## Research Article

# Prediction of Acute Inhalation Toxicity Using *In Vitro* Lung Surfactant Inhibition

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**Summary**

Private consumers and professionals may experience acute inhalation toxicity after inhaling aerosolized impregnation products. The distinction between toxic and non-toxic products is difficult to make for producers and product users alike, as there is no clearly described relationship between the chemical composition of the products and induction of toxicity. The currently accepted method for determination of acute inhalation toxicity is based on experiments on animals; it is time-consuming, expensive and causes stress for the animals. Impregnation products are present on the market in large numbers and amounts and exhibit great variety. Therefore, an alternative method to screen for acute inhalation toxicity is needed. The aim of our study was to determine if inhibition of lung surfactant by impregnation products *in vitro* could accurately predict toxicity *in vivo* in mice. We tested 21 impregnation products using the constant flow through set-up of the constrained drop surfactometer to determine if the products inhibited surfactant function or not. The same products were tested in a mouse inhalation bioassay to determine their toxicity *in vivo*. The sensitivity was 100%, i.e., the *in vitro* method predicted all the products that were toxic for mice to inhale. The specificity of the *in vitro* test was 63%, i.e., the *in vitro* method found three false positives in the 21 tested products. Six of the products had been involved in accidental human inhalation where they caused acute inhalation toxicity. All of these six products inhibited lung surfactant function *in vitro* and were toxic to mice.

Keywords: impregnation product, lung surfactant, constrained drop surfactometer, acute inhalation toxicity, OECD TG 403 and 436

## 1 Introduction

Toxicity testing is traditionally performed in experimental animals for determination of toxic and non-toxic exposure levels. However, this practice is out of pace with current legislative and ethical developments. Much of the criticism is centered on the most common parameter used by authorities: the lethal concentration 50% ($LC_{50}$), i.e., the concentration in inhaled air that is lethal to 50% of the animals. The $LC_{50}$ uses death as the endpoint, an outcome that is associated with substantial suffering. For this reason, it is no longer permitted to test for the $LC_{50}$ in Denmark (BEK no. 12 of 07/01/2016). The practice is also strongly discouraged in the rest of the EU according to Directive 2010/63/EU (EU, 2010) due to changes towards stricter regulation of experimental animal use. The bioassay used for testing of acute inhalation toxicity in the current study uses signs of potentially lethal lung damage observed during the experiment as an endpoint, rather than the death of the animal. Furthermore, acute toxicity caused by substance inhalation can have a very steep concentration-response curve, not least for the substances in the focus of this paper: impregnation products (IPs).

The development of alternative methods for testing of chemical toxicity has come a long way, but no alternative methods

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exist for assessment of acute inhalation toxicity at present (Zuang et al., 2015). Any substance that reaches the deepest parts of the lung can potentially cause acute inhalation toxicity, but the underlying mechanism is poorly understood and may vary depending on the characteristics of the substance. We have investigated the hypothesis that lung surfactant (LS) is a prime target in acute inhalation toxicity. LS covers the deepest parts of the lungs, i.e., the respiratory bronchioles and the alveoli, as a thin liquid film and is continuously formed and secreted by alveolar type II cells (Zuo et al., 2008). LS has several functions in the lungs, but the most important is to lower the surface tension at the air-liquid interface during respiration (Zuo et al., 2008). During breathing, the lungs are continuously exposed to the surrounding environment via the inhaled air, and the LS film is the first barrier that meets any inhaled substance. This interaction between substance and surfactant usually has little or no consequences for LS function, but some inhaled chemicals can disrupt the function of LS. This may lead to an increase in alveolar surface tension and subsequently alveolar collapse (Enhorning, 2001). Reopening of an atelectatic area requires energy, and breathing becomes labored. The friction, caused by the opening of the collapsed areas, may also cause damage to the airway epithelium, allowing extravasation of blood and serum proteins into the lung lumen. These proteins inhibit LS function further (Ishizaka et al., 2004).

IPs frequently cause acute inhalation toxicity in humans after accidental inhalation following application of the product. Instances where people have been injured after inhaling aerosolized products are most often found as case reports in the literature (Fagan et al., 1977; Muller-Esch et al., 1982; Schicht et al., 1982; Okonek et al., 1983; Thibaut et al., 1983; Woo et al., 1983; Christensen et al., 1984; CDC, 1993a,b; Kelly and Ruffing, 1993; Laliberte et al., 1995; Yamashita and Tanaka, 1995; Burkhart et al., 1996; Testud et al., 1998; Jacobsen et al., 1999; Ota et al., 2000; Bonte et al., 2003; Malik and Chappell, 2003; de Groot et al., 2004; Heinzer et al., 2004; Lazor-Blanchet et al., 2004; Tizzard and Edwards, 2004; Wallace and Brown, 2005; CDC, 2006; Vernez et al., 2006; Ebbehøj and Bang, 2008; Daubert et al., 2009; Hashimoto et al., 2009; Khalid et al., 2009; Epping et al., 2011; Fukui et al., 2011; Weibrecht and Rhyee, 2011; Duch et al., 2014; Nakazawa et al., 2014; Kikuchi et al., 2015; Bennett et al., 2015). These reports involve several different IPs and describe outbreaks involving from one person to hundreds of people. The symptoms are similar for the different IPs; they develop quickly, in minutes to hours after exposure. The pulmonary symptoms include shortness of breath, coughing and tightness in the chest, but may also include systemic indicators such as headache, vomiting or fever. The symptoms usually spontaneously resolve within days of exposure, but may in some cases continue for an extended period of time (months after the inhalation incident) (Schicht et al., 1982; Burkhart et al., 1996; Ota et al., 2000; Wallace and Brown, 2005; Khalid et al., 2009; Fukui et al., 2011; Kikuchi et al., 2015).

