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Research Article

An *in vitro* Method for Predicting Inhalation Toxicity of Impregnation Spray Products

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Summary

Impregnation spray products are used for making surfaces water and dirt repellent. The products are composed of one or more active film-forming components dissolved or suspended in an appropriate solvent mixture. Exposure to impregnation spray products may cause respiratory distress and new cases are reported frequently. The toxicity appears to be driven by a disruption of the pulmonary surfactant film, which coats the inside of the lungs. Due to the complex chemistry of impregnation spray products, it is impossible to predict if inhalation of an aerosolized product is toxic *in vivo*. The aim of this study was to evaluate whether disruption of the pulmonary surfactant film can be used as a predictor of the toxic effects *in vivo*. Nine impregnation products with various chemical compositions were selected for testing and the main constituents of each product, e.g., solvents, co-solvents and film-forming compounds, were identified by mass spectrometry. We used a capillary surfactometry method to assess disruption of pulmonary surfactant function *in vitro* and a mouse model to evaluate acute respiratory toxicity during inhalation. Concentration-response relationships were successfully determined both *in vitro* and *in vivo*. The true positive rate of the *in vitro* method was 100%, i.e. the test could correctly identify all products with toxic effects *in vivo*, the true negative rate was 40%. Investigation of inhibition of the pulmonary surfactant system, e.g. by capillary surfactometry, was found useful for evaluation of the inhalation toxicity of impregnation spray products and thus may reduce the need for animal testing.

Keywords: Impregnation products, pulmonary surfactant, lung toxicity, capillary surfactometry, consumer product safety, 3Rs

1 Introduction

Impregnation products are used both in commercial and private settings, and are applied to various surfaces (tiles, textiles, leather, glass etc.) to make them water and dirt repellent. These products are the cause of several cases of respiratory distress. The symptoms often develop within minutes to a few hours after exposure and include coughing, shortness of breath and chest pain (Burkhart et al., 1996; Centers for Disease Control and Prevention, 1993). The symptoms spontaneously resolve within a few days, but sometimes supportive treatment with oxygen, bronchodilators or corticosteroids is needed (Burkhart et al., 1996; Laliberte et al., 1995; Lazor-Blanchet et al., 2004). Permanent effects after exposure are rare, but have been reported (Wallace and Brown, 2005).

Pulmonary surfactant is a fluid film covering the inside of the terminal bronchioles and the alveoli. Pulmonary surfactant is composed of lipids (90%) and surfactant proteins (SP)-A, -B, -C and -D (10%) (Zuo et al., 2008). The function of the surfactant is to decrease surface tension, thus reducing the effort needed to inflate the lung during inspiration. Further, the surfactant film prevents the alveoli from collapsing during expiration. Deficiency or disruption of the surfactant film may cause severe respiratory distress. Infant respiratory distress syndrome (IRDS) is caused by an insufficient surfactant layer in the immature lungs, and the condition is treated by supplying exogenous surfactant (Polin and Carlo, 2014). Surfactant function is also disrupted in acute respiratory distress syndrome (ARDS), but the mechanisms have not yet been fully elucidated (Ware and Matthay, 2000).

Outbreaks of pulmonary illness have been reported several times in the past when the formulation of an impregnation product has been altered (Burkhart et al., 1996; Centers for Disease Control and Prevention, 1993; Heinzer et al., 2004; Laliberte et al., 1995; Lazor-Blanchet et al., 2004; Vernez et al., 2006). The potential health risk of impregnation products is well known, but pinpointing the causative agent that makes a product hazardous has proven difficult. The culprits have been suggested to be the droplet size (Yamashita et al., 1997a; Yamashita et al., 1997b), the solvent (Kelly and Ruffing, 1993) or fluororesin (Yamashita and Tanaka, 1995). More recently it has been demonstrated that the toxicity of the product may be influenced both by the film-forming

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substance and the composition of the solvent mixture (Nørgaard et al., 2014). The chemical compositions of these products are complex; therefore hazard prediction based on the individual chemical constituents does not seem feasible.

Rodent studies have been used to assess possible acute toxicity after inhalation of impregnation spray products. Both rats (Pauluhn et al., 2008), guinea pigs (Hubbs et al., 1997) and mice (Duch et al., 2014; Yamashita and Tanaka, 1995) are useful for this purpose. The mouse model used in the present study has previously been used to assess airway irritation potential of industrial chemicals (Alarie, 1973). The respiration is monitored and, based on changes in the shape of the breathing curve, it can be assessed whether a chemical affects the upper, conducting or lower airways; effects can be directly translated to the human situation due to comparable neural functions (Alarie, 1973). Inhalation of certain types of aerosolized impregnation spray products by mice has shown to lead to an irreversible reduction in both tidal volume and expiratory flow rate (Nørgaard et al., 2010, 2014). These effects have been proposed to be driven by interaction between the impregnation product and the pulmonary surfactant. The disrupted pulmonary surfactant may lead to liquid blocking of the terminal airways, which increases airway resistance. Furthermore, disruption of pulmonary surfactant may lead to development of atelectasis (Nørgaard et al., 2010). Atelectasis may progress to tissue damage and edema, and product testing may cause lethal lung damage (Hubbs et al., 1997; Nørgaard et al., 2010; Pauluhn et al., 2008). We have recently shown that a tile-coating product, “Stain repellent super”, which caused respiratory distress in 39 people in Greenland, also inhibits the function of pulmonary surfactant *in vitro* using the Langmuir balance, i.e., the classical method for studying lung surfactant properties (Duch et al., 2014), substantiating that this endpoint has relevance for effects in humans. “Stain repellent super” also induced acute pulmonary toxicity in mice similarly to the effects observed in the present study.

