# *I. Chronic obstructive pulmonary disease (COPD)*

Chronic obstructive pulmonary disease occurs among middle and older aged individuals with a long-term history of smoking and reportedly affects 15-20% of smokers. Various comorbidities are observed frequently in conjunction with smoking and age. The background of these observations suggests that, rather than an elevated incidence of comorbidities due to frequent observation of COPD among elderly individuals, COPD and various comorbidities may share a common etiology and pathology.

In a 2006 revision, the Global Initiative for Chronic Obstructive Lung Disease (GOLD), an international guideline on COPD, described a new disease concept of COPD in which "the severity of illness in individual patients also causes extrapulmonary symptoms (extrapulmonary effects) [1]." Subsequently, from a pathophysiological perspective, the 2009 revised 3rd Edition of "Guidelines for Diagnosis and Treatment of COPD [2]" by the Japan Respiratory Society also led to greater emphasis on the systemic effects of COPD.

## *Comorbidities and Systemic Inflammation in COPD*

From 1979 to 2001, the National Hospital Discharge Survey was conducted among retired military personnel in the US. Results from a study of the association between COPD status and incidence of comorbidities at hospital discharge among 47 million individuals showed that the incidence of diseases including hypertension, ischemic heart disease, diabetes, pneumonia, congestive heart failure, and respiratory failure was significantly higher in a cohort diagnosed with COPD than one not diagnosed with COPD (no coexisting COPD). In other words, comorbidities were associated with COPD pathology, represented a primary factor governing the severity of COPD, and may also have been associated with prognosis. Essentially, the results indicated that systemic comorbidities are an important target in disease management of COPD. This paper addresses COPD as a systemic disease and focuses succinctly on corrective measures for nutritional imbalances and systemic inflammation, osteoporosis, cardiovascular disease, and systemic inflammation.

### *1. Nutritional imbalances and systemic inflammation*

In 2008, Japan conducted a national status survey on COPD outpatients (survey research by the Ministry of Health, Labour and Welfare Respiratory Failure Survey Research Group) [3]. This survey found that roughly 30% of COPD outpatients had a body mass index (BMI) of less than 20 kg/m$^2$. Weight loss of this nature was also associated with severity of COPD, with body weight loss observed among approximately 60% of patients at the highest level of severity (%$FEV_1$<30%). Despite the fact that airflow limitation is more severe among COPD patients in the EU and US, the incidence of weight loss is approximately 23%, and overweight status is reportedly observed among 20% or more of such patients. The GOLD [1] also stated that approximately 25% of COPD patients with moderate to severe disease evidenced body weight loss, but weight loss is clearly observed at a higher rate in Japan than in the EU and US. The GOLD [1] also elaborates the fact that body weight loss based on many immunological research results represents an independent prognostic factor with respect to respiratory dysfunction. Besides weight loss, it is important to ascertain fat mass (FM), fat-free mass (FFM) as a quantitative index of muscle protein, and other such changes in body composition; FFM in particular is correlated to basic pathophysiological indices such as respiratory function and respiratory muscle strength, and exercise tolerance. The facts suggest that reduction in FFM is linked directly to decreases in activities of daily living (ADL) and quality of life (QOL) [4]. FFM has also been shown to be a more sensitive prognostic factor than body weight [5]. In nutritional imbalances, contributory factors include increased energy consumption at rest, systemic inflammation, and abnormalities in food intake-regulating factors such as leptin and ghrelin [4].

***Fig. 1A*** presents resting energy expenditure (REE) data for patients with stable COPD. Predicted REE ratio (%REE) is significantly higher for COPD patients versus controls adjusted for age and sex, indicating that metabolism is accelerated in COPD patients [6]. Among COPD patients as well, metabolism is further accelerated in a body weight loss cohort versus a normal body weight cohort. Values for %REE are correlated to forced expiratory volume in one second ($FEV_1$), an index of residual volume and airflow restriction, and factors such as airflow limitation and pulmonary hyperinflation, the essential state of COPD, represent likely factors causing increased REE. Consequently, the absence of an energy supply commensurate with such increased consumption indicates a nutritional imbalance. Even in study results showing good correspondence in BMI between healthy controls and a COPD cohort, fat-free mass index (FFMI), the amount of muscle protein present, is significantly lower in COPD cohort (***Fig. 1B***). Study of the association between the GOLD severity classification of COPD (severity according to $FEV_1$ by spirometry) and body components also showed that bone mineral mass and fat mass were significantly reduced in Stage III and IV severe disease cohorts versus controls, but there was no difference between Stage I and II non-severe disease cohorts. However, loss of muscle protein mass consistent with severity was observed as data progressed from non-severe disease cohorts to severe disease cohorts. These findings indicate that muscle protein mass is an important endpoint for evaluating the severity of COPD.

Examining the association between systemic inflammation and body weight loss, the elevation observed in blood concentrations of inflammatory cytokines such as TNF-α and IL-6 as weight loss progresses suggests the role of systemic inflammation in nutritional imbalance. Entities such as TNF-α and IL-6, together with leptin, also function as food intake-restricting factors.

### *2. Osteoporosis*

Osteoporosis causes problems such as spinal compression fractures and lumbar pain and is of concern as a factor decreasing ADL and QOL. Spinal compression fractures in turn cause vertebral deformation and have a direct, adverse effect on respiratory function. Sin *et al.* have studied bone mass in the femoral neck. The risk of osteoporotic complication in COPD versus that in controls free from airflow obstruction is reported as an odds ratio of 1.9. The incidence of osteoporotic complication also increases with increasing severity of airflow obstruction and is reported to rise to an odds ratio of 2.4 in severe disease [7].

Among COPD patients, factors such as smoking, hypoxia, subnutrition, diminished skeletal muscle mass, vitamin D or calcium deficiency, and systemic steroid administration can engender osteoporotic complications. A recent meta-analysis of actual COPD patients reported that the incidence of diminished bone mineral density (BMD) is high, at approximately 35% [8]. Though previous studies have not clarified whether severity

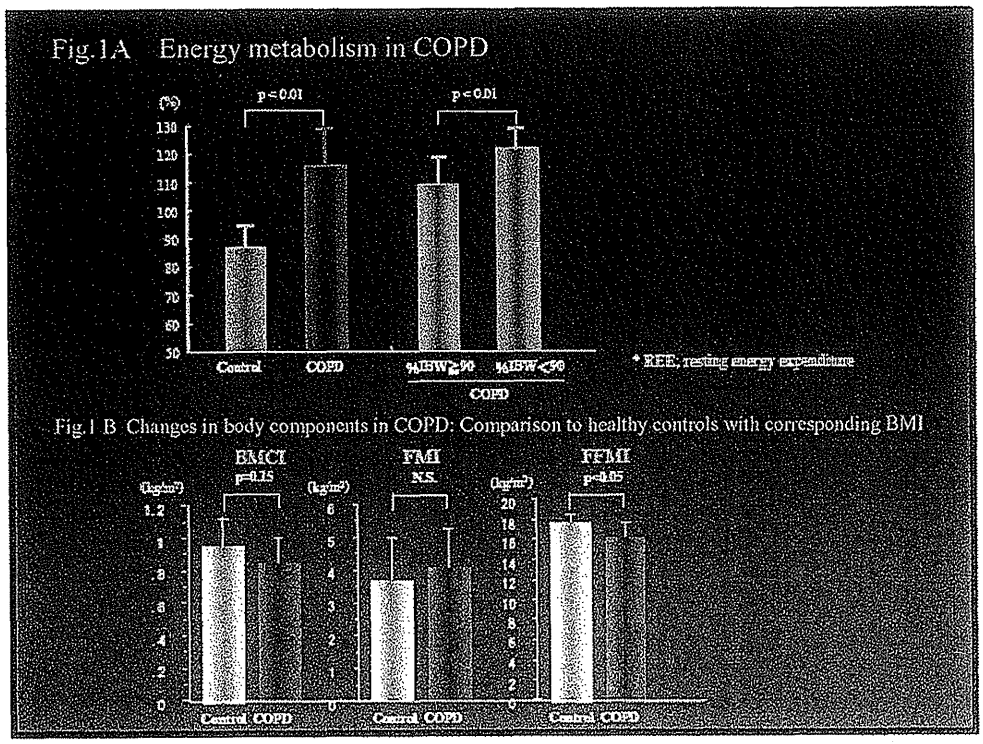


*Fig. 1.* **Energy metabolism and muscle protein mass in COPD**

of airflow obstruction presents a risk of osteoporosis, a close association has been demonstrated between decreased BMD and decreased BMI or reduced FFM.

At the same time, the extent of pulmonary emphysema on CT imaging is reported to be a regulating factor for vertebral bone density [9].

*3. Cardiovascular disease*

COPD is one of the independent risk factors for ischemic heart disease and has an observed association with elevated incidence of complicating arrhythmia and risk of cerebrovascular event. Evaluation of intimal-medial thickness (IMT) of the carotid arteries has also shown that airflow obstruction in COPD patients is a smoking-independent risk factor for arteriosclerosis [10]. Among COPD patients in the EU and US, cardiovascular disease is the cause of death in 20-30% [11], and this trend is observed notably in slight and moderate disease. In Japan, however, the rate of death from cardiovascular disease is low in comparison to the 65-70% accounted by death from respiratory failure, with deaths from heart disease reported as approximately 6% in a recent study [12] Though the related mechanism is unknown, extremely high blood concentrations of adiponectin among emphysemic COPD patients in Japan are reported to play a role, given the anti-arteriosclerotic effect and anti-inflammatory effect of this substance (***Fig. 2***) [13]. In other words, Japanese COPD patients have higher concentrations of total adiponectin in blood than healthy controls of same body weight. Further study is needed to determine whether and how elevated levels of anti-arteriosclerotic adiponectin in Japanese COPD patients play some role in cardiovascular disease in these patients.

