Carbocisteine Protects Against Emphysema Induced by Cigarette Smoke Extract in Rats

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Background

Chronic inflammation, imbalance of proteolytic and antiproteolytic activities, oxidative stress, and apoptosis of lung structural cells contribute to the pathogenesis of COPD. There is increasing evidence that carboxymethylcysteine (also known as carbocisteine) (CMC), which is commonly used for its mucoactive property, has diverse pharmacologic actions, including significant antioxidant activity. We hypothesize that CMC protects against cigarette smoke extract (CSE)-induced emphysema in rats via its antioxidant action.

Methods

Sprague-Dawley rats were divided into four groups (n = 6 in each group): control group, CSE group, CSE + 125 mg/kg/d of CMC group, and CSE + 250 mg/kg/d of CMC group. The CSE was injected intraperitoneally once a week for 3 weeks, and CMC was administered daily via a gastric gavage for the same duration. Antioxidant activity in the pulmonary and serum levels, apoptotic index, caspase-3 activity, and matrix metalloproteinase (MMP)-2 and MMP-9 activities in lung tissues were measured.

Results
CMC significantly protected against alveolar enlargement and parenchymal destruction in rats injected with CSE, resulting in prevention of the development of CSE-induced emphysema in the rats. CMC significantly protected the antioxidant activity in both the pulmonary and systemic levels, reduced pathologic apoptosis, and inhibited MMP-2 and MMP-9 activities in the lungs of rats with CSE-induced emphysema.

**Conclusions**

CMC protected against the development of CSE-induced emphysema in rats. The molecular mechanisms that were involved with stabilizing the biologic antioxidant activity resulted from the administration of CMC, which was connected to the inhibition of apoptosis and the reversal of the imbalance of proteolytic and antiproteolytic enzyme activities, eventually achieving the protection of the alveolar architecture of rats with emphysema.

**Abbreviations**

**AI**
- apoptotic index

**BAP**
- bioantioxidant power

**CMC**
- carboxymethylcysteine

**CSE**
- cigarette smoke extract

**DI**
- destructive index

**HE**
- hematoxylin and eosin

**MLI**
- mean linear intercept

**MMP**
- matrix metalloproteinase

**PBS**
- phosphate-buffered saline

**ROS**
- reactive oxygen species

**TUNEL**
- terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

**TUNEL+**
- terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-positive
COPD is a major cause of chronic morbidity and mortality throughout the world.\[1\] It is characterized by progressive airflow limitation associated with emphysema and/or small airway disease,\[2\] \[3\] which is mostly caused by cigarette smoking. The pathogenesis of emphysema has been suggested as being the result of an imbalance of proteolytic and antiproteolytic activities,\[4\] oxidative stress\[5]\] \[6\] and apoptosis\[7\] of structural cells in the lungs. One of the mechanisms by which cigarette smoke causes emphysema is through the pathway of oxidative stress, which has important consequences on several events of lung pathophysiology and can be the pathogenetic cause of COPD. Increased levels of oxidants and substrates, such as hydrogen peroxide, carbon monoxide, carboxyhemoglobin, and so on, are exhibited in the exhaled air\[8]\] \[9\] or arterial circulation\[10\] of patients with COPD. Such overwhelming levels of oxidants and substrates cause uncontrolled activity of elastases, including neutrophil elastase and $\alpha_1$-proteinase, resulting in pulmonary tissue destruction.\[11\] Oxidants not only damage DNA, lipids, and proteins\[12]\] \[13\] but also exert direct toxic effects on lung connective tissue elements, triggering the apoptosis process in the lung structures.\[5]\] \[6\] All of these activities present strong evidence that oxidative stress plays an indispensable role in the pathogenesis of COPD.

It is plausible that agents with antioxidative effects could offer promise for the prevention and treatment of COPD. Carboxymethylcysteine (also known as carbocisteine) (CMC) seems to have a role in significant antioxidation that may be more important than mucolysis itself for long-term treatment of COPD.\[14\] The addition of CMC to the BAL fluid of patients with stable COPD markedly reduced oxidative activity.\[15\] The concentration of xanthine oxidase products (superoxide and uric acid) in the supernatant fluid of endothelial cells cultured with elastase was significantly reduced by the addition of CMC to the culture.\[15\] Recent studies further demonstrated that CMC could scavenge reactive oxygen species (ROS) in vitro\[16\] and inhibit oxidant-induced apoptosis in cultured human airway epithelial tissue.\[17\] However, the majority of current evidence for the antioxidant action of CMC is extrapolated from in vitro study. The present study was designed to examine whether CMC has the potential to prevent cigarette smoke extract (CSE)-induced emphysema in rats, and if so, to further elucidate the molecular mechanisms by which CMC achieves its protective role.

