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Original Article

Toxicological evaluation of DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine)

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ABSTRACT — In the inhalation field, lipids such as egg phosphatidylcholine (PC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and dipalmitoylphosphatidylcholine (DPPC), are considered to be generally recognized as safe (GRAS), comprising materials that are endogenous to the lungs and locally present in large quantities. Indeed, PC, DSPC and DPPC may be used to form liposomes which are known to promote an increase in drug retention time and reduce the toxicity of drugs after administration. Unfortunately, published literature guidance about the safety evaluation of these lipids as pharmaceutical excipients for use in inhaled products and about application for marketing authorization, is very limited. The purpose of this article is to review the potential toxicity of DSPC for pulmonary administration. Given the use of air and vehicle controls in a range of inhalation toxicology studies as well as negative genotoxicity and also reproductive toxicity results, it is thought that the use of DSPC is shown to be safe for pulmonary administration.

Key words: Distearoylphosphatidylcholine, Toxicology, Chronic toxicity, Genotoxicity, Reproductive toxicity, Carcinogenicity

INTRODUCTION

For the treatment of asthma and chronic obstructive pulmonary disease (COPD), drugs are often delivered to the lung using an inhalation delivery device, which include pressurized metered dose inhalers (MDIs), dry powder inhalers (DPIs), soft mist inhalers (SMIs), and nebulizers. MDIs have become the most widely used delivery system for the treatment of lung diseases such as asthma and COPD (Stein *et al.,* 2014). Between 2002 and 2008, 47.5% of inhaled medications sold in Europe were MDIs (Lavorini *et al.*, 2011). Although MDIs are currently the most widely used aerosol delivery device, challenges associated with MDIs have meant that major drug classes and their combinations are not available for this inhaler type. As a result, there is a need for alternative formulation and delivery approaches (Ferguson *et al.*, 2018). Pearl Therapeutics Inc., a member of the AstraZeneca Group, has developed co-suspension delivery technology in pressurized metered-dose inhalers for multi-drug dosing, an innovative MDI formulation that suspends micronized drug crystals with spray-dried phospholipid excipient porous particles in hydrofluoroalkane (HFA) propellant (Vehring *et al.*, 2012) and this delivery technology overcomes well-known sources of variability in MDI drug delivery (Doty *et al.*, 2018). Co-suspension delivery technology was used in the development of GFF MDI, a bronchodilator fixed-dose combination (FDC)

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of glycopyrrolate (GP), a long-acting muscarinic antagonist, at a twice-daily dose of 18 µg (equivalent to 14.4 µg of glycopyrronium), and formoterol fumarate (FF), a long-acting β_2-agonist, at a twice-daily dose of 9.6 µg (equivalent to formoterol fumarate dihydrate 10 µg). GFF MDI was approved recently by the US Food and Drug Administration, European Medicines Agency and Japan Ministry of Health, Labour and Welfare, respectively, as a long-term maintenance treatment for airflow obstruction in patients with COPD (Bevespi Aerosphere®, 2016, 2018, 2019). The dual FDC MDI containing GP and FF is prepared by simultaneously suspending the micronized drug particles with spray-dried phospholipid porous particles in HFA-134a (Doty *et al.*, 2018). The manufacturing process for phospholipid porous particle has been described before (Lechuga-Ballesteros *et al.*, 2011). Briefly, an emulsion feedstock with 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and anhydrous $CaCl_2$ (equivalent to a 2:1 DSPC/$CaCl_2$ molar ratio) was prepared. During the emulsion preparation, DSPC and $CaCl_2$ were dispersed into a vessel containing heated water and perfluorooctyl bromide (perflubron) using a high-shear mixer. The coarse emulsion was then further processed with a high-pressure homogenizer before spray-drying (Ivey and Vehring, 2010).

