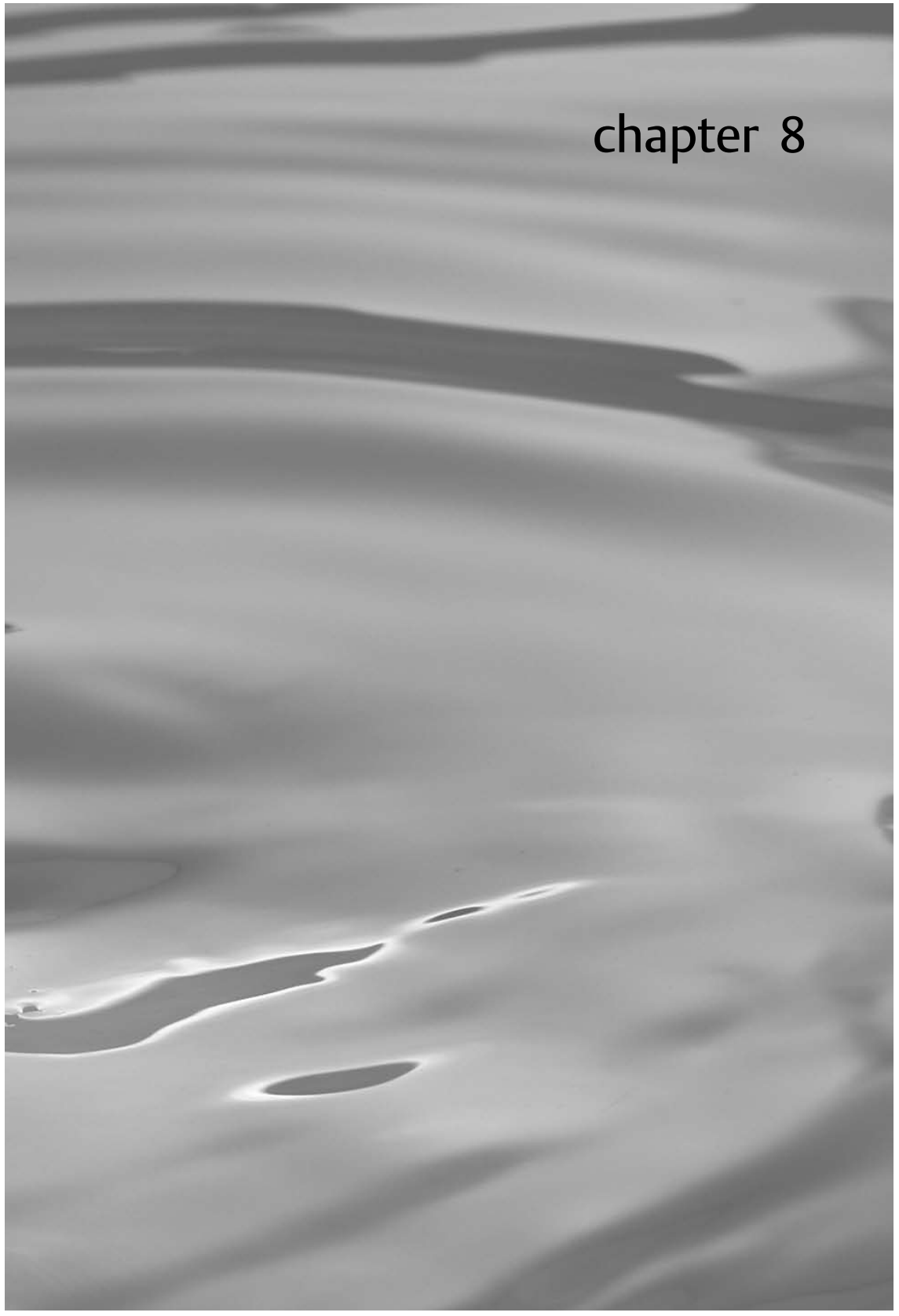
Although this rodent CPB model closely resembles current clinical standards with respect to the technical aspects of the CPB circuit, some limitations remain. These include the absence of median sternotomy and direct surgery on the heart with reperfusion of ischemic myocardium after cardioplegic arrest. Future studies should explore the longer term effects of intraoperative anticoagulants on measures of postoperative hemostasis, inflammatory response and functional outcomes.

In summary, these experiments show that the FIXa-aptamer and its complementary antidote serve as an effective reversible anticoagulant in a rat CPB model. Potential advantages of the aptamer/antidote pair over heparin and its reversal agent protamine include a moderated decline in thrombocytes and improved early postoperative cardiac contractility. This new drug-antidote pair may provide an attractive alternative to patients that cannot be exposed to heparin/protamine secondary to HIT, proven hypersensitivity to protamine or those with significantly impaired cardiac function. A phase I study has been completed on an iteration of the FIXa aptamer used in this experiment, revealing a dose-dependent increase in aPTT with aptamer infusion and return to baseline with administration of antidote.[35] There were no untoward side effects of the drug-antidote pair. Further clinical testing will be needed in order to optimize the clinical potential of these promising RNA-based therapeutics as systemic drug compounds.

## Reference List


1. Koster A, Fischer T, Praus M, Haberzettl H, Kuebler WM, Hetzer R, Kuppe H: Hemostatic activation and inflammatory response during cardiopulmonary bypass: impact of heparin management. Anesthesiology 2002; 97: 837-41
2. Ranucci M, Frigiola A, Menicanti L, Ditta A, Boncilli A, Brozzi S: Postoperative antithrombin levels and outcome in cardiac operations. Crit Care Med 2005; 33: 355-60
3. Arepally GM, Ortel TL: Heparin-induced thrombocytopenia. N Engl J Med 2006; 355: 809-17
4. Warkentin TE: New approaches to the diagnosis of heparin-induced thrombocytopenia. Chest 2005; 127: 35S-45
5. Kuitunen A, Suojaranta-Ylinen R, Raivio P, Kukkonen S, Lassila R: Heparin-Induced Thrombocytopenia Following Cardiac Surgery is Associated With Poor Outcome. J Thorac Cardiovasc Surg 2007; 21: 18-22
6. Bennett-Guerrero E, Slaughter TF, White WD, Welsby IJ, Greenberg CS, El Moalem H, Ortel TL: Preoperative anti-PF4/heparin antibody level predicts adverse outcome after cardiac surgery. J Thorac Cardiovasc Surg 2005; 130: 1567-72
7. Kress DC, Aronson S, McDonald ML, Malik MI, Divgi AB, Tector AJ, Downey III FX, Anderson AJ, Stone M, Clancy C: Positive heparin-platelet factor 4 antibody complex and cardiac surgical outcomes. Ann Thorac Surg 2007; 83: 1737-43
8. Matsuo T, Tomaru T, Kario K, Hirokawa T: Incidence of heparin-PF4 complex antibody formation and heparin-induced thrombocytopenia in acute coronary syndrome. Thromb.Res. 2005; 115: 475-81
9. Williams RT, Damaraju LV, Mascelli MA, Barnathan ES, Califf RM, Simoons ML, Deliargyris EN, Sane DC: Anti-Platelet Factor 4/Heparin Antibodies: An independent predictor of 30-day myocardial infarction after acute coronary ischemic syndromes. Circulation 2003; 107: 2307-12
10. Welsby IJ, Newman MF, Phillips-Bute B, Messier RH, Kakkis ED, Stafford-Smith M: Hemodynamic changes after protamine administration: association with mortality after coronary artery bypass surgery. Anesthesiology 2005; 102: 308-14
11. Nimjee SM, Keys JR, Pitoc GA, Quick G, Rusconi CP, Sullenger BA: A novel antidote-controlled anticoagulant reduces thrombin generation and inflammation and improves cardiac function in cardiopulmonary bypass surgery. Mol Ther 2006; 14: 408-15
12. Rusconi CP, Roberts JD, Pitoc GA, Nimjee SM, White RR, Quick G, Jr., Scardino E, Fay WP, Sullenger BA: Antidote-mediated control of an anticoagulant aptamer in vivo. Nat Biotechnol. 2004; 22: 1423-8
13. Rusconi CP, Scardino E, Layzer J, Pitoc GA, Ortel TL, Monroe D, Sullenger BA: RNA aptamers as reversible antagonists of coagulation factor IXa. Nature 2002; 419: 90-4
14. Nimjee SM, Rusconi CP, Sullenger BA: Aptamers: An emerging class of therapeutics. Annu Rev Med. 2005; 56: 555-83
15. Nimjee SM, Rusconi CP, Harrington RA, Sullenger BA: The potential of aptamers as anticoagulants. Trends Cardiovasc Med. 2005; 15: 41-5
16. Guide for the Care and Use of Laboratory Animals. Health and Human Services, National Institutes of Health Publication No.86-23, revised 1996. 2005.
17. Mackensen GB, Sato Y, Nellgard B, Pineda J, Newman MF, Warner DS, Grocott HP: Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001; 95: 1485-91
18. de Lange F, Dieleman JM, Jungwirth B, Kalkman CJ: Effects of cardiopulmonary bypass on neurocognitive performance and cytokine release in old and diabetic rats. Br J Anaesth 2007; 99: 177-83
19. Baroldi G, Mittleman RE, Parolini M, Silver MD, Fineschi V: Myocardial contraction bands. Definition, quantification and significance in forensic pathology. Int J Legal Med. 2001; 115: 142-51
20. Moscucci M: Frequency and costs of ischemic and bleeding complications after percutaneous coronary interventions: rationale for new antithrombotic agents. J Invasive Cardiol. 2002; 14 Suppl B: 55B-64B
21. Dyke CM, Smedira NG, Koster A, Aronson S, McCarthy II HL, Kirshner R, Lincoff AM, Spiess BD: A comparison of bivalirudin to heparin with protamine reversal in patients undergoing cardiac surgery with cardiopulmonary bypass: The EVOLUTION-ON study. J Thorac Cardiovasc Surg 2006; 131: 533-9
22. Kong DF, Topol EJ, Bittl JA, White HD, Theroux P, Hasselblad V, Califf RM: Clinical outcomes of bivalirudin for ischemic heart fisease. Circulation 1999; 100: 2049-53
23. Veale JJ, McCarthy HM, Palmer G, Dyke CM: Use of bivalirudin as an anticoagulant during cardiopulmonary bypass. J Extra Corpor Technol. 2005; 37: 296-302
24. Hoffman M, Monroe DM, III: A cell-based model of hemostasis. Thromb Haemost. 2001; 85: 958-65
25. Spanier TB, Oz MC, Minanov OP, Simantov R, Kisiel W, Stern DM, Rose EA, Schmidt AM: Heparinless cardiopulmonary bypass with active-site blocked factor IXa: a preliminary study on the dog. J Thorac Cardiovasc Surg 1998; 115: 1179-88
26. Tuerk C, Gold L: Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 1990; 249: 505-10
27. Portilla-De-Buen E, Ramos L, Leal C, Garcia D, Rodriguez-Reynoso S, Herta M, Intaglietta M: Activated clotting time and heparin administration in Sprague-Dawley rats and Syrian golden hamsters. Contemp Top Lab Anim Sci. 2004; 43: 21-4
28. Senden NHM, Jeunhomme TMAA, Heemskerk JWM, Wagenvoord R, van't Veer C, Hemker HC, Buurman WA: Factor Xa induces cytokine production and expression of adhesion molecules by

human umbilical vein endothelial cells. J Immunol 1998; 161: 4318-24
29. Hall RI, Smith MS, Rocker G: The systemic inflammatory response to cardiopulmonary bypass: pathophysiological, therapeutic, and pharmacological considerations. Anesth Anal 1997; 85: 766-82
30. Chung JH, Gikakis N, Rao AK, Drake TA, Colman RW, Edmunds LH, Jr.: Pericardial blood activates the extrinsic coagulation pathway during clinical cardiopulmonary bypass. Circulation 1996; 93: 2014-8
31. Laga S, Bollen H, Arnout J, Hoylaerts M, Meyns B: Heparin influences human platelet behavior in cardiac surgery with or without cardiopulmonary bypass. Artif Organs 2005; 29: 541-6
32. Belboul A, al Khaja N: The effect of protamine on the epicardial microflow and the graft flow in open-heart surgery. Perfusion 1997; 12: 99-106
33. Hird RB, Crawford FA, Jr., Mukherjee R, Zile MR, Spinale FG: Effects of protamine on myocyte contractile function and beta-adrenergic responsiveness. Ann Thorac Surg 1994; 57: 1066-74
34. Hird RB, Wakefield TW, Mukherjee R, Jones BU, Crawford FA, Andrews PC, Stanley JC, Spinale FG: Direct effects of protamine sulfate on myocyte contractile processes: Cellular and molecular mechanisms. Circulation 1995; 92: II433-II446
35. Dyke CK, Steinhubl SR, Kleiman NS, Cannon RO, Aberle LG, Lin M, Myles SK, Melloni C, Harrington RA, Alexander JH, Becker RC, Rusconi CP: First-in-human experience of an antidote-controlled anticoagulant using RNA aptamer technology. A phase 1a pharmacodynamic evaluation of a drug-antidote pair for the controlled regulation of factor IXa activity. Circulation 2006;114: 2490-7



chapter 8

# Perfluorocarbon administration during cardiopulmonary bypass in rats: an inflammatory link to adverse outcome?

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## Abstract

**Background**

Perfluorocarbon (PFC) emulsions are artificial oxygen carriers that have also been shown to attenuate the effects of air embolism. Cerebral air embolism, known to occur during cardiopulmonary bypass (CPB), may contribute to adverse cerebral outcomes after cardiac surgery. This study was designed to evaluate the effect of a 60% PFC emulsion (perfluoro-tert-butylcyclohexane; PTBCH) on the inflammatory response and neurocognitive outcome of rats after CPB.

**Methods**

28 Sprague Dawley rats subjected to 60 min of CPB were randomly divided into two groups: PTBCH CPB animals receiving 3ml/kg of PTBCH into the venous reservoirand control CPB animals receiving 3 ml/kg of 0.9% saline. At several timepoints, the cytokines IL-1β, IL-6, IL-10 and TNF-α were measured. Neurocognitive testing was planned post-operatively using the Morris water maze. Histologic samples were obtained in a separate series of experiments.

**Results**

Physiologic variables were comparable between groups, the PTBCH CPB animals required more phenylephrine compared with the controls. Cytokines in the PTBCH CPB group were significantly higher than in the control group at 2 and 4 hrs post CPB ($p<0.05$). Neurocognitive outcome could not be evaluated as none of the animals in the PTBCH CPB group survived. Myocardial histological analysis revealed increased areas of contraction band necrosis in the PTBCH CPB animals ($p=0.034$).

**Conclusions**

Administration of PTBCH during CPB was associated with an excessive release of cytokines. This enhanced inflammatory response with subsequent hypotension may have contributed to mortality in rats receiving PTBCH. The observed patterns of myocardial injury indicate global hypoperfusion and catecholamine excess.






## Introduction

Perfluorocarbons (PFC) are hydrophobic synthetic molecules that can dissolve large quantities of gases.[1] In an emulsified state, their potential as oxygen carriers (i.e. hemoglobin replacement compounds) has been investigated extensively, beginning as early as the late 1970s. Perfluorocarbons show a linear dissociation curve between $PO_2$ and oxygen content [2] that is independent of pH, 2,3-DPG, temperature or other physiologic factors. As a result, elevated arterial oxygen partial pressures are very effective in improving oxygen transport capacity of PFC emulsions in blood.[3,4] In addition, the small size of the PFC particles (<0.2 μm in diameter) enables them to enter the microvasculature not accessible to erythrocytes and hence improve local oxygen delivery. Particularly relevant to CPB is the fact that gases otherwise relatively insoluble in plasma, such as nitrogen, are considerably more soluble in PFC than in blood. This characteristic might allow for the use of PFC to absorb systemic air emboli that are routinely generated during cardiac surgery.[5]

Despite advances in perioperative care, CPB equipment, and surgical techniques, cerebral injury remains a significant source of morbidity and mortality after cardiac surgery.[6] Emboli, both gaseous and particulate, have been implicated in the etiology of adverse cerebral outcomes.[7] Gaseous emboli can result from entrainment of air from the operative field, generation in the CPB apparatus from cavitation, and from manipulations (such as perfusionist injections) on the venous site of the CPB circuit.[8] Experimental studies in pigs have previously demonstrated a significant improvement in the incidence and severity of neurologic injury after PFC administration during CPB.[9] Furthermore, PFC has been demonstrated to increase oxygen delivery to organs at risk for injury during cardiac surgery. Several experiments have shown increased in myocardial [10], gastrointestinal [11] and systemic oxygenation [12] after administration of PFC. In addition, we have previously demonstrated that PFC administration may be useful in reducing the volume of gaseous bubbles present during CPB.[13]

In several experiments we have performed CPB in rats, demonstrating the utility of this model to study neurocognitive injury as well as other organ dysfunction.[14-17] In the current experiment, our goal was to assess neurocognitive outcome, cytokine release and organ histology after administration of the PFC perfluoro-tert-butylcyclohexane (PTBCH), in an experimental rat CPB model. We hypothesized that PTBCH administration would improve postoperative neurocognitive outcome in this animal CPB model by improving oxygen delivery and attenuate ischemia-reperfusion induced inflammation.

