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Aprotinin Protects the Cerebral Microcirculation during Cardiopulmonary Bypass

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Abstract

Background and objective—We have previously reported that administration of aprotinin at a single dose protects the cerebral microcirculation. The current study was designed to identify the optimal dose for protecting the cerebral microcirculation with assessment of neurological and behavioral recovery as well as renal function after circulatory arrest and ultra low flow bypass.

Methods—Twenty-four piglets were randomly assigned to three bypass groups at risk for postoperative cerebral and renal dysfunction. Cerebral microcirculation was assessed by intravital microscopy. Rhodamine-stained leukocytes were observed for adhesion and rolling. Animals were randomized to one of four aprotinin doses. Neurological deficit score, histological score, creatinine and blood urea nitrogen were analyzed both independently for this study as well as in combination with 50 animals who were studied with the same protocol and near infrared spectroscopy.

Results—There was a dose dependent relationship resulting in fewer activated rolling leukocytes with a higher aprotinin dose. Aprotinin dose was an independent predictor of more rapid recovery of neurological and behavioral outcome. We present a linear regression model where aprotinin dose predicts neurological score. Aprotinin had no impact on renal function.

Conclusions—Aprotinin reduces cerebral leukocyte activation and accelerates neurologic recovery in a dose dependent fashion. Aprotinin has no measurable impact on standard indices of renal function in young piglets. The availability of aprotinin should be reconsidered for pediatric patients undergoing cardiopulmonary bypass.

Keywords

Cardiopulmonary bypass; Cerebrovascular circulation; Microcirculation; Inflammation; Brain protection

Introduction

Aprotinin is a broad spectrum serine protease inhibitor which has consistently been shown in clinical trials to improve hemostasis.¹ Mechanisms include inhibition of trypsin, plasmin, and kallikrein as well as preservation of platelet function.^{2, 3} Aprotinin also reduces the inflammatory effects of cardiopulmonary bypass (CPB) through reduced complement and neutrophil activation.^{4, 5} Aprotinin was widely used in pediatric cardiac surgery until recently.

In 2008 the Food and Drug Administration (FDA) suggested that Bayer, the sole worldwide supplier, consider withdrawing aprotinin from the market based on the results of a clinical trial performed in high-risk adults undergoing cardiac surgery (BART),⁶ as well as a previously published retrospective multicenter study also in adults.⁷ Bayer voluntarily agreed to do so in spite of objections by the pediatric cardiac surgical community. Twelve randomized trials in children have failed to show any increased frequency of adverse events with aprotinin.⁸ Furthermore a retrospective study reported that in 2481 pediatric patients undergoing CPB there was no association between the use of aprotinin and acute renal injury, neurological complication, and operative or late mortality.⁹

Not only has aprotinin not been demonstrated to increase morbidity in pediatric patients, previous in-vitro studies have shown that, like other serine protease inhibitors including tissue-plasminogen inhibitor,^{10, 11} aprotinin can provide direct neuroprotection against excitotoxic neuronal cell death.¹² Our previous in-vivo studies also have suggested that administration of aprotinin at a single dose level enhances recovery of cerebral energy metabolism and protects the cerebral microcirculation.^{13, 14} The purpose of this study was identify the optimal dose of aprotinin for protecting the cerebral microcirculation and to assess the relationship between dose and neurological and behavioral recovery as well as renal function after circulatory arrest and ultra low flow bypass.

Methods

Animals

Twenty-four Yorkshire piglets with a mean age 25.1 ± 1.3 days and a mean body weight 9.6 ± 1.5 kg were randomly assigned to three bypass strategies known to carry a risk for postoperative cerebral and renal dysfunction: circulatory arrest at 25°C or ultra low flow (10 ml/kg/min) at 25 or 34°C. In all animals, intravital microscopy was performed to assess the cerebral microcirculation. Animals were randomized to one of four aprotinin doses (Trasylol; Bayer, West Haven, CN): no aprotinin, low dose (LD; pump priming only of 30,000 KIU/kg), standard full dose (SF; loading of 30,000 KIU/kg, pump priming of 30,000 KIU/kg, and continuous infusion of 10,000 KIU/kg/hour), and double full dose (DF; loading of 60,000 KIU/kg, pump priming of 60,000 KIU/kg, and continuous infusion of 20,000 KIU/kg/hour). Continuous infusion of aprotinin was discontinued during circulatory arrest and after weaning from bypass.

