“Establishment and validation of inhalation towers for inhaled compound administration by aerosolation to rodents”

Masterarbeit

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Susanne Pilz

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Gutachter: Prof. Dr. rer. nat. F. Birger Anspach (HAW Hamburg)

Prof. Dr. rer. nat. Andreas Pahl (Nycomed GmbH)

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Index of content

Abbreviation 7 - 8
Summary 9

1. Introduction 10 - 23

1.1. The burden of chronic inflammatory airway diseases 10 - 11

1.1.1. Prevalence of chronic obstructive pulmonary disease (COPD) and asthma bronchiale
1.1.2. Morbidity and mortality of chronic obstructive pulmonary disease (COPD) and asthma bronchiale

1.2. Chronic obstructive pulmonary disease (COPD) 12 - 14

1.2.1. Definition and classification
1.2.2. The immunology of chronic obstructive pulmonary disease (COPD)
1.2.3. Medical treatment of COPD

1.3. *In vivo* models in preclinical drug research 15 – 23

1.3.1. COPD *in vivo* models
1.3.1.1. Tobacco smoke and elastase model
1.3.1.2. Endotoxin induced acute pulmonary inflammation model
1.3.2. Comparison between human and murine lung
1.3.3. Pulmonary drug delivery
1.3.1.1. Intratracheal instillation-advantages and disadvantages
1.3.1.2. Inhalation with whole body and nose only exposure systems

1.4. Aim of the master thesis 24

2. Material and methods 25 - 39

2.1. Material 25 - 30

2.1.1. Technical equipment
2.1.2. Chemicals
2.1.3. Reagence and solution
2.1.4. Consumables
2.1.5. Kits
2.1.6. Software
2.1.7. Technical equipment of LPS inhalation system
2.1.8. Technical equipment of EMMS inhalation system
2.1.9. Test compound and formulation
2.1.10. LPS and formulation

2.2. Methods 30 - 39

2.2.1. Direct examination of aerosol distribution in the LPS nose only exposure system 30 - 33

2.2.1.1. Filter sampling
2.2.1.2. Aerosol sampling with midget impingers
2.2.1.3. Endotoxin detection
2.2.1.4. Aerosol monitoring

2.2.2. Rat model of LPS-induced lung inflammation 34 - 39

2.2.2.1. Animals
2.2.2.2. Experimental set-up
2.2.2.3. Compound application

2.2.2.3.1. Intratracheal application
2.2.2.3.2. Nose only inhalation system for compound administration
2.2.2.3.3. Compound administration

2.2.2.4. LPS-induced neutrophilia
2.2.2.5. Bronchoalveolar lavage (BAL)
2.2.2.6. Data analysis

3. Results 40 - 66

3.1. Direct examination of aerosol distribution by filter sampling 40 - 47

3.1.1. Filter sampling
3.1.2. Calculation of inhaled dose of LPS after filter sampling
3.1.3. Filter sampling of the single inhalation tower levels

3.2. Direct examination of aerosol distribution by endotoxin detection with the limulus amebocyte lysate (LAL) assay 47 - 51

3.2.1. Quantitative endotoxin detection
3.2.2. Calculation of inhaled dose of LPS

3.3. Determination of aerosol distribution by real time monitoring 52 - 56

3.4. Indirect examination of aerosol distribution by LPS- induced neutrophilia 57 - 62

3.4.1. Comparison between the tower levels
3.4.2. Induced neutrophilia in dependency to the occupancy of the tower
3.4.3. Application of a vaccum pump

3.5. Comparison of compound administration 63 - 66

3.5.1. Intratracheal instillation
3.5.2. Nose only compound administration

4. Discussion 67 - 75

4.1. LPS lung-induced neutrophilia inhalation model 67 - 72

4.1.1. Direct examination of aerosol distribution

4.1.1.1. Endotoxin detection by filter sampling
4.1.1.2. Endotoxin detection with the limulus amebocyte lysate (LAL) assay
4.1.1.3. Aerosol monitoring

4.1.2. Indirect examination of aerosol distribution

4.2. Comparison of compound administration 73 - 75

4.2.1. Intratracheal compound administration

4.2.2. Nose only compound administration of aerosol

5. List of Literature 76 - 80

Abbreviations

ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
b.w.	Bodyweight
cAMP	Cyclic adenosine mono phosphate
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CV	Coefficient of variance
CXCL	Chemokine CXC ligand
CXCR	Chemokine CXC receptor
ECRHS	European Community Respiratory Health Survey
EGF	Epidermal growth factor
EU	Endotoxin units
GINA	Global Initiative for Asthma
GOLD	Global Initiative for chronic obstructive lung disease
HAT	Histon acetyltransferase
HNE	Human neutrophil elastase
ICS	Inhaled corticosteroids
IL	Interleukin
ISAAC	International Studie of Asthma and Allergies in Childhood
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide

MLCK	Myosin light chain kinase
MMP	Matrix metallopeptidase
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PKA	Protein kinase A
pNA	p-nitroaniline
RMV	Respiratory minute volume
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor α
TPM	Total particulate measurement
TS	Tobacco smoke
WHO	World Health Organisation

Summary

Chronic airway diseases are the fifth frequent cause of death worldwide. This trend reflects the importance and the necessity to develop new pharmaceuticals to combat the disease. Therefore preclinical *in vivo* and *in vitro* studies are necessary for this development.

The endotoxin lung-induced neutrophilia is a common *in vivo* model to mimic certain characteristics of human chronic inflammatory pulmonary diseases in rats. The administration of endotoxin as an aerosol is carried out with a nose only inhalation tower. Previous studies with this model have yield unsatisfactory results due to high variation of animal data. It was generally assumed that non-uniform aerosol distribution within the exposure system is a major factor for this effect. To verify this assumption, this thesis was aimed to determine the aerosol distribution within the inhalation system and to improve the system. To this end, the direct method of filter sampling was performed. High variation was observed with CV value of 89%. As an alternative to the filter sampling method applied, the endotoxin detection tests by limulus amebocyte lysate (LAL) assay showed as well high variation (CV value 53%). In order to improve this, the technical equipment of the system was accordingly optimised. A new application technique by introducing dummies to the tower and creating vacuum through suction was tested in order to reduce the variation in the data. Therewith, the CV value for the filter sampling was reduced from 98% to 53% whereas the CV value for the LAL assay was reduced from 39% to 8%. Furthermore, indirect examination of aerosol distribution within the exposure system was performed with vehicle treated rats. Experiments with the standard *in vivo* technique led to high variations (CV value 36% - 62%). Improvements were achieved with the new technique (dummies and vacuum pump) where CV values were reduced to 27% - 33%.

The pulmonary application of compound is still a major challenge in pulmonary drug research due to the anatomical differences between rodents and humans. Therefore, two pulmonary compound administration techniques are tested. A comparison between the intratracheal instillation technique and the nose only exposure of the corticosteroid budesonide showed that the inhibitory effect is similar for both administration techniques. Furthermore, the variation of collected data was low with CV values between 24% and 37%.

In conclusion, the inhalation tower could be optimised by means of technical improvements as seen in strong reduction in variation of collected data. In general, it should be also noted that living rats were accordingly used in regards to the national animal protection law which requires that the number of animals to be used in experiments be kept as little as possible. Based on the optimisation method by technical means, it was possible to reduce the number of used rats. Furthermore, the cost associated with rats' acquisition could be minimised.

1. Introduction

1.1. The burden of chronic inflammatory airway diseases

1.1.1. Prevalence of chronic obstructive pulmonary disease (COPD) and asthma bronchiale

The relevance of chronic inflammatory diseases has become more common due to increasing occurrence within the past decades. According to the current data of the World Health Organisation (WHO) about 235 million people suffer from asthma and 64 million individuals have chronic obstructive pulmonary disease (COPD) worldwide (WHO, 2004 Update). The main risk factor of COPD is tobacco smoke. A rise of COPD is estimated in low income countries (e.g. Western-Pacific and African regions) due to the increasing smoking behavior and unknown education of the cigarette smoking risk where men and women are equally affected (Chan-Yeung M, 2004). Environmental pollution is considered as a further risk factor. Indoor biomass cooking and heating might also be a reason for the increasing number of non smoking patients in developing countries (GOLD, 2006). Furthermore, certain studies indicate a possible genetic predisposition (Erikssen S, 1965). In addition, the medical equipment and attendance in developing countries states an obstacle in diagnosing and classifying COPD. According to this issue the Burden of Obstructive Lung Disease (BOLD) initiative performs studies with people older than 40 years in developing countries by applying the global initiative for chronic obstructive lung disease (GOLD) standardised method of spirometry to assess the prevalence (BOLD, 2011).

A prevalence study of asthma, including 20 regions of the world was published by the global initiative for asthma (GINA) in 2004. The data was obtained from two different sources where the “wheezing of the past 12 month” was questioned as an indicator for current asthma symptoms. The International Study of Asthma and Allergies in Childhood (ISAAC) project screened children in the age group of 6 - 7 and 13 - 14 years, while the European Community Respiratory Health Survey (ECRHS) analysed adults (20 - 44 years). The estimation of the studies demonstrates the highest frequency of asthma in the UK, Australia, Canada and the USA. The population in urban areas is more affected than rural regions (Masoli M, 2004). However, the prevalence of English speaking countries decreases while an increase in African and Asian countries in childhood ages is discovered (Pearce N, 2007).

About 35.5 million people in Northern America suffer from asthmatic symptoms and are diagnosed with the disease. Regarding the age, more young boys develop asthma compared to young girls. But this trend is not consistent with adults. Females >40 years develop asthma more frequently. Furthermore the prevalence of specific races in the USA reports an increase of Afro-Americans and Hispanics compared to white patients (Akinbami LJ, 2011).

1.1.2. Morbidity and mortality of chronic obstructive pulmonary disease (COPD) and asthma bronchiale

The Global Burden disease report of the WHO published in 2004 ranked respiratory diseases to the fifth frequent causes of death including all age groups. Nearly three million peopled died of the consequences of COPD which is total death of 5% in 2005 (WHO, 2011). Almost 90% of deaths caused by COPD in 2005 occur in low and middle income countries (WHO, 2011).

In contrast the mortality rate of asthma amounts 1 per 250 deaths worldwide (Pearce N, 2007). But the number of deaths decreases worldwide due to the improved medical care and long term therapies (Rogers L, 2011). The highest death rates occurs in China (36.7/100 000 asthmatics). One reason might be the suboptimal application of western medicine during an asthma attack or for long term therapy.

A projection for the next 10 years states that an overall decrease in prevalence of airway diseases is not expected. But it is proposed that inflammatory airway diseases will rank the third position of global burden death list in 2030. Two reasons might be the increasing smoking behavior of women and the aging of population.

The following course of the thesis is focused only on COPD. There is a high interest of understanding and treating the disease. Pharmacological studies in preclinical drug research are required to find appropriate therapies. But the pulmonary drug delivery remains as a big challenge due to the physiology and mechanical properties of the lung.

1.2. Chronic obstructive pulmonary disease (COPD)

1.2.1. Definition and classification

COPD was defined by the global initiative for chronic obstructive lung disease (GOLD) in 2006 as follows: "*a preventable and treatable disease with some significant extrapulmonary effects that may contribute to severity in individual patients. Its pulmonary component is characterized by airflow limitation this is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles of gases*" (GOLD, 2006).

COPD and its severity are diagnosed spirometrically through the measurement of the tidal volume and the velocity of the airflow. The ratio of the measured forced expiratory volume in one second (FEV1) and the forced vital capacity (FVC) displays the function of the lung. One classification of COPD is based on the post-bronchodilator FEV1 which is recommended for educational purposes (Barnes PJ, 2009). But the treatment depends on the symptoms which are supposed to be treated and contained. In addition, COPD is classified into five stages ranging from at risk, mild, moderate, severe to very severe. Clinical symptoms of COPD are chronic cough and sputum production (stage 0, 1). With the progress of the disease shortness of breath dyspnoea occurs (stage 2, 3). Patients describe symptoms as a sense of increased effort to breathe, heaviness, air hunger or gasping (Simon PM, 1990). At the very severe phase frequently occurring viral or bacterial infections are responsible for exacerbation which can cause a damage of the airway epithelium (emphysema). Also extrapulmonary manifestations are visible like loss of weigh. Studies show that anorexia in COPD patients is related to inflammatory immune activation which contributes to the development of cachexia (Koehler F, 2007).

1.2.2. The immunology of COPD

The pathologically classification of COPD includes chronic bronchitis, emphysema and small airway diseases. Neutrophilic granulocytes, macrophages and CD8+ T- cells accumulate in the airways and are involved in the inflammatory process. Main cytokine mediators of the inflammation are TNF-α, IL-1β and IL-6 (fig.1).
Neutrophilic granulocytes represent the key effector cells in COPD and belong to the group of the white blood cells and are an essential part of the innate immune response. They contain intracellular granula which consist of enzymes, e.g. lysozym, elastinase, collagenase.

Neutrophils circulate in the blood system and migrate in case of an inflammation to the affected organ. This migration is a multi level process where active immune cells release several chemotactic factors to attract inflammatory cells to the lung. According to study data neutrophilic granulocytes were localised in the bronchial epithelium and bronchial glands. Furthermore they were recovered in the sputum and the bronchoalveolar lavage (BAL) (Martin TR, 1985).

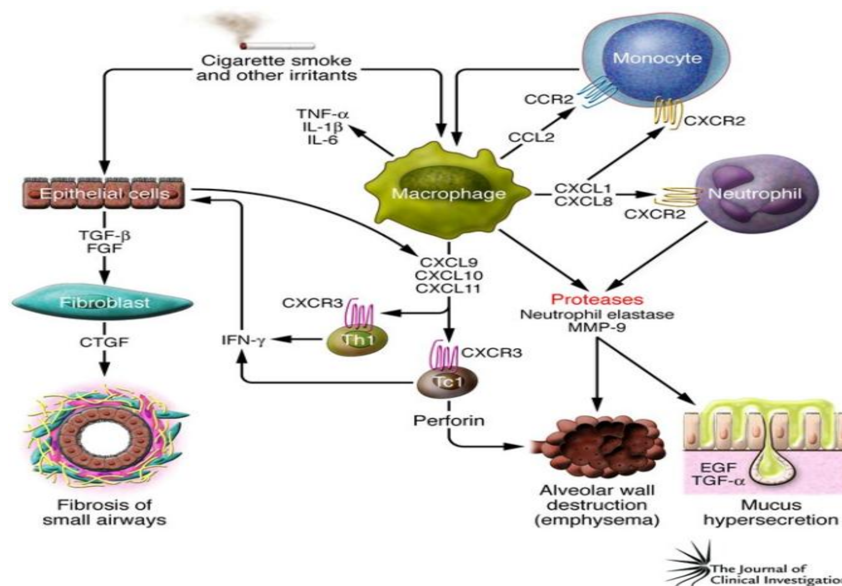


Fig.1: Involved cytokines in COPD: Inhaled irritants, such as cigarette smoke, activate epithelial cells and macrophages to release multiple cytokines and growth factors. These cells secrete the proinflammatory cytokines TNF-α, IL-1β, and IL-6 that attract circulating cells into the lungs. CXCL1 and CXCL8 act via CXCR2 to attract neutrophils and monocytes; CXCL9, CXCL10, and CXCL11 act via CXCR3 to attract Th1 cells and Tc1 cells, both of which release IFN-γ, which in turn stimulates the release of more of these CXCR3-binding chemokines.
Mucus hypersecretion is stimulated by EGF and TGF-α
(adopted from Barnes PJ, 2008)

Neutrophils release the enzyme elastase which degrades pathogens. Considering COPD, this serine proteinase causes the mucus hypersecretion which is characteristic for chronic bronchitis (Jaboff A, 1977). The neutrophilic elastase is closely associated with the development of emphysema. According to animal studies emphysema was induced through the application of neutrophile elastase (Karlinsky IB, 1978).
Macrophages are phagocytotic cells of the innate immune system. They are derived from circulating monocytes that migrate to the lungs in response to chemoattractants such as CC chemokine ligand 2 (CCL2). Macrophages initiate and regulate the inflammatory response and release cytokines IL-1ß, IL-6 and TNF-α (fig.1). An increased number of CD8+ T-cells has been detected in the airways and in the lung parenchyma. CD8+ T-cells are

cytotoxic lymphocytes and release granzyme B and perforins, which were detected at high levels in the sputum of COPD patients (Costa C, 2008).
These enzymes may induce the apoptosis of type 1 pneumocytes, thereby contributing to the development of emphysema (Majo J, 2001).
In addition, cigarette smoke and other noxious substances stimulate epithelial cells in the small airways to secrete the cytokine TGF-ß which activates the proliferation of fibroblast and results in a fibrosis of the small airways (fig.1).

1.2.3. Medical treatment of COPD

Generally accepted therapy in reducing the symptoms of COPD is seen in inhaled bronchodilators like β2-agonist (e.g. indacaterol) or anticholinergics (e.g. tiotropium bromide) as the mainstay of current treatment regimen. Inhaled corticosteroids (ICS, e.g. budesonide) are used to reduce the frequency of exacerbation.
An oral therapeutic approach through the inhibition of the enzyme phosphodiesterase (PDE) - 4 has led to the marketing authorization of Daxas® in 2010. Hereby, the active substance roflumilast reduces the exacerbation of COPD (Au DH, 2009). Although Daxas® is considered as a milestone in the COPD treatment due to high efficacy of the drug and mild side effects (Izquierdo JL, 2010). But COPD therapy remains challenging. Improvements in development of numerous medications have contributed to altering or rather slowing the progress of the disease but no cure of COPD is provided so far. In addition, improvements of therapeutic administration to patients are necessary, e.g. reduction in the daily intake dosage, easy and simple handling with the inhaled drugs as well as reduction of treatment related side effects.

1.3. *In vivo* models in preclinical research

The marketing authorisation of new pharmaceuticals always requires pharmacological studies in preclinical drug research. The approach is to examine the efficiency of new compound, to determine the delivery dose and to reduce serious side effects. For human protective reason, compound is tested *in vitro* and *in vivo*. Considering COPD, it is necessary to find at first appropriate *in vivo* models to induce characteristics of the disease.