## Methods

The study was approved by the Duke University Animal Care and Use Committee and all procedures met the National Institutes of Health (NIH) guidelines for animal care.[18] Male 350-375 g Sprague-Dawley rats (Charles River Labs, Wilmington, MA, USA) were housed three per cage with a 12 hour light-dark cycle. Food and water were available *ad libitum*.

**Anesthesia, surgical preparation and cardiopulmonary bypass**

Fasted male rats were anesthetized with 5% isoflurane in 50% $O_2$ in a plastic induction box. After orotracheal intubation with a 14G cannula (Insyte BD Medical, Sandy, UT), the animals were mechanically ventilated to a maximum airway pressure of 20 $mmH_2O$ (Harvard Model 687, Harvard Apparatus, Holliston, MA) maintaining a normal arterial carbon dioxide tension. During subsequent surgical preparation, anesthesia was maintained with 1.5- 2.0% isoflurane. A needle thermistor was inserted in the left temporal muscle adjacent to the skull to measure pericranial temperature. Body temperature was kept at 37° C throughout the procedure with both forced-air and surface heating systems

Surgical preparation consisted of cannulating the tail artery with a 20 G catheter (Insyte BD Medical, Sandy, UT), which served as the arterial inflow cannula for the CPB circuit. 150 IU of porcine heparin and 5 μg of fentanyl were administered after placement of this first catheter. Arterial blood pressure was monitored via the superficial caudal epigastric artery, which was cannulated with polyethylene tubing (PE-10 Intramedic Tubing, Becton-Dickinson, Sparks, MD). A multi-orifice 4.5 French catheter (modified Desilets-Hoffman catheter; Cook, Bloomington, IN) was advanced through the right external jugular vein into the right heart in order to serve as a conduit for the venous outflow. Repeat injections of 150 IU of porcine heparin and 5 μg of fentanyl were administered just prior to the start of CPB. In addition, 0.2 mg of pancuronium was administered. Similar anesthetic regimens without pancuronium had previously been demonstrated in this model to prevent withdrawal responses to painful stimuli. Muscle relaxation was used to prevent spontaneous ventilation that would oftentimes interfere with venous return.

The CPB circuit consisted of a 4 mL Plexiglas® venous reservoir, a roller pump (Masterflex; Cole-Parmer Instrument Co., Vernon Hills, IL, USA) and a custom-designed small-volume oxygenator. The 4 mL priming volume oxygenator was comprised of two Plexiglas® shells (12.8 cm x 12.8 cm x 2.7 cm) that carry a sterile, disposable three layer hollow-fiber membrane providing a gas exchange surface area of 558 $cm^2$.[19,20] To prevent excessive heat loss, the oxygenator had an integrated heat exchanger. An in-line flow probe (2N806 probe and T208 flowmeter, Transonics Systems Inc., Ithaca, NY) was used to continuously measure CPB flow. The entire circuit was primed with 10 mL of 6% hetastarch (Hextend®; Hospira Inc, Lake Forest, IL). All parts were connected through single use silicone tubing.

During CPB, ventilation was discontinued and 0.5 - 1% isoflurane was administered through the oxygenator. CPB was carried out for 60 minutes with a flow rate of 150 $mLkg^{-1}min^{-1}$ adjusted to maximize flow and to maintain an optimal venous reservoir blood level. Mean arterial pressure (MAP) was maintained between 45-60 mmHg, with the use of intermittent phenylephrine administered as necessary. At 30 min of CPB, a repeat dose of pancuronium (0.2 mg) was added to the bypass circuit. After 60 min of CPB, ventilation was reinitiated and CPB was discontinued and the venous cannula removed. The animals were ventilated for a further 120 min, after which the remaining cannulae were removed and the wounds closed. The rats recovered in a warmed and oxygen-enriched environment for at least 12 hr prior to return to their original cages.

After discontinuation of CPB, the remaining blood volume in the circuit was collected, 0.9% saline added, and the resulting solution centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and the resulting red blood cell suspension was reinfused into the animal. Blood gas analysis was performed before the start of CPB, at 30 min and 60 min of CPB, then at 1 hr and 2 hr post CPB, using an IL-GEM Premier 3000 blood gas analyzer (Global Medical Instrumentation, Ramsey, MI, USA).

**Perfluorocarbon description**

The perflurocarbon used was F-tert-butylcyclohexane, also known as perfluoro-tert-butylcyclohexane (PTBCH). It is a perfluorocarbon that contains only carbon and fluorine atoms ($C_{10}F_{20}$). It cannot exhibit cis-trans isomerism and thus exists as a single configuration. It is marketed as Oxycyte® (Synthetic Blood International, Costa Mesa, CA).

The dose used in this study (3mL/kg) was chosen based on information available regarding its use in other animal studies. In a previous study describing cerebral ischemia in rats, a dose of 10 mL/kg was administered without serious side-effects.[21] Safety in doses up to four times the dose we used was described in safety studies in rats by the manufacturer (data on file with manufacturer). In addition, the dose we used, albeit lower that used in rats before, was also based on PFCs being used in clinical CPB studies.[22]

**Group designation**

Immediately after insertion of all the CPB cannulae, 28 animals were randomized into one of two groups: a group exposed to 60 min of CPB with administration of 3 $mL.kg^{-1}$ i.v. PTBCH into the CPB circuit prime (PTBCH CPB group), and a control group exposed to 60 min CPB with 3 $mL.kg^{-1}$ of saline added to the CPB prime (Control CPB group).

Subsequently, in a separate series of experiments, 12 animals (5 Controls, 7 PTBCH) underwent the same protocol as described above but were euthanized 4 hrs after CPB, in order to obtain tissues for water content determination and histological analysis. In addition, these animals provided the 4 hour post CPB cytokine sample.

**Cytokine analysis**

Blood samples (0.3mL) for analysis of interleukin Il-1β, Il-6, Il-10 and TNF-α were collected from the tail artery for analysis immediately prior to bypass, 60 min after CPB, and at 2 hr and 4 hr after CPB. All samples were immediately centrifuged (at 4°C) and the supernatant frozen and stored (at -80°C) for later batch analysis. The cytokines were analyzed by multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) microtitre plate, according to the manufacturer's instructions (Endogen® - Search Light™, Inc., Woburn, MA). Results were expressed as pg/ml with the sensitivity for detection being 3.1 pg/ml for Il-β, 6.3 pg/ml for Il-6, 0.4 pg/ml for Il-10 and 3.1 pg/ml for TNFα.

**Neurocognitive testing**

Beginning on the 3rd postoperative day and continuing for one week, surviving animals underwent neurocognitive testing in the Morris water maze (MWM).[14] In brief, the MWM consisted of a 1.5 m diameter darkened pool filled with water (26 ± 1°C) with a fixed, submerged platform (submerged 1 cm) and various visual clues on the walls. The time to locate the hidden platform after placing the animal in the pool is measured and recorded as the latency. The animals were planned for daily testing with four trials per day.

**Total tissue water content**

Immediately after sacrificing the animals by decapitation, tissue samples (0.4-0.6 g) were collected from the brain, lung, heart, kidney, spleen and liver. The samples were placed in pre-weighed open vials, reweighed immediately, and then placed in a drying chamber at 40°C . The vials were weighed daily until a stable weight was reached. Tissue water content was calculated as the tissue water weight (TTW) recorded as g water/g wet weight x 100%.

**Histologic analysis**

The animals designated for histologic analysis were subjected to the same CPB procedures and randomization as described above. Four hours after CPB, they were decapitated and the tissues fixed in 3.7% phosphate-buffered neutral formaldehyde. The right kidney and right lung were dissected, and a cross-section of the heart was prepared by sectioning through the right and left ventricles approximately midway from the apex to the base of the heart. The organ samples were dehydrated with serial alcohols, embedded in paraffin, and 5 μm paraffin sections stained with hematoxylin and eosin. A pathologist (AP) blinded to group assignment evaluated the slides for any qualitative microscopic abnormalities in lungs and kidneys. Myocardial contraction band necrosis, a marker of cell death [23] and associated with catecholamine excess[24], was quantified in the subendocardial and myocardial layers. The long and short axes of areas that exhibited hypereosinophilia and





contraction bands were measured using a calibrated ocular micrometer and an estimated cross-sectional area calculated by multiplying the two diameters.

**Statistical analysis**

Physiologic parameters were analyzed using a one-way analysis of variance (ANOVA).Tissue water content between the two groups was analyzed using an independent samples t-test. Cytokine data and areas of contraction band necrosis were analyzed using the Mann-Whitney U test. All analyses were performed using SPSS 11.5 (SPSS Inc, Chicago, Il).

*Table 1. Physiologic parameters. Values represent means (SD)*

| | baseline | 30 min CPB | 60 min CPB | 1 hr post | 2 hr post |
|---|---|---|---|---|---|
| **Weight (g)** | | | | | |
| control CPB | 352 (17) | | | | |
| PTBCH CPB | 366 (22) | | | | |
| **Hematocrit (%)** | | | | | |
| control CPB | 40.1 (2.9) | 21.9 (1.2) | 20.9 (1.0) | 25.6 (3.4) | 28.5 (2.8) |
| PTBCH CPB | 39.0 (2.4) | 22.1 (1.8) | 20.8 (1.9) | 24.5 (3.3) | 28.4 (2.9) |
| **Glucose (mg/dl)** | | | | | |
| control CPB | 114 (25) | | 108 (35) | | 91.6 (32.4) |
| PTBCH CPB | 124 (41) | | 112 (15) | | 92.6 (32.5) |
| **Pericranial Temperature (°C)** | | | | | |
| control CPB | 36.6 (0.4) | 36.5 (0.2) | 36.7 (0.2) | 36.8 (0.3) | 37.1 (0.3) |
| PTBCH CPB | 36.6 (0.2) | 36.5 (0.4) | 37.0 (0.3)[a] | 36.9 (0.5) | 37.1 (0.4) |
| **CPB flow (ml/min)** | | | | | |
| control CPB | | 57 (7) | 59 (9) | | |
| PTBCH CPB | | 61 (5) | 63 (6) | | |
| **MAP (mmHg)** | | | | | |
| control CPB | 63 (12) | 57(7) | 54 (5) | 58 (7) | 53 (6) |
| PTBCH CPB | 62 (6) | 50 (13) | 49 (8) | 56 (6) | 55 (9) |
| **pH** | | | | | |
| control CPB | 7.41 (0.05) | 7.45 (0.04) | 7.44 (0.04) | 7.35 (0.06) | 7.38 (0.06) |
| PTBCH CPB | 7.42 (0.04) | 7.38 (0.11)[a] | 7.41 (0.04)[a] | 7.40 (0.05) | 7.39 (0.09) |
| **$PaCo_2$ (mmHg)** | | | | | |
| control CPB | 46 (8) | 38 (3) | 40 (2) | 50 (9) | 44 (6) |
| PTBCH CPB | 42 (5) | 45 (20) | 39 (2) | 41 (5) | 40 (5) |
| **$Pao_2$ (mmHg)** | | | | | |
| control CPB | 131 (41) | 442 (72) | 457 (97) | 181 (115) | 199 (127) |
| PTBCH CPB | 140 (27) | 491 (57) | 495 (73) | 272 (103) | 300 (129) |
| **$HCO_3^-$ (mmHg)** | | | | | |
| control CPB | 28.6 (2.3) | 26.2 (1.4) | 26.8 (2.8) | 27.0 (2.9) | 25.7 (2.1) |
| PTBCH CPB | 26.8 (1.5) | 25.2 (2.0) | 24.6 (1.8) | 25.7 (2.5) | 23.9 (2.8) |

*CPB=cardiopulmonary bypass, MAP= mean arterial pressure, PTBCH= perfluorotert-butylcyclohexane, 1 hr post= 1 hr after cessation of CPB*

*a= p<0.05 when compared to Control CPB animals*

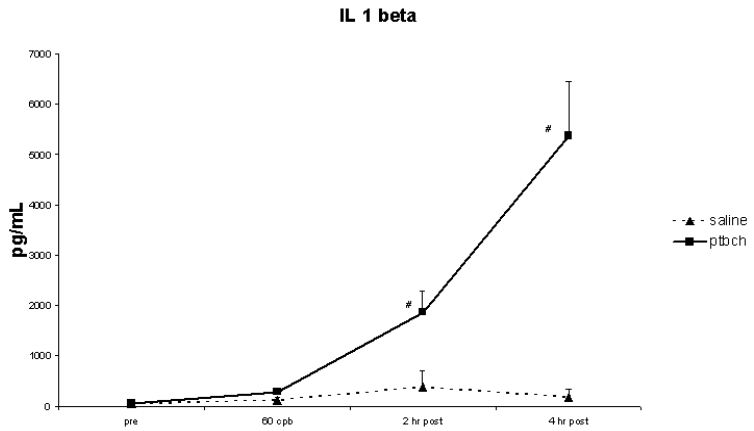



*Figure 1*

*Levels of Il-1β at four different timepoints.*
*Means + SD.*
*PTBCH= perfluoro-tert-butylcyclohexane CPB= cardiopulmonary bypass*
*Post= after cessation of CPB*
*# = p < 0.05*

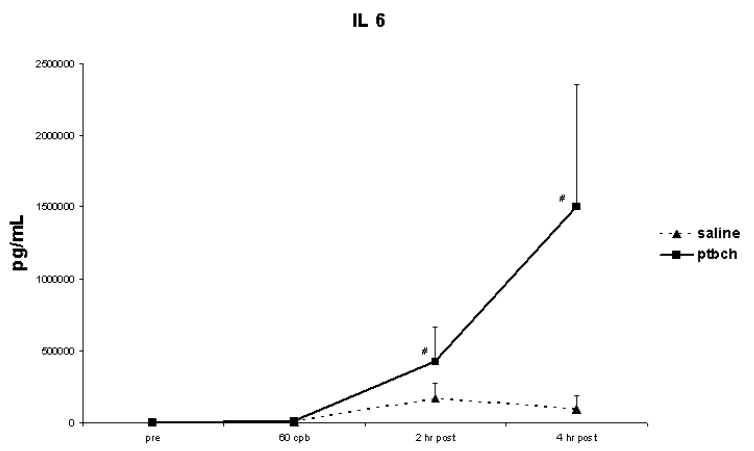



*Figure 2*

*Levels of Il-6 at four different timepoints.*
*Means + SD.*
*PTBCH= perfluoro-tert-butylcyclohexane CPB= cardiopulmonary bypass*
*Post= after cessation of CPB*
*# = p < 0.05*





