Cardiol Young 2001; 11: 532–538

ISSN 1047-9511

Original Article

Young Investigator's Prizewinner 2001

Direct visualization of the influence of normothermic as opposed to hypothermic cardiopulmonary bypass on the systemic microcirculation in neonatal piglets

Florian M.-J. Wagner,[1,2] Wolfgang Schiller,[1] Guido Dilg,[1] Christian Depner,[1] Armin Welz,[1] Francois Lacour-Gayet[2]

[1]*Department of Cardiac Surgery, University of Bonn, Germany;* [2]*Department of Paediatric Cardiac Surgery, University of Hamburg, Germany*

Abstract The direct visualization of systemic microcirculation using intravitalmicroscopy permits the classification of proinflammatory and ischemic microvascular alterations during normothermic and hypothermic cardiopulmonary bypass in neonates. We used seven newborn piglets, on average aged 9 days, and weighing 3200 g, as a control group. In addition, we studied nine piglets subjected to 60 minutes of constant non-pulsatile flow using hypothermic extracorporeal circulation at 28°C, and five piglets using normothermic conditions at 37°C. The microvascular network of the greater omentum and the subcutaneous tissue was directly visualized using intravitalmicroscopy. We analysed interactions between leukocytes and endothelial cells, microvascular morphology, and microrheological conditions, focussing on signs of ischemic and proinflammatory alterations. During normothermic cardiopulmonary bypass, the numbers of activated leukocytes were elevated compared to hypothermic cardiopulmonary bypass ($p > 0.05$). Arteriolar diameter decreased during hypothermia. Capillaries were markedly dilated during normothermia. Patterns of microvascular perfusion, for both types of cardiopulmonary bypass, showed signs of ischemic damage, revealed by a reduced functional capillary density. Perfusion dependent levels of lactate were higher during normothermic cardiopulmonary bypass ($p > 0.05$). This new experimental approach revealed that non-pulsatile cardiopulmonary bypass, independent of temperature, induces a proinflammatory and ischemic response compared to an unaltered control group. The markedly elevated numbers of activated adherent leukocytes, the reduced capillary density, and the high lactate levels in those undergoing bypass in normothermic conditions indicate a more pronounced inflammatory stimulus and tissue hypoperfusion compared to the possible protective effect of hypothermia for children undergoing cardiopulmonary bypass.

Keywords: Microcirculation; intravitalmicroscopy; cardiopulmonary bypass; inflammation; ischemia

Correspondence to: Dr Florian Max-Josef Wagner, Department of Paediatric Cardiac Surgery, University of Hamburg, Martinistr. 25, 20246 Hamburg, Germany. Tel: 49 40 42803 2440; Fax: 49 40 42803 6835; E-mail: fmjwagner@hotmail.com

This study was presented in part at the 3rd World Congress of Pediatric Cardiology and Pediatric Cardiac Surgery, May 27–31 2001, Toronto, Canada, and rewarded with the Young Investigator Award 2001. The study was supported by BONFOR Grant 0.115–0001.

Accepted for publication 2 July 2001

CARDIOPULMONARY BYPASS IN NEONATES IS STILL accompanied by a high morbidity and mortality due to the immaturity of the organism, the duration of extracorporeal perfusion, and the complexity of the surgical intervention. The inflammatory response during and after extracorporeal circulation is based on the activation of many different interacting cellular and humoral factors. The end result is a

https://doi.org/10.1017/S1047951101000774 Published online by Cambridge University Press

systemic activation of detrimental inflammatory and ischemic pathways. It is suggested that the magnitude of this response might be influenced by intraoperative temperature during cardiopulmonary bypass.[1]

Adherence of neutrophil granulocytes to endothelial cells is triggered by contact to the foreign surface of the extracorporeal circuit, increased interactions between cells, upregulation of cytokines and chemokines, and mechanical shear forces. The result is that leukocytes release specific compounds which are toxic to the tissues.[2] The development of systemic ischemic damage due to regional hypoperfusion during cardiopulmonary bypass, however, remained to be proven.[3] The present study, therefore, examined the association of inflammatory cellular interactions and ischemic damage to the tissues during cardiopulmonary bypass by direct visualization using intravitalmicroscopy in neonate piglets.

Materials and methods

This study was approved by the ethic commission authorities of Nordrhein-Westfalen and Hamburg. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals provided by the American National Institutes of Health, and was controlled by a veterinarian.

