Int J Clin Exp Med 2016;9(10):19213-19221
www.ijcem.com /ISSN:1940-5901/IJCEM0031440

Original Article

A simplified device for exposing rats to cigarette smoke

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Received April 29, 2016; Accepted August 6, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: Objective: This study aims to explore a simplified device for exposing rats to cigarette smoke. Methods: Twenty-four rats were randomized into the following four groups: Group A, B, C, and D, in which rats were exposed to the cigarette smoke produced by 5, 3, or 1 cigarette, respectively. The smoke concentration inside the device was measured with portable detectors and the cotinine level in the serum was measured using an enzyme-linked immunosorbent assay (ELISA) kit. In addition, the WBCs and differential cell counts in BALF were performed with Wright-Giemsa staining, while NQO-1 and HO-1 in carotid arteries were detected by Western blot analysis. Results: The smoke concentration inside the device, as well as the cotinine in rats, significantly increased depending on the numbers of cigarettes between groups ($P<0.01$). The WBCs mainly for macrophages in BALF and oxidant stress index (NQO-1 and HO-1) increased with the increasing of cigarettes between groups ($P<0.01$). Conclusion: The present device could be used for exposing rats to cigarette smoke with a low cost and easy to perform. More importantly, it is easy to change the smoke concentration by only altering the numbers of cigarettes used at a time.

Keywords: Device, cigarette smoke, cotinine, rats, NQO-1, HO-1

Introduction

Faced with the enormous disease burden associated with smoking, related issues have become a hot topic in research areas [1, 2]. To understand the various physiological and physicochemical processes associated with tobacco consumption, a number of experimental apparatuses aimed at exposing animals to cigarette smoke have been designed [3]. As such, there is a diversity of "whole smoke exposure" system available, ranging from commercial set-ups to bespoke machines [4, 5]. However, due to their high cost and technical demands, many existing devices are impractical for widespread use in research.

While previous studies have devised a variety of self-made apparatuses for exposing animals to cigarette smoke [6-10], there is currently no standard model used for this purpose. Furthermore, there are advantages and disadvantages to each apparatus. For example, while the device reported by Brito *et* al. [6] can be easily manufactured at a low cost, it was difficult to achieve homogeneous exposure of animals to cigarette smoke using this apparatus, as the animals typically clustered together when exposed to the smoke. Meanwhile, the device reported by Bretthauer *et* al. [9] enabled homogenous exposure, but could only be used to expose one rat at a time. Moreover, most studies related to smoking do not describe the methods used for exposure of animals to cigarette smoke. Likewise, the smoke concentration inside these devices is not always recorded, which makes it difficult to compare results between studies [11-14].

The purpose of this study was to develop a novel low cost device for exposing rats to cigarette smoke. Here, we provide a detailed description of the components, assembly, and technical use of the device. In addition, we provide validation for the use of this device by measuring the carbon monoxide (CO) and total particulate matter (TPM) concentration inside the device as well as the levels of inflammatory and antioxidant biomarkers in rats.

Methods

Animals and groups

Male 8-week-old Sprague-Dawley rats (weighting approximately 250 g) were purchased from

the Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). Rats were housed in a standard laboratory environment with ambient temperatures of 20-23°C, humidity of 43-45%, and a 12/12 h light/dark cycle. Animals had free access to standard solid claviform food and autoclaved tap water.

Twenty-four rats were acclimated for one week to the aforementioned environment and then randomized into the following four groups (6 rats per group): Group A, B, and C, containing rats exposed to the cigarette smoke produced by 5, 3, or 1 cigarettes at a time, respectively; Group D, in which rats were not exposed to cigarette smoke. The protocols used in this study were performed according to approved guidelines from the Animal Care Committee of Peking Union Medical College.

