



Original Article

An Inhalation Chamber Model for Controlled Studies of Tobacco Smoke Toxicity in Rodents

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ARTICLE INFO

Article history:

Received February 27, 2008

Accepted May 8, 2010

Keywords:

Inhalation chamber

Tobacco

Rats

Pollutants

Environmental exposure

ABSTRACT

Introduction: Smoking is a serious worldwide public health problem. Animal models act as a bridge between laboratory and human studies. The models applied are difficult to reproduce because of the use of different types of inhalation chambers and mainly because of the lack of continuous monitoring of smoke concentration.

Objective: To develop an inhalation chamber for rats (with only the nose exposed) in which the amount of carbon monoxide (CO) can be maintained and monitored constantly.

Material and methods: Male Wistar rats weighing 250 g were exposed to 50 ppm CO produced by the smoke from a filter-free cigarette. The animals were submitted to a single 2-h exposure and then sacrificed at 0, 4, 24 and 48 h. The control group was left restrained inside the small perpendicular chambers, receiving only 5 L/min of compressed air.

Results: The model was able to increase HbCO levels immediately after the end of exposure ($p < 0.001$), with a decrease being observed from 2 h onwards when compared to the levels of the control group. Plasma cotinine increased immediately after exposure, and showed still detectable levels at 2 and 4 h ($p < 0.05$).

Conclusion: We conclude that the presented inhalation chamber system is able to maintain a controlled CO concentration in a model in which small animals are exposed to the inhalation of cigarette smoke, permitting well-controlled studies, as well as investigations involving other toxic gases and air pollutants.

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Modelo de cámara inhaladora para los estudios controlados sobre la toxicidad del humo del tabaco en los roedores

RESUMEN

Introducción: El tabaquismo constituye un grave problema de salud pública en todo el mundo. Los modelos animales actúan como un paso intermedio entre los estudios de laboratorio y los estudios en seres humanos. Los modelos aplicados son difíciles de reproducir debido al uso de diferentes tipos de cámaras inhaladoras y principalmente por la falta de una monitorización constante de la concentración del humo del tabaco.

Objetivo: Desarrollar una cámara inhaladora para ratas (con la exposición exclusiva del hocico) en la que pueda mantenerse y monitorizarse constantemente la cantidad de monóxido de carbono (CO).

Palabras clave:

Cámara inhaladora

Tabaco

Ratas

Gases tóxicos

Contaminantes ambientales

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Material y métodos: Se expusieron ratas Wistar macho, de 250 g de peso, a 50 partes por millón de CO producido por el humo del tabaco de un cigarrillo sin filtro. Los animales se sometieron a una exposición de 2 h y, acto seguido, fueron sacrificados a las 0, a las 4, a las 24 y a las 48 h. El grupo de control se dejó libre dentro de pequeñas cámaras perpendiculares y solo recibió 5 l/min de aire comprimido.

Resultados: El modelo fue capaz de aumentar la concentración de carboxihemoglobina inmediatamente después del término de la exposición ($p < 0,001$), observándose una disminución desde las 2 h en adelante comparado con la concentración del grupo de control. La concentración plasmática de cotinina aumentó inmediatamente después de la exposición y todavía se detectó a las 2 y a las 4 h ($p < 0,05$).

Conclusión: Concluimos que este sistema de cámara inhaladora puede mantener una concentración controlada de CO en un modelo en el que se expone a pequeños animales a la inhalación de humo de cigarrillos, lo que permite estudios adecuadamente controlados, al igual que investigaciones sobre otros gases tóxicos y contaminantes ambientales.

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Introduction

Smoking is a serious public health problem worldwide. In Brazil it is estimated that it is the main cause of about 200,000 deaths a year. Chronic obstructive pulmonary disease (COPD) is a systemic process with a well-known physiopathology, and its main triggering mechanism is smoking.^{1,2}

Animal models are used as an intermediate step between laboratory studies and trials in humans.³ There are still only a limited number of published studies of inflammation and pulmonary emphysema in animals due to cigarette smoke intoxication.⁴ The models applied are difficult to duplicate because they use different types of inhalation chambers and mainly because of the lack of constant monitoring of the concentration of cigarette smoke. In this study we developed an inhaling chamber for rats (with exclusive snout exposure), in which we were able to constantly maintain and monitor the amount of carbon monoxide (CO).