DSPC has no precedent of use as a pharmaceutical excipient in Japan, although it is widely used as an excipient in many drugs worldwide (Bulbake *et al.*, 2017). Support for the safe use of the porous particles excipient (comprised of DSPC and $CaCl_2$) is provided by the approval of TOBI®Podhaler™ in the US (TOBI Podhaler, 2013). Further, DaunoXome® (Nexstar Pharmaceuticals, USA), a liposomal formulation of daunorubicin, containing liposomes consisting of DSPC and cholesterol, was approved by the FDA in 1996 (Bulbake *et al.*, 2017). In the inhalation field, lipids such as egg PC, DSPC and DPPC, are considered to be GRAS, comprising materials that are endogenous to the lungs and locally present in large quantities (Wauthoz and Amighi, 2014). Indeed, PC, DSPC and DPPC may be used to form liposomes which are known to promote an increase in drug retention time and reduce the toxicity of drugs after administration (Pilcer and Amighi, 2010). Unfortunately, published literature guidance about the safety evaluation of these lipids as pharmaceutical excipients for use in inhaled products and about application for marketing authorization is very limited (Baldrick, 2000). A complete toxicological evaluation of excipients individually and bridging toxicology studies in animals with the new complete formulation are generally required for regulatory approval for a new pharmaceutical excipient. However, it is surprising how little useful toxicology data is available for many well-known materials (Pilcer and Amighi, 2010), and indeed there are few reports on the toxicity evaluation of DSPC alone in the published literature. Thus in this article, the toxicological evaluation of DSPC is presented.

Pearl Therapeutics Inc., a member of the AstraZeneca Group, has conducted a range of repeated-dose inhalation toxicology studies with various formulations, including GFF MDI, GP MDI, and FF MDI, and has included vehicle and air controls in all studies. Thus, the vehicle (DSPC/$CaCl_2$) groups were compared to untreated air controls in these studies. Also, in mice and rat carcinogenicity studies using GP MDI, DSPC/$CaCl_2$ was administered by inhalation as a vehicle control, and compared with air control. The comprehensive range of preclinical toxicological studies conducted by Pearl Therapeutics Inc. were specifically designed to satisfy the requirements of pharmaceutical product registration. All studies were performed at The American Association for Accreditation of Laboratory Animal Care (AAALAC) International accredited facilities (as Contract Research Organization), in full compliance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations and OECD Principles of Good Laboratory Practice. Routine observations were made throughout each study according to national and international guideline for testing pharmaceutical products.

MATERIALS AND METHODS

Chemicals

In the 6 months repeated-dose and the carcinogenicity MDI inhalation toxicology studies, porous particles excipient (comprised of DSPC (93%) and $CaCl_2$ (7%)) was supplied as preformulated suspension (in HFA-134a) metered dose inhalers from Pearl Therapeutics Inc. (Redwood, CA, USA). In the genotoxicity studies, DSPC was supplied from Inhale Therapeutic Systems Inc. (San Carlos, CA, USA). In the reproductive toxicity studies, DSPC was supplied from Pearl Therapeutics Inc. (Morristown, NJ, USA).

Inhalation 6 months repeated-dose studies

In the 6-months repeated-dose inhalation toxicology studies with GP MDI in rats and dogs conducted by Lovelace Respiratory Research Institute (LRRI, Albuquerque, NM, USA), animals were exposed to the porous particles excipient (comprised of DSPC and $CaCl_2$) or air controls. Air control animals received filtered room air using a similar inhalation exposure system. The doses used in these studies were limited by the maximum feasible dose

from the MDI formulations, usually determined as being when the volume of propellant gas significantly reduces the oxygen availability to the animals during dosing.