Capillary surfactometry (CS) is an *in vitro* method developed by Enhorning (Enhorning, 2001; Hohlfeld et al., 1999). The method simulates the function of pulmonary surfactant in the terminal bronchioles. Surfactant function in this area of the lung is vital. If the surfactant is damaged, inactive surfactant liquid will flow into the narrow section of the bronchiole and form liquid plugs (Enhorning, 2001), causing reduced lung compliance and labored breathing. In the CS, a drop of surfactant is placed in a capillary with a narrow section that has an inner diameter that is the same as that of the terminal bronchioles (0.25 mm). The capillary is lowered into a water bath and the air pressure is raised to remove the liquid block. Airflow is measured for 2 minutes. A functioning surfactant will prevent liquid from flowing back into the narrow section, however if the surfactant is damaged the surfactant will block the narrow section and the air pressure will be raised to remove the blockage. The CS method cannot differentiate between water and surfactant (as described in detail in section 2), therefore an additional step adding POTS (hydrolysates and condensates of 1H,1H,2H,2H-perfluorooctyl trialkoxysilane) and removing solvents was introduced before measuring surfactant function in the CS. The CS method (without addition of POTS) has been used to test the effects on pulmonary surfactant of ozone exposure in mice (Currie et al., 1998a,b) and allergen challenge in asthmatic patients (Hohlfeld et al., 1999; Jarjour and Enhorning, 1999; Veldhuizen et al., 1994). The method has recently been used to test the toxic effect of a specific nanofilm impregnation product and the effect of the solvent on the product toxicity (Nørgaard et al., 2014). In addition, a strong correlation was observed between the CS and the Langmuir balance (Larsen et al., 2014). The Langmuir balance measures the surface pressure of spread films on a liquid subphase. The film is compressed and expanded and the accompanying change in surface pressure is a measure of surfactant function.

The aim of this study was to test the applicability of pulmonary inhibition *in vitro* as a predictor for lung toxicity *in vivo* and thus reduce the need for experimental animals in safety testing of commercially available impregnation products. The tests were limited to water-based and water-soluble impregnation products due to the nature of the CS method.

2 Animals, materials and methods

Chemicals

Ethanol (99.9%) and 2-propanol (99.9%) were obtained from Sigma Aldrich (Brøndby, Denmark). The lyophilized bovine-derived pulmonary surfactant formulation Alveofact® was a kind gift from Lyomark Pharma (Oberhaching, Germany). It contains phospholipids and the hydrophobic pulmonary surfactant proteins SP-B and -C (Gunther et al., 2002). 1% POTS (hydrolysates and condensates of 1H,1H,2H,2H-perfluorooctyl trialkoxysilane) in 2-propanol was used for the CS method (the product is equivalent to “Non-absorbing floor materials” from NanoCover, Tab. 1).

Impregnation products

Nine commercially available, water-soluble impregnation spray products were included in this study. The following were obtained from NanoCover (Aalborg, Denmark): “Non-absorbing floor materials”, “Textiles and leather”, “Textiles and leather concentrate”, “Bath and tiles” and “Car glass”. The product “Footwear protector” was purchased from Granger’s (Derbyshire, UK), while the products “Special textile coating” and “Rim sealer” were obtained from NanoLotus (Odense, Denmark). A product for “Wood impregnation” was obtained from a confidential source. To determine the non-volatile fraction of the products, 1 ml 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.

Chemical characterization of products

In most cases a complete product description was not available, so the supplied information for the products was complimented by mass spectrometric analysis. For each product, analysis was carried out by: 1) thermal solid phase extraction gas chromatography mass spectrometry (TSPE-GCMS) and 2) low temperature plasma ionization mass spectrometry (LTP-MS). A detailed description of the product analysis can be found in the supplementary material at <http://dx.doi.org/10.14573/altex.1408191s>. Solvents and co-solvents were identified for all products in addition to most of the film-forming components (*cf.* Tab. 1). However, the exact structures of the perfluoracrylate co-polymers in the “Footwear protector” and the “Wood impregnation” products could not be fully determined.

Surfactant function evaluation by the Capillary Surfactometer

Pulmonary surfactant function was assessed using a Capillary Surfactometer (CS, Calmia Medical Inc., Toronto, ON, Canada). This method resembles the conditions in the human terminal bronchioles (Enhoring, 2001). A surfactant sample (0.5 μ l) is introduced into the narrow section (inner diameter 0.25 mm) of a glass capillary and placed in a water bath at 37°C. The CS gradually increases air pressure until the liquid blocking the capillary is extruded. A sample with a functioning surfactant will prevent the liquid from collapsing back into the narrow section, and in this case no resistance is measured during the 120 s of airflow (Fig. 1B). However, if the surfactant is damaged, the liquid will collapse and the airflow will be blocked until the CS has raised air pressure sufficiently to remove the blockage (Fig. 1D). Surfactant function is assessed as the time of unobstructed airflow in the glass capillary (“time of open” in %).

A method was developed to quantify the amount of functional surfactant after incubation with an impregnation product. Pulmonary surfactant inhibition is determined by incubating Alveofact® with a dilution series of the impregnation products. To this mixture of pulmonary surfactant and impregnation product, a volume of POTS that only partly inhibits the surfactant function is added. This is done because the CS method cannot differentiate between water (Fig. 1A) and a functioning surfactant (Fig. 1B), both will result in a “time of open” >99%. As some of the impregnation products precipitate parts of the surfactant, leaving a “watery” liquid, the use of POTS enables discriminating the effects of the impregnation products. After addition of POTS, three possible outcomes can be observed;

- 1) If the impregnation product does not have an effect on surfactant function and the surfactant film is intact, then inhibition by POTS will be observed to the same level in the impregnation product containing samples and the control (same reduction in “time of open”; Fig. 2, black bars).
- 2) If the impregnation product inhibits the surfactant function, an additional inhibitory effect by adding POTS will be observed in the samples added impregnation product compared to the control (further reduction in “time of open”; Fig. 2, grey bars).
- 3) If the impregnation product precipitates the surfactant, POTS will not have an effect because no functioning surfactant is present (the “time of open” will be higher compared to the control). This is due to the fact that if the surfactant is precipitated it will behave like water in the CS and the capillary will be open >99% of the time (Fig. 1A).