Construction of the device used for cigarette smoke exposure

The present device was comprised of five individual components, a glass chamber (length × height × width=58 cm × 42 cm × 36 cm), a metallic shelf (with a wire mesh on top of the shelf), a wire mesh fence, a cigarette holder, and a glass cover (**Figure 1**), and was assembled as follows: the metallic shelf (**Figure 1B**) and wire mesh fence (**Figure 1C**) were sequentially placed in the glass chamber (**Figure 1A**), and the rats were placed within independent grids on the metallic shelf. Subsequently, a cigarette was placed on the cigarette holder (**Figure 1D**) and lit, and the glass cover (**Figure 1E**) was placed on top of the wire mesh fence. Importantly, the bottom of the chamber was designed to include two air inlets (red arrows shown in **Figure 1A**), and an air outlet was enclosed with the cover and the top of the chamber (red arrows shown in **Figure 1F**). In this way, the smoke inside the device could rise vertically and accumulate up to peak concentrations as the cigarettes burned out.

Cigarette smoke exposure

The rats in Groups A, B, and C were exposed to the cigarette smoke produced by 5, 3, or 1 cigarettes at a time, respectively, using the device described above. Rats were exposed to the cigarette smoke for 1 h twice a day, for a total of seven days. When the first group of cigarettes burned out (approximately 10 min), another group of cigarettes was lit to allow for continual smoke production. This process was repeated until the end of each exposure. Meanwhile, the rats in Group D were placed in a similar device, but lacking cigarette smoke, for 1 h twice a day, for seven days. The cigarettes used in this study were Huangshan brand (12 mg tar, 14 mg carbon monoxide (CO), and 1.2 mg nicotine per cigarette).

Measurement of TPM and CO levels

TPM is often used to reflect the smoke concentration, especially for the concentration of the particulate phase. In the present study, the concentration of TPM inside the device was measured using an Aerosol Monitor (DUSTTRAK II-8530, TSI, USA). Meanwhile, the concentration of CO, one of the most important components of the vapor phase of cigarette smoke, was measured using a KP-826 Gas Detector (Henan Zhongan Electronic Detection Technology Co., LTD, Henan, China). After lighting the cigarette(s), measurements were taken once per second for the first minute and then once per minute during the remainder of the exposure.

Analyses of bronchoalveolar lavage fluid (BALF)

To examine the inflammatory response within the lungs of treated rats, total and differential cell counts (macrophages, neutrophils, and lymphocytes) in BALF were performed according to previous studies [15]. Briefly, rats were anesthetized by intraperitoneal injection with 10% chloral hydrate at 3 ml/kg. Tracheas were exposed, punctured with 14-gauge needles, clamped, and the lung was then lavaged 3 times each with 4 ml normal saline. The recovery rate for each lavage was 90%-95%. The total and differential cell counts in BALF were determined by globulimeter and Wright-Giemsa staining, respectively. The relative proportion of each cell type was determined morphologically by counting 300 cells/slide and then was factored to the number ($\times 10^6$/ml) of total BALF cells collected in each group.

Measurement of cotinine in the serum

After seven days of exposure, all rats were euthanized and blood samples were harvested by puncturing the abdominal aorta. Samples were incubated at room temperature for 2 h