1.3.1. COPD *in vivo* models

1.3.1.1. Endotoxin induced acute pulmonary inflammation model

The establishment of COPD animal models is very difficult due to the differences in the respiratory system between humans and rats. Especially considering the mediators of pathology and their functional effects. However, animal models provide important knowledge about the molecular and cellular mechanisms in COPD but limitations have to be perceived. The most common murine method applied to induce a rapid pulmonary inflammation is the endotoxin (lipopolysaccharide-LPS)- induced model. It is a short term model which produces some anatomic features of human COPD. The endotoxin LPS is present in air pollution, organic dusts and occurs as a contaminant in cigarette smoke. Regarding preclinical drug research, LPS is either administered by intranasal instillation or by nose only aerosol exposure to the animals. A mixed inflammation of neutrophils and increased TNFα and IL-1β is measured in the bronchoalveolar lavage (BAL) and also detectable in the lung tissue (Fox JC, 2009).
Differences in the neutrophilic cell amount in the BAL between rats and COPD patients have been determined. Mostly, neutrophils were measured in rats whereas in COPD patients the major cells are alveolar macrophages (Rutgers SR, 2000). Despite that neutrophils are increased in COPD patients at a severe stage of the disease. The major pharmocological difference between the rat model and COPD patients was detected in the sensitivity to corticosteroids (Fox JC, 2009). The rat model is highly sensitive to steroids while the clinical efficiency of inhaled corticosteroids in COPD patients was achieved (Birrell MA, 2006). With chronic administration over several weeks, LPS instillation produces enlarged air spaces and remodeled airways with thickened walls and increased goblet cells in the larger airways in mice and guinea pigs. These changes resemble human emphysema and small airway remodeling and may be driven by mechanisms that are somehow similar to humans (Wright JL, 2008).

1.3.1.2. Tobacco smoke and elastase model

The endotoxin lung-induced model does not fully cover the whole COPD but only shows partial aspects relating to the disease. From this view, it proofs the importance of multiple models considering the characterisation of new drugs. Therefore, further models are used in preclinical drug research.

COPD is developed and caused mainly by cigarette smoking. Due to this fact, the use of the tobacco smoke (TS) model has continued to increase over the last 10 years and is routinely used as a noxious stimuli (Fox JC, 2009). Pulmonary inflammation and airway changes are induced by short-term TS exposure. Furthermore, chronic TS exposure examined emphysema development. The time to induce an emphysema varies between the animal species but generally takes approximatley 4 - 6 months (Wright JL, 2002). In comparison, the development of emphysema in human COPD patients takes decades. Guinea pigs are the most susceptible specie in the TS model whereas rats are widely used. Rats are very resistant to the induction of emphysema-like lesions, possibly because of differential sensitivity to oxidative stress and degree of inflammation (Fox JC, 2009). The most favourable laboratory animal for the TS model are mice due to their manipulation of gene expression. Furthermore, mice are known to tolerate at least two cigarettes a day for one year with minimal alterations in bodyweight (Halliwell B, 2006).

A further model which is used in preclinical drug research is the elastase emphysema model. Experiments performed with intratracheal application of human neutrophil elastase (HNE) developed inflammation of lung parenchym followed by airspace enlargement and alveolar wall destruction, resulting in increased lung volume reflecting the loss of lung elastic recoil which are characteristic features for human emphysema (Fox JC, 2009). The inflammatory cell cascade caused by elastase in the lung is similar to the TS model. A neutrophilia is obtained with an infiltration of macrophages and T-cell influx. However, both methods, TS smoke and elastase induce the emphysema by protolytic attack but the detailed mechanism of both methods is totally different (Wright JL, 2008). Even if many characteristics of human emphysema are reproduced, it is still unclear which activated molecular pathways in the elastase model are similar to human COPD patients (Fox JC, 2009).

1.3.2. Comparison between human and murine lung

The target organ in COPD treatment is the lung. Therefore, the inhalative route is used to apply compound directly to the site of action. But the anatomy and physiology between humans and rats differs.

The human lung

The human lung as a paired cone shaped organ, comprises of two main parts: left and right lung. The right lung is classified into three main sub divisions; upper, middle and lower lobars. In comparison to the right lung, the left lung is smaller in its shape and size. It is subdivided in the upper and middle lobar that enhances the more space required by the heart. The apex of the lung lies on the diaphragm (fig. 2a).

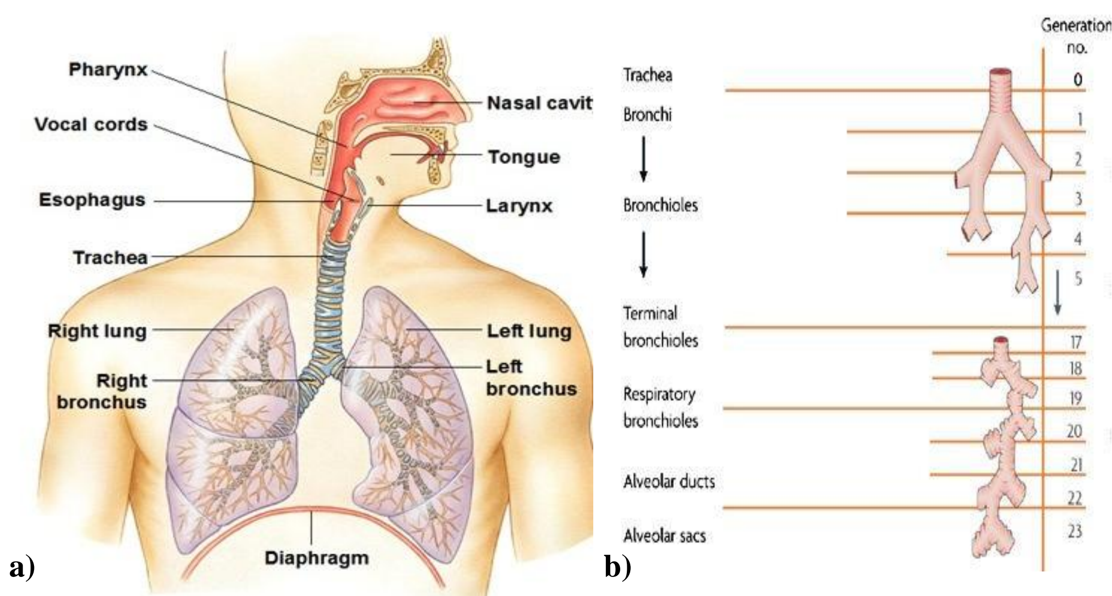


Fig. 2: The human airways: a) the respiratory system (retrieved July 17, 2011 from:http://www.colorado.edu/intphys/Class/IPHY3430-200/image/17-1.jpg); b) Branches of the airway (retried July 17,2011 from: http://www.nature.com/nrd/journal/v6/n1/images/nrd2153-f2.jpg)

The respiratory tract is a highly branched tubing system divided into the upper and lower air passages. The upper air passage consists of nasal and oral cavity, pharynx, epiglottis and larynx. The main function of the upper air passage is to conduce air while not actively participating in the gas exchange process.

The lower airpassages are segmented into a conducting and a repiratory zone. The first section of the air conducting zone is the trachea. The human trachea has between 6000 – 7000 mucus secreting goblet cells/ mm^2 (Eleffsen P, 1972). In adults, between 30 - 40% of the total cells in the large airways contain mucous cells, the amount increases in patients with chronic bronchitis and smokers.

The trachea splits into two bronchi. This merges to the lobar bronchi, the segmented bronchi and ends with the bronchiolus terminalis. Clara non ciliated bronchiolar cells are present and restricted to the bronchiolus terminalis, where their portion of the cell population amounts to nearly 20%. These cells are responsible for the production of bronchiolar surfactants and are involved in the fluid absorption (Kohlhaeufl, 2007). An increment in the lungs penetration depth leads to the multiple generation of air passages that is characterised by shorter and narrower branches (fig. 2b). The respiratory zone of the bronchial tree that is responsible for the gas exchange is the alveoli (West JB, 2008).
Almost half of the epithelial cells in the normal human airway are ciliated. The cilia is responsible for the mucociliary clearance of the upper tract. The number of cilia decreases toward the progress of the lung periphery which leads to a decrease in the ciliary transport respectively. The mucus is removed from the alveoli to the pharynx. At the pharynx, the mucus is usually swallowed or expectorated (Kohlhaeufl, 2007).

Rat lung

The murine lung is divided into a right and left lung. The left lung comprises of only one lobar. In contrast, the right lung consists of four parts (fig.3).

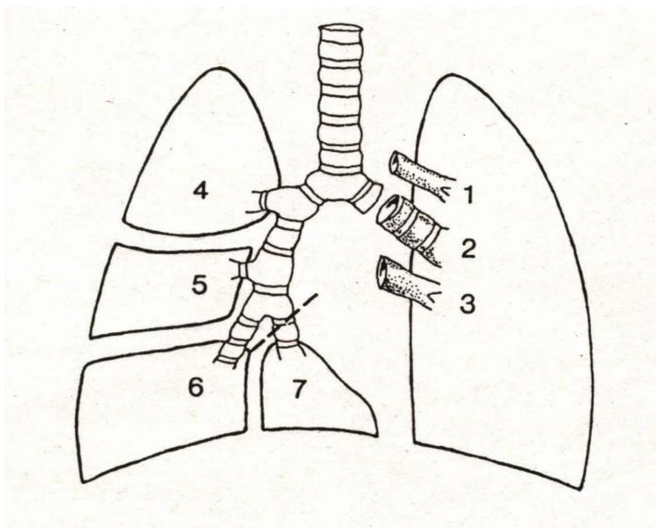


Fig.3: The anatomy of the rats lung:(1)artery pulmonalis, (2)mainbronchus, (3) vein pulmonalis, (4)lobus cranialis, (5)lobus medius, (6) lobus caudalis, (7)lobus accessorius

The murine respiratory system comprises of conducting and gas exchanging airways. The conducting airways include the nose, pharynx, larynx, trachea, bronchia and non-alveolised bronchioles. Respiratory bronchioles, alveloar ducts and alveolar air sacs are responsible for the gas exchange in the rats lung. Rats are obligate nose only breathers due to the close apposition of epiglottis to the soft palate (Krinke, 2000). The nasal cavity has a total volume of approximatley 0.25 cm^3 and a surface area of about 13 cm^2. The nasal vestibulum is lined by stratisfied epithelium. The layer of the epithelium is covered with sticky mucus to prevent the lower airways against inhaled particles and gases (Morgan DA, 1998).

Comparison between humans and rats

Anatomical differences of both species are visible when comparing the airways branching schemes. Humans are more dichotomous while rats are monopodial (Yeh HC, 1979). Studies have shown that the particle deposition in a rat was not uniform and the highest deposition was measured in the right lung lobar (Raabe OG, 1977). It could be assumed that the right lung lobar of a rat has the shortest path length between the trachea and the terminale bronchioles as well as smallest number of generation. The pulmonary ventilation is basically similar for both species and is defined as the exchange of air between the lungs and the atmosphere. The inspired enriched oxygen air is transported to the alveoli and diffuses passivly through the alveolar walls to the overlying complex pulmonary capillary system with its numerous blood vessels. The oxygen is bonded to the haemoglobin and further transported to the organs in the body. The carbondioxide enriched blood reaches via the lung artery and its capillary network the alveoli. Carbondioxide is transported in the blood vessel and diffuses through the epithelium to the lumen of the alveoli and also mixes with the remaining air before expiring. However, the human and murine lungs differ in its physiology. The major differences are illustrated in table 1.

Tab.1: Physiological comparison of rat and human lung (adapted from Snipes et al. 1988)

Parameter	Rat	Human	Unit
Tidal volume	1.4	500	mL
Respiratory rate	130	12 - 15	Min^{-1}
Respiratory Minute Volume (RMV)	200 - 220	7500	mL/min
Airway resistance	0.27	0.0016	$cmH_2O/mL^{-1} * s^{-1}$
Fractional pulmonary deposition	0.07	0.25	1 µm particles

The normal tidal volume of adult human with bodyweight (b.w.) of 70 Kg is about 500 mL per breath. With a breathing frequency of 15 breaths/min, a total ventilation of 7500 mL/min is achieved (West JB, 2008). In contrast, the respiratory minute volume (RMV) of rat is between 200 – 220 mL/min (see table 1).
It has to be considered that due to the high metabolic rate of small animals, the air turnover in relation to the rats b.w. is very high. The ratio of minute volume for rats with an average b.w. of 0.360 Kg is approximatley 0.7 mL/min/g. In comparison, the ratio of minute volume to b.w. (74 Kg) is 0.08 mL/min/g for humans. This indicates that the relative inhaled aerosol dose for small animals is much higher compared to humans (Braun A, 2007).
However, the respiration varies depending on age and gender of the species. Also the clinical status of the human has to be considered. Due to the physiological differences between humans and rats, the pulmonary drug delivery is a major challenge in pharmaceutical research.

1.3.3. Pulmonary drug delivery

The administration of compound by local application in the lung is really a challenge when being compared with oral drug delivery. Hereby the most common techniques in use to administer compound directly to the lung include the intratracheal instillation (i.t.) and the aerosol exposure respectively.

1.3.3.1. Intratracheal instillation - advantages and disadvantages

The pulmonary compound administration by intratracheal instillation (i.t.) is the most common method applied in preclinical drug research. It is the simplest method to instill water soluble compound by a syringe directly to the lung while rats are anaesthetised. The major advantage of this technique is the direct application of compound to the lower respiratory tract. Deposition of compound in nasal passages or in the fur is eliminated. Easy compound administration as well as the application of the exact volume make this method so beneficial (tab. 2). However, it has to be considered that the deposition of large particles is unnaturally deep. More so, the distribution of compound is uneven in the lung (Osier M, 1997). Due to this fact, an alternative intratracheal method performed with the microsprayer can be applied. Liquid aerosol with dropletsize of about 20 µm is generated and injected directly to the lung. Compound in form of aerosol might prevent uneven distribution. Further advantages and disadvantages of intratracheal compound application are illustrated in table 1.

Tab.2: Advantages and disadvantages of intrapulmonary drug delivery by instillation

Pros	Cons
- Administration of the exact volume and total dose delivery	- Old, ungentle method
- Handling of solution/suspension is easier compared to dried powder	- High variability
- Low amount of compound needed	- Formulation-usage of fluid& detergent
- Suitable for screening and high throughput	- Anesthesia needed
- Particle deposition independent of particle size	- Unnatural deep deposition of large particles
- Easy handling	- Mechanical irritation of airways

The intratracheal application is not an appropriate technique for humans because of the unnatural physiological route of exposure. A further important aspect is the introduction of a cannula to the lung that causes airway irritations and might lead to further problems of the respiratory tract. The method is inconvinient for humans because daily adminstration is disadvantageous and anaesthesia is always riskful. Hence, it is important to find a natural way of compound adminstration to improve the pulmonary drug delivery for humans.

1.3.3.2. Inhalation with whole body and nose only exposure systems

The most physiological way of compound administration is given by the inhalation of aerosol. Aerosol is a mixture of gas and liquid which is created by nebulisation of compound suspension or dry powder. The generated aerosol consists of droplets which range in their diameter between 1- 100 µm. Rodents are exposed either in whole body inhalation chambers or with nose only systems to aerosolised compound droplets. Both inhalation methods are not easy in handling primarly due to the different devices of the tower (tab. 3). The exposure system requires in recent time technical updates and is associated with high investment and maintance costs. Intensive manuals and on site training are also required. However, the usage of the exposure systems has to be considered as inappropriate for the administration of cost intensive biopharmaceuticals. The efficiency is also very low due to deposition of compound in the upper airways of the rat (tab. 3). As an alternative, masks are introduced in aerosolised compound administration to monkeys and dogs. The method of inhalation is though appropriate for humans but it should be considered that large inhaled particles from humans might not be respirated from rats.

Tab.3:Advantages and disadvantages of nose only inhalation to rodents

Advantages	**Disadvantages**
• Most physiologic deposition • Exact dosing calculable • High animal throughput possible • Low animal loss	• Not easy to perform • Nasal deposition • High compound consumption • Aerosol generation difficult • Delivered dose ≠ deposited dose

The administration of compound to the lung by inhalation has the advantage to act locally. But most of the compound is not received at the site of action in the respiratory tract.
Due to the mucociliary transport, compound particles are transported with the bronchial mucus to the larynx and swallowed. This indicates that about 90% of the compound reach the gastro intestinal tract. The efficiency of the mucociliary transport depends on the movement of the cilics and on the viscocity of the bronchial mucus (Luellmann H, 2001). Most pulmonary drug delivery technologies have an efficiency of less than 20% and the lung dose might change from patient to patient to about 500%. Due to this fact, not only the potency of the drug has to be considered but the efficiency as well as the reproducibility of the applied technology for the treatment have to be treated .
The most important biophysical parameter in determining the pulmonary drug delivery is the aerodynamic particle behavior. This includes the variety of the particle size, shape, density, charge and surface properties. The drug delivery and deposition also depend on the morphology and airway anatomy of the respiratory tract as well as on the breathing pattern. The greatest difficulty is the extrapolation of data from rats to humans due to the different respiratory characteristics.

1.4. Aim of the master thesis

The main purpose of the master thesis was to establish and to study pulmonary compound administration with inhalation towers. Therefore, the nose only exposure system approach was hereby employed to administer aerosolised endotoxin to induce an acute pulmonary lung inflammation in rats. Previous studies with this same approach carried out with the inhalation tower have yield unsatisfactory result due to lack of reproducibility of collected data as well as high variation with the estimated data. It was generally assumed that non-uniform aerosol distribution within the exposure system is a major factor for this effect.
Therefore, to correct this effect, the technical equipment of the system was accordingly optimised. Hence various approaches applied in respect to various useful factors as enumerated below were addressed:

1. Investigation of aerosol distribution within the nose only exposure system.

2. Does the aerosol concentration change during experiments?

3. Test of a new application technique to achieve improvement, e.g. decrease of variation of data

A direct examination of aerosol or endotoxin concentration was performed technically, e.g. filter sampling method. In addition, aerosol concentration was monitored in real time with the help of MicroDust Pro device. Furthermore, the aerosol concentration was afterwards determined by indirect method through the means of measuring the neutrophilic cell amount in the BAL in vehicle treated rats.
The second part of the thesis mainly occupied with the pulmonary administration of compound. In the course of the thesis, two common pulmonary administration techniques were compared with each other as well as question on their efficiency was raised. All experiments and the investigations were conducted on nose only exposure system and through intratracheal instillation of compound respectively.