*4. Treatment of systemic inflammation in COPD*

Inhaled corticosteroids (ICS)

When treating COPD as a systemic disease, a treatment plan must be created to control systemic inflammation. The hypothetical mechanism of onset for systemic inflammation is "spillover" to other body systems of inflammatory cytokines produced by the lungs. Inhalation of 800 μg/day of the inhaled corticosteroid (ICS) budenoside has been reported to significantly inhibit ischemic heart disease events versus placebo controls [14]. Migration of ICS throughout the body cannot be completely discounted, and these results suggest that ICS may inhibit systemic inflammation. However, inflammation in the pulmonary region and throughout the body may be controlled differently, and at present, the mechanism of onset for systemic inflammation is unclear.

Statins

Though there is currently no established treatment for inhibition of systemic inflammation, new therapeutic strategies and various other possibilities are being researched. Statins act to restrict inflammation caused by NF-κB and as an agonist to restrict inflammation caused by peroxisome proliferator-activator receptor (PPAR)-γ or PPAR-α. In COPD patients, administration of statins has been shown to inhibit reduction of $FEV_1$, and another report states that these agents are otherwise effective in reducing death rates and reducing the incidence of myocardial infarction [15]. In cigarette smoke exposure experiments, administration of statins has also been reported to alleviate elevation of pulmonary arterial systolic blood pressure and to inhibit progression to emphysema [16].

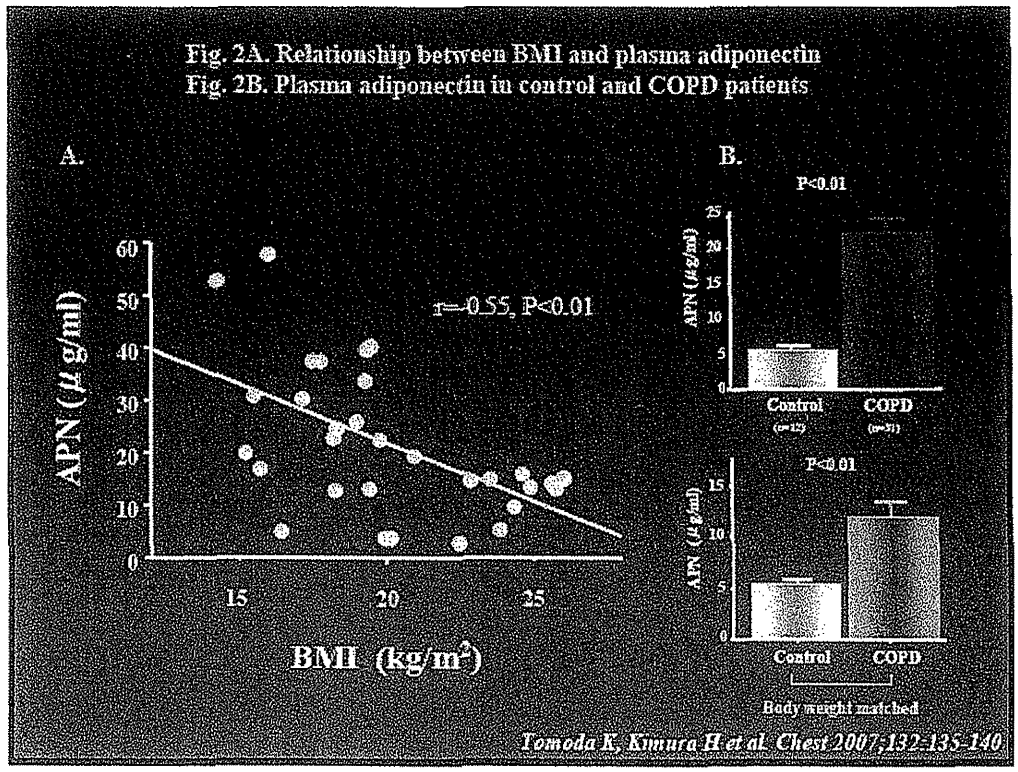


*Fig. 2.* **Plasma adiponectin levels in COPD**

Anti-TNF-α blockers

The effect of the anti-TNF-α blocker infliximab on improving motor activity and QOL has been studied [17]. Though no distinct efficacy was observed among all patient cohorts combined, a sub-analysis found improvement in a >age 65 years patient cohort and in a nutritional imbalance patient cohort with respect to the primary endpoint of 6-minute walking distance. Further study on topics including infectious disease is now awaited.

PPARs agonists

Expression of PPAR-α mRNA is decreased in the skeletal muscle of COPD patients, and such decrease is reportedly correlated with blood levels of TNF-α [18]. Inflammation or increased oxidative stress of skeletal muscle impairs skeletal muscle function, leading to diminished exercise tolerance. Administration of PPAR agonist may therefore improve these pathological conditions.

Sirtuin (SIRT1) protein

Sirtuin (SIRT1) protein is involved with deacetylation of histone proteins. The amount of SIRT1 protein in pulmonary tissue of COPD patients has been found to be lower than that in healthy individuals [19]. Decreased histone deacetylase (HDAC) activity plays a substantial role in inflammatory cytokine production. Administration of resveratrol and other such sirtuin (SIRT1) activation factors is regarded to be useful as anti-inflammatory treatment. *In vitro* experiments using monocytes/macrophages has also found that increased acetylation of NFκB accompanies decreased expression of SIRT1 caused by exposure to a tobacco-derived fluid (cigarette smoke extract (CSE)),but when siRNA is used to knock down SIRT1 and CSE exposure is then performed, acetylation of NFκB is reportedly increased further [19]. These findings suggest that maintenance and augmentation of sirtuin activity may prove to be an effective treatment strategy in smokers and COPD patients.

Ghrelin

Ghrelin is a growth hormone secretor factor expressed by gastric tissue as an intrinsic ligand for growth hormone secretagogue receptors. It has anabolic action or food intake-stimulating action as well as anti-inflammatory action. In studies on COPD patients we have clemonstrated that plasma ghrelin concentration was elevated significantly in a body weight loss COPD cohort versus a normal body weight COPD cohort and a healthy control cohort. Plasma ghrelin concentration has also been found to have a significant, negative correlation to BMI [20]. In a pilot study wherein ghrelin was administered to COPD patients presenting nutritional imbalances, improvement in QOL was observed, as was improvement in physiological functions including quantitative dietary intake, body weight, fat-free mass index, grip strength, respiratory muscle strength, and 6-minute walking distance, suggesting that administration of ghrelin could be an effective treatment strategy for nutritional imbalance in COPD [21]. In results from a multicenter, double-blind, comparative study using ghrelin administration (2 μg/kg) or placebo administration (physiological saline) in addition to respiratory rehabilitation, the ghrelin administration cohort demonstrated improvement of 6-minute walking distance maintained as a long-term trend. The ghrelin administration cohort also maintained significant improvement of symptoms on the St. George Respiratory Questionnaire (SGRQ).

## *II. Tobacco and Vascular Aging: Molecular Mechanism of Tobacco-Induced Vascular Endotheliopathy*

Cigarette smoking is well known to increase the risk for occurrence of cardiovascular events by increasing vascular aging in the form of arteriosclerosis. In the proposed mechanism, one causal factor is increased oxidative stress or inflammation caused by the cigarette smoke components nicotine and tar or by nitric acid or other gaseous components, leading to injury of the vascular endothelium. At the same time, there remains scope for debate as to the best index for measuring this vascular aging effect of cigarette smoke. Endothelial progenitor cells (EPC) contribute to regeneration of vascular endothelium or neovascularization, and their level has been proposed recently as a possible cardiovascular risk factor. Murohara *et al.* discovered that EPC counts in peripheral blood were decreased in healthy cigarette smokers and also revealed that EPC counts increased in chronic cigarette smokers after just a short duration of smoking cessation. Our paper discusses cigarette smoking and mechanisms concerning EPC and endothelial function and also mentions results from our own inquiry, *i.e.*, the protective effect of statins on endothelial function in cigarette smokers, and the role of EPC as a biomarker for vascular injury.

### *Self-Imperceptible Vascular Aging: The Importance of Surrogate Markers for Endothelial Injury*

According to death rates by primary cause of death, we have now arrived in an era where approximately one in four Japanese individuals dies from arteriosclerotic disease [22]. Cigarette smoking is a primary risk factor for non-LDL arteriosclerosis [23], and the 2010 revised guidelines of the Japanese Circulation Society also cite arteriosclerosis, a "vascular aging" pathology, as an adverse effect of cigarette smoking (***Fig. 3***) [24]. However, as in human aging, vascular aging proceeds without subjective symptoms and is well known to manifest first in an aggravated form such as myocardial infarction or stroke. This characteristic that "vascular aging is self-imperceptible" suggests the importance of what is termed biomarker-based monitoring of vascular aging and disease at the outpatient level.

### *The Molecular Mechanism of Cigarette Smoking-Induced Vascular Endotheliopathy*

The three most harmful substances in tobacco are known to be nicotine (vasodilator effect), tar (dozens of amines and other oncogenic substances, benzpyrene foremost), and carbon monoxide (induced anoxia of tissues), and among these, tar is known to play a major role in vascular injury. Production of cigarette smoking extract (CSE) is a technique for evaluating the direct effect of cigarette smoking experimentally [25,26].

Because CSE components match the profile of toxic substances in the blood of cigarette smokers, CSE can be administered directly to cultured cells to evaluate the effect of cigarette smoke *in vitro*. In this process, we ask what molecules might be useful as markers for monitoring vascular aging caused by cigarette smoking. In selecting candidates, it is essential first to understand the causal substances and their mechanisms. Cigarette smoke strongly injures the vascular endothelium [27], a structural cell of blood vessels. From studies using CSE, the related mechanisms are known [25] to center on oxidative stress (ROS ↑ NAD(P)H activity ↑ ) [28] and localized inflammation (CRP ↑ PTX3 ↑ ), and at the same time, the related molecules are reported to be useful as biomarkers for endothelial injury (***Fig. 4***).