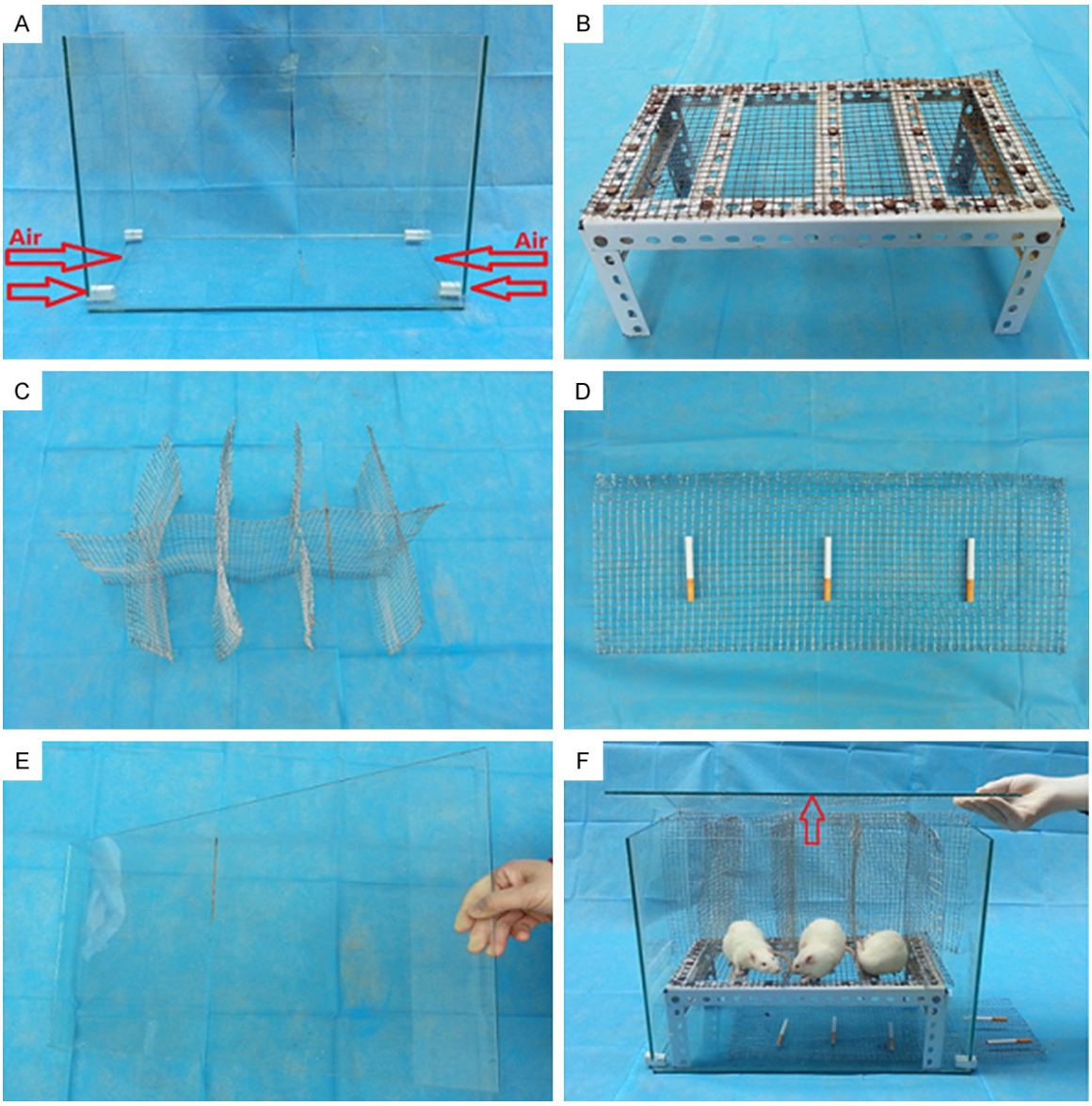

Figure 1. Components of the device utilized in this study. Letters "A-E" depict the individual components of the device, including the glass chamber (A), the metallic shelf (B), the wire mesh fence (C), the cigarette holder (D), and the glass lid (E). Letter "F" shows the assembled chamber containing rats within individual cells.

and centrifuged at 1,275 × *g* for 20 min. The serum was then collected and stored at -80°C prior to use. The concentration of cotinine in the serum was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Lot COT4596; Calbiotech, Spring Valley, CA, USA), according to the manufacturer's instruction.

Western's blot analysis of NOQ-1 and HO-1 expression levels

Total protein was extracted from the carotid arteries using lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol). Protein samples (20 μg each) were then separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were blocked in 5% skimmed milk in Tris-buffered saline + tween (TBST; 20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature, washed three times with TBST, and incubated with primary antibodies specific to NAD(P)H quinone oxidoreductase 1 (NQO-1; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and heme oxygenase-1 (HO-1) (1:500; BD Biosciences, Ltd., San Jose, CA, USA) over-

