

Urinary 8-Hydroxydeoxyguanosine Is a Potential Indicator for Estimating Pulmonary Rehabilitation-Induced Oxidative Stress in COPD Patients

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Chronic obstructive pulmonary disease (COPD) is the most common chronic lung disease and is an important cause of morbidity worldwide. The aim of this study was to evaluate whether pulmonary rehabilitation (PR) improves the oxidant/antioxidant imbalance, exercise capacity and health-related quality of life (HRQL) in patients with different stages of COPD. Eighteen stable COPD patients participated in 8-week PR; the exercise intensity was set at 70% of the $\dot{V}O_2$ peak. Subjects were divided into 2 groups: moderate to severe (stages II/III: $n = 12$) and very severe COPD with $FEV_1 < 30\%$ predicted (stage IV: $n = 6$). In patients at stages II/III, PR improved exercise capacity (6-minute walking test: 431.2 ± 26.6 vs. 489.1 ± 26.5 m, $P < 0.01$ and shuttle walking test: 329.2 ± 41.4 vs. 378.2 ± 41.5 m, $P < 0.01$) and HRQL, whereas no significant change was observed in erythrocyte lipid peroxidation and urinary 8-hydroxydeoxyguanosine, a marker for DNA damage. In contrast, PR for stage IV patients did not improve exercise capacity and HRQL, but significantly increased urinary 8-hydroxydeoxyguanosine (14.5 ± 1.7 vs. 24.3 ± 2.6 ng/mg Cr, $P < 0.05$). In both groups, erythrocyte antioxidants (superoxide dismutase, glutathione peroxidase, and catalase) did not change significantly after PR. Thus, urinary 8-hydroxydeoxyguanosine is a useful indicator for the PR-induced oxidative stress in COPD patients. In conclusion, appropriate exercise program in COPD patients can improve exercise capacity and HRQL without further increase of oxidative stress. However, PR for very severe COPD patients enhanced exercise-induced oxidative stress.

Keywords: chronic obstructive pulmonary disease; erythrocyte antioxidant enzyme; 8-hydroxydeoxyguanosine; oxidative stress; pulmonary rehabilitation

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Introduction

Chronic obstructive pulmonary disease (COPD) is the most common chronic lung disease and is an increasingly important cause of morbidity and mortality worldwide (Rabe et al. 2007). Growing evidence has indicated that there is an imbalance between oxidants and antioxidants in COPD patients and this is considered to play an important role in the pathogenesis of COPD (Repine et al. 1997; MacNee 2005). Despite bronchodilator therapy with agents such as inhaled anticholinergics and β_2 -agonists, COPD patients often experience functional deficits due to dyspnea, deconditioning and muscle weakness. On the other hand, pulmonary rehabilitation (PR) reduces the symptoms of dyspnea and improves exercise capacity and health-related quality of life (HRQL) (Nici et al. 2006). Therefore, PR

has been recommended as an integral part of the management of COPD patients (Ries et al. 2007).

Physical training is the most important component of PR. However, several studies have suggested that acute exercise may increase oxidative stress (OS), such as the increase in plasma thiobarbituric acid-reactive substance (TBARS) in COPD patients (Heunks et al. 1999; Couillard et al. 2002; Agacdiken et al. 2004). In addition, Pinho et al. (2007) reported that chronic exercise training can induce OS at baseline in COPD patients, and Barreiro et al. (2009) demonstrated that nitrosative stress in severe COPD had increased after chronic endurance exercise. To our knowledge, however, there is little information on the relationship between PR in COPD patients and OS.

Erythrocytes are expected to be one of the targets susceptible to OS during exercise for the following reasons:

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first, their membranes are rich in polyunsaturated fatty acids; second, they are continuously exposed to high concentrations of oxygen; and third, they contain ferrous ions, a potentially powerful promoter of oxidative processes (Clemens and Waller 1987). However, erythrocytes contain many antioxidant enzymes such as superoxide dismutase (SOD), mainly copper-zinc-SOD (Cu-Zn-SOD) among SOD isoenzymes, glutathione peroxidase (GPX), and catalase (CAT). SOD, GPX, and CAT form the first defense line against reactive oxygen species (ROS). SOD converts superoxide anion ($O_2^{\cdot-}$) to H_2O_2 , which is then transformed to water by GPX and CAT. If not removed, H_2O_2 may itself cause oxidative damage, or may be converted to the more harmful hydroxyl radical (OH^{\cdot}) species. These radicals may attack lipids and DNA, subsequently increasing erythrocyte TBARS and urinary 8-hydroxydeoxyguanosine (8-OHdG), a marker for DNA damage. Therefore, the aim of this study was to investigate whether PR improves the imbalance between OS and antioxidants as well as exercise capacity and HRQL in COPD patients with the different severity of airflow limitation, focusing on erythrocyte OS markers and urinary 8-OHdG.