Materials and Methods

Preparation of CSE

CSE was prepared as previously reported.\[18\] Briefly, one cigarette without filters (Marlboro; Philip Morris International Inc; New York City, New York) was burned, and the smoke was passed, using a vacuum pump, through a glass Cambridge filter (Cambridge Filter Japan, Ltd; Tokyo, Japan) with 0.20-µm pores for removing particles and bacteria into a vessel containing phosphate-buffered saline (PBS) (1 mL per one cigarette). The pH of the CSE-PBS solution was between 5.2 and 5.3, and this solution was prepared fresh for each set of experiments.

Experimental Protocols

The animal protocol was approved by the animal care and use committee of Shinshu University. Six-week-old male Sprague-Dawley rats (weight range, 200-250 g) were randomly selected and divided into four groups (n = 6 in each group): control group, CSE group, CSE + 125 mg/kg/d of CMC (low-dose) group (CSE + CMC [L] group), and CSE + 250 mg/kg/d of CMC (high-dose) group (CSE + CMC [H] group). The CMC was supplied by Kyorin Pharma, Inc, in Tokyo, Japan. The CSE group was injected intraperitoneally with 1 mL CSE-PBS solution on days 1, 8, and 15, and the control group with 1 mL of the vehicle (PBS) on days 1, 8, and 15 of the experiment. The CSE + CMC (L) and CSE + CMC (H) groups were injected intraperitoneally with 1 mL of CSE on days 1, 8, and 15 along with CMC daily at 125 mg/kg/d or 250 mg/kg/d, respectively. The CMC was prepared in distilled water and administered daily via a gastric gavage for 21 days. The dosages of CMC applied in the present experiment were determined according to studies elsewhere.\[19]\] \[20\] The CMC administration was started on the day of the first CSE injection. All of the rats were fed under a same-rearing condition for 3 weeks. At day 21 after starting the experiment, the rats were killed.

Sampling the Blood and Lung Tissues

The blood and lung tissue samples were obtained according to procedures reported previously.\[18\] The blood samples were taken from the inferior vena cava via the opened chest and centrifuged at 3,000 g for 15 min to separate the serum within 1 h after sampling. The left lung was inflated with 0.5% low-melting agarose at a constant pressure of 25 cm H$_2$O and fixed in 10% formalin for 48 h. The paraffin-embedded sections of the left lung were used for histologic examination. The right lung was homogenized immediately after harvest in lysis buffer.\[19\] The supernatant liquids were separated during two periods of centrifugation at 10,000 g for 10 min. The concentrations of proteins in the supernatant liquids were determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Inc; Rockford, Illinois). The serum and the supernatant liquids were stored at -70°C until measurements were performed.

Morphologic Assessment
To observe the emphysematous morphologic process in the lung of experimental rats, the mean linear intercept (MLI) and destructive index (DI) were measured in lung tissues stained with hematoxylin and eosin (HE). The MLI is a measurement of the mean interalveolar septal wall distance to assess the airspace enlargement, a process that is widely used to examine alveolar space size. The DI is used to estimate the parenchymal destruction and is quantified by dividing the number of identified destructive alveoli by a sample of > 3,000 alveoli randomly counted in each lung of rats. The DI was represented as the percentage of the destructive alveoli among the total alveoli. The presence of a destructive alveolus was defined if at least one of the following alveoli conditions was observed: alveolar wall defects, intraluminal parenchymal rags in alveolar ducts, obviously abnormal morphologic characteristics, or typical emphysematous changes. The morphologic assessment was repeated on coded samples.