2. Material and methods

2.1. Material

2.1.1. Technical equipment

Device	Supplier/Manufacturer
Analytical balance Mettler Toledo Excellence PlusXP	Mettler Toledo AG (Giessen,Ger)
Analytical balance CP 153	Sartorius (Goettingen, Ger)
Animal balance BP1200	Sartorius (Goettingen, Ger)
Apex personal sampling pump	Casella measurement (Bedford,UK)
Centrifuge (multifuge 3 S-R, Heraeus)	Kendro Laboratory Products (Langenselbold, Ger)
Cytometer XT-2000iV	Sysmex (Norderstedt, Ger)
Incubator	Heraeus (Wehrheim, Ger)
Isofluran nebulizer Eickemeyer Vapor 19.3	Eickemeyer (Hannover, Ger)
Oxy-Vet (oxygen concentrator)	Eickemeyer (Hannover, Ger)
Particle measurement FPIA-3000	Sysmex (Norderstedt, Ger)
Pipettes (1- 10 000 µL)	Eppendorf (Hamburg, Ger)
Pocket pumps	SKC Inc. (Dorset,UK)
Thermomixer comfort	Eppendorf (Hamburg, Ger)
Ultrasonic bath Covaris™ S2x	K Biosciences Ltd. (Hoddesdon,UK)
Ultrasonic bath SONOREX RK100H	Bandelin electronic GmbH & Co. KG (Berlin, Ger)

2.1.2. Chemicals

Substance	**Supplier/Manufacturer**
Bovine serum albumin (BSA)	Serva (Cambridge, UK)
Phosphate buffered saline tablets (PBS, pH 7.4)	GIBCO Invitrogen Cooperations (Darmstadt, Ger)
Isoflurane	ESSEX Tierarznei (Munich, Ger)
0.9 % sodium chloride solution (NaCl)	B. Braun Melsungen AG (Melsungen, Ger)
Aqua ad injectabilia	B. Braun Melsungen AG (Melsungen, Ger)
Tween 20	Merck (Darmstadt, Ger)
Dulbecco PBS with Ca^{2+} and Mg^{2+}	Biochrom (Berlin, Ger)

2.1.3. Reagents and solution

Tab.4: Reagents and solution

Lavage buffer	0.5% BSA (Serva, Cambrige, UK) with 1x PBS tablet (Invitrogen Cooperations, Darmstadt, Ger) in 1 l Aqua dest.
Vehicle	0.02% Tween 20 (Merck, Darmstadt, Ger) in Aqua ad injectabilia (B. Braun Melsungen AG, Melsungen, Ger)
LAL assay stopping reagent	Ethanol 25% v/v in water (Roth, Karlsruhe, Germany)

2.1.4. Consumables

Material	Supplier/Manufacturer
Cannula (Sterican no. 17, 0.6 x 25 mm)	B. Braun Melsungen AG (Melsungen, Ger)
Centrifuge tubes (15 and 50 mL)	VWR (West Chester, USA)
Costar 96 well plate (clear, flat bottom)	Lonza (Cologne, Ger)
EDTA monovette (2.7 mL)	Sarstedt (Nümbrecht, Ger)
Haematology tube Vacuette®	Greiner bio-one (Frickenhausen, Ger)
Omnifix syringe (5 mL)	B.Braun Melsungen AG (Melsungen, Ger)
Plastipak syringe (3 -5 mL)	Becton & Dickinson (Heidelberg, Ger)
Pipette tips (1 -10 000 µL)	Eppendorf (Hamburg, Ger)

2.1.5. Kits

Device	Supplier/Manufacturer
Limulus Amebocyte Lysate (LAL) QCL-1000®	Lonza (Cologne, Ger)

2.1.6. Software

Software	Purpose
Edacq version 1.8	EMMS inhalation tower
Graph Pad Prism version 5	Analysis of data
Magellan version 6.5	Absorption measurement
Sysmex XT-2000i/XT-1800iV	Cell count
WinDust *pro* application software version 6	Aerosol monitoring

2.1.7. Technical equipment of the LPS inhalation system

Device	Supplier/Manufacturer
Filter GF/A (diameter 37 mm)	Whatman (Kent, UK)
Filter P-050	Cryo-Technik GmbH (Seevetal, Ger)
Filter cassette	Pall (Dreieich, Ger)
Flowmeter (0-17,5 L/min)	Analyt-mtc GmbH (Muellheim, Ger)
Gravimetric dust adapter	Casella measurement (Bedford, UK)
KL 1500 LCD	Carl Zeiss (Oberkochen, Ger)
Laboport N 811KN.18 vacuumpump	knf Neuberger (Freiburg, Ger)
Microdust Pro	Casella measurement (Bedford, UK)
Microtiterplate reader Safire	Tecan (Crailsheim, Ger)
Midget impinger 25 mL	Supelco Analytical (St. Louis, USA)
Nose only aerosol exposure system	CR Equipments SA (Coppet, Switzerland)
Pari LC sprint star nebuliser	Pari Medizintechnik GmbH (Munich, Ger)
Pari Master air compressor	Pari Medizintechnik GmbH (Munich. Ger)
Perspex animal exposure tubes	Battelle Geneve (Columbus, USA)

2.1.8. Technical equipment of the EMMS system

Device	Supplier/Manufacturer
AMP 110 adaptive amplifier	EMMS (Hants, UK)
Bias airflow generator	EMMS (Hants, UK)
Filter GF/A (diameter 24 mm)	Whatman (Kent, UK)
Filterholder open (diameter 24 mm)	Pieper Filter (Bad Zwischenahn, Ger)
Nebuliser Pro	Aeroneb (Bochum, Ger)
TPF 100 flow transducer	EMMS (Hants, UK)
TPM 100	EMMS (Hants, UK)
Tower pressure balancing assembly	EMMS (Hants, UK)

2.1.9. Test compound and formulation

The corticosteroid budesonide (BYK20702-9) was used for the experiments. Aqua ad injectiablia with 0.02% Tween 20 was used as a solvent the compound as well as a vehicle.

A predefined amount of compound was weighed out in Covaris glass tubes and solubilised with a defined vehicle volume. Subsequently, the solution was treated with high energy sonification (Covaris™ S2x) for 10 min to obtain application doses as indicated in table 5.

Tab.5: Formulation of the test compounds

Compound	Application technique	Amount [mg]	Vehicle [ml]	Concentration of the suspension [mg/mL]	Application dose [µg/kg b.w.]
BYK 20702-9	i.t.	1	8	0.125	100

2.1.10. LPS and formulation

Tab.6: Product information of the inflammatory substance lipopolysaccharide (LPS)

Compound	Product number	Manufacturer
Lipopolysaccharide (LPS, *Escherichia coli* 055:B5)	L2880	SIGMA Aldrich (Steinheim,Germany)

The preparation of the LPS solution was done 30 minutes before use to ensure that LPS was completely dissolved. The LPS was solubilised in a defined volume of PBS to achieve a concentration of either 1 or 3 mg/mL and to maintain pH value of 7.4. The solution of LPS in PBS was sonificated in an ultrasonic bath (SONOREX RK110H) at ambient temperature for 10 minutes.

2.2. Methods

2.2.1. Direct examination of aerosol distribution in the LPS nose only exposure system

2.2.1.1. Filter sampling

The filter sampling is a direct method to examine the aerosol distribution in an inhalation exposure system. Glass fiber filters GF/A with a diameter of 37 mm and nominal pore size distribution of 1.6 µm were used for the filter sampling method. Prior to the experiment, the filters were pre- weighed and inserted into a closed face filter cassette holder. The inhalation tower was preconditioned for 10 min with a 3 mg LPS/mL solution (see paragraph 2.1.10.). Subsequently, eight cassette holders were connected with antistatic black carbon tubes to the ports of the inhalation system. To begin with the exposure, the nebuliser was filled with 5 mL of LPS solution. Two opposite positions of each level were sampled for 30 min with pocket pumps at a flow rate of 0.2 L/min. This rate corresponds to the respiratory minute volume (RMV) of a rat (Alexander DJ, 2008). After the exposure, the filter was weighed again and dried for one hour at 60 °C in an incubation chamber. Finally, the dried filter was weighed again to determine the mass change and the collected LPS.

2.2.1.2. Aerosol sampling with midget impinger

Fig.4: Midget impinger (source:www.zefon.com/store/fritted-midget-impingerbubbler 25ml. html)

The standard usage of midget impingers instrument is ubiquitous in environmental and building technology to investigate the amount of microorganisms in the exhausted air (Duchaine C, 2001)

The impinger used to examine the aerosol distribution in an inhalation exposure system and also acts as an alternative to other direct methods in determining the aerosol concentration. The instrument is made of glass and consists of a flask and tubing system. Autoclaved impingers were used for this experiment. The flask has a sampling volume capacity of 25 mL and was filled with volume of 5 mL pyrogenic free water during the experiment. A glass tube projects in the flask and due to capillary effect, rises 5 mm above the flask bottom (fig.4). The experiment was carried out similarly to the filter sampling method under the same condition, where the same positions of the inhalation tower were sampled. A 1 mg/mL LPS solution was prepared as described in paragraph 2.1.10. 5 mL of the LPS solution were filled into the nebuliser and nebulised by the compressor. The aerosolised LPS sample to be analysed, flowed from the inhalation tower port by a glass tubing system into the bottle through the inlet. The humid aerosol was collected in the pyrogenic free water whereas the gas in the aerosol was collected at a flow rate of 0.2 L/min by the pocket pump (SKC Ltd, Dorset, UK) suction installed at the outlet.

2.2.1.3. Endotoxin detection

The quantification of LPS in the collected sampling water was performed by using the chromogenic endpoint detection Limulus amebocyte lysate (LAL) QLC 1000® kit (fig.5).

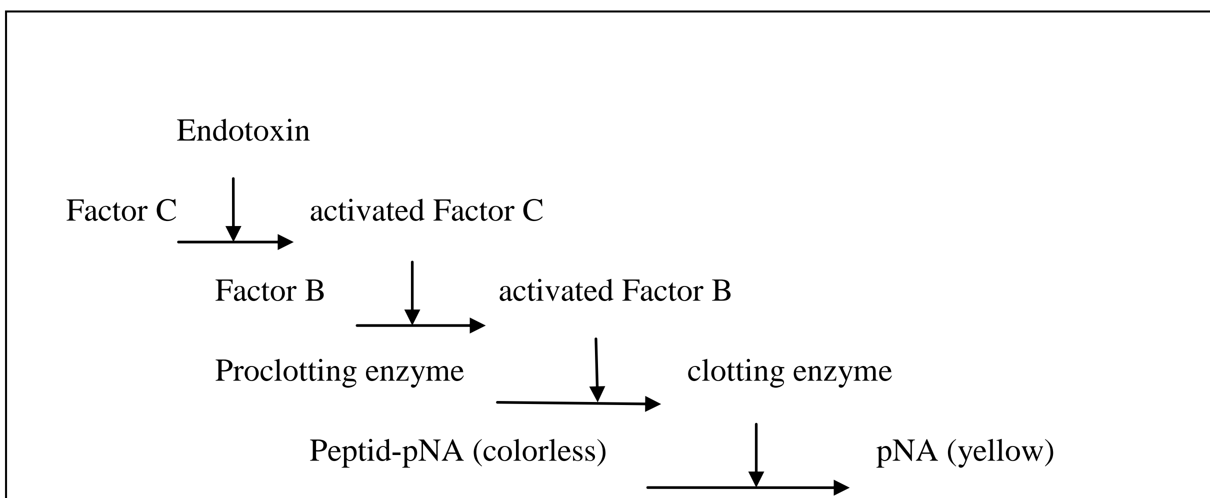


Fig.5: The coagulation cascade of the Limulus amebocyte lysate endotoxin detection test: An enzymatic reaction is initiated with the amebocyte lysate from the Limulus (horseshoe crab) and the presence of the detectable endotoxin. A generated clotting enzyme is responsible for the cleavage of the synthetic substrate into p-nitroaniline (pNA) which produces a yellow colour. The detection is executed photometrical at 405 nm (adapted from Ding,JL 2001).

The procedure was performed according to the manufactures instruction. Briefly, all samples, standards and blanks were analysed in duplicates. The standard of lyophilised endotoxin of E.coli (24 EU/mL) was reconstituted with 1 mL pyrogenic free water, pre-warmed to room temperature and vigorously shaken for at least 15 min. A serial dilution was prepared to create a standard curve. 50 µL standard/ well were carefully dispensed into the appropriate well of the 96 well plate. The endotoxin samples collected with the impingers were diluted with autoclaved PBS into autoclaved glass bottles. Subsequently, 50 µL of the sample were dispensed into the appropriate wells. Each sample was mixed with 50 µL of LAL reagent at t= 0 min. After 10 min of incubation at 37°C, 100 µL of the pre-warmed chromogenic substrate reagent (37°C, c= 2 mM) were mixed to each sample and incubated for 6 min. The reaction was stopped by adding 50 µL stop reagents 25% v/v of ethanol to each well.

The endotoxin concentration was photometrical analysed at a wavelength of 405 nm using the Magellan Version 6.5 software.

2.2.1.4. Aerosol monitoring

A further direct method to determine the aerosol concentration in the inhalation tower was performed using the Micro Dust Pro device. This method enables to examine the aerosol distribution in the exposure system within a certain time. Furthermore, the device was used to examine the change of aerosol distribution during an experiment in dependency of the occupancy of the inhalation system. The particulate concentration of an aerosolised 3 mg/mL LPS solution was measured by infrared light scattering (880 nm) within a range of 0 - 2750 mg/m^3. The measuring probe was connected by one junction of the Y-piece adapter to the inhalation tower. The other junction of the Y piece was linked to the Apex sampling pump to dilute the aerosol. The aerosol concentration was measured over a defined time and recorded. The data was read out with WinDust *pro* application software version 6 and analysed with the software Graph Pad Prism version 5.

All experiments were carried out without rats. Instead, tissue filled Perspex tubes named "dummies" were used. 40 dummies were divided into 10 groups. Table 7 shows the experimental setup of the aerosol monitoring. Four different techniques were used to examine the aerosol concentration variance during an experiment which is displayed in the results (see paragraph 3.3.).

Tab.7: Time table for experimental set-up of aerosolmonitoring

Group	t [min]
1	**0-30**
2	**5-35**
3	**10-40**
4	**15-45**
5	**20-50**
6	**25-55**
7	**30-60**
8	**35-65**
9	**40-70**
10	**45-75**

The experimental set-up was adopted according to the *in vivo* studies. A 3 mg/mL LPS solution was prepared as described in paragraph 2.1.10. The aerosol sampling was always performed at the tower level one and position 1.

2.2.2. Rat model of LPS- induced lung inflammation

2.2.2.1. Animals

Male *Sprague Dawley* (SD) rats with bodyweights ranging from 200 to 224 g were purchased from Charles River (Sulzfeld, Germany). Four rats were kept in one macrolon-typ IV cage at ambient temperature of 21 ±1 °C, relative humidity between 40 - 60 % and were kept in a dark and light cycle for 12 h. They had free access to water and food (standard rat diet 3873, NAFAG, Gossau, Switzerland). The rats were acclimatised for duration of at least five days prior to the experiment. All animal studies were performed according to the national animal protection laws and were approved by the local governmental authority (Ministerium für Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein V 312-72241.123-15 (53-6/08).

2.2.2.2. Experimental set-up

The following table 8 illustrates the experimental setup which was performed for the rat model of LPS-induced lung inflammation.

Tab.8: Experimental set-up of the LPS-induced lung inflammation

Time	***Procedure***
- 1 h	*Compound application by either/or:* • *Intratracheal instillation* • *EMMS nose only exposure system*
0 h	*LPS challenge*
+ 4.5 h	*Bronchoalveolar lavage (BAL)*

2.2.2.3. Compound application

2.2.2.3.1. Intratracheal instillation (i.t.)

The compound or vehicle was prepared as described in paragraph 2.1.9. The rats were anaesthetised by isoflurane inhalation (4-5 vol. % in oxygen) for 5 min. The front teeth of the rats were fixed with a rubber band at the application table. The tongue was pulled out by pincers and the compound was given intratracheally (i.t.). The trachea was intubated and 0.2 mL of compound suspension followed by 0.8 mL of air was administered to the lungs using a 1 mL syringe which was connected to the intubation device.
All i.t. applications were carried out within an interval of approximately one minute.

2.2.2.3.2. Nose only inhalation system for compound administration

The functional design of the nose only exposure system

The EMMS inhalation tower is a nose only exposure system (fig. 6) and is used to apply aerosolised compound to rats. The main compartments of the tower are described as followed.

Aerosol generation device

The generation of an aerosol is performed with the *Aeroneb Pro* nebuliser which consists of the OnQ^{TM}-Aerosol generator. This device includes a concave aperture plate which is surrounded by a vibrational element. The aerosol generator receives an electronic signal (128 kHz) from the control unit and causes over 120 000 vibrations per second. This rapid vibration causes a micro pumping effect. Compound is drawn through the holes to form equally sized droplets. According to the compound and droplet concentration, the aerosol droplet output is regulated by the Info Disp CND340 density control unit.

Distribution chamber

Following the aerosol generation, the compound droplets are transported with a flow rate of 10 L/min to the distribution chamber. The chamber functions as a transporting system.

Total particulate monitoring transducer TPM 100

The aerosol is further transported through the particulate measurement device TPM 100 to the inhalation tower. This unit controls the particulate concentration of the aerosol by the infrared light scattering technique (λ=880 nm). The device is integrated to the *EdacqVersion 1.8* software where the measured data is recorded and evaluated.