We divided 21 newborn piglets, aged on average 9 days, and weighing from 2500–3600 g, into three groups. We used 7 animals as control group for basic parameters of subcutaneous and greater omental microcirculation. In 9 piglets, we carried out 60 minutes of hypothermic non-pulsatile cardiopulmonary bypass at 28°C, while the remaining 5 animals were perfused under normothermic conditions at 37°C.

Operative procedure and cardiopulmonary bypass

After anesthesia induction and routine instrumentation, the animals were mechanically ventilated via the trachea. A median sternotomy was performed, the arterial duct was clipped, and cannulas for cardiopulmonary bypass were inserted via the ascending aorta and both caval veins. The circuit for extracorporeal circulation was the same as used clinically for congenital cardiac surgery, consisting of a hollow fibre membrane oxygenator combined reservoir, a non-pulsatile roller pump, and polyvinylchloride tubing. The circuit was primed with crystalloid and colloidal fluids (Osmosteril, HAES 6%, Albumin 20%, Lactated Ringers solution) to a total of 245 ml. Corpuscular blood products for priming were consciously avoided to prevent cellular interactions complicating the intravitalmicroscopical observations.

The bypass was managed following ′ -stat principles. A constant pump rate of 120–140 ml/min/kg body weight was set to stabilize systemic arterial pressure at physiological values of 55 ± 15 mmHg. The ascending aorta and pulmonary trunk were cross-clamped, and Bretschneider's cardioplegia was infused via the aortic root. The piglets were perfused for 60 minutes at the chosen temperature, and sacrificed directly after the experiment.

Intravitalmicroscopy

After institution of cardiopulmonary bypass, the subcutaneous tissues and the greater omentum were prepared surgically. The animals were fixed on a specially designed microscopic stage. The greater omentum was exposed via an extended laparatomy. The spleen was carefully mobilized and the omentum was draped over the stage.

Microcirculation in the subcutaneous tissues was studied via a 3 cm longitudinal incision along the ventral part of the upper hind leg. The subcutaneous tissues were removed step by step until a layer with a clearly intact and easily visible microvascular network was preserved.

Microscopic recordings in the control animals were performed directly after preparation of the tissues. In both groups undergoing bypass, intravitalmicroscopy was performed 45 minutes after the start, and continued to the end of the period of perfusion lasting 60 minutes.

For visualization, a Leica DM-LM fluorescence microscope was used on a swinging arm stand. The technique has been described in detail by Linke and coauthors.[4] In short, we used a 100 W mercury arch lamp attached to specific filter blocks for selective visualization of Fluorescein and Rhodamine 6 G (Leica, Bensheim, Germany). Sodium Fluorescein, given at 20 mg/kg, (Sigma, St Louis, MO) resulted in an inverse labelling of erythrocytes, while Rhodamine 6 G, at 150 mg/kg labelled the white blood cells, which were then injected intravenously. The recordings were performed in the period between 45 and 60 minutes of bypass. The digitally processed pictures were transferred from the recorder to a workstation and post-production was performed using Adobe Photoshop as described by Brunner et al.[5]

So as to analyze the pictures, the focus was set upon interactions between leukocytes and endothelial cells to describe activation of inflammation, and patterns of microvascular perfusion patterns to quantify ischemic alterations. The stage of activation of the leukocytes was classified by quantification of their behavior of flow. Circulating cells without adhesive contact to the vascular wall were called free-flowing leukocytes. Leukocytes that were slowed down due to contact with the vascular wall, or were temporary adherent to endothelial cells for less than

https://doi.org/10.1017/S1047951101000774 Published online by Cambridge University Press