To assess pulmonary surfactant inhibition by the impregnation products, Alveofact® (4 mg/mL in milliQ water) was incubated with the impregnation products diluted in their original solvents, or the solvent alone (control), at 37 °C for 30 min. An optimal dose of POTS (see below) was subsequently added to the mixtures, mixed and solvents evaporated immediately after. Solvents on their own may significantly inhibit the surfactant function in this method (Fig. 1C), therefore the solvents were removed by evaporation at reduced pressure (200 mbar) and a temperature of 36 °C for 30-60 min. Controls containing 2-propanol (solvent of POTS) and the solvent of the impregnation product were always included to determine if the evaporation step had removed the solvents satisfactorily. Following evaporation, samples only containing solvents resulted in a measurement of >95% “time of open” of the capillary (Fig. 1E), whereas evaporation of the sample with POTS resulted in 2.6% “time of open” (Fig. 1F). Thus, the effect of solvent or the entire impregnation product may be distinguished after evaporation (Fig. 1 C-F). The solvent may facilitate interaction between the active ingredient of the product, but this effect is obtained during the first step.

After evaporation, 0.5 μ L of the sample was analyzed in the CS. Multiple replicates (3-20) were measured for each sample. In the outer points of the “time of open” (>95% or < 5%), the variation is very small, so fewer replicates were measured, whereas when the capillary was open 50 \pm 20% of the time, the variation was very high and more replicates were performed. To normalize for possible time-dependent variations in the method, a positive control (POTS) was included in each experiment. The volume of POTS added to a final test volume of 60 μ l ranged from 6 to 10 μ l. The volume of POTS added was adjusted so that the surfactant was inhibited to a degree that resulted in the capillary being open ~50% of the measured time. At 50% “time of open” a change in surfactant function (and thus “time of open”) can be observed if the impregnation product of interest affects the surfactant: the impregnation product is then able to either reduce (inhibit the surfactant) or increase (precipitate the surfactant) the “time of open.” If the inhibition by POTS is too high or too low these effects will be masked. The effect of the impregnation products on surfactant function was determined in at least two independent experiments.

Concentration-response relationships for the inhibitory effects of impregnation products on the pulmonary surfactant function were established for each of the nine products. The products were diluted in the same solvent as used in the product (*cf.* Tab. 1). The potency of the impregnation products with respect to surfactant inhibition was assessed by identifying the highest concentration of the product that did not have a significant inhibitory effect on the surfactant function (no-observed effect level, NOEL). The product was considered to have no effect on the surfactant function when there was no statistically significant difference between the sample and solvent control ($p < 0.05$).

Animals

The mouse bioassay data for “Non-absorbing floor materials” and “Bath and tiles”, as well as “Textile and leather concentrate” and “Special textile coating” were published in Nørgaard et al., 2010 and 2014, respectively, therefore these animal data are not included below. However for the new experiments, mice were of the same strain and age as in previous experiments and kept under the same housing conditions. Thus, further 90 inbred BALB/cA male mice aged 5-8 weeks at arrival were purchased from Taconic M&B (Ry, DK) and housed in polypropylene cages (380x220x150 mm). The mice were randomly assigned to cages and acclimatized for minimum one week. Generally, mice from the same cage were used for one experiment as the different dose setting experiments of each spray product were performed on different days. The weight was 24.8 \pm 1.9 g when entering the experiments. The cages, housing 4-8 mice each, were furnished with Lignocel® bedding material (Brogaard, DK), gnaw sticks and transparent red plastic nesting houses. The photo-period was from 6 a.m. to 6 p.m., and the temperature and mean relative humidity in the animal room were

21°C ± 2 and 55% ± 5 (mean ± SD), respectively. Cages were sanitized twice weekly. Food (Altromin no. 1324, Altromin, Lage, DE) and municipal tap water were available *ad libitum*.

Ethical statement

Treatment of the animals followed procedures approved by The Animal Experiment Inspectorate, Denmark (Permissions no. 2006/561-1123-C3 and 2012-15-2934-00616-C1). 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. 88 af 30/01/2013), which includes guidelines on care and use of animals in research. Anesthesia was not used during the experiments, because the bioassay depends on fully awake animals. At the time of testing all animals were naïve and unconditioned to experimental procedures. The acute lung toxicity was observed as an irreversible depression of the tidal volume, i.e., during the recovery period lung function did not return to normal. Based on our first publication, where this led to the animals being in a moribund state or death within 24 h (Nørgaard et al., 2010), we assumed in subsequent tests that the observation of irreversible tidal volume depression during the recovery period would lead to the animals' death. Therefore, mice were killed immediately after the recovery period by cervical dislocation. No acute fatalities were observed. In our previous publications (Nørgaard et al., 2010, 2014; Duch et al. 2014), however fatalities were documented with two water-based products in the present study. We have no explanation for this except that this may be ascribed to the specific product formulation and thus may not have been preventable. Until its death, the mouse's respiration was included in the calculation of the group mean, but after its death, the group mean was calculated based on the remaining mice. As explained below, we have refined the test method, so fewer animals are used for each product testing. The exact number of animals used is given in Tab. S1 at <http://dx.doi.org/10.14573/altex.1408191s>.

