Table 2. Concentrations of inflammatory mediators in the autologous blood

	ANH (n = 23)	PD (n = 8)
Neutrophil elastase (ng·ml^{-1})	251 (151–280)	506 (479–633)*
IL-6 (pg·ml^{-1})	4.9 (2.3–4.7)	5.3 (2.0–5.5)
IL-8 (pg·ml^{-1})	10.0 (2.8–11.7)	18.1 (8.2–22.0)
IL-10 (pg·ml^{-1})	11.9 (5.9–18.2)	28.0 (11.4–42.6)*

*P < 0.05 vs ANH group
Concentration was determined at the time of retransfusion. Data values are expressed as means (25th–75th percentiles)

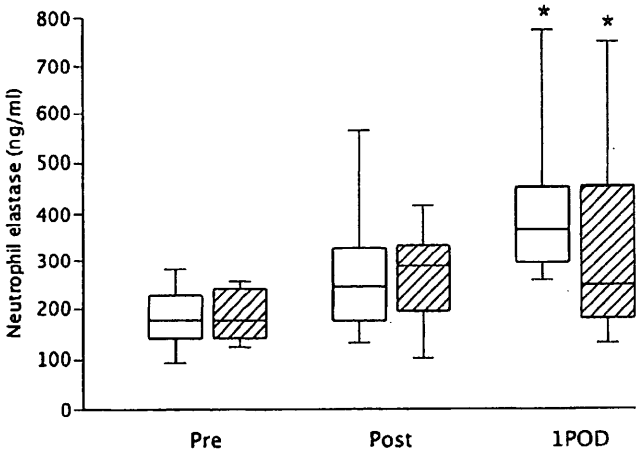

Fig. 1. The plasma concentrations of neutrophil elastase in the acute normovolemic hemodilution (ANH) group (n = 23; *open boxes*) and PD group (n = 8; *shaded boxes*) are summarized in this box plot. The median values, 25th–75th percentiles, and 10th–90th percentiles are given. *Pre*, after anesthetic induction; *Post*, at the end of operation; *1POD*, the morning after the operation. *P < 0.01 vs preoperative value with Wilcoxon rank sum test. No significant differences were noted between the ANH group and the PD group

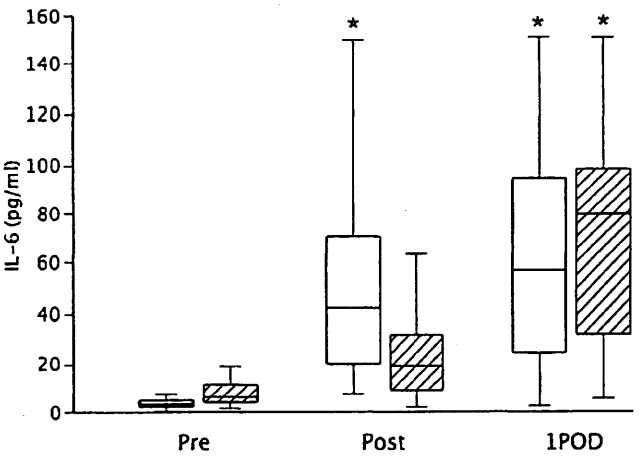

Fig. 2. The plasma concentrations of interleukin-6 (*IL-6*) in the ANH group (n = 23; *open boxes*) and PD group (n = 8; *shaded boxes*) are summarized in this box plot. The median values, 25th–75th percentiles, and 10th–90th percentiles are given. *Pre*, after anesthetic induction; *Post*, at the end of operation; *1POD*, the morning after the operation. *P < 0.01 vs preoperative value with Wilcoxon rank sum test. No significant differences were noted between the ANH group and the PD group

marized in Table 2. The concentrations of NE and IL-10 in the donated blood were significantly higher in the PD group than in the ANH group. However, neither IL-6 nor IL-8 levels in the donated blood differed between the two groups.

Changes in the plasma concentrations of the studied inflammatory mediators during the perioperative period are summarized in Figs. 1 to 4. There was a significant increase in the plasma NE concentration on POD 1 compared to the preoperative value in both groups, but there was no significant difference between the groups (Fig. 1). Plasma IL-6 also steadily increased immediately postoperatively and on POD 1 in the ANH group (Fig. 2). In the PD group, it remained unchanged immediately postoperatively, but was significantly increased on POD 1. Plasma IL-8 was significantly increased immediately postoperatively in the ANH group (Fig. 3) and then decreased significantly on POD 1 and returned to the preoperative level. In the PD group, no significant change in plasma IL-8 was noted during the study period. The plasma IL-10 level did not change during the study period in either of the groups (Fig. 4). Although the changing profiles of these inflammatory mediators in the two groups were somewhat different over time, the plasma concentrations of NE, IL-6, IL-8, and IL-10 at equivalent time points were not significantly different between the two groups.

Discussion

This study demonstrated that NE and IL-10 were significantly higher in PD blood than in ANH blood (Table 2). This finding indicates that the generation of inflammatory mediators is, to some extent, affected by storage conditions. The risks associated with allogeneic blood transfusions have been well recognized, and autologous

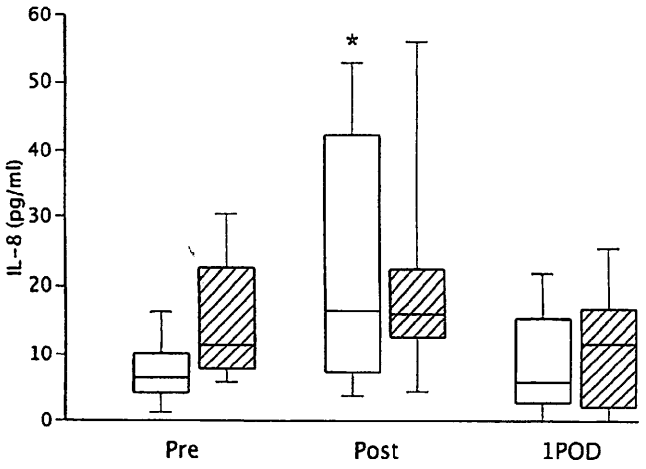

Fig. 3. The plasma concentrations of IL-8 in the ANH group ($n = 23$; *open boxes*) and the PD group ($n = 8$; *shaded boxes*) are summarized in this box plot. The median values, 25th–75th percentiles, and 10th–90th percentiles are given. *Pre*, after anesthetic induction; *Post*, at the end of operation; *1POD*, the morning after the operation. $*P < 0.01$ vs preoperative value with Wilcoxon rank sum test. No significant differences were noted between the ANH group and the PD group

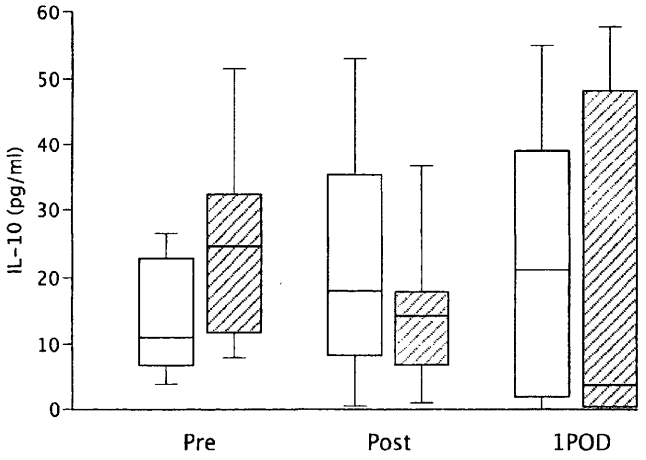

Fig. 4. The plasma concentrations of IL-10 in the ANH group ($n = 23$; *open boxes*) and the PD group ($n = 8$; *shaded boxes*) are summarized in this box plot. The median values, 25th–75th percentiles, and 10th–90th percentiles are given. *Pre*, after anesthetic induction; *Post*, at the end of operation; *1POD*, the morning after the operation. No significant differences were noted between any time points and the preoperative value, nor were any significant differences noted between the ANH group and the PD group

blood transfusion is now widely preferred [16]. One of the major advantages of autologous transfusion is that it causes less of a proinflammatory response when compared to allogeneic blood transfusions.

Recently, much attention has focused on the accumulation of inflammatory mediators during the retrieval and storage of donated blood. Possible relationships between the accumulation of inflammatory mediators and the side effects of blood transfusions, such as nonhemolytic febrile reaction, transfusion-related lung injury, and multiple organ failure after a massive transfusion have been investigated. For example, several studies have reported increased concentrations of NE, IL-1β, IL-6, and IL-8 during storage [17–20]. Additionally, Biedler et al. [13] reported that banked whole blood had an immunosuppressive effect that was largely attributable to storage-dependent factors. Jensen et al. [4] reported an increased IL-6 concentration 3 days after colorectal surgery in patients who underwent allogeneic transfusion, and they noted that the increase was attenuated by leukocyte depletion before storage.

Because these findings suggest that the presence of leukocytes during storage significantly augments the inflammatory response, leukocyte depletion in autologous transfusion may have the potential to attenuate the inflammatory response. Additionally, the storage duration is obviously longer in PD blood than in ANH blood. This storage period may have a significant impact on the transfusion-related inflammatory response. Recent investigations, which have reported that the prolonged storage of allogeneic blood might increase morbidity, may support this possibility [21–23]. The temperature during storage may also affect the inflammatory response. ANH blood is typically stored at room temperature in order to preserve platelet function [24,25]. Based on these possibilities, we hypothesized that the methods of autologous transfusion may affect the perioperative inflammatory response, because different storage durations and conditions may cause a distinct pattern of inflammatory mediator generation.

