Dynamics of Oxidative Stress Evoked by Myocardial Ischemia Reperfusion After Off-Pump Coronary Artery Bypass Grafting Elucidated by Bilirubin Oxidation

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Background: Revascularization therapy relieves myocardial ischemia, but can also result in ischemia-reperfusion injury caused by oxidative stress. However, the biokinetics of oxidative stress after myocardial ischemia-reperfusion are uncertain. This study aimed to evaluate the dynamics of oxidative stress after off-pump coronary artery bypass grafting (OPCAB) by measuring urinary biopyrrin levels. Biopyrrin is an oxidative metabolite of bilirubin thought to reflect oxidative stress, along with reactive nitrogen species (RNS).

Methods and Results: The study included 18 patients who underwent OPCAB; patients were divided into effort angina pectoris (EAP; n=11) and unstable angina pectoris (UAP; n=7). Urinary biopyrrin and RNS levels were measured during the perioperative period (<48h after surgery). Biopyrrin levels transiently increased 4–12h post-surgery (early phase), followed by a prolonged increase approximately 24–32h post-surgery (late phase). The delayed increase in biopyrrin tended to be higher in patients with UAP, with a simultaneous increase in RNS. The patients in the UAP group had generally high pulmonary capillary wedge pressure (PCWP), although the cardiac index was within a normal range during the delay phase.

Conclusions: The dynamics of biopyrrin levels revealed a biphasic pattern of oxidative stress after OPCAB. Delayed production of oxidative stress may be influenced by preoperative severity of myocardial ischemia and delayed RNS production.

Key Words: Bilirubin oxidation; Biopyrrin; Coronary artery bypass grafting; Oxidative stress

Insufficient blood flow causes deficient oxygen and energy supply to tissues, resulting in malperfusion and necrosis. Restoration of blood flow and re-oxygenation improves metabolism in ischemic cells, thus salvaging the tissue. However, it has previously been reported that reperfusion of ischemic tissue can also cause ischemia-reperfusion (IR) injury. Oxidative stress evoked by re-oxygenation may be a contributing factor in the tissue injury. Tissue damage from reactive oxygen species (ROS) has been suggested as one mechanism underlying IR injury. Several products of oxidative stress are known to be associated with IR (Figure 1). Angina pectoris (AP) is caused by widespread myocardial ischemia from transient or continuous interruption in coronary blood flow. Complete coronary revascularization, such as coronary artery bypass grafting (CABG), may cause major oxidative stress while improving the widespread myocardial ischemia. Some patients experience postoperative complications such as lung injury 1 or 2 days after cardiac surgery. We have focused on the fact that ROS increase again one day after MIR. It is important to understand whether oxidative stress is increased during the perioperative period, especially in the late phase after CABG.

In this study, we used biopyrrin, a marker of oxidative stress, to investigate the dynamics of oxidative stress. Some reported ROS scavengers include allopurinol, statins, and edaravone. Bilirubin is also a suicidal ROS scavenger. Oxidation of bilirubin results in the formation of biopyrrin. One of the advantages of biopyrrin as an oxidative stress marker is that it is excreted in the urine and thus reflects oxidative stress in real time. Before acute heart transplant rejection, urinary levels of biopyrrin increase earlier than those of troponin, a marker of myocardial damage. It has been reported that the reaction of ROS and nitric oxide (NO) results in the production of reactive nitrogen species (RNS), which can cause severe
was confirmed using near-infrared fluorescence angiography and transit time flowmetry under stable hemodynamics before administration of protamine.24,25

Although this study was designed to study the dynamics of oxidative stress after OPCAB, the oxidative stress dynamics after extended thymectomy (EXT) for thymoma and myasthenia gravis were also measured, as both OPCAB and EXT patients underwent median sternotomy and the same maneuvers, apart from coronary artery anastomosis. It was hypothesized that EXT patients would not have significant oxidative stress because they were not exposed to tissue injury.7 RNS may be involved in MIR injury as inflammatory mediators, although the detailed mechanism is unknown. It has been confirmed that bilirubin oxidation by RNS can produce biopyrrin.15

The aim of this study was to investigate the urinary levels of biopyrrin for evaluating oxidative stress levels in the perioperative period following OPCAB for AP. Furthermore, this study also assessed the dynamics of RNS and their role in biopyrrin production.