**Measurements of Antioxidant Activity in Pulmonary Level and Systemic Level**

The antioxidant activity in the supernatant liquids of the lung homogenates and the serum was measured using the bioantioxidant power (BAP) test and the Free Radical Elective Evaluation FRAS4 system (Diacron International SRL; Grosseto, Italy). The BAP test was performed in each rat according to the measurement procedures in the manufacturer's instructions. The results of the BAP test provided the biologic antioxidant potential, which was expressed in µmol/L.

**Apoptotic Assay**

To detect the apoptotic status in the lung parenchyma of the experimental rats, the apoptotic index (AI) and expression of activated caspase-3 (a marker of apoptosis) were examined in the lung tissues of the rats.

**Measurement of the AI**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was performed to label the DNA-damaged cells in the lungs of the experimental rats. The TACS 2 TdT DAB kit (Trevigen, Inc; Gaithersburg, Maryland) was used for this TUNEL experiment, following the manufacturer's instructions. The AI was calculated as the percentage of TUNEL-positive (TUNEL+) nuclei in a sample of > 3,000 nuclei randomly counted for each lung of rats (magnification ×400).

**Immunohistochemical Findings for Activated Caspase-3**

The expression of activated caspase-3 was determined in the lungs of the experimental rats by immunohistochemical examination using a rabbit polyclonal antibody against the cleaved caspase-3 (Cell Signaling Technology, Inc; Danvers, Massachusetts) according to the manufacturer's instruction.

**Gelatin Zymographic Examination**

To estimate the imbalance of proteolytic and antiproteolytic activities in the experimental rats, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) activities were determined in the lungs of the experimental rats by gelatin zymographic examination. The supernatant liquids from each lung homogenate were electrophoresed on 10% zymogram (gelatin) gels (Invitrogen; Carlsbad, California). Proteolysis bands were visualized and quantified using Printgraph (ATTO Corporation; Tokyo, Japan).

**Statistical Analysis**

A software package (SPSS 15.0; SPSS Inc; Chicago, Illinois) was used to perform all statistical analyses. Continuous data were expressed as mean ± SD. Any differences among the four groups were evaluated by one-way analysis of variance. A value < .05 was considered statistically significance.

**Results**

**Morphologic Findings**

Intraperitoneal injection of CSE in rats caused emphysematous destruction of the lungs within 3 weeks. Compared with the normal alveolar architecture in the control group (Fig 1A,a), once-a-week injection of CSE for 3 weeks induced enlargement of alveolar airspaces and destruction of lung parenchyma in the CSE group (Fig 1A,b). The MLI and DI were significantly increased in the CSE group compared with the values in the control group (Fig 1B) (MLI: 106.6 ± 8.4 µm vs 69.8 ± 6.6 µm, P < .001; and DI: 67.6% ± 15.6% vs 13.9% ± 2.7%, P < .0001). As expected, CMC significantly reduced airspace enlargement and parenchyma destruction in the rats injected with CSE (Fig 1A,c,d). The DI was significantly reduced, from 67.6% ± 15.6% in CSE-injected rats to 38.1% ± 16.5% (P < .005) by administration of 125 mg/kg/d of CMC and to 36.6% ± 16.4% (P < .005) by administration of 250 mg/kg/d of CMC. The MLI was significantly reduced, from 106.6 ± 8.4 µm in CSE-injected
rats to 84.9 ± 9.7 µm ($P < .001$) by administration of 250 mg/kg/d of CMC (Fig 1B). However, the MLI and DI in the CMC-treatment groups did not completely recover to the levels in the control group ($P < .05$), indicating that CMC incompletely protected against the emphysematous morphologic changes in rats injected with CSE. There were no significant differences in the MLI and DI between the two CMC-treatment groups ($P > .05$).

**Figure 1**  
A, Histologic examination of rat lung stained with hematoxylin and eosin (HE) showed normal alveolar architecture in (a) the control group, (b) airspace enlargement and alveolar destruction in the CSE group, and (c) improvement of airspace enlargement and alveolar destruction in the CSE + CMC (L) group and in (d) the CSE + CMC (H) group (original magnification ×100). B, Morphometric measurements of MLI and DI in lung tissues stained with HE as described in the text. * = $P < .001$ vs the control group; # = $P < .005$ vs the CSE group; ‡ = $P < .05$ vs the control group. CMC = carboxymethylcysteine; CSE = cigarette smoke extract; CSE + CMC (H) = cigarette smoke extract + high-dose carboxymethylcysteine; CSE = CMC (L) = cigarette smoke extract + low-dose carboxymethylcysteine; DI = destructive index; MLI = mean linear intercept.