We found no differences in the IL-6 and IL-8 concentrations in the stored blood between our two groups. These cytokines are, presumably, released from the neutrophils and monocytes contained in the stored blood. Kristiansson et al. [18] reported increased concentrations of these cytokines in red blood cell concentrates during storage. Interestingly, they reported that the increase of IL-6 was independent of the length of storage, but the increase of IL-8 was dependent on the length of storage. In contrast, we found that the IL-10 concentration in the PD blood increased significantly during storage (Table 2). Hodge et al. [26] reported that IL-10 production during storage was decreased at room temperature and increased at 4°C. Our data correspond with their conclusion that temperature plays an important role in IL-10 production during storage.

The plasma concentrations of the investigated cytokines increased at some points of measurement in each study group, as shown in Figs. 1 through 4. However, there were no significant differences in the plasma concentrations of IL-6, IL-8, and IL-10 between the two

groups. Additionally, there was no apparent relationship between the concentration in the stored blood and the plasma sampled from the patients. Avall et al. [12] found that patients who were transfused with PD blood demonstrated higher IL-6 and IL-8 concentrations in plasma than patients who received allogeneic blood transfusion. They concluded that an attenuated cytokine response to allogeneic transfusion was a sign of immunosuppression. Heiss et al. [11] reported a significant increase in plasma IL-10 after an allogeneic transfusion but not after an autologous transfusion in patients undergoing colorectal cancer surgery. Tylman et al. [27] reported that reinfusion of salvaged blood resulted in an increased plasma IL-10 concentration. These results suggest that the difference between PD and ANH had less of an impact on the transfusion-triggered inflammatory response compared to allogeneic transfusion or the reinfusion of salvaged blood.

There are some limitations in the present study. First, the present study population varied in terms of background and surgical procedure. These differences were mainly caused by the fact that the decision to apply autologous transfusion was at the discretion of the surgeons and was based on their clinical preferences. These differences make the interpretation of the data somewhat difficult. The different baseline characteristics of the subjects, especially the presence of malignancy, may have affected the results. Previous investigations have demonstrated that preoperative values of the inflammatory cytokines that we investigated were similar in patients undergoing either surgery for cancer removal or orthopedic reconstructive surgery [4,11,12,27]. However, it is still possible that the transfusion-related inflammatory response was actually less in our ANH group, but the difference may have been undetected due to the influence of a more stressful surgical procedure in the ANH group. Second, the numbers of participants in the two groups were small and unevenly distributed. Because the cytokine concentrations were not normally distributed, formal power analysis was not feasible for our study. However, we do not think that increasing the number of participants would drastically change the results. Third, other medications may also affect the inflammatory response. For example, erythropoietin [28] and $FeSO_4$ [29] were administered to the PD group, while HES 70/0.5 was infused to maintain normovolemia in the ANH group. Thus, the anti-inflammatory properties of the HES solutions may have contributed to the results [30]. However, the preparations used in the present study have characteristics different from those of commonly used HES preparations, such as HES 130/0.4 or 200/0.5, so this possibility remains to be clarified [31]. Despite these limitations, the present study provided previously unknown information about the differences in inflammatory mediator generation during storage and the inflammatory response elicited in the recipients of PD blood and ANH.

In conclusion, this study demonstrated higher NE and IL-10 concentration over time in predonated autologous blood than in instantaneous autologous blood obtained by normovolemic hemodilution. This difference may be related to the differences in storage conditions between the two methods. However, the plasma concentrations of these inflammatory mediators were not different between the study groups after retransfusion, indicating that the inflammatory response was not affected by the method of autologous transfusion.

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An individualized recruitment maneuver for mechanically ventilated patients after cardiac surgery

Ryohei Serita[1], Hiroshi Morisaki[2], and Junzo Takeda[2]

[1] Department of Anesthesiology, Tokyo Dental College Ichikawa General Hospital, 5-11-13 Sugano, Ichikawa 272-8513, Japan
[2] Department of Anesthesiology, School of Medicine, Keio University, Tokyo, Japan

Abstract

Purpose. The recruitment maneuver (RM) has been shown to improve oxygenation for post-cardiopulmonary bypass (CPB) patients; however, sustained inflation of the lung gives rise to hypotension. The primary goal of our study was to evaluate the safety and efficacy of our proposed RM, defined on the basis of dynamic lung compliance (Cdyn).

Methods. Twenty-eight patients undergoing elective cardiac surgery with CPB were assigned to two treatment groups: an individualized RM group, in which a pressure equal to 15 ml × real body weight/Cdyn + positive end-expiratory pressure (PEEP) cmH_2O was applied for 15 s; and a control RM group, in which a pressure of 20 cmH_2O was applied for 25 s. Arterial blood pressure, cardiac output, pulmonary artery pressure, and heart rate (HR) were monitored. Tidal volume (Vt), and airway pressure were continuously obtained from an expiratory flow meter and pressure monitor. Blood samples were obtained and analyzed with a blood gas analyzer.

Results. The changes in HR, mean arterial pressure, mean pulmonary artery pressure, and cardiac index at the end of the RM were not significantly different between the two groups. The mean airway pressure of sustained inflation was 28.3 ± 1.3 cmH_2O in the individualized RM group. The individualized RM significantly improved the Cdyn and partial pressure arterial oxygen/inspiratory fraction of oxygen (P/F) ratio compared with values in the control RM group ($P = 0.026$ and $P = 0.012$, respectively).

Conclusion. The present study indicates that the individualized RM resulted in minimum changes of hemodynamics and brought about improvement in oxygenation and lung compliance.

Key words Recruitment maneuver · Dynamic compliance · Cardiopulmonary bypass · Cardiac surgery

Introduction

The recruitment maneuver (RM) has been shown to improve oxygenation for patients with cardiopulmonary bypass (CPB) [1]. However, sustained inflation of the lung often gives rise to a decrease in venous return and cardiac output (CO) in patients ventilated after CPB [2–6]. A previous study demonstrated that the RM using a sustained pressure technique with continuous inspiratory pressure of 40 cmH_2O for 10 s and 20 s reduced CO by more than 50%, reduced left ventricular end-diastolic area by about 45%, and reduced mean arterial pressure (MAP) by 20% in cardiac patients [6]. The hemodynamic effects of positive airway pressure will depend on the degree of lung inflation and holding time [3,4]. Furthermore, the inflation volume of the lung will contribute to lung compliance and chest wall elastance [5]. Although lung compliance varies among mechanically ventilated patients, in previous studies the level of the sustained inflation pressure used in RMs has been the same constant pressure [1,7]. The optimal pressure and duration of inflation have not been documented for RMs, so that the most effective technique for RM remains undetermined, despite many studies of acute respiratory distress syndrome (ARDS) [8].

Individualization of RM to respiratory mechanics may improve oxygenation without causing hemodynamic effects. Traditionally, static pulmonary mechanics has been used to assess lung mechanics [5]. However, recent studies have indicated that the application of dynamic respiratory mechanics in ventilated patients is more appropriate than the use of static lung mechanics [9–11]. Therefore, we have proposed the concept of an individualized RM that was defined on the basis of dynamic compliance (Cdyn), which is easily obtained from the ventilator as a bedside diagnostic tool [9–11].

The purpose of the present study was to assess the safety and efficacy of our individualized RM in patients after cardiac surgery. The primary endpoint of our study was to verify the hemodynamic effects induced by the individualized RM and to evaluate the improvement in oxygenation and Cdyn.

Address correspondence to: R. Serita
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Methods

Patients

With the approval of our institutional ethics committee and informed consent, from the patients, patients who had undergone elective cardiac surgery with CPB and received overnight mechanical ventilation were enrolled in this prospective and randomized study. Patients were admitted to the 14-bed medical and surgical intensive care unit (ICU) at a university hospital. All patients were mechanically ventilated with an 840 Ventilator System (Nellcor Puritan Bennet, Boulder, CO, USA). Patients with chronic obstructive lung disease (percent volume exhaled during the first second of a forced expiratory maneuver ([$FEV_{1.0\%}$] < 70%), intraoperative pulmonary trauma, and hemodynamic instability (cardiac index [CI] < 2.2 $l \cdot min^{-1} \cdot m^2$ and/or arterial systolic pressure < 80 mmHg) were excluded. Patients with a pacemaker, intraaortic balloon pumping, or percutaneous cardiopulmonary support were also excluded.

Protocol

All patients were ventilated in the pressure-regulated control mode with 5 cmH_2O of positive end-expiratory pressure (PEEP). Other ventilatory parameters were set as follows: inspiratory plateau pressure (≤30 cmH_2O) was set to obtain a tidal volume (VT) of 10–12 $ml \cdot kg^{-1}$, or inspiratory plateau pressure (>30 cmH_2O) was set to obtain a VT of 6–8 $ml \cdot kg^{-1}$; respiratory rate (RR) and inspiratory fraction of oxygen ($F_{I_{O_2}}$) were set to obtain a Pa_{CO_2} of 38–42 mmHg and a Pa_{O_2} of 150–180 mmHg; inspiratory time was set at 1.0 s. A standard-size tracheal tube was used, with a 7.5-mm inner diameter (ID) for women and an 8.5-mm ID for men. A standard ventilator tubing set (Universal Ventilator Tubing Set; Hudson Respiratory Care, Durham, NC, USA) was used for the respiratory circuit. The patients were well-sedated with a continuous infusion of propofol (50–100 $mg \cdot h^{-1}$), and were observed for 3 to 4 h after intensive care unit (ICU) admission so as to ascertain hemodynamic stability, which was defined as less than 15% variation of hemodynamic parameters with no clinically relevant bleeding (<100 $ml \cdot h^{-1}$).