To validate the chamber we analysed two parameters, carboxyhaemoglobin (HbCO) and cotinine. CO mostly binds to haemoglobin, producing HbCO, with an affinity for haemoglobin that is 220 times that of oxygen. The nicotine in cigarette smoke is metabolised in the liver, producing cotinine, which is detectable in plasma by means of different techniques.⁵ The enzyme responsible for its metabolism is isoenzyme 2A6 of the cytochrome P450 system.⁶

The objective of this investigation was to develop an inhaling chamber to study diseases related to smoking and environmental pollution, in which the concentration of cigarette smoke could be maintained constant according to monitoring of CO concentration and in which the rats were exposed in a uniform manner to known and controlled quantities of CO.

Materials and Methods

The study was approved by the research committee of the University of São Paulo. Inhaling chamber: We designed an acrylic, cylindrical, 30 cm diameter and 50 cm high chamber (fig. 1) with a total capacity of 35.3 l for the exposure of rodents to toxic gasses. We inserted eight small perpendicular chambers in the body of the chamber that contained the rats (6 × 25 cm), and each chamber had an internal piston to confine the animals. We made an opening 1 cm in diameter between the body of the chamber and each individual chamber, so that only the animal's snout was exposed to the main chamber. Said system was equipped with a Venturi three-directional valve (Intermed, Brazil). In the afferent portion of the valve (part generating the flow) compressed air was connected with a high sensitivity flow-meter of up to 1 l/min, with a flow rate that varied from 50-100 ml/min; in the lateral portion a filterless lit cigarette was introduced (air aspiration) and the efferent part was connected to an extension that reached the hole in the lateral and inferior part

of the main chamber (propulsive portion). This system made aspiration of cigarette smoke possible and its conduction to the main chamber. An additional amount of compressed air was injected into the chamber through another low sensitivity flow-meter of up to 15 l/min, with a flow rate of 5 l/min, to maintain the desired concentration of CO. Within the chamber we introduced an air mixer to homogenise the compressed air and the cigarette smoke with the aim of maintaining a homogeneous concentration of smoke within the chamber. CO concentration, registered as parts per million (ppm), was monitored with a co-oxymeter (Toxi-Biosystems™, USA) that was in the main chamber. The smoke was constantly produced and expelled through the middle of the upper part of the main chamber.

"Exposure of animals to cigarette smoke". Male Wistar rats, weighing 250 g, were exposed to cigarette smoke or compressed air. The exposed group received cigarette smoke at a target CO concentration of 50 ppm. To standardise animal exposure, all the exposures were carried out with the same brand of cigarette. Each cigarette used contained 0.8 mg nicotine, 10 mg tar and 10 mg CO. The animals underwent a 2 hour exposure, and, immediately after, were killed at 0, 4, 24 and 48 hours. The control group was left free in the interior of small perpendicular chambers and only received 5 l/min of compressed air.

"Slaughter of the animals and blood sample taking". After completing exposure during the pre-determined time periods, the animals were slaughtered using standard techniques according to the Guide for the Care and Use of Laboratory Animals (National

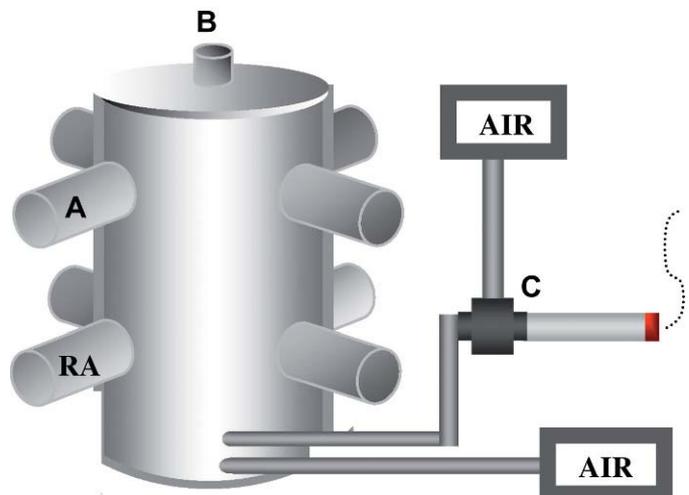


Figure 1. Chamber. A) Individual cylinders to place the animals. B) Output of cigarette smoke. C) Venturi valve.