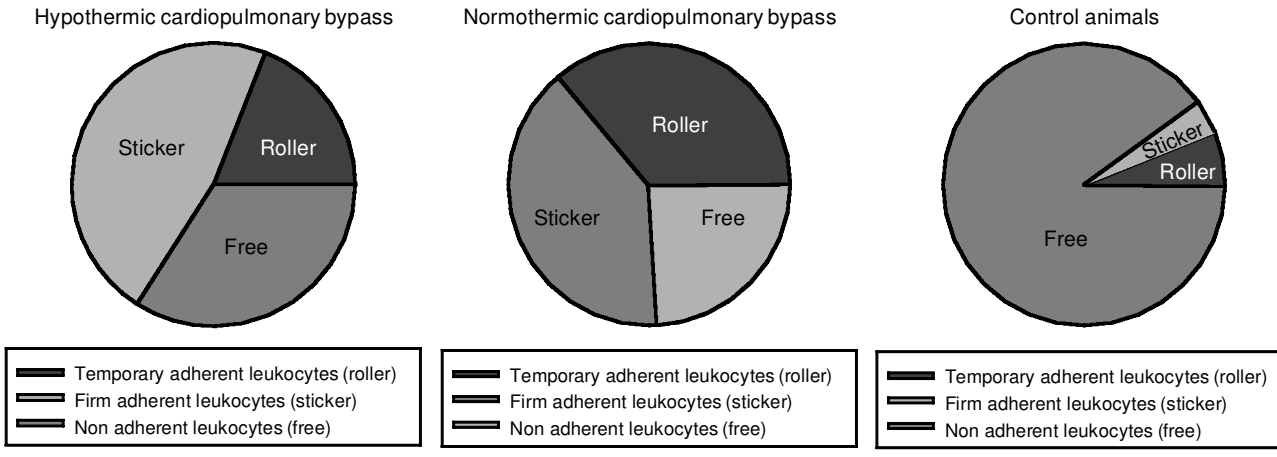

Figure 1.
Pie chart of the classification of leukocytes into free-flowing, rolling and sticking forms in the three experimental groups.

10 seconds, were deemed to be "rolling". Leukocytes that were firm adherent to the vascular wall without any movement in 10 seconds were considered "stickers". The proportions of each stage of leukocytes was expressed as a percentage of all visible circulating leukocytes. The pattern of perfusion of the tissues was rated according to the calculation of the functional capillary density. This parameter is related only to the length of regularly perfused capillaries in a region of interest, and is expressed as mm/mm². Capillary, venular, and arteriolar diameters were measured, and the state of contraction or dilation of the vessels was described.

Hematology

Arterial blood samples were taken in the control animals after induction of anesthesia, and at the end of the videorecordings. In the animals undergoing cardiopulmonary bypass, samples were collected after the sternotomy, 15 minutes after the commencement of bypass, and at the end of the experiment. Of the blood obtained, 3 ml was immediately centrifuged, and samples of serum stored at −70°C for measurement of IL-2 and TNF-′ using the ELISA method.

Differentiation of leukocytes, the thrombocytic count, measurements of hematocrit and hemoglobin, and the levels of lactate and electrolytes were automatically performed at the Institute of Clinical Chemistry.

Statistics

Data are given as mean and standard deviation. Variances were tested with the Kolmogorov-Smirnov test. Data were analyzed with the unpaired t-test. Statistical significance was taken when the p value was smaller than 0.05.

Results

The data obtained using the intravitalmicroscope revealed the onset of an inflammatory reaction in all animals undergoing cardiopulmonary bypass when compared to the native animals. Leukocytes were activated, and adhesion to endothelial cells was significantly increased, after 45 minutes of cardiopulmonary bypass (Figs 1 and 2). The percentage of rolling and sticking leukocytes was higher, and free-flowing cells were significantly reduced in the setting of normothermia as compared to hypothermia. The inflammatory reaction was confirmed by the finding of increased levels of IL-2 and TNF-′, with a more pronounced rise during hypothermic perfusion (Fig. 3).

The microvascular perfusion, indicated by the functional capillary density, was dramatically reduced, falling to less than 20% of normal values in all animals undergoing cardiopulmonary bypass. This observation was independent of the temperature of perfusion (Fig. 4). The levels of lactate in the blood were increased with extracorporeal circulation, but had reached a higher peak at the end of cardiopulmonary bypass under conditions of normothermia (Fig. 5). A low functional capillary density, and high levels of lactate, indicate the development of ischemic damage to the tissues.