**Effect of CMC on Antioxidant Activity in Pulmonary Level and Systemic Level in Rats With Emphysema Induced by CSE**

The biologic antioxidant activity was protected in the CSE-injected rats by the administration of CMC (Fig 2). The antioxidant activity in lung homogenates was significantly reduced in the rats injected with CSE (4,415.1 ± 417.4 µmol/L, $P < .05$) compared with that in the control rats (4,884.1 ± 462.1 µmol/L). However, CMC treatment significantly prevented this reduction (CSE + CMC [L] group: 4,800.1 ± 388.8 µmol/L, $P < .05$; and CSE + CMC [H] group: 4,881.9 ± 305.6 µmol/L, $P < .01$, vs the CSE group). The BAP in the lung in the two CMC-treatment groups was completely recovered to the same level as in the control group. There was no significant difference in the pulmonary BAP activity between the two CMC-treatment groups.
In parallel with the status of the antioxidant activity in lung homogenates, the antioxidant activity in the serum was also significantly decreased in the CSE-treated rats (1,725.6 ± 498.7 µmol/L, \( P < .01 \)) compared with that in the control rats (2,438.9 ± 235.6 µmol/L). Expectedly, CMC treatment significantly prevented this reduction (CSE + CMC [L] group: 2,557.4 ± 668.9 µmol/L, \( P < .05 \); and CSE + CMC [H] group: 2,695.2 ± 397.7 µmol/L, \( P < .05 \), vs the CSE group). There was no significant difference in the serum antioxidant activity between the CMC-treatment groups and the control group, indicating that the serum antioxidant activity was completely protected in the emphysematous rats by the administration of CMC.

**Effect of CMC on Apoptosis in Lungs of Rats With Emphysema Induced by CSE**

CMC inhibited pathologic apoptosis in the emphysematous lungs induced by CSE in rats (Fig 3). The TUNEL+ cells were frequently localized in the peribronchiolar, intraalveolar, and septal structures in the lungs of CSE-challenged rats (Fig 3A,b). However, the number of TUNEL+ cells in the emphysematous lungs was markedly reduced by the administration of CMC (Fig 3A,c,d), compared with that in CSE-challenged rats (Fig 3A,b). The protective effect of CMC on apoptosis was evaluated using the AI, which quantitatively identified the number of DNA-damaged alveolar cells, namely, TUNEL+ cells in the lung parenchyma of rats (Fig 3B). The AI was 2.9% ± 0.9% in the control rats, whereas it significantly increased to 20.3% ± 1.3% in the CSE-injected rats (\( P < .0001 \)). CMC significantly hampered the AI increase in the emphysematous lungs, with results of 8.7% ± 3.8% in the CSE + CMC (L) group (\( P < .0001 \), vs the CSE group) and 6.9% ± 2.1% in the CSE + CMC (H) group (\( P < .0001 \), vs the CSE group). However, the AI in the two CMC-treatment groups was still significantly higher than that in the control group (\( P < .05 \)), suggesting that CMC treatment did not provide complete protection against apoptosis in the emphysematous parenchyma induced by CSE in rats.
A, Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) stain to detect apoptotic nuclei showed occasional distribution of TUNEL-positive (TUNEL+) cells in the septal structure of the lungs in (a) control rats, (b) frequent localization of TUNEL+ cells in the peribronchiolar, intraalveolar, and septal structures in the lungs of CSE-challenged rats, and (c) marked reductions of the TUNEL+ cells in the emphysematous lungs of the CSE + CMC (L) group and (d) the CSE + CMC (H) group (original magnification ×400). B, AI was calculated as described in the text. * = P < .0001 vs the control group; # = P < .0001 vs the CSE group; ‡ = P < .05 vs the control group. AI = apoptotic index. See Figure 1 legend for expansion of other abbreviations.