The present study aimed to investigate whether disruption of LS function *in vitro* can be used as an alternative method to test for acute inhalation toxicity caused by IPs. An alternative method will firstly reduce the need for experimental animal testing by identifying potentially toxic products *in vitro*, allowing for removal of toxic products before undertaking *in vivo* studies. Secondly, testing impregnation products *in vitro* will lead to better consumer safety by easing identification of potentially hazardous products before human health is jeopardized.

As the alternative method, we use the constant flow through set-up of the constrained drop surfactometer (cf-CDS) (Valle et al., 2015; Sørli et al., 2015a) as a screening tool to assess the effect of IPs on LS function. The cf-CDS method is a novel *in vitro* method that mimics the conditions for the LS in the lungs (Sørli et al., 2015a). A surfactant drop is placed on a hollow pedestal with a sharp edge, the volume (and so the surface area) is adjusted by introducing and removing liquid through the base of the pedestal by a syringe connected to a computer-controlled stepping motor. This simulates the movement of the LS layer during breathing. Images of the drop are collected as it is cycled between the set minimum and maximum volume, and based on these images a computer program, ADSA (axisymmetric drop shape analysis) (Zuo et al., 2004; Saad and Neumann, 2016), calculates the surface tension of the drop continuously. To determine if the cf-CDS method can predict whether IPs are toxic to inhale, we exposed mice to the same IPs by inhalation while continuously monitoring their respiration pattern to determine the effect on lung function *in vivo*.

The mouse model used in the present study has previously been used to assess the airway irritation potential of industrial chemicals (Alarie, 1973; Nielsen et al., 2005). The effect of the test substance is assessed based on changes in the breathing pattern during respiration (Alarie, 1973). Inhalation of some aerosolized IPs leads to an irreversible reduction in tidal volume (Nørgaard et al., 2010, 2014; Duch et al., 2014; Sørli et al., 2015b). This effect has been proposed to be driven by interaction between the IP and the LS, which may lead to development of atelectasis (Nørgaard et al., 2010). Atelectasis may progress to tissue damage and edema, and product testing may therefore cause irreversible and lethal lung damage (Hubbs et al., 1997; Pauluhn et al., 2008; Nørgaard et al., 2010). We refined the mouse model during the course of the experiments to keep the potential suffering of the animals at the lowest possible level. The refinements are described in the section "Refinement of the *in vivo* model".

Our aim was to determine whether LS inhibition could be used as an alternative method for testing acute inhalation toxicity of IPs. In the long run, this method may prove to be an alternative to the currently regulatory accepted OECD guidelines OECD TG 403 and 436 for acute inhalation toxicity using animals (OECD, 2009a,b). 21 IPs were tested using the cf-CDS method, whereof 6 have been involved in human inhalation accidents. As 10 of the products had been previously been tested for acute inhalation toxicity in mice, only the other 11 products were tested in the *in vivo* bioassay in the present study. The results from the *in vitro* method were subsequently compared to the *in vivo* toxicity in both mice and humans.

## 2 Animals, materials and methods

*Generation of IP aerosols*

Aerosols of the tested IPs were generated in the same way for *in vitro* and *in vivo* experiments. The product was led from a glass syringe into a Pitt no. 1 jet nebulizer (Wong and Alarie, 1982) by an infusion pump (New England Medical Instruments Inc., Medway, MA, USA). In the *in vitro* experiments, the exposure air-stream was led through glass columns and into the 1.9-l chamber of the cf-CDS and sucked out through the baseplate. For the *in vivo* mouse bioassay, the IP aerosols entered a 20-l exposure chamber of glass and stainless steel (Clausen et al., 2003), with an air exchange rate of approximately 1 per min. Outlet air was passed through a series of particle and active coal filters before exhaust to the atmosphere.