The venular diameter was unaffected by extracorporeal circulation or temperature. Arterioles were contracting during hypothermia, but remained normal during normothermia. Capillaries, in contrast, were dilated with normothermia, but normal during hypothermia (Fig. 6).

https://doi.org/10.1017/S1047951101000774 Published online by Cambridge University Press

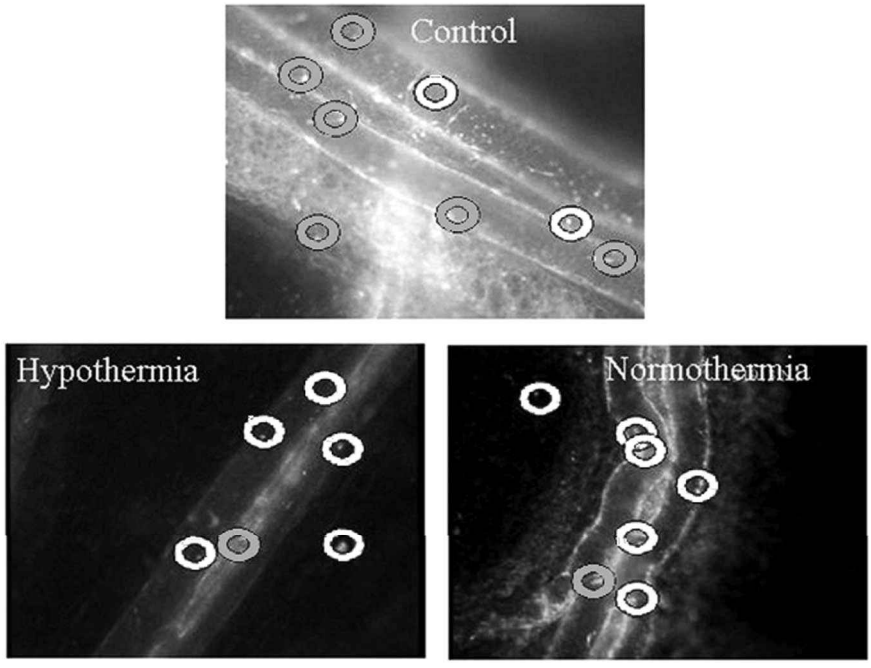

Figure 2.
Intravitalmicroscopic pictures of the greater omental microvascular network stained with Rhodamin 6G to visualize white blood cells and mark the vascular endothelial cell layer. Free-flowing leukocytes are circled in green, while temporary or firmly adherent leukocytes are shown in red.

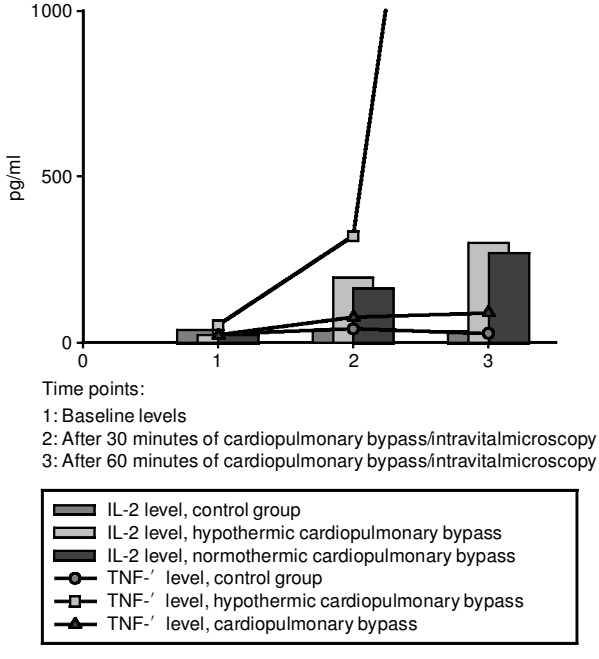

Figure 3.
Changes in levels of interleukin-2 (bar graph) and tumor-necrosis factor (line graph) in the control group and the animals subjected to cardiopulmonary bypass as visualized by intravitalmicroscopy.

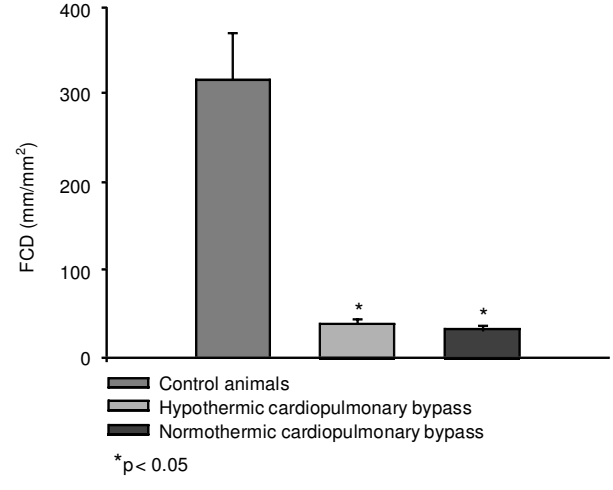

Figure 4.
Bar graph showing the influence of cardiopulmonary bypass and temperature on changes of microvascular perfusion, indicated by the functional capillary density assessed by intravitalmicroscopy.