Methods

Participants

The subjects were 18 patients with clinically stable COPD, as defined by Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (Rabe et al. 2007). All subjects were required to have a smoking history (> 10 pack-years) and to have quit smoking at least 6 months before the study. All 18 patients received anticholinergic agents and/or β_2 -agonists as bronchodilator therapy, and some of them used inhaled steroids. None of the patients had taken oral steroid medication, antioxidants, or long-term oxygen therapy. Exclusion criteria were respiratory disorders other than COPD, malignancy, overt cardiac failure, and hepatic or renal disease. The classification of severity was as follows: moderate COPD (stage II), $50\% \leq FEV_1 < 80\%$ predicted; severe COPD (stage III), $30\% \leq FEV_1 < 50\%$ predicted; and very severe COPD (stage IV), $FEV_1 < 30\%$ predicted (Rabe et al. 2007). According to this classification, COPD patients were divided in two groups: moderate to severe (II/III: $n = 12$) and very severe (IV: $n = 6$). The subjects were informed of the procedures involved and any possible risks and discomfort associated with the study before giving written consent. The study was conducted according to the Declaration of Helsinki and was approved by the local ethics committee of Tokyo Medical University.

Experiment Protocol

All patients participated in comprehensive PR for 8 weeks. A pulmonary function test, 6-minute walking test (6MWT), and shuttle walking test (SWT) were administered before and after PR. HRQL was assessed using the St. George's Respiratory Questionnaire (SGRQ) (Jones et al. 1992), and peripheral venous blood and urine samples were obtained prior to and after PR.

Comprehensive Pulmonary Rehabilitation Program

An introduction to PR, in which exercise training and education were the major components, was given to all subjects for the initial two weeks (four times a week). The aerobic training was the main

part of the exercise session; the walking speed was set at 70% of the peak oxygen consumption ($\dot{V}O_2$ peak) obtained in the SWT before PR. The session also included upper limb and respiratory muscle training, stretching, breathing techniques, energy conservation technique, and stress management. After the introduction, the session was performed twice per week for the rest of the program period. The walking exercise was maintained for as long as tolerated, and for at least 20 minutes per session. These subjects were encouraged to exercise at home on days when they did not visit the hospital.

Pulmonary Function Tests

Pulmonary function tests were performed with a CHESTAC-55V spirometer (Chest Corp., Tokyo), based on the American Thoracic Society recommendations for acceptability and reproducibility (American Thoracic Society 1995). Three technically acceptable measurements were performed in each patient and the highest value was used in all analyses.

Exercise Capacity

Participants performed 6MWT and SWT as described previously (Singh et al. 1992; ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories 2002). The regression equation described by Singh et al. (1994) was used to predict the $\dot{V}O_2$ peak from the SWT distance. Values for $\dot{V}O_2$ equivalent to 70% of the predicted $\dot{V}O_2$ peak were calculated and the corresponding walking speeds were determined.

Health-Related Quality of Life

HRQL was assessed using the validated Japanese version of the SGRQ (Hajiro et al. 1998). The questionnaire includes four component scores for total, symptoms, activity and impact (Jones et al. 1992). A change of 4 points is generally considered to be a minimum clinically important difference (MCID) (Jones et al. 1991).

Preparation of Hemolyzed Erythrocytes

Heparinized venous blood was obtained from each patient before and after PR. The blood was centrifuged (750 g, $4^\circ C$, 10 min), and the plasma and buffy coat were removed. Erythrocytes were washed three times with 0.9% NaCl and centrifuged after each washing. Washed erythrocytes were stored at $-80^\circ C$ until analysis. For measurements of antioxidant enzyme activities and oxidative damage, the erythrocytes were hemolyzed by thawing and adding approximately five volumes of phosphate-buffered saline (PBS). The concentration of hemoglobin (Hb) was assayed using a HemoCue analyzer (HemoCue, Angelholm) and expressed in g/dl.