In vitro *method measuring LS inhibition*

LS inhibition *in vitro* was tested using the cf-CDS method (Sørli et al., 2015a) by exposing a drop of LS to increasing amounts of IP. A drop of LS (Curosurf®, 10 µl of 2.5 mg/ml) was placed on a hollow based pedestal with a sharp edge, and subjected to dynamic cycling at 40 cycles/min and less than 30% compression. The cf-CDS and aerosol generation setup was kept at 37°C inside a heating box. In short, a steady stream of air (containing the aerosolized IP) flowed from the top to bottom of the chamber to expose the LS to an increasing concentration of the tested product. The exposure concentration was monitored by a quartz crystal microbalance (QCM) placed close to the pedestal.

The LS was cycled prior to exposure to obtain a baseline value for the surface tension, and any experiment with a minimum surface tension of > 5 mN/m was discarded. The cycling of the LS was stopped at intervals and the drop was refilled with buffer to replace liquid that had evaporated. Images were continuously taken of the drop and analyzed by ADSA. The primary output was the surface tension of the LS drop. If the IP inhibited the LS (as described below), the aerosolization rate was reduced. Thus, IPs were tested with the lowest aerosolization rate that caused inhibition within 5 min. All non-inhibiting IPs were tested for 10 min at the highest possible aerosolization rate. After each experiment, the exposure was stopped and the chamber was left for 5 to 10 min to allow the volatile fraction of the IP to evaporate from the QCM and the deposited material to reach a stable plateau.

A surface tension plot, where each dot corresponds to a single captured drop image, was created by ADSA. Representative surface tension profiles of LS subjected to inhibitory and non-inhibitory IPs can be found in Figure S1[1]. Inhibition of LS activity was defined as at least seven consecutive minimum surface tensions of ≥ 10 mN/m during compression. Atelectasis is thought to occur *in vivo* at this minimum surface tension (Tashiro et al., 1998). Inhibition of LS function could, alternatively, be defined by an IP film forming on the drop (see below, Fig. 1).

Most of the inhibitory IPs inhibited the LS function by preventing the cycling LS from reaching a minimum surface tension below 10 mN/m. However, some products inhibited the LS function by forming a thick film on the surface of the surfactant droplet during dynamic cycling. The IP film held together the droplet and increased the surface viscosity, thus resulting in the top of the droplet being "flattened" during compression (Fig. 1). The thickness of the IP film seemed to gradually increase with time. For the products "Stain repellent nano" and "Liquid stain protection", the LS was inhibited as a low surface tension could not be reached (the minimum surface tension increased to > 10 mN/m after 9 and 30 s of exposure, respectively). In addition, a film appeared 3 and 10 min after the start of exposure, respectively. For "HG textile" and "HG leather" the minimum surface tension did not increase to levels above 10 mN/m, however a film formed on the LS drop after less than 2 min of exposure. The "flattened" images are analyzed as having a low surface tension by ADSA, but with continuous cycling the IP film distorted the axisymmetry of the drop by wrinkling the surface or skewing the drop. The latter images cannot be analyzed by ADSA and it gives a warning.

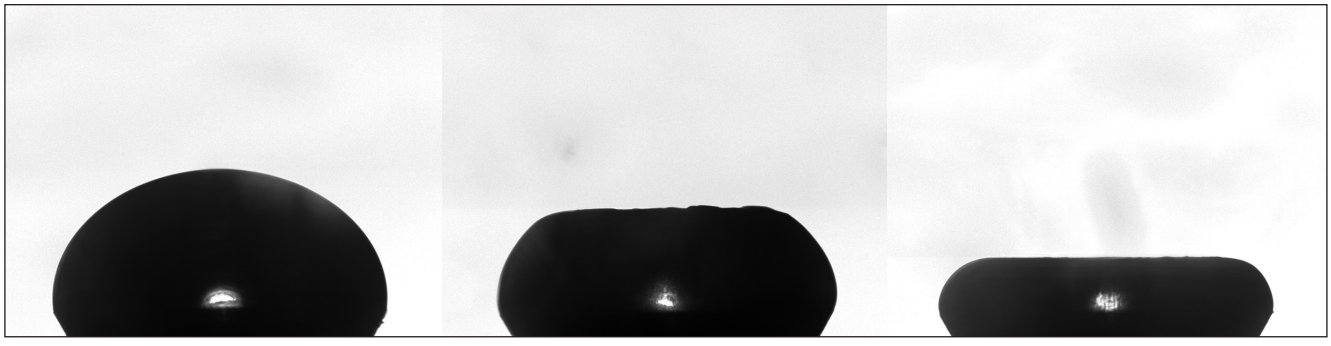

**Fig. 1: Images of the surfactant drop with IP film formation during compression**

An LS drop not exposed to IP (left) has a rounded shape. When an IP film forms on the surfactant drop, the top is "flattened". LS drop exposed to "Liquid stain protection" (middle) and "Stain repellent nano" (right). The irregularities seen on the top stem from the film being wrinkled during compression.