Rat

Sprague Dawley Rats, 5-6 weeks of age on arrival, were purchased from Charles River Laboratories (Portage, MI, USA), individually identified by microchip, and were randomized by body weight into two individual groups (each group consisted of 30 rats (15 males and 15 females)). Animals were exposed to the porous particles (comprised of DSPC and $CaCl_2$) or filtered air, 120 min per day/seven days/week for 6 months (181 days) using a flow-past, nose-only rodent inhalation exposure system (LRRI). The target aerosol concentration of porous particles was 0.17 ± 0.08 mg/L (estimated average total inhalation dose:12 mg/kg/day). The air control group received filtered air only. The porous particles were determined to be maximum stable achievable concentrations. The overall mean total mass aerosol concentration ± standard deviation achieved on the study was 0.00 ± 0.00 and 0.17 ± 0.04 mg/L for the air and the porous particles groups. Average particle size of the porous particles MDI aerosol delivered to the animals, expressed as mass median aerodynamic diameters (MMAD) was 4.45 μm, with a geometric standard deviation (GSD) of 1.59.

Study endpoints included clinical observations, body weights, clinical pathology, ophthalmology, organ weights, gross pathology and histopathology.

Daily detailed clinical observation of all animals were performed and recorded during the twice daily cage-side observation. Body weights were measured weekly and at necropsy. Ophthalmic examination was administered 1% tropicamide ophthalmic drops (Falcon Pharmaceuticals, Ltd. Fort Worth, TX, USA) and evaluated with a hand-held slit lamp (Kowa SL-15, Kowa Optimed, Inc., Torrance, CA, USA) and indirect ophthalmoscopy utilizing a Keeler Indirect Ophthalmoscopy Headset (Keeler Ltd., Windsor, UK) and Volk 40 Diopter lens (Volk Optical Inc., Mentor, OH, USA).

All animals had terminal blood collected in K_3EDTA and serum tubes for hematology and clinical chemistry indices. For hematology, whole blood (≤ 2 mL) was collected into K_3EDTA vacutainers and evaluated using an automated hematology analyzer (ADVIA™120 Hematology System, Siemens, Tarrytown, NY, USA) for standard hematology parameters. For serum chemistry, whole blood (≤ 4 mL) was collected into serum tubes and evaluated using an automated chemistry analyzer (Hitachi Modular Analytics Clinical Chemistry System, Roche Diagnostics, Indianapolis, IN, USA) for standard serum chemistry parameters.

At termination, all animals were euthanized with an overdose of Euthasol® (Virbac AH Inc., Fort Worth, TX, USA) and weighed. Body surface, orifices, cranial, thoracic puncture cavities were examined for abnormalities and lesions. Blood was collected via cardiac puncture and placed into 2- and 4-mL K_3EDTA vacutainers and serum tubes. Tissues were examined, collected, weighed, and/or fixed. The tissues collected for histological analysis were fixed in 10% neutral buffered formalin or Bouin's. Fixed tissues were trimmed for placement into tissue cassettes and submitted to histology. Tissue were processed routinely, paraffin embedded, sectioned at 4 μm, and stained with hematoxylin and eosin for microscopic examination.

Statistical calculations were performed using the SAS® software system 9.1 (Cary, NC, USA). Two-way analysis of variance (ANOVA) was used to assess body weights for different treatment and days. Generalized estimating equations (GEE) were used to correct the correction of repeated measurements for the same rat. Dunnett's multiple comparisons were performed to assess different between the air control and the porous particle group. One-way ANOVA was used to assess the effects at each different endpoint (hematology, serum chemistry and organ weight).

Dog

Beagle Dogs, 5-7 months of age on arrival, were purchased from Covance Laboratories (Cumberland, VA, USA), identified by ear tattoo, and were randomized by body weight into two individual groups (each group consisted of 8 dogs (4 males and 4 females). Animals were exposed to the porous particles (comprised of DSPC and $CaCl_2$) or filtered air, 30 min per day/seven days/week for 6 months (180 days) using a face mask dog inhalation exposure system (LRRI). The target aerosol concentration was 0.09 ± 0.02 mg/L (estimated average total inhalation dose:0.9 mg/kg/day). The air control group received filtered air only. The porous particles were determined to be maximum stable achievable concentrations. The overall mean total mass aerosol concentration ± standard deviation achieved on the study was 0.00 ± 0.00 and 0.08 ± 0.05 mg/L for the air and the porous particles groups. Average particle size of the porous particles MDI aerosol delivered to the animals, expressed as mass median aerodynamic diameter (MMAD) was 4.23 μm, with a geometric standard deviation (GSD) of 1.77.