Inhalation tower

The nose only exposure of aerosols to rats is carried out with a 12 port inhalation tower. The functional design of the tower consists of an inner and outer tower. The inner tower conveys the aerosol through the system. The outer tower connects the inner tower with the rats in the Perspex tub*es (EMMS, Hants, UK).*

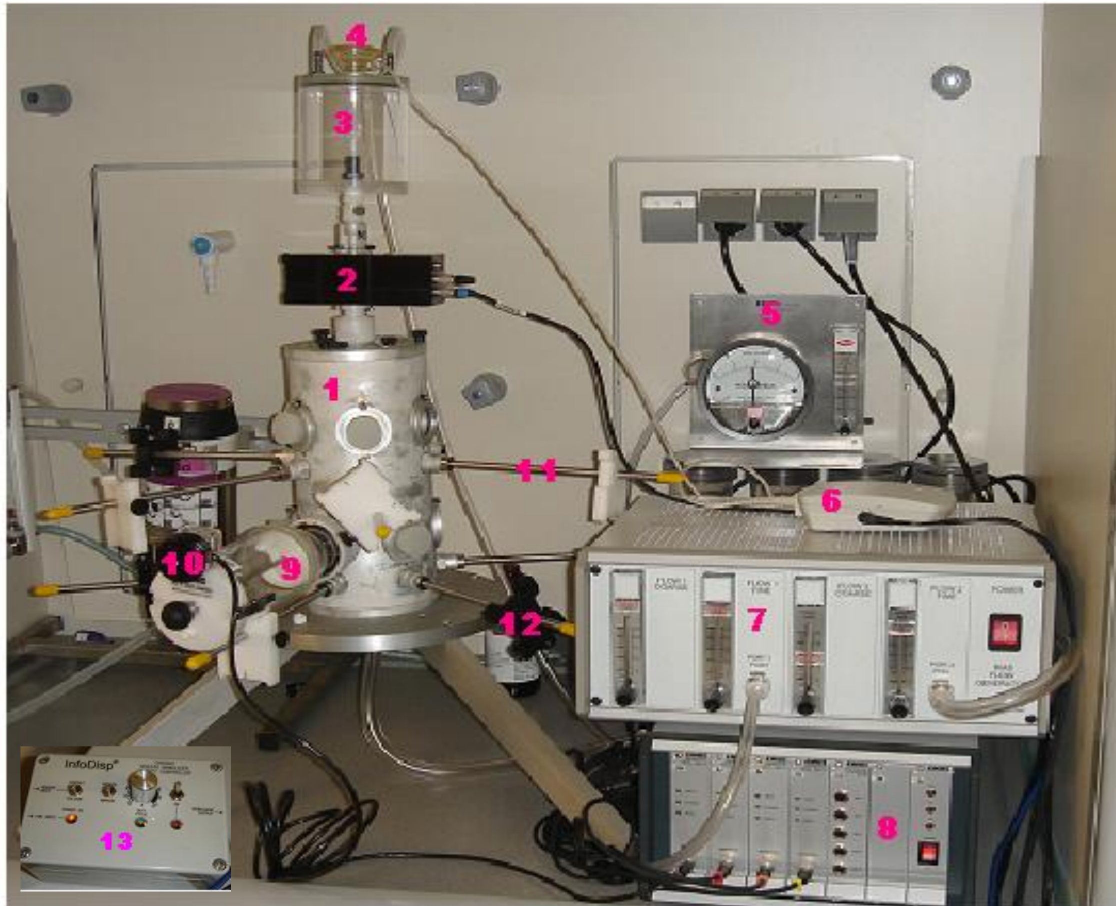


Fig.6: EMMS nose only exposure system for compound application: (1) 12 port inhalation tower, (2) TPM 100 aerosol monitoring, (3)Aerosol distribution chamber, (4) Aeroneb nebulizer, (5) Tower pressure balancing control, (6) Aeroneb Pro control unit, (7) Bias airflow generator, (8) AMP 110 adaptive amplifier, (9) Perspex tubes, (10) Bodyplethysmograph with TPF 100 flow transducer, (11) Support for the head out plethysmograph, (12) Support for the Perspex tubes, (13) InfoDisp density control unit

Bias airflow generator and tower pressure balancing assembly

An even distribution of aerosol in the tower is achieved by the generation of a constant flow. This is carried out by a bias airflow generator that transports compressed air with a flow rate of 10 L/min from the aerosol distribution chamber to the inhalation tower. Additionally, 5 L/min of aerosol is sucked out of the tower and further transported to the cleaning station. The in- and output flow control is performed by a tower pressure balancing assembly.

Cleaning station

It is required to eliminate the aerosolized compound from the exposure system to avoid contamination of the airflow generator. The cleaning station, consisting of four attached polycarbonate columns filled with orange silica gel beads operate at a maximum pressure of 6 bare. Silica beads have a large surface area and absorb water due to hygroscopic

characteristics. The absorbance of water induces a colour change from orange into white silica beads.

Head out plethysmography

The head out plethysmography is a method to determine the lung function of the rat during the experiment. The rats are placed into the Perspex tubes which are equipped with latex rubber neck seals to ensure that the chamber is widely closed. Due to the air tightness the thoraxic movement of the rat inside the chamber is determined. The developed pressure differential inside the chamber is measured and transduced into an electrical signal by the TPF 100 flow transducer which is connected to the wall of the plethysmographic tube.

AMP 110 adaptive amplifier

The AMP 110 adaptive amplifier is a connection between the TPF 100 flow transducer and the graphic data output *Edacq version 1.8* program. The amplifier has the function to convert the measured signal into an imaging signal.

2.2.2.3.3. Nose only compound application

The nose only exposure system is a technique used for direct compound administration to the lung. The nose only compound application (tab.9) was carried out with two groups, each consisting of 12 SD rats.

Tab.9: Compound application by the nose only exposure system

Group	**Compound**	**Dose [µg/kg b.w.]**
1	Vehicle	-----
2	BYK20702-9	100

A group of 12 SD rats was placed into Perspex tubes five minutes before the exposure started to provide a short acclimatisation time. During this time, 1 mL of the compound solution was filled into the nebuliser. The rats were placed into the inhalation system individually and exposed nose only to the aerosolised compound for 10 min. During the exposure, the nebuliser was refilled to achieve a constant aerosol distribution. The input droplet concentration of the compound aerosol was controlled by the TPM 100 aerosol monitoring device to 5 mg droplets/m^3. In addition, four of the Perspex tubes were connected with the TPF 100 flow transducer to perform a head out plethysmography. This device controls the respiratory minute volume and the tidal volume in order to calculate the amount of inhaled compound for each animal.

2.2.2.4. LPS- induced pulmonary neutrophilia

Exposure of rats to aerosolised LPS causes a neutrophil-dominated airway inflammation that can be assessed in the bronchoalveolar lavage (BAL). The nose only LPS challenge was conducted exactly 60 minutes after the compound application. The LPS solution with a concentration of 3 mg/mL was prepared as described in paragraph 2.1.10. The LPS challenge was carried out with the nose only exposure (inhalation tower) system where every rat was exposed for 30 min (fig. 7).

Fig.7 : LPS-COPD nose only exposure system: (1) compressor, (2) PariNeb, (3) Aersoldistribution-orifice, (4) inhalation tower consisting of four level (level 1 to 4 top down), (5) application of rats in exposure tubes (Batelle,Geneve), (6) tower outlet pipe (7) flowmeter

The inhalation tower has 32 ports and comprises of four separated levels. The *PariLC* sprint star nebuliser was filled with 5 mL of LPS solution. From the LPS solution, the aerosol was generated by passing 6 L/min of compressed air from the Pari Master compressor to the nebuliser. The aerosol was further transported by a tubing system to the tower system. Along the path to the tower system, the aerosol was diluted with a controlled air flow of 10 L/min.

Prior to the experiment, the inhalation tower was preconditioned with aerosolised LPS for 10 min, where all ports were plugged to achieve an even distribution in the system. Five min before the exposure start, rats were placed into the Perspex tubes to provide a short acclimatisation time.

2.2.2.5. Bronchoalveolar lavage (BAL)

4.5 hours after the LPS exposure rats were euthanized by isoflurane inhalation (4-5 vol. % in oxygen) for 5 min followed by cervical dislocation. The trachea was prepared. A plastic cannula was inserted into the trachea and a volume of 5 mL of BAL buffer was injected. The injected fluid was aspirated with a sterilised syringe and the lavage was repeated once again with 5 mL of BAL buffer. The accumulated sampling fluid was analysed using the cytometer XT- 2000iV and the total amount of cells was counted. In addition, the cell differentiation was performed to distinguish between by total white blood cells, neutrophils, monocytes and total lymphocytes in the BAL.

2.2.2.6. Data analysis

All data were analysed and illustrated using GraphPad Prism 5.0 software. Unless otherwise indicated all data were analysed by mean ± SD. Statistical analysis was done testing the neutrophil numbers of each drug-treated group vs. the vehicle-treated control group using One-Way ANOVA and Dunnett's multiple comparison test. P values < 0.05 were considered statistically significant. The mean of neutrophil numbers of the negative control group served as the baseline level. Baseline correction was done for each sample according to the formula:

Baseline-corrected neutrophil count = observed neutrophil count – mean (neutrophil count of negative control group)

All further calculations were performed with baseline-corrected values.

The inhibitory effect of the drug on neutrophil numbers is given in percent (% effect) and calculated according to the formula:

% effect = (Y-K)/K*100

With defining:

Y= Mean of the compound-treated group

K= Mean of the vehicle-treated group

3. Results

The aim of the thesis was the establishment of the nose only inhalation system that is used generally to apply LPS to rats. The tower comprises of four levels numbered 1 to 4 top down in which each of the levels is interconnected internally by a connecting tube going through the centre of the hollow inhalation tower. Each level is separated from the outside of the tower by means of a metallic disc for stability reasons during experiment with rats (see fig. 8a). The connecting tube is branched off into eight smaller tubes per level that end at the outside of the inhalation tower. These are the ports where each rat is inserted and also exposed.
In total, the inhalation tower consists of 32 ports where restrained rats in Perspex tubes are exposed to compound (see fig. 8b). Aerosolised compound is generated by a nebuliser and transported by compressed air via the tower inlet to the exposure system (fig. 8a). The aerosol streams passively out of the tower. Prior to every exposure, all tower ports are sealed by rubber plugs (fig. 8c).

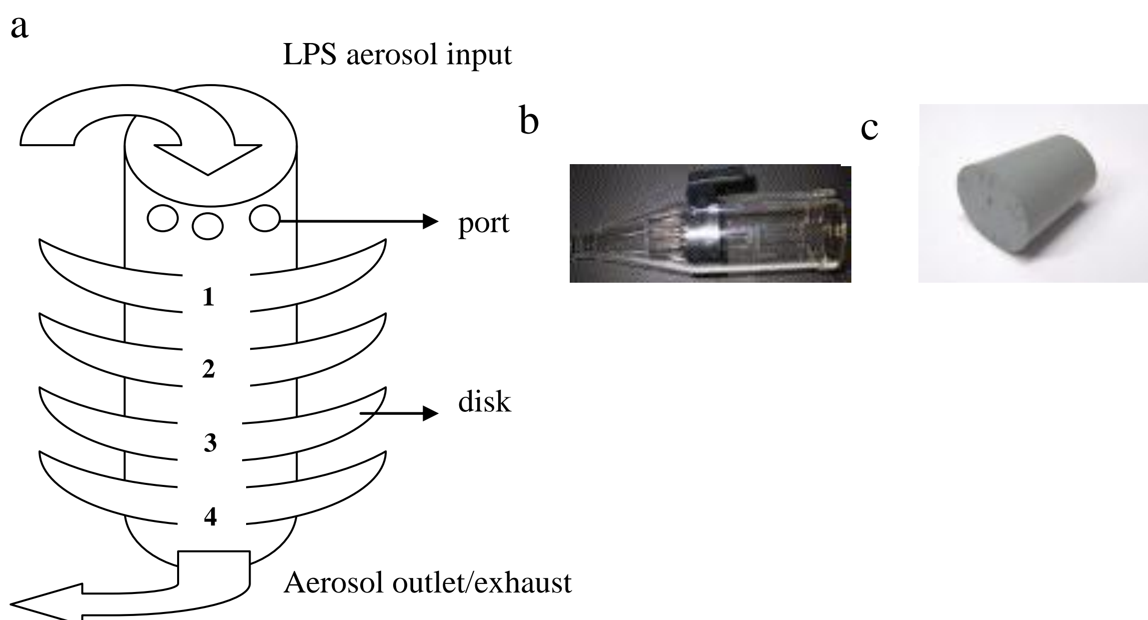


Fig.8: Equipment of the inhalation tower: (a) nose only exposure system with four levels and each level numbered 1 to 4 top down, (b) Perspex tubes to restrain rats and expose them nose only (source: Sinswat P,2008); (c) rubber plugs to close the ports

3.1. Direct examination of aerosol distribution by filter sampling

3.1.1. Filter sampling

The filter sampling was performed, as described in paragraph 2.2.1.1. to examine the aerosol distribution within the nose only exposure system. On the tower, two positions opposite each other on a level were sampled. Every single filter, fixed in a filter holder and connected to a pocket pump (flow rate of 0.2 L/min) was exposed to LPS aerosol for 30 min. Three different techniques were at one hand applied to determine the LPS distribution and on the other hand to improve the tower system (tab. 11). All samples were taken from the same positions of the tower.

Tab.11: Application techniques of filter sampling

Technique	Conditions
1	All ports are plugged
2	All ports are open
3	All ports are occupied with dummies*

**dummies are tissue filled Perspex tubes*

The first technique performed with closed ports (fig. 9a) showed a drop in the median value of collected mass from level 1 to 4. The mass of filter samples from the first level has a range of 0.2 – 0.9 mg. In contrast, a slight mass change of value 0.1 – 0.2 mg as well as no mass change was weighed on the last level (level 4). The second experiment performed with the open ports has a median with significant variation in the tower levels (fig. 9b). After introducing dummies (third technique) to the inhalation tower, the recorded value for the mass changes of the sampled filters was between 0.1 – 0.6 mg (fig. 9c). The median value of all sampled levels is approximately similar.

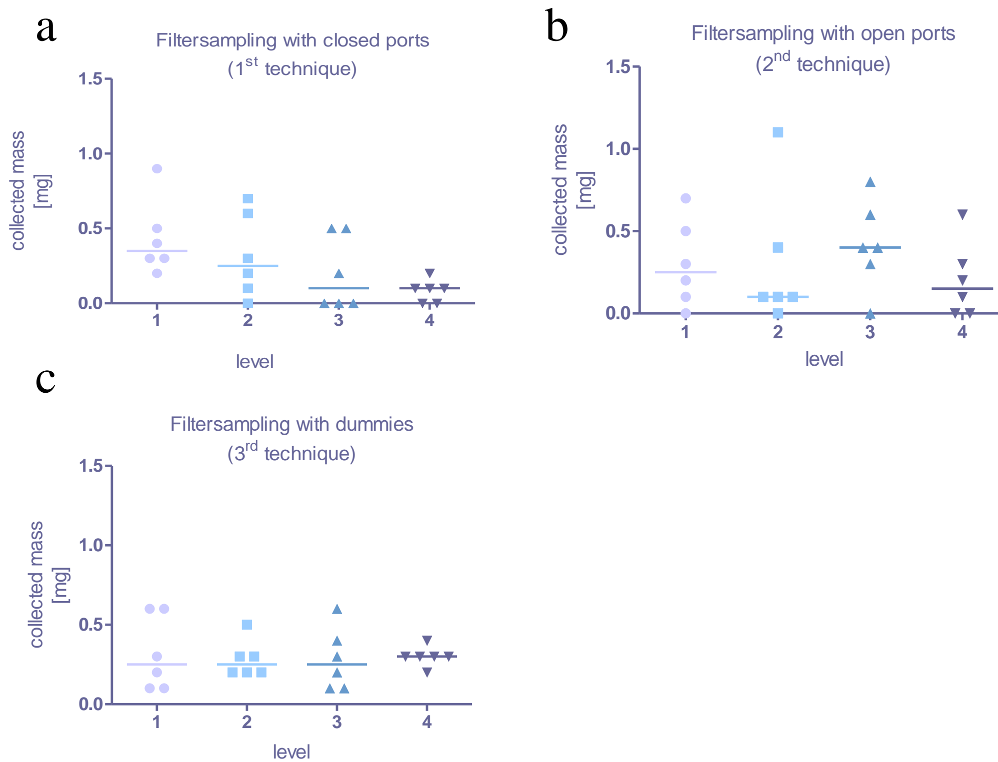


Fig.9: *LPS mass collection by filter sampling method: The tower comprises four levels and is numbered top-down; two filters are sampled per level in triple replicates, all six data points are illustrated with the median; (a) inhalation system with plugged ports, (b) inhalation system with unplugged port, (c) inhalation system occupied with dummies*

From the result, it could be shown that the lowest median variations between the tower levels were achieved by the third technique with the dummies. This shows and proofs the importance of applying improved techniques.

To determine the results precisely, the inhalable dose for each tower position as well as sampling technique would hereby be considered and examined.

3.1.2. Calculation of inhalation dose of LPS after filter sampling

Data from filter sampling illustrated in paragraph 3.1.1. was used in evaluating the corresponding inhalation dose of LPS (in µg). Afterwards data of the filter sampled position was summarised for one of the levels in order to clarify the quantity of LPS received at each tower level.

The inhalation dose for one rat was calculated according to the following formula:

$$\text{Inhalation dose} = \frac{(\text{aerosol concentration} * \text{sampling flow} * \text{sampling time})}{1000}$$

whereas:

(Aerosol concentration = filter mass / collected sampling volume)

and

collected sampling volume = ((sampling time * sampling flow)/ 1000) = 0.00594 mg/m^3

sampling time = 30 min

sampling flow = 0.2 L/min

The estimated statistical mean value as well as the standard deviation, SD of LPS doses in µg together with the coefficient of variation, CV are illustrated in the following table 12.

Tab.12: Determination of inhalation doses (in µg, mean ± SD, CV, n=6) of three application techniques through filter sampled mass change

Level	Application techniques (mean ± SD,CV)		
	1st technique closed ports	2nd technique open ports	3rd technique dummies
1	453.52 ± 290.75 64%	333.33 ± 289.74 86%	399.99 ± 265.68 66%
2	351.85 ± 309.65 88%	333.33 ± 460.80 138%	333.33 ± 129.89 38%
3	222.22 ± 272.16 122%	462.96 ± 301.57 65%	355.55 ± 215.64 60%
4	92.59 ± 83.64 90%	222.22 ± 253.37 114%	311.11 ± 70.27 22%
Mean ± SD, CV of level 1-4	280.04 ± 275.82 98%	337.96 ± 325.14 96%	326.96 ± 174.26 53%

The first applied technique with closed ports showed a drop of inhalable doses across level 1 to 4 (tab. 12). An inhalation dose with a mean value of 453.52 µg and a SD of 290.75 µg was estimated for the first tower level whereas in the fourth level an estimated mean value of 92.59 µg and a SD 83.64 µg for the inhalation dose was achieved. Invariably, with this technique, all the levels in the tower have a total estimation mean value of 280.04 µg and a high standard deviation of 275.85 µg. This high SD value is presumably an effect from some filter samples without any mass changes.

On the other hand, the estimated inhalation dose for the second technique performed with open ports has a mean value of 337.96 µg and a SD of 325.14 µg. Though a lower evaluated CV value of 96% was measured by this technique but still a high variation between all levels is significant.

However, with the introduction of dummies to the tower (third technique) nearly similar amount of the inhalation dose was recorded in all the four levels of the inhalation tower. This is observed and seen firstly by the low SD value of 174.26 µg. In comparison to the first technique, a drastic drop in the CV value from 98% to 53% with the third technique is hereby evaluated.

The other important factor to be considered after the determination of LPS doses is the LPS loss within the exposure system. Hence the measurement of every LPS output was hereby conducted. To generate this, the nebuliser was weighed before and after each exposure experiment. In table 13 below is illustrated the estimated value of the statistical parameter (mean and SD) from the nebuliser output as well as the corresponding LPS output during the 30 min exposure of 3 mg/mL LPS solution.