Neurological deficit score (NDS), histological score, serum creatinine (Cr) and blood urea nitrogen (BUN) were analyzed independently and in combination with 50 animals who were studied previously with the same protocol using near infrared spectroscopy rather than intravital microscopy.¹⁵

All animals received human care in accordance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996. The study was approved by the Institutional Animal Care and Use Committee of the Children’s National Medical Center.

Bypass Technique

The surgical preparation and the details of bypass technique have been described previously.^{14, 16} The pH-stat strategy was used and 30% hematocrit level was maintained in all animals. After baseline recordings CPB at a flow rate of 100 mL/kg/minute was begun and was continued for 10 minutes at normothermia. Piglets underwent 40 minutes of cooling to an

esophageal temperature of 25°C or 34°C. After cooling, circulatory arrest or ultra low-flow perfusion was initiated for 60 minutes. During 40 minutes of rewarming, animals were warmed to 37°C with a flow rate of 100 mL/kg/minute.

Mean arterial pressure (MAP) and esophageal and rectal temperature were recorded every 10 minutes. Arterial Po₂, Pco₂, pH, hematocrit, and lactate level and mixed venous oxygen saturation were measured every 15 minutes on CPB, at 30 minutes after CPB, and once an hour until extubation with a blood gas analyzer (Rapidlab 1200; Siemens, New York, NY). Animals were mechanically ventilated under sedation and monitored continuously for 12 hours after CPB. The chest tubes were removed, and the animals were extubated on postoperative day (POD) 1.

Leukocyte-endothelial Cell Interactions

The animals were placed prone into a stereotactic frame. After removal of the scalp, a hole was drilled over the right parietal cortex 3 mm inside the sagittal and coronal sutures. A Leica stereo epifluorescence microscope (Model MZFL III, Leica, Heerbrugg, Switzerland) with a 100 W mercury gas discharge lamp equipped with a rapid filter exchanger (including 3 sets of filters) was placed above the cranial window. The images from the CCD camera (Dage DTI) were displayed on a high-resolution 12-inch monitor. The final magnification on the monitor was ×400. The analysis of the recorded images was performed offline on a computer. The diameter of venous cerebrocortical microvessels was measured from video still images by use of an image analysis program after calibration of the software with a video caliper. On average, 4 to 5 arterioles and 5 to 6 venules per observation area were measured. More detail of the preparation and materials for intravital microscopy have been described previously.^{14, 16}

Plasma was labeled with 0.05 mL/kg of fluorescein isothiocyanate–dextran 5% before each measurement to visualize microvessels within the cerebral microcirculation. Twenty to 40-μm diameter postcapillary venules were observed for the assessment of circulating leukocytes which were labeled with 0.05 mL/kg of rhodamine-6G 0.2%. Adherent leukocytes were defined as leukocytes that attached to the endothelium and did not detach within a 30-second observation period. The number of adherent leukocytes was expressed per area over a 100-μm length and 30-μm diameter. Rolling leukocytes were distinguishable by their slower velocity than free-flowing leukocytes. The number of rolling leukocyte was counted for 30 seconds and expressed as number per minute per area with 100-μm length and 30-μm diameter. Total activated leukocytes were defined as the sum of adherent and rolling leukocytes.

Intravital fluorescence microscopy was performed at baseline; at 10 minutes of normothermic CPB; at 20 minutes of cooling; at the end of cooling; at every 15 minutes during the ultra low-flow period; at 5, 15, 30, and 40 minutes of rewarming; and 30, 60, 120, and 180 minutes after weaning from CPB.