Study endpoints included clinical observations, body weights, clinical pathology, ophthalmology, electrocardiography, organ weights, urinalysis and histopathology.

Clinical observation, body weights, ophthalmic exami-

nation, and hematology and clinical chemistry used same methods as described for the rat 6 months repeated-dose study.

Electrocardiograms (ECG) were collected after the first exposure (Day 1) and after the last exposure (Day 180). ECG tracings of leads I, II, III, AVR, AVL and AVF were qualitatively evaluated for rhythm, morphologic, or apparent functional changes. Heart rate (PR interval) was calculated from the number of ECG complexes in a 5 sec period at the same time point for each ECG time point.

At termination, all animals were tranquilized with acepromazine (0.1 mL/animal) and torbugesic (0.33 mg/kg), administered 1 mL/9 kg of 1:1 mixture of ketamine (100 mg/mL) and diazepam (5 mg/mL), and euthanized with an overdose of Euthasol®. Body surfaces, orifices, cranial, thoracic, and abdominal cavities were examined for abnormalities and lesions. Tissue were examined, collected, weighted, and/or fixed. The method of histopathology examination used was the same described for the rat 6 months repeated-dose study.

Statistical analyses methods were same as described for the rat 6 months repeated-dose study.

Genotoxicity studies

All genotoxicity studies used DSPC only, and were conducted at SITEK Research Laboratories (Rockville, MD, USA: Facility no longer in business).

Salmonella typhimurium/Escherichia coli plate incorporation mutation assay

The experimental procedures used to perform this study were essentially those of Ames *et al.* (1975), Maron and Ames (1983), Green and Muriel (1977), and Venitt and Parry (1984). The *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were originally obtained from Dr. Bruce N. Ames, University of California, Berkeley. The *Escherichia coli* strain WP2 *uvrA* was obtained from Ms. Judy Mayo of Pharmacia and Upjohn Co. (Kalamazoo, MI, USA). DSPC dissolved in ethanol, was tested at 313, 625, 1250, 2500, and 5000 µg/plate with and without metabolic activation system consisting of Aroclor-induced rat liver S-9 in 0.15M KCl plus cofactors (S-9 mix). The S-9 was made by SITEK. The doses were selected based on the results of the range finding test (Data was not shown). Treatment was performed by adding either 500 µL of deionized, distilled water or 500 µL of rat S-9 cofactor mix to tubes containing 2.0 mL of top agar supplemented with 1x histidine-biotin or 1x tryptophan solution. Immediately thereafter, 100 µL of strains TA98, TA100, TA1535, TA1537, or WP2 *uvr*A were added, followed by 50 µL of the appropriate DSPC or solvent. Each tube was vortexed, and the contents were evenly distributed over a Vogel-Bonner bottom agar plate. The test article concentrations of 1250, 2500, and 5000 µg/plate formed precipitate in the treatment tubes. Each plate was placed on a level surface until the top agar solidified. The plates then were inverted and incubated at 37 ± 1°C for approximately 67 hr. After the incubation period was completed, the plates, starting with the highest test article concentration, were observed for the presence of precipitate. Plates having no interfering precipitate were counted using an automatic colony counter (Model 880, Artek Systems, Farmingdale, NY, USA). Plates with precipitate that interfered with automatic counting were counted by hand. Three counts were taken by rotating the plate on the counter stage and median count was entered into a validated, Lotus 123 (version 3.4) spreadsheet program.