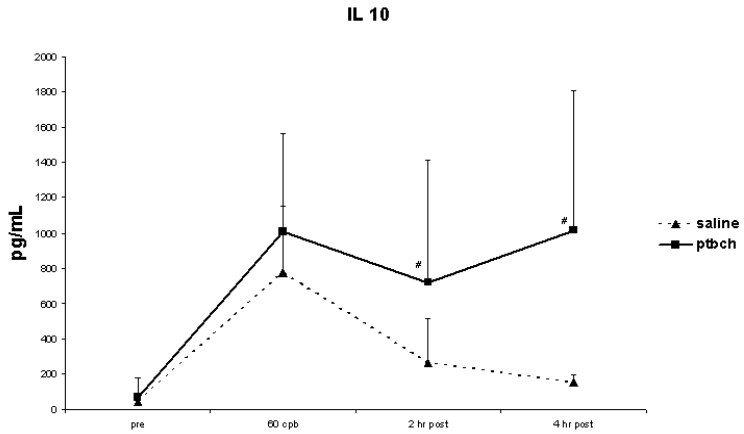



*Figure 3*

*Levels of Il-10 at four different timepoints.*
*Means + SD.*
*PTBCH= perfluoro-tert-butylcyclohexane CPB= cardiopulmonary bypass*
*Post= after cessation of CPB*
*# = p < 0.05*

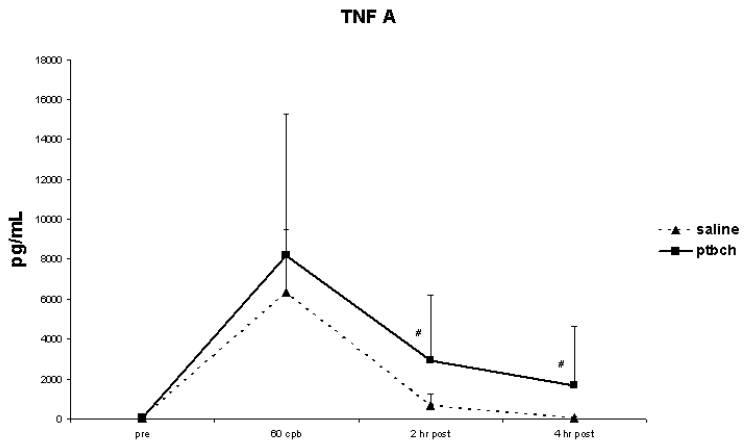



*Figure 4*

*Levels of TNF-Ð at four different timepoints. Means + SD.*
*PTBCH= perfluoro-tert-butylcyclohexane CPB= cardiopulmonary bypass*
*Post= after cessation of CPB*
*# = p < 0.05*

## Results

The physiological parameters in the two groups are presented in Table 1. During CPB, the pH was significantly lower in the PTBCH CPB group, but was still within normal physiological ranges (at 30 min CPB, 7.45 ± 0.04 Control CPB group *vs* 7.38 ± 0.11 PTBCH CPB group; and at 60 min CPB, 7.44 ± 0.04 Control CPB group *vs* 7.41 ± 0.04 PTBCH CPB group, p= 0.032 and p= 0.038 respectively). There was also a small difference in pericranial temperature at the end of CPB (36.7 ± 0.2 °C control CPB group *vs* 37.0 ± 0.3°C PTBCH CPB group, *p*= 0.041). In addition, the PTBCH CPB group required more phenylephrine to maintain the targeted MAP (total dose in the PTBCH CPB group 280 mcg vs 20 mcg in Control CPB group , *p*=0.03).

Of the 14 animals randomized into the PTBCH CPB group, 4 (29%) experienced significant hypotension and died during or shortly after the procedure. Of the 10 surviving animals, only one survived for 3 days while the other nine animals died within the first 24 postoperative hours. Of the 14 animals randomized to the Control CPB group, 2 died perioperatively due to cannulation and/or equipment related problems. Of the remaining 12 animals, one died on day one and one died on the second postoperative day; the remaining 10 animals survived through the duration of the experimental protocol. Due to the excessive mortality in the PTBCH CPB group, no comparisons between group neurocognitive performances in the Morris water maze were possible.

At the end of the experiment, cytokines (Il-1β, Il-6, Il-10 and TNF-α) were analyzed in 12 Control CPB and 10 PTBCH CPB animals with 5 animals in each group completing the 4 hr timepoint cytokine measurements (Figures 1-4). For all four cytokines, there was a significant difference between groups at the 2 hr and 4 hr post-CPB time points. There were no significant differences at the baseline and 60 min CPB time points.

The tissue water content data are presented in Table 2. In the PTBCH CPB animals, the water content in kidneys and spleen was significantly lower than in the Control CPB group (74.9% vs 76.1%, *p*=0.01 and 73.8% vs 76.0 %, *p*=0.03 respectively). There were no differences in water content between the groups for the brain, heart, lung and liver specimens.

Histologic sections of the lungs revealed only small areas of normal lung tissue. Most of the lung tissue had alveolar capillaries engorged with polymorphonuclear leukocytes (PMNs) and macrophages, along with lesser number of lymphocytes. There were no differences between the two groups. In the kidneys, the PTBCH CPB animals all demonstrated finely vacuolated tubular epithelial cells, while no histological abnormalities were seen in the Control CPB group. Foci of hypereosinophilic myocytes with scattered contraction bands were seen in four of the five hearts in both the PTBCH and Control CPB groups. However, the areas of contraction band necrosis were larger in the PTBCH CPB rats than in the Control CPB rats ( *p*=0.034: Fig 5). Some foci of

*Table 2. Tissue water content in different organs. Values represent*

| | brain | lung | heart | kidney | liver | spleen |
|---|---|---|---|---|---|---|
| control CPB | 76.4 (0.6) | 79.3 (0.4) | 76.9 (0.9) | 76.1 (0.6) | 69.9 (1.1) | 76.0 (1.3) |
| PFC CPB | 76.4 (0.4) | 79.1 (0.8) | 76.9 (0.5) | 74.9 (0.6)[a] | 71.1 (2.2) | 73.8 (1.4)[a] |
| *p value* | | | | *P*=0.01 | | *P*=0.04 |

*CPB=cardiopulmonary bypass, PTBCH= perfluoro-tert-butylcyclohexane*
*a= p<0.05 when compared to Control CPB animals*

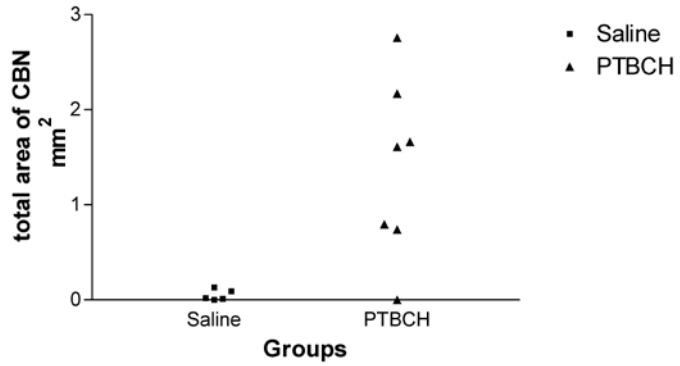



*Figure 5*

*Total area of myocardial contraction band necrosis.*
*PTBCH= perfluoro-tert-butylcyclohexane*
*MCBN= myocardial contraction band necrosis*
*# = p < 0.05*

contraction band necrosis, usually the larger foci, had a mild infiltrate of neutrophils and macrophages.

## Discussion

The aim of the present study was to investigate the effects of perfluorocarbon administration during CPB with a particular focus on its potential effects in modulating the inflammatory response to bypass as well any influence on neurocognitive outcome. The administration of PTBCH, a 60% PFC emulsion to the rats during CPB resulted in a significantly exaggerated inflammatory response, as demonstrated by increased serum cytokine levels and loss of vasomotor tone represented by higher vasopressor needs. Due to insufficient survival in the PTBCH CPB group, neurocognitive performance could not be assessed in the animals receiving PTBCH. Histologic examination revealed increased multifocal contraction band necrosis in the myocardium of the PTBCH CPB animals.

The degree to which the cytokines were elevated in the PTBCH group was unexpected. Previous experiments in our rat model of CPB have demonstrated that after 60 min CPB, a predictable rise in cytokine levels occurs, with Il-1β, Il-6 and TNF-α normally reaching their peak levels approximately 2 hr after cessation of CPB before slowly returning to baseline levels within a few hours.[25] Il-10 usually has its peak at the end of CPB. As can be seen in Figures 1-4, the control CPB group replicates this expected pattern very closely. The PTBCH CPB group, however, demonstrated an exaggerated rise in Il-1β and Il-6 (up to tenfold the otherwise expected increase) with the levels still rising at 4 hr post CPB, suggesting the possibility of even higher peak levels occurring later. For Il-10, the levels in the PTBCH group were also elevated when compared to the control group. For the TNFα levels, they were also elevated compared to the control group, but the groups demonstrated a similar pattern over time.

An inflammatory response, though not to this degree, has been previously demonstrated with PFCs. A clinical study in human volunteers examining the effects of another PFC (Oxygent, Alliance Pharmaceutical Corp., San Diego, CA), demonstrated that the infusion of this PFC in the healthy volunteers lead to a dose dependent increase in Il-6 at 8 h after infusion.[26] Perfluorocarbons, particularly earlier generation compounds, have been identified previously as having marked febrile and flu-like responses, demonstrating their ability to activate the immune system.[4] It is unclear as to the effects of later generation PFCs, such as the one we used, on the inflammatory response. Two earlier animal experimental studies[9,12] used a second-generation PFC emulsion in combination with CPB, but no inflammatory responses were measured and survival was not an endpoint of those studies.

Several processes are thought to play a role in cytokine release after CPB, including direct blood contact with the surface of the CPB circuit, surgical trauma, the occurrence of ischemia and reperfusion, and endotoxemia.[27-29] Because in this study both groups were treated equally in regard to contact activation and surgical trauma, this can be excluded as a cause of additional inflammation in the PTBCH CPB group. Perfluorocarbons, due to their microscopic size and high oxygen carrying capacities, especially during high partial pressures, have shown to improve tissue oxygen delivery [4] Therefore, the development of ischemia or the translocation of endotoxin across an ischemic gut wall seems unlikely. In the many studies investigating the inflammatory response to CPB, Il- 6 is most consistently increased.[27] We speculate that the large cytokine rise seen in this present study was due to a PFC-specific amplication of the inflammatory response normally seen in CPB.

Myocardial contraction band necrosis is a marker of cell death[23] and also is associated with catecholamine excess.[24] The myocardial contraction band necrosis as seen in the ventricular myocardium of PTBCH treated animals could very well be a result of the generalized and significant myocardial hypoperfusion. However, it may also be a direct result of an endogenous catecholamine response to hypotension in combination with the catecholamine administration needed to maintain stable hemodynamics.[24] We cannot completely exclude this possibility as we noted larger requirements for phenylephrine in the PTBCH CPB group to maintain a target MAP. Due to removal of the cannula two hours after the end of CPB, MAP in the later postoperative period could not be determined. The other finding, vacuoles in the renal tubular epithelium, most likely reflects tubular reabsorption of PTBCH since such vacuoles are not a typical effect of renal ischemia. The pulmonary effect of 60 min CPB (capillary engorgement with leukocytes) was seen in the lungs of both groups suggesting that these did not contribute to the excessive mortality.

Tissue water analysis revealed that the kidney and spleen in the PTBCH CPB group had a lower water content compared to control animals. There are two possible explanations for this. Firstly, there may have been less edema accumulation in these tissues. However, differences in edema were not observed in any of the other tissues. A more likely explanation is that accumulation of lipid containing PTBCH in these tissues resulted in overall net lower tissue water content. Emulsion particles of PFCs are cleared from the blood by the macrophages of the reticuloendothelial system (RES) in the spleen, which may have resulted in increased lipid content in this organ.[30] The reason for an accumulation of lipid in the kidney is not as clear, but likely reflects tubular reabsorption of PTBCH manifesting as vacuolization of renal tubular epithelial cells.

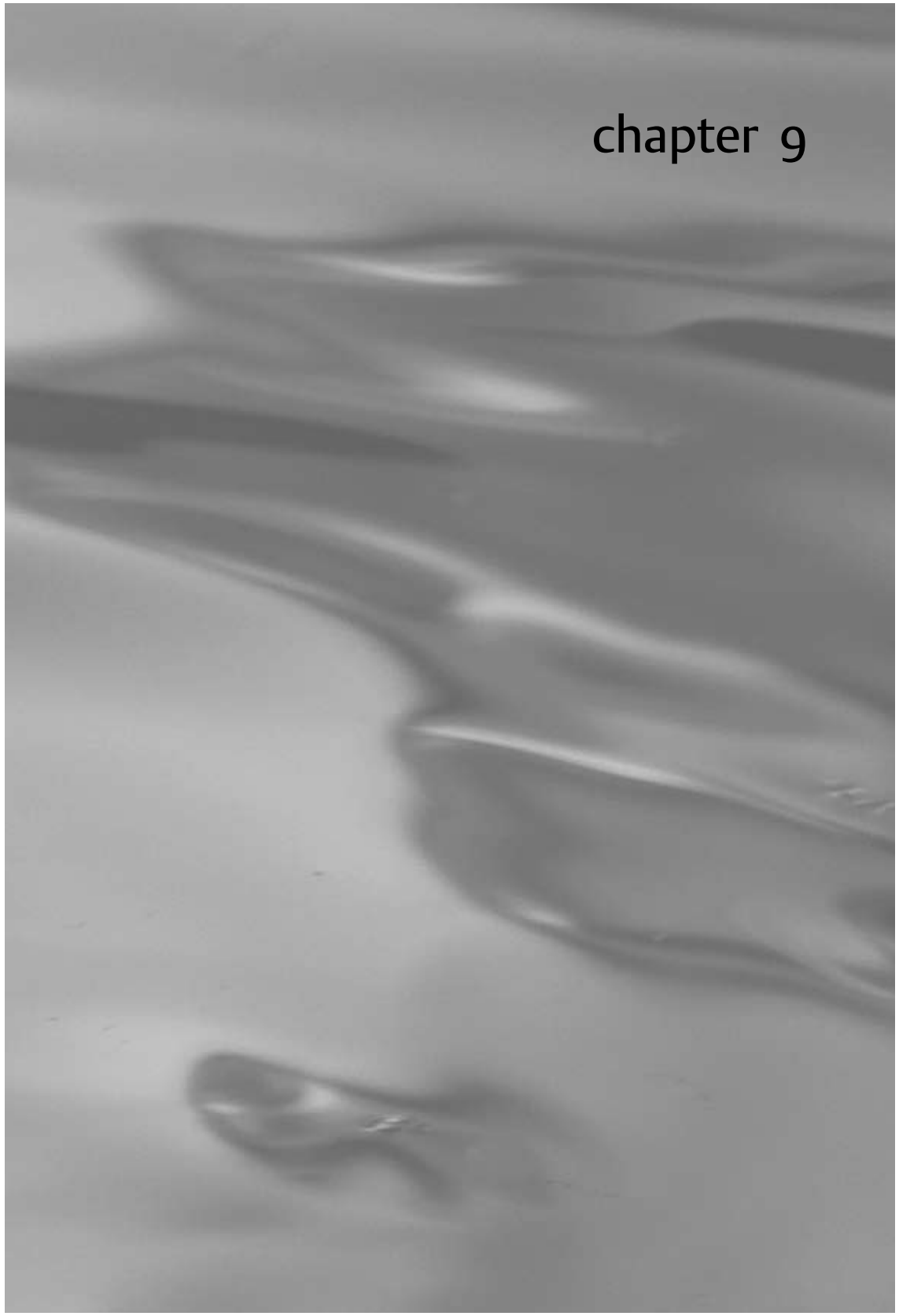
There were several limitations to the present study. We did not administer PTBCH to a group of non-CPB rats. This would have allowed us to study the effects on cytokine release in the absence of bypass. Nonetheless, after the unexpected deaths in the PTBCH CPB group, we did inject two animals with the PTBCH emulsion and found them to be physiologically

indistinguishable from other animals (data not shown). In addition, the manufacturer has administered this particular PFC to several different animal species during the development phase of the drug and reported no clear adverse effects. Slight immune activation was seen as a consequence of macrophage clearance of emulsion particles, but caused only some flu-like symptoms (data on file at Synthetic Blood International). The decision to determine Il-1β, Il-6, Il-10 and TNFα was made because of their role in the inflammation process during CPB, which is thought to contribute to neurocognitive dysfunction after cardiac surgery.[7,28] More extensive immunological evaluation of the effect of PFC during CPB would have added additional information to this issue.

