Effects of Leukocyte-Depleted Warm Blood Cardioplegia on Cardiac and Endothelial Function

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Summary: It has been reported that neutrophils and platelets have deleterious effects on myocardium and endothelium during and after ischemia. In this study we evaluated the effects of a leukocyte-depleting filter (Sepacell PLX, Asahi medical, Tokyo) during warm blood cardioplegia and early reperfusion on cardiac and endothelial function in the blood-perfused rat heart. Hearts (n=7 per group) from donor rats were excised and perfused with blood at 37 °C from a support rat. After 10 min of stabilization, the hearts were arrested for 60 min with warm blood cardioplegia given at 20 min intervals. This was followed by 60 min of reperfusion. A leukocyte-depleting filter was used during the cardioplegia and the initial 10 min of reperfusion in the experimental group (Group F) and it was not used in the control group (Group N). Left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), maximum rate of left ventricular pressure rise (+dp/dt) and maximum rate of left ventricular pressure fall (−dp/dt) were measured as indices of left ventricular function before and after cardioplegic arrest. Coronary sinus effluent was obtained and the levels of MB isozyme of creatine kinase (CKMB), malondialdehyde (MDA), elastase and thromboxane B2 (TXB2) were measured as indices of myocardial and endothelial injury. After 60 min of reperfusion, acetylcholine (Ach.) was administered to the coronary perfusate and the difference of nitric oxide (NO) concentration between inflow and outflow, and coronary blood flow were measured as an indication of endothelial function. Group F showed significantly lower LVEDP than Group N at 10 min of reperfusion. The elastase levels were significantly (p <0.05) lower and the CKMB levels tended (p<0.1) to be lower in Group F at 60 min of reperfusion. The administration of Ach. to the coronary perfusate showed significantly (p <0.05) greater coronary blood flow and NO production in Group F. The results suggested that the use of a leukocyte-depleting filter during warm blood cardioplegia and early reperfusion preserves endothelial function and left ventricular diastolic compliance. The technique may provide beneficial effects by reducing reperfusion injury in patients undergoing cardiac surgery.

Key words nitric oxide, leukocyte-depleting filter, cardioplegia, cardiac function, endothelial function, coronary blood flow, reperfusion injury

INTRODUCTION

Myocardial protection is an essential technique for cardiac surgery. Although various studies have been undertaken concerning myocardial protection during cardiac surgery, reperfusion injury is still one of the major concerns [1]. Growing evidence shows that local and general ischemia impairs endothelium-dependent reactions [2,3]. This malfunction may be caused by reduced endothelium-derived relaxing factor (EDRF) [4,5], which is thought to be NO or S-nitrosocysteine. NO acts not only as a vasodilator but also as an inhibitor of platelet aggregation and neutrophil activation [6]. NO has also been shown to improve metabolic dysfunction during myocardial ischemia [7] and to reduce the area of cardiac necrosis after reperfusion following myocardial ischemia [8]. Therefore, preservation of endothelial production
of NO during ischemia and reperfusion may lead to improved cardiac function after cardioplegic arrest and reperfusion. Pathologically, leukocyte infiltration of the tissue, adhesion to the endothelium, accumulation into, and plugging of the microvessels were observed during and after myocardial ischemia [9]. Another study has also shown endothelial edema, detachment and cellular death after ischemia [3]. During myocardial ischemia, neutrophils are activated and produce superoxide radicals, elastase and myeloperoxidase [10]. The production of these cytotoxic chemical substances results in cellular membrane destruction. It has also been suggested that platelets aggregate and cause vasoconstriction and microemboli when they are activated [11]. Therefore, we hypothesized that depletion of neutrophils and platelets during ischemia and reperfusion would reduce endothelial damage and preserve endothelial function to release NO. Thus this strategy may preserve cardiac function by maintaining NO release. This study was designed to evaluate the influence of neutrophils and platelets depletion during cardioplegia and early reperfusion on cardiac function, metabolism and endothelial function of the isolated, perfused, rat heart.

MATERIALS AND METHODS

Experimental groups

Adult male Wistar rats (400-500 g) were used in this experiment. The isolated hearts were divided into two groups according to the use of a leukocyte removal filter (Sepacell PLX, Asahi Medical Co., Ltd., Tokyo). The filter was used during the administration of cardioplegia and during the initial 10 min of reperfusion in Group F (n=7) and it was not used in Group N (n=7). Cardiac arrest was achieved with the administration of 20 ml/kg/min of warm blood cardioplegic solution [12] into the aortic root for 2 min. Supplemental cardioplegia was delivered for 2 min at 20 min intervals. After 60 min of cardiac arrest, the heart was reperfused for 60 min.