Chromosomes aberrations in cultured Chinese Hamster Ovary (CHO) cells with and without activation

The clone CHO-W-B1 of the CHO cell line, used in this study, was obtained by SITEK through the Environmental Health Research and Testing Laboratories (Lexington, KY, USA). The modified McCoy's 5A medium used in this study was obtained in liquid form. The metabolic activation mixture consisted of phenobarbital/β-naphthoflavone-induced rat liver homogenate (S-9) and cofactor pool. Two replicate cultures, seeded with 500,000 cells each approximately 20-24 hr earlier, were treated at each concentration level in the non-activated and activated systems. The cells were treated at concentration of 300, 400, 500, and 1000 µg/mL of DSPC based on range finding test (Data was not shown) in both the non-activated and activated systems. Mitomycin-C (MMC) was used as the positive control at 0.4 and 0.8 µg/mL in the non-activated system, and Cyclophosphamide (CP) was used at 7.5 and 12.5 µg/mL in the activated system. Solvent controls were included in each system. One hundred metaphases were scored for chromosome aberrations from each of the two duplicate flasks, thus providing 200 metaphases per concentration level. The data for the percentage of cells with aberrations for each concentration were compared to the solvent control values using the Chi-square test. A validated statistical package (Epistat) was used to calculate the p values for the Chi-square test. Results were considered significant if $p \leq 0.05$.

Micronucleated polychromatic erythrocytes in mouse bone marrow cells

DSPC was tested for its potential to induce micronu-

cleated polychromatic erythrocytes (MPCE) in the bone marrow cells of CD-1 mice. Approximately 42-day-old, male and female, CD-1 mice were purchased from Harlan Sprague Dawley, Inc (Indianapolis, IN, USA). DSPC was suspended in dimethyl sulfoxide (DMSO), which was also used as the vehicle control. The test article and vehicle control were administered by intraperitoneal injection. The dose volume was 4.0 mL/kg for the test article and vehicle control. Based on the results from the range finding test (data was not shown), the micronucleus assay was performed at doses of 6.25, 12.5 and 25 mg/kg for both the males and females. A total of 90 mice (45 males, 45 females) were used for the standard micronucleus assay and an additional 9 male mice were used for blood sampling. The test article and vehicle control were administered as a single dose. The positive control group received a single, oral gavage dose of cyclophosphamide (CP) at 80 mg/kg. Animals were sacrificed approximately 24 or 48 hr after dose administration. Positive controls were included in the 24-hr harvest only. After the animal was sacrificed, the femurs were exposed by cutting into the skin and muscle of the thighs. The bone marrow from the femurs was flushed into a culture tube, containing 1.0 mL of fetal bovine serum, using a 1 mL syringe fitted with a 25-gause, needle. The tubes were centrifuged, the supernatant was removed, a small drop of cell suspension was placed on a microscope slide and spread along the length of the slide. The slides were allowed to air dry, then fixed in methanol for 15 min and allowed to air dry again. The slide were stained in Wright-Giemsa stain for 3-4 min, then rinsed in distilled water. The slides were scored "blind" in order to avoid bias on the part of the scores. First, the number of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) among 200 erythrocytes (PCE+NCE) per animal was determined. The number of micronucleated polychromatic erythrocytes (MPCE) then was determined for 2000 PCE per animals. In addition to the standard micronucleus assay, three male mice for each dose level were dosed for blood sampling. The dosing procedure was the same as for the standard micronucleus assay. The blood was collected 4 hr after the dose administration. The frequency of MPCE in each dose group was compared to that in the respective vehicle control group using a one-tailed Student's t-test (Lovell *et al.*, 1989).

Inhalation carcinogenicity studies in mice and rats

Carcinogenicity studies with GP MDI in mice and rats were conducted by LRRI.

Animals were exposed to the porous particles excipient (comprised of DSPC and $CaCl_2$) or air controls. Air control animals received filtered room air using a similar inhalation exposure system. The doses used in these studies were limited by the maximum feasible dose from the MDI formulations, usually determined as being when the volume of propellant gas significantly reduces the oxygen availability to the animals during dosing.