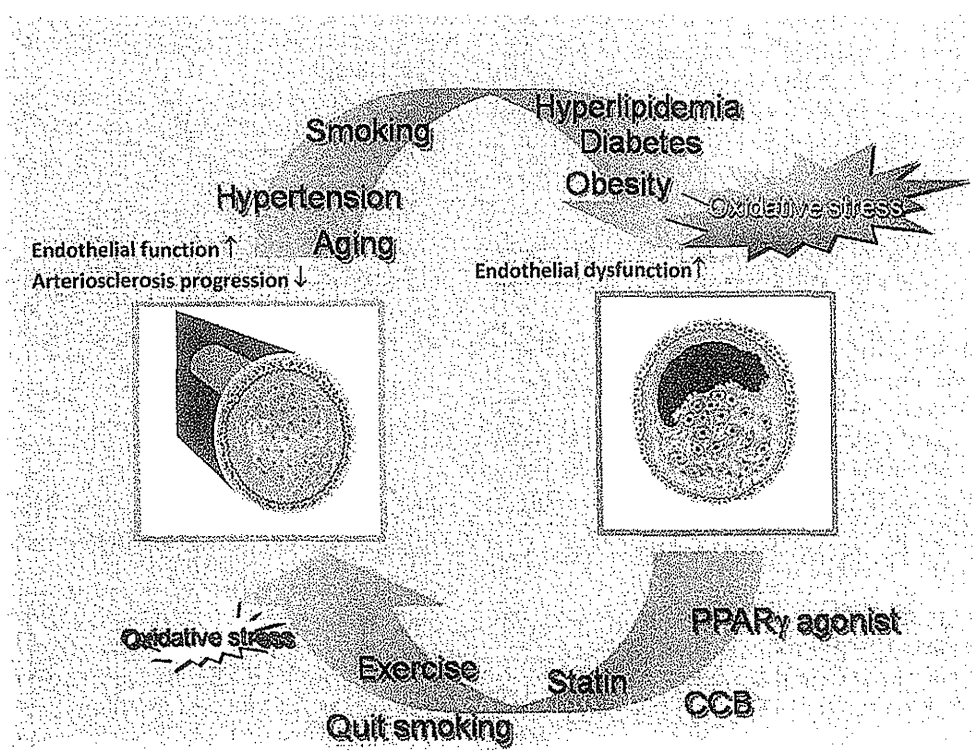


***Fig. 3.*** **Oxidative stress is essential for progression of vascular aging**

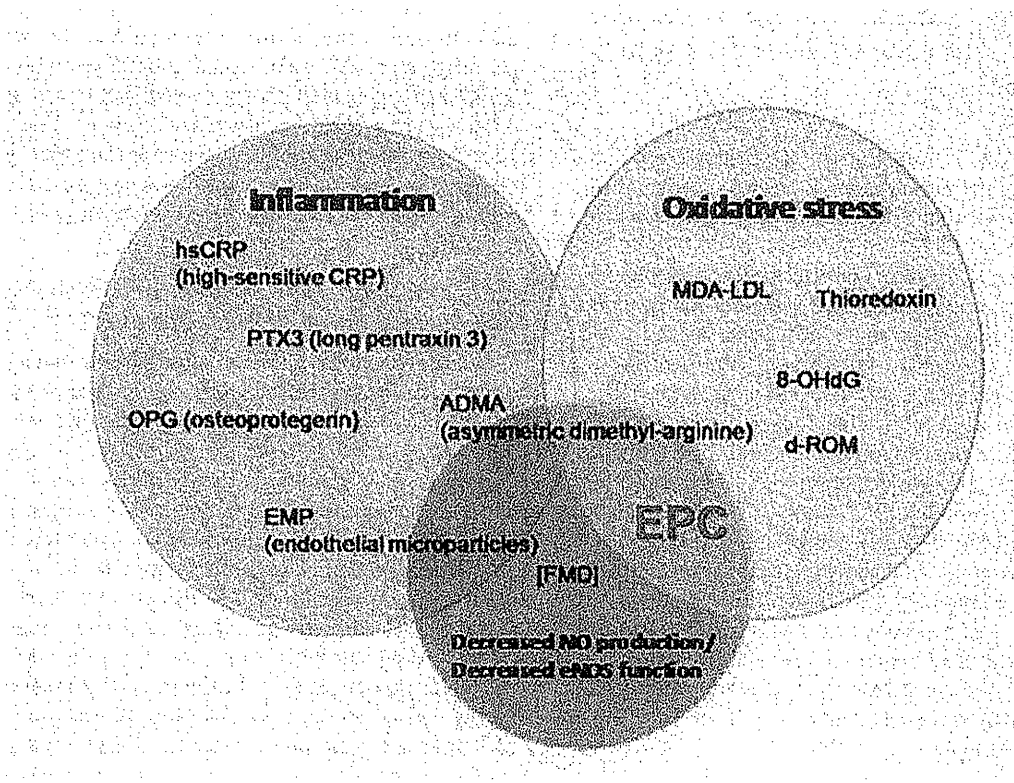


*Fig. 4.* **Biomarkers for endothelial dysfunction**

*Endothelial Injury and Endothelial Progenitor Cells (EPC): The Role of EPC as a Biomarker*

Endothelial progenitor cells (EPC) are a type of undifferentiated somatic stem cell endowed with capacity for differentiation and reproduction, the role of which is determined almost entirely by the vascular endothelial system [28,29]. A 2001 pilot study in the New England Journal of Medicine reported that EPC counts in peripheral blood were decreased among effort angina patients [30], and a 2003 study published in the same also reported that EPC counts in peripheral blood were inversely proportional to cardiovascular risk [31]. EPC were subsequently acknowledged as an important biomarker for monitoring endothelial injury [32].

## *Corrective Measures for Cigarette Smoking-Induced Cardiovascular Injury: Smoking Cessation and Statins*

What methods are effective for inhibiting vascular injury caused by cigarette smoking? Cumulative findings to date have shown the usefulness of statin administration in smoking cessation. Smoking cessation has been shown to increase EPC counts and improve function in smokers (*Fig. 4*) [33]. Of greater interest, smoking cessation has been reported to improve patient prognosis after myocardial infarction both in Japan [34] and abroad [35], though the detailed molecular mechanism requires further study.

Apart from smoking cessation, statins, a class of therapeutics for dyslipidemia, have also shown potential for anti-arteriosclerosis as a mitigation strategy for vascular injury. In 1994, articles in the Lancet reported that statins were effective in lowering the total death rate among hypercholesterolemia patients with a history of IHD (4S study, Lancet, 1994), while subsequent research reported a secondary preventive effect on cardiovascular events among IHD patients with complicating mild dyslipidemia (less than 240 mg/dL; CARE, 1996), and a secondary preventive effect on cardiovascular deaths, total deaths, and cerebral stroke among patients with average cholesterol levels (cholesterol less than 213 mg/dL in 42%; LIPID, 1998). Statins were subsequently shown to have a primary and secondary preventive effect for cardiovascular events which is independent of their lipid-lowering action, and these actions are recognized as pleiotropic effects beyond the original therapeutic effect of statins for dyslipidemia.

Statins are reported to increase mobilization of EPCs to peripheral blood by promoting cell differentiation [30], and to inhibit remodeling of injured blood vessels by promoting recruitment of EPCs to injured endothelium and thus contributing to endothelial repair [36]. Our research group also studied outcomes of pitavastatin administered to 30 male smokers diagnosed with dyslipidemia and found that pitavastatin had an improving effect on vascular endothelial function in smokers mediated by an antioxidative effect [37].

## *III. Tobacco and the New Oxidized/Modified LDL Marker α1-Antitrypsin-LDL (AT-LDL)*

Cigarette smoking is one principal cause of lipid peroxidation, and oxidized LDL plays an important role in onset and progression of arteriosclerosis. Recently, two novel oxidized/modified LDL markers have been identified: serum amyloid A/LDL complex (SAA-LDL) and α1-antitrypsin-LDL complex (AT-LDL). SAA-LDL is a complex in which low-density lipoprotein (LDL) and serum amyloid A (SAA), an acute phase reactive protein present in bound form with HDL, are bound by reactive oxygen species derived from activated,

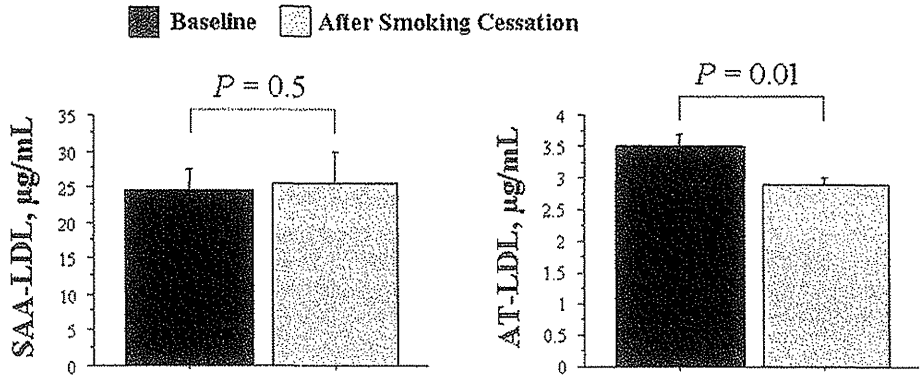


*Fig. 5* **Serum levels of AT-LDL significantly decreased after successful smoking cessation**

inflammatory cells at sites of endovascular inflammation. This complex is regarded as LDL oxidatively altered by oxidation of lipids in the complex and by fragmentation of apoB, and the complex is reported to be associated closely with obesity and inflammation. In contrast, AT-LDL is a complex formed from low-density lipoprotein (LDL) and α1-antitrypsin, and is regarded as a type of oxidized LDL subjected to oxidative alteration by macrophage uptake at atherosclerotic lesions, due to the degree of lipid oxidation involved. Oxidized LDL is present at atherosclerotic lesions and contributes to formation of atheroma by undergoing macrophage uptake and forming foam cells; it also has a variety of arteriosclerosis-promoting effects, such as activation of endovascular cells. In this light, AT-LDL is regarded as a useful marker in arteriosclerosis research. We discovered that SAA-LDL and AT-LDL are both significantly elevated in the serum of cigarette smokers; that 3 months of smoking cessation did not change levels of SAA-LDL but reduced levels of AT-LDL significantly (*Fig. 5*); and through multivariate analysis, that AT-LDL, together with age and HDL-C, is closely associated with current cigarette smoking. AT-LDL may be useful as an arteriosclerosis marker closely associated with cigarette smoking and aging.