Blood-perfused rat heart preparation

The perfusion system used in these experiments is shown in Fig. 1. The circuit was primed with 40 ml heparinized blood (obtained from two rats of the same strain as group F and N) and lactated Ringer’s solution. The hematocrit was maintained 25-30% throughout the experiment. After intramuscular administration of sodium pentobarbital (100 mg/kg), the support rat was fixed in the supine position on a hot plate and body temperature was maintained at 38 ºC. The rat was ventilated through a tracheostomy tube with a volume ventilator (ASR-830, CWE Co., Ltd., Ardmore, PA) in order to maintain PaO2 above 200 mmHg, and PaCO2 within 30-40 mmHg. The fraction of inspired oxygen (FiO2) concentration was maintained at 1.0. Heparin sodium (1000 U/kg) was given intravenously. The femoral artery and vein were cannulated with 24 gauge Teflon tube to supply arterial blood to the donor heart and allow for the return of blood to the support animal. Blood pressure of the support rat was monitored with a pressure transducer (UK4006, Baxter Co., Ltd., Tokyo) and maintained above 80 mmHg by intravenous infusion of lactated Ringer’s solution.

Isolated heart

Donor rats were anesthetized with sodium pentobarbital (100 mg/kg) administered intramuscularly and inhalation of diethyl ether. The femoral vein was exposed and heparin sodium (1000 U/kg) was administered intravenously. Median sternotomy and pericardiomyotomy were performed and the heart was excised rapidly. The aorta was cannulated and perfused in the Langendorff mode with blood at a constant perfusion pressure of 100 cmH2O through a thermostatically controlled delivery line from the
LEUKOCYTE-DEPLETED WARM BLOOD CARDIOPLEGIA

Fig. 2. Experimental protocol. Leukocyte removal filter was used during the administration of blood cardioplegia and during the initial 10 min of reperfusion in group F. Acetylcholine chloride was administered to the coronary perfusate to evaluate endothelial function at 60 min of reperfusion.

Fig. 3. Cardiac function (LVSP, LVEDP). Left ventricular function observed at the left ventricular balloon volume of 0.1 ml showed significantly greater LVEDP in group N compared with group F at 10 min of reperfusion.

support rat. A pacemaker electrode was fixed on the apex, and heart rate was set at 270 beat/minute. After 10 min of stabilization, a latex balloon attached to a pressure transducer (UK4006, Baxter Co., Ltd., Tokyo) was inserted into the left ventricle through the mitral valve.

Measurements

Left ventricular function: Left ventricular systolic pressure (LVSP: mmHg), left ventricular end-diastolic pressure (LVEDP: mmHg), maximum rate of left ventricular pressure rise (+dp/dt: mmHg) and maximum rate of left ventricular pressure fall (−dp/dt: mmHg) were measured (MINGOGRAF 7, Fukuda Denshi, Tokyo) by stepwise inflation of the intraventricular balloon at the end of the stabilization period, as well as at 10 and 60 min of reperfusion.

Biochemical tests: Coronary sinus blood samples were obtained and the levels of MB isozyme of creatine kinase (CKMB: IU/L), malondialdehyde (MDA: nmol/ml), elastase (ng/dl) and thromboxane B2 (TXB2: pg/ml) were measured at the end of stabilization, at 10 and 60 min of reperfusion. The levels of CKMB, MDA, elastase, TXB2 were measured with chemiluminescent immunoassay, thiobarbituric acid reaction, radioimmunoassay, and radioimmunoassay polyethylene glycol method, respectively.

Endothelial function: Coronary blood flow (C. flow) and the difference in NO concentration between coronary perfusate and coronary sinus effluent (DasNOconc.) were measured so as to evaluate the endothelial function at the end of stabilization, at 10 and 60 min of reperfusion. Blood samples for the determination of NO concentration were centrifuged immediately at 3000 rpm for 5 min at 4 °C and the plasma was immediately measured by Griess reaction (ENO-20, Eicom Co., Ltd., Kyoto). NO concentration was determined by reconverting its oxidation end-products (nitrite, NO2−) and nitrate (NO3). NO production (NO prod.) was calculated by the following formula: NO prod. = C. flow × DasNOconc.

Acetylcholine chloride was administered to the coronary perfusate so as to achieve a final concentration of 1×10−8 M/L (Ach. 1) or 1×10−7 M/L (Ach. 2) or 5×10−7 M/L (Ach. 3), at 60 min of reperfusion. C. flow and DasNOconc. were measured at each concentration of acetylcholine chloride. The experimental protocol is shown in Fig. 2.

Statistical methods

Data are expressed as mean ± standard error of the mean. Repeated measures two-way analysis of
 течение не значительных различий между группой F и группой N в +dp/dt, и -dp/dt в любой момент времени.