Generation of test atmospheres and particle characterization

During animal exposure, the impregnation products were led from a glass syringe into a Pitt no. 1 jet nebulizer (Wong and Alarie, 1982) by means of an infusion pump (New England Medical Instruments Inc., Medway, MA, US). The exposure air-stream (25 L/min) was subsequently led through a Vigreux column and then directed into a 20 l exposure chamber of glass and stainless steel (Clausen et al., 2003) resulting in an air exchange rate of approximately 1 min⁻¹. Exposure concentrations were obtained from gravimetric filter sampling as described previously (Clausen et al., 2003) combined with the measurement of the non-volatile part of the products to calculate the wet weight of the product exposure (Tab. 1). Outlet air was passed through a series of particle- and active coal filters before the exhaust to the atmosphere. Characterization of particle size distributions were done separately using and Electrical Low Pressure Impactor (ELPI+, Dekati, Finland) which measures aerodynamic diameters between 6 nm and 10 µm in 14 channels. Sampling was done through 6 mm copper tubing inserted into a central position in the exposure chamber.

Mouse bioassay for evaluation of acute lung toxicity

To assess the acute effects on respiration of the impregnation products, groups of mice (n =4-10, cf. Tab. S1 at <http://dx.doi.org/10.14573/altex.1408191s> for exact groups size) were placed in body plethysmographs and exposed head-out only. First, a 15-min baseline period was recorded for each mouse while inhaling laboratory air. The exposure period (up to 60 min) was followed by a 15-min recovery period in which the mice were exposed to laboratory air. To assess exposure-related effects, the respiratory parameters during exposure were compared 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.

The mouse bioassay data from "Non-absorbing floor materials", "Bath and tiles", "Textiles and leather concentrate" and "Special textile coating" were collected from our previous publications (Nørgaard et al., 2010, 2014). Because these first publications showed a very uniform toxic response on respiration of the mice, we were able to reduce the number of animals in the following studies. "Rim sealer" was tested with 6 mice per group per concentration. For the remaining products, an initial screening was performed by exposing 4 mice to increasing concentrations of impregnation product. This was done by doubling the infusion flow rate after 15 min if no effects were observed. After additionally 15 min exposure, the infusion flow rate was doubled again. If no effects were observed after a total of 60 min exposure, a new group of mice were exposed for 60 min to the highest air concentration possible to generate. If on the contrary, effects were observed during the screening study, dose-response and NOEL determinations were performed with other groups of mice. Due to the nature of the toxic response, which eventually leads to the death of the animals, we decided to determine the NOEL as the highest concentration not causing a significant difference in tidal volume between the baseline and the recovery periods and where no single animal had an irreversible reduction of the tidal volume or died.

Collection of respiratory parameters

The Notocord Hem (Notocord Systems SA, Croissy-sur-Seine, France) data acquisition software was used to collect respiratory parameters. The acquisition program calculates the respiratory frequency (breaths/min), tidal volume (VT, ml), time of inspiration (ms), time of expiration (ms), time from end of inspiration until the beginning of expiration, termed time of brake (TB, ms), time from end of expiration until beginning of the next inspiration, termed time of pause (TP, ms) and mid-expiratory flow rate (VD, ml/s). Stimulation of the trigeminal and vagal nerve endings causes increases in TB and TP, which are markers of upper/sensory and lower airway irritation, respectively. 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 analysis have been made elsewhere (Alarie, 1973; Nielsen et al., 1999; Larsen and Nielsen, 2000; Vijayaraghavan et al., 1993). Data acquisition and calculations were performed as described previously (Larsen et al., 2004).

Statistics

The *in vitro* effect of different impregnation product concentrations was evaluated in a one-way ANOVA. In the case of significant difference between the groups, pairwise comparison with Tukey Simultaneous Test was performed. For all analyses, a significant

difference was accepted at $p < 0.05$. Calculations and power analyses were performed using the Minitab Statistical Software version 15 (Minitab Inc, State College, PA). Effects of the impregnation products on the respiratory parameters were performed by comparing recovery values with baseline values within the same group of exposed mice, i.e., each mouse is an experimental unit. Statistical significance was accepted if the 95% confidence interval of the response induced by impregnation product exposure was not included in the 95% confidence interval of the mean baseline values for all animals.

3 Results

Effects of impregnation product on surfactant function in the in vitro method

The effects of the 9 tested impregnation products on surfactant function are summarized in Table 1. Two products, “Textiles and leather” and “Special textile coating” had no inhibitory effect on pulmonary surfactant. The products “Car glass” and “Bath and tiles” had a high NOEL (>8% impregnation product added to the pulmonary surfactant before any effects were observed), one product (“Rim sealer”) had a NOEL of 4%. Four impregnation products had low NOELs of <2%, two of these “Footwear protector” and “Wood impregnation” contained perfluoracrylate in a water and glycol solution. The remaining two products with a low NOEL contained perfluorsilan/siloxane in water (“Textiles and leather concentrate”) or 2-propanol (“Non-absorbing floor materials”). The results of two impregnation products are shown exemplarily in Fig. 3. “Special textile coating” did not have an effect on the surfactant function, and “Rim sealer” had an inhibitory effect on the surfactant function. Similar dose-response relationships were determined for all products, and their effects (none, inhibitory or precipitating) are indicated in Table 1.

