

Comparison of Serum Adiponectin in Smoke-induced Pulmonary Emphysema Rats Fed Different Diets

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Abstract

Background: Smoking and body mass index (BMI) are the key risk factors for chronic obstructive pulmonary disease (COPD). Adiponectin with both anti-inflammatory and pro-inflammatory properties is a vital modulator of inflammatory processes, which is expressed in epithelial cells in the airway in COPD-emphysema. The aim of this study was to examine the effects of adiponectin on tobacco smoke-induced emphysema in rats, which were fed different diets.

Methods: Seventy-six adult (6–8 weeks old) male Sprague-Dawley rats (average weight 220 ± 20 g) were exposed to smoke or smoke-free room atmosphere and fed different diets (regular, high-fat, or low-fat diets) for 6 months. The rats were randomly divided into six groups. They are nonsmoke-exposed regular diet ($n = 10$), nonsmoke-exposed high-fat diet ($n = 14$), nonsmoke-exposed low-fat diet ($n = 14$), smoke-exposed regular diet ($n = 10$), smoke-exposed high-fat diet ($n = 14$), and smoke-exposed low-fat diet groups ($n = 14$). A full 2^3 factorial design was used to evaluate the effect of independent variables on smoke exposure and different rearing methods. Serum adiponectin and inflammatory cytokines were measured by the enzyme-linked immunosorbent assay (ELISA).

Results: Serum adiponectin levels in rats fed low-fat and regular diets exposed to smoke exposure were remarkably higher than that of rats exposed to room air while serum adiponectin levels of fat-rich diet rats exposed to tobacco smoke were lower than that of rats exposed to room air. Compared with regular diet or low-fat diet group, serum adiponectin levels in high-fat diet rats exposed to tobacco smoke were lower ($t = 6.932, 11.026$; all $P < 0.001$). BMI was inversely correlated with serum adiponectin levels ($r = -0.751, P = 0.012$). Serum interleukin 6 (IL-6), tumor necrosis factor- α (TNF- α), and 4-hydroxy 2-nonenal (HNE) levels in rats exposed to low-fat or fat-rich diets were remarkably higher than that of rats exposed to normal diets (IL-6, $t = 4.196, 3.480$; $P < 0.01, P = 0.001$; TNF- $\alpha, t = 4.286, 3.521$; $P < 0.01, P = 0.001$; 4-HNE, $t = 4.298, 4.316$; all $P < 0.001$). In nonhigh-fat diet rats exposed to tobacco smoke, serum adiponectin levels correlated positively with serum IL-6, TNF- α , and 4-HNE, bronchoalveolar lavage cell count, and mean linear intercept. In contrast, in high-fat diet rats, serum adiponectin levels correlated inversely with these parameters.

Conclusions: In smoke-induced emphysema and fat-rich diet rat model, serum adiponectin level was decreased, and the anti-inflammatory effect was attenuated. By contrast, nonhigh-fat diet elevated serum adiponectin and enhanced the role of pro-inflammatory.

Key words: Adiponectin; Diet; Pulmonary Emphysema; Smoke

INTRODUCTION

Chronic bronchitis and emphysema are the main pathological characteristics of chronic obstructive pulmonary disease (COPD).^[1] Smoking is an important risk factor for the development and progression of COPD, which triggers the inflammation and oxidative stress, and interrupts the balance between protease and antiprotease. In addition, body mass index (BMI) is another risk factor for COPD. Guerra *et al.*^[2] revealed a U-shaped pattern of risk between BMI and COPD in a longitudinal cohort

study. They demonstrated that both lower and higher BMI increased the risk of COPD. Lower body weight is associated with emphysema while obesity is associated with

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Received: 06-09-2015 **Edited by:** Li-Min Chen

How to cite this article: Wang RY, Liu H, Ma LJ, Xu JY. Comparison of Serum Adiponectin in Smoke-induced Pulmonary Emphysema Rats Fed Different Diets. *Chin Med J* 2016;129:187-93.