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**Tab. 1: The source of the 21 impregnation products used in this study and references to previously published *in vivo* mouse bioassay data and known human toxicity**

| Impregnation product | Source | *In vivo* data published | Human toxicity |
|---|---|---|---|
| "Wood impregnation" | Dr Scheepers, Radboud University Nijmegen (The Netherlands) | Yes (Sørli et al., 2015b) | Yes (Scheepers et al., 2016) |
| "Stain repellent super" | Akemi GmbH (Nürnberg, Germany) | Yes (Duch et al., 2014) | Yes (Duch et al., 2014) |
| "Liquid stain protection" | Dorothee Walter, Fraunhofer ITEM (Hannover, Germany) | | Yes (Hahn et al., 2015) |
| "Faceal oleo MG" | PSS Interservice (Geroldswil, Switzerland) | | Yes (personal communication, Danish poison center) |
| "HG textile"[a] | HG International (Almere, The Netherlands) | | Yes (personal communication, Dutch poison center) |
| "HG leather"[a] | HG International (Almere, The Netherlands) | | Yes (personal communication, Dutch poison center) |
| "Antismuds" | ENC Natursten A/S (Svendborg, Denmark) | | |
| "Footwear protector" | Granger's (Derbyshire, UK) | Yes (Sørli et al., 2015b) | |
| "Nakano impregnation" | Harald Nyborg (Odense, Denmark) | | |
| "Non-absorbing floor materials" | NanoCover (Aalborg, Denmark) | Yes (Nørgaard et al., 2010) | |
| "Rim sealer" | NanoLotus (Odense, Denmark) | Yes (Sørli et al., 2015b) | |
| "Stain repellent nano" | Akemi GmbH (Nürnberg, Germany) | | |
| "Stain repellent" | Akemi GmbH (Nürnberg, Germany) | | |
| "Bath and tiles" | NanoCover (Aalborg, Denmark) | Yes (Nørgaard et al., 2010) | |
| "Faceal oleo HD" | PSS Interservice (Geroldswil, Switzerland) | | |
| "Special textile coating" | NanoLotus (Odense, Denmark) | Yes (Nørgaard et al., 2014) | |
| "Textiles and leather concentrate" | NanoCover (Aalborg, Denmark) | Yes (Nørgaard et al., 2014) | |
| "Textiles and leather" | NanoCover (Aalborg, Denmark) | Yes (Sørli et al., 2015b) | |
| "Car glass" | NanoCover (Aalborg, Denmark) | Yes (Sørli et al., 2015b) | |
| "Footwear repel" | Granger's (Derbyshire, UK) | | |
| "Performance repel" | Granger's (Derbyshire, UK) | | |

[a] full names "HG water, oil, fat & dirt proof for textile" and "HG water, oil, fat & dirt proof for leather", respectively

The warning, combined with visual confirmation of the "flattening" of the drop, defined the products as inhibitory to LS. If the IP film "only" flattened the drop, the determination had to be done visually. The drop image is followed visually throughout the experiment, and the flattening is clearly noticeable.

*Animals*

The mouse bioassay data for 10 of the IPs have been published previously (see Tab. 1). For the 11 additional IP bioassays, mice were of a similar strain (BALB/cJ, as the BALB/cA strain is no longer available) and age as in previous experiments and were housed under the same conditions. The mice were kept behind a specific pathogen free (SPF) barrier. Thus, 212 inbred BALB/cJ male mice aged 5-8 weeks at arrival were purchased from Taconic M&B (Ry, Denmark) and housed in polypropylene cages (1290D Eurostandard type III from Scanbur, 425 x 266 x 155 mm) furnished with aspen bedding material (Tapvei, Estonia), enriched with a mouse house (80-ACRE011, Techniplast, Italy) and small aspen blocks (Tapvei, Estonia). The mice were 6-12 weeks old when they were used in the bioassay. The photo-period was from 06:00 to 18:00, the temperature 21°C and relative humidity 55%. Cages were sanitized twice weekly. Food (Altromin no. 1324, Altromin, Lage, Germany) and municipal tap water were available *ad libitum*. The mice

were randomly assigned to cages upon arrival, 3-4 mice per cage, and acclimatized for a minimum of one week. Generally, mice from the same cage were used in the same experiment; the mice had not been used for any other procedures prior to the bioassay. The experiments were performed between 09:00 and 15:00. The breathing pattern of each mouse was monitored in real time and the mice were visually monitored throughout the experiment.

*Ethical statement*

Treatment of the animals followed procedures approved by the Animal Experiment Inspectorate, Denmark (Permissions No. 2006/561-1123-C3, 2012−15−2934−00616-C1 and 2014-15-2934-01042-C2). All experiments were performed by trained personnel and conformed to the Danish Regulations on Animal Experiments (LBK nr. 474 af 15/05/2014 and BEK nr 1589 af 11/12/2015), which include guidelines on care and use of animals in research. Anesthesia was not used during the experiments, because the bioassay depends on the animals being fully awake with uncompromised breathing. Acute inhalation toxicity was observed as a rapid depression of the tidal volume. The mouse bioassay has gone through several rounds of refinement as described below. The number of animals used to test the toxicity of each product is given in Table S1[1].