**Fig. 5. CKMB, MDA, ELASTASE, TXB2.** MDA и TXB2 уровни не были значимо различны между двумя группами. Уровни CKMB тенденциально были ниже в группе F, чем в группе N после 60 минут реперфузии. Уровни эластазы были значительно ниже в группе F по сравнению с группой N после 60 минут реперфузии.

Версия была использована для анализа функции сердца, коронарного кровотока и данных NO. Когда p значение было меньше 0.05, неупарный t-тест использовался для сравнения двух групп. Для сравнения других непрерывных переменных, неупарный t-тест был использован. A p значение меньше 0.05 было рассмотрено статистически значимым.

**RESULTS**

**Leukocyte and platelet counts**

Количество лейкоцитов в коронарном полости уменьшилось значимо с фильтрацией (6.92±0.99 vs. 0.38±0.15×10³/µL, p<0.01). Количество тромбоцитов также уменьшилось значимо (40.9±3.8 vs. 4.3±1.4×10⁴/µL, p<0.01).

**Left ventricular function**

Данные, полученные при объеме миокардиального баллона 0.1 мл, показаны на рисунках 3 и 4. Группа N показала значимое большее LVEDP по сравнению с группой F (27.3±13.7 mmHg versus 3.7±2.4 mmHg; p<0.05). Величины LVSP, +dp/dt, и -dp/dt в любой момент времени не демонстрировали значительных различий между группами F и N.

**Biochemical tests**

CKMB, MDA, эластаза, и TXB2 уровни в коронарной синусной крови перед кардиальной аритией и во время

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reperfusion are shown in Fig. 5. The levels of MDA and TXB₂ did not differ between the two groups. The levels of CKMB tended to be lower in Group F than those in Group N (431±97 IU/L vs. 694±130 IU/L; p<0.10) at 60 min of reperfusion. The levels of elastase were significantly lower in Group F compared with those in Group N (63.8±6.5 ng/dl versus 97.5±6.5 ng/dl; p<0.05) at 60 min of reperfusion.

**Coronary blood flow and NO production**

Coronary blood flow (C. flow), difference of nitric oxide levels between coronary inflow and outflow (DasNOconc.), and NO production (NO prod.) are shown in Figs 6, 7 and 8. There were no significant differences in C. flow, DasNOconc. and NO prod. between the two groups at any time. The administration of acetylcholine chloride to the coronary perfusates at 60 min of reperfusion (Figs 9-11) resulted in significantly greater C. flow in Group F (3.9±0.3 ml/min vs. 2.9±0.4 ml/min; p<0.05) and a tendency for NO production to be greater in Group F (3.88±1.56×10⁻⁸ M/min vs. 0.77±0.76×
Fig. 10. Difference of nitric oxide concentration between coronary artery and coronary sinus. The coronary arterio-venous difference of NO concentration tended to be greater in group F when Ach. was administered into the coronary perfusate at the concentration of $5 \times 10^{-7}$ M/L.

Fig. 11. Nitric oxide production. Administration of acetylcholine to the coronary perfusate at 60 min of reperfusion indicated a tendency to greater NO production in group F when Ach. concentration was $1 \times 10^{-7}$M/L and a significantly greater NO production in group F when Ach. concentration was $5 \times 10^{-7}$ M/L.

$10^{-8}$ M/min; $p < 0.10$) when Ach. concentration was $1 \times 10^{-7}$ M/L. DasNOconc. and NO production were significantly greater in Group F than those in Group N (1.64±0.35×$10^{-8}$ M/L vs. 0.96±0.16×$10^{-8}$ M/L; $p < 0.05$ and 6.35±1.43×$10^{-8}$ M/min vs. 2.34±0.73×$10^{-8}$ M/min; $p < 0.05$, respectively), when Ach. concentration was $5 \times 10^{-7}$ M/L.

DISCUSSION

Although the use of cardioplegia has provided satisfactory clinical results in cardiac operations [13-15], the occurrence of reperfusion injury after cardioplegic arrest is still one of the major concerns. Thus, further improvement of cardioplegic technique is required [16]. Cardiac arrest with cardioplegia and following reperfusion have been reported to be associated with delayed recovery of cardiac and endothelial function [2,3]. Endothelial dysfunction induced by reperfusion injury causes reduction of NO production from endothelium [4]. NO is synthesized in the endothelium from L-arginine by NO synthase [17]. NO acts not only as a vasodilator but also as an inhibitor of platelet aggregation and neutrophil adhesion to the endothelium [18]. It has also been suggested that NO reduces anaerobic myocardial metabolism by depressing myocardial contractility during ischemia [6,7]. Therefore, these effects of NO may prevent reperfusion injury and preserve cardiac function after ischemia.

Neutrophils have been reported to be closely associated with myocardial tissue damage during reperfusion. Engler and colleagues [10] reported that myocardial stunning was caused by the following three main consequences of granulocyte activation.