The VT, RR, and airway pressure were obtained from the eligible patients, and the Cdyn in each patient was calculated as VT/(end-inspiratory pressure—PEEP), breath-by-breath. Then the eligible patients were randomly assigned to two treatment groups: an individualized RM group and a control RM group. In the individualized RM group, an inflation pressure (cmH_2O) equal to 15 × real body weight/Cdyn + PEEP (previously used) was applied for 15 s. We allowed a maximum inflation pressure of up to 45 cmH_2O. In the control RM group, an inflation pressure of 20 cmH_2O was applied for 25 s; this has been reported to have minimal effects on CO and arterial pressure in cardiac surgery patients [2,4]. A table of random numbers, generated by computer software, was utilized for patients' randomization into the two groups (Fig. 1).

The baseline variables of hemodynamics and respiration were obtained before the initiation of RM, and then hemodynamic variables were measured at the end of RM. Respiratory measurements were repeated at 15, 60, and 180 min after RM. The percent change (Δ%) in the variables was calculated [(variables post-RM–the variables at baseline) × 100/ variables at baseline].

Measurements and calculations

Arterial blood pressure was monitored through a radial artery catheter (20-G Arterial Line Kit; Argon Medical, Athens, TX, USA). Cardiac output (CO) and pulmonary arterial pressure (PAP) were measured by the thermodilution method, using a continuous CO catheter (Swan-Ganz CCO mbo; Edwards Life Sciences, Irvine, CA, USA). Heart rate (HR) was monitored by an electrocardiogram.

The VT, RR, and airway pressure were continuously obtained from the expiratory flowmeter and pressure monitor of the ventilator system. Calibrations of the flowmeter and oxymeter, and correction of the respiratory circuit, were performed daily by a biomedical engineer. Respiratory variables, which were obtained from

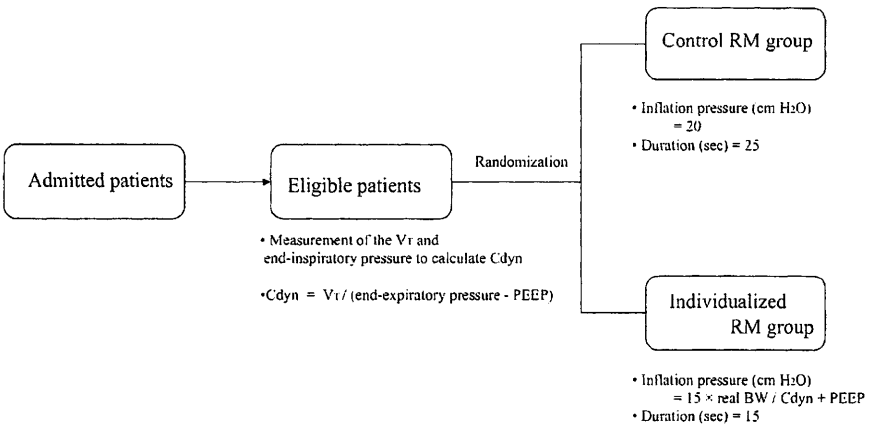

Fig. 1. Protocol for the setting of the recruitment maneuver (RM). *VT*, Tidal volume; *Cdyn*, dynamic compliance; *real BW*, real body weight; *PEEP*, positive end-expiratory pressure

the flowmeter and pressure monitor, were calculated by an average of five breaths. Arterial blood samples were analyzed with a blood gas analyzer (ABL 700; Radiometer, Copenhagen, Denmark). Dynamic compliance was calculated on a breath-by-breath basis.

Statistical analyses

All data values are expressed as means ± SEM unless otherwise described. Before starting the present study, we determined the number of subjects based on a power calculation; this showed that 28 subjects would be needed to achieve an 80% power to detect a difference of 10% in the $Pa_{O_2}/F_{I_{O_2}}$ (P/F) ratio, with $\alpha = 0.05$. The patient characteristics were analyzed using Student's *t*-test, the Mann-Whitney *U*-test, and the Kruskal-Wallis test for differences between the groups. The data were analyzed by two-way repeated-measures analysis-of-variance for differences between the groups, followed by the Mann-Whitney *U*-test. $P < 0.05$ was considered to be statistically significant.

Results

Thirty-nine post-CPB patients were admitted our ICU, and 11 patients were excluded (1 patient with chronic obstructive lung disease, 3 patients with intraoperative pulmonary trauma, 3 patients with hemodynamic instability, 2 patients with a pacemaker, and 2 patients with intraaortic balloon pumping). In total, 28 patients were randomly allocated to the individualized RM group ($n = 14$) or the control RM group ($n = 14$).

The patients' characteristics, including the duration of CPB, were not significantly different between the groups (Table 1). Both hemodynamic and respiratory parameters in the two groups had similar values at baseline and there were no significant differences (Table 2). No patient was ventilated with an inspiratory plateau pressure of more than 30 cmH_2O in either group. The P/F ratio in 4 of the 14 patients in the RM control group and in 6 of the 14 patients in the individualized RM group ranged between 200 and 300. There was no patient with a P/F ratio of less than 200 in either group.

Table 3 shows the percent changes in HR, MAP, mean pulmonary arterial pressure (MPAP), and cardiac index (CI) at the end of the RM. There were no significant differences between the groups. During the RM, no patient in either group was observed with hypotension (MAP < 50 mmHg), arrhythmia, or a low CI ($<2.2\ l \cdot min^{-1} \cdot m^2$). There were significant improvements in the Δ Cdyn and the Δ P/F in the individualized RM group, compared with values in the control RM group ($P = 0.026$ and $P = 0.012$, respectively; Fig. 2). The mean airway pressure of sustained inflation was 28.3 ± 1.3 (21.4–33.8) cmH_2O in the individualized RM group. In the six patients with a P/F ratio ranging between 200 and 300, this pressure was 28.6 ± 1.8 cmH_2O range, 24.2–32.9 cmH_2O. Two of the 14 patients in the control RM group were ventilated with a noninvasive positive-pressure ventilator within 24 h of extubation due to hypoxemia, whereas none of the patients in the individualized RM group needed such ventilation.

There was no correlation between the inflation pressure and the changes in hemodynamic parameters, involving the HR, MAP, MPAP, and CI, in the individualized RM group ($P > 0.1$; linear regression analysis). Also the inflation pressure was not correlated with the improvement of the P/F ratio (Fig. 3; $r^2 = 0.07$, $P = 0.18$, linear regression analysis).

Discussion

The present study indicates that the individualized RM, defined on the basis of dynamic compliance, improved pulmonary oxygenation and slightly increased lung compliance in post-CPB patients. In addition, there was no difference in hemodynamic stability between the two groups, and both groups were stable and safe. These findings suggest that our individualized RM, which is optimized for each patient's dynamic compliance, is appropriate for post-CPB patients without hemodynamic instability.

Table 1. Patients' characteristics

	Control RM group ($n = 14$)	Individualized RM group ($n = 14$)	*P* value
Age (years)	64.6 ± 3.4	66.7 ± 3.4	0.67
Height (cm)	161.8 ± 1.9	158.5 ± 2.9	0.36
Weight (kg)	62.8 ± 2.4	59.6 ± 2.6	0.38
Sex (M/F)	10/4	10/4	1
Surgery			
CABG	7	6	0.56
AVR	3	3	
MVR	1	3	
Ao graft	3	2	
Duration of CPB (min)	111.1 ± 9.4	111.1 ± 12.6	1

Values are means ± SEM
CABG, coronary artery bypass graft; AVR, aortic valve replacement; MVR, mitral valve replacement; Ao graft, aortic graft surgery; CPB, cardiopulmonary bypass

Table 2. Baseline hemodynamic and respiratory parameters

Parameter	Control RM group (n = 14)	Individualized RM group (n = 14)	P value
Heart rate (bpm)	81.1 ± 3.0	87.6 ± 3.5	0.18
Mean arterial pressure (mmHg)	73.3 ± 2.9	75.0 ± 3.1	0.70
Mean pulmonary pressure (mmHg)	15.6 ± 1.1	15.6 ± 1.6	0.98
Cardiac index ($l \cdot min^{-1} \cdot m^2$)	3.1 ± 0.2	3.5 ± 0.2	0.20
$F_{I_{O_2}}$	0.51 ± 0.02	0.50 ± 0.02	0.78
Pressure (cmH_2O)	20.7 ± 1.0	20.7 ± 1.7	1.00
Respiratory rate (bpm)	11.6 ± 0.6	12.1 ± 0.9	0.64
Tidal volume (ml)	650.4 ± 32.7	610.1 ± 28.3	0.36
Dynamic compliance ($ml \cdot cmH_2O^{-1}$)	41.5 ± 2.3	39.2 ± 2.1	0.46
Pa_{O_2} (mmHg)	163.3 ± 6.4	157.0 ± 9.3	0.58
Pa_{CO_2} (mmHg)	38.3 ± 1.9	39.9 ± 1.2	0.45
$Pa_{O_2}/F_{I_{O_2}}$	326.8 ± 18.4	313.7 ± 19.5	0.63

Values are means ± SEM
$F_{I_{O_2}}$, inspiratory fraction of oxygen; Pa_{O_2}, partial pressure of arterial oxygen; Pa_{CO_2}, partial pressure of arterial carbon dioxide

Table 3. Hemodynamic changes at the end of the RMs

Percent change in parameter	Control RM group (n = 14)	Individualized RM group (n = 14)	P value
Heart rate (%)	7.9 ± 4.8	1.8 ± 1.2	0.25
Mean arterial pressure (%)	0.3 ± 1.8	−2.7 ± 6.3	0.64
Mean pulmonary artery pressure (%)	17.3 ± 7.8	28.2 ± 13.5	0.49
Cardiac index (%)	−1.0 ± 0.7	1.0 ± 1.2	0.14