Several clinical studies of PFC administration in this perioperative setting have been undertaken. Hill *et al* [22] reported increased cerebral blood flow and total cerebral embolic load in cardiac surgical patients treated with a PFC emulsion. Although with transcranial Doppler no differentiation between gaseous or particulate emboli could be made, this has potential significant clinical sequelae and may outweigh the potential benefits of absorption of gaseous emboli or enhanced tissue oxygen delivery distal to an atheromatous occlusion. In addition, a phase 3 trial in cardiac surgery was terminated prematurely due to a higher incidence of neurologic complications in the groups treated with the PFC (Oxygent®) emulsion.[22] Our experimental results corroborate these earlier safety concerns and suggest that additional information regarding the inflammatory response to PFC administration during CPB is needed before advancing PFC emulsions into the sphere of clinical CPB.

## Reference List


1. O'Brien RN, Langlais AJ, Seufert WD. Diffusion coefficients of respiratory gases in a perfluorocarbon liquid. Science 1982; 217: 153-5
2. Spahn DR, Pasch T. Physiological Properties of Blood Substitutes. News Physiol Sci 2001;16:38-41
3. Lowe KC. Perfluorinated blood substitutes and artificial oxygen carriers. Blood Rev. 1999; 13: 171-84
4. Spahn DR. Blood substitutes Artificial oxygen carriers: perfluorocarbon emulsions. Critical Care 1999; 3: R93-R97
5. van der Linden J, Casimir-Ahn H. When do cerebral emboli appear during open heart operations? A transcranial Doppler study. Ann Thorac Surg 1991;51:237-41.
6. Roach GW, Kanchuger M, Mangano CM, Newman M, Nussmeier N, Wolman R, Aggarwal A, Marschall K, Graham SH, Ley C. Adverse cerebral outcomes after coronary bypass surgery. Multicenter Study of Perioperative Ischemia Research Group and the Ischemia Research and Education Foundation Investigators. N Engl J Med 1996;335:1857-63.
7. Grocott HP, Homi HM, Puskas F. Cognitive dysfunction after cardiac surgery: revisiting etiology. Semin Cardiothorac Vasc Anesth 2005;9:123-9.
8. Borger MA, Peniston CM, Weisel RD, Vasiliou M, Green REA, Feindel CM. Neuropsychologic impairment after coronary bypass surgery: Effect of gaseous microemboli during perfusionist interventions. J Thorac Cardiovasc Surg. 2001; 121: 743-9
9. Cochran RP, Kunzelman KS, Vocelka CR, Akimoto H, Thomas R, Soltow LO, Spiess BD. Perfluorocarbon Emulsion in the Cardiopulmonary Bypass Prime Reduces Neurologic Injury. Ann Thorac Surg 1997; 63: 1326-32
10. Spiess BD, McCarthy RJ, Tuman KJ, Ivankovich AD. Protection from coronary air embolism by a perfluorocarbon emulsion (FC-43). J Cardiothorac Anesth 1987; 1: 210-5
11. Frumento RJ, Mongero L, Naka Y, Bennett-Guerrero E. Preserved Gastric Tonometric Variables in Cardiac Surgical Patients Administered Intravenous Perflubron Emulsion. Anesth Analg 2002; 94: 809-14
12. Holman WL, Spruell RD, Ferguson ER, Clymer JJ, Vicente WVA, Murrah CP, Pacifico AD. Tissue oxygenation with graded dissolved oxygen delivery during cardiopulmonary bypass. J Thorac Cardiovasc Surg 1995;110:774-85.
13. Yoshitani K, de Lange F, Ma Q, Grocott HP, Mackensen GB. Reduction in Air Bubble Size Using Perfluorocarbons During Cardiopulmonary Bypass in the Rat. Anesth Analg 2006; 103: 1089-93
14. Mackensen GB, Sato Y, Nellgard B, Pineda J, Newman MF, Warner DS, Grocott HP. Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001; 95: 1485-91
15. Podgoreanu MV, Michelotti GA, Sato Y, Smith MP, Lin S, Morris RW, Grocott HP, Mathew JP, Schwinn DA. Differential cardiac gene expression during cardiopulmonary bypass: ischemia-independent upregulation of proinflammatory genes. J Thorac Cardiovasc Surg 2005; 130: 330-9
16. Ma D, Yang H, Lynch J, Franks NP, Maze M, Grocott HP. Xenon attenuates cardiopulmonary bypass-induced neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2003; 98: 690-8
17. Grocott HP, Mackensen GB, Newman MF, Warner DS: Neurological injury during cardiopulmonary bypass in the rat. Perfusion 2001; 16: 75-81
18. Guide for the Care and Use of Laboratory animals. Health and Human services, National Institutes of health publication No.86-23, revised 1996. 2005.
19. Jungwirth B, Mackensen GB, Blobner M, Neff F, Reichart B, Kochs EF, Nollert G. Neurologic outcome after cardiopulmonary bypass with deep hypothermic circulatory arrest in rats: Description of a new model. J Thorac Cardiovasc Surg. 2006; 131: 805-12
20. De Lange F, Dieleman JM, Jungwirth B, Kalkman CK. Effects of cardiopulmonary bypass on neurocognitive performance and cytokine release in old and diabetic rats. Br J Anaesth. 2007;99(2):177-83.
21. Woitzik J, Weinzierl N, Schilling L. Early administration of a second-generation perfluorochemical decreases ischemic brain damage in a model of permanent middle cerebral artery occlusion in the rat. Neurol Res 2005;27:509-15.
22. Hill SE, Grocott HP, Leone BJ, White WD, Newman MF. Cerebral Physiology of Cardiac Surgical Patients Treated with the Perfluorocarbon Emulsion, AF0144. Ann Thorac Surg 2005; 80: 1401-7
23. Buja LM. Myocardial ischemia and reperfusion injury. Cardiovasc Pathol. 2005; 14: 170-5
24. Baroldi G, Mittleman RE, Parolini M, Silver MD, Fineschi V. Myocardial contraction bands. Definition, quantification and significance in forensic pathology. Int.J Legal Med 2001; 115: 142-51
25. Jones WL, Clark J, Smith BE, Grocott HP. Hypothermia Attenuates the Inflammatory Response to Cardiopulmonary Bypass in the Rat. Anesthesiology 2004; 100, A-875 .
26. Noveck RJ, Shannon EJ, Leese PT, Shorr JS, Flaim KE, Keipert PE, Woods CM. Randomized Safety Studies of Intravenous Perflubron Emulsion. II. Effects on Immune Function in Healthy Volunteers. Anesth Analg 2000;91:812-22.
27. Hall RI, Smith MS, Rocker G. The systemic inflammatory response to cardiopulmonary bypass: pathophysiological, therapeutic, and pharmacological considerations. Anesth Analg 1997;85:766-82.
28. Paparella D, Yau TM, Young E. Cardiopulmonary bypass induced inflammation: pathophysiology and treatment. An update. Eur J Cardiothorac Surg 2002; 21: 232-44
29. Hayashi Y, Sawa Y, Nishimura M, Satoh H, Ohtake S, Matsuda H. Avoidance of full-sternotomy: effect on inflammatory cytokine production during cardiopulmonary bypass in rats. J Card Surg 2003;18:390-5.
30. Flaim SF. Pharmacokinetics and side effects of perfluorocarbon-based blood substitutes. Artif Cells Blood Substit Immobil Biotechnol 1994;22:1043-54



chapter 9

# A pooled analysis of Morris water maze data of CPB and sham animals from five different studies

## Introduction

In 2001 Grocott and Mackensen at Duke University Medical Center (Durham, NC, USA) described a rat survival CPB model with neurologic and neurocognitive outcome measures.[1] In this original model, a commercially available neonatal oxygenator (Micro® neonatal oxygenator, Cobe Cardiovascular, Arvada, CO) was used. When used for rat CPB, this oxygenator is approximately 20 times oversized in reference to total oxygenator surface area to blood volume ratio and thus might have augmented the deleterious effects of CPB. Several years later, a custom-made disposable membrane oxygenator especially designed for rodents became available, thus reducing priming volume and blood-membrane contact area and ratio.[2] Since that time, all experiments in rat CPB have been performed using these appropriately sized oxygenators. In *Chapter 3* we describe experiments in which rats with a known susceptibility for cognitive dysfunction (for example, old rats and diabetic rats) were subjected to 90 min of CPB. In this study on the short-term effect of CPB on cognitive outcomes, assessed using the Morris water maze (MWM), no differences between CPB and sham groups could be detected. We speculate that the new smaller sized oxygenator could have resulted in an attenuated effect of CPB on MWM performance. Coupled with insufficient statistical power (as group sizes were calculated based on the differences noted in the original study), this might have also contributed to the lack of effect of CPB on cognition as seen in later experiments.

In an effort to address this statistical shortcoming of potential insufficient power, we pooled all studies performed by the same investigator using this small oxygenator in the rat CPB model and investigated the effect of CPB (with the appropriately sized oxygenator) on cognitive performance in the MWM when compared to an identical sham group.

## Methods

Of all the rat CPB studies performed exclusively by one investigator (FdL), only data meeting the following three criteria were considered eligible for inclusion in the post hoc pooled analysis: i) the appropriately sized oxygenator was used in the study: ii) short-term (up to 12 days) cognitive outcomes were assessed using the MWM: iii) only CPB and sham animals from the same study using the same protocol were included, so the only difference was the actual use of CPB. From each qualifying animal, the perioperative physiological values and mean latency per day in the MWM were collected. Because two differently sized swimming pools were used (2.1 and 1.5 in diameter, swimming surface 3.46 and 1.76 $m^2$) latencies of the smaller pool were multiplied by 3.46/1.76 to correct for difference in pool size. 120 s was the maximum allowed swimming time. Physiological values (before CPB, 30 min CPB, end CPB, 1 hr postoperatively) were compared using the Student's -t test. MWM latencies were analyzed using repeated measurements analysis of variance. $p < 0.05$ was considered significant.





## Results

The studies meeting the eligibility criteria and the number of rats thus included in the pooled analysis are depicted in Table 1. Because a neonatal oxygenator was used in the young rat study (*Chapter 2*) and the temperature study (*Chapter 4*) no animals from these studies were included. All old animals and diabetic animals (*Chapter 3*) were included. Despite the high mortality in the treatment group in the perfluorocarbon (PFC) study (*Chapter 8*), complete data sets were available for saline CPB and saline sham groups and thus these animals were also included. From a pilot study investigating the effect of CPB on cerebral embolization, a CPB and a sham group without cerebral embolization (sham embolization) were included. Finally, from a study investigating the effect of a proprietary anti-inflammatory drug on CPB, the saline CPB and sham group were also considered.

Overall, data of 44 sham animals and 41 CPB animals could be included. All were male Wistar or Sprague-Dawley rats. In most studies MWM testing was initiated on the third postoperative day, however, in the study of old animals testing was started on postoperative day 8. For analysis purposes, the first day of cognitive testing was considered identical in all experiments. All animals underwent at least five days of MWM testing and only these days were included for analysis.

Physiological parameters at several different time points are depicted in Table 2.

Before the start of CPB, physiological parameters were comparable for all groups.

*Table 1. Studies included in the pooled analysis. Numbers of animals, postoperative starting-day of MWM testing and length of testing are tabulated.*

| study name | sham animals | CPB animals | start day MWM | days of testing | comments |
|---|---|---|---|---|---|
| Old | 11 | 9 | 8 | 7 | |
| Diabetic | 7 | 7 | 4 | 5 | |
| PFC | 10 | 10 | 3 | 7 | no perfluorocarbon |
| Embolization | 5 | 5 | 5 | 5 | no emboli |
| Inflammation | 11 | 10 | 3 | 7 | no active drug |
| **total** | **44** | **41** | | | |

*Table 2. Physiological values at different time points; means (SD)*

| | baseline | CPB 30 min | CPB end | 1 hr post |
|---|---|---|---|---|
| **Weight (g)** | | | | |
| sham | 482 (150) | | | |
| CPB | 435 (136) | | | |
| **Hematocrit (%)** | | | | |
| sham | 41 (3) | 27 (7) | 29 (6) | 27 (4) |
| CPB | 42 (4) | 24 (3)[a] | 23 (3)[a] | 29 (4) |
| **Glucose (mmol/L)** | | | | |
| sham | 9.7 (7.1) | | 11.5 (7.8) | |
| CPB | 10.1 (7.9) | | 10.2 (6.7) | |
| **Pericranial Temperature (˚C)** | | | | |
| sham | 37.2 (0.7) | 37.1 (0.4) | 37.1 (0.7) | 37.4 (0.7) |
| CPB | 37.0 (0.6) | 36.7 (0.4)[a] | 37.0 (0.4) | 36.9 (0.3)[a] |
| **CPB flow (ml/min)** | | | | |
| sham | | – | – | |
| CPB | | 60 (11) | 61 (12) | |
| **MAP (mmHg)** | | | | |
| sham | 84 (27) | 68 (14) | 67 (11) | 66 (10) |
| CPB | 81 (22) | 62 (11) | 65 (12) | 71 (9) |
| **pH** | | | | |
| sham | 7.43 (0.05) | 7.38 (0.06) | 7.38 (0.07) | 7.37 (0.05) |
| CPB | 7.42 (0.06) | 7.41 (0.05)[a] | 7.39 (0.09) | 7.36 (0.05) |
| **$PaCO_2$ (mm Hg)** | | | | |
| sham | 43.2 (8.3) | 45.5 (10.9) | 45.0 (11.1) | 42.6 (5.3) |
| CPB | 46.3 (8.4) | 39.4 (5.2)[a] | 42.8 (9.7) | 47.0 (7.9)[a] |
| **$PaO_2$ (mm Hg)** | | | | |
| sham | 173 (46) | 224 (75) | 231 (92) | 257 (94) |
| CPB | 150 (54) | 328 (137)[a] | 345 (146)[a] | 156 (93)[a] |
| **$HCO_3^-$ (mmol/L)** | | | | |
| sham | 28.0 (2.0) | 26.0 (2.6) | 25.9 (2.5) | 24.5 (2.3) |
| CPB | 28.9 (2.1) | 24.8 (2.5)[a] | 24.8 (3.0) | 26.0 (2.0)[a] |

CPB=cardiopulmonary bypass, MAP= mean arterial pressure
a= p<0.05 when compared to sham animals

However, at different time points during the extracorporeal circulation or in the postoperative period, Hct, pericranial temperature, $PaCO_2$, $PaO_2$ and bicarbonate were different between the two groups. All values were well within their physiological range. The difference in Hct values during CPB can be explained by the deliberately low Hct values in the PFC CPB group as these animals served as controls in a study evaluating an artificial oxygen carrier. The corresponding sham group was not hemodiluted to the same extent. Glucose levels were relatively high in both groups due to the inclusion of diabetic





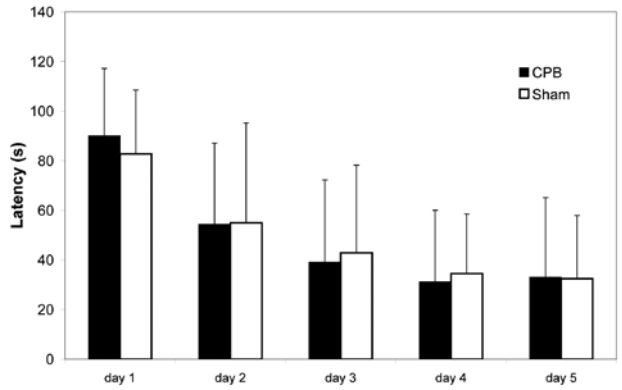



*Figure 1*

Latency of sham and CPB animals groups in the Morris water maze during five consecutive days. Bars represent means (sd)

animals. Due to the nature of CPB, temperatures in the CPB group were slightly lower than in the sham group.

The latencies are depicted in Figure 1. Both groups show a decrease in latency over time ($p<0.001$), however there were no differences between the groups ($p= 0.96$)

## Discussion

In the present analysis the MWM results from five studies by the same investigator in two laboratories were pooled to investigate the effect of CPB on MWM performance. There were in total 44 CPB and 41 identical sham animals included. Both groups showed improved performance in the MWM over the five day time span, but no difference between the groups could be detected. This supports the hypothesis that CPB as performed in these particular experiments, with the use of an appropriately sized oxygenator, is only a subtle stressor of the central nervous system and not sufficient to consistantly produce the type of cerebral injury we are attempting to model.