Antioxidant Enzymes in Erythrocytes

Erythrocyte SOD activity was determined using the method of Crapo et al. (1978). Determination of the Cu-Zn-SOD concentration was performed using a commercial kit for human Cu-Zn-SOD based on a sandwich ELISA (Kamiya Biomedical Company, Seattle). GPX activity was measured using the spectrophotometric assay described by Tappel (1978). CAT activity was assayed using the method of Aebi (1984). All values are expressed relative to Hb concentration.

Oxidative Damage in Erythrocytes

Lipid peroxidation was measured as the TBARS level using the spectrophotometrical method of Ohkawa et al. (1979). Erythrocyte TBARS levels are expressed in $\mu M/g$ Hb.

Oxidative Damage in Urine Samples

Prior to exercise tests, spot urine samples of all subjects were collected before and after PR. Urinary 8-OHdG was measured by an immunochromatographic assay (ICR-001; Selista Inc., Tokyo) using a designated test card which measured 8-OHdG (immunochromatography) and urinary creatinine (Jaffe method) levels simultaneously. Urinary 8-OHdG levels are expressed in ng/mg Cr.

Muscular Damage and Nutritional Status in Serum Samples

Before and after PR, antecubital venous blood samples were collected. Serum was obtained by centrifugation (750 g, 4°C, 10 min), and creatine kinase (CK) as a marker of muscular damage and albumin as a marker of nutritional status were determined using standard laboratory techniques.

Statistical Analysis

All data are expressed as means \pm SEM. A paired Student *t* test was used to compare measurements before and after PR. The Mann-Whitney U test was used to estimate the statistical significance of the differences in characteristics and outcome measurements between the "II/III" and "IV" groups and Fisher's exact test used to compare categorical variables. A *p* value < 0.05 was considered statistically significant. Analysis of all data was performed using SPSS version 11.0 for Windows (SPSS Inc., Chicago).

Results

Patient Demographics

Eighteen men with COPD (7 at stage II, 5 at stage III, and 6 at stage IV) were enrolled into PR programs, and were divided into 2 groups: II/III group (*n* = 12) and IV group (*n* = 6). The baseline characteristics of the both COPD groups are shown in Table 1. The younger patients were categorized in the more severe group and there were

significant differences in ages between II/III group and IV group. There were no difference between the II/III and IV group in body mass index (BMI), PaO₂, PaCO₂, and medication use. The levels of Hb, albumin and CK were within normal values in both groups.

Effects of PR on Physiological Parameters and Pulmonary Function

No major complications occurred during PR. In both group, there were no significant differences in measures of nutritional status such as BMI, body weight, Hb and albumin before and after PR (Table 2). After PR, serum CK levels were not elevated in either group. Therefore, PR did not cause any overt muscular damage in COPD patients. Pulmonary function data on pre- and post-PR in both groups are shown in Table 2. At baseline, FEV₁, FEV₁ % predicted, FVC, FVC % predicted, FEV₁/FVC and inspiratory capacity were lower in IV group than in II/III group. PR did not change any parameters in pulmonary function test in either group.

Exercise Capacity

The results of both exercise tests are shown in Fig. 1. PR markedly increased the distance in both the 6MWT (from 431.2 \pm 26.6 to 489.1 \pm 26.5 m, *P* < 0.01) and SWT (from 329.2 \pm 41.4 to 378.2 \pm 41.5 m, *P* < 0.01) in II/III group patients. In contrast, no significant improvement was seen in either exercise tests after PR in IV group patients.

Health-Related Quality of Life

In II/III group, analysis of the mean SGRQ data showed a MCID change of 4 points after PR for all scores

Table 1. Characteristics of COPD patients at baseline.

Variables	II/III group	IV group
Gender, male	12	6
Mean age (years)	71.5 \pm 1.31	65.7 \pm 1.52*
Smoking history (pack-years)	57.6 \pm 8.3	77.5 \pm 19.1
BMI (kg/m ²)	21.4 \pm 0.98	20.6 \pm 0.67
Weight (kg)	57.8 \pm 2.74	55.5 \pm 1.98
Hemoglobin (g/dl)	14.5 \pm 0.31	15.8 \pm 0.30
Albumin (g/dl)	4.15 \pm 0.06	4.43 \pm 0.21
Creatine Kinase (IU/L)	148.2 \pm 24.01	84.0 \pm 11.29
PaO ₂ (mmHg)	75.9 \pm 1.8	72.2 \pm 3.7
PaCO ₂ (mmHg)	40.0 \pm 0.7	43.6 \pm 3.5
Medication (<i>n</i>)		
Bronchodilator therapy	12/12	6/6
Anticholinergics	8/12	6/6
β_2 -agonists	7/12	4/6
Theophylline	6/12	3/6
Inhaled corticosteroid	4/12	3/6

Data are presented as the mean \pm SEM or number of patients. COPD, chronic obstructive pulmonary disease; BMI, body mass index.