Mouse

B6C3F1 Mice, 9 weeks of age on arrival, were purchased from Charles River Laboratories (Portage, MI, USA), identified by microchip, and were randomized by body weight into two individual groups (each group consisted of 120 mice (60 males and 60 females)). Animals were exposed to the porous particles (comprised of DSPC and $CaCl_2$) or filtered air 120 min per day/seven days/week for up to 104 (±1) weeks using a flow-past, nose-only rodent inhalation exposure system (LRRI). The target aerosol concentration was 0.17 ± 0.08 mg/L (estimated average total inhalation dose: 20 mg/kg/day). The air control group received filtered air only. The porous particles were determined to be maximum stable achievable concentrations. The overall mean total mass aerosol concentration ± standard deviation achieved on the study was 0.00 ± 0.00 and 0.17 ± 0.03 mg/L for the air and the porous particles groups. Average particle size of the porous particles MDI aerosols delivered to the animals, expressed as MMAD, was 4.36 μm, with a GSD of 1.53.

Study endpoints included clinical observations, body weights, ophthalmology, gross pathology, organ weights, and histopathology.

Daily detailed clinical observation of all animals was the same as described for the rat 6-months repeated-dose study.

At scheduled necropsy, early termination, or in cases of morbidity, animals were euthanized by intraperitoneal injection of an overdose of Euthasol®. Blood was collected first, then the organs collected, weighed, and preserved for histopathology. Detailed gross necropsies were performed on all animals and consisted of a complete external and internal examination including body orifices (ears, nostrils, mouth, anus, etc.) and cranial, thoracic, and abdominal organs and tissues. Representative samples of tissues were harvested and fixed in 10% neutral buffered formalin or Bouin's. Tissue were processed routinely, paraffin embedded, sectioned and stained with hematoxylin and eosin for microscopic examination.

Data were analyzed as described for the rat 6 months repeated-dose study. The incidence of tumors was analyzed by Peto's mortality-prevalence method (Peto *et al.,* 1980) without continuity correction, incorporating the context (incidental or fatal) in which tumors were

observed. For all organs, the incidence of each tumor type was analyzed with a 2-sided comparison of the porous particle group versus the Air control group.

Rats

Sprague Dawley (CD) rats, 5-6 weeks of age on arrival, were purchased from Charles River Laboratories (Portage, MI, USA), identified by microchip, and were randomized by body weight into two individual groups (each group consisted of 140 rats (70 males and 70 females)). Animals were exposed to the porous particles (comprised of DSPC and $CaCl_2$) or filtered air 120 min per day/seven days/week for up to 82 weeks using a flow-past, nose-only rodent inhalation exposure system (LRRI). The target aerosol concentration was 0.15 ± 0.08 mg/L (estimated average total inhalation dose:10 mg/kg/day). The air control group received filtered air only. The porous particles were determined to be maximum stable achievable concentrations. The overall mean total mass aerosol concentration ± standard deviation achieved on the study was 0.00 ± 0.00 and 0.17 ± 0.08 mg/L for the air and the porous particles groups. Average particle size of the porous particles MDI aerosols delivered to the animals, expressed as MMAD, was 4.20 μm, with a GSD of 1.68.

Study endpoints included clinical observations, body weights, ophthalmology, gross pathology, organ weights, and histopathology.

Daily detailed clinical observation of all animals were the same as described for the rat 6 months repeated-dose study. The post-necropsy method and statistical treatments were the same as described for the mouse carcinogenicity study.

Reproductive toxicity studies

All reproductive and developmental toxicity studies used DSPC only and were conducted at Experimur (Chicago, IL, USA).