In all five separate studies included, no difference between CPB and sham animals was observed in MWM testing. However, the group sizes were limited and ranged from 5 to 11 animals. Statistical power could have been an issue, as most studies were powered to detect a 45% difference in latency. In the present analysis with 44 animals per group, a

20% difference in latency can be detected (power: 0.8). To detect an even more subtle difference of 10% in MWM latency, 142 animals per group would be needed. As such numbers of experimental animals are practically impossible, the addition of superimposed insults such as cerebral emboli to obtain a model in which putative neuroprotective strategies can be tested is necessary. Jungwirth *et al* recently described such a model.[3] This thesis also describes a pilot study examining a model for bilateral cerebral embolization that quantifies actual cerebral embolic delivery (*Chapter 5*).

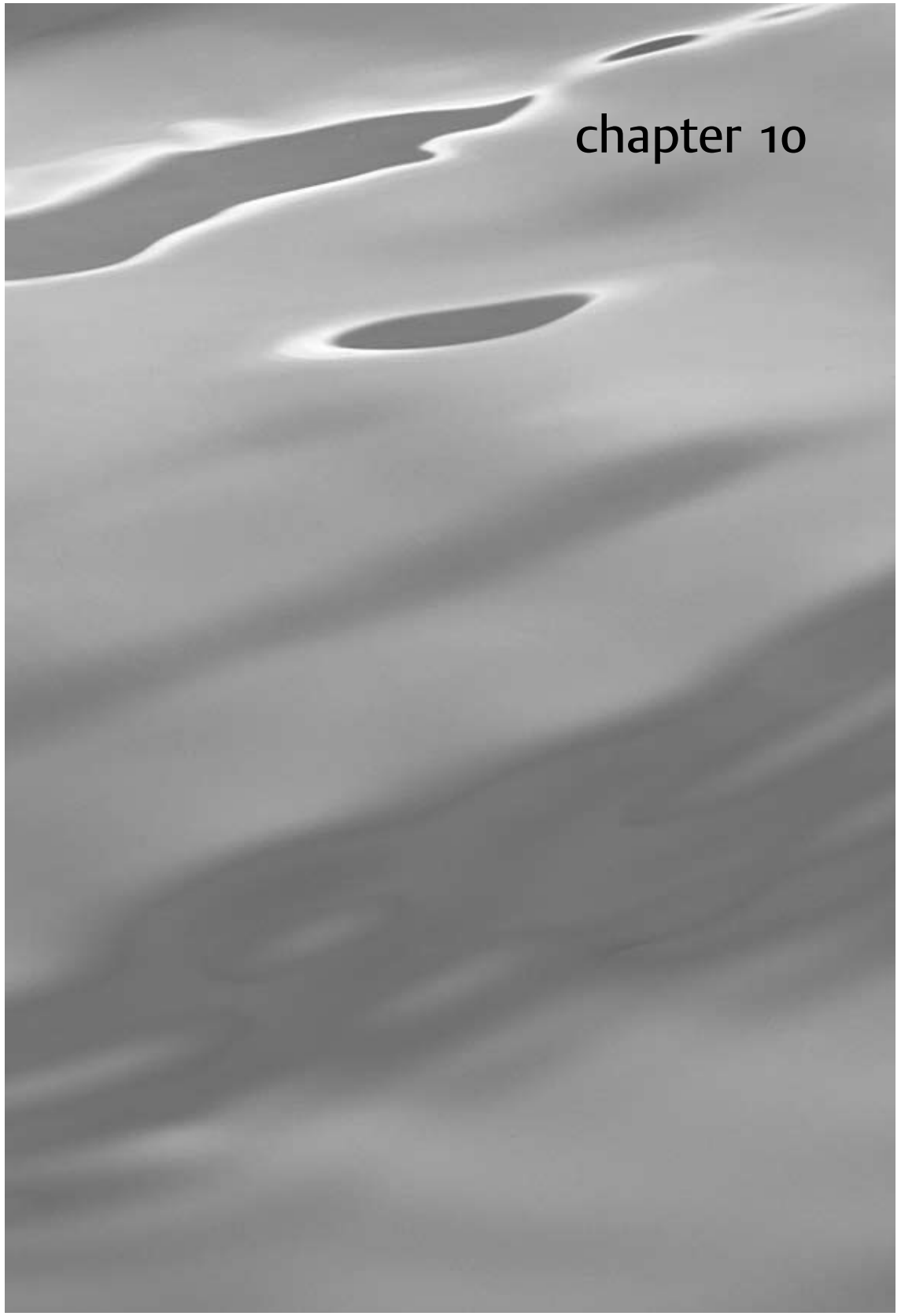
This post-hoc pooled analysis does have several potential limitations. It does not include postoperative days 6 and 7 of MWM testing as performed in three of the five included studies. We chose to only use the complete data sets and thus excluded days 6 and 7. However, as can be seen in Figure 1, a plateau in MWM latency is reached on day 4 and no further decrease in latency occurs. As a result, it is very likely that days 6 and 7 would have had the same plateau levels as days 4 and 5 and therefore their omission probably will not have influenced the results of this analysis. Secondly, the MWM testing was performed in two different laboratories with two differently sized pools. The surface area of one pool was 3.46 $m^2$, the other was 1.76 $m^2$. As size of the pool is related to at random chance to find the platform, we decided to correct for difference in pool size by multiplying the latencies obtained in the smallest pool by 1.97 (=3.46/1.76). As both platforms were equally sized no correction for platform size seemed necessary.

It is remarkable that in the smallest sized pool all animals were able to find the hidden platform within the maximum set time limit, whereas in the large pool several animals reached the maximum without finding the platform. One explanation for this observation could be the fact that the laboratories did not only differ in pool size but also in rat strains used. Overall swimming speeds for Wistar rats has been shown to be significantly lower than for Sprague-Dawley rats.[4] The laboratory with the 1.76$m^2$ pool used Sprague-Dawley rats (faster swimmers) for their experiments and this may have contributed to the lower latencies in the smaller pool. For example, in the diabetic study (Wistars) the swimming speeds averaged 20.9 ± 2.9 cm/s , whereas in the perfluorocarbon study( Sprague-Dawleys) the swimming speeds averaged 40 ± 2.2 cm/s. Importantly, in none of the studies included in this analysis did CPB and sham groups differ in swimming speeds. We corrected for pool size but did not correct for swimming speeds as assumed similar effects for sham and CPB groups.

In summary, this pooled analysis of MWM data from five different rat CPB studies suggests that minor impairments in MWM performance that might be caused by CPB *per se* using the appropriately sized oxygenator are likely too subtle to be detected with group sizes smaller than 40 animals per group.

## Reference List


1. Mackensen GB, Sato Y, Nellgard B, Pineda J, Newman MF, Warner DS, Grocott HP: Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001; 95: 1485-91
2. Jungwirth B and Kochs EF, Mackensen GB. Development of a small volume oxygenator: An essential improvement to a model of cardiopulmonary bypass in the rat. Anesthesia Analgesia 96, SCA 72. 2003.
3. Jungwirth B, Kellermann K, Blobner M, Schmehl W, Kochs EF, Mackensen GB: Cerebral air emboli differentially alter outcome after cardiopulmonary bypass in rats compared with normal circulation. Anesthesiology 2007; 107: 768-75
4. Wyss JM, Chambless BD, Kadish I, van Groen T: Age-related decline in water maze learning and memory in rats: strain differences. Neurobiology of Aging 2000; 21: 671-81



chapter 10

# General discussion

## CPB and cognitive decline; rationale for the animal model and thesis hypotheses

In 1996, halfway into what was later known as "the decade of the brain" [1] , Roach *et al* published the results of a prospective observational study on neurological outcomes after coronary bypass surgery (CABG).[2] They reported an 6.1% incidence of adverse cerebral outcomes (coma, stroke, TIA, seizures or cognitive deficits) after CABG surgery. This study led to widespread recognition of a problem that had been described in identical settings since the early 80s.[3,4] In response, investigators have trialed several agents, shown to have neuroprotective effects in animal models of cerebral ischemia, in human cardiac surgery settings. A study on nimodipine, effective in subarachnoid hemorrhage, was discontinued because of excessive mortality in the treatment group [5] and another study showed that propofol titrated to EEG burst-suppression during surgery produced hypotension but did not improve neurologic outcomes.[6] Arrowsmith et al evaluated the effect of a 9-day course of the NMDA antagonist remacemide.[7] Although there was no difference in incidence of cognitive deficits, the learning ability in the remacemide group was better. Partly due to the severe psychomimetic and other side-effects (including ataxia and dizziness), as well as internal decisions by the manufacturer, this drug was not further pursued for this indication. Overall, it must be concluded that although dozens of different agents have been investigated for their neuroprotective potential in cardiac surgery, none have proven sufficiently efficacious to warrant clinical application.[8]

These results illustrate the special difficulties of research focusing on neuroprotection in the complex setting of cardiac surgery where many factors may play a role in the etiology of neurocognitive dysfunction (NCD). Major theories regarding this etiology center around cerebral microembolization, global hypoperfusion, inflammation, hyperthermia, cerebral edema, blood brain barrier dysfunction and genetic susceptibility.[9] Due to its unique complexities, therapies that are effective against cerebral ischemic insults in the absence of cardiopulmonary bypass (CPB) may not necessarily result in better outcomes in the presence of CPB.[1,10] Before any neuroprotective therapy is tested in humans, it must have been shown to be effective and safe in an appropriate animal model.

To increase the chances of clinical effectiveness and safety, promising neuroprotective agents should ideally be tested in a primate model before initiating clinical trials. Large animal models of CPB in dogs, sheep, pig and rabbits have been used but their use is limited to experiments with small sample sizes due to the costs of operating in a full-scale environment and the personnel involved. In small animal models utilizing rats however, a single investigator can perform the entire experiment. Moreover, rats are widely used in neuroscience research and a variety of neurobehavioral and cognition test





are well described. Therefore, an appropriate rat model for CPB resulting in measurable cognitive outcomes would be an effective tool to facilitate translational research. Several models of CPB in rats have been described [11], but Grocott and Mackensen at Duke University Medical Center (Durham, NC, USA) were the first to actually describe a rat survival CPB model with neurologic and neurocognitive outcome measures.[12,13] The aim of this thesis was to validate this model and to conduct mechanistic studies investigating the effect of physiological factors such as perfusion pressure, temperature, age and cerebral embolization on cognitive outcome, thus progressing the study of putative pharmacological neuroprotective strategies.

**Findings from Utrecht University and Duke University**

In collaboration with Grocott and Mackensen from Duke University, a rat CPB set-up almost identical to the Duke model was established in Utrecht. The initial studies at Duke University focused on short-term outcomes (up to day 12 postoperatively) Consistent visuo-spatial impairments in Morris water maze (MWM) testing after CPB were found.[12-14] In Utrecht, the initial goal was to study long-term cognitive outcomes (1-3 months). However, in the first study from the Utrecht laboratory, 60 min of CPB did not cause detectable cognitive deficits in healthy young rats (*Chapter 2)*. In this study, longitudinal long-term outcomes were assessed 4-7 weeks after CPB using several different test paradigms of the Can test. Twelve weeks after CPB visuo-spatial learning capabilities were also tested in the Morris water maze (MWM). The difference between the findings might be explained by the difference in focus of the experiments: short-term versus long-term. It is conceivable that the deficits were transient and no longer evident after one to three months. However, if cognitive dysfunction after CPB in the young healthy rat is only transient and not long-lasting, the clinical relevance of the model to test protective strategies for CPB is questionable. In addition, one might question the use of young healthy animals in a model for a condition that mainly affects the elderly.[2] Therefore, the model needed tailoring to the clinical situation.

Several human studies have identified old age and diabetes mellitus as independent risk factors for neurocognitive decline after cardiac surgery.[15,16] In animal models of neurological injury, increased vulnerability of the aging brain to various insults has been demonstrated repeatedly. Not only do aged rats demonstrate increased infarct volumes in stroke models [17,18], they also have a reduced capacity for neurogenesis after ischemia [19] and demonstrate increased apoptosis and impaired MWM performance after intermittent episodes of hypoxia.[20] This reduced capacity for regeneration and loss of neurons and synapses, especially in the hippocampus, leads to deficits in spatial learning and memory.[21,22]

Diabetes mellitus can be induced in rats by injecting streptozotocin two months before

the start of an experiment.[23] Experimentally induced diabetes in young rats causes irreversible cerebral microvascular changes, decreased cognitive performance and increased infarct volumes in experimental stroke models.[24] In addition, using the MWM, it has been shown that 8 weeks of untreated streptozotocin-induced diabetes produced impairments in visuo-spatial learning in diabetic rats.[25] We decided to test the hypothesis that 90 min of CPB in these two animal models with increased susceptibility for CNS injury would lead to impaired MWM performance. Neither old nor diabetic animals subjected to CPB had consistent short-term or long-term deficits when compared to identical sham animals (*Chapter 3)*. Despite the lack of difference in cognitive outcomes, CPB did lead to an increase in plasma IL-6 levels in diabetic animals compared to diabetic non-CBP animals. This observed absence of visuo-spatial deficits after CPB is in apparent contradiction with the previous studies from Duke [12-14] and this warrants further discussion.

Although the conduct of bypass was the same in both laboratories, the bypass set-up showed several differences that might have contributed to the differences observed. Firstly, at the time the model was originally developed, no miniature oxygenators were available. As a result, the smallest (volume 40 mL) clinically available neonatal oxygenator had to be used. This membrane oxygenator was approximately 20 times oversized for rats when compared with the clinical situation (0.66 vs 0.02-0.04 $m^2 kg^{-1}$). Both laboratories used the oversized oxygenator in the early studies with the young rats, however for the old/diabetic study in Utrecht a novel appropriately sized miniature oxygenator was used, thus reducing priming volume and blood-membrane contact area. Secondly, in the previous studies at Duke University, the oxygenator and tubing were cleaned, sterilized and reused. It is thus conceivable that, despite cleaning, the membranes were still partly coated with protein residues which could have resulted in an exaggerated inflammatory response or dislocation of organic aggregates from the system into the animal. In Utrecht, a bubble trap was inserted in the arterial line, whereas no such device was used at Duke. A possibly larger embolic load and more pronounced inflammatory response may thus have accounted for the described visuo-spatial impairments found in the Duke laboratory.

To investigate the effect of CPB on inflammation, we chose to measure the pro-inflammatory cytokine IL-6 in the old/diabetic study because it is released relatively downstream in the inflammatory cascade and consistently increased after cardiac surgery.[26] The observed increase in IL-6 levels in the diabetic animals after exposure to CPB was in accordance with the literature. The lack of a between-group difference in the IL-6 levels in the aged animals might possibly be attributed to the larger variability in the data as well as undiagnosed underlying illnesses.

In the first study performed in Utrecht, healthy young rats underwent 60 min of CPB





*(Chapter 2)*, but surprisingly no differences in IL-6 levels were found between CPB and sham animals. One hypothesis is that the lack of an inflammatory response after CPB can be explained by continued ventilation of the lungs during CPB in that early study. During full CPB in humans the lungs are typically excluded from the circulation and only minimal nutritive flow to the lungs via the bronchial arteries continues. This ischemia/reperfusion injury to the lung has been shown to be a major contributor to cytokine release after CPB (beside myocardial ischemia and contact activation in the CPB circuit).[27] In the rat CPB model however, complete CPB is difficult to achieve as venous flow is passively drained by a single cannula, visual assessment of cardiac volumes is impossible and electrically-induced cardiac arrest cannot be sustained. As a result, variable residual pulmonary blood flow may remain, thus attenuating the ischemia-reperfusion of the lungs and lowering the inflammatory response. Ventilation may even elicit additional pulmonary circulation and hence could have led to even lower cytokine levels, making differences harder to detect. In the clinical situation, continued pulmonary flow can be achieved with the Drew-Anderson technique. In this technique right heart bypass is achieved by cannulating the right atrium and the pulmonary artery. The left atrium and aorta are canulated for left heart bypass and the patient's own lungs can serve as oxygenators while on CPB, thus reducing the inflammatory responses and improving pulmonary outcomes.[28-30] Aiming to mimic the clinical scenario as closely as possible, we decided to discontinue ventilation during CPB in all subsequent rat CPB studies.