In vivo pulmonary toxicity after inhalation of impregnation product

The 9 different impregnation products were tested in mice and the NOEL determined for each of the products (Tab. 1). For the products “Textiles and leather concentrate”, “Special textile coating”, “Bath and tiles”, “Textiles and leather” and “Car glass” the maximum concentration of the generating system was used to expose the mice for 60 min without effects on their respiration, thus dose-response curves cannot be shown, but data for the three first products is published (Nørgaard et al. 2010, 2014). Measurements of particle size distributions showed that respirable particles were generated for all products (*cf.* Fig. S1 at <http://dx.doi.org/10.14573/altex.1408191>), and thus aerosols from all products are able to reach the alveolar level.

Detailed dose-response evaluation and determination of a NOEL of 16.1 mg/m³ (2958 mg/m³ wet weight of product) was published for “Non-absorbing floor materials” (Nørgaard et al., 2010). Dose-response evaluations were made for “Footwear protector”, “Wood impregnation” and “Rim sealer” (Tab. 1 and Fig. 3). An air concentration of 103 mg/m³ “Footwear protector” caused irreversible depression of the tidal volume in the mice and one mouse died during the exposure. No effects were observed at 6 mg/m³ (Fig. 3A). The “Wood impregnation” product also caused an irreversible depression of the tidal volume at 114 mg/m³, and two mice died during the exposure (Fig. 3B). An exposure concentration of 39 mg/m³, only affected one mouse, which had a severe depression of the tidal volume, leading to its death. The concentration below 39 mg/m³ did not cause any effects on respiration in the mice. “Rim sealer” caused irreversible depression of the tidal volume in all 6 mice at 1612 mg/m³. Three of 6 mice were severely affected at 591 mg/m³, whereas no effect was observed at 269 mg/m³ (Fig. 3C). None of the products caused upper or lower airway irritation (data not shown and (Nørgaard et al., 2010, 2014)).

Correlation of in vivo and in vitro outcomes

The true positive rate of the CS method was 100%, i.e., all products that were toxic in mice upon inhalation also inhibited the pulmonary surfactant function *in vitro*. Two of the five products that did not have an effect in the mouse bioassay were also negative in the CS method, thus the true negative rate was 40%. However, two of the three false positives only had effects in the CS method at very high concentrations (>8%). The negative predictive value was 100%, i.e., the method does not label any toxic materials as non-toxic. The positive predictive value of the CS method was 57%, largely influenced by the false positive results mentioned above.

4 Discussion

This study shows that products based on organic solvent as well as water-based products may be toxic upon inhalation. The film-forming components in the toxic products may be fluorinated or non-fluorinated. Our data clearly shows that assessment of pulmonary surfactant inhibition is a good predictor for acute pulmonary toxicity after inhalation of an aerosolized impregnation spray product. Hence, all products that were toxic in mice also inhibited pulmonary surfactant function *in vitro*.

Although the CS method was applied in the present study, other assays for pulmonary surfactant function and pulmonary surfactant formulations other than Alveofact® may be useful. Thus, Tashiro et al. (1998) used surfactant isolated from pigs and extracted with organic solvents in the pulsating bubble surfactometer to show the effects of a fabric protector on pulmonary surfactant. This effect was linked to the toxicity of the product in rats, and the affected rats could subsequently be rescued by introducing exogenous surfactant to the lungs (Tashiro et al., 1998). Fisher et al. (2012) used an isolated perfused rat lung model to assess the effect of waterproofing sprays. The observed effects were comparable to those found in live rats and indicated that the spray products damaged the surfactant film. We have shown inhibition of pulmonary surfactants using the Langmuir technique (Duch et al., 2014; Larsen et al., 2014) and the interactions have been demonstrated using porcine (HL-10 (Vermehren et al., 2006) and Curosurf® (Bernhard et al., 2000)) as well as bovine (Alveofact® (Gunther et al., 2002)) pulmonary surfactant formulations. Results from these relatively diverse pulmonary surfactant formulations were in overall agreement, suggesting that pulmonary surfactant inhibition is a robust and reliable endpoint.

Due to the uniform response of lung collapse observed for the tested products, only four mice were used for the initial screening. We performed power calculations of some of our experiments (*cf.* Table S1 at <http://dx.doi.org/10.14573/altex.1408191s>). With the observed SD and when all mice in the group are affected to a 50% reduction in tidal volume, a group size of 5 is sufficient to obtain a power > 0.9. Obviously, larger group sizes are needed if only half of the mice in a group are affected, leading to a mean reduction of 25% (as for example seen in the 591 mg/m³ “Rim sealer” group). Further, if the product exposure affects all animals in the group, the exposure may be stopped and the recovery period initiated. This will cause shorter exposure time for the individual animal. Another refinement is to kill the mice right after the exposure instead of, e.g., collecting lung tissue 24 h later for histology. For risk assessment of spray products, the value of observing lung edema and/or inflammation is limited.

The methods applicable for the study of pulmonary surfactant inhibition all have advantages and limitations. The Langmuir technique provides insight into the mechanics of the inhibition process. This method was recently used to identify that the main target of an impregnation product was surfactant-associated protein-B (Larsen et al., 2004). Furthermore, the Langmuir technique allows assessment of impregnation products with a high boiling point (Duch et al., 2014). On the other hand, the Langmuir technique is time-consuming and it requires relatively large amounts of pulmonary surfactant. The CS method, in contrast, can be used to study surfactant inhibition in samples of only a few microliters, and is therefore also potentially applicable for pulmonary surfactants extracted from, e.g., broncho-alveolar lavage from small animals (Currie et al., 1998a,b). However there are limitations related to the CS method: the first being the inability to manipulate the atmosphere surrounding the surfactant film, rather the product of interest has to be mixed with surfactant prior to analysis. Another limitation is that the method is not able to distinguish between a functioning surfactant and a precipitated surfactant (comparable to water). This problem has been overcome by adding POTS to the sample as described in section 2. Lastly, the CS method used in this study is strictly limited to water-based or water-soluble products, since the solvents have to be removed prior to assessment in the CS.