Discussion

We have found three major features when comparing the microcirculation of neonatal piglets subjected to normothermic and hypothermic cardiopulmonary bypass. First, the tone of the walls of the arterioles and capillaries is affected, indicating a difference in the regulation of vascular resistance with changing temperatures. Second, leukocytes are activated and adhere to the vascular endothelium under conditions of cardiopulmonary bypass. In this respect, hypothermia confers a partial protection against massive rolling and sticking of the leukocytes when compared to normothermic perfusion. Third, the temperature of

https://doi.org/10.1017/S1047951101000774 Published online by Cambridge University Press

perfusion has no effect on microvascular perfusion during non-pulsatile cardiopulmonary bypass.

The activation of both the humoral and cellular components of the inflammatory response caused by cardiopulmonary bypass has been extensively documented in earlier studies,[6] and seems to be ruled by other mechanisms in children when compared to adults.[7] The management of cardiopulmonary bypass in neonates, therefore, is correspondingly different because of these physiological and pathological considerations.[8]

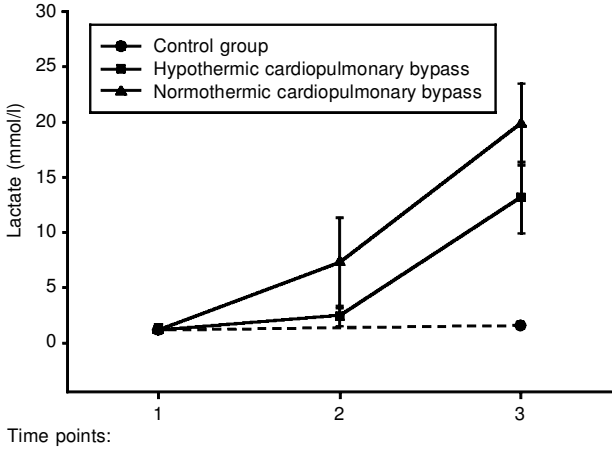

Figure 5.
Line graph showing the influence of cardiopulmonary bypass and temperature on systemic levels of lactate.

Mild hypothermic non-pulsatile cardiopulmonary bypass is the strategy used most often during congenital cardiac surgical procedures. Hypothermia offers some important advantages, such as a decreased systemic metabolic demand, and reduced extravasation of fluids during the surgical procedure.[9] Further more, the lower temperatures reduce the cytotoxic potential of activated leukocytes and proinflammatory cytokines.[10] The essential supply of oxygen to the brain has been shown to be increased with mild hypothermic cardiopulmonary bypass when compared to normothermia, at least when following the ′-stat principles of acid-base management.[11] Levels of lactate found during cardiopulmonary bypass are related to the length of perfusion, and have been shown to be an early indicator for morbidity and mortality.

The sources of lactate production are manifold, but always reveal a process of anaerobic energy production in response to hypoperfusion of the tissues, decreased extraction of oxygen, and increased metabolic demand, as in typical inflammatory reactions.[12] The increased levels of lactate which we measured in this study are comparable to values observed clinically. Levels were slightly higher during normothermic perfusion. The adequacy of perfusion during extracorporeal circulation, however, is difficult to monitor, as the available parameters, such as output of urine or extraction of oxygen, are not reliable.

Intravitalmicroscopy, in contrast, offers the golden standard with which to observe the patterns of microvascular perfusion, offering the unique possibility of visualizing directly the microcirculatotion.