*Significantly different from II/III group at baseline: *p* < 0.05 .

Table 2. Effects of pulmonary rehabilitation on physiological parameters and pulmonary function in the two groups of COPD severity.

Variables	II/III group (n = 12)		p-value	IV group (n = 6)		p-value
	Pre-PR	Post-PR		Pre-PR	Post-PR	
BMI (kg/m ²)	21.4 ± 0.98	21.4 ± 1.02	0.963	20.6 ± 0.67	20.6 ± 0.50	0.693
Weight (kg)	57.8 ± 2.74	57.9 ± 2.78	0.662	55.5 ± 1.98	55.7 ± 1.90	0.695
Hemoglobin (g/dl)	14.5 ± 0.31	14.5 ± 0.41	0.876	15.8 ± 0.31	15.6 ± 0.40	0.631
Albumin (g/dl)	4.15 ± 0.06	4.18 ± 0.07	0.745	4.43 ± 0.21	4.51 ± 0.10	0.211
Creatine Kinase (IU/L)	148.17 ± 24.0	180.3 ± 33.3	0.301	84.0 ± 11.3	89.5 ± 6.40	0.530
FEV ₁ (L)	1.52 ± 0.12	1.43 ± 0.11	0.133	0.70 ± 0.17*	0.69 ± 0.10	0.349
FEV ₁ , % predicted (%)	55.6 ± 4.11	52.7 ± 3.78	0.122	24.2 ± 2.01*	23.5 ± 1.30	0.757
FVC (L)	3.30 ± 0.26	3.28 ± 0.21	0.878	2.20 ± 0.26*	2.26 ± 0.30	0.333
FVC, % predicted (%)	96.8 ± 6.47	96.0 ± 4.63	0.836	61.9 ± 5.79*	63.5 ± 6.67	0.836
Inspiratory capacity (L)	2.25 ± 0.17	2.36 ± 0.17	0.294	1.81 ± 0.20*	1.86 ± 0.25	0.757

Data are presented as the mean ± SEM. COPD, chronic obstructive pulmonary disease; BMI, body mass index; PR, pulmonary rehabilitation; FEV₁, forced expiratory volume in one second; %pred, %predicted; FVC, forced vital capacity.

*Significantly different from II/III group at pre-PR: $p < 0.05$.

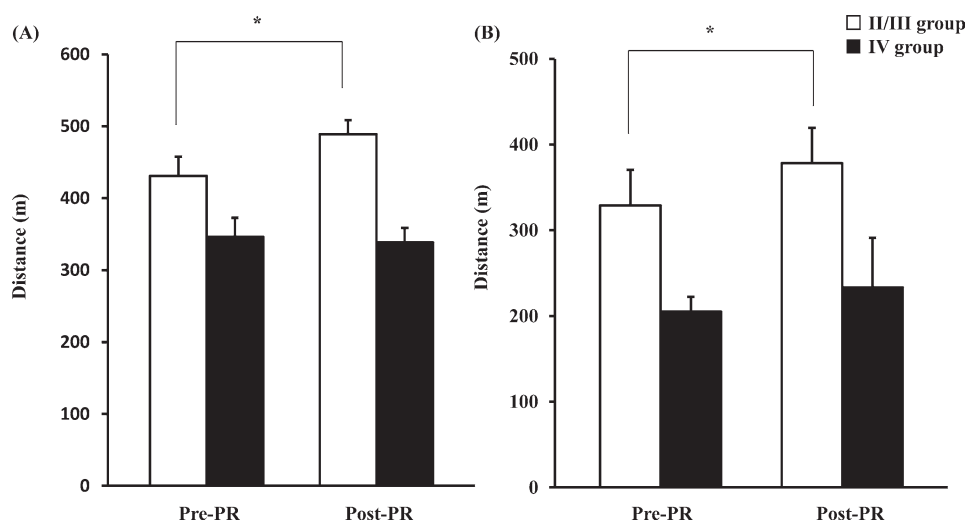


Fig. 1. Mean walking distance in the 6MWT and SWT before and after pulmonary rehabilitation.