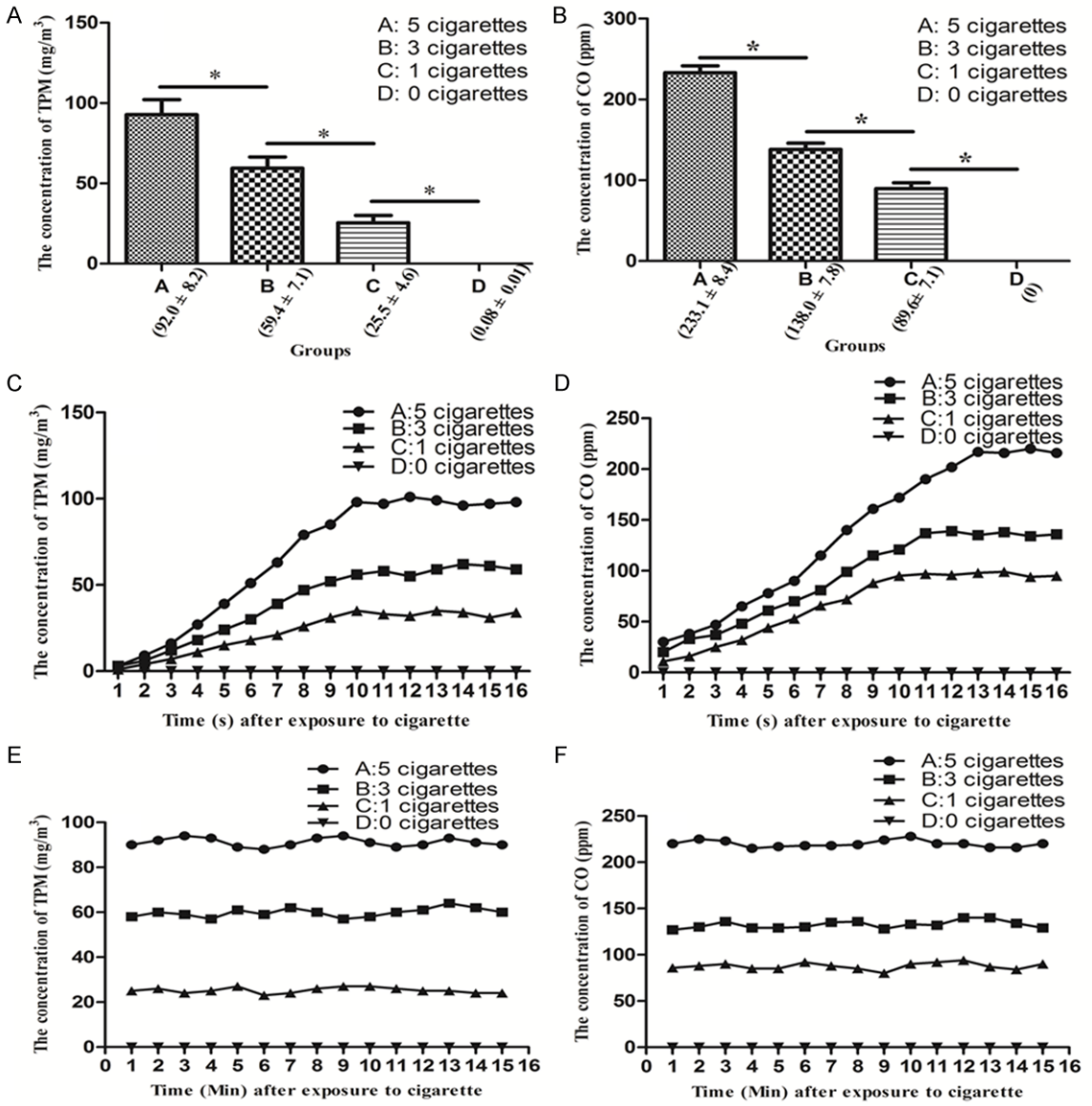

Figure 2. Graphic depiction of the total particulate matter (TPM) and carbon monoxide (CO) concentrations in each treatment group over the course of the experiment. *P<0.01.

night at 4°C. An antibody specific to GAPDH (glyceraldehyde 3-phosphate dehydrogenase; 1:5000; Proteintech, Chicago, IL, USA) was used as a loading control within each individual experiment. Following three washes, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse/anti-rabbit IgG (1:7000; Zhongshan Jinqiao Biotechnology Co., Ltd, Beijing, China) for 1 h at room temperature. Antigen-antibody complexes were detected by enhanced chemiluminescence. Images were obtained, and the gray values for each target protein were analyzed using an Alpha EaseFC system (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis

Continuous variables were expressed as means ± standard deviations (SD). Comparisons between groups were analyzed by one-way analysis of variance (ANOVA). All analysis was performed using SPSS 20.0 (SPSS Statistics Inc., Chicago, IL, USA). For all comparisons, P<0.05 was considered statistically significant.