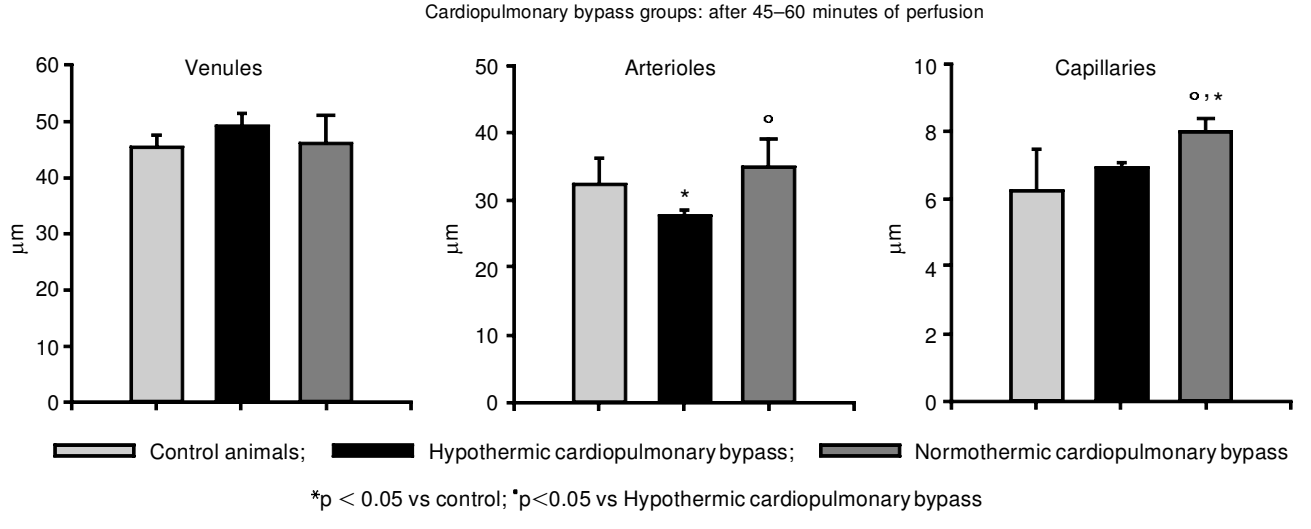

Figure 6.
Microvascular diameters of arterioles, capillaries and venules.

https://doi.org/10.1017/S1047951101000774 Published online by Cambridge University Press

Hypoperfusion was unequivocally evident in our studies as shown by the massively reduced functional capillary density. This reduction was comparable in all animals undergoing cardiopulmonary bypass, irrespective of the differences in inflammatory activation of the leukocytes. The "plugging" of activated leukocytes in the capillaries was thought to play a major role in the development of ischemic injury. But pharmacological blockade of such cells plugging the capillaries did not improve the functional capillary density, nor reduce the damage to tissues, in a skeletal muscular model of ischemia.[13] The benefit of hypothermia in reducing leukocytic adhesion is shown by the higher percentage of free-flowing leukocytes observed in the intravitalmicroscope when compared to normothermic perfusion. Earlier studies examined biochemical markers of leukocytic activation, and found complementing results. There was reduced release of leukocytic elastase,[14] and reduced expression of adhesion molecules CD11b and L-selectin[15] under hypothermic conditions. The pattern of perfusion during cardiopulmonary bypass, therefore, seems to be independent of the inflammatory leukocytic traffic. Hence, it is necessary to look at the perfusion deficit as an independent pathophysiologic entity beside the inflammatory response. Ischemia seen in the stage of hypoperfusion during cardiopulmonary bypass can have multiple causes: capillary leakage, interstitial edema, swelling of endothelial cells, thrombocytic plugging, disorders of coagulation, and masked hypovolaemia to name but a few. The altered distribution of kinetic energy caused by non-pulsatile perfusion can be of additional importance. Pulsatility has been shown to provide superior flow of blood to vital organs as assessed by injection of labeled microspheres when compared to non-pulsatile perfusion.[16]

In conclusion, our study has revealed the importance of leukocytes as mediators of the inflammatory activation during cardiopulmonary bypass. We have shown, by direct visualization, the development of ischemic damage in hypoperfused and non-perfused compartments. Hypothermia offers a partial protection against the massively increased activation of the leukocytes compared to normothermic perfusion. The mechanism of ischemia observed during cardiopulmonary bypass is independent from the inflammatory response, as dramatic hypoperfusion is present in all animals undergoing cardiopulmonary bypass. Thus, in neonates who, subsequent to a good surgical repair of congenital cardiac malformations, have an adequate cardiac output and no signs of an inflammatory reaction after cardiopulmonary bypass, but still remain in an unsatisfactory condition, the possibility of isolated systemic ischemic damage, with consequent reperfusion injury, must always be considered in the differential diagnosis, particularly when there are coexisting elevated levels of lactate.