The CS method has proven useful in determining the toxicity of water-based and water soluble impregnation spray products. Three false positive results lower the true negative rate (40%) and positive predictive value (57%) of the CS method. Of these three products, two are positive at a very high concentration (>8% of the product added). It is likely that this concentration may not be reached in the alveoli or the terminal bronchioles, where the surfactant is found, explaining the discrepancy between *in vivo* and *in vitro* results. Future improvements to methods for investigating pulmonary surfactant inhibition by impregnation spray products may bring this concept a step further. For instance, real-time monitoring of surfactant properties during aerosol exposure of a pulmonary surfactant film by an impregnation product may be highly valuable, since this better reflects a realistic exposure.

Figure 4 compiles a flow chart of our proposed testing strategy to be used during development of novel impregnation spray products. In principle, only the hazard identification steps may be performed in, e.g., product screening, but for risk assessment purposes, dose-response evaluation and exposure assessment may be included. Our *in vitro* model for testing novel impregnation sprays will have a certain risk (43%) of misclassifying non-toxic products as toxic since the positive predictive value is only 57%, but this strategy will require the fewest tests in animals.

Currently, no regulation exists on inhalation toxicity of impregnation sprays although they are widely available in consumer and occupational settings and despite numerous cases of respiratory distress have been registered world-wide. A standardized *in vitro* screening tool, such as the CS method, may be able to prevent pulmonary toxicity following human exposure. When using an impregnation product, the application mode (by brush, pump spray or pressurized spray can) is decisive as to whether respirable droplets are generated. Further, the toxicity of impregnation sprays depends on the total formulation – not solely the active compound. All points should be included in product safety testing. Thus, the CS method may assist in the formulation steps during the development of new products and not only while testing existing products.

Importantly, the development of an *in vitro* method based on surfactometry may greatly reduce the number of animals used for toxicity testing and formulation of new products. In conclusion, this study presents a proof-of-principle for using pulmonary surfactant inhibition as a predictor for toxicity of inhaled impregnation spray products in mice.

Conflict of interest

None of the authors has potential conflicts of interest.

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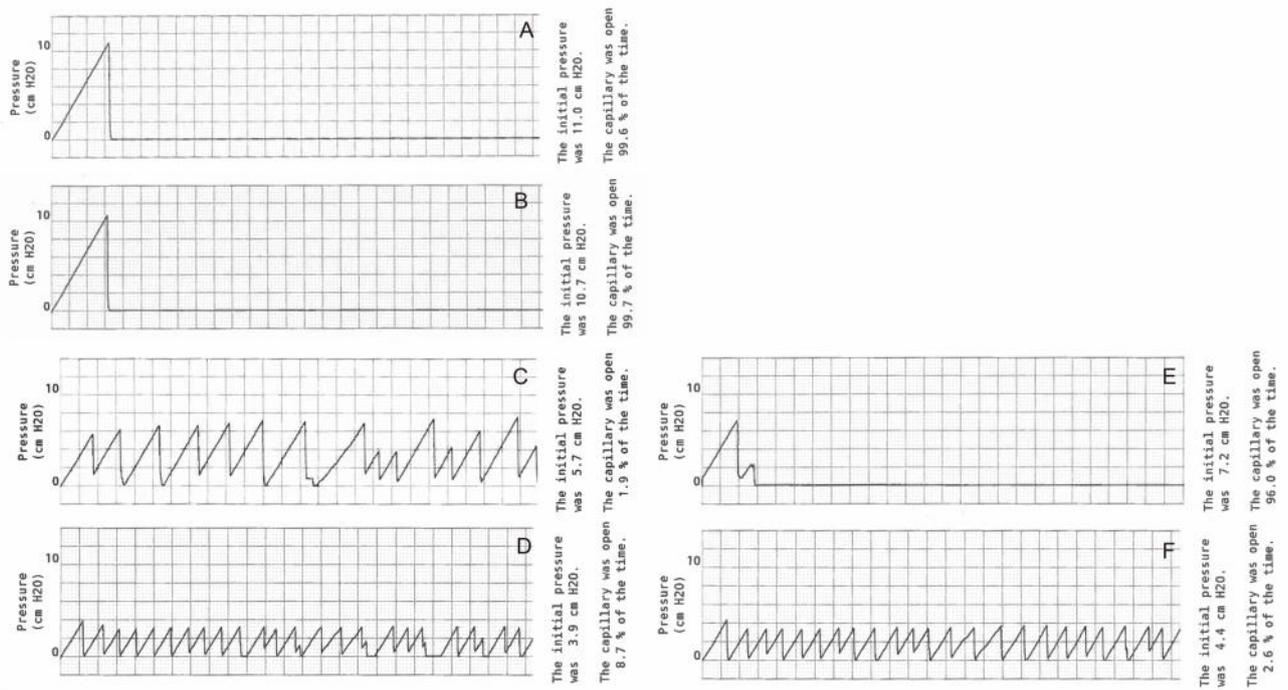


Fig. 1: Original printouts from the CS instrument

The instrument measures whether the capillary is blocked or open during 120 s measuring time and calculates the “time of open” in percent. Adding water (A) or 4 mg/ml Alveofact® (B) to the capillary resulted in the capillary being open >99% of the time. Adding 10 µl propanol (C) or 10 µl POTS (D) to 4 mg/ml Alveofact® resulted in inhibition of the surfactant seen as a reduction in the “time of open”. After the solvent was removed by evaporation, the inhibition was abrogated in the sample where 2-propanol was added (E), but the inhibition remained in the sample containing POTS (F).