**Assessment of neurocognitive function**

To assess whether CPB induces NCD in the rat model, an appropriate test, validated in rodent behavioural studies, had to be used. Besides the Can test [31], which had proven its value by being able to detect subtle cognitive impairments in diabetic rats, the Morris water maze was used for cognitive evaluation. First described by Morris in 1982 [32], it is a widely accepted test investigating visuo-spatial learning and memory in rats and mice.[33] Functional hippocampal integrity is essential for normal performance.[34] As the CA-1 region of the hippocampus is most vulnerable to injury, the Morris maze is sensitive to detect subtle differences even after non-surgical insults in rats.[22]

The execution of reliable neurocognitive tests in small animals is a difficult task. It preferably needs a totally controlled environment without distracting internal or external influences. Furthermore it requires an experienced experimenter that consistently performs the tests exactly the same way every time with each animal being approached and handled identically.[35,36] In the study from Utrecht subjecting young rats to CPB *(Chapter 2)* the Morris maze tests were performed by a very experienced technician from the Rudolf Magnus Institute of Neurosciences. In the study of old and diabetic animal groups the tests were performed in a dedicated room after rigorous training of the

experimenter at the Rudolf Magnus Institute. This gives us reason to believe that small changes in cognitive performance were unlikely to be missed due to improper execution or lack of sensitivity of the test. Based on the experiments performed in Utrecht we also have to conclude that CPB, using an appropriately sized, disposable oxygenator, did not lead to demonstrable short-term or longterm visuo-spatial impairments in old or diabetic animals with increased susceptibility to NCD.

**General considerations in rodent models**

In contrast to the previous paragraph one might argue that CPB, using a disposable system and an appropriately sized oxygenator, does cause deficits in cognitive performance but that these deficits are too subtle to detect. When we consider the study in old rats performed in Utrecht, power analysis shows that this study was sufficiently powered to detect gross differences in MWM latency, but lacked power to detect subtle abnormalities. The laboratory in Utrecht based its sample size estimation on the differences described in the early Duke University studies. For example, 10 animals per group would allow detection of a 45% difference in MWM latency at day 2 with 80% power (mean latencies 100 and 55 s, SD 35 s). However, to detect a more subtle difference of 25% (mean latencies 100 and 75 s, SD 35 s) group size would need to be increased to 32 animals per group. To detect a 10% latency decrease (latencies 100 and 90 s, SD 35 s) at least 193 animals per group are needed (calculations performed by Powercalculations, www.stat.ubc.ca). In *Chapter 9* of this thesis we present a pooled analysis of five rat CPB studies, all performed by the same investigator. Although each group consists of over 40 animals it is powered to detect a 20% difference in latency. False negative interferences, also known as type II errors, can occur when statistical test are unnecessarily stringent or sample sizes too small to test the hypothesis under consideration.[37]

The identical situation can be observed in the clinical setting. The Octopus trial compared postoperative cognitive decline (POCD) in on-pump and off-pump CABG patients.[38] When this randomized trial was designed, a high incidence (21%) of POCD was expected after on-pump CABG, and a sample size of 280 patients was proposed to be sufficient to demonstrate a cognitive benefit of off-pump CABG. The true incidence of POCD three months after on-pump CABG, however, appeared to be less than 12%, and a statistically significant difference in POCD favoring off-pump CABG could only be demonstrated in secondary analyses. Thus, the incidence of POCD was clearly overestimated during the design of the Octopus study as power calculations were based on older studies, reporting higher incidences of POCD. There seems to be a more general principle that the first studies describing a new phenomenon (in this case POCD) report more pronounced effects than later studies on the same topic. This has been named the Proteus phenomenon and





may reflect poor study design, small sample sizes, unsuitable definitions of outcome measures, lack of control groups, inadequate randomization procedures, and publication bias.[39] All these sources of bias may also be present in animal studies. Van der Worp *et al* found in a meta-analysis of 45 preclinical (animal) studies whose positive outcomes resulted in large clinical trials with thousands of patients, that the median group size was 9, the maximum 23.[40] Moreover, methodological flaws were observed in all but one study. These methodological inaccuracies very well could be a fundamental source of bias in preclinical studies. Bias is defined as the combination of various design, data, analysis and presentation factors that tend to produce research findings when they should not be produced.[41] Although well-known but rarely described in clinical studies [42], experimenter bias has been repeatedly described in laboratory studies testing animal behaviour. Experimenters who were told to expect a certain behaviour in rats, recorded this behaviour more often than experimenters who were not.[43] And in their meta-analysis, Van der Worp showed that random treatment allocation was only reported in 42% of the animal studies, blinded administration of an agent only in 22% and blinded assessment of outcome in only 40%.[40] Based on their meta-analysis, Van der Worp concluded that in state of the art preclinical research procedures used to prevent experimenter bias should be published in more detail. In our studies, we tried to minimize bias by randomization of treatment allocation, blinding of the experimenters performing the neurocognitive tests and histology, computerized analysis of Water maze results and excluding the "surgeon" from performing cognitive testing. Especially in a one-experimenter set-up as in the rat CPB model, where the experimenter can be the surgeon and the behavioural investigator, caution is warranted to prevent bias.

**The temperature study (chapter 4); conflicting results?**

Laboratory settings have often demonstrated the beneficial effect of hypothermia on experimental cerebral ischemia.[44-47] However, in the setting of cardiac surgery its neuroprotective properties remain unclear. Hypothermic bypass has been examined in a number of trials focusing on neurocognitive outcome but showed no beneficial effects.[48-51] A study by Grigore *et al* suggested that active rewarming at the end of CPB might play a role as slow rewarming resulted in improved cognitive outcomes when compared to conventional rewarming.[52]

The rat CPB model as used in our laboratories is well suited to study temperature regimens, especially those that cannot be performed in the clinical setting. In *Chapter 4* we describe the effect of four different perioperative temperature strategies on Morris maze performance in the rat CPB model. The animals were subjected to either a normo (37.5°C) - or hypothermic (32°C) CPB period of 90 min in combination with a 6 h hypothermic (35°C) or normothermic (37.5°C) postoperative period. In comparison to the

other groups, the hypothermic CPB group with the hypothermic postoperative period performed significantly better in the MWM. These results corroborate the findings of Nathan *et al* who demonstrated beneficial effects of limited rewarming protocols (34°C) during surgery with just surface rewarming after arrival in the ICU unit.[53]

The improved outcome in the hypothermic group does imply an inducible neurocognitive deficit that, as stated before, was difficult to induce in our earlier studies. However, the experimental conditions during these temperature experiments more consistently matched the orginal Mackensen conditions -and MWM findings- with no arterial line filtration (with the chances of larger cerebral embolic load) and larger volume membrane oxygenators, as previously discussed. This apparent contradiction in findings might also be explained by the temperature conditions during CPB in our other studies. In those studies, temperature during CPB was about 0.5-1°C lower than 37.5°C. The animal was placed on a heating pad that was intermittently activated. A drop in temperature of blood passing through the oxygenator was prevented by an integrated heat exchanger. In the temperature study however, active (re)warming to 37.5°C was continuously accomplished by use of heating blankets, convective air systems, water jacketed venous reservoirs and arterial lines and a heated operating platform. It might therefore be possible that the true hypothermic CPB/postoperative group had improved outcomes because strict normothermic bypass (that is, the active rewarming needed to maintain the normothermia) may have had deleterious effects on the brain that manifests more easily in demonstrable cognitive deficits in the MWM. This assumption is supported by experimental evidence showing that very small differences in temperature may significantly alter cerebral outcome following ischemia.[54] In the earlier studies, lower normothermic temperatures during CPB and in the postoperative period may have had a coincidental protective effect. However, the lack of a sham group with an identical temperature regimen makes comparison to the other studies difficult.

**Limitations of the model and resolutions**

The rat CPB model set-up as originally described by Grocott and Mackensen[12,13] displayed several limitations. Most importantly, the cognitive dysfunction endpoint was inconsistently demonstrated, varying with methodological differences in the chosen experimental conditions. This raised the question as to what the value of a partial CPB model is for cognitive dysfunction when cognitive impairments are either too subtle to detect or inconsistently induced in the majority of animals.

From the clinical setting it is known that dislodging of aortic atheromatous material, air and other debris can lead to cerebral embolization.[55,56] Pugsley et al demonstrated that postoperative neuropsychologic deficits are related to the number of cerebral emboli after routine CPB.[55] In the rat CPB model, however, arterial inflow is via the tail artery and





not in the proximal aorta. This may limit embolization to the brain or lead to inconsitent cerebral embolic loads. To study the effect of microembolization during CPB on cerebral outcomes such as cognition, a model of direct cerebral embolization was required. Therefore, we developed a model of unilateral intracarotid injection of MRI detectable microspheres, thus enabling quantification of embolic delivery *(Chapter 5)*. By clamping the contralateral carotid artery, blood flow to the brain was dependent on the carotid artery in which the emboli were injected. This approach allowed for bilateral distribution of the microspheres; as visualized by MRI, approximately 33% of microspheres were lodged in the contralateral hemisphere. In this manner, delivery failures (common when working with microspheres) can be quickly assessed and variations in outcomes may be reduced. Jungwirth et al from Munich described the effect of escalating volumes of carotid air emboli during CPB on survival but were unable to properly assess cognitive function due to the dose-finding design of that study.[57] However, these authors were able to show that significantly larger volumes of air were tolerated (using survival as an endpoint) in the absence of CPB. In a second study using the same model, Jungwirth et al were able to show that cerebral air emboli superimposed during CPB result in measurable endpoints such as infarct volumes as well as cognitive deficits.[58] In conclusion, the addition of emboli, either gaseous or particulate, to the CPB model likely improves its overall validity. This opens up the possibility of investigating potential neuroprotective compounds in the setting of CPB.

Another disadvantage of the original model was the absence of cardiac arrest resulting in continued ejection of blood from the heart during CPB, leading to incomplete (partial) CPB with parallel circulations. In rats, long-lasting cardiac arrest can only be induced and maintained by continuous alternating current to the endocardium or epicardium [59], both leading to thermal cardiac lesions, possible tamponade and other complications.[60] For optimal survivability and postoperative functioning of the animals, sternotomy (with its associated partial disruption of the upperlimb musculature) in a four-legged animal should be avoided. We therefore developed a model of cardioplegic cardiac arrest during CPB without with the need for sternotomy *(Chapter 6)*. In analogy to the Heart-Port™ minimally invasive procedure in humans [61] endoaortic crossclamping was achieved in the rat by retrogradely inserting an inflatable balloon tipped catheter just above the aortic valve. At the moment of balloon inflation, cardioplegia is administered through the integrated central lumen distal to the balloon with subsequent cardioplegic arrest being achieved. In the Duke laboratory, several studies using this model are now being conducted, investigating new cardioplegia and cardioprotective strategies.

**Applications of the cpb model not related to behavioral outcomes**

While these adaptations of the model were being developed, the original model was also

used to address other clinically relevant CPB-related research questions. Despite advances in CPB technology, including coating of systems and applying cell saving techniques, activation of the inflammation and coagulation cascades still remains a relevant clinical problem. Hence the original model is well fitted to investigate coagulation related research.

In the clinical environment, the "historical" first choice for anticoagulation, heparin, is now being reconsidered. The use of heparin may lead to formation of antibodies directed to complexes containing heparin and the endogenous platelet factor 4 ($PF_4$). Not only are these $HepPF_4$ antibodies related to various adverse outcomes after cardiac surgery themselves, they also can result in heparin-induced thrombocytopenia with thrombosis.[62]

In *Chapter 7* the anticoagulant, inflammatory and cardio-depressive properties of a novel anticoagulant-antidote pair have been compared with heparin-protamine. This newly developed RNA molecule with antagonistic activity against factor IXa was shown to be as effective as heparin regarding anticoagulation and resulted in similar degrees of inflammation, but improved cardiac performance three hours after CPB.

In addition, we used the rat CPB model to investigate an artificial oxygen carrier.

A perfluorocarbon (PFC) solution, able to carry supra-physiological amounts of oxygen into the microvasculature and to absorb gaseous emboli, was thought to possibly improve postoperative neurocognitive outcome by improving oxygen delivery and attenuating ischemia-reperfusion induced inflammation. Further, PFC administration had previously been shown to decrease gaseous microbubble volumes during experimental CPB in rats.[63] Therefore, we tested this compound in the rat CPB model hypothesizing that cognitive function following CPB would improve *(Chapter 8)*. However, administration of PFC caused excessive release of cytokines, increased areas of myocardial necrosis and 100% mortality in the treatment group. The reason for this is still unclear, but we speculate that the large cytokine increase seen in this present study was due to a perfluorocarbon specific amplification of the inflammatory response normally seen in CPB. Use of this PFC solution in the rat CPB model alerted us to a potentially lethal complication not seen in rat studies without CPB, thus proving the validity of the model for assessing effectiveness and safety of agents in the setting of CPB.

**Future studies**

We originally anticipated that this rat model for CPB would allow us to produce consistent NCD that could be detected with standard neurocognitive testing such as the MWM. Combining the results of several studies with this model from the Duke and Utrecht laboratories, we conclude that CPB *per se* in rats is not a consistent stressor for the central nervous system to induce reproducible deficits (*Chapters 2, 3, 9*). However, the model is extremely useful to test novel anticoagulants, artificial blood products or





anti-inflammatory agents. In experienced hands, 90% survival can be obtained, the opportunities for blood sampling are ample and histology can be performed when desired. The combination of the model with cerebral (air) emboli has now facilitated studies investigating possible neuroprotective agents.[58] Because our investigations focused on the brain , the effect of (partial) CPB on other organ systems is still unknown. The effect of CPB on intestinal motility in the same model is now being investigated (Epema, Groningen, the Netherlands) and renal outcomes are evaluated by Mackensen (Duke, North Carolina) and Mazer (Toronto, Canada). In addition the laboratory in Munich (Jungwirth) developed a model for deep hypothermic cardiac arrest using the CPB set-up.[64]

The CPB model with cardioplegic cardiac arrest, as described in *Chapter 6*, creates a new era of possibilities. This novel CPB model is the first rodent cardioplegic cardiac arrest model with good survivability in a field that until today used isolated heart models. It allows for both the study of myocardial ischemia-reperfusion injury as well as the evaluation of new cardioprotective strategies and mimics the spectrum of injuries associated with clinical cardiac surgery. Models such as the one described will likely be utilized to not only assess longer-term cardiac functional outcomes (using echocardiography) but also to characterize the enzymatic, genetic, and histologic response to myocardial injury and protective strategies. We anticipate that the models that we developed will continue to contribute to a better understanding of pathophysiologic mechanisms and help to develop protective strategies leading to improved perioperative outcomes.