Academy Press, 1996, Washington, D.C.). The rats were anaesthetised with pentobarbital (30 mg/kg) by the intraperitoneal route so that profound anaesthesia was induced without respiratory depression. The animals were placed lying down horizontally with their legs extended by fixating them with string until they were slaughtered by haemorrhage by puncturing the abdominal aorta to obtain blood through a laparotomy. The blood samples were stored in two types of tubes, one set with anticoagulant and the other without anticoagulant. The samples used to determine cotinine were stored frozen at -70°C .

"Determination of HbCO". The concentration of HbCO was determined by differential spectrometry in the variable region according to the technique described by Beutler and by West.⁷ The heparinised blood samples were stored in refrigerators until they were analysed.

"Determination of cotinine". Plasma cotinine was determined by duplicate by means of radioimmunoanalysis using an antibody according to the Langone method.⁸ Crossed reactivity of the anticotinine antibody with other nicotine metabolites was $< 5\%$. Detectable variation of the nicotine curve was 0.2-20 ng/ml with a variation coefficient of 6-10%. Blood samples were kept at -70°C until they were analysed.

"Statistical analysis". Results were registered as mean \pm standard error of the mean. Statistically significant differences were determined by means of variance analysis (ANCOVA) and a *posthoc* analysis was carried out using the Dunnet test for multiple comparisons with the control group. A value of $p < 0.05$ was considered significant.

Results

The animals were housed and in individual chambers and remained relaxed throughout the experiment, showing no signs of stress.

This exposure model was tested on 20 rats and the results were compared with four control rats. In this model the concentration of HbCO increased immediately after the end of exposure ($p < 0.001$), with a decrease observed from 2 hours onwards, in comparison with the concentration in the control group. Plasma cotinine increased immediately after exposure showing detectable concentrations at 2 and 4 hours ($p < 0.05$) and decreasing from that moment on (table 1). During the time of exposure 4-6 cigarettes were used to maintain a stable concentration of CO.

Discussion

In published studies different inhaling chamber systems have been described as models for exposure to cigarette smoke, cylindrical chambers⁹ or square¹⁰ ones, but not all studies describe the shape of the chamber used. Some researchers refer to exposure by means of method in which only the snout is exposed, but do not describe any additional details.^{11,12}

Table 1

Determinations of plasma concentrations of carboxyhaemoglobin and cotinine at different times in rats exposed to cigarette smoke for 2 hours ($n = 4$ in each group)

	HbCO (%)	Cotinine (ng/ml)
Control	0 \pm 0	0 \pm 0
0 h	3 \pm 0*	17 \pm 1.9**
2 h	0 \pm 0	12 \pm 4.5**
4 h	0.25 \pm 0.25	12.7 \pm 2.7**
24 h	0 \pm 0	0.25 \pm 0.25
48 h	0.25 \pm 0.25	0.75 \pm 0.47

HbCO indicates carboxyhaemoglobin.

Values are expressed as mean+standard error of the mean.

* $p < 0.05$.

