

TOXIC EFFECT IN THE LUNGS OF RATS AFTER INHALATION EXPOSURE TO BENZALKONIUM CHLORIDE

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Abstract

Background: Benzalkonium chloride (BAC) is a quaternary ammonium compound (QAC) toxic to microorganisms. Inhalation is one of the major possible routes of human exposure to BAC. **Materials and Methods:** Experiments were performed on female Wistar rats. The rats were exposed to aerosol of BAC water solution at the target concentration of 0 (control group) and 35 mg/m³ for 5 days (6 h/day) and, after a 2-week interval, the animals were challenged (day 21) with BAC aerosol at the target concentration of 0 (control group) and 35 mg/m³ for 6 h. **Results:** Compared to the controls, the animals exposed to BAC aerosol were characterized by lower food intake and their body weight was significantly smaller. As regards BAC-exposed group, a significant increase was noted in relative lung mass, total protein concentration, and MIP-2 in BALF both directly after the termination of the exposure and 18 h afterwards. Significantly higher IL-6 and IgE concentrations in BALF and a decrease in the CC16 concentration in BALF were found in the exposed group immediately after the exposure. The leukocyte count in BALF was significantly higher in the animals exposed to BAC aerosol compared to the controls. In the lungs of rats exposed to BAC the following effects were observed: minimal perivascular, interstitial edema, focal aggregates of alveolar macrophages, interstitial mononuclear cell infiltrations, thickened alveolar septa and marginal lipoproteinosis. **Conclusion:** Inhalation of BAC induced a strong inflammatory response and a damage to the blood-air barrier. Reduced concentrations of CC16, which is an immunosuppressive and anti-inflammatory protein, in combination with increased IgE concentrations in BALF may be indicative of the immuno-inflammatory response in the animals exposed to BAC aerosol by inhalation. Histopathological examinations of tissue samples from the BAC-exposed rats revealed a number of pathological changes found only in the lungs.

Key words:

Benzalkonium chloride, Rats, Inhalation, Lungs, Biomarkers, Histopathological examinations

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INTRODUCTION

Benzalkonium chloride (BAC) belongs to the group of quaternary ammonium compounds (QAC). In recent years, numerous works have been published describing the toxic activity of BAC in the *in vitro* and *in vivo* studies [1–13]. Scientific interest in BAC toxicity to the living organisms has resulted from a wide range of its use as a bactericide and preservative. It has been found that BAC used as a component of eye drops may adversely affect the different structures of the eye [1,2,5,7–10]. The widespread use of BAC also involves utilization of its bactericidal activity in the treatment of teeth [14] and the development of new nanocomposite structures with a built-in BAC molecule [15].

In some cases, the use of a BAC-containing product for its intended purpose caused a severe allergic reaction of the skin [16] and eye conjunctiva [17]. There are a number of reports of accidental exposure of humans to BAC, which led to death or a serious injury [18–20].

A major route of human exposure to this toxic compound is inhalation. Inhalation exposure of humans to preparations which include BAC results from the widespread use of BAC in various nasal sprays [21,22]. It was also found that BAC may play a role in occupational exposure [23].

In recent years, it has been demonstrated that BAC may cause an increased immune response in mice. The authors suggest that the exposure by inhalation can result in an increased risk of allergy caused by BAC [24]. In an inhalation study performed in mice, it was found that inhalation of BAC at higher concentrations induced deep lung effects and also lung inflammation [25].

In previous years we assessed toxic effects of BAC to the respiratory system and changes in the concentrations of catecholamines in the brain of rats exposed by inhalation to BAC aerosol [26,27]. The present study is an extension of our earlier works assessing the toxic effects of exposure to aerosol of BAC water solution in laboratory animals under conditions of repeated inhalation exposure.

MATERIALS AND METHODS

Animals

Female NIOM outbred Wist Wistar rats aged from 2 to 3 months obtained from the breeding farm of the Nofer Institute of Occupational Medicine, Łódź, Poland, were used in the experiment.

Chemicals

The animals were exposed in exposure chambers to Fluka BAC (CAS No. 8001-54-5, purity $\geq 95\%$) aerosol.

Inhalation exposure and experimental design

The chemical to be tested was injected to the exposure chamber with dynamic air flow to ensure 15 air changes per hour, where rat's head/nose was exposed to a direct contact with the BAC aerosol. BAC was injected into the chamber in the form of aqueous aerosol. The BAC concentration in the chamber was monitored by HPLC [26,28].