## *IV. Rejuvenation From Smoking Cessation*

Smoking cessation has come to be seen as an important strategy and an essential part of treatment and prevention of many diseases [38-44]. In this context, it is apparent from copious data that cigarette smoking promotes aging, and there is now a focus on the importance of smoking cessation therapy to facilitate the transition from smoking to smoking cessation.

Our paper addresses and comments in turn on three recent smoking cessation-related topics: "Passive smoking," "smoking cessation therapy," and "social support and treatment for women, children, and smokers with psychiatric disorders."

### *Passive Smoking*

Though active smoking is a major risk factor for cardiovascular disease, ischemic heart disease foremost therein, it has become clear recently that passive smoking, as well as active smoking, is such a risk factor.

The nicotine in tobacco smoke stimulates the sympathetic nerve system, causing contraction of the peripheral vessels, elevation of blood pressure, and increased heart rate. Tobacco smoke contains approximately 4% carbon monoxide, which binds firmly to hemoglobin in the blood, producing a state of chronic hypoxia or promoting alteration of cholesterol and injury of the vascular endothelium, while also diminishing HDL cholesterol levels and thus promoting arteriosclerosis [39-41].

A review encompassing the health effects from passive smoking shows that the rate of death from myocardial infarction among non-smokers is increased by a factor of 1.3 or more by even minor amounts of passive smoking, and that 1-3% of persons subjected to higher levels of passive smoking die of myocardial infarction caused by passive smoking. Passive smoking is also not prevented by smoking outdoors or with ventilation [42-44].

A 2010 study on the effects of smoking outdoors showed that passive smoking can occur even at a location separated [from the source] by 17 meters. It is not surprising then that indoor smoking, from the outset, and outdoor smoking too, have come under more stringent regulation.

### *Smoking Cessation Therapy*

For quite some time, smoking has been regarded as a matter of habit, and smoking cessation an act of will power, but nicotine has been shown to create dependence and is the major cause of tobacco dependence. Prochaska encompassed the path of the smoker leading to smoking cessation in the Transtheoretical Model (TTM) theory [45]. At present, smoking cessation in health care facilities typically combines drug treatment with behavioral therapy such as "avoiding smoking materials" or "changing behavior patterns."

In Japan, health insurance coverage for smoking cessation as a "nicotine dependence control cost" was initiated in April 2006. On notification, health care facilities fulfilling 5 criteria, including prohibition of smoking on the facility property, can provide smoking cessation therapy using health insurance, upon approval from the competent authorities [46]. Patient requirements include a score of 5 points or higher on the "Tobacco Dependence Screener" (TDS), a tobacco dependence screening test. Insurance benefits for smoking cessation therapy ("nicotine dependence control costs") cover outpatients, and treatment initiated after hospitalization is out-of-pocket care; this and other aspects differ from ordinary insurance care. Note should also be taken that after one session of smoking cessation therapy covered by insurance is provided, the cost cannot be recalculated for a year or more from the initial date of calculation.

There are two smoking cessation aids currently available in Japan: nicotine patches and varenicline. Support for smoking cessation is adjusted individually for the severity (nicotine dependence level) and the living environment of the case, with drug therapy provided as appropriate. When a smoking cessation aid is used, the success rate of smoking cessation is said to increase roughly 2 to 3-fold.

Nicotine replacement therapy

Nicotine substitutes include nicotine patches requiring a physician prescription and nicotine patches (small size) and nicotine gum available for purchase without prescription at pharmacies and drugstores.

Nicotine substitutes contain nicotine which is absorbed into the body gradually from contact surfaces of the skin or oral mucosa, the purpose of which is to support smoking cessation by alleviating withdrawal symptoms that occur during smoking cessation. Outside of Japan, aids are sold in a variety of forms, but in Japan, only two types of aids are available for use: nicotine gum and nicotine patches.

Use of such aids during pregnancy is not permitted in Japan. Prudent use is also needed immediately after contraction of a disease increasing the risks imposed by nicotine, such as myocardial infarction or cerebral infarction. The most frequent adverse effect is a rash at the application site or stomatitis.

Oral Smoking Cessation Aids

The $\alpha_4\beta_2$ receptor site agonist varenicline can be used as an internal medicine. This agent binds with nicotine receptors to prevent nicotine binding and at the same time releases a low dosage of dopamine. This treatment alleviates the withdrawal symptoms accompanying smoking cessation as well as craving for tobacco. Because it does not contain nicotine, it can be used readily for smoking cessation therapy for ischemic heart disease patients not readily able to use conventional nicotine formulations such as patches. Given its mechanism of action, this agent and a nicotine formulation cannot be used concomitantly. Because the agent is excreted by the kidneys, caution is needed in persons with poor renal function. The agent can also be used during pregnancy or lactation.

The most frequent adverse effect is nausea; headache, constipation, insomnia, and nightmares are also noted. Because the agent can also cause drowsiness, prudent administration to drivers was also deemed necessary in July 2011.

Outcomes of insured treatment for smoking cessation, including drug therapy, have been published as a study by the Central Social Insurance Medical Council, which reported a success rate of approximately 80% when the specified number of treatments (5) was completed by the end of the 12-month period of insured treatment.

*Social Support and Smoking Cessation Support for Women*

As smoking cessation therapy becomes more widely practiced, smoking recidivism after the end of the insured treatment period has become a problem. The psychological dependence arising in memory persists long after smoking has ceased and can manifest to trigger recidivist smoking. Many smokers overcome by a temptation for just one taste return in a short time to the smokers they were. For long-term continuation of smoking cessation, it is important to utilize social support from the family, workplace, and other settings to complement smoking cessation support in a health care setting. Even in Japan, the Internet "Quit Smoking Marathon" and other programs that alleviate the work of health care providers have been developed for long-term smoking cessation support, with some indications of a preventive effect on recidivist smoking [47]. The self-help mailing list "Quit Smoking Health Net KK" is also offered free of charge for smoking cessation support (sign up from the Quit Smoking Marathon home page) [48], and we would hope to see active use.

Other non-health smoking cessation benefits such as "my grandchildren started visiting me more" and "my efficiency at work improved" are deemed useful for continuing long-term smoking cessation [49].

Smoking cessation is reputedly more difficult for women than men. Most women smoke few cigarettes and are reluctant to visit a smoking cessation therapy setting, but smoking even a small number of cigarettes often engenders strong nicotine cravings or depressions of mood. Even if insurance coverage is difficult, the use of smoking cessation aids is recommended.

The reason smokers relate most frequently for not quitting smoking is weight gain after quitting. Weight gain after smoking cessation is generally seen within 3 months of cessation, and two-thirds of former smokers who have quit are said to experience weight gain. The following three causes of weight gain have been suggested: 1) Increased appetite or oral fixation as a symptom of nicotine abstention, or increased food intake due to change in flavor, 2) Increased absorption due to recovery of gastrointestinal function accompanying smoking cessation, and 3) Change in metabolic pathways formerly involving nicotine. Of these, items 2) and 3) are causes of weight gain arising without any change in quantitative dietary intake, and body weight gain for these reasons is generally limited to 3 kg or less. Consequently, weight gain on the order of 3 kg after smoking cessation is not regarded as pathological.

Even in a short period, weight gain attenuates the will to cease smoking. There is also a negative effect on biochemical data, such as HbA1c and neutral fats.

Dietary advice and enhanced exercise are essential to counter these phenomena. Use of a nicotine patch during smoking cessation also restricts weight gain after cessation. Though the internally-administered drug varenicline is not recognized to have a weight-restricting effect, based on the observation that nausea as a frequently occurring adverse effect does restrict appetite, smoking cessation drug therapy is also recommended for preventing weight gain after cessation.

## *V. Conclusion*

Oxidative stress and inflammation caused by cigarette smoking injure pulmonary alveolus and vascular endothelium and cause cardiopulmonary vascular disease. These effects are sustained systemically, not only in the cardiopulmonary vascular system, and manifestly contribute to aging in individuals. COPD, representative of tobacco-related disease, has recently come to be regarded as a systemic inflammatory disease. As an independent risk factor for cardiovascular disease, COPD is deemed to raise cardiovascular risk synergistically with smoking through vascular inflammation. From the standpoint of inhibiting occurrence of life-threatening cardiovascular events, physicians must keep in mind the need to raise awareness and adoption of smoking cessation, and the fact that smoking cessation therapeutics are useful for alleviating withdrawal symptoms in nicotine-dependent smokers; their application in treatment and advice for cigarette smokers is essential.