Fertility and early embryonic development in rats

DSPC was administered parenterally by intraperitoneal injection in a vehicle of corn oil at dose levels 0 (vehicle control), 1, 25, and 50 mg/kg/day to male and female Sprague-Dawley rats (from Charles River, males received at 6-7 weeks of age and females were 5-6 weeks of age) approximately 8 weeks old at treatment initiation. Each group consisted of 25 Core rats/sex plus 3 Toxicokinetic (TK) rats/sex. Rats were dosed with a volume of 1 mL/kg daily, males were dosed for 4 weeks prior to and during mating and until their scheduled necropsy, while females were dosed for at least 2 weeks prior mating, during mating and through Gestation Day 6 (*i.e.,* implantation). Blood samples for TK were collected from TK rats/sex/time-point prior to and 0.5, 1, 2 and 4 hr following the first and last doses prior to mating (for males this corresponded to study Days 1 and 28 ± 2, while for females it corresponded to study Day 1 and 14 (± 1 day) of the premating period). Dams underwent cesarean sections on Gestation Day 13 after receiving a minimum of 28 daily doses, while males received at least 45 daily doses and were then sacrificed after mating. Rats were mated 1:1 within group. Toxicology parameters evaluated included body weights, food consumption, clinical observations, mating performance, estrus cyclicity, reproductive performance, sperm analyses, organ weights and gross pathology. The 50 mg/kg/day dose was considered the maximum feasible and was based on a range-finding study (Data was not shown). The highest dose administered was intended to provide systemic exposure with little to no accumulation of DSPC in the peritoneal cavity.

Moribundity/Mortality Observation: During the quarantine period, animals were observed at least once daily for moribundity and mortality. During the study, all animals were observed twice daily (*i.e.,* cage-side observations) on weekdays and at least once daily on weekends and holidays for mortality or evidence of moribundity, and any abnormal clinical signs were recorded. Each animal was evaluated during dosing for general appearance as well as overt signs of toxicity, any abnormal clinical signs were noted.

Clinical Observations: A detailed clinical examination was performed on all animals during the quarantine period (pretest), and in conjunction with body weights during the study.

Body Weights: Rats were weighted during quarantine (pretest) and their weights used for randomization. Following randomization, rats were weighted weekly until cohabitation (mating), but were not weighted during the mating period. Males were weighed once prior to their scheduled sacrifice. Dams (*i.e.,* impregnated females) were weighed on Gestation Days 0, 3, 6, 9, 12, and prior to scheduled necropsy (*i.e.,* Day 13).

Food Consumption: Food consumption measurement corresponded with body weight collection and was measured for each rat weekly prior to mating and for dams on day 0, 3, 6, 9 and 12 of gestation.

Vaginal Smears: Collection of smears from the first 10 females/group began approximately two weeks prior to mating to ensure and evaluate cyclicity; collection of smears from all 25 females/group began 2 days prior to initiation of mating. Smears were also collected during the cohabitation period until the presence of sperm

or sperm-plug during the cohabitation period indicated a positive mating and that day was designated as Gestation Day 0.

Mating: Rats were mated 1:1 (one male to one female) within group for a maximum of 2 weeks. Successful mating was determined by detecting the presence of sperm in the vaginal smear. Cohabitation ceased immediately after mating was observed.

Toxicokinetics: Blood specimens were collected from the sublingual vein into tubes containing K_2EDTA as anticoagulant from 3 rats/sex/group at 0, 0.5, 1, 2, and 4 hr. Rats were bled prior to dosing and at the aforementioned times on the first and last days of treatment prior to mating; for males this would correspond to study day 1 and 28 (± 1 day), while for females this would correspond to study day 1 and day 14 (± 1 day). Approximately 0.5 mL of whole blood/animal were collected at each time-point.

Preserved Tissues: Reproductive organ were fixed in 10% neutral-buffered formalin, except for testes and epididymides with were fixed in Davidson's solution. Tissue collected at necropsy were retained for possible future histopathological evaluation.

Histopathology: Based on the lack of adverse effects on reproductive outcome, the tissue were not evaluated but were stored for possible future evaluation.