1. Oxygen radical production causing cell membrane, sarcoplasmic reticulum, or enzyme damage.
2. Capillary plugging by granulocytes, resulting in continued ischemia.
3. Degranulation leading to enzyme damage in the cells.

Activated neutrophils produce superoxide radicals which dismutate to hydrogen peroxide and can generate hydroxyl radicals in the presence of iron. Granulocytes and possibly histiocytes contain the enzyme myeloperoxidase, which forms hypochlorous acid, a powerful oxidant, from hydrogen peroxide and chloride ions [18]. Various experimental evidences suggest that stimulated neutrophils contribute to free radical-mediated myocardial ischemia and reperfusion injury [19]. The proteolytic enzyme elastase is also generated by activated neutrophils. Elastase acts as a mediator of tissue injury by hydrolyzing collagen structures and thus damaging
endothelial cells, basement membrane structures, and myocardial tissue and promoting extravasation of neutrophils adhering to the endothelium and influences the adhesion process itself [20].

In order to minimize the myocardial damage by activated leukocytes, a technique of leukocyte-depleted terminal cardioplegia was investigated in an experimental model [21]. In the report, adhesion of neutrophils to the endothelium and neutrophil plugging of capillaries were observed in a group receiving whole blood cardioplegia, while they were significantly inhibited in a group using a leukocyte-depleting filter.

From these evidences, we hypothesized that leukocyte depletion during cardioplegia and early reperfusion prevents adhesion of neutrophils to the endothelium and reduces endothelial injury.

In this study, the levels of elastase were significantly lower, and CKMB values tended to be lower, in Group F at 60 min of reperfusion. This finding is presumably the consequence of the reduced neutrophil adhesion to the endothelium and extravasation resulting from the leukocyte depletion produced by the filter. Meanwhile, the levels of MDA were essentially unchanged in both groups throughout the experiment. Sawa and colleagues [21,23,24] reported a greater rise of MDA in a group receiving terminal whole-blood cardioplegia compared to a group using leukocyte-depleted terminal blood cardioplegia in experimental and clinical studies. In contrast, Johnson and colleagues [25] observed that although total load of activated neutrophils was greater after whole blood cardioplegia, no significant release of MDA was observed during and after aortocoronary bypass grafting in a leukocyte-depleted group. This discrepancy regarding MDA levels during reperfusion might be explained by the fact that Sawa and colleagues used either newborn animals or patients with hypertrophied left ventricle, which are known to be more susceptible to reperfusion injury. MDA is a breakdown product of spontaneous fragmentation of peroxides formed from polyunsaturated fatty acids. Since these breakdown reactions to give rise to MDA are complex, MDA levels might be an indirect index of free radical activity. Therefore, further investigations are required to elucidate the effect of leukocyte filtration on free radical activity.

With regard to platelets, they release TXA2 when they are activated. TXA2 causes contraction of vascular smooth muscle, lysis of cellular membranes, and acceleration of intravascular aggregation of platelets [26]. TXB2 is the metabolite of TXA2. It causes local coronary vasoconstriction by acting on vascular smooth muscle and mechanical obstruction in small vessels as a result of TXB2-induced platelet aggregation [27,28]. Therefore, we hypothesized that the filter would reduce TXB2 levels during reperfusion by causing platelets depletion. However, the levels of TXB2 did not differ between the two groups, while platelets were depleted significantly with the filter. This result may be partly explained by the fact that platelets may be damaged and activated by the filter, with resulting TXB2 release. Moreover, Seneri and colleagues [29] have reported that TXB2 is produced not only from platelets but also from arterial wall. Therefore, there is a possibility that the TXB2 levels measured in the present study may reflect its release from arterial wall as well as platelets.

With regard to the endothelial function, the present study demonstrated greater coronary flow and NO release from endothelium in Group F when acetylsalicylic acid was administered. The results suggested that the leukocyte-depleting filter reduced endothelial reperfusion injury and preserved its function to release NO. On the other hand, the endothelium is known to regulate the permeability of vessels. Wilson and colleagues [30] showed a superior preservation of left ventricular compliance in a leukocyte-depleted group. They also demonstrated that the changes in ventricular diastolic function were associated with a significant increase in left ventricular water content. In this study, we also found lower end diastolic pressure in Group F during reperfusion. Therefore, it is suggested that the use of a leukocyte filter during cardioplegia and early reperfusion prevents hyper permeability and myocardial tissue edema through preservation of the endothelium.

In conclusion, the use of a leukocyte-depleting filter during cardioplegia and early reperfusion may reduce endothelial and cardiac dysfunction after ischemia. The technique may provide beneficial effects by reducing ischemia-reperfusion injury in patients undergoing cardiac surgery.

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