Methods

Subjects

We hypothesized that patients who undergo OPCAB for AP produce oxidative stress during reperfusion of myocardial ischemia. A total of 23 patients admitted to Kochi Medical School Hospital underwent OPCAB over a period of 18 months. OPCAB was used unless the patient was critically ill, as in cardiogenic shock; however, none of the study patients with ischemic heart disease (IHD) were deemed critical. Subjects included patients undergoing isolated OPCAB (OPCAB group; n=18; age 71.1±2.3 years; Table 1) during this study period. All the patients who agreed to participate in this study were included (n=18); 5 patients did not participate for various reasons. Another 26 patients underwent CABG with CPB during this period. One IHD patient underwent on-pump beating CABG for a severely dilated left ventricle and reduced ventricular function. Another 25 CPB-supported CABG patients had concomitant valve procedures (24 patients) or constrictive pericarditis (1 patient). No OPCAB patient had CPB or steroid administration, as these can influence oxidative stress production after MIR. The surgical approach used during OPCAB was the classical median sternotomy, and intracoronary shunt tubes were used during the formation of anastomoses to maintain coronary arterial blood flow; side-to-side or end-to-side anastomoses were made with the internal thoracic artery, radial artery, and saphenous vein. After the anastomoses were completed, patency of the grafts

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**Oxidative Stress Pathway**

1. Xanthine oxidase system
   - Deficient ATP + re-oxygenation
   - ATP → ADP → AMP → Adenosine → Xanthine
   - Xanthine → Hypoxanthine
   - Endothelial cell
   - O$_2$ (reperfusion) → H$_2$O$_2$ → OH$ullet$

2. NADPH oxidase system
   - Leukocyte infiltration
   - NADPH → O$_2$ → O$_2$ → H$_2$O$_2$ → Myeloperoxidase
   - H$_2$O $\rightarrow$ H$_2$O

3. Nitric oxide
   - NO + O$_2$ $\rightarrow$ ONOO$^-$

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**Biopyrrin and bilirubin oxidation**

Myocardial Ischemia Reperfusion

- Reactive Oxygen Species
- Heme oxygenase-1 (HO-1)

- Scavenger
- Biliverdin
- Bilirubin
- Biopyrrin

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**Figure 1.** Hypothesized production pathway of oxidative stress after myocardial ischemia-reperfusion. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide.

**Figure 2.** Biokinetics of biopyrrin and bilirubin oxidation after myocardial ischemia-reperfusion (MIR). The reactive oxygen species (ROS) induces heme oxygenase-1 that produces bilirubin from heme and biliverdin after MIR. Bilirubin quenches ROS as a suicidal radical scavenger, which results in the formation of biopyrrin.
Ethical Considerations

The study protocol was approved by the Ethics Committee of Kochi Medical School and was carried out according to the Declaration of Helsinki. Written informed consent was given by every patient and/or family.

Collection of Samples

Urine samples were collected before surgery, during the start of the operation, 1 and 2 h after starting surgery, at the end of surgery, and every 4 h thereafter for the following 48 h in all groups. Individual urine samples were collected from urethral catheters and cryopreserved at −80°C prior to assay within approximately 1 month.

Measurement of Biopyrrin and RNS in Urine Samples

After centrifugation of the urine samples at 3,000 g, biopyrrin levels were measured by enzyme-linked immunosorbent assay (ELISA), using antibody 24G7 (Biopyrrin EIA Kit; Shino-Test, Tokyo, Japan), according to the manufacturer’s protocol. Bilirubin and biopyrrin levels were measured with the Biopyrrins EIA Kit, and the total bilirubin concentration in the sample was measured separately. Total urine bilirubin concentration was measured using Vanadate Oxidation Total Bilirubin E-HR (Wako Pure Chemical Industries, Osaka, Japan; detection limit, 0.2 mg/dL). The urinary biopyrrin levels were corrected for urinary creatinine levels, as measured using an enzymatic assay. RNS were measured by concentrations of nitrite/nitrate (NO2/NO3)
Results

Patients’ Characteristics

Patients’ characteristics are shown in Table 1. There were no significant differences in age among the groups. The prevalence rates of risk factors, including diabetes mellitus, hypertension, dyslipidemia, smoking, and comorbidities, including previous cerebrovascular attack and chronic renal failure, were not significantly different among the OPCAB, EAP, UAP, and EXT groups.

The prevalence rate of LMT disease was 44.4%, 27.3%, and 71.4% of patients in the OPCAB, EAP, and UAP groups, respectively. The rate of 3VD was 77.8%, 72.7%, and 85.7% in the OPCAB, EAP, and UAP groups, respectively. There were no differences in the frequency of LMT disease and 3VD between the EAP and UAP groups. Moreover, the prevalence rate of severe ischemia (LMT >90% or branches >99%) was 45.5% and 100% in the EAP and UAP groups, respectively.