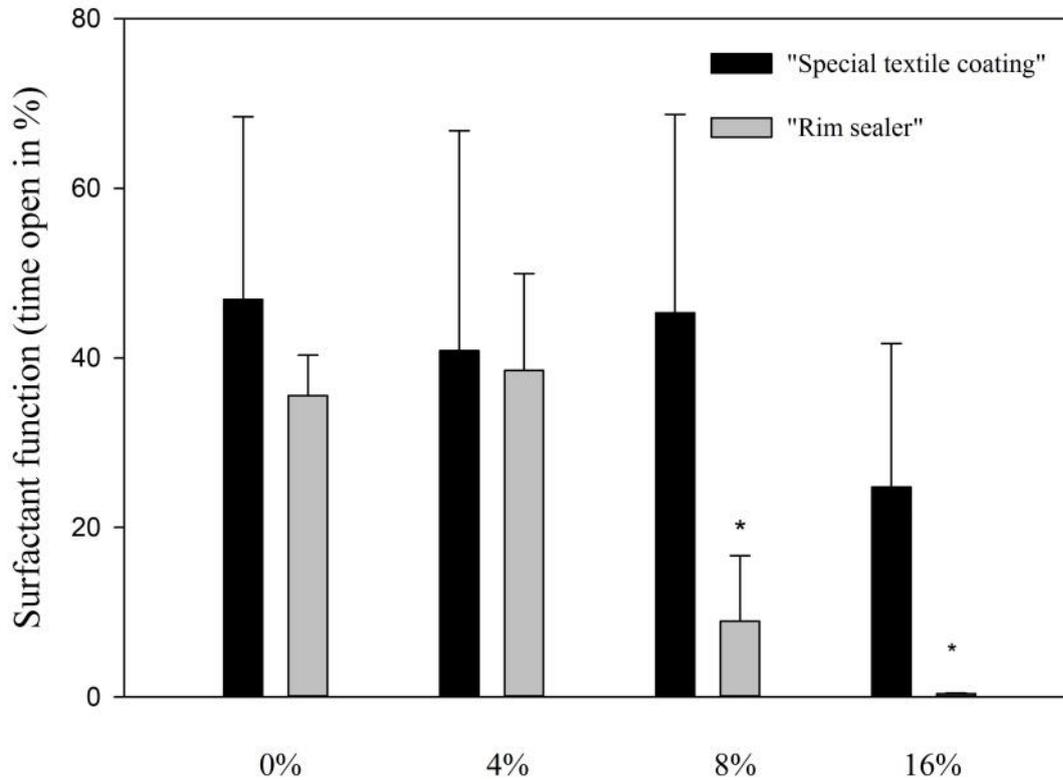


Fig. 2: The effect of two impregnation products on surfactant function

Alveofact® (final concentration of 4 mg/ml) was mixed with different concentrations of impregnation products in their original solvent. POTS was then added to this mixture and the solvents removed by evaporation. The surfactant function was measured in the CS. The product "Special textile coating" had no effect at the tested concentrations, whereas the product "Rim sealer" inhibited the surfactant at concentrations greater than 4%. * represent significant difference between samples containing 0 and 4 % "Rim sealer". Mean and SEM of 3-20 repetitions is shown.

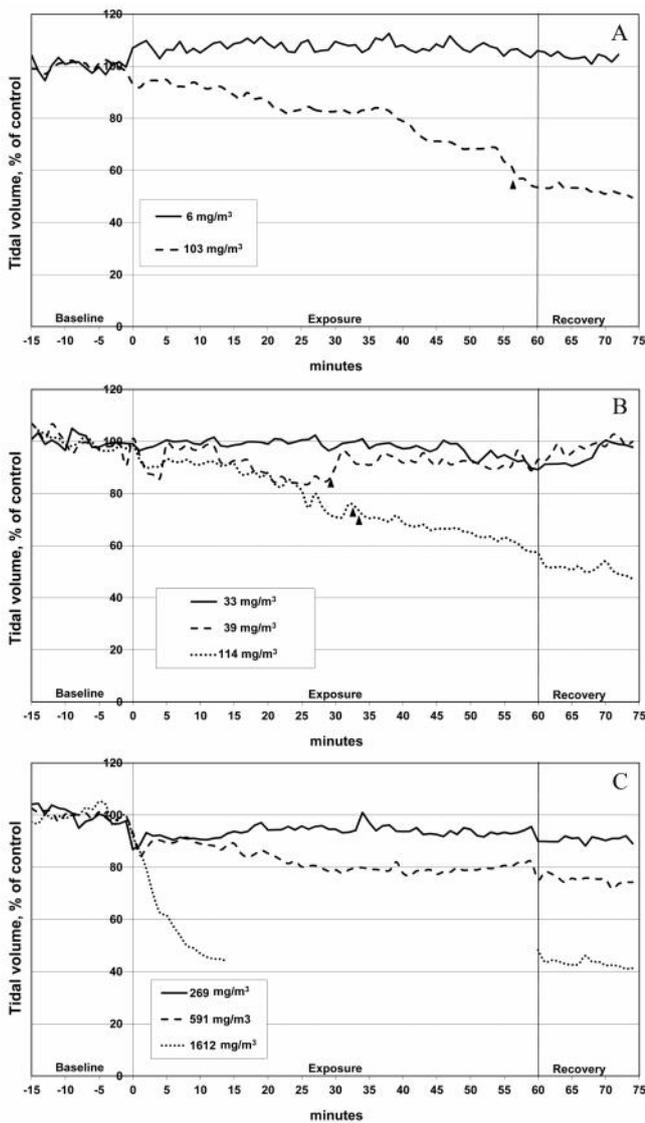


Fig. 3: Effect on tidal volume following inhalation of the products “Footwear protector” (A), “Wood impregnation” (B) and “Rim sealer” (C)

Mice ($n = 6-8$ per group) were exposed for 15 min to laboratory air to record individual baseline values. Then the animals were exposed to the aerosolized product for up to 60 min, followed by a 15-min laboratory air recovery period. The “Rim sealer” exposure to 1612 mg/m³ was stopped after 15 min because all mice had a severe tidal volume depression and the recovery period was started subsequently. Arrow heads designate death of one mouse. The group mean value of 1-min periods are shown.