The Local Ethics Committee for Experiments on Animals approved the study protocol (Opinion No. Ł/BD/247). Figure 1 is a schematic representation of the experimental protocol. Rats were exposed to BAC aerosol at the target concentration of 0 (control group) and 35 mg/m³ for 5 days (6 h/day) and, after a 2-week interval, the animals were challenged (day 21) with BAC aerosol at the target concentration of 0 (control group) and 35 mg/m³ for 6 h. Body weights and food intakes were measured weekly. The animals were given standard laboratory food and water *ad libitum*, except for the time during which they were exposed to BAC aerosol in the dynamic inhalation chambers. The chamber relative temperature and humidity were maintained at 20–22°C and 35–45%, respectively.

Biological materials were collected from the exposed and control animals at two time-points: immediately after termination of exposure (day 21) and 18 h after the BAC challenge inhalation ended (Figure 1). The rats were

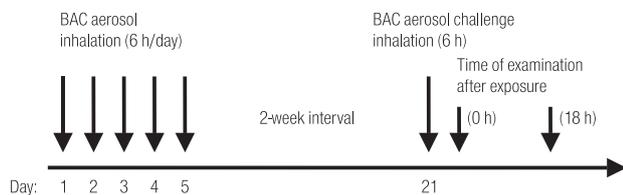


Fig. 1. Schematic diagram of the experimental protocol

anesthetized by an intraperitoneal injection of pentobarbital sodium at 50 mg/kg body weight and their lungs were lavaged with 2×5 ml of 0.9% NaCl solution. Bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 200 g for 10 min at 4°C. The supernatant of BALF was used for biochemistry. Clara cell protein (CC16) was determined with the use of latex immunoassay [29]. Specific rabbit antibodies against CC16 and a standard for CC16, based on the purified protein, were obtained as described earlier [30]. Hyaluronic acid (HA) was measured in non-concentrated BALF by enzymatic-immunoassay (ELISA) kit, including hyaluronic acid binding protein (HABP) capture molecule, Chungai-test (Japan). Lactate dehydrogenase (LDH) activity [31] and levels of matrix metalloproteinase-9 (MMP-9) (Calibrochem), tumor necrosis factor (TNF- α), interleukin 6 (IL-6), macrophage inflammatory protein (MIP)-2 (Biosource) and total IgE (Bethyl) were measured in BALF (IgE BALF) and serum (IgE-S) using the ELISA kit. GSH-S-transferase (GST) (EC. 2.5.1.18) in postmitochondrial supernatants of rat lung samples was assessed with 1-chloro-2,4-dinitrobenzene (CDNB) [32]. The quantity of CDNB (nmol) reacting during 1 min was assumed to be a unit (u).

The total and differential cell numbers were measured using standard procedures. Lymphocytes, neutrophils and macrophages were counted (%) under microscope in sediment cells from BALF after staining them with Giemsa and May-Grünwald reagents. For the estimation of leukocytes, sediment cells from BALF were suspended in PBS treated with Türk reagents and counted by optical microscopy in a Bürker chamber. Viability (%) was

estimated for 100 cells after staining the samples with trypan blue.

The necropsy was performed in all animals. Lungs, liver, spleen and kidneys removed during necropsy were fixed in 10% phosphate-buffered formalin, processed for embedding in paraffin, sectioned and stained with hematoxylin and eosin according to the van Gieson's method.

Statistical analysis

Statistical analysis was performed with the use of the Kruskal-Wallis test extended with all pairwise comparisons (STATISTICA.PL). Differences were regarded as significant when the probability of the null hypothesis was < 0.05.

RESULTS

All animals exposed to BAC aerosol at ca. 35 mg/m³ survived the inhalation exposure. Table 1 shows true BAC aerosol concentration in exposure chamber atmosphere, mean body weight and food intake during the experiment. At all measurement time points (day 7, 14 and 21 of the experiment), food intake and body weight of the animals exposed to BAC aerosol were significantly lower compared to the controls. The reduction of food intake and body mass in the BAC-exposed animals was particularly evident during the first week of the experiment: the corresponding values were ca. 64% and 22% compared to the controls, respectively. During the 2nd and 3rd week, the rats exposed to BAC aerosol consumed ca. 28% and 18% less food, and their body weight was ca. 11% and 10% lower compared to the controls, respectively. Macroscopic examinations did not reveal any significant changes between the controls and the exposed animals. A significant reduction of the absolute liver mass was recorded in all rats exposed to BAC aerosol compared to the control group. A significant increase of the absolute and relative mass of the lungs was noted in

Table 1. Body weight and food intake parameters in study groups

Parameters	Control group (N = 7)	Exposed group (N = 10)
Concentration of BAC (mg/m ³), M±SD	0	35.4±6.3
Body weight (g), M±SD		
day 1 of measurement	162±4.5	170±10.4
day 7 of measurement	205±5.0	160±13.5***
day 14 of measurement	225±8.7	201±13.5**
day 21 of measurement	220±14.1	199±13.4*
Food intake (g/rat/week), M		
day 1 of measurement	–	–
day 7 of measurement	124	45
day 14 of measurement	133	95
day 21 of measurement	120	98

M – mean; SD – standard deviation.