Operative and Postoperative Data

The OPCAB group had longer surgical duration, greater amounts of bleeding, and greater amounts of blood administered during surgery compared with the EXT group (Table 1). Although the operation time was longer in the UAP group than in the EAP group, there was no significant difference between these groups in bleeding or transfusion amounts. The number of anastomoses performed was 3.7±0.3 per patient in the OPCAB group, and there was not a significant difference in the number of anastomoses performed between the EAP and UAP groups (3.6±0.3 and 3.7±0.2). Intraoperative near-infrared fluorescence angiography revealed patency for all grafts. Furthermore, ECG revealed no postoperative ST changes in the groups. No patients had complications of cerebral infarction, respiratory failure, or renal failure after surgery. Neither group had any cases of surgery-related or hospitalization-related deaths. There were no differences in systolic and diastolic arterial blood pressures between the OPCAB and EXT groups, including the EAP and UAP subgroup (Table 2).

Statistical Analysis

All group differences in the urinary and hematological data and the results of the pressure study were statistically analyzed by Mann-Whitney U-test. The unpaired t-test was used to compare the patient characteristics. P<0.05 was considered statistically significant. All results are expressed as mean ± standard error of the mean. JMP statistical software version 11 (SAS Institute, Cary, NC, USA) was used to perform all statistical analyses. A sample size calculation was not performed because this was a pilot study.
There was also no significant difference in preoperative urinary NO₂/NO₃ concentration among the groups (Figure 4). Urinary NO₂/NO₃ concentration increased transiently in the OPCAB and EXT groups immediately after surgery, and tended to increase again at 24–32 h (Figure 4A). The NO₂/NO₃ concentration showed a tendency of a higher value in the UAP group than in the EAP group, although there were no significant differences (Figure 4B). However, only at 32 h after surgery did NO₂/NO₃ concentrations show a greater increase in the UAP group than in the EAP group (P=0.015).

**Measurement of Serum Levels of Total Bilirubin, CRP, CPK, LDH, and Creatinine**

Although there was no significant difference in the preoperative serum levels of total bilirubin and CRP between the groups, CPK and LDH levels were significantly higher in the UAP group than in the EAP and EXT groups (Figure 5). The levels of CPK-MB isozyme were also higher in the UAP group than in the EAP group before the operation. Moreover, increases in all enzymes were observed immediately after surgery and at 12 and 36 h after surgery in the UAP and EAP groups, although there were significant differences between the EAP and UAP groups. Creatinine values were significantly increased in the EAP and UAP groups; however, values were ≤1 mg/dL. Thus, there was no effect on urinary excretion, including urinary excretion of biopyrrin.

In addition, the UAP group showed high PAP 12 h after the operation. Both the CI and PCWP of all patients in this study were within normal ranges.

**Urinary Biopyrrin Dynamics**

The urinary biopyrrin levels of the OPCAB and EXT groups were assessed (Figure 3A). Urinary biopyrrin levels were measured to understand the time course of systemic bilirubin oxidation in the perioperative period. The levels transiently increased around 4–12 h (early phase), followed by a prolonged increase until 24–32 h (late phase); the levels then decreased 48 h after surgery. Conversely, patients in the EXT group did not have a significant increase during the observation period. The biopyrrin levels were prone to be higher in patients with OPCAB than in the EXT group, although there were no significant differences between the 2 groups. A comparison between the UAP and EAP groups showed a tendency of higher values in the UAP group, although not significant (Figure 3B). The early phase increase of biopyrrin was prolonged until 12 h after surgery in the UAP group and the late phase of biopyrrin elevation was longer in the UAP group. However, the biopyrrin level (24 h after surgery, P=0.014) showed significance. Urinary bilirubin was below detectable levels in all groups.

**Urinary NO₂/NO₃ Dynamics**

There was also no significant difference in preoperative urinary NO₂/NO₃ concentration among the groups (Figure 4). Urinary NO₂/NO₃ concentration increased transiently in the OPCAB and EXT groups immediately after surgery, and tended to increase again at 24–32 h (Figure 4A). The NO₂/NO₃ concentration showed a tendency of a higher value in the UAP group than in the EAP group, although there were no significant differences (Figure 4B). However, only at 32 h after surgery did NO₂/NO₃ concentrations show a greater increase in the UAP group than in the EAP group (P=0.015).
Oxidative Stress of OPCAB