Tab.13: Determination of the nebuliser output (mean ± SD) in a 30 min exposure of 3 mg/mL LPS solution

Technique	Nebuliser output in ml (mean ± SD)	LPS output in mg (mean ± SD)
1 (closed ports)	3.65 ± 0.66	10.95 ± 1.95
2 (open ports)	4.11 ± 0.36	12.33 ± 1.08
3 (dummies)	3.53 ± 0.21	10.59 ± 0.75

The mean value of LPS output in mg for each technique was normalised in order to calculate the inhaled dose in percent (%) and also to subsequently determine the entire LPS consumption in the exposure system during the sampling of eight ports.

The inhalable LPS dose in % per level was calculated according to the following formula:

$$\text{Inhalation dose per level (in \%)} = \frac{x}{y} * 100$$

with *x: mean inhaled amount of LPS for one level (in mg)*

y: total nebulised amount LPS during one exposure (in mg)

and *y: nebuliser output during 30 min exposure (mL) * concentration of LPS solution (in mg/mL)*

Example for the calculation of the inhaled dose per level (in %) with data from the first level and first technique:

x = 0.45352 mg
y = 3.65 mg*3 mg/mL=10.95 mg

Inhalation dose per level (in %) = (0.45352/10.95)*100 = 4.14 %

Each technique was at first evaluated and then summarised in order to determine a total consumption of LPS (%) during one exposure. From this, the LPS loss can be determined. The inhaled dose of LPS per level (%) is illustrated in the following table 14. From the table 14, it could be taken that the same trend of aerosol distribution as already described above in measured mass changes on filter and the corresponding inhaled doses in µg exists.

Tab.14: Evaluation of LPS output of three different application techniques (in %, mean ± SD)

level	**inhaled dose of LPS per level [in %, mean ± SD]**		
	1st technique closed ports	2nd technique open ports	3rd technique dummies
1	4.14 ± 2.65	2.70 ± 2.34	3.77 ± 2.50
2	3.21 ± 2.85	2.70 ± 3.73	3.14 ± 1.22
3	2.02 ± 2.48	3.75 ± 2.44	3.35 ± 2.03
4	0.85 ± 0.76	1.80 ± 2.05	2.96 ± 0.67
Σ level 1- 4	**10.23 ± 8.73**	**10.96 ± 10.58**	**13.22 ± 6.43**
Loss of compound	**89.76 ± 1.49**	**89.04 ± 0.37**	**86.77 ± 6.78**

Considering the inhalation system as an entire piece, the application of the first technique resulted in a total mean value of 10.23% collected LPS on eight filters and a significantly high SD of 8.73%. The data evaluated with the open ports (second technique) varied slightly to the closed ports with the total mean value of 10.96% and SD of 10.58%. The total LPS collected on the third technique using dummies amounts to a mean value of 13.22% and a reduced SD of 6.43%. The low SD is attributed to little or no mass change on the sampled filter.

However, loss of LPS highly depends on the construction of the inhalation tower and is hardly preventable. In addition, the application technique might as well influence the loss of compound. The highest loss of compound was generated with the first and second technique whereas the dummy technique had the lowest loss of LPS recorded.

In conclusion, it was shown that the aerosol distribution within the inhalation tower strongly depends on the applied technique. Partly non-uniform distribution of aerosol in the tower has led to high variation between the ports and the levels. Due to this reason, experimental studies were performed to precisely examine the aerosol distribution within the single ports and the levels of the tower.

3.1.3. Filter sampling of the single inhalation tower levels

The filter sampling was performed to determine the aerosol distribution of every single level. Every level was sampled separately while the rest of the ports were plugged. To start with, the closed port technique (first technique) was chosen because highest mass changes between the levels were obtained in the previous filter sampling experiments and a more precise examination is necessary.

All eight ports of one level were connected with filters, filter holders and sampling pumps (flow rate = 0.2 L/min) in one row and sampled for 30 min (fig.10).

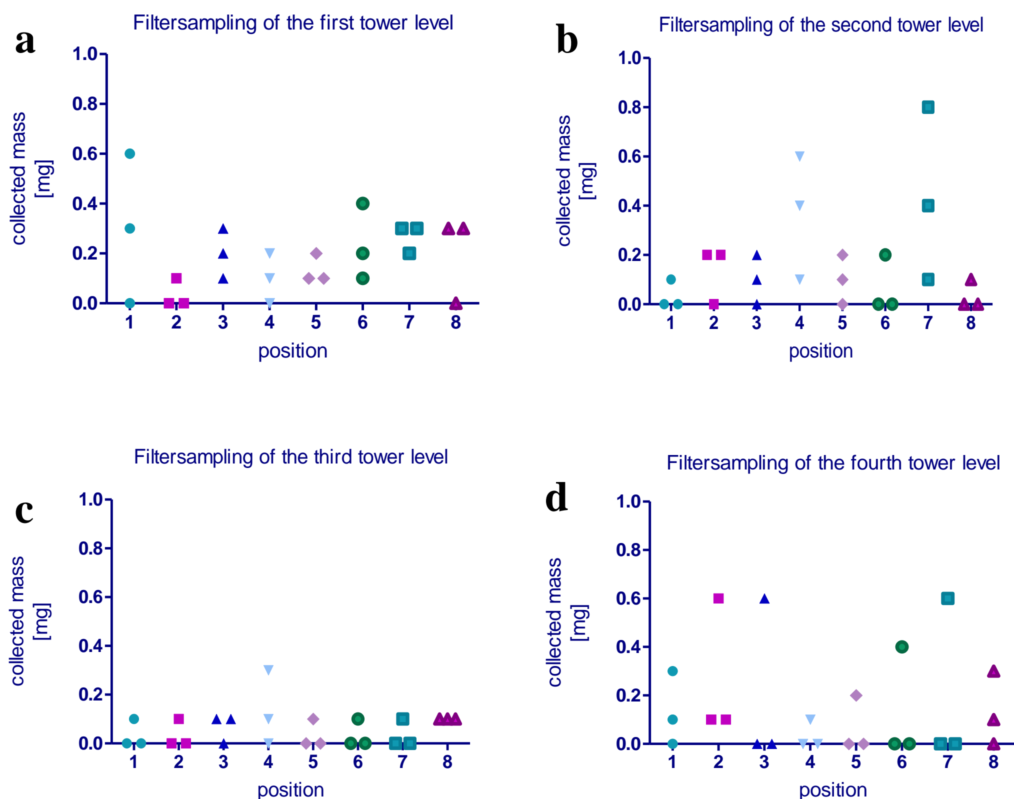


Fig.10: LPS mass collection by filter sampling: All experiments were performed as triple replicates; n=3; (a) tower level 1, (b) tower level 2, (c) tower level 3, (d) tower level 4

Results from the first level had a variation in collected mass in the range of 0 - 0.6 mg although frequent data were measured in the range of 0 - 0.4 mg. Considering the second tower level, high variation in the data of collected mass between 0 - 0.9 mg was estimated.

The lowest variation of mass change was obtained within the third level where most of the data show either no mass change or only 0.1 mg. The collected mass from the fourth level weighed between 0 - 0.6 mg even though half of the samples after weighing show no mass change. Due to the high variation in filter mass changes of all levels, it was assumed that the aerosol distribution within the levels is not uniform especially for levels 1, 2 and 4.
In summary, it could be assumed that the filter sampling method might not be an appropriate and reliable method in analysing and determining the aerosol concentration due to the low mass quantity collected on the filter. Therefore, an alternative method has to be considered to quantify the LPS output of the inhalation system.

3.2. Direct examination of aerosol distribution by endotoxin detection with the limulus amebocyte lysate (LAL) assay

3.2. 1. Quantitative endotoxin detection

The quantitative endotoxin detection with LAL Assay was used as an alternative method to the filter sampling. The approach was to examine the LPS output and the aerosol distribution within the nose only exposure system. Instead of filters, impingers were used to collect the nebulised LPS in a sampling fluid which was subsequently analysed with the LAL assay. Firstly, two impingers per tower level filled with pyrogenic free water were connected opposite each other to the tower ports. A sampling pump with a flow rate of 0.2 L/min was connected to each impinger to collect the aerosol into fluid and to simulate the RMV of the rats. 1 mg/ml LPS solution was aerosolised for 30 min.
A further approach of the applied method was to determine if new techniques, e.g. application of dummies or vacuum pump improve the inhalation system and whether a reduction in the high variations in aerosol distribution between the tower levels could be reached. Hence, the in table 15 described techniques were performed.

Tab.15: Applied techniques for LPS quantification by LAL endotoxin detection assay

Technique	Condition	Abbreviation
4	All ports are plugged	Closed ports
5	All ports are plugged and a vacuum pump is attached (flow rate = 9 L/min)	Closed ports and vacuum
6	All ports are occupied with tissue filled Perspex tubes (dummies)	Dummies
7	All ports are occupied with tissue filled Perspex tubes (dummies) and a vacuum pump was attached (flow rate =9 L/min)	Dummies and vacuum

Sampling performed with closed ports (fourth technique) achieved the endotoxin concentration mean values between 17.49 x 10^5 EU/mL and 32.55 x 10^5 EU/mL whereas the endotoxin concentration increases from level 1 to 4 (tab.16). Due to high variability estimated for the entire inhalation system with a CV value of 39%, it is assumed that differences in the atmosphere between the tower levels still persist.

Tab.16: Determination of endotoxin concentration of three application techniques (mean ± SD, CV)

Level	endotoxin concentration [x 10^5 EU/mL; mean ± SD, CV]			
	4th technique	**5th technique**	**6th technique**	**7th technique**
1	17.49 ± 17.90 102%	27.03 ± 3.41 12%	25.43 ± 1.21 4%	20.45 ± 0.64 3%
2	22.89 ± 3.52 15%	23.60 ± 6.16 26%	27.19 ± 0.69 2%	21.47 ± 0.65 3%
3	25.51 ± 9.46 37%	28.94 ± 0.36 26%	25.22 ± 3.97 15%	21.95 ± 0.39 2%
4	32.55 ± 0.02 0.65%	26.49 ± 4.73 17%	22.44 ± 3.97 35%	19.20 ± 3.59 18%
Mean ± SD, CV of level 1 - 4	**24.61 ± 9.69 39%**	**26.51 ± 3.80 14%**	**25.07 ± 3.83 15%**	**20.77 ± 1.79 8%**

To optimise the experimental set-up and thereby reducing the high variations in the data, a vacuum pump was applied to the tower outlet (fifth technique). The results achieved for the endotoxin concentration from the fifth technique is in the order of 23.60 x 10^5 EU/mL and 28.94 x 10^5 EU/mL. The highest concentration was determined in the third level and the lowest in the second level (tab. 16). Comparing this result with the fourth technique, it can be assumed that a more uniform aerosol distribution with a drastic drop in the CV value from 39% to 14% was herewith achieved though the LPS concentrations still varies. A further technique to improve aerosol distribution within the tower and to reduce the variability where dummies were applied to the remaining unused ports (sixth technique). The result of the endotoxin concentrations for the sixth technique ranged between 22.44 x 10^5 EU/mL and 27.19 x 10^5 EU/mL. A change in endotoxin concentration from the second level to the fourth level is hereby generated (fig. 11c). The approach to reduce the variation in the estimated data was only partly possible as differences seen between the estimated CV values for this technique could not be neglected. The first and second tower levels have relatively low values of CV ranging between 2% and 4% whereas the third and fourth level show higher CV values between 15% and 35% (tab. 16).

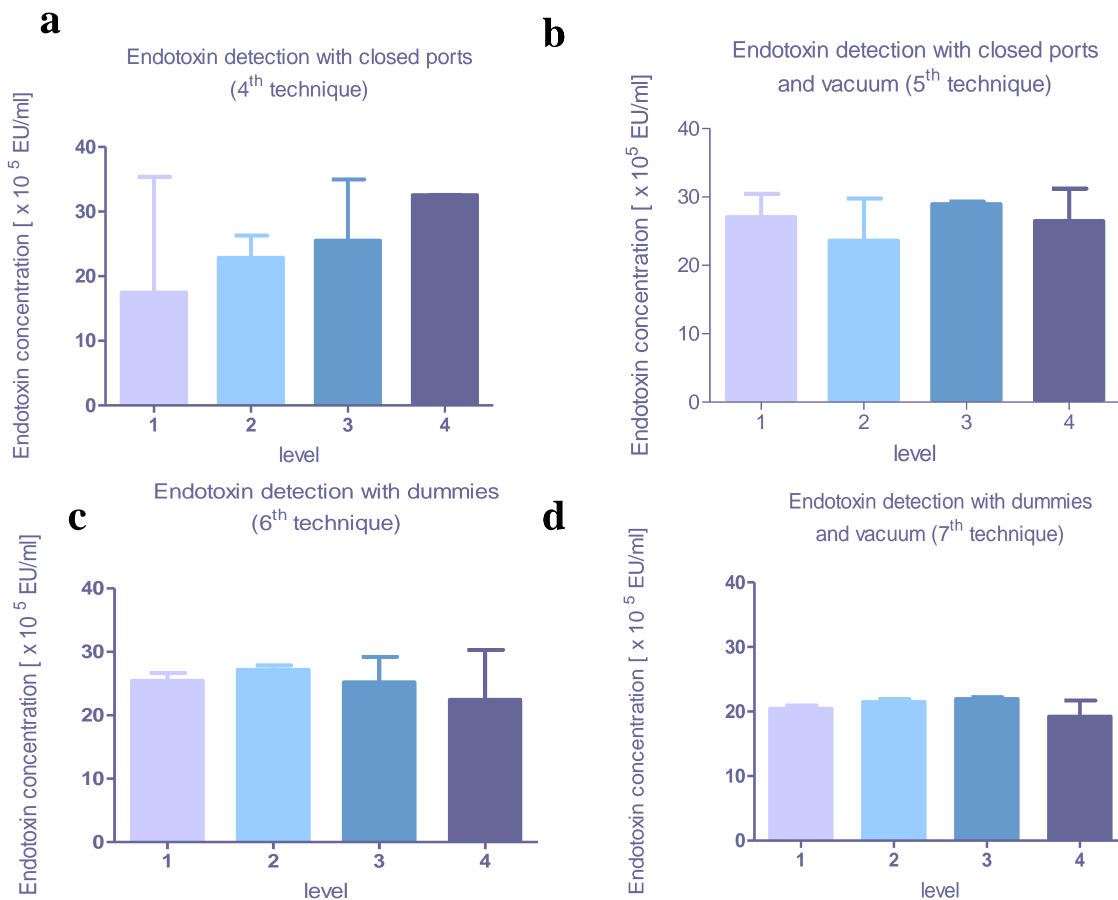


Fig.11: Endotoxin quantification of LPS aerosol: Eight impingers were connected to the inhalation tower. Two impingers per level were located opposite to each other. A 1 mg/mL LPS solution was nebulised for 30 min and collected into pyrogenic free water in impinger; subsequent the collected fluid was diluted and analysed with the LAL Assay QCL 100 (a) closed ports (b) closed ports with vacuum pump (c) dummies (d) dummies with vacuum pump

The necessity to improve the variation in the estimated CV value of the endotoxin concentration by improving the sixth technique was considered. This was achieved by installing a suction pump to the tower (seventh technique). From the generated results, a mean value in the range between 19.20 x 10^5 and 21.95 x 10^5 EU/mL of the endotoxin concentration was estimated. A similar concentration between 20.45 x 10^5 EU/mL and 21.95 x 10^5 EU/mL was measured in all the levels in the tower which led to a low CV value of 8%.

From this result, uniform distribution of aerosol within the tower is hereby assumed. Therefore, it was demonstrated that the application of dummies and a vacuum pump reduced the high variation and improvements was achieved. Considering the fourth and seventh technique, a substantial drop in the variation value from 39% to 8% was achieved (tab. 16).

However, it is of high interest that a more precise determination of the inhalable dose for each tower position and corresponding sampling technique is to be calculated.

3.2.2. Calculation of inhalation dose of LPS

The approach enables to determine the corresponding inhalation dose in percentage and to evaluate the consumed LPS at each level of the tower as well as the loss of quantity. Therefore, the determination of LPS output for every exposure was required. The nebuliser was weighed before and after the exposure. The table 17 below illustrates the nebuliser output (in mL). 1 mg/mL LPS solution was nebulised for 30 min.

Tab.17: Determination of the nebuliser output in 30 min exposure

Technique	**Nebuliser output [in ml]**
4 (closed ports)	3.9
5 (closed ports/pump)	4.15
6 (dummies)	4.03
7 (dummies/pump)	3.98

The LPS output of each technique was normalised to calculate the inhaled dose in percentage and to subsequently determine the entire LPS consumption in the exposure system during the sampling of eight ports. The inhaled dose of LPS per level in percent is illustrated in the following table 18. From this, it could be seen that the same trend of aerosol distribution as already described above in measured mass changes with the endotoxin detection test was shown.

Tab.18: Determination of inhaled dose of LPS in % (mean ± SD) per level and for each technique; determination of total amount of consumed LPS

Level	**inhalation dose [in %]**			
	4th technique	**5th technique**	**6th technique**	**7th technique**
1	4.48 ± 4.59	6.51 ± 0.86	6.31 ± 0.30	5.13 ± 0.16
2	5.85 ± 0.88	5.68 ± 1.48	6.74 ± 0.17	5.39 ± 0.16
3	6.54 ± 2.42	6.97 ± 0.08	6.26 ± 0.98	5.51 ± 0.09
4	8.34 ± 0.01	6.38 ± 1.13	5.56 ± 1.96	4.82 ± 0.89
Σ level 1 - 4	**25.23 ± 2.01**	**25.56 ± 3.53**	**24.89 ± 0.82**	**20.87 ± 0.38**
Loss of compound	**74.77 ± 2.01**	**74.44 ± 3.53**	**75.11 ± 0.82**	**79.13 ± 0.38**

The mean value of LPS inhalation doses varied between 20.87 - 25.56% in all the four different application techniques. The highest amount of LPS was consumed in the first and second technique. In comparison, the lowest LPS consumption was achieved at the fourth technique. This indicated that the dummy technique together with the installed vacuum pump improved the aerosol distribution as well aid the spread uniformly within the tower system as seen from the evaluated mean value of 20.87% and the very low SD of 0.38%.

It was demonstrated that the aerosol distribution within the inhalation tower depends strongly on the applied technique. However, to consider the closed port technique, the

aerosol was partly distributed non-uniformly through the tower which has the adverse effect on the high variations between the levels and ports. But the improved technique with the dummies and the creation of vacuum at the tower outlet led to uniform distribution of aerosol as shown in the drop of variation between the levels in the tower.
A further aspect to be considered with the use of the nose only exposure system is the determination of changes in aerosol concentration during an experiment. Therefore, the following experiments were monitored in real time.