Neurologic and Behavioral Evaluations

Postoperative neurologic and behavioral evaluations were performed by a senior assistant blinded to the experimental protocol on POD1 and continued at 24-hour intervals up to POD4. Neurologic scoring was adapted from modified NDS. In modified NDS, four general components of the neurologic examination are evaluated and a score of 100 is assigned to each category, which includes level of consciousness, respiration, motor and sensory function, and overall behavior such as drinking or walking. A total score of 400 indicates brain death, whereas a score of 0 is normal.¹⁷

Histological Analysis

The brain was harvested on POD4. The preparation of the cerebral specimens and the details of histologic analyses have been described previously.¹⁸ Histologic damage was scored using the following criteria: 5, cavitated lesions with necrosis; 4, significant damage to neurons; 3, large clusters of injured neurons; 2, small clusters of damaged neurons; 1, isolated neuronal damage; 0, normal. A single neuropathologist (H.G.W.L.) examined all specimens in a blinded fashion.

Hematological Assessments and Renal Functions

Systemic hematological studies including leukocyte and platelet counts were performed at baseline, at the end of cooling, at the end of CPB, and at 3 hrs after CPB, respectively. Serum Cr, BUN, urine protein, and urine Protein/Cr were measured at baseline, POD1 and POD4, respectively.

Statistical Analysis

Continuous variables are expressed as mean $\pm$ standard deviation. The Kolmogorov-Smirnov test of normality indicated that leukocyte data, MAP, renal function data, and NDS all followed a normal (Gaussian-shaped) distribution. Paired *t*-tests were used for evaluating changes in serum Cr and BUN within experimental groups. Two-way repeated-measures analysis of variance (ANOVA) with F-tests for time and group was used to compare number of leukocytes, PCO₂, SVO₂, MAP, arterial pH, lactate, hematocrit, and platelets to test differences over time (baseline, cooling, rewarming, end of bypass) as well as between aprotinin doses at a fixed time point. In addition, one-way ANOVA was utilized to compare histological scores between aprotinin doses and CPB settings with Bonferroni adjustment.¹⁹ In order to develop a dose-response model that incorporated both CPB setting and aprotinin dose as predictors of NDS POD1, a general equation of the form $y = \beta_1 X_1 + \beta_2 X_2 + C$ was derived by multiple linear regression analysis, where X_1 is CPB setting, X_2 is dose and C is the fitted y-intercept.²⁰ The nonparametric Spearman rho correlation coefficient was used to measure the association between aprotinin dose and leukocytes during bypass. Statistical analysis was performed using the SPSS software package (version 16.0, SPSS Inc., Chicago, IL). Power analysis indicated that a minimum sample size of 18 animals randomized to each aprotinin dose (no aprotinin control, low dose, standard full, double full) would provide 80% power to detect an effect size of 30 points in NDS POD1 and a mean difference in BUN of 4 mg/dL on POD1 and POD4 between groups (nQuery Advisor, Statistical Solutions, Saugus, MA).

Results

Experimental Conditions and Hematological Assessments

There were no significant differences between aprotinin dose groups with respect to all experimental conditions and the number of systemic leukocytes and platelets in both studies (N=24 and N=74).

Mean Arterial Pressure

Mean arterial pressure in each aprotinin dose group was significantly higher than in the No Aprotinin group during rewarming ($F=3.22$, $P<0.03$, $N=74$).

Leukocyte-endothelial Cell Interactions

Adherent Leukocytes (N=24)—Adherent leukocytes were significantly increased between baseline and cooling (1.1 ± 1.0 to 2.0 ± 1.6 , $P<0.01$), stabilized during CPB, and then decreased after CPB (1.9 ± 1.9 to 1.4 ± 1.7 , $P<0.05$). There were no significant differences in numbers of adherent leukocyte between aprotinin dose groups at baseline ($F=1.8$, $P=0.18$), cooling ($F=2.0$,

$P=0.15$), late phase of rewarming ($F=2.2$, $P=0.12$), and post CPB ($F=1.2$, $P=0.34$), respectively. During the early phase of rewarming, adherent leukocytes in each aprotinin dose group were significantly lower than in the No Aprotinin group (Figure 1A). Aprotinin dose ($F=4.4$, $P<0.05$) but not bypass conditions ($F=2.2$, $P=0.15$) was an independent predictor of mean number of adherent leukocytes during CPB.