Acknowledgements

We appreciate very much the cooperation with Christine Peigney and Mario Sitzia throughout the experiments. FMJ Wagner is supported by a BONFOR grant at the University of Bonn, Germany.

References

1. Hashimoto K, Sasaki T, Hachiya T, Onoguchi K, Takakura H, Oshiumi M, Takeuchi S. Influence of cardiopulmonary bypass temperature on circulatory pathophysiology and clinical outcomes. Jpn Circ J 2000; 64: 436–444.
2. Le Deist F, Menasche P, Kucharski C, Bel A, Piwnica A, Bloch G. Hypothermia during cardiopulmonary bypass delays but does not prevent neutrophil-endothelial cell adhesion. A clinical study. Circulation 1995; 92: II354–358.
3. Pathi VL, Morrison J, MacPhaden A, Martin W, McQuiston AM, Wheatley DJ. Alterations in renal microcirculation during cardiopulmonary bypass. Ann Thorac Surg 1998; 65: 993–998.
4. Linke R, Wagner F, Terajima H, Thiery J, Teupser D, Leiderer R, Hammer C. Prevention of initial perfusion failure during xenogeneic ex vivo liver perfusion by selectin ihibition. Transplantation 1998; 66: 1–8.
5. Brunner J, Krummenauer F, Lehr HA. Quantification of videotaped images in microcirculation research using inexpensive imaging software (Adobe Photoshop). Microcirculation 2000; 7: 103–107.
6. Finn A, Dreyer WJ. Neutrophil adhesion and the inflammatory response induced by cardiopulmonary bypass. Cardiol Young 1993; 3: 244–250.
7. Seghaye MC, Duchateau J, Grabitz RG, Nitsch G, Marcus C, Messmer BJ, von Bernuth G. Complement, leukocytes and leukocyte elastase in full term neonates undergoing cardiac operation. J Thorac Cardiovasc Surg 1994; 108: 29–36.
8. Lazenby DW, Krieger KH. Cardiopulmonary bypass – organ blood flow and metabolism in the pediatric patient. Cardiol Young 1993; 3: 232–243.
9. Christakis GT, Koch JP, Deemar KA, Fremes SE, Sinclai L, Chen E, Salerno TA, Goldman BS, LichtensteinSV. A randomized study of the systemic effects of warm heart surgery. Ann Thorac Surg 1992; 54: 449–459.
10. Qing M, Vazquez-Jimenez JF, Klosterhalfen B, Sigler M, Schumacher K, Duchateau J, Messmer BJ, von Bernuth G, Seghaye MC. Influence of temperature during cardiopulmonary bypass on leukocyte activation, cytokine balance and postoperative organ damage. Shock 2001; 15: 372–377.
11. Okano N, Owada R, Fujita N, Kadoi Y, Saito S, Goto F. Cerebral oxygenation is better during mild hypothermic than normothermic cardiopulmonary bypass. Can J Anaesth 2000; 47: 131–136.
12. Munoz R, Laussen PC, Palacio G, Zienko L, Piercey G, Wessel DL. Changes in whole blood lactate levels during cardiopulmonary bypass for surgery for congenital cardiac disease: an early indicator of morbidity and mortality. J Thorac Cardiovasc Surg 2000; 119: 155–162.
13. Harris AG, Steinbauer M, Leiderer R, Messmer K. Role of leukocyte plugging and edema in skeletal muscle ischemia-reperfusion injury. Am J Physiol 1997; 273: H989–H996.

14. Menasche P, Peynet J, Haeffner-Cavaillon N, Carreno M, de Chaumaray T, Dillisse V, Bouchaib F, Piwnica A, Bloch G, Tedgui A. Influence of temperature on neutrophil trafficking during clinical cardiopulmonary bypass. Circulation 1995; 92 (suppl II): II-334– II-340.
15. El Habbal MH, Carter H, Smith LJ, Elliott MJ, Strobel S. Neutrophil activation in paediatric extracorporeal circuits: effect of circulation and temperature variation. Cardiovasc Res 1995; 29: 102–107.
16. Ündar A, Masai T, Yang S, Goddard-Finegold J, Frazier OH, Fraser Jr CD. Effects of perfusion mode on regional and global organ blood flow in a neonatal piglet model. Ann Thorac Surg 1999; 68: 1336–1343.