In parallel with the AI results, when compared with the stained cells distributed occasionally in the normal lungs of the control rats (Fig 4A), the alveolar septal cells immunostained with caspase-3, a marker of apoptosis, were abundantly distributed in the emphysematous parenchyma of rats injected with CSE (Fig 4B). The number of caspase-3-stained cells was largely lessened in the CSE-injected rats after administration of CMC (Figs 4C, 4D). The amount of capase-3-stained cells in the CMC-treatment groups was visually equivalent to that in the lungs of the control rats.
Figure 4 Immunohistochemical examination to detect activated caspase-3 showed occasional staining of activated caspase-3 in the alveolar septa. A, In control rats. B, Abundant staining in the emphysematous parenchyma in rats injected with CSE. C, Sporadic staining in the lungs of the CSE + CMC (L) group. D, Sporadic staining in the lungs of the CSE + CMC (H) group (original magnification ×1,000). See Figure 1 legend for expansion of abbreviations.

Effect of CMC on MMP-2 and MMP-9 Activities in Lungs of Rats With Emphysema Induced by CSE

CMC significantly inhibited the increases of MMP-2 and MMP-9 activities in the emphysematous lungs induced by CSE (Fig 5A). Relative to 100% of the density of MMP-2 and MMP-9 in the control group displayed on gelatin zymographic examination (Fig 5B), the density of MMP-2 increased to 112.5% ± 10.2% (P = .092), and the density of MMP-9 increased to 114.1% ± 7.4% (P < .05) in the CSE group. Whereas CMC significantly inhibited such increases of MMP-2 and MMP-9 activities, resulting in 84.6% ± 6.3% of MMP-2 activity (P < .01) and 85.0% ± 14.5% of MMP-9 activity (P < .01) in the CSE + CMC (L) group and 82.7% ± 11.6% of MMP-2 activity (P < .01) and 62.5% ± 7.4% of MMP-9 activity (P < .001) in the CSE + CMC (H) group.

Figure 5 A, Gelatin zymographic findings on the expression of MMP-2 and MMP-9 activities showed enhanced MMP-9 and MMP-2 activities in lung tissue in rats injected with CSE and reduced MMP-9 and MMP-2 activities in both the CSE + CMC
COPD. Systemic delivery of CSE in rats produced an excess of oxidant substrates and reduced the biologic antioxidant activity in both the pulmonary and systemic levels and inhibited pathologic apoptosis and MMP-2 and MMP-9 activities in emphysematous lungs of rats. It is highly suggested that CMC protected against CSE-induced emphysema in rats through the molecular mechanisms of inhibition of apoptosis and MMP-2 and MMP-9 activities in emphysematous lung parenchyma, which was realized by stabilizing biologic antioxidant activity locally in the lung and systemically in the circulating blood, and that these results were achieved by the administration of CMC.

Cigarette smoke is the most important source of noxious and oxidant-radical-rich gas, which represents a major risk factor for COPD. Systemic delivery of CSE in rats produced an excess of oxidant substrates and reduced the biologic antioxidant activity in both the lung and serum. Excessive levels of oxidants result in altered cell signaling, leading to injuries in structural proteins, lipids, and DNA, triggering apoptosis. The cells undergoing apoptosis enhance oxidative stress in a vicious circle as well. Simultaneously, oxidants derived from cigarette smoke inactivate antiproteinases (particularly α1-antitrypsin), resulting in uncontrolled elastase activity and pulmonary tissue destruction by direct toxicity to protein and lipid structures. The enhanced elastase activity promotes the conversion of endogenous pulmonary xanthine dehydrogenase to xanthine oxidase, which acts on purine substrates and molecular oxygen to produce superoxide. Such loops of pathophysiologic molecular pathways eventually brought about the emphysema in the rats injected with CSE in the present study, in which the biologic antioxidant activity in both the lung and serum was reduced, apoptosis in the pulmonary parenchyma was amplified, and MMP-2 and MMP-9 activities were augmented. Therefore, the strategy to antagonize oxidative stress may avert or ameliorate the robust destructive cascade that contributes to emphysema.