*Collection of respiratory parameters*

The Notocord Hem (Notocord Systems SA, Croissy-sur-Seine, France) data acquisition software was used to collect and calculate several mouse respiratory parameters. We used the tidal volume (VT, mL) and respiratory frequency (breaths/min), but the program also calculates parameters linked to airway irritation and other parameters not reported in this study. Atelectasis may be observed as an irreversible decrease in VT, concurrent with a compensatory increase in respiratory frequency (Nørgaard et al., 2010, 2014). Comprehensive descriptions of the breathing parameters and their interpretation have been made elsewhere (Alarie, 1973; Vijayaraghavan et al., 1993; Larsen and Nielsen, 2000). Data acquisition and calculations were performed as described previously (Larsen et al., 2004).

*Mouse bioassay for evaluation of acute inhalation toxicity*

To assess the acute effects of IPs on respiration, groups of mice (n = 4-10, see Tab. S1[1]) were placed in individual, whole body plethysmographs and exposed head-out. First, a 15-min baseline period was recorded for each mouse while inhaling laboratory air. Then, the mice were exposed to the IP until the breathing pattern was affected, or for a maximum of 60 min. To assess exposure-related effects, the respiratory parameters during exposure were compared in real time to baseline levels, i.e., each mouse served as its own control. For each mouse, mean values of each minute during the experiment were calculated. Examples of concentration- and time-dependent effect curves can be found in (Nørgaard et al., 2010, 2014; Duch et al., 2014; Sørli et al., 2015b)

For the products "Stain repellent", "Stain repellent nano" and "Antismuds", the No Observed Adverse Effect Concentration (NOAEC) was found by exposing a group of 8-10 mice to a high starting concentration, and then decreasing the concentration used to expose other groups of mice until no effect on the VT was seen. For the IP "Liquid stain protection", groups of 8-10 mice were subjected to a high starting concentration, followed by lower concentrations, but the NOAEC was determined after doing a range-finding experiment, followed by a NOAEC experiment (see below). For the remaining seven IPs the following was done: an initial range-finding experiment was performed by exposing a group of mice (n = 4-5) to increasing concentrations of IP. The start concentration was set based on data from the cf-CDS method, i.e., an IP that inhibited LS function started at a lower concentration than a product that did not inhibit LS *in vitro*. This was done to ensure that the first concentration would not cause acute inhalation toxicity. Following recording of the baseline, the start concentration was used during the first 15 min of exposure, and if no effect was observed at the previous concentration the infusion flowrate was then doubled every 15 min. If no effect was observed after a total of 60 min exposure (and testing of four concentrations), a second range-finding experiment was done using a new group of mice and the flow rate was increased until the highest concentration that could be generated in the system was reached. If no effect occurred during any of the range-finding experiments, a group of mice (n = 5-7) was exposed to the highest concentration that could be generated and this concentration was designated the NOAEC. If, on the other hand, an effect occurred in the range-finding experiment, a group of mice (n = 5-7) was subjected for 60 min to the concentration previous (lower) to the one causing the effect in the range-finding experiment. If no reduction in VT was observed during this experiment, this concentration was denoted the NOAEC. However, if an effect did occur during the 60-min period, the concentration was reduced again by half and this exposure concentration was generated for 60 min as described above.

*Refinement of the* in vivo *model*

We have worked with the acute airway effect of IPs for several years (Nørgaard et al., 2010, 2014; Duch et al., 2014; Sørli et al., 2015b), and during this period, the *in vivo* bioassay has gone through several rounds of refinement.

From the first sets of experiments (Nørgaard et al., 2010, 2014), we know that the toxic response to IPs is very uniform, and manifests as a rapid reduction in VT. Animals experiencing a toxic response to an IP are in a moribund state and will die within 24 h (Nørgaard et al., 2010). The reduction of VT is irreversible and recovery does not occur (Nørgaard et al., 2010, 2014; Duch et al., 2014; Sørli et al., 2015b). A severe reduction in VT during the experiment (> 50% reduction compared to baseline) can lead to death during exposure (Nørgaard et al., 2010, 2014; Duch et al., 2014; Sørli et al., 2015b). Based on these observations, we reduced the group size in each experiment from n = 10 to n = 4-5 during range-finding, and to n = 5-7 for determination of the NOAEC. The animals were removed after the experiment and killed immediately by cervical dislocation without a period of recovery to reduce the time a single animal was exposed and restrained. In addition, animals

with a rapid reduction in VT were removed from the exposure chamber and killed immediately. Finally, data from the cf-CDS method was used to determine the start concentration in the range-finding experiment, so that exposure to products inhibiting LS *in vitro* started at a lower concentration and accidental induction of acute inhalation effects was prevented.