Values are means ± SEM

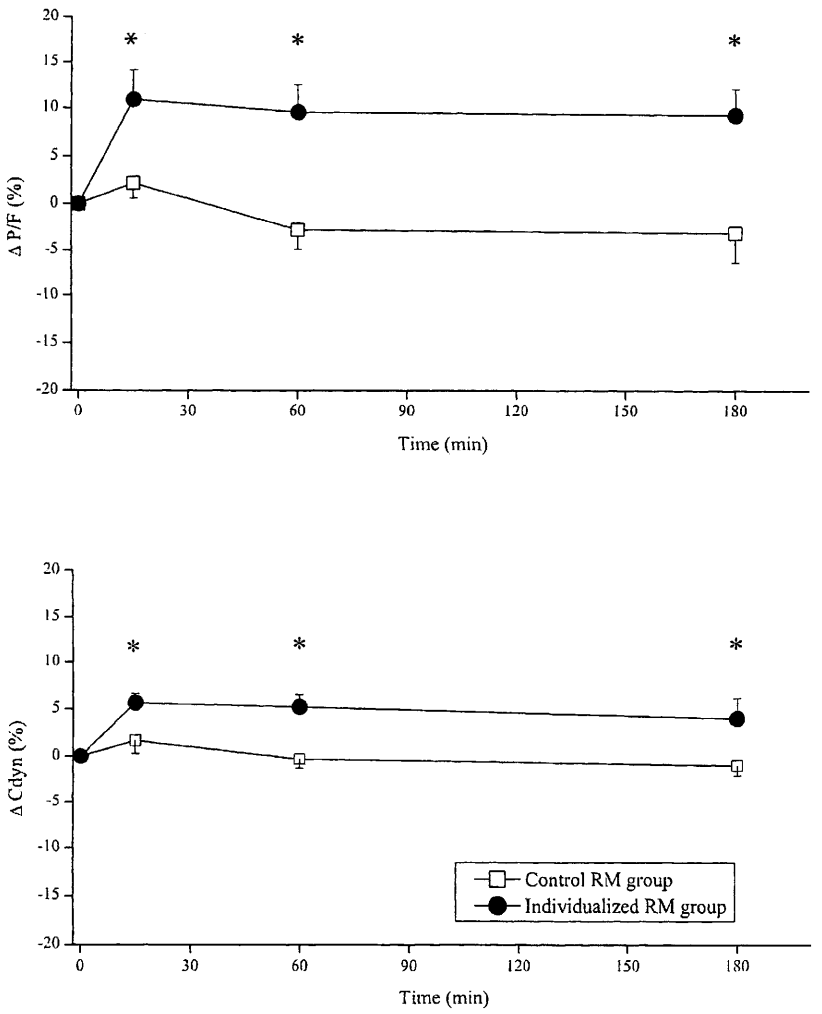

Fig. 2. Effects of control *vs* individualized recruitment maneuver (*RM*) on the percent change in partial pressure arterial oxygen/inspiratory fraction of oxygen (*P/F*) and dynamic compliance (*Cdyn*). Changes in both parameters decreased beyond 15 min after the control RM, whereas significant improvements were found in the individualized RM group immediately after RM, and these were preserved for 3 h. There were significant improvements in the percent changes in P/F and Cdyn in the individualized RM group compared with values in the control RM group (P = 0.026 and P = 0.012, respectively). *P < 0.05 vs control group, at each time point

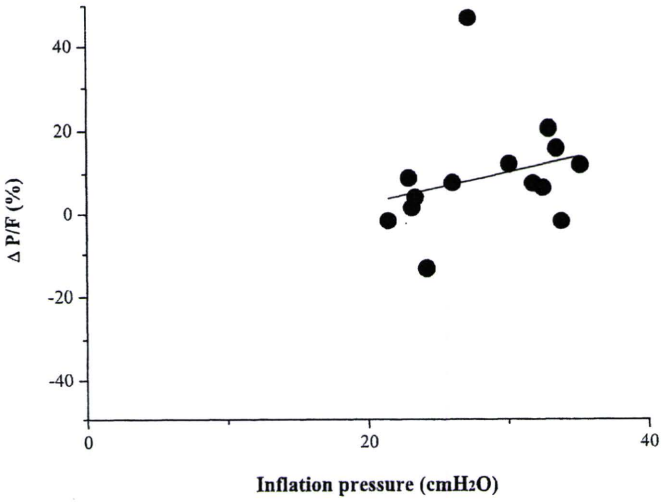

Fig. 3. The inflation pressure was not correlated with the improvement of the P/F ratio at 15 min after RM ($r^2 = 0.07$; $P = 0.18$; linear regression analysis). *P/F*, partial pressure arterial oxygen/inspiratory fraction of oxygen

The preservation of hemodynamic stability should be the first priority for the postoperative management of cardiac surgery. The RM should be used to avoid hemodynamic changes after cardiac surgery because cardiac compensation is insufficient in post-CPB patients, owing to myocardial stunning [6,12]. Indeed, in our clinical experience, a substantial decline in CO and MAP occurs in some patients. Therefore, the application of up to 20 cmH_2O continuous positive airway pressure (CPAP) for 25 s, associated with minimal and short-term changes in CO, was selected for the control procedure. It was obvious that this application was insufficient to improve pulmonary oxygenation or compliance in our pilot study.

The inflation pressure of the individualized RM was relatively higher than that of the control method (28 cmH_2O vs 20 cmH_2O); however, hemodynamic changes were similar in the two groups. The individualized RM in our study reduced MAP by only 3% and changed CI by 1% and HR by about 2%, while an RM with high inflation pressure (40 cmH_2O, 10 s or 20 s) has been reported to reduce CO by more than 50%, left ventricular end-diastolic area by about 45%, and MAP by 20% [6]. This discrepancy between the studies may be explained in terms of intrathoracic pressure (ITP) and sympathetic withdrawal [13]. Increasing airway pressure is elevated in ITP and this leads to decreased venous return and CO. Decreasing lung compliance, however, has been shown to decrease the transmission of the airway pressure to the ITP [13]. In our patients, in whom lung injury was mild to moderate, there was not much difference in ITP, even though there were about 8-cmH_2O differences in inflation pressure between the groups. On the other hand, large-volume inflation of the lung (>15 $ml \cdot kg^{-1}$) decreased HR in an animal model [14]. Lung volume in an RM with 40 cmH_2O may reach more than 15 $ml \cdot kg^{-1}$, and lead to sympathetic withdrawal in post-CPB patients. Indeed, an RM with high inflation pressure (40 cmH_2O, 20 s), as opposed to our methods, was shown to decrease HR by about 20% [6]. Therefore, an RM with high inflation pressure may contribute to hemodynamic instability.

An injured lung often shows nonhomogeneous alveolar distension and high airway pressure is needed to recruit alveoli. Previous studies have shown that, if inflation volume is constant, ITP will be equally increased, which will not reflect a change in the cardiovascular status, although the alveolar distention is not homogeneous [13,15]. In our study, inflation pressure varied, while the inflation volume of the individualized RM was theoretically constant (= 15 × real body weight). Accordingly, the individualized RM would change hemodynamic stability only slightly.

The impairment of pulmonary gas exchange after cardiac surgery contributes to the requirement for prolonged mechanical ventilation [16]. A previous study showed that the P/F ratio in patients with extubation failure was only 7% lower than that in the patients without extubation failure after cardiac surgery [17]. Another study demonstrated that the relative risk of delayed extubation was 0.935 when the P/F ratio increased by 10 [18]; in that study, the patients' P/F ratio at baseline was similar to that in our study. Determining the effect of the RM on long-term outcome after the procedure was not the purpose of our study; however, 2 of the 14 patients in the control RM group were ventilated with a noninvasive positive-pressure ventilator as a result of hypoxemia, whereas none of the patients in the individualized RM group needed such ventilation. Therefore, this slight improvement, without hemodynamic instability, could have some relevance for patients with CPB, although our individualized RM increased the P/F ratio by only about 38 from baseline.

The present study has some limitations. First, our ventilation setting of V_T was relatively high and may be unsuitable for the management of patients with acute lung injury. Pulmonary dysfunction in most patients with CPB is reported to range from subclinical functional changes to moderate lung injury [19,20]. In our patient population, lung injuries were not severe but mild, and no patient had a P/F ratio of less than 200. All patients had been ventilated with an inspiratory plateau pressure of about 20 cmH_2O. It remains controversial whether or not V_T should be reduced when the inspiratory plateau pressure is lower than 30 cmH_2O [21,22]. Therefore, we used a V_T of 10–12 ml to avoid increasing pulmonary atelectasis. Second, we did not try to obtain static pressure-volume loop values because it was our desire to simplify the individualized RM in terms

of daily management. We considered that static pressure—volume loop values did not always provide clear inflection points without neuromuscular agents and the use of these parameters was not necessarily of advantage for post-CPB patients [23]. Recent studies indicate that the application of dynamic respiratory mechanics as a diagnostic tool in ventilated patients could be more appropriate than using static pressure-volume curves [9]. Similarly, we did not correct for the influence of the tracheal tube on airway pressure, and this influence possibly modifies the measurement of dynamic lung compliance. Correction of the airway pressure could make it possible to accurately calculate the dynamic compliance of the respiratory system. By the monitoring of airway pressure in the trachea, respiratory mechanics can be assessed more accurately [24]. Third, in the present study, we chose the pressure to be 15 × real body weight/dynamic compliance + PEEP (cmH_2O), and did not investigate other pressures or hold-times. There may be another combination of pressure and hold-time which is even more effective for the improvement of oxygenation. Nevertheless, we believe that our method, based on dynamic compliance, facilitates the identification of the optimal pressure and hold-time. Finally, based on the P/F ratio, lung injuries in our patient population were not severe but mild, and no patient had a P/F ratio of less than 200. Therefore, it remains unclear whether our individualized RM would be effective for patients with ARDS.