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*Original Article*

# Suppressed anti-oxidant capacity due to a cellulose-free diet declines further by cigarette smoke in mice

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**ABSTRACT** — Dietary fiber, maintaining the gut environment, contributes to better lung function among smokers. This study was aimed to investigate the role of dietary fiber on the anti-oxidant capacity and gut environment during exposure to cigarette smoke. The anti-oxidant capacity as well as caecal levels of organic acids and population of micro-flora in the gut was measured after 4 months' exposure to cigarette smoke in mice (C57BL/6NcrSlc) fed with a cellulose-free diet. Animals were divided into control diet (AIN-93G)/non-smoke, cellulose-free diet/non-smoke, control diet/smoke, and cellulose-free diet/smoke groups. The anti-oxidant capacity in plasma was significantly suppressed by the cellulose-free diet in the non-smoke exposed mice. The suppression in the anti-oxidant capacity further declined following exposure to cigarette smoke. Both these changes in the anti-oxidant capacity were accompanied with changes in some organic acids levels in caecal contents. The anti-oxidant activity was significantly inversely correlated to succinic acid / acetic acid levels balance in caecal contents. In conclusion the cellulose-free diet suppressed the anti-oxidant capacity in mice, and the suppression further decreased by exposure to cigarette smoke. These changes in the anti-oxidant capacity may be related with changes in the gut environment.

**Key words:** Cigarette smoke, Cellulose-free diet, Anti-oxidant capacity, Organic acid, Gut environment

## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive disease mainly caused by cigarette smoke. There is no consensus of prophylaxis for progression of COPD except for cigarette smoke cessation (Scanlon *et al.*, 2000). Recently, some epidemiological studies demonstrated that dietary fiber mitigated cough and sputum in smokers and second hand smokers (Butler *et al.*, 2004; David *et al.*, 2005; Butler *et al.*, 2006). Moreover, it has been demonstrated that intake of dietary fiber was associated with a better lung function and reduced prevalence of COPD (Kan *et al.*, 2008). These reports speculated that these effects may partially come from anti-oxidant capacity by dietary fiber (Eastwood, 1999; Larrauri *et al.*, 1996). However, it has not been elucidated how dietary fiber changes the anti-oxidant capacity during exposure to cigarette smoke. In addition to the anti-oxidant activity, dietary fiber is crucial in maintaining the gut environment. Dietary fibers pass through the small intestine without being digested, and then are metabolized to organic acids by micro-flora in the large intestine (Roediger, 1980). In particular, acetic acid, propionic acid and butyric acid, which are short chain fatty acids (SCFAs) in organic acids, are absorbed via the colon mucosa and are utilized not only as a mucosa energy substrate but also as systemic energy sources (Roediger, 1980). Not only the energy substrate for intestinal mucosa, but also the SCFAs have an inhibitory effect on the growth of a pathogenic bacillus in the gut (Sugawara *et al.*, 2006).

In patients with ulcerative colitis (UC), an inflammatory bowel syndrome (IBS), the organic acid levels in the gut are changed (Vernia *et al.*, 1988), while UC has

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been recognized as a systemic disease based on systemic inflammation (Zilberman *et al.*, 2005). In severe UC, anti-oxidant capacity is decreased, contributing in turn to increased systemic oxidative stress (D'Odorico *et al.*, 2001; Koutroubakis *et al.*, 2004). The changes of proportion in organic acids in the gut are thought to be closely related with not only the inflammation in intestinal mucosa but also systemic effects in UC (Rodriguez-Cabezas *et al.*, 2003). Recently, our study has demonstrated that cigarette smoke exposure to rats for 4 weeks altered the gut environment (Tomoda *et al.*, 2011).

In this study we hypothesized that the changes in anti-oxidant capacity by dietary fiber may be partially related with those in organic acids levels in the gut environment. In this context, we investigated whether a cellulose-free diet changes the anti-oxidant capacity and organic acid levels and the population of micro-flora in the gut in mice exposed to cigarette smoke to clarify the role of dietary fiber on anti-oxidant capacity and organic acids levels in the gut during exposure to cigarette smoke.

## MATERIALS AND METHODS

### Animals and diets

Twelve-week-old, male C57BL/6NcrSlc mice purchased from Japan SLC, Inc. (Shizuoka, Japan) were used for the cigarette smoking experiment after preconditioning for two weeks. The animals were fed with AIN-93G (Oriental Yeast, Co., Ltd., Tokyo, Japan) or the cellulose-free diet and water *ad libitum* throughout the preconditioning and the experimental period in the laboratory animal research center at Nara Medical University. They were kept in a limited-access barrier housing maintained at a room temperature of 22 ± 1°C, with the humidity level at 55 ± 10%, and a 12-hr light/dark cycle, the illumination extending from 08:00 to 20:00. All procedures performed during these animal experiments were carried out under the control of our committee, in accordance with The Guidelines for Animal Experiments in Nara Medical University, and Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

The animals were separated into the 4 groups as follows: non-smoking group fed with the control diet (AIN-93G), non-smoking group fed with the cellulose-free diet (cellulose-free AIN-93G), smoking group fed with the control diet and smoking group fed with the cellulose-free diet.

The cellulose-free AIN-93G, in which cellulose in the AIN-93G contents was replaced quantitatively with sucrose, was prepared as the cellulose-free diet (Table 1). The quantitatively replaced sucrose, which is all digested in the small intestine, has only few effects on the production of organic acids in the large intestine and permeability of the intestine.

### Method of cigarette smoke exposure

The animals were compulsorily exposed to cigarette

**Table 1.** The components of AIN-93G (control diet) and the cellulose-free AIN-93G

| Ingredient | AIN-93G (Control diet) | Cellulose-free AIN-93G (Cellulose-free diet) |
|---|---|---|
| Cornstarch | 397.486 | 397.486 |
| Casein | 200.000 | 200.000 |
| Dextrinized cornstarch | 132.000 | 132.000 |
| Sucrose | 100.000 | 150.000 |
| Soybean oil | 70.000 | 70.000 |
| Cellulose | 50.000 | 0.000 |
| Mineral mix | 35.000 | 35.000 |
| Vitamin mix | 10.000 | 10.000 |
| L-Cystine | 3.000 | 3.000 |
| Choline bitartrate | 2.500 | 2.500 |
| Tert-butylhydroquinone | 0.014 | 0.014 |

(g/kg)

smoke using a tobacco smoke exposure apparatus (MIPS, Inc., Osaka, Japan) based on the method of Tomoda *et al.* (2011). All smoke exposure experiments were carried out using filtered tipped Long Peace cigarettes (Japan Tobacco Industry Co., Ltd., Tokyo, Japan), the nicotine and tar contents of which are 2.3 mg and 28 mg per cigarette, respectively.

The animals (fed with the control diet n = 5, with the cellulose-free diet n = 4) were exposed to smoke from 30 cigarettes for 20 min between 08:00 and 10:00 A.M. for 5 days a week, (Monday to Friday). The ten animals (fed with the control diet n = 5, with the cellulose-free diet n = 5) in the non-smoking group were also kept for 20 min in the apparatus holders.

Smoke was generated by inhaling the fired cigarette with compressor in the apparatus. The generated smoke was mixed with 7 volumes of air and the mixture was used as exposed smoke. The smoke was moved via a soft flexible tube from the apparatus to a chamber connected with holders in which 9 animals were kept separately. 2 sec of the smoke, followed by 2 sec of air was inhaled to each animal via their noses at a rate of 15 puffs per minutes.

The body weight change was recorded during the experimental period. The animals were decapitated under anesthesia by an intraperitoneal injection with sodium pentobarbital (50 mg/kg) 24 hr after the last cigarette smoke exposure, and anti-oxidant capacity in the plasma and the gut environment were evaluated.

### Total anti-oxidant capacity in plasma

The systemic anti-oxidant condition was indirectly evaluated by measuring total anti-oxidant capacity in plasma using the OXY-adsorbent Test (OXY Diacron, Grosseto, Italy) in a plasma sample (Cesarone *et al.*, 1999; Vassalle *et al.*, 2008). This test is based on the capacity of hypochlorous acid (HClO) to oxidize the physiological antioxidants. Total anti-oxidant capacity can be obtained by evaluating the capacity to inactivate the oxidant solution (HClO) added in excess to the sample. As HClO reacts with a chromogenic substrate (N, N-diethyl-paraophenylendiamine), a colored complex develops that can be measured photometrically. The spectrophotometric measurement was determined within 1 min of incubation at room temperature, at a wave length of 540 nm. The concentration of the colored complex was directly proportional to the concentration of HClO and indirectly proportional to the anti-oxidant capacity. The results were expressed as μmol of HClO consumed by 1ml of sample (μmol HClO/ml).

### Duration of smoke exposure

We have reported that a significant decrease in body weight gain and an increase in antioxidant capacity after 4 weeks' exposure of cigarette smoke to rats (Tomoda *et al.*, 2012). To investigate the effects on the antioxidant capacity, more than 4 weeks' exposure is needed. Moreover the non-exposed mice with control diet were not able to be inserted into the holder for their body size beyond 4 months after the exposure was started. For these two reasons the animals were exposed to cigarette smoke during four months.

### Quantitative analysis of organic acids in caecal contents

The examination of organic acids in caecal contents was performed according to the previously reported method (Asahara *et al.*, 2001). Briefly, after cigarette smoke exposure for four months, caecal contents were obtained from mice after sacrifice. The caecal contents were homogenized in 1 ml of distilled water, and homogenate was centrifuged at 13,000 x g at 4°C for 10 min. A mixture of 0.9 ml of the resulting supernatant and 0.1 ml of 1.5 mol/l perchloric acid in a glass tube was mixed well and allowed to stand at 4°C for 12 hr. The suspension was then passed through a filter with a pore size of 0.45 μm (Millipore Japan Ltd., Tokyo, Japan). The sample was analyzed for organic acids by high-performance liquid chromatography, as described in a previous report (Kikuchi and Yajima, 1992). The high-performance liquid chromatography was performed with a Waters system (Waters 432 Conductivity Detector; Waters, Milford, MA, USA) equipped with two columns (Shodex Rspack KC-811; Showa Denko Co. Ltd., Tokyo, Japan). The concentrations of organic acids were calculated using external standards, and the reproducibility and stability of these measurements have been reported (Kikuchi and Yajima, 1992).