*Exposure monitoring*

Exposure concentrations in the bioassay were calculated by gravimetric filter sampling (described in Clausen et al., 2003) combined with measurement of the non-volatile compounds of the products to calculate the wet weight of the product exposure. To determine the non-volatile fraction of the products, approximately 1 ml of test IP was transferred to a pre-weighed 2 ml glass vial and purged to dryness at ambient temperature by a gentle stream of nitrogen. The non-volatile fraction was determined gravimetrically in duplicate. Aerosol particle size distribution was measured for the 10 previously published bioassays (Nørgaard et al., 2010; Duch et al., 2014; Sørli et al., 2015b) (Tab. S2[1]). These IPs contain a variety of solvents and active ingredients, and aerosolization consistently produced inhalable droplets. The same aerosolization technique was used in the present publication. We therefore assumed that the additional products tested for this publication also produced respirable droplets.

*Human toxicity*

Six of the tested products have accidently been inhaled by and associated with toxicity in humans.

1) The product "HG leather" had been used in an unventilated room and the woman who had used the product felt like she was going to faint, but there were no respiratory complaints at the time of the emergency call. Information on this case was provided by the Dutch poison center.
2) The product "HG textile" was involved in two poisoning cases. In the first case, a woman complained of dyspnea, cough, dizziness, tiredness and myalgia the day after using the product. On examination, there were no signs of pneumonia or fever, and her oxygen saturation was normal. In the second case, a woman had sprayed two whole cans (2 x 300 ml) of the product and two days later complained of headache, dyspnea and cough. Upon examination, she did not have a fever and her oxygen saturation was 97%. Information on these cases was provided by the Dutch poison center.
3) A worker sprayed 10-15 l of the product "Faceal Oleo MG" on a tile surface using a low-pressure spraying device; the application took approximately 30 min. The location, a staircase leading down to a metro station, was partly open to ambient air, but without active ventilation. The person did not wear respiratory protection during the application. The worker started coughing 20 min after the spraying, developed chills and was taken to hospital where he presented with slightly decreased $O_2$ saturation. The symptoms resolved after 24 h, but the patient subsequently developed non-allergic asthma (personal communication, Danish poison center).
4) The "Wood impregnation" product was involved in an inhalation toxicity accident involving 10 workers. One liter of the product was sprayed in a workshop, and one person who entered the workshop shortly after the application rapidly developed respiratory symptoms and was diagnosed with severe chemical pneumonitis. Nine people, working in the room next door, who were exposed 15 hours after the spraying incidence, experienced dry cough and chest tightness (Scheepers et al., 2016).
5) The "Liquid stain protection" product caused 11 described cases of intoxication between 2003 and 2011 that were related to application of the product. Symptoms ranged in severity from minor to severe, but all cases presented with initial severe cough (Hahn et al., 2015).
6) The product "Stain Repellent Super" was the cause of a large inhalation exposure accident in Greenland when the IP was sprayed on the ground floor of a supermarket using an airless spray gun. In the hours following the application, 43 people contacted the local hospital with respiratory symptoms, and 39 thereof were clinically examined. Their symptoms included coughing, tachypnoea, chest pain, general malaise and fever. The physical examination revealed perihilar lung infiltrates on chest radiographs and reduced blood oxygen saturation. The acute symptoms resolved gradually within 1-3 days and no delayed symptoms were observed. The incident is described in detail by Duch et al. (2014).

## 3 Results

### 3.1 Lung surfactant inhibition *in vitro*

21 IPs were tested in the cf-CDS method. Five IPs had no inhibitory effect, whereas 16 products inhibited LS function (Tab. 2).

### 3.2 *In vivo* toxicity in mice

The NOAEC of 10 of the IPs had been determined in the mouse bioassay previously (see Tab. 1), thus for this work, only the other 11 IPs were tested. NOAEC was determined *in vivo* and defined as the highest concentration at which there was no change in VT compared to baseline. Of the total of 21 products, 8 did not affect VT, even at the highest exposure concentration that could be generated (Tab. 2).

### 3.3 Correlation of *in vitro, in vivo,* and human data

The effect of IPs on LS function *in vitro* is summarized in Table 2, alongside their *in vivo* effects in mice and their involvement in human toxicity accidents, if any. Overall there is correlation between classification of a product as inhibitory or not *in vitro* and the presence or absence of toxicity to mice for 18 of the 21 IPs. Importantly, all 13 products that were toxic to mice also inhibited LS *in vitro*. Thus, the sensitivity (true positive rate) of the *in vitro* method is 100%. There were no false negatives, i.e., no products that were toxic to mice were classified as "not inhibitory" to LS. Of the 8 products that were non-toxic to mice, only 5 did not inhibit LS function *in vitro*, therefore