Conclusion

In conclusion, this preliminary study suggests that an individualized RM, defined on the basis of dynamic compliance, slightly improves oxygenation and lung compliance, without hemodynamic instability, for post-CPB patients. We expect to propose a new RM concept by seeking to optimize the inflation pressure for each individual patient, but a large-scale study will be required to determine the optimal pressure and hold-time, and other parameters.

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Recurrent ST-segment elevation on ECG and ventricular tachycardia during neurosurgical anesthesia

Yoshifumi Kotake[1], Midori Matsumoto[2], Tomoko Yorozu[3], and Junzo Takeda[4]

[1]Department of Anesthesiology, Toho University School of Medicine, 6-11-1 Ohmori-nishi, Ohta-ku, Tokyo 143-8541, Japan
[2]Department of Anesthesiology, Tachikawa Kyosai Hospital, Tokyo, Japan
[3]Department of Anesthesiology, Kyorin University School of Medicine, Tokyo, Japan
[4]Department of Anesthesiology, Keio University School of Medicine, Tokyo, Japan

Abstract
This article reports an unusual case of repeated intraoperative myocardial ischemia and ventricular arrhythmia during neurosurgical anesthesia. The presentation was clinically diagnosed as coronary spasm after successful resuscitation. Intraoperative prostaglandin E_1 and β-adrenergic blockade, as well as vagal stimulation due to surgical manipulation, may have contributed to the episode.

Key words Coronary spasm · Anesthesia · Neurosurgical · Prostaglandin E_1 · Propranolol

Introduction

Coronary vasospasm may occur intraoperatively and cause serious ventricular arrhythmia and hemodynamic instability [1,2]. However, recurrent episodes of intraoperative coronary spasm in one patient during a single procedure is very rare. We present a case of multiple intraoperative episodes of ST change and ventricular tachycardia in a neurosurgical patient. These episodes were clinically diagnosed as coronary spasm, and the administration of a β-blocker and prostaglandin E_1 for deliberate hypotension may have been involved in the pathophysiology of the recurrent symptoms.

Case report

A 60-year-old, 54-kg, 157-cm-tall woman underwent neck clipping of an unruptured cerebral aneurysm. She had already undergone neck clipping of a ruptured cerebral aneurysm 10 years prior to this procedure, without any neurological sequelae. She had no history of coronary artery disease, and all the preoperative tests, including chest radiograph and ECG, revealed no abnormalities. Premedication consisted of oral famotidine and intramuscular meperidine, midazolam, and atropine sulfate. General anesthesia was induced with intravenous propofol and fentanyl, supplemented with vecuronium and maintained with continuous infusion of propofol and inhaled N_2O-O_2 (fractional inspired oxygen; $F_{I_{O_2}} = 0.33$). The maintenance dose of propofol was 4 $mg \cdot kg^{-1} \cdot h^{-1}$. The patient was mechanically ventilated to maintain Pa_{CO_2} at 30 to 35 mmHg. Before incision, administration of intravenous propranolol (0.6 mg) and continuous infusion of prostaglandin E_1, at a rate of 0.03 $\mu g \cdot kg^{-1} \cdot min^{-1}$, were started for deliberate hypotension. Twenty minutes after incision, a brief episode of bradycardia (heart rate [HR], 45 bpm), atrioventricular (AV) block, ST elevation, and T wave inversion with hypotension (arterial pressure, 74/48 mmHg) was noted (Fig. 1, trace 1A). Two minutes later, these changes disappeared, and only ST depression persisted (Fig. 1, trace 1B). After another minute, the ECG spontaneously returned to normal (Fig. 1, trace 1C). At this time, the HR was 74 bpm, and the blood pressure was 94/56 mmHg. The attending anesthesiologist diagnosed this episode as coronary vasospasm, and a transdermal isosorbide dinitrate patch was applied as a prophylactic measure. Prostaglandin E_1 administration was temporarily stopped and then restarted at the same dose 20 min later when microscopic manipulation was started. Eighty minutes after the first episode and during the microscopic manipulation of cerebral aneurysm, significant ST elevation and premature ventricular contractions were noted (Fig. 1, trace 2). At this time, the HR was 84 bpm, and the blood pressure was 89/46 mmHg. One minute later, ventricular tachycardia was noted on the ECG and was successfully treated with 60 mg of intravenous lidocaine. Two minutes after this event, the ECG returned to normal following transient ECG evidence of ST depression and T wave inversion. After this

Address correspondence to: Y. Kotake
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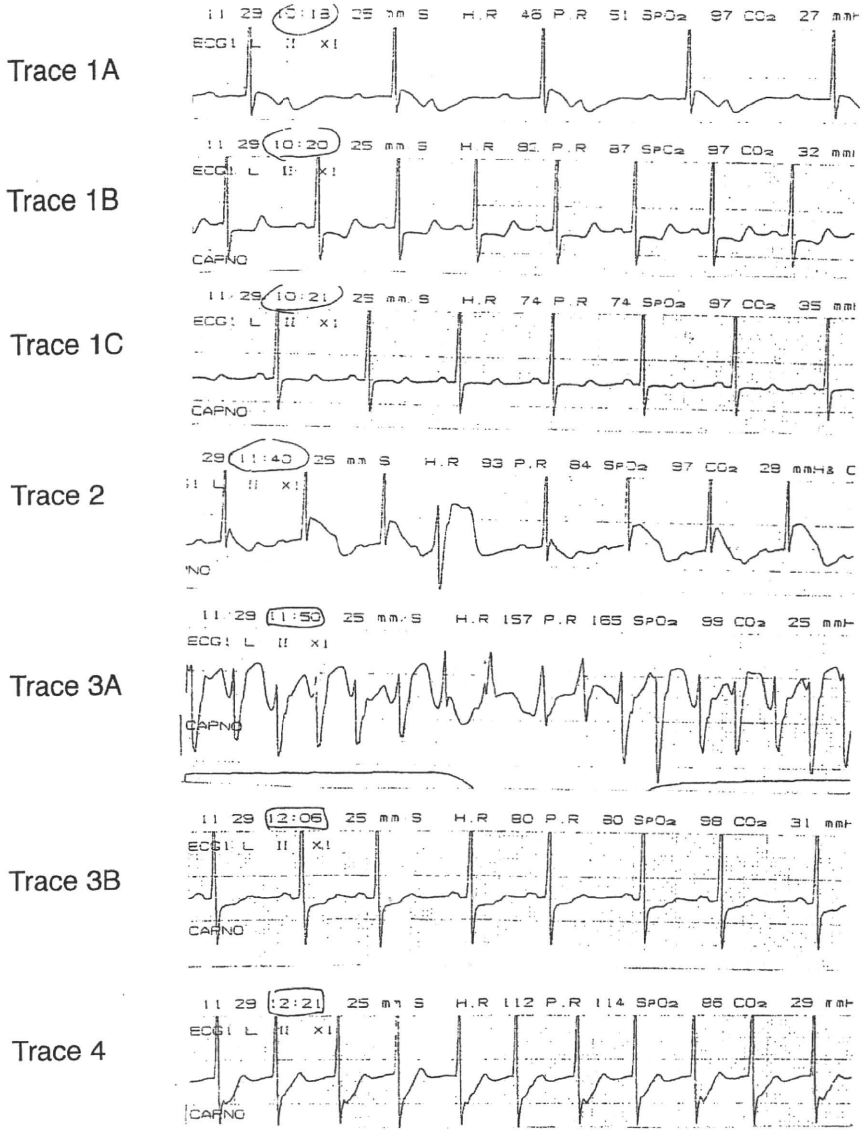

Fig. 1. Electrocardiographic recordings of repeated intraoperative ST changes and ventricular arrhythmia. Time of the recording and the heart rate (HR) of each trace are shown in the *upper left corner of each trace. Traces 1A through 1C.* First episode of electrocardiographic (ECG) change (trace 1A); subsequent ST depression and T wave inversion (trace 1B) spontaneously normalized (trace 1C). *Trace 2.* Second episode of ECG change. ST elevation and ventricular premature contractions were successfully treated with intravenous lidocaine. *Traces 3A and 3B.* The third episode of ECG change. Ventricular tachycardia, which necessitated DC defibrillation, temporary chest compression, and epinephrine administration, was recorded in lead II at 10 min after the second episode (*trace 3A*). Only slight ST depression was noted in the ECG after treatment (*trace 3B*). *Trace 4.* The fourth episode of ECG change. Junctional rhythm, ST depression, and severe hypotension, which required external chest compression and intravenous epinephrine administration, were noted 30 min after the episode of coronary spasm

second event, the prostaglandin administration was terminated. At this time, the HR was between 80 and 90 bpm, and the systolic blood pressure was between 90 and 110 mmHg. The surgical procedure proceeded after consultation with the neurosurgeon, as the second episode of ECG change had been successfully treated with lidocaine. The rest of the anesthetic regimen remained constant during these periods, and arterial blood gas analysis revealed no abnormalities (pH, 7.45; Pa_{CO_2}, 38 mmHg; Pa_{O_2}, 170 mmHg; hemoglobin [Hb], 13.1 $g \cdot dl^{-1}$). Ten minutes after the second episode, significant ST elevation and pulseless ventricular tachycardia was noted (Fig. 1, trace 3A). This life-threatening arrhythmia did not respond to 100 mg intravenous lidocaine and was immediately treated with DC defibrillation and 1 mg of intravenous epinephrine. The ECG returned to sinus rhythm with a rate of 90 bpm and moderate ST depression. The systolic blood pressure was stabilized around 120 mmHg after transient hypertension due to intravenous epinephrine administration (Fig. 1, trace 3B). After this event, N_2O was terminated but propofol was continued at the same rate as previously. Cardiovascular support and vasospasm prophylaxis consisted of a continuous infusion of dopamine, nicardipine, lidocaine, and diltiazem, and the surgery was postponed due to these adverse cardiovascular conditions. Multiple episodes of hypotension with systolic blood pressure around 80 mmHg occurred during dural and cranial closure and these were treated with intravenous ephedrine and phenylephrine. Thirty minutes after the third event, clinical cardiac arrest following severe hypotension (systolic blood pressure below 60 mmHg) occurred and was successfully treated with 1.5 min of chest compression and repeated epinephrine administration. ECG monitoring at this time revealed an AV junctional rhythm with a rate of 112 bpm and marked