Access this article online

Quick Response Code:



Website:
www.cmj.org

DOI:
10.4103/0366-6999.173486

chronic bronchitis.^[2,3] However, the underlying mechanisms are still largely unknown.

Adiponectin is secreted by adipocytes which exist as oligomers, including trimeric (low molecular weight), hexameric (middle molecular weight), and high molecular weight (HMW) complexes and globular adiponectin. A previous study^[4] suggested that serum adiponectin levels were negatively correlated with BMI, means the serum adiponectin levels are lower in obesity, while it is higher in underweight patients. Carolan *et al.*^[5] reported that adiponectin played a role in the development of emphysema. Interestingly, low levels of total adiponectin are present in smokers without emphysema^[6] while high levels of adiponectin are observed in emphysema patients.^[7] Summer *et al.*^[8] reported that genetically induced adiponectin gene-deficient mice showed an increase in tumor necrosis factor (TNF)- α and matrix metalloproteinases in alveolar macrophages. These mice also contain enlarged alveolar spaces, which are a typical characteristic of emphysema. In addition, Nakanishi *et al.*^[9] reported that adiponectin supplementation significantly improved the symptoms of COPD in mice. They found that adiponectin inhibited the development of emphysema. When mice were additionally exposed to tobacco smoke, they showed no sign of increased lung inflammation or enlarged air spaces, indicating that adiponectin exhibits the anti-inflammatory effect.^[10]

Smoking and diet could have a profound impact on COPD.^[11,12] Previous studies of adiponectin were focused on the single factor such as weight or smoking in the etiology of emphysema. We explored the role of adiponectin on tobacco smoke-induced emphysema in rats fed different diets.

METHODS

Animal studies

This study was approved by the Animal Welfare and Ethics Committee of our university. Seventy-six adult (6–8 weeks old) male Sprague-Dawley rats (average weight 220 ± 20 g) were kindly provided by the Laboratory Animal Center of Shanxi Medical University in China. They were randomly divided into six groups based on exposure to smoke (smoke or room-air exposed) and different diets (regular, high-fat, and low-fat). They are nonsmoke exposed regular diet ($n = 10$), nonsmoke exposed high-fat diet ($n = 14$), nonsmoke exposed low-fat diet ($n = 14$), smoke-exposed regular diet ($n = 10$), smoke-exposed high-fat diet ($n = 14$), and smoke-exposed low-fat diet groups ($n = 14$).

In smoke-exposed group, rats were exposed to tobacco smoke for 6 months after adapting to conditions for approximately 1 week. Rats were subjected to chronic tobacco smoke environment (15 cigarettes/each time, twice per day, and 6 days/week). One cigarette contains 11 mg tar and 0.9 mg nicotine. The cigarettes were purchased from Anyang Cigarette Factory in Henan Province of China. Nonsmoke-exposed mice were placed under room atmosphere without smoke. Regular diet

group included rats fed a standard diet (10% calories from fat, D12450B, $10 \text{ g} \cdot 100 \text{ g}^{-1} \cdot \text{d}^{-1}$); high-fat diet group included rats treated with fat-rich diet (45% calories from fat, D12451, $10 \text{ g} \cdot 100 \text{ g}^{-1} \cdot \text{d}^{-1}$); and low-fat group was fed minimal fat-containing diet (10% calories from fat, D12450B, $6 \text{ g} \cdot 100 \text{ g}^{-1} \cdot \text{d}^{-1}$). Fodder was purchased from Guangdong Medical Laboratory Animal Center of China. Rats were housed in plastic cages, maintained under standardized conditions of light (12/12-h light/dark cycle) and room temperature ($20\text{--}25^\circ\text{C}$). Water was available arbitrarily. All animal handling procedures and experiments were performed in accordance with established protocols. Experiments were performed at the Experimental Animal Center of Shanxi Medical University.