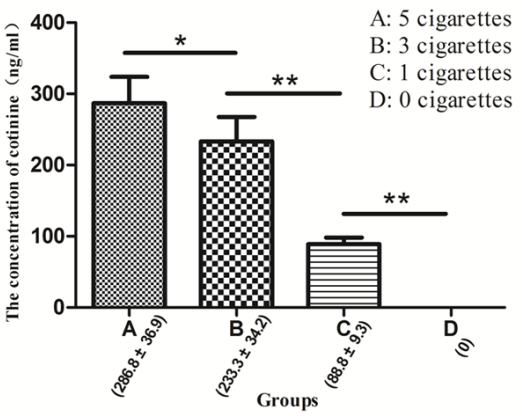

Figure 3. Graphic depiction of the serum concentrations of cotinine in the rats in each treatment group. **P*<0.05; ***P*<0.01. Data presented depict the average values from 6 biological replicates for each group.

Results

Analysis of the concentrations of TPM and CO in each treatment group

As shown in **Figure 2A**, the peak concentration of TPM increased depending on the numbers of cigarettes used with the values of TPM in group A, B, C, and D of 92.0 ± 8.2 mg/m^3; 59.4 ± 7.1 mg/m^3; 25.5 ± 4.6 mg/m^3; 0.08 ± 0.01 mg/m^3; respectively, (*P*<0.01). Similarly, as shown in **Figure 2B**, the peak concentration of CO elevated with the numbers of cigarettes used and its values in group A, B, C, and D was 233.1 ± 8.4 ppm; 138.0 ± 7.8 ppm; 89.6 ± 7.1 ppm; 0 ppm; respectively, (*P*<0.01). Notably, peak TPM and CO concentrations were achieved within only 12 s (**Figure 2C** and **2D**), and the peak value was stably maintained throughout the exposure process (**Figure 2E** and **2F**).

Analysis of the serum cotinine concentrations in the rats in each treatment group

As shown in **Figure 3**, there was a significant increase in the serum concentration of cotinine in rats exposed to cigarette smoke compared to that in the control group (*P*<0.01). Moreover, the serum concentration of cotinine increased depending on the number of cigarettes used (Group A=286.8 ± 36.9 ng/ml; Group B=233.3 ± 34.2 ng/ml; Group C=88.8 ± 9.3 ng/ml; respectively, *P*<0.05).

Total white blood cell (WBC) and differential cell count in the BALF of rats in each treatment group

There was a significant increase in the number of total WBCs (**Figure 4A**) and macrophages (**Figure 4B**) depending on the number of cigarettes to which the rats were exposed (*P*<0.05). Furthermore, differential count analyses indicated that macrophages were the primary inflammatory cells recruited to the lungs of rats upon smoke exposure. Meanwhile, there were no changes in the neutrophil (**Figure 4C**) and lymphocyte (**Figure 4D**) counts between treatment groups (P>0.05).

Analysis of the expression levels of NOQ-1 and HO-1 in the carotid artery of rats in each treatment group

As shown in **Figure 5**, there were marked increases in the expression levels of the antioxidant proteins HO-1 and NQO-1 in rats exposed to the smoke produced by one cigarette compared to that in the control group (*P*<0.01). In contrast, there was a significant decrease in the expression levels of these proteins in the rats exposed to the smoke produced by three or five cigarettes compared to the control group (*P*<0.01). However, no difference was detected in the expression levels of HO-1 and NQO-1 between Groups A and B (P>0.05).

Discussion

The development of a simplified, standard device for exposing rats to cigarette smoke would not only contribute to a better understanding of smoke toxicity and the mechanisms of smoke-related diseases, but would also facilitate comparisons of results between studies [16, 17]. The design of this smoke exposure device was inspired by the coal stoves that are often used to provide warmth during the winters in china, and the underlying theory behind the device was based on the “chimney effect” concept [18].