### Examination of caecal bacterial flora

The examination of caecal bacterial flora was performed according to previously reported method (Asahara *et al.*, 2001). Briefly, caecal contents were obtained from the killed mice. One gram of caecal contents was placed in grinding tubes containing 1 ml of sterilized anaerobic transfer medium, and then homogenized with a Teflon grinder. After serial dilution of the caecal suspensions with an anaerobic buffer solution, 50 μl or 500 μl portions of the diluents were spread onto the following culture media (agar plate: 50 μl, roll tube agar: 500 μl). Heart infusion agar, supplemented with 0.2 mg/ml neomycin (Sigma, St Louis, MO, USA), 0.01%

(w/v) brilliant green, 0.1% (w/v) sodium taurocholate, 0.03% (w/v) L-cysteine hydrochloride, and 5% (w/v) defibrinated horse blood (modified NBGT agar), was used for selective isolation of the Bacteroidaceae. CPLX agar was used for selective isolation of *Bifidobacterium*. LBS agar (Becton Dickinson and Company, Cockeysville, MD, USA), supplemented with 0.8% (w/v) Lab Lemco powder (Oxoid Ltd., Basingstoke, UK), 0.1% (w/v) sodium acetatetrihydrate and 0.37% (w/v) acetate, was used for selective isolation for *Lactobacillus*. COBA agar was used for selective isolation for *Enterococcus*. DHL agar (Nissui Pharamaceutical Co., Ltd., Tokyo, Japan) was used for selective isolation for Enterobacteriaceae. *Staphylococcus* medium No. 110 agar (Nissui Pharmaceutical Co., Ltd.) was used for selective isolation for *Staphylococcus* and *Bacillus*. VL-G roll tube agar supplemented with 0.2% (w/v) cellobiose and 0.2% (w/v) maltose (modified VL-G roll tube agar) was used for determination of total anaerobic counts. Modified NBGT agar, CPLX agar, and LBS agar were cultured under anaerobic conditions in an atmosphere of 7% $H_2$, 5% $CO_2$ in $N_2$ at 37°C for 72 hr. After incubation, the colonies on the plates were counted and Gram stained. Species and biotypes of the bacteria were identified with API systems (bioMerieux S.A., Montalieu-Vercieu, France): rapid ID 32 A for the Bacteroidaceae and Lactobacillaceae, API 20 STREP for the Enterococcaceae, API 20 E for the Enterobacteriaceae and API 20 STAPH for the Staphylococcaceae. The lower limit of bacterial detection with this procedure was 100 cfu/g caecal contents.

Scanning for anaerobic fujiform bacteria was carried out by microscopic bacterial counts. For quantification, 10 µl portions of the diluents were put into a 10-well immunofluorescent slide (Flow Laboratories, Inc., McLean, VA, USA), fixed and Gram-stained. Fujiform bacteria were counted with the aid of an ocular grid containing 100 squares (calibrated with a stage micrometer), a 100 × objective, and a 10 × ocular lens. Acceptable slides considered for analysis had to meet the following two criteria. (i) The bacteria appeared to be evenly distributed, and (ii) the number of bacteria per 100 grid squares was between 20 and 300. Counts were made in 10 fields chosen randomly.

The number of viable bacteria per gram wet weight caecal contents was calculated. The lower limit of bacterial detection with this procedure was 100 cfu/g caecal contents. Results were expressed as means ± standard deviation (S.D.) numbers of cfu per 1 g of caecal contents.

### Statistics

Comparisons of parameters between the two groups were made by Mann-Whitney U test. A $p$ value less than 0.05 was considered to indicate a statistically significant difference. Comparisons of body weight between the two groups were performed with two-way analysis of variance.

## RESULTS

### Changes in body weight of each group during the entire experimental period

Figure 1 shows changes in body weight during cigarette smoke exposure. Cigarette smoke significantly inhibited body weight gain in both groups fed with the cellulose-free diet and the control diet from the fourth week of the experimental period ($p < 0.05$). In the smoking groups the decrease in body weight gain was accelerated by feeding with the cellulose-free diet from the 11th week of the experimental period ($p < 0.05$). However, in the non-smoking groups there was no significant difference.

This result suggests that there was no significant effect of the replaced sucrose in the cellulose-free diet on calorie balance during this experimental period.

### Effects of the cellulose-free diet on the anti-oxidant capacity

Figure 2 shows the anti-oxidant capacity in plasma after cigarette smoke exposure for four months. The anti-oxidant capacity in animals fed with the cellulose-free diet was significantly lower than those fed with the control diet in both the non-smoking groups (390.8 ± 38.7 vs. 297.0 ± 64.1µmol/ml, respectively, $p = 0.047$) and the smoking groups (404.6 ± 17.5 vs. 209.8 ± 31.2 µmol/ml, respectively, $p = 0.014$). The suppression in the anti-oxidant capacity in the cellulose-free diet was further decreased by cigarette smoke (297.0 ± 64.1 vs. 209.8 ± 31.2 µmol/ml, respectively, $p = 0.014$), however in the control diet groups cigarette smoking tended to increase the anti-oxidant capacity (390.8 ± 38.7 vs. 404.6 ± 17.5 µmol/ml, respectively).

Based on these results at first we investigated independent effects by cigarette smoke and the cellulose-free diet on the organic acid levels, pH and population of micro-flora in caecal contents. After these investigations, the additive effects of cigarette smoke on these changes by the cellulose-free diet as well as those of cellulose-free diet on these changes by cigarette smoke were investigated.

### Changes in organic acids levels and pH in caecal contents

The independent effects of cigarette smoke and the cel-

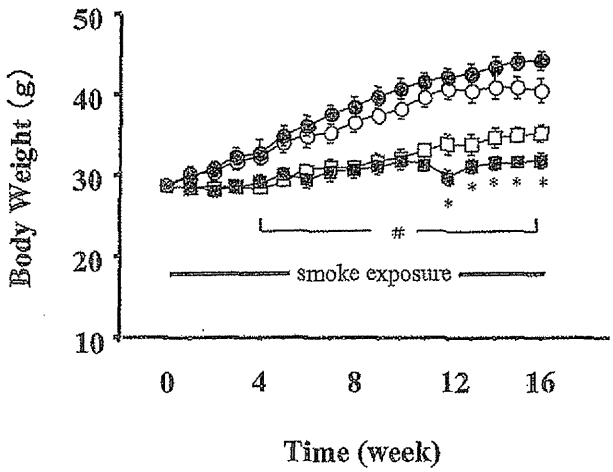


**Fig. 1.** Changes in body weight of each group during the entire experimental period. Each point indicates the mean ± S.E. of 4 to 5 animals. Symbols; Group A ○: non-smoking group fed with control diet (n = 5), Group B ●: non-smoking group fed with the cellulose-free diet (n = 5), Group C □: smoking group fed with control diet (n = 5), Group D ■: smoking group fed with the cellulose-free diet (n = 4). #$p < 0.05$: Group A vs. Group C, and Group B vs. Group D from the 4th week to the last week. *$p < 0.05$: Group C vs. Group D from the 11th week to the last week. Data were statistically analyzed by the Mann-Whitney U test and two-way analysis of variance (ANOVA).

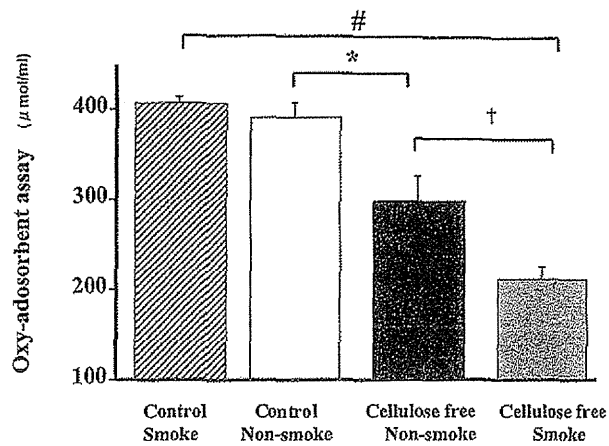


**Fig. 2.** Effect of the cellulose-free diet on the anti-oxidant capacity in plasma after four months' exposure of cigarette smoke. Each point indicates the mean ± S.D. of 4 to 5 animals. Non-smoking group fed with control diet (Group A: outlined bar; n = 5), non-smoking group under fed with the cellulose-free diet (Group B: solid bar, n = 5), smoking group fed with control diet (Group C: hatched bar, n = 5), smoking group fed with the cellulose-free diet (Group D: shaded bar, n = 4). *$p < 0.05$: Group A vs. Group B. #$p < 0.05$: Group C vs. Group D. †$p < 0.05$: Group B vs. Group D. Data was statistically analyzed by Mann-Whitney U test.