*, **, *** Significantly different from the control group at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

the group of animals exposed to BAC aerosol compared to the controls.

Table 2 shows the changes in the lung weight/100 g b.w. as well as GST in the lung samples and IgE in serum and other biomarkers in BALF of the rats exposed to BAC aerosol in relation to the control group. As regards BAC-exposed group, a significant increase was noted in the relative lung mass, total protein concentration, and MIP-2 in BALF, both directly after the termination of the exposure and 18 h afterwards. Significantly higher IL-6 and IgE concentrations

in BALF and a decrease in the CC16 concentration in BALF were found in the exposed group immediately after the exposure. No significant differences were noted for HA, GST, MMP-9 and TNF- α in BALF and IgE-S levels between the exposed group and the controls.

Table 3 specifies cellular characterization of BALF data concerning the control group and the rats exposed to BAC aerosol. Leukocyte count in BALF was significantly higher in the exposed animals compared to the controls. No significant differences were noted in the

Table 2. Comparison of examined parameters in study groups at different time points

Parameters	Control (N = 7)	Exposed group	
		time point (0 h) (N = 5)	time point (18 h) (N = 5)
Body weight (g)	220.00±14.10	203.00±6.70*	196.00±18.20*
Lung weight/100 g b.w.	0.66±0.12	0.96±0.11**	0.89±0.13*
Total protein (g/l)	0.31±0.22	4.52±1.91**	3.29±1.93*
CC16 (mg/l)	7.73±1.25	4.28±2.25*	5.96±1.29

Table 2. Comparison of examined parameters in study groups at different time points – cont.

Parameters	Control (N = 7)	Exposed group	
		time point (0 h) (N = 5)	time point (18 h) (N = 5)
HA ($\mu\text{g/l}$)	6.60 \pm 4.19	11.83 \pm 10.09	4.35 \pm 5.87
LDH (U/l)	151.60 \pm 58.60	321.40 \pm 94.90**	217.00 \pm 77.00
MMP-9 (ng/ml)	0.79 \pm 0.05	0.78 \pm 0.09	0.77 \pm 0.05
TNF- α (pg/ml)	29.30 \pm 20.40	38.80 \pm 41.10	23.60 \pm 15.50
IL-6 (pg/ml)	71.20 \pm 69.30	837.60 \pm 601.10**	496.40 \pm 670.40
MIP-2 (pg/ml)	248.10 \pm 56.90	608.60 \pm 138.30***	376.60 \pm 48.30*
IgE BALF (ng/ml)	5.34 \pm 4.21	71.36 \pm 98.60*	15.10 \pm 26.80
IgE-S (ng/ml)	140.80 \pm 48.20	128.20 \pm 6.30	162.80 \pm 29.30
GST (u/mg protein)	125.60 \pm 53.90	164.10 \pm 107.50	65.40 \pm 36.00

CC16 – Clara cell protein; HA – hyaluronic acid; LDH – lactate dehydrogenase; MMP-9 – matrix metalloproteinase 9; TNF- α – tumor necrosis factor α ; IL-6 – interleukin 6; MIP-2 – macrophage inflammatory protein 2; IgE BALF – immunoglobulin E in bronchoalveolar lavage fluid; IgE-S – immunoglobulin E in serum; GST – glutathione S-transferase.

*** ** Significant difference from the control group at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Results expressed as a mean \pm standard deviation.