Measurement of body mass and body length

The body mass and body length (from the tip of the nose to anus) of rats were measured at the end of 6 months of feeding period. $\text{BMI} (\text{kg}/\text{m}^2) = \text{body mass}/\text{body length}^2$.

For the determination of serum adiponectin, interleukin 6 (IL-6), tumor necrosis factor- α (TNF- α) and 4-hydroxy 2-nonenal (HNE), 5 ml of blood sample was drawn from abdominal aorta. Blood samples for serum collection were immediately centrifuged at 3000 r/min for 15 min and aliquots were stored at -80°C . The concentrations of serum adiponectin (R and D Systems; Minneapolis, USA), IL-6 (Blue Gene; Shanghai, China), TNF- α (Blue Gene; Shanghai, China), and 4-HNE (Blue Gene; Shanghai, China) were determined by enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturers.

Cell counting and classification of bronchoalveolar lavage

Bronchoalveolar lavage (BAL) fluid was collected by lavaging the lung with 2.5 ml of saline (37°C), which was repeated on 5 occasions via a tracheal catheter. After clipping the right main trachea, it was repeated once to ensure that the recovery of BAL fluid was more than 80%. BAL fluid was centrifuged at 1500 r/min for 10 min at 4°C , and the cell pellet was re-suspended in 1 ml of Hank's medium. The cell number in BAL fluid was counted under an inverted microscope (Leica Micro-systems Wetzlar GmbH, Germany). Then, BAL fluid was centrifuged, and the supernatant was discarded. To perform differential cell count, cellular slime was smeared onto slides using a cytospin ($500 \text{ r}/\text{min}$ for 5 min) and air-dried. Slides were stained with Wright-Giemsa, and differential cell counts (macrophages and neutrophils) were performed under a light microscope. Two-hundred cells were counted to calculate the macrophage and neutrophil proportions.

Lung histology

Lungs in the different groups of rats were equally inflated with the same volume of 4% paraformaldehyde solution to preserve the pulmonary architecture. Inflated right lungs were fixed for 48 h before lungs were embedded

in paraffin. The extent of emphysema was assessed by calculating the mean linear intercept (MLI) and mean alveoli number (MAN) in lung sections stained with hematoxylin and eosin. MLI and MAN were quantitated using a light microscope (Leica DMLS, Solms, Germany) attached to an image analysis system (Image-Pro Plus, Chengdu Thai Union Technology Limited Company, China). A cross line was drawn at the center of each field, the number of alveolar septa (NS) intersected the cross lines and the number of alveoli (Na) in each field were counted, and the length of the cross-hairs (L) and the area of each field (S) were measured. Finally, MLI and MAN were calculated as follows: $MLI = L/NS$, representing the average diameter of the alveoli, and $MAN = Na/S$, reflecting the alveolar density. MLI and MAN were quantitated for each rat in 2 sections, with three random fields per section and avoiding large vessels and bronchi ($\times 200$ magnification).

Statistical analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used to process data. All quantitative data were presented as mean \pm standard deviation (SD). The analyses were performed using factorial design analysis of variance. The significant correlations were determined using the Pearson test. The difference was considered statistically significant at $P < 0.05$.

RESULTS

The statistics analysis of the detection index met the homogeneity of variance ($P > 0.1$). Smoke exposure and feeding methods only had an interactive effect on the level of serum adiponectin in rats ($F = 10.327$, $P < 0.001$). However, single smoking or feeding factors showed a significant impact on all detection indices ($P < 0.01$).

Effects of tobacco smoke and diet on body mass index of rats

Irrespective of smoke exposure, compared with regular diet group (6.54 ± 0.27), BMI was significantly up-regulated in the high-fat group (7.53 ± 0.45 , $P < 0.01$), but down-regulated in low-fat groups (5.81 ± 0.48 , $P < 0.01$).

Compared with the groups, in the room environment, BMI was significantly lower in groups exposed to smoke ($F = 15.591$, $P < 0.01$).