** $p < 0.001$, control group in comparison with the exposed group.

The chamber we have described has a cylindrical shape so that the animals could be exposed to smoke in the most homogeneous manner possible, with individual transparent chambers for rats so that they could be seen during the whole time of exposure. Different studies have described rectangular chambers, with the rats housed inside the chamber as a group during exposure. Normally, these animals have a tendency to form groups and some can be on top of others, so that not all of them receive the same amount of smoke. Furthermore, another problem with this type of exposure is that products derived from tobacco are deposited on the animals' skins. And when the animals lick themselves they ingest these cigarette components which modify the exposure information. In a previous study our group of researchers used a model in which all the rats remained in the same chamber and we found a lot of problems with that model.¹⁰ In this study, the animals were housed outside the main chamber in individual units, so that only their snouts were exposed to cigarette smoke. Within the chamber a ventilator was installed so that the smoke-air mix was as homogeneous as possible.

With reference to smoke generation, different published studies used a fixed number of cigarettes per exposure,^{10,11,13,14} others determined the volume (ml) of the smoke flow output in the chamber and its duration in seconds^{9,11,13,15-17} or the quantity (mg) and diameter (μm) of the particles generated^{11,12,15,16} and, finally, some researchers determined the amount of CO released in a certain time, but not continuously.^{12,16}

We consider that a model that generates a constant concentration of CO is the most efficient method of exposure of animals to cigarette smoke, since it makes it possible to maintain the same concentration during the whole exposure. During the period of exposure we used 8-12 cigarettes to maintain a concentration of CO of 50 ppm. This variation was due to the introduction of cigarette smoke in the chamber. As result, to standardise exposure to smoke we monitored CO concentration throughout exposure. In a preliminary study we saw that, during combustion, a cigarette produces different concentrations of CO: it is lower at the beginning of the combustion of the cigarette and increases suddenly towards the end (data not shown). To maintain the same CO concentration throughout the experiment we examined different types of pumps, without any practical results. Finally, we developed a Venturi valve, which, connected to a synthetic air tank and controlled by a high sensitivity flow-meter, was able to maintain a constant flow of cigarette smoke. We were able to maintain appropriate aeration and CO concentration within the main chamber with a variable flow of synthetic air through a 15 l/min flow-meter. To homogenise the smoke we used a ventilator within the chamber. We introduced a co-oxymeter within the chamber, which made it possible to have constant CO measurement.

In general, the chamber does not preserve the smell of smoke, but, if it should do so, as it is of acrylic material it can be washed with water and detergent. Furthermore, technicians must keep in mind that the use of products that contain ether, acetone or abrasive substances may affect the acrylic material.

The increase in HbCO concentration in animals slaughtered 2 hours after exposure and the subsequent decrease lead us to conclude that the rats inhaled the cigarette smoke. The aim of the experiment was to produce a model to simulate the concentration of HbCO during the daily life of a smoker without reaching toxic levels. A high concentration of HbCO may cause death in rates due to hypoxemia. Furthermore, this double flow model makes it possible to achieve any concentration of CO in the exposure chamber just by varying the rate of flow of the high sensitivity flow-meter and adjusting it to the flow of the low sensitivity flow-meter. The use of another marker, plasma concentration of cotinine, clearly supports the information that the rats had inhaled cigarette smoke.

Basic research with animal models may determine disease mechanisms, especially chronic obstructive pulmonary disease, and,

in this case, cigarette smoke may contribute to a greater understanding of its physiopathology. This model of an inhaling chamber makes it possible to carry out dose-response studies that may produce more precise information on these inflammatory phenomena. We examined the stability of CO concentrations of up to 500 ppm during the same time period (2 hours) (data not shown). To achieve these high concentrations we only had to increase the flow of air through the Venturi valve.

We concluded that this system of using an inhaling chamber can maintain controlled concentrations of CO in a model in which small animals are exposed to the inhalation of cigarette smoke, which makes it possible to carry out appropriately controlled studies, as also investigations on toxic gasses and environmental pollutants.

Conflict of Interest

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Programa de Apoio a Núcleos de Excelência (PRONEX).

Acknowledgements

The authors wish to express their acknowledgement to Mauricio Levi-Orozco (Hospital del Mar, Barcelona, Spain), Carlos Colonell (Hospital del Mar, Barcelona, Spain) and Simona Pichini (Laboratorio Di Biochimica Clinica, Istituto Superiore Di Sanità, Roma, Italia) for their expert help and contributions to the study.

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