Notably, the function of the present device is not dependent on any electric machine, which not only contributes to energy conservation, but also avoids any impact on the experimental results due to the noise associated with mechanical devices. Notably, only approxi-

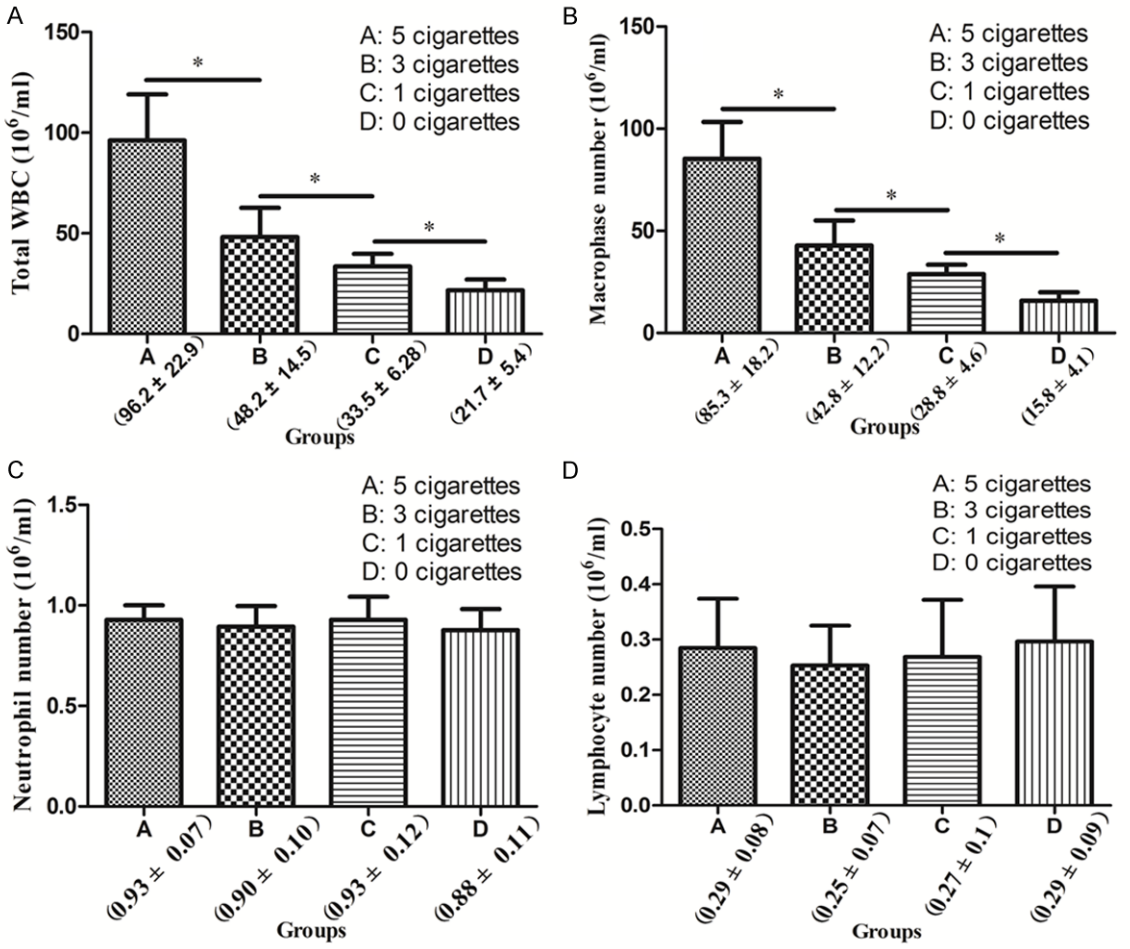

Figure 4. Total and differential cell counts in bronchoalveolar lavage fluid (BALF) of rats exposed to cigarette smoke. *P<0.05. Data presented depict the average values from 6 biological replicates for each group.

mately 12 s were needed to reach the peak concentration within the device after lighting the cigarettes. More importantly, however, the peak smoke concentration was stably maintained throughout the subsequent exposure process, even while changing cigarettes. Because the cigarette holder can easily be pulled out or pushed in through the air inlets when changing cigarettes, the smoke concentration inside the device was not diminished.

To keep the rats separate, we included a wire mesh fence within the device. Each grid was large enough to accommodate one rat, which ensured that all rats were homogeneously exposed to the cigarette smoke by preventing clustering of the animals. To verify the stability of present device, we measured the smoke concentration and responses of rats when exposed to different numbers of cigarettes.