CMC acts as a scavenger of hypochlorous acid and hydroxyl radicals by oxidation of its thioether group and the subsequent generation of sulfoxide. It scavenges ROS in vitro and has a protective effect on human respiratory cells during oxidative stress. CMC activates Akt phosphorylation, which inhibits the activation of caspase-3 and caspase-9 induced by hydrogen peroxide, inhibiting oxidant-induced apoptosis in cultured human airway epithelial cells. Moreover, the scavenger capacity of CMC on hypochlorous acid is paralleled by its protection of α1-antitrypsin, which is a major protease inhibitor that controls the overactivity of proteolytic elastase to protect against the alveolar destruction observed in chronic emphysema. With such knowledge and information about CMC, it is thinkable that the protective effectiveness of CMC against emphysema is realized by protecting the antioxidant activity in the lung in priority, which then, in cascade, inhibits apoptosis and proteases in the lung parenchyma.

Currently, CMC remains a well-tolerated mucolytic drug for the reduction of the exacerbation rate of COPD, with a favorable safety profile. The exceptional antioxidant activity of CMC has been consistently reported in vitro studies elsewhere. Hypothetically, therefore, CMC may offer a potential way to modify oxidative stress in the pathogenesis of emphysema, beyond aiding in sputum clearance. Our results provided suggestive evidence that the available mucolytic CMC may be a proper candidate drug in therapeutic regiments for the protection of antioxidant activity in patients with emphysema and/or COPD, apart from its clinical use for sputum clearance in the treatment of airway diseases.

In conclusion, this study demonstrated that CMC protected against the development of CSE-induced emphysema in rats, probably as the result of its antioxidant action, which was achieved by the inhibition of apoptosis and the reversal of the imbalance between proteolytic and antiproteolytic enzyme activities in emphysematous lung tissues. Further studies are required to investigate the relative clinical importance of the antioxidant action of CMC in the treatment of emphysema and COPD and the optimum dosing regimen for exploiting its antioxidant activity.

**Discussion**

The most remarkable finding of this study is that CMC, characterized by its antioxidant property in addition to its mucolytic activity, defended against the development of emphysema induced by systemic injection of CSE in rats. CMC significantly protected antioxidant activity in both the pulmonary and systemic levels and inhibited pathologic apoptosis and MMP-2 and MMP-9 activities in emphysematous lungs of rats. It is highly suggested that CMC protected against CSE-induced emphysema in rats through the molecular mechanisms of inhibition of apoptosis and MMP-2 and MMP-9 activities in emphysematous lung parenchyma, which was realized by stabilizing biologic antioxidant activity locally in the lung and systemically in the circulating blood, and that these results were achieved by the administration of CMC.

CMC is a mucoactive agent primarily prescribed for long-term COPD treatment. The structure and mechanism of action of CMC differs from that of other commonly available mucolytic drugs such as N-acetylcysteine, which bear free sulphydryl (thiol) groups via which they split glycoprotein bonds in mucus and also play an antioxidant role in the treatment of emphysema. In contrast, CMC does not carry a free thiol group, but its thioether group can be oxidized by ROS to form the sulfoxide by-product. The publication of several trials demonstrating the positive effects of CMC on the exacerbation frequency of COPD and quality of life of patients evidenced that this drug, commonly used for its mucoactive property, has diverse mechanisms of action, including significant antioxidant activity. The present study evidenced that administration of CMC to CSE-injected rats prevented emphysematous morphologic changes in rats, with inhibition of pathologic cellular apoptosis and attenuation of MMP-2 and MMP-9 proteinase activities. The mechanism is not yet fully understood, however, the antioxidant action of CMC, especially on the pulmonary level, is likely to be the active agent in the attack on the pathogenetic cascade of emphysema.

**Figure 1**

(L) group and the CSE + CMC (H) group. B, Relative density of the bands of MMP-2 and MMP-9 on zymographic examination. * = P < .05, vs the control group; # = P < .01, vs the CSE group. MMP = matrix metalloproteinase. See Figure 1 legend for expansion of other abbreviations.
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Dr Droma: contributed to the acquisition, analysis, and interpretation of the data; and drafted the submitted manuscript.

Dr Chen: contributed to the laboratory work, acquisition of the data, and revision of the manuscript.

Dr Agatsuma: contributed to the laboratory work, acquisition of the data, and revision of the manuscript.

Dr Kitaguchi: contributed to the laboratory work, acquisition of the data and revision of the manuscript.

Dr Voelkel: contributed to interpretation of the data and critically revised the manuscript.

Dr Kubo: contributed to the supervision of the study and revision of the manuscript.

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