Shown are the data (m) of the 6MWT (A) and SWT (B) before and after pulmonary rehabilitation in the two groups of COPD patients.

6MWT, 6-minute walking test; SWT, shuttle walking test; COPD, chronic obstructive pulmonary disease; PR, pulmonary rehabilitation.

*Significant difference between pre- and post-PR: $p < 0.01$.

(Fig. 2). In IV group, only the score for the symptoms was clinically improved after PR, but not the score for the activities, impacts, or total.

Antioxidants in Erythrocytes

At baseline, Cu-Zn-SOD concentration level was significantly lower in IV group patients compared with II/III group patients (Table 3). In both II/III and IV group patients, PR did not significantly alter either SOD, GPX, or CAT activities, or the Cu-Zn-SOD concentration.

Oxidative Damage in Erythrocytes and Urine Samples

Baseline values of OS were not significantly different between both groups (Fig. 3). PR-induced oxidative damages such as erythrocyte TBARS and urinary 8-OHdG were not seen in II/III group patients. In IV group patients, TBARS levels did not differ significantly, but urinary 8-OHdG levels significantly increased after PR.

Discussion

The current study shows that PR programs used in this study improved exercise capacity and HRQL with no further increase of OS in moderate to severe COPD patients.

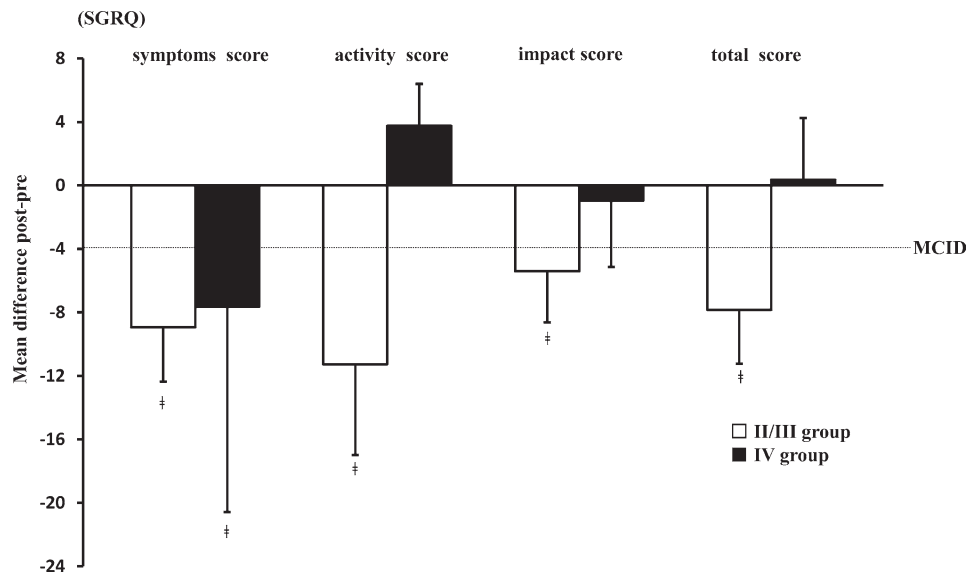


Fig. 2. Effect of pulmonary rehabilitation on health-related quality of life in COPD patients.

COPD, chronic obstructive pulmonary disease; SGRQ, St. George's Respiratory Questionnaire; MCID, minimum clinically important difference.

#MCID between pre- and post-PR.

Table 3. Effects of pulmonary rehabilitation on erythrocyte antioxidants in the two groups of COPD severity.

Antioxidants	II/III group (n = 12)		p-value	IV group (n = 6)		p-value
	Pre-PR	Post-PR		Pre-PR	Post-PR	
SOD activity (U/gHb)	1,889.6 ± 102.7	1,823.8 ± 147.3	0.373	1,961.4 ± 92.9	1,916.8 ± 73.3	0.655
Cu-Zn-SOD (μg/gHb)	445.5 ± 73.4	486.6 ± 78.0	0.131	219.8 ± 91.7*	207.3 ± 68.6	0.668
GPX (U/gHb)	40.0 ± 3.09	40.2 ± 3.24	0.940	45.5 ± 2.63	46.8 ± 5.20	0.820
CAT (K/gHb)	165.9 ± 8.12	177.6 ± 8.23	0.101	174.1 ± 14.3	185.9 ± 12.3	0.309

Data are presented as the mean ± SEM. COPD, chronic obstructive pulmonary disease; PR, pulmonary rehabilitation; Cu-Zn-SOD, copper-zinc-SOD; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; Hb, hemoglobin.