The concentrations of CO and TPM are the most frequently used index for reflecting the concentration of the vapor phase and particulate phase, respectively. Florek *et al*. [19] reported a new mechanical machine with which they examined the severity of smoke exposure by only modulating the CO concentrations within the device. Another machine reported by Haussmann *et* al. [20] was used to expose rats to different TPM concentrations. However, there are several disadvantages to this machine, including a high cost and complicated procedures. Meanwhile, we observed distinct CO (233.1 ± 8.4 ppm for 5 cigarettes, 138.0 ± 7.8 ppm for 3 cigarettes, and 89.6 ± 7.1 ppm for 1 cigarette) and TPM (92.0 ± 8.2 mg/m^3 for 5 cigarettes, 59.4 ± 7.1 mg/m^3 for 3 cigarettes, and 25.5 ± 4.6 mg/m^3 for 1 cigarette) concentrations depending the number of cigarette used, and there were differences in

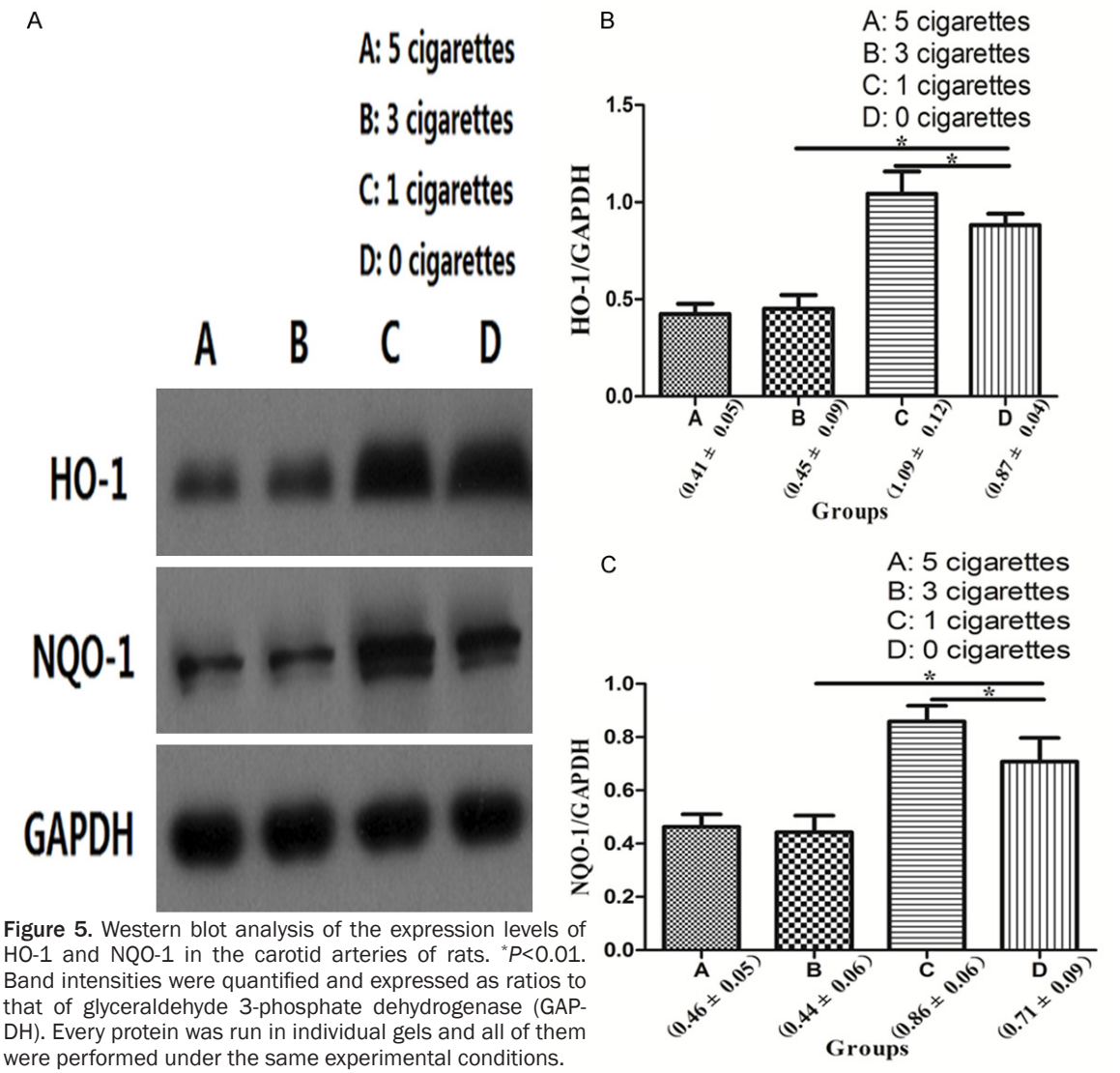

Figure 5. Western blot analysis of the expression levels of HO-1 and NQO-1 in the carotid arteries of rats. *P<0.01. Band intensities were quantified and expressed as ratios to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Every protein was run in individual gels and all of them were performed under the same experimental conditions.

the peak CO and TPM values among groups. These findings demonstrate that our device would enable researchers to easily modulate the exposure level of rats to cigarette smoke by altering the number of cigarettes used per treatment and would reflect the smoke concentration by the index of CO and TPM at the same time. In addition, to achieve distinct concentrations of CO and TPM, researchers could likely adjust the size of the air inlets or outlets on the device; however, confirmation of this hypothesis requires further study.

Due to its long half-life, cotinine, a metabolic product of nicotine in cigarette smoke, is one of the most commonly used biomarkers to reflect smoke exposure in animals and humans [21]. Based on the observed increase in the cotinine concentration in rats exposed to smoke, compared to that in the control group, we concluded that the rats had indeed inhaled the cigarette smoke. Moreover, the cotinine concentration significantly increased depending on the number of cigarettes to which the rats were exposed. While the cotinine concentrations observed here were different from those obtained in previous studies [8, 22], these discrepancies could likely be explained by differences in the method used to expose animals to smoke or in the duration of smoke exposure.

Previous studies have shown that oxidative stress is the primary mechanism through which cigarette smoke exposure leads to the development of various diseases [23, 24]. In our present study, there was a significant