Rolling Leukocytes (N=24)—Rolling leukocytes were significantly increased from cooling to the early phase of rewarming (0.8 ± 1.0 to 2.7 ± 2.5 , $P<.001$) and decreased after CPB (2.2 ± 2.4 to 1.3 ± 1.7 , $P=0.01$). There were no significant differences in numbers of adherent leukocyte between aprotinin dose groups at baseline ($F=0.9$, $P=0.48$) and during the late phase of rewarming ($F=1.8$, $P=0.19$). During cooling and the early phase of rewarming, rolling leukocytes in group DF were significantly lower than in the No Aprotinin group (Figure 1B). After CPB, rolling leukocyte in group LD were significantly higher than them in group DF ($F=4.6$, $P<0.008$). Aprotinin dose ($F=9.9$, $P<0.01$) but not bypass conditions ($F=0.1$, $P=0.88$) was an independent predictor of the mean number of rolling leukocyte during bypass. A significant inverse correlation was found between aprotinin dose and rolling leukocytes in each bypass period; cooling ($\rho=-0.67$, $P<0.01$), early phase of rewarming ($\rho=-0.70$, $P<0.01$), and late phase of rewarming ($\rho=-0.46$, $P<0.05$). The mean number of rolling leukocytes during CPB had a significant inverse correlation with a higher aprotinin dose (Figure 1C).

Activated Leukocytes (N=24)—The number of activated leukocytes during cooling in group SF and DF was significantly lower than in the No Aprotinin group ($F=4.6$, $P<0.008$), and the number during the early phase of rewarming in each aprotinin group was significantly lower than in the No Aprotinin group ($F=10.7$, $P<0.008$). There were no significant differences during the late phase of rewarming ($F=2.9$, $P=0.06$) and post CPB ($F=1.2$, $P=0.34$). Aprotinin ($F=10.4$, $P<0.01$) but not bypass conditions ($F=1.3$, $P=0.30$) was an independent predictor of activated leukocytes. A significant inverse correlation was found between aprotinin dose and activated leukocytes in each bypass period; cooling ($\rho=-0.54$, $P<0.05$), early phase of rewarming ($\rho=-0.72$, $P<0.001$), and late phase of rewarming ($\rho=-0.48$, $P<0.05$). Mean number of activated leukocyte during CPB in group SF and DF was significantly lower than in the No Aprotinin group (Figure 1D).

Neurologic and Behavioral Evaluations

Both aprotinin dose ($t=-4.81$, $P<0.0001$, $N=74$) and bypass conditions ($t=8.24$, $P<0.0001$, $N=74$) were independent predictors of NDS on POD1. The two variable regression model had good accuracy ($R_2=0.62$, $P<0.0001$, $N=74$), and aprotinin predicted NDS on POD1 based on linear dose-response model. The predictive equation was: Predicted Score = $67X_1 - 28X_2 + 33$, where X_1 is CPB setting ($1=25^\circ\text{C}$, $10\text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $2=34^\circ\text{C}$, $10\text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $3=25^\circ\text{C}$, Circulatory arrest) and X_2 is aprotinin dose ($0=\text{No Aprotinin}$, $1=\text{LD}$, $2=\text{SF}$, $3=\text{DF}$). For a given bypass setting, each increase of one level in aprotinin dose was associated with a predicted NDS that was 28 points lower (Table 1, Figure 2).

Histopathologic Analysis

There was no significant differences in histological score among aprotinin dose groups (No Aprotinin; 7.5 ± 6.6 , LD; 5.7 ± 5.2 , SF; 5.7 ± 5.3 , DF; 6.7 ± 6.1 , $F=0.4$, $P=0.75$, $N=74$). Bypass conditions ($F=26.7$, $P<0.0001$, $N=74$) but not aprotinin dose ($F=0.7$, $P=0.53$, $N=74$) were predictive of histological score.