ST depression (Fig. 1, trace 4). High-dose continuous epinephrine ($0.5\ \mu g \cdot kg^{-1} \cdot min^{-1}$) administration was added to the medications, and stable hemodynamics was achieved during craniotomy closure. At the end of the surgery, these intraoperative events were diagnosed as coronary vasospasm by a consulting cardiologist. Subsequent echocardiographic study demonstrated no pathologic lesion, no signs of inadequate preload, and well-preserved ventricular contractility. Postoperative chest radiograph and blood gas analysis revealed no abnormal findings, and the patient was transferred to the neurological intensive care unit (ICU) and mechanically ventilated. On arrival in the ICU, the HR was 86 with a sinus rhythm, and the systolic blood pressure was between 120 and 140 mmHg with dopamine infusion at a rate of $3\ \mu g \cdot kg^{-1} \cdot min^{-1}$. Neurologically, the patient showed dilated pupils with a sluggish light reflex at the end of surgery, but she had regained consciousness 60 min later without any neurological signs or symptoms. The patient was extubated after the administration of epinephrine was terminated. During and after this recovery period, no ECG abnormality was found, and the patient was discharged without invasive diagnostic testing.

Discussion

The three episodes of ST segment elevation and ventricular arrhythmia described in this report were most likely caused by coronary spasm, because the patient had no signs of preoperative myocardial ischemia and no apparent imbalance of the myocardial oxygen demand-and-supply relationship at the time of these events [3]. Based on our MEDLINE literature search, 17 relevant case reports written in English about intraoperative coronary spasm during noncardiac surgery were located. Additionally, 21 case reports with English abstracts were found in the Japanese literature. The case we have described is characterized by the fact that the multiple events of coronary spasm took place during a single anesthesia course. From this perspective, we believe this report may provide some additional information about the interaction between coronary spasm and anesthesia. Although the pathophysiology of coronary spasm remains to be elucidated, involvement of endothelial dysfunction and the autonomic nervous system is suggested [4]. In the context of anesthetic management, sympathetic activation due to inadequate depth of anesthesia, parasympathetic activation due to vagal stimulation, neostigmine and neuraxial blockade, alkalosis, and hypotension have been implicated as triggers of coronary spasm [2,4,5]. We believe that the HR and blood pressure in our patient precluded inadequate anesthetic depth or myocardial hypoperfusion at the time of the coronary spasm. At this time, the blood gas analysis revealed no hypocapnia or alkalosis. It is not readily known whether all episodes were triggered by the same mechanism, however, three possible causes may have been involved. First, the administration of propranolol and prostaglandin E_1 may have triggered a coronary spasm. Several reports implicate propranolol as a triggering agent of coronary spasm, by blocking sympathetic activity and causing parasympathetic dominance [6–8]. Although the majority of investigations have revealed a protective effect of β-blockade on myocardial ischemia [9], β-blockade may cause spastic vasoconstriction under certain conditions. Whether β-blockade may trigger coronary spasm or protect the post-ischemic myocardium warrants further investigation. Prostaglandin E_1 is generally regarded to have a myocardial protective effect [10]. However, several anecdotal reports in the Japanese literature have demonstrated a temporal coincidence between prostaglandin E_1 administration and the occurrence of coronary spasm [11–13]. In our patient, the fact that ECG change relevant to coronary spasm occurred only during prostaglandin E_1 infusion may suggest this possibility. Second, stimulation of the vagal nerve during neurosurgical manipulation may be involved as an underlying mechanism of coronary spasm [12]. As each episode in our patient occurred during craniotomy and during surgical exposure of the cerebral aneurysm, it is possible that the vagal nerve may have been stimulated at the time of each episode. Third, propofol-based anesthesia may have contributed to the coronary spasm. One laboratory investigation demonstrated that propofol was less protective against coronary spasm than sevoflurane [14]. Of the 115 cases of coronary spasm reported during the period from 1968 to 1998, 32% of the patients were anesthetized with an inhalational agent, while 11% were anesthetized with an intravenous agent [2]. However, the contribution of anesthetic choice to the occurrence of coronary spasm is not readily understood, because the total number of cases is not known.

In summary, we have reported a case of recurrent episodes of ST elevation and ventricular tachycardia during neurosurgical anesthesia. Coronary spasm is most likely implicated as an underlying mechanism of these symptoms. Although the precise mechanisms remain unclear, multiple factors, such as β-blockade, prostaglandin E_1 administration for deliberate hypotension, vagal stimulation elicited by surgical manipulation, and propofol may have been involved. This case reminds us that even transient ST elevation and a few ventricular premature contractions that are spontaneously alleviated may be a sign of more clinically significant coronary spasm. Meticulous attention is needed to circumvent possible triggering conditions and to provide definitive prophylaxis after these episodes.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Orengedokuto and berberine improve indomethacin-induced small intestinal injury via adenosine

Yoko Watanabe-Fukuda · Masahiro Yamamoto · Naoko Miura · Masato Fukutake · Atsushi Ishige · Rui Yamaguchi · Masao Nagasaki · Ayumu Saito · Seiya Imoto · Satoru Miyano · Junzo Takeda · Kenji Watanabe

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Abstract

Background Recent endoscopic technology has revealed that small intestinal injury is a serious threat to patients receiving nonsteroidal anti-inflammatory drugs (NSAIDs). We previously showed that Japanese herbal medicine, Orengedokuto (OGT; Huang-Lian-Jie-Du-Tang in Chinese), protects mice from lethal indomethacin (IND)-induced enteropathy. To elucidate the mechanism of the protective effect of OGT, we performed microarray analyses and high power statistical analyses of microarray data using new bioinformatics tools.

Methods Female BALB/c mice were subcutaneously injected with IND (20 mg/kg) once a day for 2 days. OGT-treated mice received a diet containing OGT from the first IND injection until the end of the experiment. Gene expression signals of small intestine were obtained with GeneChip®. Analyses for overrepresentation of Gene Ontology categories were conducted using MetaGene Profiler (MGP) and the changes were visualized by Cell Illustrator Online (CIO). Furthermore, active ingredients of OGT were investigated.

Results MGP and CIO suggested a critical role for the adenosine system, especially adenosine deaminase (ADA), a key enzyme of adenosine catabolism. Quantitative real time RT-PCR and in situ hybridization showed that OGT decreased the expression of ADA, which possibly resulted in the elevation of the anti-inflammatory nucleoside adenosine. Blockade of the adenosine A2a receptor abrogated the protective effect of OGT. Berberine, a major ingredient of OGT, suppressed ADA expression and reduced the incidence of lethality.

Conclusions OGT may prevent IND-induced enteropathy by decreasing ADA which results in the elevation of adenosine. Modulation of the adenosine system may be an efficient therapeutic strategy for NSAID-induced enteropathy.

Keywords Nonsteroidal anti-inflammatory drug · Herbal medicine · Small intestinal injury · Adenosine deaminase · Microarray

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Y. Watanabe-Fukuda · J. Takeda
Department of Anesthesiology,
Keio University School of Medicine,
Tokyo 160-8582, Japan

Y. Watanabe-Fukuda · M. Yamamoto · N. Miura · A. Ishige · K. Watanabe (✉)
Center for Kampo Medicine,
Keio University School of Medicine,
35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: toyokeio@sc.itc.keio.ac.jp

M. Yamamoto · N. Miura · M. Fukutake
Tsumura Laboratory, Tsumura & Co., Ibaraki 300-1192, Japan

R. Yamaguchi · M. Nagasaki · A. Saito · S. Imoto · S. Miyano
Human Genome Center, Institute of Medical Science,
University of Tokyo, Tokyo 108-8639, Japan

Introduction

The gastroduodenal toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs) is well recognized. Meanwhile recent advances in diagnostic methods, such as video capsule endoscopy (VCE) have revealed a high prevalence (2/3) of small intestinal lesions among long-term NSAID users [1–3]. NSAID may present a variety of small intestinal lesions including ulcers [4], and may cause lethal

outcomes, such as bowel obstruction, perforation, and sepsis. Thus, prevention of NSAID-induced small intestinal lesions, even those judged "subclinical" by conventional diagnosis, may be an important issue for long-term NSAID users. However, various suggested treatments [e.g. co-administration of misoprostol (PGs) or rebamipide] [5, 6] are yet to be established as clinical strategy for NSAID-induced small intestinal lesions, and furthermore COX-2 selective agents, which were anticipated to be effective according to theories concerning the pathogenesis of NSAID-induced gastrointestinal lesions, did not reduce the occurrence of small intestinal lesions in long-term NSAID users [7].

Previously we reported that Orengedokuto (OGT; Huang-Lian-Jie-Du-Tang in Chinese), a Kampo (Japanese traditional herbal) medicine, prevents indomethacin (IND)-induced lethal enteropathy in a rodent model [8]. In Japan, Kampo medicines, which are pharmaceutical grade herbal medicines manufactured on a modern industrial scale under strict quality control, are integrated into the national medical system and are ethically used by physicians educated in modern Western medicine. OGT is one such medicine and has been used for various indications including gastric ulcers, gastritis and melena. We demonstrated that the beneficial effects of OGT in the IND-induced enteropathy model were accompanied by an increase in the number of COX-2 expressing cells in lamina propria and in the production of PGE2 by isolated lamina proprial mononuclear cells. However, it is known that the pathogenetic mechanism of IND-induced enteropathy depends not only on COX-PGE2 association but also on other factors including intestinal bacteria and nitric oxide [9, 10]. Furthermore, OGT is a mixture comprising four crude herbs, each of which contains an enormous number of pharmacological compounds. The biological effect of OGT is thought to be mediated by a combination of various pharmacological actions via diverse signaling pathways driven by a number of different compounds. Thus, we anticipated that additional mechanisms might be involved in the beneficial effect of OGT on the enteropathy model. Consequently, a comprehensive analysis by "omics" technologies, such as functional genomics, proteomics and metabolomics, is an essential prerequisite for investigating such a complex system.