3.3. Determination of aerosol distribution by real time monitoring

The aerosol monitoring with the MicrodustPro (Casella, Bedford, UK) device is a further method to examine the aerosol distribution within the exposure system. Hence, the following experimental set-up was performed to compare the standard *in vivo* method with improved techniques in consideration to the aerosol concentration in real time (tab. 19). All experiments were simulated and instead of rats, tissue filled Perspex tubes (dummies) were employed. All measurements were taken at the first level and first port number.

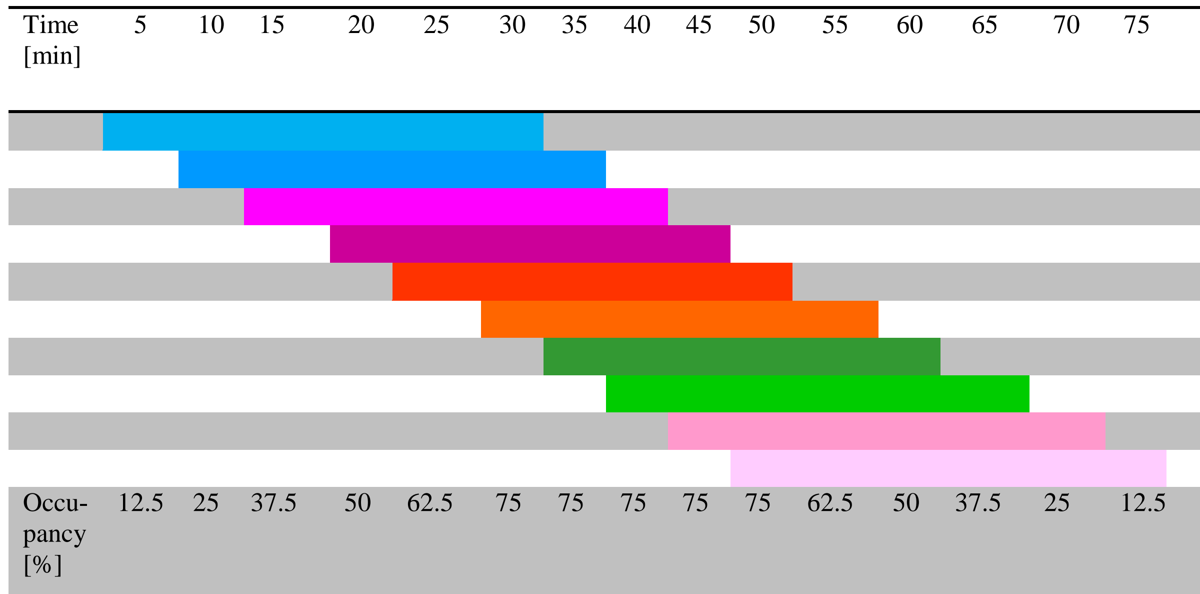
Tab.19: Experimental set-up for all aerosol monitoring experiments

Technique	**8**	**9**	**10**	**11**
Preconditioning [min] with c= 3 mg/ml LPS	10	10	10	10
Exposure time [min]	75*	75*	75*	75*
Number of dummies	40	40	40	40
Setup	all ports were plugged (standard *in vivo* method)	all ports were plugged and a vacuum pump was attached to the tower outlet	all ports were occupied with dummies (improved method)	all ports were occupied with dummies and a vacuum pump was attached to the tower outlet (improved method)
Experimental Set-Up	every 5 minutes applicati on of four dummies (one per level)	every 5 minutes application of four dummies (one per level)	every 5 minutes application of four dummies (one per level)	every 5 minutes application of four dummies (one per level)

** The exposure time relates to the entire exposure time for the aerosol monitoring; every dummy was exposed to LPS for 30 min*

A further approach of the experiments was to examine the influence of occupancy to aerosol distribution in the system. The entire exposure time was 75 min whereas four dummies were applied to the tower in a 5 min rhythm and each one was exposed for 30 min. The following scheme (tab. 20) illustrated the changes of occupancy that occur during the entire exposure time.

Tab.20: Occupancy of the inhalation tower for all experiments



Time [min]	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
Occu-pancy [%]	12.5	25	37.5	50	62.5	75	75	75	75	75	62.5	50	37.5	25	12.5

The inhalation tower was employed within the first five min of exposure with four dummies and hereby resulted in total tower occupancy of 12.5%. With the further application of dummy groups in five min rhythm, the tower occupancy increased to 75% within the first 30 min of exposure. After 55 minutes of experimental time, the total occupancy decreased within every 5 min by 12.5% until the tower was occupied for last five min of the experiment (70-75 min) by 12.5%.

Firstly, the standard *in vivo* method (technique 8) was simulated with dummies to determine the real time aerosol changes while the rest of the ports were plugged (fig.12).

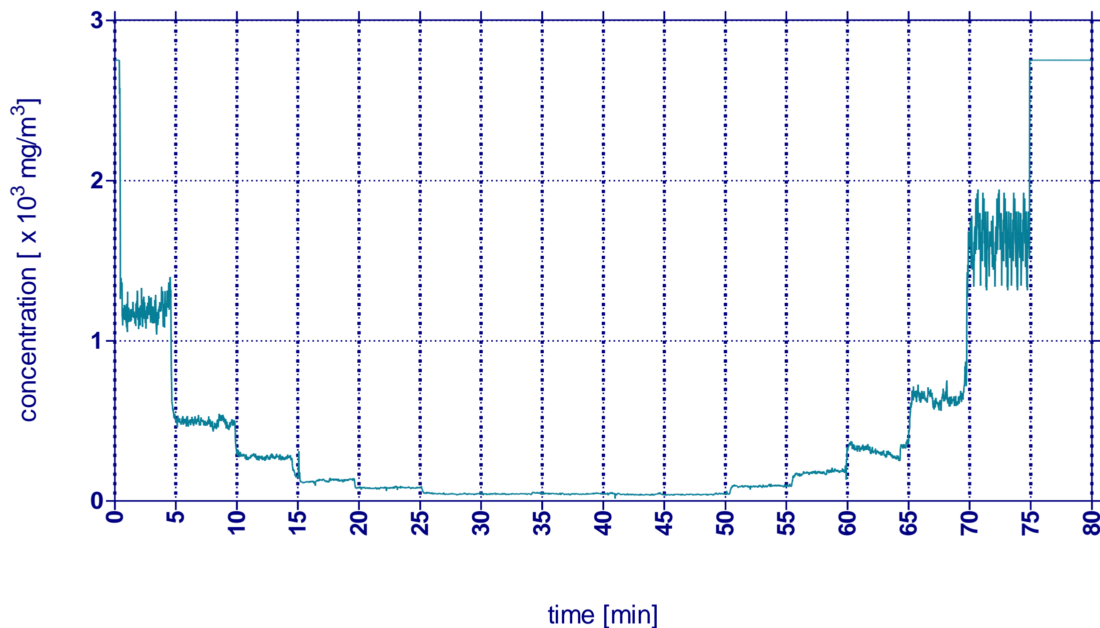


Fig.12: Aerosol monitoring with the simulation of a standard in vivo method: all ports were plugged, plugged ports were exchanged with dummies in a 5 min interval where a group of four dummies was inserted to the tower; each dummy had an exposure time of 30 min. The aerosol concentration was measured by the MicroDust Pro measuring device. The TPM average value is 1.0498 x 10^3 mg/m^3.

Fig. 12 showed the course of the aerosol concentration during the entire exposure time of the first experiment. The particulate concentration decreased with the first application of four dummies where 12.5% of the tower was occupied (tab. 20). With the further application of dummy groups, the aerosol concentration continuously decreased until the lowest concentration of approximately 0.1 x 10^3 mg/m^3 was reached while 75 % of the tower was occupied. After the first four dummies were exposed for 30 min to LPS, the first group was removed from the tower. The following groups were removed in a 5 min rhythm. The empty ports were plugged and the aerosol concentration started to rise continuously. The aerosol concentration course indicated an uneven aerosol distribution in the tower during the experiment. It could be assumed, that the first and last groups of dummies receive more LPS compared to the groups in the middle.

To provide a constant aerosol concentration during the entire experiment a new improved technique was tested. Hence, the tenth technique of aerosol monitoring (tab. 19) was performed. At the beginning of the experiment all unused ports were occupied with dummies. Dummy groups were inserted into the tower in a 5 min rhythm and exposed for 30 min. The entire experimental time was 75 min with a tower occupancy illustrated in table 20.

Fig. 13 showed the course of the particulate concentration which had an approximate constant value of 0.1 x 10^3 mg/m^3 during the entire experimental time.

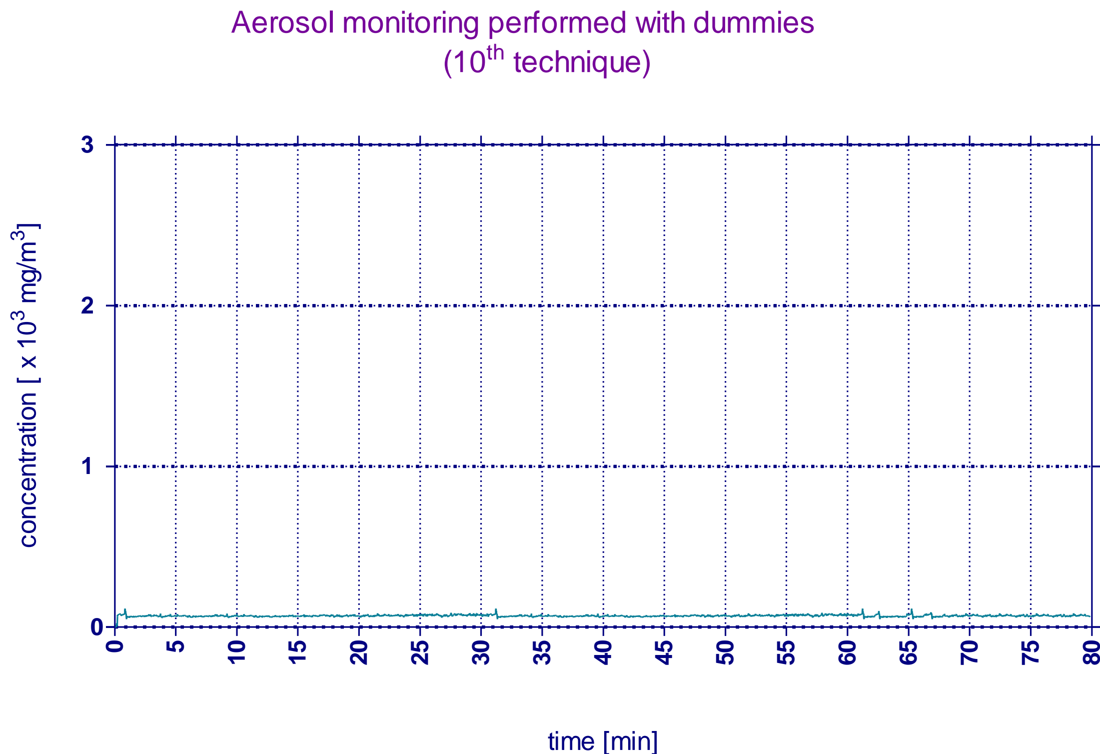


Fig.13: Aerosol monitoring of a new experimental technique with dummies: All ports were applied with dummies, dummies were exchanged with dummies in a 5 min interval where a group of four dummies was inserted to the tower; each dummy had an exposure time of 30 min. The aerosol concentration was measured by the MicroDust Pro measuring device (Casella, Bedford, UK). The TPM average value is 0.141 x 10^3 mg/m^3.

Considering the occupancy of the tower, no changes in aerosol concentration occurred neither with the application of dummies to the tower nor with the removal of dummies from the tower. It could be assumed that an improvement was achieved with the application of the second technique due to the constant average TPM value of 0.141 x 10^3 mg/m^3.

A further aspect that was considered is the air flow within the nose only exposure system. But to achieve a constant flow, an active aerosol output was necessary. Hence, suction with a vacuum pump was generated to improve the inhalation system. The ninth technique was performed with closed ports and a suction pump (vacuum). In contrast, the eleventh technique was carried out with dummies and a vacuum. Results illustrate in table 21 summarises all average TPM values of the four applied techniques.

Tab.21: Average TPM values [x 10^3 mg/m^3] for all four application techniques

Technique	closed ports	dummies	vacuum pump [9 L/min]	TPM average [x 10^3 mg/m^3]
8	+	-	-	1.048
9	+	-	+	1.021
10	-	+	-	0.141
11	-	+	+	0.115

Considering the eighth technique (standard *in vivo* experiment) no real difference was obtained compared to the ninth technique with the application of the vacuum pump. In general, this result is neglected because of variation in TPM values during the entire exposure time. The measured average TPM value from the tenth technique was 0.141 x 10^3 mg/m^3. With the application of the vacuum (eleventh technique), a slight reduction by 18.44% to a mean TPM value of 0.115 x 10^3 mg/m^3 was achieved. Although that might indicate that the application of a vacuum pump does not really reduce the aerosol concentration. But it was shown that the application of dummies achieved a constant aerosol distribution during the entire exposure time with a relatively low average particulate concentration.

All together it was shown with the aerosol monitoring experiments that the aerosol distribution depends on the occupancy of the inhalation tower considering the standard *in vivo* method. In addition, it was also shown that the improved technique with dummies led to independency of occupancy in the tower which hereby results in a constant aerosol concentration during the entire experimental time.

To validate the results achieved by direct examination of aerosol distribution within the inhalation system, further experiments are hereby performed. The LPS lung-induced neutrophilia *in vivo* model is used whereas placebo treated rats are exposed in the inhalation tower to LPS. Subsequently, the number of neutrophils in the BAL is measured which is an indicator for the endotoxin aerosol distribution within the tower system.

3.4. Indirect examination of aerosol distribution by LPS-induced pulmonary neutrophilia

3.4.1. Comparison between the tower levels

The determination of aerosol distribution inside the inhalation exposure system was performed indirectly with the LPS- induced pulmonary neutrophilia model. The approach was to examine the correlation between the four tower levels. This examination was carried out with the application of the standard *in vivo* technique (technique 12, tab. 22) and by vehicle treated rats.

Tab.22: Application technique for vehicle treated rats

Technique	Rats	Compound application	Technique applied for the nose only LPS exposure
12	40	Vehicle (i.t.)	All ports were closed with plugs. Four rats were applied to the inhalation tower within an interval of five minutes and exposed for 30 min.

The number of endotoxin induced neutrophils in the BAL of vehicle treated rats was analysed and is shown in figure 14.

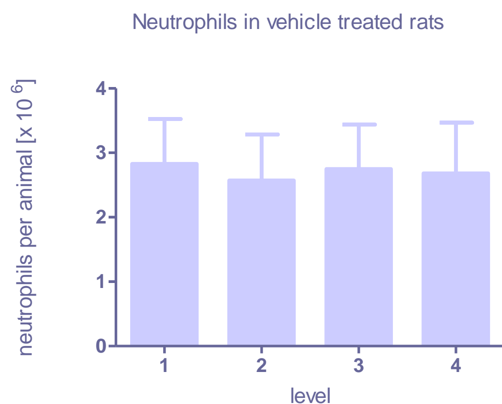


Fig.14: Examination of aerosol distribution by LPS- induced pulmonary neutrophilia in vehicle treated rats considering the inhalation tower levels(n=10, mean ± SD)

Tab.23: Illustration of estimated data from the indirect examination of aerosol distribution with vehicle treated rats within the inhalation tower system (mean ± SD, CV, n=10)

Level	Mean ± SD [x10^6 neutrophils per animal]	CV
1	2.82 ± 0.69	24%
2	2.57 ± 0.71	27%
3	2.74 ± 0.69	25%
4	2.68 ± 0.78	29%
$\bar{x}$	2.71 ± 0.70	26%

From the data shown in fig. 14, it could be assumed that an approximate similar mean value for all tower levels was evaluated. But considering the estimated data in tab. 23 it is shown that the variation between all levels is very high due to the CV value of 26%. Therefore, the next approach of the following experimental study was to determine if the high variation of neutrophils per animal depends on the applied technique and also on the occupancy of the tower.

3.4.2. Induced neutrophilia in dependency to the occupancy of the tower

The following experiments with the model of LPS- induced pulmonary neutrophilia were performed to determine a correlation between the occupancy of the inhalation tower and neutrophilic lung infiltration. Therefore, 32 rats were divided into four groups and all of them were vehicle treated by i.t. application before nose only LPS exposure. The LPS exposure was performed as illustrated in table 24 by the thirteenth technique where all ports are plugged. This technique refers to the standard *in vivo* technique. In addition, a new technique was applied with 32 rats which were as well divided into four groups. The nose only LPS exposure was performed by the fourtheenth technique (tab. 24), where dummies were inserted to the tower ports. For every exposure, 3 mg/mL LPS solution was nebulised within 30 min.

Tab.24: Application techniques to determine the correlation between the lung neutrophils per animal and the occupancy of the inhalation tower

Technique	Group 1	Group 2	Group 3	Group 4	Σrats	Condition
13	8 rats, 8 exposures with 1 rat each	8 rats, 4 exposures with 2 rats each	8 rats, 2 exposures with 4 rats each	8 rats, 1 exposure with 8 rats	32	All ports were plugged
14	8 rats, 8 exposures with 1 rat each	8 rats, 4 exposures with 2 rats each	8 rats, 2 exposures with 4 rats each	8 rats, 1 exposure with 8 rats	32	All ports were occupied with dummies

During the 30 min of LPS exposure to induce pulmonary neutrophilia in the lung, the inhalation system was occupied as illustrated in figure 15.

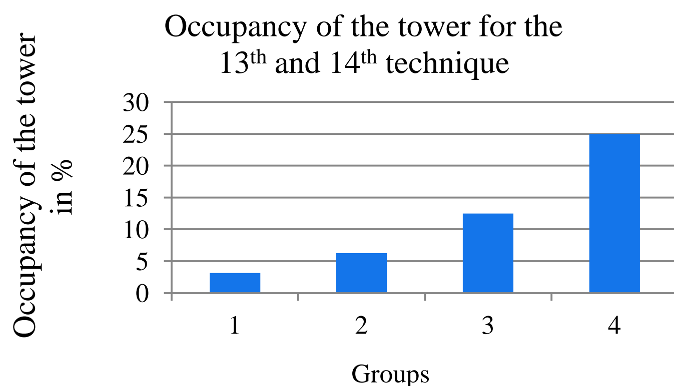


Fig.15: Occupancy of the inhalation tower in %

All groups consisted of 8 rats. Considering the first group, only one rat was exposed to LPS which resulted in tower occupancy of 3.125%. To achieve enough data points the experiments were repeated eight times. Experiments of the second group were performed with two rats and repeated four times and the tower was occupied by 6.25% during every exposure. Regarding the third group, four rats were exposed and the experiment was repeated twice. The tower occupancy increased to 12.5%. The last group (fourth group), eight rats were exposed nose only and the tower was occupied by 25%.