Effects of tobacco smoke and diet on serum adiponectin level

Smoke exposure or feeding methods had significant effect on the serum adiponectin levels ($P < 0.05$). Smoke exposure and feeding methods had an interactive effect on the level of serum adiponectin in rats ($F = 10.327$, $P < 0.001$).

In the rats, under normal air environment, serum adiponectin levels were 7.80 ± 1.14 $\mu\text{g/ml}$ (low-fat diet), 6.57 ± 1.15 $\mu\text{g/ml}$ (regular diet), and 5.49 ± 0.71 $\mu\text{g/ml}$ (high-fat diet). In the rats exposed to tobacco smoke, serum adiponectin levels were 9.31 ± 1.25 $\mu\text{g/ml}$ (low-fat diet),

8.14 ± 1.17 $\mu\text{g/ml}$ (regular diet), and 4.40 ± 0.78 $\mu\text{g/ml}$ (high-fat diet). Compared with regular diet or low-fat diet group, serum adiponectin levels in high-fat diet rats exposed to tobacco smoke were lower ($t = 6.932$, 11.026 ; all $P < 0.001$). Serum adiponectin levels in rats fed low-fat and regular diets exposed to smoke exposure were remarkably higher than that of rats exposed to room air, while serum adiponectin levels in high-fat diet rats exposed to tobacco smoke were lower than that of rats exposed to room air.

In brief, smoking exposure affected the level of serum adiponectin in rats; along with the increase in dietary fat content, the level of serum adiponectin was gradually decreased. It is highest in the low-fat diet rats exposed to tobacco smoke and lowest in the high-fat diet rats exposed to tobacco smoke [Table 1 and Figure 1].

Effects of tobacco smoke and diet on systemic inflammation

Smoke exposure and feeding methods showed no interactive effect on the level of IL-6, TNF- α , and 4-HNE ($P > 0.05$). However, single factor either smoke exposure or feeding methods had significant effect on these inflammatory cytokines. (IL-6, $F = 40.150$, 9.115 ; TNF- α , $F = 58.096$, 9.378 ; 4-HNE, $F = 29.961$, 11.317 ; all $P < 0.001$).

Compared with the regular diet group, the levels of IL-6, TNF- α , and 4-HNE in rats fed low-fat diet or high-fat diet were remarkably higher (IL-6, $t = 4.196$, 3.480 ; TNF- α , $t = 4.286$, 3.521 ; 4-HNE, $t = 4.298$, 4.316 ; all $P < 0.01$). However, there was no significant difference between the low-fat diet group and the high-fat diet group ($P > 0.1$).

Effects of tobacco smoke and diet on bronchoalveolar lavage inflammation

Compared with the regular diet group, rats fed low-fat or high-fat diet exhibited a significant up-regulation in total BAL cells, BAL macrophages, and BAL neutrophils ($t = 4.561$, 2.798 ; $P < 0.001$, $P = 0.007$). However, there was no significant difference between the low-fat and the high-fat dietary groups ($P > 0.05$).

Average diameter of alveoli in smoke- and diet-induced rats

The alveolar diameter of the smoking group was significantly increased. Regardless of smoke exposure, the levels of MLI in rats fed low- or high-fat diets were significantly higher than those in rats fed regular diets ($t = 6.863$, 3.715 ; $P < 0.001$, $P = 0.001$). The low-fat dietary group showed remarkably higher levels than the high-fat dietary group ($t = 3.503$, $P = 0.001$). MLI reflected the degree of emphysema.

Smoke- and diet-induced effects on mean alveolar numbers

The mean alveolar numbers of the smoking group were significantly decreased. MAN in rats fed low- or high-fat diets were remarkably lower than those in rats fed regular diets ($t = 4.491$, 2.259 ; $P < 0.001$, $P = 0.028$). The low-fat

diet group was remarkably lower than the high-fat diet group ($t = 2.683, P = 0.01$). MAN also reflected the extent of emphysema.

Altered lung pathology of rat models [Figure 2]

Correlation of adiponectin with other parameters

Serum adiponectin levels were inversely correlated with BMI in rat fed high-fat diets exposed to tobacco smoke ($r = -0.751, P < 0.05$).