In the present study, we performed microarray analyses using the Affymetrix GeneChip platform. High power statistical analyses of microarray data by new bioinformatics tools MetaGene Profiler (MGP) [11] and Cell Illustrator Online (CIO) [12] suggested that the adenosine system, especially adenosine deaminase (ADA), may be involved in the effect of IND and/or OGT on the small intestine. Validation by biological experiments and investigation of active ingredients were also described.

Materials and methods

Drugs and chemicals

OGT consists of crude ingredients extracted with boiling water from the following four medicinal herbs in the ratio given in parentheses: Ogon (*Scutellariae radix*; 3.0), Oren (*Coptidis rhizoma*; 2.0), Sanshishi (*Gardeniae fructus*; 2.0) and Obaku (*Phellodendri cortex*; 1.5). Spray-dried extract powders of OGT and its constituent herbs were prepared by Tsumura & Co. (Tokyo, Japan). IND and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Treatment of mice

Female BALB/c mice (7-weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were housed in an air-conditioned room with a 12-h light–dark cycle under specific pathogen-free conditions. All mice were given AIN-93M powder diet (CLEA Japan, Inc., Tokyo, Japan) and water ad libitum. Prior to each experiment the mice were fasted for 24 h and then re-fed the normal diet or diets containing OGT at concentrations of 2%, which corresponds to the effective dose established in our previous study. These diets were administered throughout the experimental period. Enteropathy was induced as described in a previous study. In brief, 1 h after re-feeding, mice were subcutaneously injected (sc) with freshly prepared IND (20 mg/kg) once a day for two days.

Tissue dissection

For preparation of tissue samples, mice were sacrificed by cervical dislocation 24 h after the second IND injection and the small intestines were immediately dissected. Tissues used for morphological studies were rinsed with saline, opened longitudinally, spread flat on filter paper and fixed in 15% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Tissues used for RNA extraction were washed in ice-cold PBS and then immediately frozen in liquid nitrogen. Tissues used for in situ hybridization (ISH) were dissected, cut longitudinally and fixed with Tissue Fixative (Genostaff Co., Ltd) before being embedded in paraffin. All animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals for research, which conform to the guidelines of the Science Council of Japan.

Morphological studies

Using a stereoscopic microscope, we counted the number of ulcers and quantified the sum of ulceration areas in the

whole small intestines by tracing the outline of the areas with an image processing program, Image J (National Institutes of Health, Bethesda, MD).

RNA extraction

Mice were sacrificed and the dissected small intestines were used for the preparation of total RNA. Each of the frozen samples was homogenized in a 1 ml/0.1 g tissue of TRI REAGENT (Sigma-Aldrich Japan, Tokyo, Japan) with a POLYTRON tissue homogenizer (Kinematica, Littau-Lucerne, Switzerland) and incubated for 10 min at room temperature. A conventional chloroform extraction and isopropanol/ethanol precipitate technique were used to isolate RNA.

Microarray analysis

Total RNA was re-purified by RNeasy spin columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. All samples were monitored using an Agilent Bioanalyzer (Agilent Biotechnologies, Boeblingen, Germany) and consistently demonstrated high-quality RNA (28S/18S ratio, ~2). GeneChip analysis using an MG-U74Av2 array (Affymetrix, Santa Clara, CA) was performed according to the Affymetrix protocol. Data were analyzed using the Affymetrix Microarray Suite (MAS) v.5.0 with all of the parameters set at default values (a global normalization was applied). Probe sets that had three present A MAS detection calls per group (3 samples) in at least one of the groups were included in the analysis.

MetaGene Profiler and Cell Illustrator Online

MetaGene Profiler has been developed to evaluate the significance of predefined sets of genes from transcriptome data (http://metagp.ism.ac.jp/) [11]. The method accumulates statistical evidence from a set of genes in order to build a more powerful test than can be achieved by analyzing individual genes. In the present study, we first predefined the group of genes for each gene ontology (GO) term [all three categories, i.e., biological process (BP), cellular component (CC) and molecular function (MF), were used]. The number of gene sets annotated by GO terms was over 20,000. To obtain the *P*-values for individual genes, Welch's *t* test was performed. The individual *P*-values of the genes included in the gene set were integrated to obtain "the integrated *P*-value for the gene set", as described previously (http://metagp.ism.ac.jp/). A gene set containing too small a number of genes is, in principle, unsuitable for evaluation of over-represented gene sets. Preliminary examination suggested GO terms containing more than 100 genes provided relatively little information because these terms represent too broad a concept to give a foothold for further biological investigation. Therefore MGP analysis was applied to the gene sets consisting of 4–99 genes. As described in the results section, the above-mentioned analysis suggested a possible involvement of the adenosine system in the pathogenesis of IND-induced enteropathy and its amelioration by OGT. Therefore additional analysis using MGP was performed on the gene sets representing distinct facets of the adenosine system, that is, purine metabolism, adenosine metabolism and/or signaling, three types of adenosine-related apoptosis and literature analysis of ADA. CIO is a graphic platform for modeling and simulating signaling pathways (http://www.cellillustrator.com) [12].

Real-time RT-PCR

Real time RT-PCR was performed in two laboratory sites where different PCR methodologies were used: the TaqMan® Gold RT-PCR Kit without controls (Applied Biosystems, Foster City, CA), and the QuantiFast™ SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. These two assays gave essentially the same results. For TaqMan® assay, combinations of probes and primers of Mm01247822_m1 for ADA and Mm00607939_s1 for beta-actin were used. Real time PCR analysis was performed using an ABI Prism 7900HT (Applied Biosystems) with the following thermal cycling conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All samples were run in triplicate. Data was normalized against beta-actin. For the QuantiFast™ assay, the primers for ADA (forward: 5′-GAGCTGCGCAACATT ATCG-3′, reverse: 5′-GCCTTCATCTCCACAAACTC-3′) and GAPDH (forward: 5′-AGGAAGCTCACTGGCATG G-3′, reverse: 5′-CCTGCTTCACCACCTTCTTG-3′) were used. The cycle parameters involved an initial activation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 10 s then annealing and extension at 60°C for 30 s. After amplification, samples were kept at 55°C for 1 min and the temperature was gradually raised by 0.5°C every 10 s to perform the melt-curve analysis. All procedures of real time PCR were performed on the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Tokyo, Japan) All reactions were performed in triplicate. The threshold cycles (Ct) were used to quantify the mRNA expression levels of samples using GAPDH for normalization.

In situ hybridization

In situ hybridization was performed at Genostaff Co., Ltd. Detailed protocols are described in Supplementary Materials.

Effects of adenosine receptor blockade on IND-induced enteropathy with OGT

We wanted to test the role of the adenosine system on the effect of OGT during IND-induced enteropathy. Thus, we examined the effect of adenosine receptor antagonists on the lethality induced by IND with or without OGT. An adenosine receptor A2a antagonist 8-(3-chlorostyryl) caffeine (CSC; 10 mg/kg) or an equal volume of vehicle were administered intraperitoneally (i.p.) for five consecutive days from one day before the first IND treatment.

Results

Effect of Orengedokuto on indomethacin-induced enteropathy

As previously reported [8], IND induced lethal small intestinal ulceration and OGT prevented the ulcerations and the lethality. OGT rescued mice from IND-induced death (Fig. 1a). OGT reduced the number and area of IND-induced ulcerations (Fig. 1b, c).

Gene expression in the murine small intestine

For the gene expression studies, the mice used for this experiment were divided into four groups: N (normal), I (IND-treated), N/O (OGT-treated) and I/O (IND- and OGT-treated). Preparation of tissue samples was performed 48 h after the first injection of IND when more than 90% of mice still survived even in group I. Three comparison sets were made, that is, EXP_IND: N versus I (effect of IND), EXP_OGT: N versus N/O (effect of OGT), EXP_OGT_IND: I versus I/O (effect of OGT in IND-enteropathy model), and *P*-value for each probe set was calculated by Welch's *t* test. A list of genes that displayed increased or decreased levels of expression in each comparison set is shown in Supplementary Table S1–S3. For nine genes, the changes in gene expression induced by IND were partially abrogated by OGT (highlighted in bold font in Table S1, S2). OGT decreased ADA expression both in EXP_OGT and EXP_OGT_IND (highlighted in bold font in Table S2, S3).