increase in expression levels of the antioxidant proteins NQO-1 and HO-1 within the carotid arteries of rats exposed to the smoke produced by one cigarette compared to control group, which was in keeping with the results obtained in our previous study [25]. In contrast, there was a significant decrease in the expression levels of NQO-1 and HO-1 in rats exposed to the smoke produced by three and five cigarettes compared to control group, which was inconsistent with previous findings [26, 27]. The increased severity of smoke exposure in our present study may account for this difference; however, further studies are needed to confirm this conclusion.

Regarding the inflammatory response within the lungs of rats exposed to cigarette smoke, the total WBC count in the BALF of these animals increased with numbers of cigarettes used, and macrophages comprised the predominant inflammatory cell type. Furthermore, the magnitude of the increase of these parameters was smoke dose-dependent, which is consistent with the results of a previous study [28]. In contrast, another previous report indicated that neutrophils were the primary inflammatory cell type recruited to the lungs after smoke exposure [29]; however, we detected no significant differences in the number of neutrophils in the BALF samples harvested from distinct treatment groups. In our present study, the duration of smoke exposure was one week while the duration of exposure in previous studies ranged from 2 weeks to 36 weeks [28, 29]. As such, these differences in exposure lengths could explain the distinct results obtained in each study. However, further research is needed to confirm this possibility.

In summary, there are many advantages to our device. First, the severity of smoke exposure can readily be adjusted by altering the numbers of cigarettes used. Second, because no mechanical component was involved in the device, it avoids negative effects due to noise. More importantly, the present device could be made up with a low cost and easy to perform. In addition, our device could be utilized to expose a variety of other small animals, such as mice, to cigarette smoke. There were also some limitations to our study. First, a limited numbers of rat were included in each treatment group. Second, the researchers need extra protection from smoke exposure when using the present device. Lastly, the cigarette smoke examined in the present study was sidestream smoke, not the mainstream smoke as humans.

Disclosure of conflict of interest

None.

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