In rats fed high-fat diets and exposed to tobacco smoke, serum adiponectin levels correlated inversely with serum IL-6, TNF- α , 4-HNE levels, BAL cell count, the percentage of macrophages, and MLI and correlated positively with MAN [Table 2].

In rats exposed to nonhigh-fat diet and tobacco smoke, serum adiponectin levels correlated positively with serum IL-6, TNF- α , 4-HNE levels, BAL cell count, the percentage

Diets	Smoking exposure				Total	Diets	
	Nonsmoking	<i>n</i>	Smoking	<i>n</i>		<i>F</i>	<i>P</i>
Low-diet	7.80 ± 1.14	14	9.31 ± 1.25	14	8.64 ± 1.39* [†]	61.415	<0.0001
Regular diet	6.57 ± 1.15	10	8.14 ± 1.17	10	7.35 ± 1.38 [‡]		
High-diet	5.49 ± 0.71	14	4.40 ± 0.78	14	4.97 ± 0.92		
Total	6.49 ± 1.36	38	7.22 ± 2.43	38			
Smoking exposure	<i>F</i>		5.498		Smoking exposure diet*	<i>F</i>	10.327
	<i>P</i>		0.023			<i>P</i>	<0.0001

*Compared with regular diet: $P < 0.01$; [†]Compared with high-diet: $P < 0.001$; [‡]Compared with high-diet: $P < 0.001$.

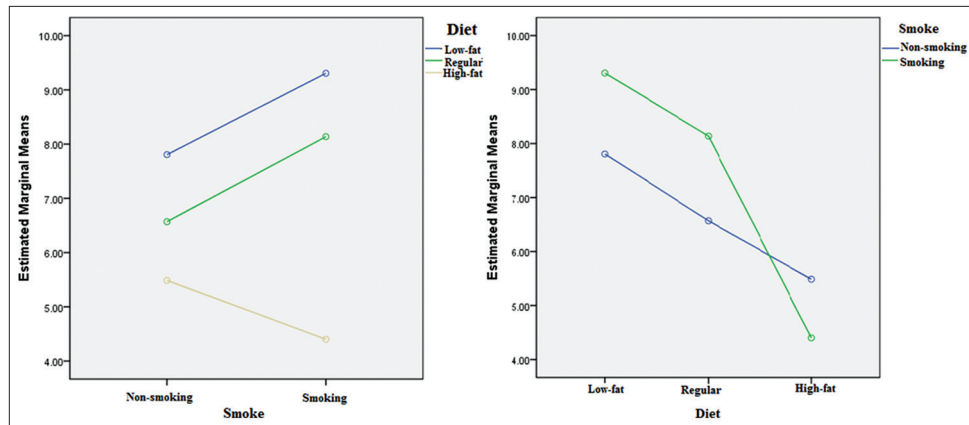


Figure 1: Profile plots of interactive effect about serum adiponectin level (smoke exposure and feeding methods had an interactive effect on the level of serum adiponectin in rats).