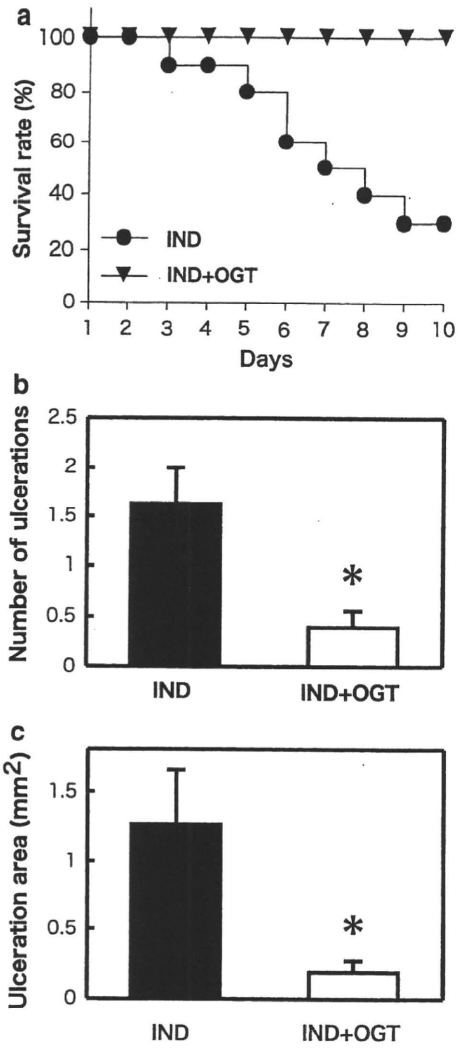

Fig. 1 **a** Orengedokuto (OGT) rescued mice from indomethacin (IND)-induced death. Survival curves were drawn using the Kaplan–Meier method. The difference in survival ratio at day 10 was analyzed by Fisher's exact test, $*P < 0.01$ ($n = 10$ per group) **b**, **c** OGT reduced IND-induced small intestinal lesions. Small intestines were harvested at 24 h after the second injection of IND. The number and areas of ulcerations were calculated macroscopically.$*P < 0.01$, data represent the means ± SEM ($n = 10$ per group)

Gene set analyses by MetaGene Profiler on GO terms

Filtering genes by setting the threshold for expression level, fold-changes, statistical significance, etc. is not ideal because the setting of the threshold cannot eliminate the problem of arbitrary selection. Furthermore, single-gene analysis has been known to have a number of major limitations. MGP, a recent developed method to evaluate microarray data at the level of gene sets, gives the "integrated *P*-value" for a given gene set by integrating *P*-values of all probe sets contained in the gene set. MGP facilitates an evaluation of the statistical significance of the given gene set. Initial analysis was performed using the

gene sets defined by the GO consortium. The "integrated *P*-values" for all possible gene sets defined by all GO terms (>20,000 terms) were calculated and the top 20 GO terms in MF, BP and cell component (CC) categories for EXP_IND are shown in Table 1 and Supplementary Table S4. A detailed discussion of these results is given in the Supplementary Materials. As shown in Table 1, among the GO terms denoting relatively broad and general concepts, those containing a small number of genes specific for particular biological activity, such as "rhodopsin-like receptor activity" and "ADA activity", were ranked. The gene list of "rhodopsin-like receptor activity" contains 4 probe sets for adenosine receptors among 19 probe sets in total. The results of MGP analysis for EXP_IND_OGT are shown in Table 2 and Supplementary Table S5. For the MF category, "deaminase activity" whose major members are ADAs, "rhodopsin-like receptor activity" and "ADA activity" were ranked. These MGP analyses prompted us to hypothesize a possible involvement of the adenosine system in the enteropathy and recovery by IND and OGT, respectively. Because the spectrum of adenosine functions is extremely wide, we defined several subsets of adenosine system-related genes. Further MGP analyses were then performed on the newly defined gene sets. This secondary MGP analyses suggested that the genes involved in adenosine metabolism were the most affected gene set by IND and/or OGT treatment (Table 3; details have been described in Supplementary Materials). MGP analysis for EXP_OGT gave only a few GO terms (7 GO terms in 3 categories with integrated *P*-value <0.05) suggesting that OGT has little or no effect on the expression profile of normal small intestines (data not shown).

Table 1 MGP analysis on EXP_IND: effect of IND

MF (molecular function)		
GO ID	GO term	Integrated P
0003779	Actin binding	1.48E-14
0008565	Protein transporter activity	1.65E-11
0004930	G-protein coupled receptor activity	2.40E-11
0004842	Ubiquitin-protein ligase activity	9.41E-11
0008234	Cysteine-type peptidase activity	1.76E-10
0003682	Chromatin binding	4.00E-10
0004197	Cysteine-type endopeptidase activity	5.36E-10
0030145	Manganese ion binding	1.33E-09
0005200	Structural constituent of cytoskeleton	1.98E-09
0008289	Lipid binding	5.90E-09
0005529	Sugar binding	1.76E-08
0046983	Protein dimerization activity	2.82E-08
0008026	ATP-dependent helicase activity	4.28E-08
0001584	*Rhodopsin-like receptor activity*	4.45E-08
0004000	*Adenosine deaminase activity*	5.04E-08
0030528	Transcription regulator activity	5.48E-08
0008083	Growth factor activity	5.65E-08
0004386	Helicase activity	5.93E-08
0042802	Identical protein binding	8.37E-08
0046982	Protein heterodimerization activity	1.24E-07

Biological investigation of a possible involvement of the adenosine system in the effects by IND and/or OGT

In the next step, the possible involvement of the adenosine system was examined biologically. Firstly, ISH was performed to clarify the localization of ADA, which was most clearly affected by IND/OGT treatment (Fig. 2). ADA expression was observed specifically at the luminal surface of intestinal villous tips. It was increased in IND-treated mice, which was reduced by co-treatment with OGT. ADA signals were absent in the site of ulceration or perforation where villi had been destroyed because of the exclusive localization of ADA to villous tips (data not shown). Next, we performed real time RT-PCR to confirm the change of ADA gene expression of the small intestine. In accordance with microarray and ISH data, IND increased the expression of ADA mRNA in the small intestine whereas OGT decreased the expression level of ADA mRNA (Fig. 3). Administration of OGT alone also decreased ADA mRNA (Fig. 3). To determine whether adenosine signaling is involved in the effects elicited by IND and/or OGT, we administered an adenosine A2a receptor antagonist, CSC, to IND-treated mice with or without OGT and observed lethality. Treatment with CSC alone gave no significant effect on IND-treated/untreated mice (data not shown) but abrogated the OGT's preventive effect on IND-induced enteropathy (Fig. 4).

Investigation of the active ingredients of OGT for IND-induced death

To gain an insight into the active ingredients responsible for OGT's beneficial effect, we prepared three fractions consisting mainly of (a) alkaloids, (b) high molecular weight substances (e.g. polysaccharides) or (c) the residues, whose contents were equivalent to those contained in 2% OGT. Mice treated with the alkaloid fraction survived IND-induced death. By contrast, the high molecular weight fraction and the residue fraction had no preventive effect and only a modest preventive effect, respectively (Fig. 5a). Therefore we next tested the effects of four major alkaloids contained in OGT on the lethality induced by IND. Diets containing each alkaloid at twice the concentration corresponding to that in 2% OGT (2%OGT includes 0.0703% berberine, 0.00706%

Table 2 MGP analysis on EXP_IND_OGT: effect of OGT in IND_enteropathy

MF (molecular function)		
GO ID	GO term	Integrated P
0003924	GTPase activity	2.94E-05
0046873	Metal ion transporter activity	6.23E-04
0019239	*Deaminase activity*	2.74E-03
0004445	Inositol-polyphosphate 5-phosphatase activity	3.05E-03
0004601	Peroxidase activity	5.46E-03
0004907	Interleukin receptor activity	6.01E-03
0003847	1-Alkyl-2-acetylglycerophosphocholine esterase activity	9.05E-03
0001584	*Rhodopsin-like receptor activity*	1.31E-02
0005044	Scavenger receptor activity	1.56E-02
0004252	Serine-type endopeptidase activity	1.68E-02
0004000	*Adenosine deaminase activity*	1.81E-02
0043022	Ribosome binding	1.89E-02
0004930	G-protein coupled receptor activity	2.10E-02
0016772	Transferase activity, transferring phosphorus-containing groups	2.34E-02
0003779	Actin binding	2.39E-02
0005375	Copper ion transporter activity	2.47E-02
0005385	Zinc ion transporter activity	2.52E-02
0005200	Structural constituent of cytoskeleton	2.76E-02
0000049	tRNA binding	2.96E-02
0004263	Chymotrypsin activity	3.12E-02

Table 3 MGP analysis on selected gene sets of adenosine system

Gene set	Description	Effect of IND	Effect of OGT in IND-model	Effect of OGT
1	Purine metabolism	2.896E-02	1.019E-02	9.341E-01
2	Adenosine metabolism	1.935E-06	1.860E-02	6.567E-01
3	Adenosine signaling	2.977E-02	2.829E-01	8.654E-01
4	Adenosine metabolism/signaling	1.307E-05	5.245E-02	8.718E-01
5	T cell apoptosis subset	3.513E-01	4.211E-02	7.906E-01
6	Other cell apoptosis subset	1.824E-04	6.813E-03	9.530E-01
7	AdoHcy-mediated apoptosis	6.306E-01	1.630E-01	9.086E-01
8	Literature analysis	1.182E-07	8.794E-07	5.397E-01

Definition of gene sets (Table S6 in supplementary material)

coptisine, 0.00129% palmatine and 0.00176% magnoflorine) were administered from the first injection of IND until 10 days after the first IND injection ($n = 10$ per group). Our results showed that berberine and coptisine, but not palmatine and magnoflorine, possibly reduced the incidence of IND-induced death (data not shown). Then we focused our attention on berberine, which has been ethically used. Berberine suppressed IND-induced lethality in a dose-dependent manner (Fig. 5b). Real time RT-PCR showed that berberine treatment reduced IND-induced increase in ADA mRNA expression in the small intestine (Fig. 6).

Schematic representation of changes in gene expression of adenosine system by CIO (http://www.cellillustrator.com)

We represent the changes in gene expression of the adenosine system by a visualization method using CIO (Fig. 7). The genes involved in the adenosine system were classified into three categories; intracellular adenosine metabolism, extracellular adenosine metabolism and adenosine signal transduction. Intracellular adenosine is provided by SAH hydrolase (Ahcy) from *S*-adenosylhomocysteine and degraded by ADA or adenosine kinase (ADK). In the