Renal Function

Serum Cr and BUN were significantly increased from baseline to POD1 (Cr; 0.9 ± 0.2 to 1.0 ± 0.4 , $P<0.001$, BUN; 9.3 ± 3.6 to 26.9 ± 10.4 , $P<0.0001$, $N=74$) and decreased from POD1 to

POD4 (Cr; 1.0 ± 0.4 to 0.7 ± 0.1 , $P < 0.01$, BUN; 26.9 ± 10.4 to 13.3 ± 5.6 , $P < 0.0001$, $N = 74$), respectively. Urine Protein and urine Protein/Cr were significantly decreased from baseline to POD1 (Protein; 10.9 ± 7.9 to 7.4 ± 5.4 , $P < 0.05$, Protein/Cr; 0.9 ± 1.1 to 0.2 ± 0.1 , $P < 0.05$, $N = 24$), and increased from POD1 to POD4 (Protein; 7.4 ± 5.4 to 53.0 ± 34.0 , $P < 0.0001$, Protein/Cr; 0.2 ± 0.1 to 0.5 ± 0.4 , $P < 0.05$, $N = 24$), respectively. There were no significant differences between aprotinin dose groups in both studies (Table 2).

Discussion

Aprotinin reduces cerebral leukocyte activation

This study confirms the anti-inflammatory action of aprotinin during cardiopulmonary bypass as documented by reduced white cell activation. Leukocyte adhesion during the early phase of re-warming in each aprotinin group was significantly inhibited, suggesting that even pump priming alone with aprotinin can reduce leukocyte-endothelial cell interaction. Differences in leukocyte adhesion shown in Figure 2A also suggest that pump priming with aprotinin can reduce the massive increase in adhesion which occurs at the start of bypass and can inhibit the aggravation of adhesion seen with rewarming.

On the other hand, leukocyte rolling during cooling and the early phase of rewarming was significantly inhibited by the double full dose regimen but not the low dose. Leukocyte rolling had an inverse correlation with a higher aprotinin dose, suggesting that continuous infusion of aprotinin can reduce leukocyte rolling.

Both the standard and double full dose regimen, but not the low dose regimen, significantly reduced leukocyte activation, which we define as the total of leukocyte adhesion and rolling during CPB. Both standard and double full dose regimens are recommended to inhibit the inflammatory response of cerebral endothelial cells.

Aprotinin accelerates neurologic recovery

Aprotinin was associated with more rapid recovery of neurological and behavioral outcome after bypass in a dose-dependent manner. The principal mechanism of the accelerated recovery may be protection of the endothelial function of cerebral microvessels by the anti-inflammatory effect of aprotinin. In a previous study we showed an inverse correlation between leukocyte activation and eNOS expression in cerebral endothelial cells.²¹ Protection of endothelial cell function such as eNOS expression would contribute to a reduced impact of cytokine attack or free radical attack on neurons or glia, and also would reduce brain edema. Recent reports have also suggested that the protease tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke.²² The serine protease inhibitor aprotinin may protect the blood-brain barrier by inhibition of the plasminogen plasmin system.

Although aprotinin accelerated functional neurologic recovery in our study, it was not effective in reducing histological neuronal damage. If enough amount aprotinin could pass the brain-blood barrier, it should reduce ischemic neuronal injury¹² suggesting that aprotinin did not pass the blood-brain barrier in our model. This might be because of insufficient protective effect on the blood-brain barrier or insufficient insult to the barrier by the bypass conditions. More studies of the permeability of the brain-blood barrier during bypass are needed.

In this study, we assessed neuronal injury but not injury of glial cells such as astrocytes and oligodendrocytes. In the developing brain oligodendrocytes are known to be much more vulnerable than neurons to inflammation or ischemia/reperfusion injury. Cerebral palsy is frequently a result of oligodendrocyte injury which has resulted in white matter injury usually adjacent to the lateral ventricles (periventricular leukomalacia). Recent reports have confirmed the susceptibility of the developing brain to white matter injury after bypass.²³ Future studies

are also needed to investigate the impact of CPB on the function and development of glial cells such as oligodendrocytes.

Aprotinin's impact on renal function

The observational study in adults published by Mangano et al suggested that renal failure was an important risk of aprotinin use during bypass in adults.⁷ In the prospective BART study, however the use of aprotinin did not increase the risk of renal failure.⁶ Other recent reports have also concluded that there is no direct correlation between aprotinin and renal dysfunction^{24, 25} which has also been the experience in children. In the current study aprotinin even with a double full dose regimen had no impact on Cr, BUN, Urine protein, and urine protein Cr ratio after CPB. We conclude that aprotinin had no measurable impact on standard indices of renal function in a young piglet model. This is consistent with the clinical experience that aprotinin does not increase the risk of renal failure in pediatric patients.