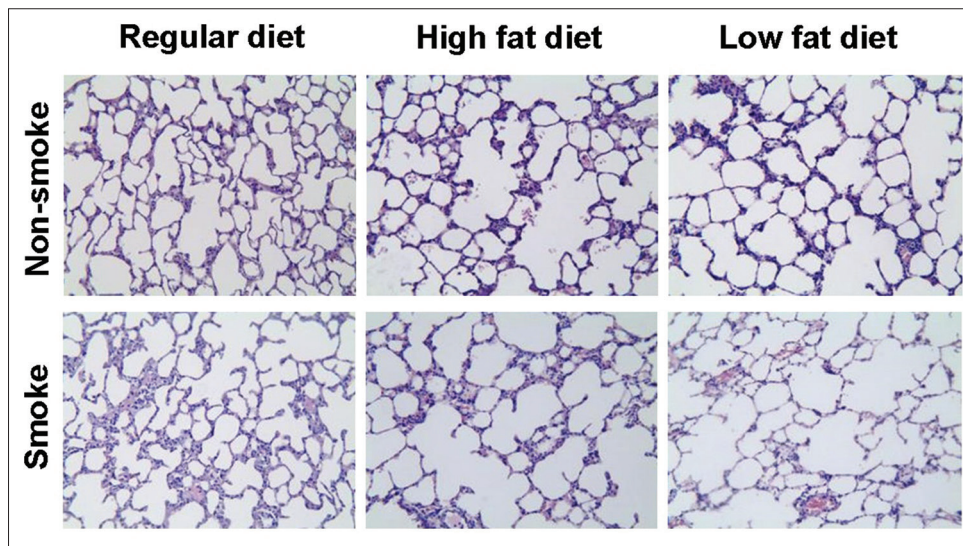


Figure 2: Pulmonary histology of rat exposed to different diets under smoking and nonsmoking environment. Smoke exposure can promote the formation of emphysema. The high- and low-fat diets intensified the pathological changes (H and E staining, original magnification $\times 200$).

of macrophages, and MLI and correlated negatively with MAN [Table 3].

DISCUSSION

Our results showed that a combination of diet and smoking aggravated emphysema in rat models. We found that either low- or high-fat diet affected serum adiponectin levels in smoke-induced pulmonary emphysema rats and BMI was inversely correlated with serum adiponectin levels in rats without smoke exposure. It may be caused by necrosis of adipocytes resulting from obesity and hypoxia.^[13] Necrotic adipocytes attract macrophages to collect and form syncytia around the adipocytes^[14] and produce TNF- α and IL-6, which in turn may inhibit the local production of adiponectin in a paracrine fashion.^[15] Consistently, this phenomenon was also reported in Arita *et al.*^[4] study.

Miller *et al.*^[15] reported that Wild type (WT) mice exposed to tobacco smoke for 6 months had significantly higher levels of BAL adiponectin, compared with mice that were not exposed to tobacco smoke. This result is consistent with our finding that serum adiponectin levels of rats exposed to low-fat and normal diet and tobacco smoke were higher than that of rats exposed to room air. It is perhaps because that rats with emphysema have an additional mediator (not adiponectin),

which can up-regulate adiponectin expression and counteract the inhibitory effect of tobacco smoke on suppressing adiponectin expression. In addition, we observed that serum adiponectin levels in rats exposed to high-fat diet and tobacco smoke were lower than that of rats exposed to room air. The cause is probably systemic oxidative stress induced by tobacco smoke, and high-fat diet results in aggravating the inhibitory effect of adiponectin generated, which leads to the decrease of adiponectin level further. However, the possible mechanisms remain to be investigated further.

In the present study, we observed that rats exposed to high-fat diets and tobacco smoke developed significant emphysema and decrease of serum adiponectin. Serum adiponectin is negatively correlated with systemic inflammatory response, oxidative stress, and the severity of emphysema, suggesting that adiponectin has anti-inflammatory effect. Our findings are consistent with previous studies from Summer *et al.*^[8] and Nakanishi *et al.*^[9] They reported that adiponectin may exert anti-inflammatory effects during the development of emphysema. The effect is possibly mediated by several pathways, including inhibiting production of TNF- α and inducing the generation of anti-inflammatory cytokines receptor antagonist, such as IL-10 and IL-1; as well as promoting the removal of early apoptotic cells by acting on macrophages.^[15,16] The serum adiponectin levels of high-fat diet rats in tobacco smoke environment are lower than that of rats in room air. Therefore, its anti-inflammatory, anti-apoptotic, and insulin-sensitizing effects were weakened, leading to the reduction of its protective effects on the cardiovascular system. This may explain the clinical phenomenon of the death observed in obese patients with COPD due to cardiovascular diseases in the USA.^[17,18]