**Table 2.** Effects of exposure to cigarette smoke and the cellulose-free diet on organic acid levels and pH in caecal contents

| Organic acid and pH | Control diet Non-smoke (n = 5) (Control) | Control diet Smoke (n = 5) (Effect of smoke) | Cellulose-free diet Non-smoke (n = 5) (Effect of cellulose-free diet) |
|---|---|---|---|
| Acetic acid | 52.7 ± 7.2 | 65.8 ± 12.2# | 72.1 ± 15.2 |
| Propionic acid | 19.0 ± 1.6 | 15.4 ± 2.5# | 14.7 ± 3.2# |
| Butyric acid | 11.0 ± 3.5 | 29.6 ± 35.3 | 12.8 ± 5.5 |
| Isobutyric acid | 4.7 ± 2.7 | 4.1 ± 2.9 | 8.3 ± 5.8 |
| Valeric acid | 7.2 ± 4.3 | 3.6 ± 1.5 | 3.1 ± 4.3 |
| Isovaleric acid | 6.5 ± 2.1 | 3.9 ± 2.2 | 3.7 ± 3.3 |
| Succinic acid | 1.8 ± 2.0 | 1.1 ± 0.8 | 15.6 ± 9.7§ |
| pH | 7.2 ± 0.3 | 7.6 ± 0.3 | 6.9 ± 0.3 |

(μmol/g caecal contents)

Each point indicates the mean ± S.D. of 5 animals. #$p < 0.05$, §$p < 0.01$: significant relative to control by the Mann-Whitney U test.

lulose-free diet on the organic acids levels and pH in caecal contents are shown in Table 2. Cigarette smoke significantly increased acetic acid levels (52.7 ± 7.2 vs. 65.8 ± 12.2 μmol/g caecal contents, respectively, p = 0.028) and significantly deceased propionic acid levels (19.0 ± 1.6 vs. 15.4 ± 2.5 μmol/g caecal contents, respectively, p = 0.028) while the cellulose-free diet significantly increased succinic acid levels (1.8 ± 2.0 vs. 15.6 ± 9.7 μmol/g caecal contents, respectively, p = 0.009) and significantly decreased propionic acid levels (19.0 ± 1.6 vs. 14.7 ± 3.2 μmol/g caecal contents, respectively, p = 0.028). On exposed to cigarette smoke, the cellulose-free diet significantly decreased acetic acid levels (65.8 ± 12.2 vs. 45.5 ± 4.6 μmol /g caecal contents, respectively, p = 0.014) and significantly increased isovaleric acid levels (3.9 ± 2.2 vs. 7.5 ± 1.1 μmol/g caecal contents, respectively, p = 0.014) (Table 3), while on the cellulose-free diet, cigarette smoke induced a significant decrease in acetic acid levels (72.1 ± 15.2 vs. 45.5 ± 4.6 μmol/g caecal contents, respectively, p = 0.014) and elevated the pH of caecal contents (6.9 ± 03 vs. 7.6 ± 0.2, respectively, p = 0.028) (Table 4).

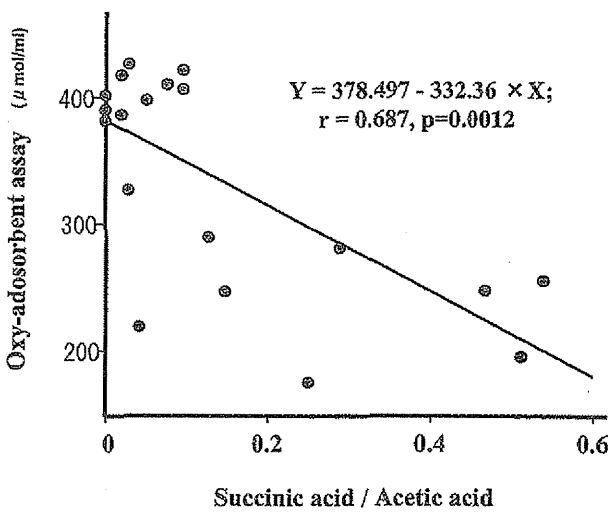


Fig. 3. Relationship between the anti-oxidant capacity and the ratio of succinic acid to acetic acid levels in caecal contents.

## Relationship between the anti-oxidant capacity and succinic acid / acetic acid balance in the caecal contents

The anti-oxidant capacity in plasma was significantly inversely correlated to the ratio of succinic acid levels to acetic acid levels in the caecal contents (r = 0.687; p = 0.0012) as shown in Fig. 3. This result suggests that systemic anti-oxidant capacity may be closely related to proportion of organic acids especially succinic acid/acetic acid balance in the gut.

## Changes in the population of micro-flora in the caecal contents

The independent effects of cigarette smoke and the cellulose-free diet on the population of micro-flo-

**Table 3.** Additive effects of the cellulose-free diet on changes in organic acid levels and pH in caecal contents by exposure to cigarette smoke

| Organic acid and pH | Smoke Control diet (n = 5) | Smoke Cellulose-free diet (n = 4) | p value |
|---|---|---|---|
| Acetic acid | 65.8 ± 12.2 | 45.5 ± 4.6# | 0.014 |
| Propionic acid | 15.4 ± 2.5 | 14.2 ± 1.4 | 0.327 |
| Butyric acid | 29.6 ± 35.3 | 14.0 ± 3.4 | 0.624 |
| Isobutyric acid | 4.1 ± 2.9 | 5.0 ± 2.9 | 0.462 |
| Valeric acid | 3.6 ± 1.5 | 7.9 ± 6.0 | 0.221 |
| Isovaleric acid | 3.9 ± 2.2 | 7.5 ± 1.1# | 0.014 |
| Succinic acid | 1.1 ± 0.8 | 14.3 ± 10.6 | 0.066 |
| pH | 7.6 ± 0.3 | 7.6 ± 0.2 | 0.462 |

(μmol/g caecal contents)

Each point indicates the mean ± S.D. of 4 or 5 animals. # $p < 0.05$: significant relative to animals fed with the control diet by the Mann-Whitney U test.

**Table 4.** Additive effects of cigarette smoke on changes in organic acids and pH in caecal contents by the cellulose-free diet

| Organic acid and pH | Cellulose-free diet Non-smoke (n = 5) | Cellulose-free diet Smoke (n = 4) | p value |
|---|---|---|---|
| Acetic acid | 72.1 ± 15.2 | 45.5 ± 4.6# | 0.014 |
| Propionic acid | 14.7 ± 3.2 | 14.2 ± 1.4 | 0.807 |
| Butyric acid | 12.8 ± 5.5 | 14.0 ± 3.4 | 0.807 |
| Isobutyric acid | 8.3 ± 5.8 | 5.0 ± 2.9 | 0.221 |
| Valeric acid | 3.1 ± 4.3 | 7.9 ± 6.0 | 0.270 |
| Isovaleric acid | 3.7 ± 3.3 | 7.5 ± 1.1 | 0.111 |
| Succinic acid | 15.6 ± 9.7 | 14.3 ± 10.6 | 0.806 |
| pH | 6.9 ± 0.3 | 7.6 ± 0.2# | 0.028 |

(μ mol/g caecal contents)

Each point indicates the mean ± S.D. of 4 tor 5 animals. # $p < 0.05$: significant relative to animals without exposure by the Mann-Whitney U test.

**Table 5.** Effects of exposure to cigarette smoke and the cellulose-free diet on the population of micro-flora in caecal contents

| Organisms | Control diet Non-smoke (n = 5) (Control) | Control diet Smoke (n = 5) (Effect of smoke ) | Cellulose-free diet Non-smoke (n = 5) (Effect of cellulose-free diet) |
|---|---|---|---|
| Fusiform bacteria | 10.2 ± 0.1 | 10.1 ± 0.3 | 10.6 ± 1.0 |
| Bacteroidaceae | 9.7 ± 0.4 | 9.6 ± 0.5 | 10.0 ± 0.1 |
| *Bifidobacterium* | 6.0 ± 0.3 | 5.3 ± 0.8 | 5.0 ± 0.4* |
| *Lactobacillus* | 6.8 ± 0.5 | 6.9 ± 1.0 | 7.5 ± 0.4 |
| *Enterococcus* | 7.1 ± 0.4 | 6.9 ± 0.2 | 7.3 ± 0.1 |
| Enterobacteriaceae | 6.7 ± 0.8 | 6.5 ± 0.4 | 6.0 ± 0.6 |
| *Staphylococcus* | 6.7 ± 0.7 | 7.0 ± 0.1 | 6.5 ± 0.3 |

($\log_{10}$ CFU/g caecal contents )

Each point indicates the mean ± S.D. per one g of caecal contents for 5 animals. *$p < 0.05$: significant relative to control by the Mann-Whitney U test.

ra in caecal contents are shown in Table 5. Cigarette smoke tended to decrease the population of *Bifidobacterium* (6.0 ± 0.3 vs. 5.3 ± 0.8 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.175), while the cellulose-free diet significantly decreased the population of *Bifidobacterium* (6.0 ± 0.3 vs. 5.0 ± 0.4 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.016) and tended to increase *Lactobacillus* (6.8 ± 0.5 vs. 7.5 ± 0.4 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.050 ) (Table 5). On exposed to cigarette smoke, the cellulose-free diet tended to decrease the population of Staphylococcus (7.0 ± 0.1 vs. 6.6 ± 0.4 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.050) and inrease the population of *Bifidobacterium* (5.3 ± 0.8 vs. 6.2 ± 0.5 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.080) (Table 6), while on the cellulose-free diet, cigarette smoke induced significant increase of the population of *Bifidobacterium* (5.0 ± 0.4 vs. 6.2 ± 0.5 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.020) (Table 7).

**Table 6.** Additive effects of the cellulose-free diet on changes in the population of micro-flora in caecal contents by exposure to cigarette smoke

| Organisms | Smoke Control diet (n = 5) | Smoke Cellulose-free diet (n = 4) |
|---|---|---|
| Fujiform bacteria | 10.1 ± 0.3 | 10.3 ± 0.2 |
| Bacteroidaceae | 9.6 ± 0.5 | 10.0 ± 0.4 |
| *Bifidobacterium* | 5.3 ± 0.8 | 6.2 ± 0.5 |
| *Lactobacillus* | 6.9 ± 1.0 | 7.5 ± 0.4 |
| *Enterococcus* | 6.9 ± 0. 2 | 7.1 ± 0.4 |
| Enterobacteriaceae | 6.5 ± 0.4 | 5.8 ± 0.9 |
| *Staphylococcus* | 7.0 ± 0.1 | 6.6 ± 0.4 |

($\log_{10}$ CFU/g caecal contents)

Each point indicates the mean ± S.D. per one g of caecal contents for 4 or 5 animals.