Tab.25: Determination of neutrophilic cell number per animal (mean ± SD, CV, n=8)

	Technique 13		**Technique 14**	
Group	Mean ± SD [x10^6 neutrophils per animal]	CV	Mean ± SD [x10^6 neutrophils per animal]	CV
1	6.89 ± 2.85	41%	5.44 ± 1.98	36%
2	4.03 ± 2.51	62%	4.66 ± 1.39	29%
3	3.61 ± 1.52	42%	4.53 ± 2.19	48%
4	4.24 ± 1.54	36%	6.45 ± 2.46	38%

Results achieved by the thirteenth technique were shown in table 25 and figure 16 a. The first group had the highest number of induced neutrophils with a mean value of 6.89 x 10^6 and a SD value of 2.85 x 10^6 neutrophils per animal and a CV of 41%. In this experiment, only one rat at a time was occupied in a port in the whole tower which led to tower occupancy of 3.75%. Experiments with the second group were carried out with eight rats. Two rats were applied in sequence to the tower and the experiment cycle was repeated four times. A mean value of 4.03 x 10^6 and SD of 2.51 x 10^6 neutrophils per animal was achieved. The lowest mean of 3.61 x 10^6 neutrophils per animal was achieved for the third group with four rats where 12.5% of the tower was occupied. Compared to this, an increase of neutrophils was measured for the fourth group with eight rats (mean/SD with 4.24/1.54 x 10^6 neutrophils per animal) where 25% of the tower was occupied.

It could be assumed that the number of neutrophils measured in the BAL depends on the occupancy of the tower especially regarding the first group. However, from the estimated CV values between 36% and 62% (tab. 25) high variations in the group still exist. To avoid the dependency of tower occupancy an improved technique was used and tested.

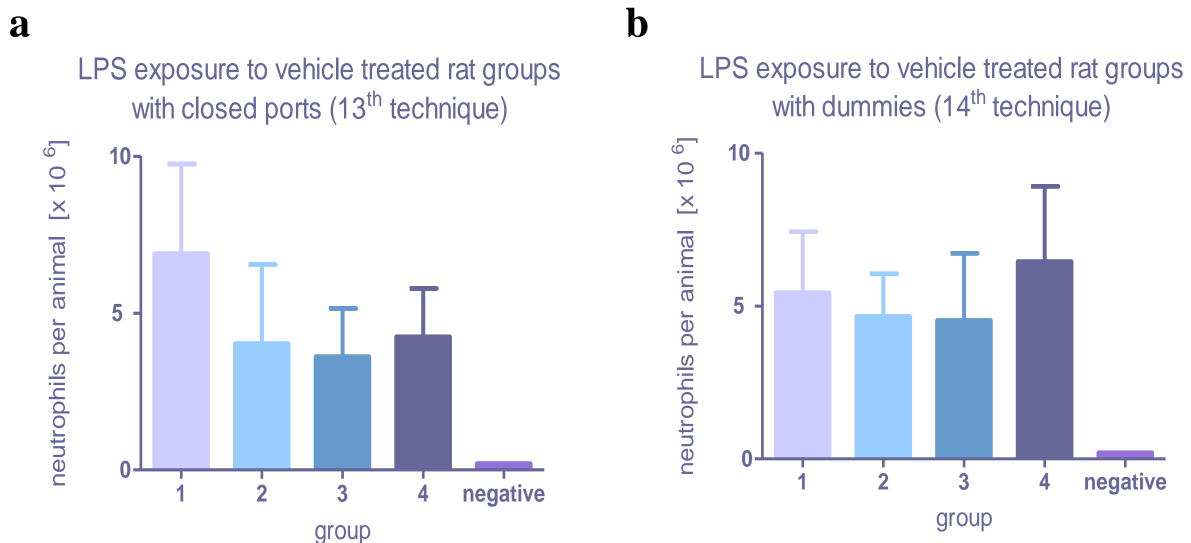


Fig.16: Neutrophilic cell count in BALF of vehicle treated rats in neutrophils per animal (mean ± SD, n=8); (a) The thirteenth technique was performed where the remaining ports were plugged (b) The fourteenth technique was performed where remaining ports were occupied with dummies

Hence, experiments with dummies (fourteenth technique, tab. 24) were carried out and the results are displayed in fig. 14 b. The first group had a mean value of 5.4 x 10^6 and a SD of 1.9 x 10^6 neutrophils per animal (tab. 25). A slight reduction of the mean neutrophilic number per animal was achieved in the second group (tab. 25 and fig. 14b). The third group had a mean value of 4.5 x 10^6 and SD of 2.1 x 10^6 neutrophils per animal whereas the highest mean of neutrophils in the BAL was measured within the fourth group (mean /SD with 6.4/2.4 x 10^6 neutrophils per animal). The difference in the mean values of all groups indicates that no associated relationship exists between the occupancy of the tower and amount of the neutrophilic cell. Nonetheless the high variation (CV value between 26% – 48%) remains a conflicting factor to be studied.

All together, it was shown that the application of dummies to the inhalation improved the nose only exposure system. But only the variation of the first and second group was reduced with the application of dummies to the inhalation tower. The variation for the third and fourth group remained almost equal. This shows that further improvement technique is required.

3.4.3. Application of a vacuum pump

Although with the existing high variation, it is worth to mention that slight improvements were achieved in the previous technique with dummies. To validate these results, experiments were repeated and executed only with a smaller group of rats (tab. 26). It was assumed that the variation from the evaluated data of former experiments would be minimised by the installation of vacuum pump to generate suction and to aid constant flow inside the tower. Therefore, experiments were performed simultaneously with and without the vacuum pump (tab. 26).

Tab.26: Application techniques for vehicle treated rat groups

Technique	Group 1	Group 2	Σ Rats	Condition
15	8 rats, 8 exposures with 1 rat each	8 rats, 1 exposures with 8 rats	16	All ports were occupied with dummies
16	8 rats, 8 exposures with 1 rat each	8 rats, 1 exposures with 8 rats	16	All ports were occupied with dummies and a vacuum pump was attached

All positions of the inhalation tower were occupied with dummies. The first group consists of eight rats whereas one single rat was exposed to LPS for 30 min and the experiment was repeated eight times. The second group consists of eight rats and all of them were exposed at one time. The division of the first and second group is identical for the fifteenth and sixteenth techniques (tab. 26). The only difference in both techniques is with or without vacuum pump.

Tab.27: Determination of neutrophilic cell number per animal (mean ± SD, CV, n=8)

	Technique 15		Technique 16	
Group	Mean ± SD [x 10^6 neutrophils per animal]	CV	Mean ± SD [x 10^6 neutrophils per animal]	CV
1	5.32 ± 1.55	29%	3.44 ± 0.93	27%
2	4.62 ± 2.17	47%	3.97 ± 1.32	33%

Results illustrated in figure 17a were achieved from the fifteenth application technique (tab. 27). The first group has a mean value of 5.32 x $10^6$and a SD value of 1.55 x 10^6 neutrophils per animal. The mean value generated by the second group was 4.62 x 10^6 with SD of 2.1 x 10^6 neutrophils per animal (tab. 27). Even if the mean values are approximately equal, the high variation is non negligible. The first group has a CV value of 29% whereas the second group has a CV value of 47%.

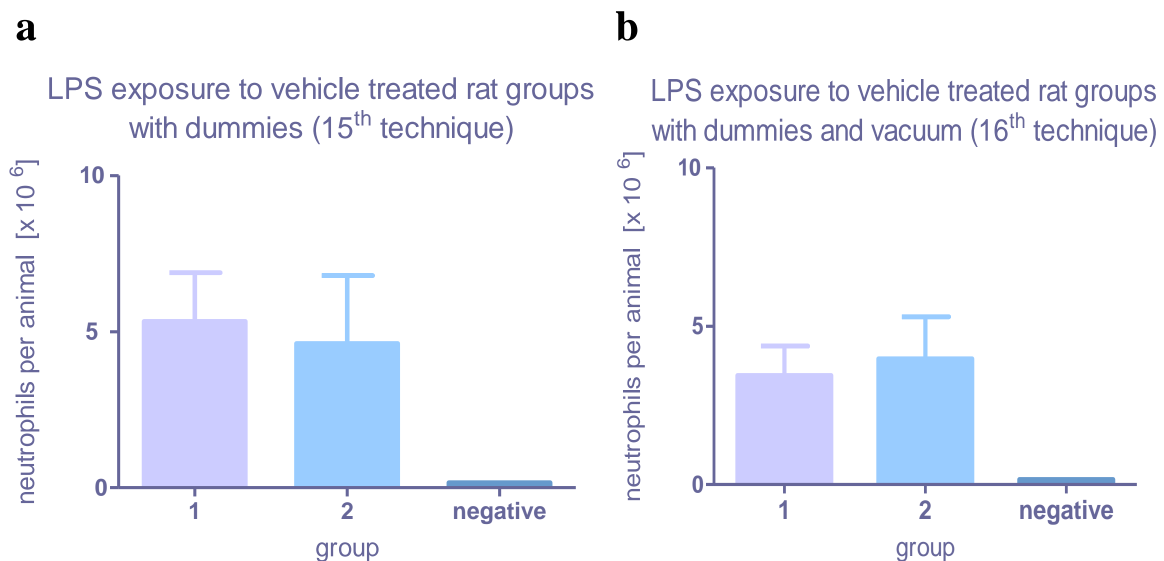


Fig.17: Neutrophilic cell count of BALF of vehicle treated and untreated (negative) animal groups in neutrophils per animal. First group: one rat exposed and repeated eight times, second group: eight rats exposed at one time (a) The fifteenth technique was performed where dummies are placed to the tower ports (b) The sixteenth technique was performed where remaining ports were occupied with dummies and an vacuum pump is applied to the outlet tower system

Data in figure 17b were achieved with the sixteenth application technique. The first group has a mean value of 3.4 x 10^6 and SD of 0.9 x 10^6 neutrophils per animal. The mean value of 3.9 x 10^6 and a SD value of 1.3 x 10^6 neutrophils per animal increased slightly with the application of eight rats at one time (second group). By installing a vacuum pump in this technique, the variation was positively minimised. The CV value from the first group was reduced from 29% to 27% and the second group from 47% to 33% (tab. 27).

In summary, it could be demonstrated that through the application of dummies and installation of a vacuum pump to the LPS inhalation tower exposure to rats, gross improvement was reached. Even though the neutrophilic cell number as well as the variation was generally through this technique reduced but the inflammation could still be induced. In addition, it was however discovered that no correlation exists between the occupancy of the inhalation tower and the neutrophilic cell number in BAL for experiments performed with dummies.

3.5. Compound administration

The LPS lung- induced neutrophilia *in vivo* model is used to analyse the effect of pharmaceutical compound in the lung. Therefore, various administration techniques are used to deliver compound directly to the respiratory tract. Subsequent to compound application, animals are exposed to LPS to induce an inflammation which is measured by the number of neutrophilic cells in the BAL. It has to be considered that not all compound administration techniques achieve a high efficiency and might not be appropriate for rats and humans. Therefore, two different compound administration techniques are applied and compared with each other.

3.5.1. Intratracheal instillation (i.t.)

The first application technique was the intratracheal instillation (i.t.). It was performed as described in paragraph 2.2.2.3.1. A compound group of 12 rats was divided into three subgroups, each consisting of four rats. Each subgroup was administered within a time range of five min. Subsequent to the administration rats were exposed nose only to LPS to induce a neutrophilia in the rats lung. The conditions of the inhalation tower and the performance of the LPS exposure are shown table 28.

Tab.28: Application technique for nose only LPS exposure of i.t. treated rats

Technique	Rat amount	Compound application	Technique applied for the nose only LPS exposure
17	12	Vehicle, BYK20702-9 (i.t.)	All ports were occupied with dummies. A vacuum pump was attached to the outlet of the inhalation tower. 12 rats were exposed for 30 min to LPS.

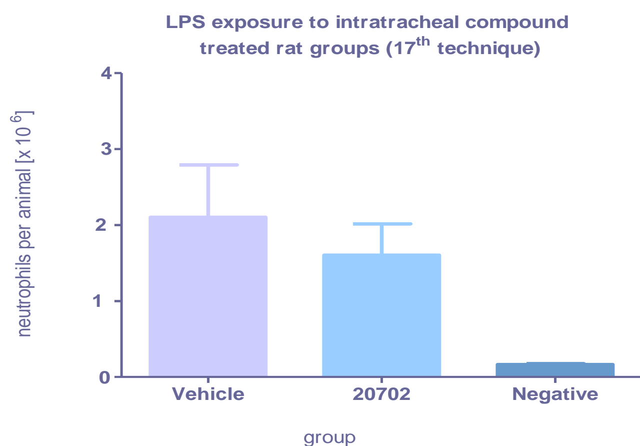


Fig.18: Cell count of neutrophils per animal in BALF: Intratracheal compound administration (i.t.) and subsequent LPS exposure to induce a lung inflammation(mean ± SD, n=12)

Analysis of BAL resulted in mean value of 2.1 x 10^6 and SD of 0.6 x 10^6 neutrophils per animal and a CV value of 28% for the LPS vehicle treated group (fig. 18). Results for compound treated rats (BYK20702-9) are lower with a mean value of 1.6 x 10^6 and SD of 0.6 x 10^6 neutrophils per animal (CV value 37%). Due to the decrease of inflammatory cells by the application of budesonide an inhibitory effect of 23.7% was examined.

3.5.2. Nose only compound administration

In comparison to the i.t. compound administration the nose only exposure by an inhalation tower was performed. Vehicle and compound BYK20702-9 were administered to rats. One group of 12 rats was exposed to vehicle and another group of 12 rats was exposed to aerosolised budesonide. Both exposures were carried out for 10 min.

Due to well equipped nose only exposure system the respired volume of air during the exposure was recorded for four rats. The RMV of each animal is shown in table 29. It was shown that the RMV varies between the rats in a range of 25.7 – 210.5 mL/min.

Tab.29: Accumulated volume of four rats generated with the bodyplethysmograph during nose only exposure

Rat	**RMV [mL/min] Vehicle group**	**RMV [mL/min] BYK20702-9**
1	210.5	144
2	25.7	50.2
3	155.0	305.7
4	76.2	201.0
ΔRMV [mL/min]	116.85±82	175.225±107
DD[mg/kg]*	--------	0.095*

**delivered dose (DD) calculated with C=0.0135 mg/L, BW=0.25 Kg*

The delivered dose budesonide (BYK20702-9) was calculated according to the following formula:

$$DD = \frac{C\ x\ RMV\ x\ D}{BW}$$

as 0.095 mg/Kg

with DD= delivered dose (mg/Kg), C= concentration of compound in air (mg/L), RMV= respiratory minute volume of air inhaled in one minute (L/min), D=duration of exposure (min) and BW=bodyweight (Kg). For the calculation of DD the mean RMV was used. Subsequent to the compound administration, the lung inflammation was induced by the nose only inhalation of LPS. The inhalation tower conditions of the applied technique are illustrated in table 30. The number of neutrophils in the BAL was analysed.

Tab.30: Application technique for nose only LPS exposure of nose only compound treated rats

Technique	Rat amount	Compound application	Technique applied for the nose only LPS exposure
18	12	Vehicle, BYK20702-9 (nose only exposure)	All ports were occupied with dummies. A vacuum pump was attached to the outlet of the inhalation tower.12 rats were exposed for 30 min to LPS at the same time.

Results in fig. 19 were achieved by the nose only compound administration and the subsequent nose only LPS exposure.

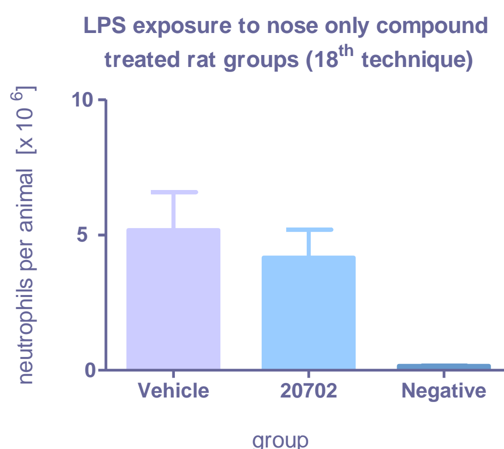


Fig.19: Cell count of neutrophils per animal in BALF: Nose only compound application and subsequent LPS exposure to induce lung inflammation (mean ± SD, n=12)

A mean value of 5.1 x 10^6 and SD of 1.4 x 10^6 neutrophils per animal was measured for the vehicle treated group with a CV value of 27%. The second nose only exposure to the compound budesonide (BYK20702-9) resulted in a mean value of 4.16 x 10^6 and SD of 1.0 x 10^6 neutrophils per animal and a CV value of 24%. Due to the reduction in neutrophilic cell amount after the compound administration, an inhibitory effect of 19.6% was obtained. All together, the efficiency of budesonide was demonstrated for both compound administration techniques. The inhibitory effect applied by intratracheal instillation was higher in rats compared to the nose only exposure. However, the number of measured cells in the BAL of the i.t. technique was in general way much lower for both compound groups compared to the nose only exposure.

4. Discussion

4.1. LPS lung-induced neutrophilia inhalation model

The following scheme (fig. 20) illustrates the experimental set-up to determine the aerosol distribution and to establish the LPS nose only inhalation tower.