The use of aprotinin in pediatric patients has greater benefit than the risk

In conclusion aprotinin reduces cerebral leukocyte activation and accelerates neurologic recovery in a dose dependent fashion in a piglet model of high stress cardiopulmonary bypass. Aprotinin has no significant effects on standard indices measuring renal function in young piglets. The availability of aprotinin should be reconsidered for pediatric patients undergoing CPB who could benefit from its use.

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Figure 1.

A. Administration of aprotinin reduced leukocyte adhesion at early phase of rewarming. **B.** Rolling leukocytes in group DF were significantly lower than the number in the No Aprotinin group. **C.** Aprotinin reduced leukocyte rolling during bypass in a dose dependent fashion. **D.** Group SF and DF reduced the mean number of activated leukocytes during bypass.

* $P < 0.0083$ vs. No Aprotinin; ANOVA with Bonferroni comparison, † $P < 0.001$ vs. No Aprotinin; ANOVA with Bonferroni comparison.

Figure 2.

Aprotinin predicted NDS on POD1 based on linear dose-response regression model: Predicted Score = $67X_1 - 28X_2 + 33$, where X_1 is CPB setting (1=25°C, 10 mL·kg⁻¹·min.⁻¹, 2=34°C, 10 mL·kg⁻¹·min.⁻¹, 3=25°C, Circulatory arrest) and X_2 is aprotinin dose (0=No Aprotinin, 1=LD, 2=SF, 3=DF).

Table1

Predicted Neurological Deficit Scores Based on Aprotinin Dose for Each Bypass Condition

		Ultra-Low Flow Bypass Groups		HCA Group
		25°C, 10 mL·kg ⁻¹ ·min. ⁻¹	34°C, 10 mL·kg ⁻¹ ·min. ⁻¹	25°C, Circulatory arrest
Aprotinin		(n=24)	(n=25)	(n=25)
No Aprotinin	(n=20)	100	167	234
Low Dose	(n=18)	72	139	206
Standard Full	(n=18)	44	111	178
Double Full	(n=18)	16	83	150

HCA; Hypothermic circulatory arrest

Table 2

Renal Function

	No Aprotinin	LD	SF	DF	F-test	P-value
Serum Cr (mg/dL)						
Baseline	0.8 ± 0.2	0.9 ± 0.3	0.8 ± 0.2	0.9 ± 0.3	1.49	0.23
POD1	1.0 ± 0.4	1.1 ± 0.4	1.1 ± 0.6	1.0 ± 0.2	0.54	0.66
POD4	0.7 ± 0.1	0.8 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	1.52	0.24
BUN (mg/dL)						
Baseline	9.7 ± 3.0	10.0 ± 4.5	8.5 ± 2.7	9.3 ± 4.2	0.54	0.66
POD1	28.1 ± 10.8	29.0 ± 10.1	26.9 ± 13.2	23.6 ± 6.4	0.90	0.45
POD4	12.7 ± 7.2	10.4 ± 3.7	16.8 ± 6.0	12.6 ± 2.7	1.39	0.28
Urine Protein (mg/dL)						
Baseline	7.6 ± 3.2	14.3 ± 10.0	10.0 ± 5.9	13.0 ± 12.0	0.68	0.58
POD1	7.2 ± 3.9	10.2 ± 10.2	6.0 ± 0.0	6.3 ± 0.8	0.72	0.55
POD4	46.2 ± 13.3	72.8 ± 54.6	38.8 ± 16.4	54.2 ± 39.7	0.85	0.48
Urine Protein/Cr						
Baseline	1.4 ± 1.9	0.5 ± 0.1	0.6 ± 0.3	1.1 ± 1.1	0.74	0.54
POD1	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	1.47	0.26
POD4	0.5 ± 0.4	0.4 ± 0.2	0.5 ± 0.2	0.6 ± 0.6	0.27	0.85

LD; Low dose, SF; Standard full dose, DF; Double full dose

Cr; serum creatinine, BUN; blood urea nitrogen, POD; Postoperative day