**Tab. 2: Summary of the effect of impregnation products on LS function *in vitro*, on the breathing pattern of mice, correlation between *in vitro* and *in vivo* results, and involvement in human acute inhalation toxicity accidents**

| Impregnation product | *In vitro* LS inhibition | *In vivo* toxicity | Correlation *in vitro – in vivo* | Human toxicity |
|---|---|---|---|---|
| "Wood impregnation" | Yes | Yes | Yes | Yes |
| "Stain repellent super" | Yes | Yes | Yes | Yes |
| "Liquid stain protection" | Yes | Yes | Yes | Yes |
| "Faceal oleo MG" | Yes | Yes | Yes | Yes |
| "HG textile" | Yes | Yes | Yes | Yes |
| "HG leather" | Yes | Yes | Yes | Yes |
| "Antismuds" | Yes | Yes | Yes | – |
| "Footwear protector" | Yes | Yes | Yes | – |
| "Nakano impregnation" | Yes | Yes | Yes | – |
| "Non-absorbing floor materials" | Yes | Yes | Yes | – |
| "Rim sealer" | Yes | Yes | Yes | – |
| "Stain repellent nano" | Yes | Yes | Yes | – |
| "Stain repellent" | Yes | Yes | Yes | – |
| "Bath and tiles" | No | No | Yes | – |
| "Faceal oleo HD" | No | No | Yes | – |
| "Special textile coating" | No | No | Yes | – |
| "Textiles and leather concentrate" | No | No | Yes | – |
| "Textiles and leather" | No | No | Yes | – |
| "Car glass" | Yes | No | No, false positive | – |
| "Footwear repel" | Yes | No | No, false positive | – |
| "Performance repel" | Yes | No | No, false positive | – |

the specificity (true negative rate) of the cf-CDS method was 5/8 = 63%. In other words, the false positive rate *in vitro*, i.e., the likelihood of labeling a product as toxic *in vitro* when it would not have an effect *in vivo*, is 37%. In humans, 6 of the 21 investigated IPs have given rise to cases of acute inhalation toxicity. All of these 6 products also caused *in vivo* toxicity in mice and inhibition of LS *in vitro*.

## 4 Discussion

Impregnation products consist of very complex chemical mixtures. They contain active ingredients, which form a water and dirt repellent surface film after application, solvents that carry the active ingredient, and in some cases also a propellant. Each product may contain several different substances from each of these categories. The cause of the observed toxicity of IPs has been suggested to relate to the overall chemical composition of the products, rather than to individual chemicals (Nørgaard et al., 2014). We and other researchers have not been able to identify a clear relationship between the content of specific groups of chemicals and the toxicity of IPs. As an example, fluorinated compounds are found both in toxic and non-toxic products (e.g., "Rim sealer" and "Textiles and leather", respectively, see Tab. S2[1]). A strategy to make products safe has been to use water as a solvent, however, "Footwear protector" is toxic whereas "Performance repel" is not – though both are water-based (Tab. S2[1]). To complicate matters further, it has previously been shown that different solvents may modify the toxicity of an active ingredient – or may even be a prerequisite for its toxicity (Nørgaard et al., 2014). Thus, the toxicity of a particular IP is hard to predict, and several different chemical compositions can induce a toxic response. Our current knowledge does not allow prediction of inhalation toxicity of an IP based on its chemical composition. Safety testing of all possible combinations of substances by the conventional *in vivo* bioassay is not a rational option. Regulation of specific products is further hampered by the name of the toxic product often being omitted in case reports. Even when the product name is given, this does not grant access to the complete chemical composition as Material Safety Data Sheets are often incomplete. Finding a good predictor of the acute and serious lung reaction will ease the identification of hazardous products both during product development and upon suspicion of toxicity of specific products.

IPs may cause acute inhalation toxicity to consumers and the effects of the inhalation can be moderate to severe. In this paper, we describe an *in vitro* method that can be used to screen for toxicity of IPs. The method detected all the products that were toxic to mice upon inhalation. More importantly, all products that have been associated with inhalation toxicity in humans were detected in the *in vitro* model. The method has proven useful for determining the inhalation toxicity of IPs, however we do not know if it can be used with the same success with other chemical classes. We will continue the work with other substances to determine if the method can be used to predict the inhalation toxicity of other inhaled substances. As part of developing the cf-CDS method, we tested commercially available pharmaceutical formulations intended for inhalation (Sørli et al., 2015a). These formulations have proven to be safe for humans to inhale, and we found no effect on LS function, even at extreme concentrations. This and testing the method with IPs are the first steps in the process towards establishing an alternative method to acute inhalation toxicity testing in animals, i.e., OECD TG 403 or 436 (OECD, 2009a,b).

The cf-CDS method only screens for acute inhalation toxicity related to disruption of LS function. There may be other mechanisms associated with inhalation toxicity that are not related to LS inhibition, such as cytotoxicity or systemic toxicity; these mechanisms would not be picked up by the LS inhibition method. This has to be taken into consideration before the assay can be accepted by regulators. Addition of an *in vitro* method that can measure cytotoxicity and systemic toxicity may be required before the current guidelines (OECD, 2009a,b) can be completely replaced.