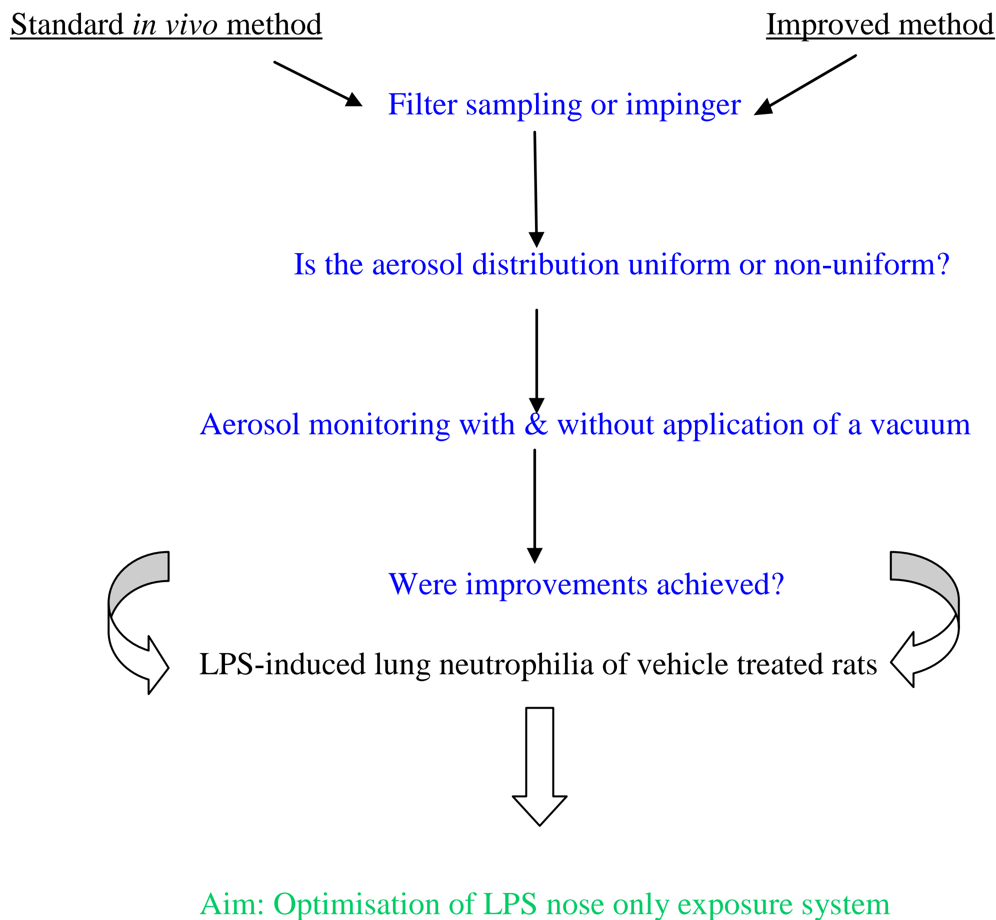


Fig.20: Pattern of experimental set-up for determining the aerosol distribution within the LPS inhalation system. Blue: direct sampling method, black: indirect sampling method

4.1.1. Direct examination of aerosol distribution

4.1.1.1. Endotoxin detection by filter sampling

Due to its flexibility and simplicity, the gravimetric determination of endotoxin is the most widely utilized aerosol measurement technique. The filter sampling is performed by collecting LPS on glass fiber filters that is connected to a sampling pump (flow rate = 0.2 L/min). A comparison between the standard *in vivo* method with closed ports as well as the improved method with dummies (without suction) was performed. By considering the tower level using the standard *in vivo* method, less LPS was collected from the lower tower levels than at the upper levels (see fig.9a). Also, a single sampling of each tower port showed high variance relative to mass changes (see fig. 10). This effect is presumably traced to the non constant aerosol flow as well as the non consistent distribution of aerosol in the tower. The result from the filter mass changes with the improved method (dummies) obtained after the filter sampling indicated a uniform aerosol flow and regular distribution of aerosol in the inhalation tower (see fig. 9c). Nonetheless, a high variance is obtained for both methods in the estimated collected mass. This may be caused by the inequality in the high loss of compound between 86.77 – 89.76 % in the inhalation tower. The high loss of compound is effected by long path of aerosol transport through the tubing system to the filter holder. More to this, the particles collision to the filter holder cassettes caused by mechanisms of sedimentation, impaction and gravitational settling contribute to loss of compound (Lee KW, 1993). Experiences with open faced filter holders show a uniform deposition and also lower sampling loss of compound occurs (Lee KW, 1993). However, the usage of open face filter holders is technically not appropriate for the nose only inhalation system. Furthermore, a reason for the loss of compound might be that the filter holder inlet surface is coated with a polymer film (plastic) as LPS adheres to plastic and glass material (Rosner MR, 1979).

According to the product information from Pari Master LC star nebuliser, an aerosol particle size distribution between 0.5 - 20 µm is achieved with a flow rate of 6 L/min of compressed air. This indicates invariably that particles less than 1.6 µm might not be collected on the fibrous glass filter. However, chemical analysis of filter sampling has also shown that the pore size of the filter has no effect on the collected LPS amount (Li, 1999).

Since the gravimetric method is less sensitive and only minor changes in the weight of filter are measured, the weighing procedure has to be performed carefully to avoid effects of humidity from the environment (Lee KW, 1993). To achieve a weight increase of the filter, an increase of sampling flow rate as well as the sampling time has to be considered even if this does not correspond to the RMV of rats. Nevertheless, the sampling flow rate of 0.2 L/min is not uncommon in filter sampling (Cooper A, 2010). However, aerosol sampling studies obtained no significant influence on the flow rate and the sampling time (Li, 1999). An alternative method to weighing the filter is the use of chemical analysis.

With the chemical analysis, an analytical measurement is carried out on the particulate collected by the extraction of the particles (Lee KW, 1993).

4.1.1.2. Endotoxin detection with the limulus amebocyte lysate (LAL) assay

An alternative to the filter sampling method are impingers. These devices collect LPS aerosol in liquid that is subsequently analysed by LAL assay. The LPS concentrations collected by the standard *in vivo* and improved method (without suction) are higher compared to the filter sampling method. A lower amount of compound loss between 20.87 – 25.56 % is estimated. The chromogenic endpoint LAL detection assay is a highly sensitive quantification test to the extent that even low amounts of endotoxin can be determined (Ding JL, 2001). Environmental aerosol sampling studies performed in swine barns to determine the bacterial amount in the air achieved similar results. Endotoxin collection with impingers and subsequent analysis with the LAL assay lead to a significantly higher endotoxin concentration compared to the filter sampling (Duchaine C, 2001). A further reason for the high quantity of compound could be traced to the vertical connection of the impinger to the sampling ports. The positioning of the device indicates that the physical mechanism of gravitational force has a positive effect to the sampling. Furthermore, LPS that adheres, due to impaction or sedimentation mechanism, at the surface of the glass impinger wall is removed by vortexing.

However, the tower outlet is built of a small hole which indicates that an optimal aerosol transport to the environment is not provided. The tower might be overloaded with aerosol in the lower tower level after a certain time of exposure due to recirculation of aerosol. The estimated data from the standard *in vivo* method confirmed this assumption as the highest LPS concentrations were herein measured at the lower tower level (see fig. 11a). A high CV value of 39% confirms the variation between the tower levels. The vacuum creation achieves nearly equal endotoxin concentration between 23.60 x 10^5 EU/mL and 28.94 x 10^5 EU/mL for all levels which indicates an elimination of aerosol overloading in the system (see fig. 11b) as well as a reduction in the CV value from 39% to 14% (see tab. 16). Taking the improved method with the application of dummies and suction into account, the best results are achieved. First, the use of dummies resulted in regular aerosol distribution and with the vacuum pump a constant flow was achieved within the tower. This is shown by the reduction in the CV value from 15% to only 8% (see tab. 16). However, it has to be considered that the aerosol output of each tower port is minimised due to the fact that 16 L/min of aerosol are transported inside the system and 9 L/min are being sucked by the pump. In the course of this, 7 L/min remains and spread accordingly within the tower system. Therefore an output of approximately 0.2 L/min for each port is conveyed.

4.1.1.3. Aerosol monitoring

Direct portable aerosol measurement devices have been commercially available for many years and are widely employed to determine the intensity of dust in the environmental field (Thorpe A, 2002). Regarding inhalation towers, the aerosol concentration measurement by infra red light scattering belongs nowadays to the integrated standard equipment of inhalation system to determine the aerosol concentration for quantitative purposes. But the goal of this experiment is to question, if the aerosol concentration changes during experiments and also with the occupancy of the system. Therefore, two applied techniques are compared with each other (see fig. 12 and 13). The first technique, the standard *in vivo* technique with closed ports (see tab. 19) was performed where changes in aerosol concentration as depended on changes in tower occupancy were measured (see fig. 12). The first application of four dummies (occupancy 12.5%) caused a drop of aerosol concentration from 2.8 x 10^3 mg/m^3 to 1.1 x 10^3 mg/m^3. With the further application of dummy groups, the aerosol concentration continuously decreased until the lowest concentration of approximately 0.1 x 10^3 mg/m^3 was reached while 75% of the tower was occupied. After the 30 min exposure of each dummy, the groups were removed from the tower in a 5 min interval and the unused ports were plugged. A continuous increase of the aerosol concentration to the starting concentration value was measured. At this point, it is shown that the entire aerosol distribution depends on the number of open ports and on the occupancy of the tower (fig. 11). With the closure of all ports and the outlet of the tower being small in diameter, an over pressure is hereby built up inside the system. With the opening of one port, the entire aerosol streams out of this port which leads to a high aerosol concentration at this particular position. With further opening of more ports a decrease of aerosol concentration in the inhalation tower was measured. To achieve equal condition for all tower positions, an improved technique is hereby performed. All ports are occupied with dummies before the exposure begins (see tab.19). During the exposure of 75 min and with the increase in the number of occupied ports, a steady aerosol concentration of an average value of 0.1 x 10^3 mg/m^3 was achieved (see fig. 13).

4.1.2. Indirect examination of aerosol distribution

The LPS induced lung inflammation model is used as a short term *in vivo* method to produce anatomic features of COPD and cause acute exacerbation (Wright JL, 2008). In consideration to the physiological aspects, the nose only exposure achieves the most homogenous aerosol deposition (Sakagami M, 2003). However, studies in preclinical drug research have shown high variations in the number of neutrophils in the BAL. This leads to difficulties to receive reproducible data. Due to the outmoded exposure system, it was questioned if the aerosol distribution is uniform within the tower. Therefore, the aerosol

distribution was determined indirectly by quantifying the neutrophilic cells amount in BAL in vehicle treated rat (see fig. 14). Obtained estimated data shows no significant differences in the mean value of neutrophilic cells per animal between each tower level but the variation is high with a CV value of 26% (see tab. 23). One reason might be traced to the application technique where unused ports are closed and four rats are applied with an interval of 5 min. It should also be considered that the inhalation dose of each animal varies due to uncontrollable experimental parameters. Rats are restrained in tubes during 30 min of exposure and this could tend to be a stressful situation for the rats as each individual has different adaptation abilities. Not only the volume of respired air per minute (RMV) of each individual has to be considered but change in the breathing pattern plays a vital role. A small number of deep breaths in one minute can achieve the same RMV as a large number of shallow breaths but the amount of deposited compound depositing deep in the lung may differ (Alexander DJ, 2008). A further aspect considering the inhalation dose is the bodyweight. Even if every individual inhales the same amount of compound, the actual dose per body weight of each animal differs (Alexander DJ, 2008). Although, simulation exposure studies performed with F344 rats (male & female, age: 3-24 months) show that the respiratory frequency decreases with rats age and increasing bodyweight (JL, 1986). This respiratory data was obtained by bodyplethysmographs which is a standard lung measurement. Due to the fact, that the determination of an inhaled compound is very complex, new nose only inhalation tower system are well equipped with these devices to monitor the respiration of each rat and to further predict the inhalable dose of each individual. However, in this case the LPS-COPD nose only exposure system is not well established.

The following experiments were performed to examine if the standard *in vivo* application technique with closed ports is responsible for the high variation of BAL data. The highest neutrophilia was induced with the first group of vehicle treated rats with a mean value of 6.89×10^6 and SD value of 2.85×10^6 neutrophils per animal and a CV value of 41% (see fig. 16a). The first group contained eight single rats and was exposed consecutively. With the flow of aerosol into the system pressure is build up inside the inhalation tower. By opening of one of the ports, a reduction of the pressure in the inhalation system is hereby noticed. However, there is little or no significance in the neutrophilic cell amount, if two, four or eight rats are applied to the tower at the same time. But the variation between the groups is still high as seen in the differences in the CV values between 36% - 62 % (see tab. 25). It is noticeable that collected results from the first and second group have the highest standard deviation. A reason for this might be on one hand the consecutive performance of experiments. It can be assumed that the LPS concentration in the tower increases after a certain amount of exposures compared to first exposure. As an option, cleaning and venting of the system after each every exposure could be considered as this could possibly improve by bringing equal condition in the inhalation system. On the other

hand, not all experiments were performed on the same day which implies that the bodyweight and age of rats herewith vary. Also the LPS solution could be considered as a source of error because the LPS solution is stored in a glass bottle during the entire exposure time. The active substance in the solution could sediment to the bottom of glass bottle or on the arteries of the glass wall. LPS adheres to glass and this might cause change in endotoxin concentration during exposures (Rosner MR, 1979). It is recommended and very important to vortex the solution over the entire experimental time to avoid sedimentation and adherence of LPS.

Simultaneously to standard *in vivo* experiments, a new improved technique with tissue filled Perspex tubes named “dummies” was used to achieve a uniform aerosol distribution in the inhalation system (technique 14, see tab. 24). The estimated mean value of neutrophils per animal varies between 4.53 x 10^6 and 6.45 x 10^6 for all groups whereas the highest number of cells was measured for the fourth group (see fig. 16b). It is also seen, that the variation is very high with CV values in the range between 29% and 38%. To reduce the variation, a suction vacuum was generated (technique 16, see tab. 26). The variation in percent was hereby reduced to values between 27% - 33% and also a reduction in the amount of neutrophils in BAL was achieved (see fig. 17b). Due to the aerosol input of 16 L/min and the suction of 9 L/min only 7 L/min remain in the tower. So less amount of aerosol is flowing through each port and less LPS is consumed by the rats. A reduction in the variation of the neutrophilic cell number was verified with this improved method. The reason could be traced to the created vacuum that has the function to maintain a constant flow within the exposure system.

In conclusion, the indirect examination of aerosol distribution within the exposure system by vehicle treated rats led to the following results. Experiments performed with the standard *in vivo* technique resulted in high variations (CV value between 36% - 62%). A reason for this might be the non-uniform aerosol distribution in the inhalation system as partly dependent on the occupancy already shown earlier with the direct method of aerosol monitoring (see paragraph 3.3). Improvements were achieved with the new technique (dummies and vacuum pump) where CV values were reduced from 36% - 62% to 27% - 33%.

4.2. Comparison of compound administration

Nowadays, oral therapeutic treatment reliefs the symptoms and decelerates the process of COPD. But the major goal in pharmaceutical research is the compound administration directly to the lung. However, the administration via the inhalative route to the target organ remains challenging. Two common pulmonary administration techniques were performed and compared with each other. The LPS-lung induced pulmonary neutrophilia *in vivo* model was applied to test the efficiency of compound budesonide.

4.2.1. Intratracheal compound administration

The intratracheal compound instillation of rats and the subsequent induced neutrophilia led to an inhibitory effect of +23.7% (see fig. 18). In general, the application technique has been proven to be efficient. The advantage of the technique lies with direct compound application to the lung. But it has to be considered that this technique is challenging due to the narrow oral cavity and glottis of the rat. Therefore, skilled personnel are highly necessary to ensure a successful instillation, to visualise the trachea and to reduce the failure rate. A large cannula is inserted into the trachea which might lead to irritations in the respiratory tract of the rat. However, the compound is applied to the target organ with a very low loss of compound. But this method is not natural and large particle deposit unnaturally deep in the lung. Considering the LPS exposure system performed with the improved technique (dummies and vacuum) a reduced neutrophilic cell amount in the BAL compared to former experiments was measured. The generated active exhaust of aerosol causes a decrease in endotoxin consumption of each tower position. From an aerosol input of 16 L/min and an outlet of 9 L/min, 7 L/min remain in the tower. Regarding the entire tower with 32 ports, only 0.22 L/min stream out of each port. But an inhibitory effect was still achieved. In comparison to previous studies, the variation value of the vehicle and compound treated groups is low with the CV values between 28% and 37%.

4.2.2. Nose only compound administration of aerosol

The nose only compound exposure is a more physiological technique which enables a homogeneous aerosol deposition in the lung. Rats were exposed nose only to vehicle and budesonide (see tab. 30). Subsequently, the lung inflammation by LPS exposure was induced and the number of neutrophils in BAL was measured. A low variation (CV value between 24% - 27%) and an inhibitory effect of +19.6% were estimated. This is even lower compared to i.t. technique (+23.9%). But in general, the neutrophilic cell amount in

BAL is higher for the compound and vehicle group (see fig. 19). A reason might be an insufficient generation of the suction by the vacuum pump.
Considering the reduced inhibitory effect, loss of compound could be a factor affecting the achieved result. Fractions of compound deposit in the nasal as well as in the tracheabronchial airways of the rat (Raabe OG, 1988). Simulation models have shown the importance and effect of the particles size during inhalation whereas particle deposition in the lung decreases linearly with increasing particle size (diameter 2 - 4 µm). The particle size output of the Aeroneb Pro nebuliser has an average particle size diameter of 3.6 ± 2.2 µm. Studies with fluorescin aerosol and mono disperse particles (size diameter 3.6 µm) has resulted in a lung deposition of 12.5% (Sakagami M, 2003). It can be assumed that due to the high inhibitory effect, the deposition of compound in the lung was higher than 12.5%. The reasons might be the nose only exposure tower conditions as well as the aerosol concentration. In addition, according to recent studies, particles with a diameter less than 1 µm achieved the highest deposition in the rats' lung (Braun A, 2007). Therefore, a nebuliser that generates a smaller particle size distribution could be considered for further experiments since the particle diameter of 3.6 µm is more appropriate for the human lung (Sakagami M, 2003). In addition, a determination of particle size distribution by an impactor is essential for further studies.
Furthermore, the particle deposition depends on the breathing pattern of each individual. An average RMV of 175.225 ± 107 mL/min of four rats was recorded by bodyplethysmographs whereas the variation is very high (see tab. 29). Even though the nose only exposure system was well equipped, difficulty with software limitations in recording data was unpreventable. Slight movements from rats in restrainer irritate the record and it takes a certain time until the record could proceed or the software system to even out and the data collected during this time were termed void. In addition, it has to be considered that not all rats are monitored during the 10 min compound exposure and a determination of an average RMV of all exposed rats is really difficult. From this view, it could only be assumed that all rats have a similar breathing pattern which is really disadvantageous for the determination of compound delivery and deposition.
A further important issue to be considered in aerosol therapy is the variation of ventilation in the lung. Due to the physiological characteristics of the respiratory system, regional differences in ventilation occur. The lower respiratory zone is better ventilated compared to the upper part due to the smaller resting volume and the lungs are easier to inflate. Other reasons for uneven ventilation are airway resistance or regional alterations in lung distensibility. The pattern of ventilation inequality under these circumstances will depend on the frequency of breathing (Braun A, 2007).

It can be summarised that the compound budesonide inhibited the pulmonary inflammation in the lung with both application techniques. It was shown, that the inhalation tower was optimised by the means of technical improvement as seen in variation of collected data (CV values between 24% and 37%). Furthermore, it should be noted that living rats were accordingly used in regards to the national animal protection law which requires that the number of animals to be used in experiments be kept as little as possible. Based on the optimisation method, it was possible to reduce the number of used rats. Furthermore, the cost associated with rats' acquisition could be minimised.

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