Table 3. Bronchoalveolar lavage fluid (BALF) parameters in both study groups

Parameter	Control (N = 7)	Exposed group	
		time point (0 h) (N = 5)	time point (0 h) (N = 5)
Leukocytes ($\times 10^3/\text{mm}^3$)	1.74 \pm 0.73	3.64 \pm 2.03	5.56 \pm 4.69*
Viability (%)	97.00 \pm 0.60	97.20 \pm 0.50	97.60 \pm 0.60
Macrophages (%)	93.00 \pm 0.00	75.60 \pm 24.00	87.80 \pm 8.40
Neutrophils (%)	7.00 \pm 0.00	24.40 \pm 24.00	11.60 \pm 8.40
Lymphocytes (%)	0.00 \pm 0.00	1.80 \pm 4.00	0.60 \pm 0.90

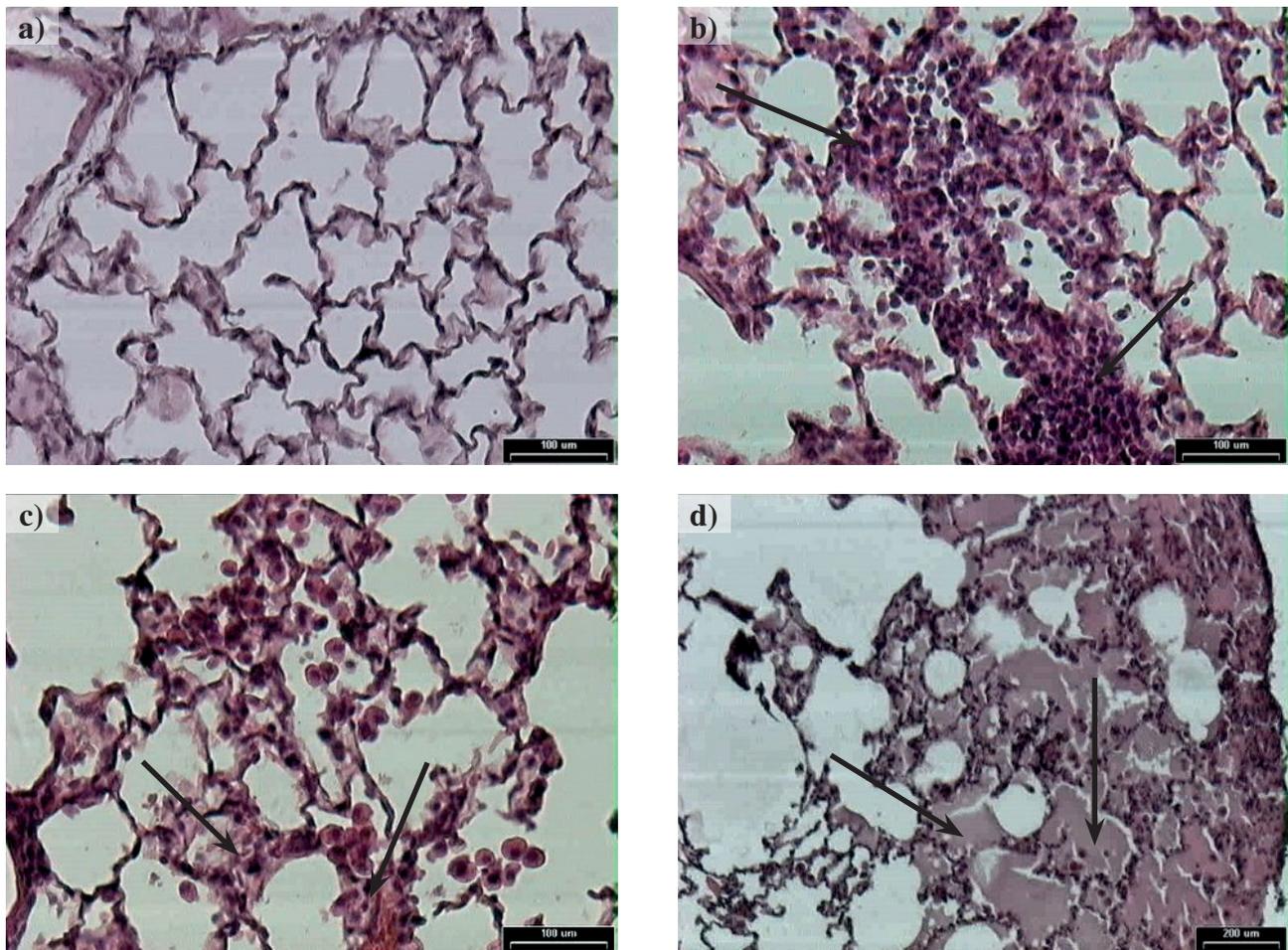
* Significantly different from the control group at $p < 0.05$.

Results expressed as a mean \pm standard deviation.

number of macrophages, neutrophils, lymphocytes or the viability of cells from BALF between the control and the exposed rats.