The cf-CDS method did not falsely identify acutely toxic products as safe, i.e., no false negative results were observed. The cf-CDS method did however identify some products as toxic even if no reaction was observed in the mouse bioassay (false positives). Curosurf®, the LS used in the *in vitro* tests, does not contain all the components found in natural lung surfactant, such as the proteins SP-A and SP-C and cholesterol. This difference may make the surfactant more sensitive to inhibition, and may be the reason for the false positives predicted by the *in vitro* method. However, even if natural surfactant may be a better approximation to lung function *in vivo*, it is difficult to obtain in sufficient and reliable amounts. An alternative method to the existing OECD guidelines using animals cannot rely on laborious collection of surfactant, when there are commercially, well characterized and controlled LS preparations readily available. We have therefore chosen to base the cf-CDS method on this commercial LS preparation.

We have previously shown that when IPs were tested in other *in vitro* models of LS inhibition (in the Langmuir trough or using the Capillary surfactometer), the results correlated well with inhalation toxicity in mice (Duch et al., 2014; Sørli et al., 2015b). However, neither method could mimic physiologically relevant conditions, such as the frequency of cycling between the maximum and minimum surface area of the LS film and manipulation of the atmosphere that the LS film was exposed to. One of the methods (the Capillary surfactometer (Sørli et al., 2015b)) was limited to the study of water soluble products. The cf-CDS method is much more suitable for testing the interaction of chemicals with the LS, as the method mimics the physiological conditions of the lung, such as cycling frequency, the extent of the compression, and temperature. The cf-CDS method can test products of any composition, the exposure concentration can be continuously increased, and the exposure concentration can be monitored (Sørli et al., 2015a).

Of the 21 products tested, 13 were toxic to mice, and also inhibited LS function *in vitro*. It is however not possible to use the *in vitro* ranking to predict the NOAEC exposure concentration *in vivo*. There are several reasons for this, but one important factor is the difference in exposure concentration measurement. For the mouse bioassay, the concentration is calculated by combining filter measurements of the exposure atmosphere and the dry weight of the non-volatile fraction after drying with nitrogen. For the cf-CDS method, the concentration is calculated as the non-volatile fraction of the IP that settles on the QCM and has not evaporated after drying for 5 min under a stream of air. Depending on whether an IP is dried under a flow of nitrogen, a flow of air in the animal exposure chamber, or in the CDS chamber, the drying is different; therefore, the measured exposure concentrations are not directly comparable. Instead, the *in vitro* method can be used as a qualitative toxic/non-toxic screening method prior to or instead of the mouse bioassay. As we develop the cf-CDS method further, we will try to make more comparable measurements of exposure concentration, e.g., by measuring the aerosol composition in both chambers.

The concentration of LS used in the *in vitro* assay (2.5 mg/ml) is lower than the concentration in the lung lining fluid. The surfactant concentration in the alveolar hypophase is estimated to range from 30 to 100 mg/ml, depending on the specific mammalian species (Zuo et al., 2008). However, we and others have found the same equilibrium, minimum and maximum surface tension of a range of surfactant concentrations (0.5 to 28 mg/ml), surfactant preparations (Infasurf, BLES and Curosurf®) and method of analysis (pulsating bubble surfactometer, captive bubble surfactometer or CDS) (Bachofen et al., 2005; Acosta et al., 2007; Zuo et al., 2008; Valle et al., 2014, 2015). If the existing guideline for measuring acute inhalation toxicity using animals is going to be replaced, the replacement has to be cheap and easy to perform. Using 2.5 mg/ml as the test concentration in the *in vitro* method is a good approximation to the lungs when measured according to the surface tension.

The relatively high false positive rate (the *in vitro* method predicted 37% of the non-toxic IPs as toxic) may be a drawback of the method. However, if the positive products are tested in animals for confirmation, there is a high risk of testing toxic products and causing suffering to the animals.

Based on the knowledge of the current project and earlier work (Sørli et al., 2015a), we would recommend that potential products first be tested *in vitro* and that the results from this test will determine the progression to animal testing. Products

that inhibit LS function should be discarded or reformulated before a new *in vitro* test is performed. IPs that do not inhibit LS function will still need to be tested in animals at the moment. However, as the method is tested with more potentially inhaled substances, we believe that the cf-CDS, possibly in combination with other *in vitro* assays, will be able to completely replace the currently accepted acute inhalation toxicity test. Using this approach will reduce the suffering that would otherwise have occurred during testing of toxic products, and will reduce the number of animals needed for testing.

Six of the tested products have been involved in incidents in which up to 43 people were exposed to aerosols of the product and subsequently fell ill. Human acute inhalation toxicity often occurs when consumers do not use the product as intended by the manufacturer, e.g., by spraying a product that should be applied with a mop or brush, or using a nozzle producing small droplets (case number 6 and 3, respectively). However, in some of the inhalation toxicity cases, the products were intended for spraying (e.g., case 1 and 2).

In summary, testing whether an impregnation product causes inhibition of lung surfactant *in vitro* is an excellent way of screening products before they are marketed and potentially can cause harm to humans. The cf-CDS method is a promising model for such screening.

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## Conflict of interest

The authors declare that they have no conflicts of interest.

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