## Reference List


1. Hindman BJ, Todd MM. Improving neurologic outcome after cardiac surgery. Anesthesiology 1999;90:1243-7.
2. Roach GW, Kanchuger M, Mangano CM, et al. Adverse cerebral outcomes after coronary bypass surgery. Multicenter Study of Perioperative Ischemia Research Group and the Ischemia Research and Education Foundation Investigators. N Engl J Med 1996;335:1857-63.
3. Breuer AC, Furlan AJ, Hanson MR, et al. Central nervous system complications of coronary artery bypass graft surgery: prospective analysis of 421 patients. Stroke 1983;14:682-7.
4. Sotaniemi KA. Cerebral outcome after extracorporeal circulation. Comparison between prospective and retrospective evaluations. Arch Neurol 1983;40:75-7.
5. Legault C, Furberg CD, Wagenknecht LE, et al. Nimodipine Neuroprotection in Cardiac Valve Replacement : Report of an Early Terminated Trial. Stroke 1996;27:593-8.
6. Roach GW, Newman MF, Murkin JM, et al. Ineffectiveness of burst suppression therapy in mitigating perioperative cerebrovascular dysfunction. Multicenter Study of Perioperative Ischemia (McSPI) Research Group. Anesthesiology 1999;90:1255-64.
7. Arrowsmith JE, Harrison MJG, Newman SP, et al. Neuroprotection of the Brain During Cardiopulmonary Bypass : A Randomized Trial of Remacemide During Coronary Artery Bypass in 171 Patients. Stroke 1998;29:2357-62.
8. Grocott HP, Yoshitani K. Neuroprotection during cardiac surgery. J Anesth 2007;21:367-77.
9. Grocott HP, Homi HM, Puskas F. Cognitive dysfunction after cardiac surgery: revisiting etiology. Semin Cardiothorac Vasc Anesth 2005;9:123-9.
10. Hindman BJ, Dexter F, Enomoto S, et al. Recovery of evoked potential amplitude after cerebral arterial air embolism in the rabbit: a comparison of the effect of cardiopulmonary bypass with normal circulation Anesthesiology 1998;88:696-707.
11. Ballaux PK, Gourlay T, Ratnatunga CP, Taylor KM. A literature review of cardiopulmonary bypass models for rats. Perfusion 1999;14:411-7.
12. Grocott HP, Mackensen GB, Newman MF, Warner DS: Neurological injury during cardiopulmonary bypass in the rat. Perfusion 2001; 16: 75-81
13. Mackensen GB, Sato Y, Nellgard B, et al. Cardiopulmonary bypass induces neurologic and neurocognitive dysfunction in the rat. Anesthesiology 2001;95:1485-91.
14. Ma, D. Xenon attenuates cardiopulmonary bypass-induced neurologic and neurocognitive dysfunction in the rat. Anesthesiology 96, A807. 10-15-2002.
15. Newman MF, Croughwell ND, Blumenthal JA, et al. Predictors of cognitive decline after cardiac operation. Ann Thorac Surg 1995;59:1326-30.
16. Ridderstolpe L, Ahlgren E, Gill H, Rutberg H. Risk factor analysis of early and delayed cerebral complications after cardiac surgery. J Cardiothorac Vasc Anesth 2002;16:278-85.
17. Kharlamov A, Kharlamov E, Armstrong DM. Age-Dependent Increase in Infarct Volume Following Photochemically Induced Cerebral Infarction: Putative Role of Astroglia. J Gerontol A Biol Sci Med Sci 2000;55:B135-B141.
18. Davis M, Mendelow AD, Perry RH, et al. Experimental stroke and neuroprotection in the aging rat brain. Stroke 1995;26:1072-8.
19. Jin K, Minami M, Xie L, et al. Ischemia-induced neurogenesis is preserved but reduced in the aged rodent brain. Aging Cell 2004;3:373-7.
20. Gozal D, Row BW, Kheirandish L, et al. Increased susceptibility to intermittent hypoxia in aging rats: changes in proteasomal activity, neuronal apoptosis and spatial function. J Neurochem 2003;86:1545-52.
21. Gallagher M, Rapp PR. The use of animal models to study the effects of aging on cognition. Ann Rev Psych 1997;48:339-70.
22. Wyss JM, Chambless BD, Kadish I, van Groen T. Age-related decline in water maze learning and memory in rats: strain differences. Neurobiol Aging 2000;21:671-81.
23. Biessels GJ, Gispen WH. The impact of diabetes on cognition: what can be learned from rodent models? Neurobiol Aging 2005;26 Suppl 1:36-41.
24. Duverger D, MacKenzie ET. The quantification of cerebral infarction following focal ischemia in the rat: influence of strain, arterial pressure, blood glucose concentration, and age. J Cereb Blood Flow Metab 1988;8:449-61.
25. Kamal A, Biessels GJ, Duis SE, Gispen WH. Learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: interaction of diabetes and ageing. Diabetologia 2000;43:500-6.
26. Hall RI, Smith MS, Rocker G. The systemic inflammatory response to cardiopulmonary bypass: pathophysiological, therapeutic, and pharmacological considerations. Anesth Analg 1997;85:766-82.
27. Massoudy P, Zahler S, Becker BF, et al. Evidence for inflammatory responses of the lungs during coronary artery bypass grafting with cardiopulmonary bypass. Chest 2001;119:31-6.
28. Richter JA, Meisner H, Tassani P, et al. Drew-Anderson technique attenuates systemic inflammatory response syndrome and improves respiratory function after coronary artery bypass grafting. Ann Thorac Surg 2000;69:77-83.
29. Massoudy P, Piotrowski JA, van de Wal HC, et al. Perfusing and ventilating the patient's lungs during bypass ameliorates the increase in extravascular thermal volume after coronary bypass grafting. Ann Thorac Surg 2003;76:516-21.
30. Glenville B, Ross D. Coronary artery surgery with patient's lungs as oxygenator. Lancet 1986;2:1005-6.
31. Popovic M, Biessels GJ, Isaacson RL, Gispen WH. Learning and memory in streptozotocin-induced diabetic rats in a novel spatial/object discrimination task. Behav Brain Res 2001;122:201-7.
32. Morris RG, Garrud P, Rawlins JN, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. Nature 1982;297:681-3.

33. Hodges H. Maze procedures: the radial-arm and water maze compared. Brain Res Cogn Brain Res 1996;3:167-81.
34. D'Hooge R, De Deyn PP. Applications of the Morris water maze in the study of learning and memory. Brain Res Rev 2001;36:60-90.
35. Sousa N, Almeida OF, Wotjak CT. A hitchhiker's guide to behavioral analysis in laboratory rodents. Genes Brain Behav 2006;5 Suppl 2:5-24.
36. Lewejohann L, Reinhard C, Schrewe A, et al. Environmental bias? Effects of housing conditions, laboratory environment and experimenter on behavioral tests. Genes, Brain Behav 2006;5:64-72.
37. Hartung J, Cottrell JE. Statistics and hypothermia. J Neurosurg Anesthesiol 1998;10:1-4.
38. Van Dijk D, Jansen EW, Hijman R, et al. Cognitive outcome after off-pump and on-pump coronary artery bypass graft surgery: a randomized trial. JAMA 2002;287:1405-12.
39. Ioannidis JP. Evolution and translation of research findings: from bench to where? PLoS Clin Trials 2006;1:e36.
40. Worp H, Haan P, Morrema E, Kalkman C. Methodological quality of animal studies on neuroprotection in focal cerebral ischaemia. J Neurology 2005;252:1108-14.
41. Ioannidis JP. Why most published research findings are false. PLoS Med 2005;2:e124.
42. Eisenach JC, Lindner MD. Did experimenter bias conceal the efficacy of spinal opioids in previous studies with the spinal nerve ligation model of neuropathic pain? Anesthesiology 2004;100:765-7.
43. Rosenthal R, Lawson R. A longitudinal study of the effects of experimenter bias on the operant learning of laboratory rats. J Psychiatr Res 1964;69:61-72.
44. Barone FC, Feuerstein GZ, White RF. Brain Cooling During Transient Focal Ischemia Provides Complete Neuroprotection. Neurosci Biobehav Rev 1997;21:31-44.
45. Busto R, Dietrich WD, Globus MY, et al. Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. J Cereb Blood Flow Metab 1987;7:729-38.
46. Minamisawa H, Nordstrom CH, Smith ML, Siesjo BK. The influence of mild body and brain hypothermia on ischemic brain damage. J Cereb Blood Flow Metab 1990;10:365-74.
47. Minamisawa H, Smith ML, Siesjo BK. The effect of mild hyperthermia and hypothermia on brain damage following 5, 10, and 15 minutes of forebrain ischemia. Ann Neurol 1990;28:26-33.
48. Randomised trial of normothermic versus hypothermic coronary bypass surgery. The Warm Heart Investigators. Lancet 1994;343:559-63.
49. Grigore AM, Mathew J, Grocott HP, et al. Prospective randomized trial of normothermic versus hypothermic cardiopulmonary bypass on cognitive function after coronary artery bypass graft surgery. Anesthesiology 2001;95:1110-9.
50. McLean RF, Wong BI, Naylor CD, et al. Cardiopulmonary bypass, temperature, and central nervous system dysfunction. Circulation 1994;90:II250-II255.







51. Mora CT, Henson MB, Weintraub WS, et al. The effect of temperature management during cardiopulmonary bypass on neurologic and neuropsychologic outcomes in patients undergoing coronary revascularization. J Thorac Cardiovasc Surg 1996;112:514-22.
52. Grigore AM, Grocott HP, Mathew JP, et al. The Rewarming Rate and Increased Peak Temperature Alter Neurocognitive Outcome After Cardiac Surgery. Anesth Analg 2002;94:4-10.
53. Nathan HJ, Wells GA, Munson JL, Wozny D. Neuroprotective Effect of Mild Hypothermia in Patients Undergoing Coronary Artery Surgery With Cardiopulmonary Bypass: A Randomized Trial. Circulation 2001;104:851-91.
54. Warner DS, McFarlane C, Todd MM, et al. Sevoflurane and halothane reduce focal ischemic brain damage in the rat. Possible influence on thermoregulation. Anesthesiology 1993;79:985-92.
55. Pugsley W, Klinger L, Paschalis C, et al. The impact of microemboli during cardiopulmonary bypass on neuropsychological functioning. Stroke 1994;25:1393-9.
56. Abu-Omar Y, Balacumaraswami L, Pigott DW, et al. Solid and gaseous cerebral microembolization during off-pump, on-pump, and open cardiac surgery procedures. J Thorac Cardiovasc Surg 2004;127:1759-65.
57. Jungwirth B, Kellermann K, Blobner M, et al. Cerebral air emboli differentially alter outcome after cardiopulmonary bypass in rats compared with normal circulation. Anesthesiology 2007;107:768-75.
58. Jungwirth B, Gordan ML, Blobner M, et al. Xenon impairs neurocognitive and histologic outcome after cardiopulmonary bypass combined with cerebral air embolism in rats. Anesthesiology 2006;104:770-6.
59. von Planta I, Weil MH, von Planta M, et al. Cardiopulmonary resuscitation in the rat. J Appl Phys 1988;65:2641-7.
60. Chen MH, Liu TW, Xie L, et al. A simpler cardiac arrest model in rats. Am J Emerg Med 2007;25:623-30.
61. Reichenspurner H, Gulielmos V, Wunderlich J, et al. Port-Access Coronary Artery Bypass Grafting With the Use of Cardiopulmonary Bypass and Cardioplegic Arrest. Ann Thorac Surg 1998;65:413-9.
62. Bennett-Guerrero E, Slaughter TF, White WD, et al. Preoperative anti-PF4/heparin antibody level predicts adverse outcome after cardiac surgery. J Thorac Cardiovasc Surg 2005;130:1567-72.
63. Yoshitani K, de Lange F, Ma Q, et al. Reduction in Air Bubble Size Using Perfluorocarbons During Cardiopulmonary Bypass in the Rat. Anesth Analg 2006;103:1089-93.
64. Jungwirth B, Mackensen GB, Blobner M, et al. Neurologic outcome after cardiopulmonary bypass with deep hypothermic circulatory arrest in rats: Description of a new model. J Thorac Cardiovasc Surg 2006;131:805-12.

# Summary

**Chapter 1 - Introduction**

After twenty years of research, Dr John H. Gibbon performed the world's first successful open heart procedure in which total heart-lung bypass was employed. In later years, the initial 'screen' oxygenators were replaced by 'bubble' oxygenators. In the 1980s several investigators reported that large quantities of micro- and macrobubbles were present in the blood leaving these oxygenators, therefore possibly attributing to the high incidence of central nervous system complications observed after cardiac surgery. Hence these bubble oxygenators were slowly replaced by 'membrane' oxygenators. However, even in modern cardiac surgery, subtle central nervous system complications remain a problem. To elucidate the pathophysiological mechanisms underlying these neurological and neurocognitive complications, laboratory research in an appropriate animal model is necessary. A recently at Duke University Medical Center developed rat cardiopulmonary bypass model formed the basis for this thesis.

**Chapter 2 - Cardiopulmonary bypass in young rats**

To investigate the long-term effects of cardiopulmonary bypass on cognitive performance in animals and to validate the model, young male rats were exposed to either 60 min of cardiopulmonary bypass (CPB) or to cannulation only (sham animals). Long-term neurocognitive function was assessed at 4 to 7 weeks after CPB using the Can test, and again after 12 weeks using the Morris water maze.
No differences beteen the groups were found in cognitive performance, hippocampal cell counts or cytokine release patterns.These results were in contrast with previous investigations.

**Chapter 3 - Cardiopulmonary bypass in old rats and diabetic rats**

As age and diabetes are independent risk factors for cognitive decline after cardiac surgery, we evaluated the effects of CPB on short-term and long-term cognitive performance in aged and diabetic rat groups.
Aged rats (26 mo) and rats with streptozotocin-induced chronic diabetes were subjected to 90 min of CPB or an identical sham period. No differences in Morris water maze performances could be detected between the CPB and the sham animals. IL-6 assays showed an increased inflammatory response after CPB in the diabetic animals, but not in the aged rats. We conclude that CPB per se did not lead to detectable differences in Morris water maze performance in these animal groups with increased susceptibility for cognitive decline.

**Chapter 4 - Effect of perioperative CPB temperature strategies on cognitive function in rats**

Even though in many settings hypothermia is shown to be neuroprotective, in the setting of cardiac surgery clinical studies have failed to demonstrate its effect. It is hypothesized





that rapid rewarming with potential hyperthermia might attenuate the protective benefits of hypothermia. In this study we investigated the relative influence of CPB temperature, rewarming strategies and postoperative temperature in the rat CPB model.
Single episodes of hypothermia, either during or after CPB, did not improved cognitive performance. However, hypothermia, when induced during CPB and continued into the postoperative period, demonstrated a significant improvement in water maze performance. No differences in brain histology could be demonstrated between the groups.

**Chapter 5 - Cerebral embolization using MRI detectable holmium microspheres**

Several different animal models aim to mimic the dislodging of particles -as occurs during cardiac, orthopedic or cerebrovascular surgery- by injecting microspheres in the cerebral circulation. However, these models have been hindered by the fact that injection in the carotid artery only results in an unilateral distribution and unpredictable delivery which cannot be quantified directly. Using MRI-detectable holmium microspheres, we developed a method to obtain a bilateral distribution of the spheres by temporarily occluding the contralateral carotid artery. On post-injection MRI scans, one third of the dose was consistently located on the contralateral side. The procedure resulted in a reproducible administration regarding distribution and number of microspheres.

**Chapter 6 - Cardioplegic cardiac arrest during cardiopulmonary bypass in rats**

As a result of rapidly expanding surgical techniques a growing population with impaired preoperative cardiac function will undergo cardiac surgical procedures. To improve myocardial protection continuing efforts are warranted. Previous research was limited to either large animal models or ex vivo preparations. We developed a model in which a balloon catheter, retrogradely inserted from the common carotid artery to above the aortic valve, serves as crossclamp. To initiate cardiac arrest, the balloon is inflated and a cardioplegic solution injected. Rats were subjected to 30 min of complete arrest and 75 min of CPB. Fourteen days after surgery, functional assessment revealed no neurologic deficits or histological abnormalities. Due to the good survivability, this model allows for the study of myocardial ischemia-perfursion injury as well an new cardioprotective strategies.

**Chapter 7 - A Factor IXa aptamer and its antidote to replace heparin and protamine**

Heparin is the anticoagulant of choice in cardiac surgery, mainly because its effects are easily reversible. However, the clinical benefits of heparin and protamine are now being questioned because of serious side effects such as allergic reactions and heparin-induced thrombocytopenia. We investigated a single stranded RNA molecule (aptamer) and its antidote against factor FIXa and compared them with heparin/protamine in the rat CPB model. It was shown that factor IXa aptamer can replace heparin as the sole anticoagulant during CPB. The

inflammatory profiles between the aptamer/heparin animals were identical, but the aptamer group showed less decrease in platelet count and an improved cardiac performance.