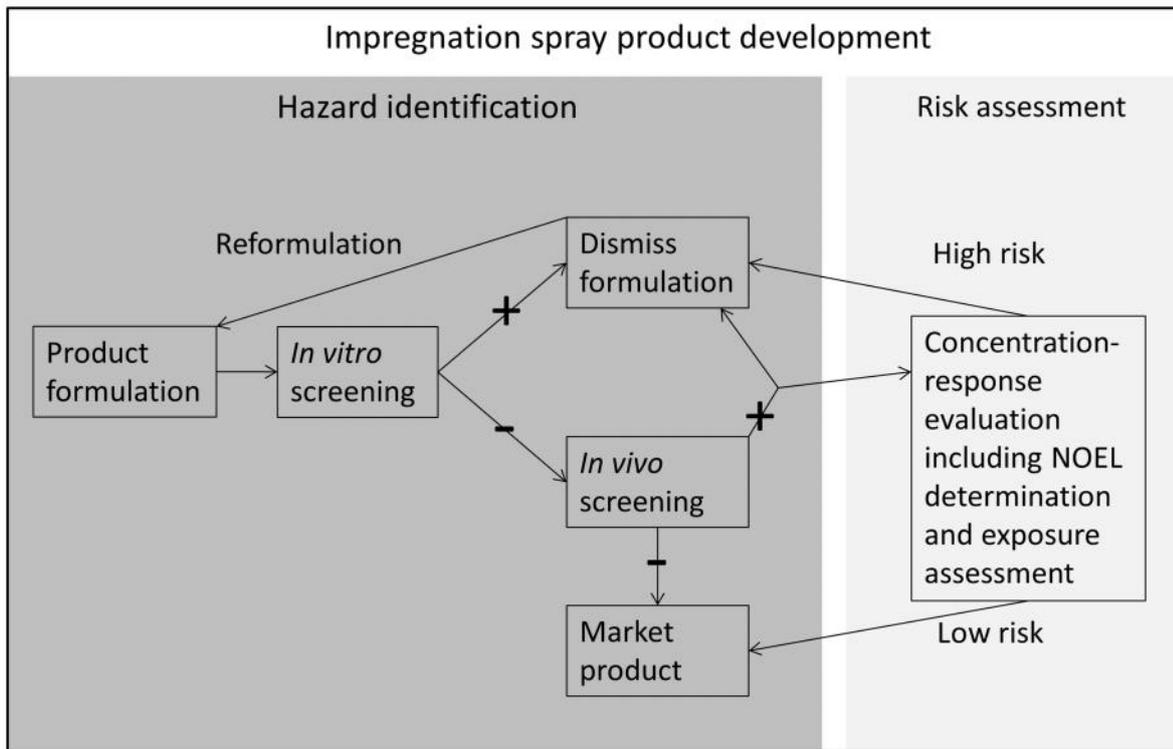


Figure 4: Example of test strategy for development of non-toxic impregnation spray products

The first step of the hazard identification is testing the product formulation in the *in vitro* method. Formulations that are toxic *in vitro* (positive) are dismissed and can be reformulated. If a product is negative in the *in vitro* method, an *in vivo* screening is performed. If the product is non-toxic, it may be considered safe to be marketed. Formulations that are positive in the *in vivo* screening are either reformulated to reduce the toxicity or undergo risk assessment, including concentration-response evaluation and an exposure assessment. Products with low risk may be ready to be marketed, whereas high risk products should be reformulated. NOEL: no observed effect level

Tab. 1: The chemical composition of nine spray products, and their *in vitro* and *in vivo* effects

Product	Active ingredient	Solvent	Effect <i>in vitro</i> , NOEL (% wet product in Alveofact®), effect on surfactant	Effect <i>in vivo</i> , NOEL (mg/m ³ wet product)
“Footwear protector”	Perfluoracrylate	Water and glycolethers	Yes, 2, precipitate	Yes, 6
“Wood impregnation”	Perfluoracrylate	Water and glycolethers	Yes, 2, precipitate	Yes, 33
“Car glass”	Alkylsilan/siloxan	Ethanol	Yes, 8, inhibit	No, 1253
“Bath and tiles”	Alkylsilan/siloxan	Ethanol	Yes, 8, inhibit	No, 22161
“Textiles and leather”	Perfluorsilan/siloxan	Water	No, 16, none	No, 259
“Special textile coating”	Perfluorsilan/siloxan	Water	No, 16, none	No, 1364
“Textiles and leather concentrate”	Perfluorsilan/siloxan	Water	Yes, 2, precipitate	No, 6676
“Non-absorbing floor materials” (POTS)	Perfluorsilan/siloxan	2-propanol	Yes, 2, inhibit	Yes, 2958
“Rim sealer”	Perfluorsilan/siloxan	Mixture of 2-propanol, 1-methoxy-2-propanol and ethylacetate	Yes, 4, inhibit	Yes, 269