**Table 7.** Additive effects of cigarette smoke on changes in the population of micro-flora in caecal contents by the cellulose-free diet

| Organisms | Cellulose-free diet Non-Smoke (n = 5) | Cellulose-free diet Smoke (n = 4) |
|---|---|---|
| Fujiform bacteria | 10.6 ± 1.0 | 10.3 ± 0.2 |
| Bacteroidaceae | 10.0 ± 0.1 | 10.0 ± 0.4 |
| *Bifidobacterium* | 5.0 ± 0.4 | 6.2 ± 0.5* |
| *Lactobacillus* | 7.5 ± 0.4 | 7.5 ± 0.4 |
| *Enterococcus* | 7.3 ± 0.1 | 7.1 ± 0.4 |
| Enterobacteriaceae | 6.0 ± 0.6 | 5.8 ± 0.9 |
| *Staphylococcus* | 6.5 ± 0.3 | 6.6 ± 0.4 |

($\log_{10}$ CFU/g caecal contents)

Each point indicates the mean ± S.D. per one g of caecal contents for 4 or 5 animals. *$p < 0.05$: significant relative to animals without exposure to cigarette smoke by the Mann-Whitney U test.

## DISCUSSION

This study demonstrated that the cellulose-free diet suppressed the anti-oxidant capacity in mice and that the suppression was exacerbated by cigarette smoke. Both these changes in the anti-oxidant capacity were accompanied with changes in the proportion of organic acids in the gut. This study is the first experimental report that supports the results of epidemiological studies about the beneficial effects of dietary fiber among subjects exposed to cigarette smoke. The most impressive finding in this study is that the suppression in the anti-oxidant capacity by feeding with the cellulose-free diet was further decreased by cigarette smoke while in the control diet group the anti-oxidant capacity tended to be increased by cigarette smoke. In smokers the anti-oxidant levels were elevated in serum and the lung. Erythrocytes from smokers contain more glutathione and catalase and protect endothelial cells from hydrogen peroxide (Toth *et al.*, 1986). Ascorbic acid levels in alveolar macrophages from smokers are increased (McGowan *et al.*, 1984). These anti-oxidant levels are suggested to increase to compensate for increased oxidative stresses by cigarette smoke. Cavarra *et al.* (2001) demonstrated that in mice, which

are sensitive to cigarette smoke and develop to emphysema by cigarette smoke, the anti-oxidant activity during exposure to cigarette smoke was decreased to about 70% of that of non-exposed mice while in resistant mice strains the anti-oxidant activity was increased during exposure to cigarette smoke. In patients with COPD the anti-oxidant capacity was decreased to about two-thirds of that of healthy control subjects (MacNee, 2005). In the present study the anti-oxidant capacity in the smoke exposed mice with the cellulose-free diet was decreased to about a half of that in non-exposed mice with control diet. This result demonstrated that dietary fiber especially cellulose is a crucial factor in maintaining the anti-oxidant capacity to reduce oxidative stress due to cigarette smoke and preventing subjects exposed to cigarette smoke from developing COPD.

This study did not fully clarify the mechanism how the anti-oxidant capacity was suppressed by the cellulose-free diet. Dietary fiber contributes to maintain the gut environment. In patients with IBS, alterations of organic acid levels and micro-flora population, and a decrease of the anti-oxidant capacity are reported. The changes in the gut environments supposedly contribute to systemic effects in IBS (Rodriguez-Cabeza *et al.*, 2003). Additionally, dietary fiber is known to contribute to maintaining the gut environment in a normal state. Given this background, we focused on alteration of the gut environment, especially changes in the proportion of caecal organic acids besides the anti-oxidant capacity. This study demonstrated that the cellulose-free diet suppressed the anti-oxidant capacity and the suppressed anti-oxidant capacity was further declined by cigarette smoke while under feeding with control diet cigarette smoke tended to increase the anti-oxidant capacity. Therefore, at first we investigated the independent effects of the cellulose-free diet and cigarette smoke on the organic acid pattern and pH. After these investigations the additive effects of cigarette smoke on the changes by the cellulose-free diet as well as those of the cellulose-free diet on the changes by cigarette smoke were investigated.

At first, in the non-exposed mice the cellulose-free diet significantly decreased propionic acid levels and significantly increased succinic acid levels in caecal contents. Succinic acid is seldom detected under normal fermentation in the large intestine (Morita *et al.*, 1998), because it is a typically intermediate metabolite and is quickly converted to propionate or acetate by acid-utilizing bacteria. However, an abnormal fermentation in the large intestine leads to succinic acid accumulation such as in short bowel syndrome, diarrhea and acute weaning diets (Tsukahara and Ushida, 2002). On the other hand, it has been reported that succinic acid inhibited epithelial cell proliferation of colonic mucosa in rats and had cytotoxic effects on cultured cell lines (Inagaki *et al.*, 2007), and also caused lesions in ligated rabbit ileum loops resembling those of ulcerative colitis (Gaginella *et al.*, 1977). Increased succinic acid levels may contribute to alter the gut environment. Propionic acid is one of the short chain fatty acids and has some effects on the host. Dietary propionic acid decreases cholesterol levels, while enemas consisting of propionic acid, acetic acid and butyric acid improve ulcerative colitis (Patz *et al.*, 1996). However, the role of propionic acid has not been fully elucidated. In this study the suppressed the anti-oxidant capacity by the cellulose-free diet was further declined by cigarette smoke while under feeding with control diet cigarette smoke tended to increase the anti-oxidant capacity. Thinking of these results into changes in organic acids levels, acetic acid levels may be related to changes in the anti-oxidant capacity during the exposure to cigarette smoke, because cigarettes smoke under feeding with control diet increased the acetic acid levels while under feeding with the cellulose-free diet cigarette smoke decreased the acetic acid levels. Acetic acid is primarily utilized by intestinal epithelial cells as energy substrates (Clark *et al.*, 2003; Goto *et al.*, 2005; Oba *et al.*, 2004) and contributes to maintaining the gut environment.

The anti-oxidant capacity in plasma significantly inversely correlated to the ratio of succinic acid levels to acetic acid levels in the caecal contents as shown in Fig. 3. Changes in the gut environment especially proportion of organic acids may contribute to changes in the anti-oxidant capacity. To clarify the effect of proportion of organic acids on the anti-oxidant capacity further investigations are needed.

Another interesting result about the additive effects of cigarette smoke on the changes by cellulose free diet was an elevation in pH level in the caecal contents. The elevation in pH may be linked to a decrease in acetic acid levels (Shimizu *et al.*, 2006). The pH in the colon of patients with colonic cancer is more alkaline, indicating a reduction in colonic carbohydrate fermentation by organic acids (Fallingborg, 1999). Cigarette smoke promoted growth of colon cancer in a mouse model (Wong *et al.*, 2009). The elevation in pH in the colon and the decreases in certain organic acids levels by cigarette smoke during feeding with the the cellulose-free diet may be related to the development and growth of colon cancer.

In this study the cellulose-free diet further reduced the decrease in body weight gain by cigarette smoke. In smoke exposed mice cellulose-free diet significantly decreased the acetice acid levels in ceacal contents.

Acetic acid is absorbed into hepato-portal flow to the liver and utilized as systemic energy sources. It has been reported that about 10% of systemic energy sources are obtained from organic acids in humans. In this study, cigarette smoke exposure deceased body weight gain. This effect of the cellulose-free diet on body weight gain during exposure to cigarette smoke may be related to a decrease in acetic acid levels in the caecum.

Although the present study did not elucidate how organic acid balance was changed, food contents, gut movement, population of micro-flora and fermentation of micro-flora *et al.* may contribute to changes in organic acid levels in the gut. In the present study we evaluated micro-flora in the caecal contents. The cellulose-free diet decreased propionic acid levels and increased succinic acid levels while the cellulose-free diet decreased the population of *Bifidobacterium* which produces mainly acetic acid, but the population of Bacteroidaceae, which mainly produces succinic acid, proved to be unchanged. Cigarette smoke increased acetic acid levels while cigarette smoke did not significantly change the population of the micro-flora. On exposed to cigarette smoke the cellulose-free diet decreased acetic acid levels and increased isovaleric acid levels while it did not significantly change the population of the micro-flora. With the cellulose-free diet cigarette smoke decreased acetic acid levels, while cigarette smoke increased the population of *Bifodobacterium*. Therefore, the changes in the organic acid levels in the present study were not able to be explained only by changes in the population of micro-flora. Other factors besides the changes in the population micro-flora in the gut, may contribute to connect with organic acid levels in caecal contents. Further investigations about the affects of the cellulose-free diet and cigarette smoke on fermentation of organic acids by micro-flora in the gut are needed to gain further insights into the changes organic acid levels in caecal contents by the cellulose-free diet and exposure to cigarette smoke.

One of the limitations of the present study is the small size of the animal groups. However, the anti-oxidant capacity remarkably decreased with statistical significance (390.8 ± 38.7 vs. 209.8 ± 31.2 μmol/ml, respectively, $p = 0.0143$). We therefore feel that the sample size of this study, although limited, is enough to provide the insight into the change in the anti-oxidant capacity by the cellulose-free diet and cigarette smoke.

In conclusion, the cellulose-free diet suppressed the anti-oxidant capacity in mice and that the suppression was further exacerbated by cigarette smoke. Both these changes in the anti-oxidant capacity were accompanied with changes in proportion of organic acids in the gut. The changes in the anti-oxidant capacity by the cellulose-free diet and cigarette smoke may be related with changes in the gut environment.

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