**Chapter 8 - Perfluorocarbon administration during cardiopulmonary bypass in rats**

Perfluorocarbons are substances able to dissolve large quantities of gasses. In the setting of CPB this would not only lead to increased delivery of oxygen to the tissues, but also to attenuation of the number of air emboli as these can be absorbed by the perfluorocarbon. This study was designed to evaluate the effect of a 60% perfluorocarbon emulsion on the inflammatory response and neurocognitive outcomes in rats subjected to CPB. Administration of perfluorocarbon emulsion during CPB was associated with an excessive release of cytokines which may have contributed to the high mortality in rats receiving perfluorocarbon emulsion. Myocardial histology revealed increased areas of contraction band necrosis in the perfluorocarbon group. Further investigations are warranted to investigate the full effects of perfluorocarbons in the setting of CPB.

**Chapter 9 - Pooled analysis of Morris water maze data**

When the rat CPB model was first described, a neonatal (and thus oversized) oxygenator was used. However, later studies employed a newly developed miniature oxygenator. This small oxygenator reduced priming volume and blood-membrane contact area and might have attenuated the effect of CPB on Morris maze performance in rats. Coupled with insufficient statistical power (power calculations were based on the first results) this might have contributed to the lack of effect of CPB on cognition. We pooled all studies performed by the same investigator using the small oxygenator. Even though both sham and CPB groups each contained more than 40 animals, no differences in MWM performance could be detected, thus suggesting that CPB with the use of an appropriately sized oxygenator is only a subtle stressor of the central nervous system.

**Chapter 10 - General discussion**

This chapter describes the rationale behind the animal model and the thesis hypothesis we started with. It describes in detail several differences between studies with conflicting outcomes from the laboratories involved, along with the general line of thought on how the rat CPB model can be adapted to investigate several relevant research questions. In addition, we describe some of the pitfalls that we encountered. Finally, the role the rat models may play in a better understanding of pathophysiological mechanisms and development of protective strategies leading to improved perioperative outcomes, is addressed.

## Samenvatting in het Nederlands voor niet-ingewijden

**Hoofdstuk 1 - Introductie**

In 1953 werd de eerste succesvolle open-hartoperatie in de mens uitgevoerd door de chirurg John. H. Gibbon. De hart-long machine en de oxygenator (gasuitwisselaar) die daarbij gebruikt werden, waren het resultaat van twintig jaar experimenteren met proefdieren in zijn laboratorium. De plaat-oxygenator zoals door Gibbon ontwikkeld, werd later vervangen door een bubble-oxygenator. In de tachtiger jaren verschenen verschillende wetenschappelijke publicaties die beschreven dat het gebruik van deze bubble-oxygenator leidde tot aantoonbare belletjes in het bloed en dit zo zou kunnen bijdragen aan het veelvuldig voorkomen van neurologische schade bij patiënten na hartoperaties. In de jaren daarna werden de bubble-oxygenatoren vervangen door membraan-oxygenatoren. Echter, zelfs in de hedendaagse hartchirurgie kan er nog steeds subtiele neurologische uitval optreden (geheugenstoornissen bijvoorbeeld). Om de oorzaken hiervan verder te onderzoeken is gedegen onderzoek in een goed diermodel noodzakelijk. Het recent in Duke University Medical Center (North Carolina, VS) ontwikkelde rattenmodel voor het gebruik van de hart-long machine vormt de basis voor dit proefschrift.

**Hoofdstuk 2 - Hart-long machine blootstelling in jonge ratten**

Om de langetermijneffecten van het gebruik van de hart-long machine op het brein te onderzoeken, vergeleken we jonge ratten die 60 minuten aan de hart-long machine gelegen hadden met ratten die evenlang geopereerd waren maar niet aan de hart-long machine blootgesteld waren. Vier tot zeven weken na de operatie werden het leervermogen en het geheugen getest met behulp van de Blikjes test (Can test). Twaalf weken na de operatie volgden testen in een zwembad waarbij ze een verborgen eiland moesten vinden (Morris water maze). Na analyse van de resultaten bleek er geen verschil tussen beide groepen ratten te zijn; niet in de leer-en geheugentesten, niet in de hoeveelheid hersencellen en niet in de ontstekingsparameters in het bloed.

**Hoofdstuk 3 - Hart-long machine gebruik in oude ratten en in diabetische ratten**

Omdat bewezen is dat hoge leeftijd en het hebben van diabetes risicofactoren zijn voor het ontwikkelen van neurologische complicaties na hartchirurgie, onderzochten wij het effect van hart-long machine (HLM) gebruik op het geheugen en leervermogen van oude ratten en diabetische ratten. Oude ratten en ratten die diabetes ontwikkeld hadden na inspuiting van streptozotocine, werden onderworpen aan 90 minuten hart-long machine gebruik. In vergelijking met identieke ratten werd er geen verschil in geheugen en leervermogen gevonden. Wel waren de infectie parameters in de diabetische HLM-ratten verhoogd, maar niet in de oude HLM-ratten. We concludeerden dat HLM-gebruik ook in dieren die





verhoogd gevoelig zijn voor het optreden van hersenschade, niet tot aantoonbare defecten leidt.

**Hoofdstuk 4 - Het effect van temperatuur tijdens hart-long machine gebruik**

Hoewel in verscheidene ziektebeelden het gunstige effect van koeling voor het brein is bewezen, is het beschermende effect van koeling tijdens hartchirurgie voor het brein nog onduidelijk. De reden daarvoor is dat de beschermende werking van koeling teniet gedaan zou kunnen worden door het relatief snelle “opwarmen” aan het einde van de operatie. In dit hoofdstuk bestudeerden we het effect van ondertemperatuur tijdens en na de operatie in het ratten HLM-model. We toonden aan dat een enkele periode van ondertemperatuur, tijdens of na de operatie, geen verbetering geeft op het geheugen en leervermogen. Indien echter ondertemperatuur, gestart tijdens de operatie, voortgezet wordt in de eerste uren na de operatie, heeft dit wel een beschermend effect. In de hersenen van al deze dieren zagen we overigens geen verschil.

**Hoofdstuk 5 - Embolisatie naar het brein van MRI-aantoonbare microbollen**

In de onderzoekswereld zijn verscheidene diermodellen bekend die embolisatie nabootsen. Embolisatie is het losraken van kalkdeeltjes, vet of bloedstolsels zoals dat kan gebeuren tijdens hart-, hersen- of botchirurgie. Deze diermodellen worden echter beperkt door het feit dat éénzijdige injectie in een aanvoerend bloedvat naar de hersenen slechts zorgt voor vastlopen in één hersenhelft en niet in beide. Bovendien was de aankomst van de geïnjecteerde deeltjes niet voorspelbaar en kon deze niet gemakkelijk geëvalueerd worden. Wij beschrijven een methode om bij éénzijdige injectie van deeltjes toch een tweezijdige verdeling (ongeveer 2/3- 1/3) over de hersenhelften te krijgen. De door ons geïnjecteerde microbollen zijn zichtbaar op MRI-beelden en zo kan de aankomst en verdeling gecontroleerd worden.

**Hoofdstuk 6 - Stilzetten van het hart tijdens hart-long machine gebruik**

Door de toegenomen chirurgische behandelmethodes en de almaar ouder wordende bevolking, zal er een toenemende vraag zijn naar manieren om het (stilliggende) hart tijdens hartoperaties te beschermen. Onderzoek naar bescherming van het hart tijdens HLM-gebruik en daarna was echter alleen mogelijk in grote proefdieren en losse preparaten. We pasten ons bestaande HLM-model in ratten aan, zodat het hierin mogelijk was het hart stil te leggen zonder de borstkas te openen. We beschrijven hoe, via een ballon catheter tijdens HLM-gebruik in de halsvaten ingebracht, vloeistoffen toegediend kunnen worden die het hart stil leggen. Na 30 minuten komt het hart weer spontaan op gang en daarna kan de HLM-periode weer beëindigd worden. Er bleken op dag 14 na de operatie geen afwijkingen in de motoriek van de dieren te zien te zijn en ook waren er geen aantoonbare

grove afwijkingen in de hersenen te zien. Vanwege de goede overleving kan dit model zeer geschikt zijn om het ontstaan van en beschermende strategieën voor hartschade tijdens HLM-gebruik te onderzoeken.

**Hoofdstuk 7 - Een nieuw RNA-molecuul dat heparine kan vervangen tijdens hart-long machine gebruik**

Om klontering van bloed tijdens HLM-gebruik te voorkomen wordt van oudsher heparine toegediend tijdens hartchirurgie. De werking van heparine kan ongedaan gemaakt worden door toediening van protamine, waarna het bloed dus weer kan stollen. Aan het gebruik van heparine en protamine kleven echter verscheidene nadelen zoals het kunnen optreden van allergische reacties en een mogelijke daling van het aantal bloedplaatjes. In ons ratten HLM-model vergeleken we heparine/protamine met een nieuw ontwikkeld RNA-molecuul dat een bepaalde factor van het stollingsmechanisme onwerkzaam maakt. Na toediening van een ander RNA-molecuul dat het werkzame deel weer wegvangt, kan de factor zijn werk weer doen en het bloed weer stollen. We toonden aan dat het RNA-molecuul even goed het bloed kan ontstollen als heparine, maar dat de bloedplaatjes minder dalen en het hart nadien krachtiger klopt.

**Hoofdstuk 8 - Toediening van een kunstmatige zuurstofdrager tijdens hart-long machine gebruik**

Perfluorocarbonen zijn kunstmatige zuurstofdragers die naast het vervoeren van zuurstof ook gasbelletjes, zoals die kunnen ontstaan bij HLM-gebruik, kunnen absorberen. We testten het effect van een perfluorocarbon emulsie op de ontstekingsparameters en het geheugen en leervermogen in het ratten HLM-model. Toediening van de perfluorocarbon emulsie resulteerde in een enorme stijging van de ontstekingsparameters en hoge sterfte van de ratten. Tevens vonden we bij microscopisch onderzoek toegenomen weefselschade in de hartspier van de perfluorocarbon dieren. Meer onderzoek is noodzakelijk voordat perfluorocarbonen in de kliniek kunnen worden toegepast.

**Hoofdstuk 9 - Samenvoeging van data uit verschillende studies met de Morris water maze**

Bij de eerste beschrijvingen van het ratten HLM-model werd gebruik gemaakt van de kleinste commercieel verkrijgbare (kinder)oxygenator, die voor een rat nog altijd ongeveer 20x te groot is. Bij latere studies kon een speciaal ontwikkelde ratten-oxygenator gebruikt worden, waarbij dus het opvul-volume en het contact oppervlak duidelijk kleiner waren. Deze aanpassing zou echter het schadelijke effect van de HLM op de het brein verminderd kunnen hebben. Omdat het geteste aantal ratten gebaseerd was op berekeningen met de grote oxygenator zou het dus kunnen dat kleinere verschillen in leervermogen, veroorzaakt





door de aangepaste oxygenator, niet opgemerkt werden omdat we te weinig ratten getest hebben. In dit hoofdstuk voegden we de resultaten van de Morris water maze (eiland zwemtest) van ratten uit vijf verschillende studies samen. Ook met deze grote groepen ratten bleek er geen verschil te zijn tussen HLM-ratten en niet-HLM-ratten in de resultaten van de Morris water maze. Dit suggereert dat HLM-gebruik met een evenredig geproportioneerde oxygenator mogelijk alleen tot subtiele schade leidt in het brein.

**Hoofdstuk 10 - Algemene beschouwing**

In de algemene beschouwing bekijken we het oorspronkelijke uitgangspunt van de onderzoeken en beschrijven de wijzigingen in het model in verloop van de tijd. De verschillen in uitkomsten tussen de studies worden besproken en mogelijke verklaringen gegeven.
Tevens worden enkele algemene methodologische aspecten, belangrijk in dierexperimenteel onderzoek, belicht. Ter afsluiting bespreken we verdere onderzoeksmogelijkheden met het ratten HLM-model, bedoeld om de mechanismen achter hersenschade tijdens hartoperaties te begrijpen en zo te komen tot mogelijk beschermende maatregelen.

# Dankwoord

Toen ik in 2001 begon met de combinatie van onderzoek en opleiding, had ik geen idee welke intense jaren mij te wachten stonden. Deze intensiteit werd ruimschoots gecompenseerd door alle leuke en verrijkende ervaringen die ertegenover stonden. Het ontmoeten van nationaal en internationaal gerenommeerde onderzoekers uit verschillende vakgebieden, de samenwerking met allerlei uiteenlopende specialismen, het kijken buiten je eigen Nederlandse ziekenhuis en natuurlijk de bijna twee jaar in de Verenigde Staten zijn voor mij van grote waarde geweest. Zonder dit proefschrift was dat alles niet mogelijk geweest. Er zijn ontelbare mensen aan wie ik mijn dank verschuldigd ben, helaas kan ik slechts een aantal daarvan hier noemen.

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De leden van de beoordelingscommissie: Prof. Dr. P.R. Bär, Dr. R.M. Dijkhuizen, Prof. Dr. S.G. de Hert, Prof. Dr. L.A. van Herwerden en Prof. Dr. F. Ohl wil ik hartelijk bedanken voor de bereidwilligheid in deze commissie plaats te nemen en mijn manuscript in recordtempo door te lezen.

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Dankwoord

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Curriculum Vitae

## Curriculum Vitae

Fellery de Lange was born on September 4, 1972 in Leiden, the Netherlands. After graduating from high school at the 'Christelijk Lyceum' in Alphen aan den Rijn, she studied medicine at the Utrecht University from 1990 to 1997 where she received her Medical Degree.

From 1997 to 2000 she worked as a resident-not-in-training in the Department of Cardiothoracic Surgery (chair Prof.dr. J.J. Bredée). The last two years of this period, she worked in the Cardiac Surgery Intensive-Care Unit (head Dr. H. Wesenhagen).

In 2000, Fellery began working as a resident not-in-training in the Department of Anesthesiology at the Utrecht University Medical Center. In 2001, she initiated the research described in this thesis (promotor Prof. Dr. C.J. Kalkman) and simultaneously began her specialist training in Anesthesiology (chair Prof. Dr. J.T.A. Knape).

In 2004 and 2005, she worked as a Research Associate at the Duke University Medical Center, North Carolina, USA (co-promotores, Drs. H.P. Grocott and G.B. Mackensen). As her ambition is to become a TEE-certified cardiac anesthesiologist, she will start a 1-year fellowship in Cardiothoracic and Vascular Anesthesia in the Department of Anesthesia at the Duke University Medical Center in July 2008.

# Publications

Bakker P, de Lange F, Hauer R, Derksen R, De Bakker J. Sequential map-guided endocardial resection for ventricular tachycardia improves outcome. Eur J Cardiothorac Surg 2001;19:448-454.

Houston RJ, de Lange F, Kalkman CJ. A new miniature fiber oxygenator for small animal cardiopulmonary bypass. Adv Exp Med Biol. 2003;540:313-6.

De Lange F. Society of Neurosurgical Anesthesia and Critical Care Scientific Meeting Report. Anesthesiology 2005;102(5):1074-5.

Dieleman JM, de Lange F, Houston RJ, Biessels GJ, Bär PR, Mackensen GB, Grocott HP, Kalkman CJ. Cardiopulmonary bypass and long-term neurocognitive dysfunction in the rat. Life Sci. 2006;79(6):551-8.

Yoshitani K, de Lange F, Ma Q, Grocott HP, Mackensen GB: Reduction in air bubble size using perfluorocarbons during cardiopulmonary bypass in the rat. Anesth Analg 2006 ;103(5):1089-93.

Welsby IJ, Jones WL, Arepally G, de Lange F, Grocott HP, Becker R, Mackensen GB: Effect of combined anticoagulation using heparin and bivalirudin on the hemostatic and inflammatory responses to cardiopulmonary bypass in the rat. Anesthesiology 2007; 106(2): 295-301.

De Lange F, Dieleman JM, Jungwirth B, Kalkman CJ: Effects of cardiopulmonary bypass on neurocognitive performance and cytokine release in old and diabetic rats. Br J Anaesth 2007; 99 (2):177-83.

De Lange F, Yoshitani K, Proia AD, Mackensen GB, Grocott HP: Perfluorocarbon administration during cardiopulmonary bypass in rats; an inflammatory link to adverse outcome? Anesth Analg 2008;106(1):24-31.

De Lange F, Jones WL, Mackensen GB, Grocott HP: Effect of limited rewarming and postoperative hypothermia on cognitive function in a rat CPB model. Anesth Analg 2008; 106(3):739-45.