The aim of this exploratory study was to investigate the changes in oxidative stress following OPCAB by measuring urinary biopyrrin levels. A transient increase in biopyrrin levels was observed 4–12 h after OPCAB surgery (early phase), followed by a prolonged increase until 48 h (late phase); the levels then decreased 48 h after surgery. Thus, biopyrrin levels exhibited a biphasic pattern of increase. There was no significant difference between the OPCAB and EXT groups statistically, as this study had a small sample size. However, this study showed that the urinary biopyrrin value tended to be higher in the OPCAB group at all observation points. Therefore, it is expected that the urinary biopyrrin level will be a useful oxidative stress maker after revascularization therapy, including CABG. The increase in biopyrrin level was greater in the UAP group than in the EAP group, which suggested that the difference in oxidative stress during the late phase depends on the severity of the myocardial ischemia before reperfusion therapy. Fortunately, there were no patients with lung edema or congestion observed on radiography. The systolic PAP tended to be higher and the CI lower after surgery in the UAP group. In addition, lung congestion may cause oxidative stress,33 even mild oxidative stress, because elevated CPK-MB isozyme levels suggest several myocardial injuries. Another experimental study suggested that biopyrrin synthesis was by inflammatory cells that invaded the alveolar wall in the lungs after MIR, as in systemic inflammatory response syndrome (SIRS).13 However, further study is required to elucidate the relationship between lung congestion and oxidative stress, as the lung is a target in various inflammatory situations and because the mechanism by which oxidative stress is generated in the lung is not well understood.33 There was no significant difference between the UAP and EAP groups statistically, as this study had a small sample size. However, this study showed that the urinary biopyrrin value tended to be higher in the UAP group at all observation points. Further examination is required to determine whether the urinary biopyrrin value reflects ischemic severity before myocardial IR.

There was a slight increase in \( \text{NO}_2/\text{NO}_3 \) in the UAP patients after surgery, although we had previously hypothesized that the reaction between ROS and NO results in RNS production.7 The RNS evaluated by their urinary concentrations were higher in the UAP patients than in the EAP group; this gap may be related to the severity of preoperative myocardial ischemia. NO itself is not toxic and does not induce tissue injury, even at high doses, though NO can react with ROS to produce harmful RNS such as peroxynitrite, resulting in tissue injury,33–38 and there was a lag in the increase time between biopyrrin and \( \text{NO}_2/\text{NO}_3 \) in the UAP group. The urinary biopyrrin value may indicate ongoing changes in oxidative stress more rapidly and sensitively than either an oxidative stress marker or an enzyme.15 Furthermore, this clinical study was designed to assess oxidative stress in patients undergoing OPCAB surgery.
such that we could not consider the influence of nitrates, including nitroglycerin, which are generally administered in the perioperative period following coronary re-vascularization therapy. A nitrate was administered to the OPCAB group during the observation period; however, in a preliminary experiment in rats, the administration of Milisrol (Nippon Kayaku Co., Ltd., Tokyo, Japan), a nitrate preparation, did not increase urinary NOx/NO. There is utility in evaluating biopyrrin levels because they clearly reflect ROS and RNS levels, although verification is needed. RNS may affect lung injury, but this study only focused on the increase in urinary biopyrrin levels and RNS in the late phase of OPCAB. Further study is required to elucidate the relevance of RNS in lung congestion. Furthermore, it is possible that inhibiting RNS prevents pneumopathy after MIR, although this series had no patients with severe lung congestion or edema after surgery. Although within the normal limits, the slight difference in PAP and PCWP between the UAP and EAP patients suggests an area of focus for the treatment of postoperative patients.

**Study Limitations**

We assessed the change in oxidative stress after OPCAB for AP, and there were some limitations. The first limitation was the small sample size, and the second limitation was that a sample size calculation was not performed, because this was a pilot study. The third limitation was the lack of a true control group, although there was comparison with the EXT group. The fourth limitation was that OPCAB is surgically invasive, especially considering surgical duration and the amount of blood administered during surgery. In short, the possibility that oxidative stress occurs because of invasive surgery cannot be excluded and further conclusions are difficult to draw based on this study. However, the patients in the EXT group without myocardial IR did not show an increase in biopyrrin levels. Therefore, it may not be wrong that myocardial IR causes an increase in biopyrrin levels. The fifth limitation was that postoperative treatment and hemodynamics were not factored in for each patient.

**Conclusions**

This study evaluated the dynamics of oxidative stress caused by MIR after OPCAB by measuring urinary biopyrrin levels. A transient increase in biopyrrin levels was observed shortly after surgery, followed by a prolonged increase in the late phase. Therefore, urinary biopyrrin levels may be useful for monitoring oxidative stress after OPCAB. Delayed production of oxidative stress may be influenced by preoperative severity of myocardial ischemia and delayed RNS production.

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**Conflict of Interest Statement**

M.Y. and other co-authors have no conflicts of interest.

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Oxidative Stress of OPCAB