Previously, Tomoda *et al.*^[19] reported that serum adiponectin levels in COPD patients with lower BMI and normal weight were significantly higher than those in control subjects. Similarly, in our animal experiments, we also found that serum adiponectin levels in tobacco smoke exposed rats fed low-fat or normal diets were remarkably elevated, indicating that adiponectin may be a pro-inflammatory mediator. We observed that rats exposed to tobacco smoke and low-fat diets showed increasingly significant emphysema than rats fed normal diets. However, serum adiponectin levels were elevated. The serum adiponectin levels were positively correlated with systemic inflammatory response, oxidative stress, and the severity of emphysema, suggesting that adiponectin plays a role in promoting the progression of inflammation. These findings are consistent with the report that adiponectin expressed by airway epithelial cells in COPD-emphysema elicited functional responses in the lung via autocrine^[20] or paracrine pathways.^[21] In A549 cells, a human alveolar epithelial cell line, Nigro *et al.*^[22] found that adiponectin increases apoptosis by regulating extracellular regulated protein kinases (ERK) 1/2 and Serine/threonine Kinase (AKT) pathways. These studies suggest that many COPD

Table 2: Pearson test between adiponectin and inflammatory factors (high-fat and smoke-exposed rats)

Factors	<i>r</i>	<i>P</i>
BMI	-0.751	0.012
TNF- α	-0.760	0.011
IL-6	-0.739	0.015
HNE	-0.685	0.029
Cells	-0.804	0.005
Macrophage	-0.750	0.012
MLI	-0.699	0.024
MAN	0.715	0.020

TNF- α : Tumor necrosis factor- α ; IL-6: Interleukin 6; BMI: Body mass index; HNE: 4-hydroxy 2-nonenal; MLI: Mean linear intercept; MAN: Mean alveoli number.

Table 3: Pearson test between adiponectin and inflammatory factors (nonhigh-fat and smoke-exposed rats)

Factors	<i>r</i>	<i>P</i>
BMI	-0.392	0.119
TNF- α	0.700	0.002
IL-6	0.694	0.002
HNE	0.519	0.033
Cells	0.535	0.027
Macrophage	0.806	<0.001
MLI	0.699	0.002
MAN	-0.667	0.003

TNF- α : Tumor necrosis factor- α ; IL-6: Interleukin 6; BMI: Body mass index; HNE: 4-hydroxy 2-nonenal; MLI: Mean linear intercept; MAN: Mean alveoli number.

patients suffering from malnutrition frequently died from respiratory failure induced by chronic hypoxia in Japan.^[23]

In summary, both high- and low-fat diets promote the formation of emphysema. Under experimental conditions, unfortunately, all rats with emphysema were not tested for pulmonary function. Therefore, we speculate that in the obese COPD patients, serum adiponectin level is decreased and the anti-inflammatory effect is weakened, which mainly affect the cardiovascular system. In the skinny COPD patients, serum adiponectin level is elevated and the pro-inflammatory effect is enhanced, which mainly affect the respiratory system. The mechanisms underlying the specific anti-inflammatory and pro-inflammatory roles of adiponectin are unknown. We hypothesized that it might be associated with the diverse effects of adiponectin under different internal environments via the formation of different oligomers. The oligomerization pattern of adiponectin is altered in COPD; the elevated levels of adiponectin are associated with a specific increase in HMW.^[24,25] Studies also show that HMW has anti-inflammatory effects^[26] while gAd has pro-inflammatory effects.^[27] Another limitation of our study relates to the absence of research involving oligomers and receptors of adiponectin.

In conclusion, smoke exposure and different feeding mode can promote the formation of emphysema. In fat-rich group, serum adiponectin level was decreased and the anti-inflammatory effect was weakened while in nonhigh-fat groups, serum adiponectin level was elevated and the pro-inflammatory effect was enhanced, laying the foundation for future experiments.

Financial support and sponsorship

This work was supported by a grant from National Natural Science Foundation of China (No. 81141041).

Conflicts of interest

There are no conflicts of interest.

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