*Significantly different from II/III group at pre-PR: $p < 0.05$.

On the other hand, in very severe COPD patients, the same PR programs induced the increase of DNA oxidation products, that is, urinary 8-OHdG, without sufficient improvements of exercise capacity and HRQL. Therefore, the PR for COPD with very severe airflow limitation may induce the exercise-induced OS in COPD patients. Taken together, urinary 8-OHdG levels might be a reliable marker for suspending exercise training.

Several studies have shown improvements in exercise capacity and HRQL following PR, regardless of the severity of the COPD patients (Berry et al. 1999; Takigawa et al. 2007). Since the MCID in the 6MWT has been proposed to be about 50 m (Redelmeier et al. 1997), the increase of 57.9 m in II/III group of our study was significant clinically as well as statistically. We also evaluated the exercise capacity using the SWT, since Green et al. (2001) showed that the correlation of the SWT with the $\dot{V}O_2$ peak is better than that for the 6MWT. The results showed that the walking distance of SWT in II/III group patients was improved after PR. The results also showed that the change of all SGRQ

scores in II/III group patients exceeded the MCID. However, there were no improvements in pulmonary function after PR. In general, impaired skeletal muscle in COPD is associated with reduced exercise capacity, and exercise training induces an adaptation phenomenon of skeletal muscle (American Thoracic Society and European Respiratory Society 1999). Therefore, our results may suggest that an improvement in skeletal muscle function after PR results in gains in exercise capacity, despite the absence of changes in pulmonary function. Moreover, this may reduce exertional dyspnea, thereby inducing the improvements in HRQL. On the other hand, our results indicated that PR was not beneficial enough to improve exercise capacity and HRQL in IV group. Possible explanations for the discrepancy between the current results and the previous reports may be due to the differences of PR programs used, or the time points examined.

In general, exhaustive exercise increases the generation of ROS (Davies et al. 1982), resulting in the increase of oxidative products such as TBARS and 8-OHdG. In the

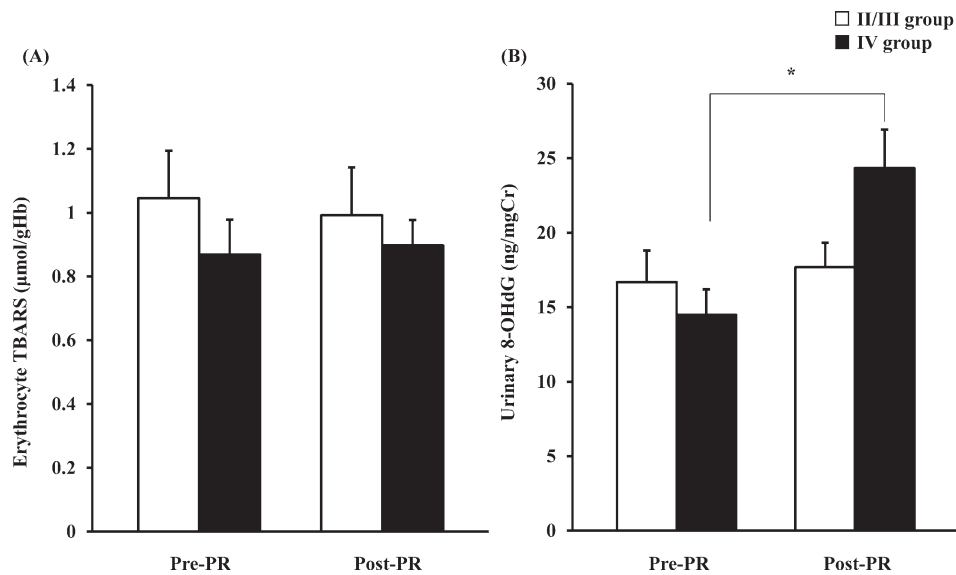


Fig. 3. Effects of pulmonary rehabilitation on erythrocyte TBARS and urinary 8-OHdG in COPD patients. Shown are the changes in erythrocyte TBARS (A) and urinary 8-OHdG (B) in the two groups of COPD patients after pulmonary rehabilitation. TBARS, thiobarbituric acid-reactive substance; 8-OHdG, 8-hydroxydeoxyguanosine; COPD, chronic obstructive pulmonary disease; PR, pulmonary rehabilitation; Hb, hemoglobin; Cr, creatinine. *Significant difference between pre- and post-PR: $p < 0.05$.