No signs of histopathological changes were observed in the liver, kidneys and spleen of rats. Photo 1 shows a typical lung of a control rat and the changes in the lungs of the

rats exposed to BAC aerosol that were observed 18 hours after the inhalation. Minimal perivascular interstitial edema, focal aggregates of alveolar macrophages, interstitial mononuclear cell infiltration, thickened alveolar septa and marginal lipoproteinosis were seen in the lungs of those rats.



b) Interstitial mononuclear cell infiltrations, thickened alveolar septa, and focal aggregates of alveolar macrophages.
 c) Minimal perivascular and interstitial edema. Focal aggregates of alveolar macrophages.
 d) Marginal lipoproteinosis and focal aggregates of alveolar macrophages.
 H&E Hematoxylin and eosin staining.

Photo 1. Lungs of the studied rats: a) control group. b–d) group exposed to BAC aerosol at 18 h after the inhalation

DISCUSSION AND CONCLUSIONS

Multiple inhalation exposures to ca. 35 mg/m³ BAC aerosol produced severe toxic effects in rats. The animals exposed to BAC aerosol consumed less food and their body weight continued to be lower compared to the controls throughout the experiment.

Similarly to earlier study assessing the pneumotoxic effect of BAC aerosol on laboratory animals, this research revealed a significant influence of the exposure on the level of lung biomarkers. In the majority of cases, a similar

trend could be observed in that respect [26]. The adverse effect of exposure to BAC aerosol was not found to be time-dependent. The exposure to BAC aerosol produced a strong pneumotoxic effect manifested by necrosis of the respiratory cells (increased LDH activity) and lung inflammation (higher level of IL-6 and MIP-2) in BALF of the inhalation-challenged rats. Some authors suggest that quaternary ammonium compounds (QAC), such as BAC, may produce proinflammatory changes due to oxidative stress [33–35]. The irritant respiratory effects observed during our

experiment were attributable probably to the BAC-induced oxidative stress. It is not clear whether the process paves the way for the changes leading finally to the development of asthma, the more so that MMP-9, a marker of severe asthma-like respiratory abnormalities, was at a similar level in the exposed and control animals [36].

On the other hand, the damage to Clara cells, reflected by a decreased concentration of CC16 showing immunosuppressive and anti-inflammatory activity, accompanied by significant increase in the IgE level after exposure to BAC may point to an immuno-inflammatory response [37,38]. The more so that the CC16 protein released by Clara cells in the respiratory system acts as a controller of the immunosuppressive and anti-inflammatory processes preventing an adverse immune response. It is generally recognized that CC16 controlling the inflammatory process is an equivalent of uteroglobulins [39]. Reduced CC16 concentrations may be indicative of asthmatic changes. Asthmatic patients have been shown to be characterized by lower levels of phospholipase A2, towards which the CC16 protein acts as an inhibitor [40], and reduced levels of CC16 protein [41,42]. A significant increase of the leucocyte count in BALF and numerous focal aggregates of alveolar macrophages observed in the microscopic images of the lungs also point to the immunoinflammatory processes occurring in the lungs of the exposed animals.

BAC exposure induces a massive influx of proteins from blood vessels to the alveolar space, which is manifested by increased total protein concentration in BALF. Increased total protein concentration accompanied by simultaneously reduced CC16 protein concentrations may point to the damaged integrity of the blood-air barrier [43–46]. Increased total protein levels in the BALF of rats exposed to BAC could be directly responsible for the lack of an immune response in serum. Possibly, during inhalation exposure, a small amount of BAC enters the bloodstream of the rat and most of it is retained in the respiratory tract, where it can be attached to the proteins infiltrating from

the bloodstream. Quaternary ammonium compounds are known to have a high affinity to proteins [47]. There is no immediate data analyzing the distribution of BAC under inhalation exposure conditions.

It can be assumed that the intravenous or intra-arterial administration may to some extent illustrate the distribution which occurs during the inhalation exposure. During the experiment on rats, 2 min after the introduction of BAC to the bloodstream by intravenous or intra-arterial injection, it was noted that the BAC concentrations in the lungs were several times higher compared to the levels observed in the liver, kidneys, and even in the blood itself [48]. This suggests that intravascular administration of BAC causes its rapid penetration into the lumen of the lungs, and the lungs are the critical organ. Summing up, inhalation of BAC produced a strong inflammatory response and damage to the blood-air barrier. Decreased serum concentrations of CC16, a protein with immunosuppressive and anti-inflammatory activity, and a parallel increase in the IgE levels in BALF may point to the immuno-inflammatory response of animals exposed by inhalation to BAC aerosol. Histopathological examination of the tissues removed from the rats exposed to BAC shows that pathological changes were found only in the lungs. This indicates that inhalation exposure to BAC results mainly in the damage to the respiratory tract.

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