present study, PR for IV group patients induced a significant increase in urine 8-OHdG. Since hypoxic exercise is reported to generate oxidative DNA damage in human (Moller et al. 2001), this result suggests that the intensity of PR used to COPD patients with an advanced disease might be too high that it became an anaerobic exercise. Our results, however, showed that erythrocyte TBARS levels did not differ significantly in IV group patients, and TBARS might be not so sensitive to detect the change of OS in the current study. On the other hand, PR did not alter either erythrocyte TBARS or urine 8-OHdG in II/III group of the current study. These findings were consistent with the findings of Mercken et al. (2005) for urinary malondialdehyde (MDA) as lipid peroxidation. However, these findings are in contrast to the previous report that exercise training caused a significant increase of plasma MDA in COPD patients (Pinho et al. 2007). This discrepancy may be explained by differences in disease severity, rehabilitation programs used, and samples used. First, II/III group patients in this study had better airflow obstruction (FEV_1 predicted of $55.6 \pm 4.1\%$) than those (FEV_1 predicted of $35.8 \pm 10.1\%$) in Pinho et al. (2007). Second, the exercise program for II/III group might have been not so strong to increase OS, compared with the cycle exercise at $60\% \dot{V}O_2$ peak for up to 1 h (three times a week) used in the study of Pinho et al. (2007). Third, Pinho et al. (2007) measured TBARS in plasma, whereas we chose to examine TBARS in erythrocytes.

In this study, there was no difference in the baseline values of SOD activity level between both groups. This result was consistent with previous studies (Kluchová et al. 2007; Ahmad et al. 2013). On the other hand, we found

that Cu-Zn-SOD concentration level was significantly lower in IV group patients compared with II/III group patients at baseline. Although there were no sufficient data about the erythrocyte Cu-Zn-SOD concentration level in COPD patients, Ookawara et al. (1992) reported that Cu-Zn-SOD undergoes a site-specific and random fragmentation by ROS formed by the Maillard reaction. Therefore, although further clinical studies are needed, one reason for this result may be related to the conformational change in the active site region of Cu-Zn-SOD, which could not be measured accurately in an ELISA method used in this study, by OS or hypoxemia or unknown reason in IV group patients compared with II/III group patients.

Since OS usually means that free radical generation exceeds the level of antioxidant defense, that level is easily expected to be influenced by exercise-induced OS. Azizbeigi et al. (2013) have shown the upregulation of erythrocyte SOD activity with 8-week resistance training in untrained men. Therefore, 8-week program was expected to be enough for inducing the upregulation of erythrocyte antioxidant enzymes. However, the current PR program failed to show the upregulation of antioxidant enzymes in erythrocytes. These observations were consistent with several studies showing that antioxidants enzymes (SOD and CAT) activities were not increased after exercise training in the muscles of COPD patients (Barreiro et al. 2009; Rodriguez et al. 2012). One possible explanation for the lack of induction of antioxidants in erythrocyte after PR is that the duration of PR used in this study may be shorter than the life span of an erythrocyte in the circulation (90 to 120 days). In fact, previous report used in the training programs with longer duration than our study showed that,

although in the healthy subjects, erythrocyte antioxidants enzymes (SOD and GPX) activities were increased after training (Miyazaki et al. 2001). Therefore, further studies will be necessary to elucidate the effect of PR on erythrocyte antioxidant enzymes in COPD patients.

In conclusion, the current findings suggest that appropriate exercise program in COPD patients can improve exercise capacity and HRQL without further increase of OS. However, there seems to be the possibility that PR for COPD patients with advanced stages increases OS without sufficient improvements of exercise capacity and HRQL. The exercise intensity of the PR program seems to be a key factor in the current investigation, and 8-OHdG examined in this study might be a useful indicator for estimating PR-induced OS in COPD patients. Further clinical studies, however, are needed to establish the effect of PR on oxidants and antioxidants in COPD patients.

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Conflict of Interest

The authors declare no conflict of interest.

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