Statistics: Body weight, and food consumption data were collected using an on-line data acquisition system (LabPractica). Reproductive (vaginal cytology) and postmortem (organ weight, sperm evaluations, corpora lutea and implantation counts) data were collected and manually entered into Excel® spreadsheets, and, means and standard deviations were calculated for all parameters using the same software. Data were analyzed for homogeneity of variance using Levene's test. When the variances were homogeneous ($p > 0.001$), the data were further analyzed by one-way analysis of variance (ANOVA). When a significant F value was observed ($p \leq 0.05$), each treatment groups was compared to the vehicle control group using Dunnett's two-tailed t-test. Statistical significance for the Dunnett's test was declared at $p \leq 0.05$. When Levene's test was significant ($p \leq 0.001$), the data were log-transformed and the analyses were performed on the transformed data. When log-transformed data were not homogeneous ($p \leq 0.001$), then the Kruskal Wallis test was performed, followed by Mann-Whitney U test. The incidence of abnormal sperm (detached or misshapen heads) was also analyzed using Chi-Square. Minimal statistical significance was $p \leq 0.05$ in all cases. All non-descriptive statistical analyses were performed with Systat® 10 software (Systat Software Inc., San Jose, CA, USA).

Embryo-fetal development in rats and rabbits

Rats

DSPC was administered parenterally by intraperitoneal injection in a vehicle of corn oil at dose levels of 0 (vehicle control), 1, 25, and 50 mg/kg/day to 23 time-mated Sprague-Dawley female rats/group. The rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA), were at least 8 weeks old. Rats arrived at the testing facility on or before Gestation Day 5. Doses were administered once daily at a constant volume of 1 mL/kg during the period of major organogenesis (Gestation Days 6 through 17) and covered implantation until closure of the hard palate. The objective of this study were to determine the potential developmental toxicity and TK profile of DSPC following parenteral administration. As such, maternal body weight, body weight gain and food consumption were measured throughout the gestation period. Dams were euthanized on the 21st day of gestation and subjected to a cesarean section and gross necropsy. The uteri were weighed, opened and inspected for implantation sites; fetuses were harvested, weighed, given a gross external examination. One-half of the fetuses in each litter were subjected to visceral examinations, while control and high dose fetuses were subjected to skeletal and/or cephalic examinations. The 50 mg/kg/day dose was considered the maximum feasible and was based on a range-finding study (Data was not shown). The highest dose administered was intended to provide systemic exposure with little to no accumulation of DSPC in the peritoneal cavity.

Moribundity/Mortality and Clinical Observation: Methods are the same as described for the Fertility and early embryonic development study in rats.

Study Termination: Core dams were euthanized by CO_2 asphyxiation on their respective Gestation Day 21; TK dams were euthanized after last scheduled blood collection.

Body Weight: Each dam was weighted at receipt for randomization and on Gestation Days 6, 9, 12, 15, 18, and 21.

Food Consumption: Food consumption measurement corresponded with body weight collection and was measured for each dam over Gestation Days 6-9, 9-12, 12-15, 15-18, and 18-21.

Tokicokinetics (TK): Blood specimens were collected from the sublingual vein into tubes containing K_2EDTA as anticoagulant. Samples were collected prior to treatment initiation pre-dose and 0.5, 1, 2, and 4 hr post-dose on Gestation Day 6 and 17 (± 2 days). Rats were bled from the sublingual vein prior to dosing and at the aforementioned times on Gestation Days 6 and 17 (± 2 days).

Approximately 0.5 mL of whole blood/animal was collected at any one time.

Tissue Collection: Fetuses were removed from the dam by cesarean section on Gestation Day 21 (sperm-positive day/plug day is Gestation Day 0). Tissues and suspect lesions were dissected, if present, sliced into appropriate-sized sections, and fixed in 10% neutral buffered formalin.

Gross External Evaluation and Cesarean sections: The uterus was weighed with the ovaries. After weighing, the corpora lutea were counted and recorded for the left and right ovaries. Uterine weights collected from animals that died, aborted or delivered prematurely were excluded from calculations of the means and standard deviations. Fetuses were removed, counted and identified in a systematic fashion and were given a gross external morphological examination.

Visceral Examinations: Visceral examinations were performed on designated fetuses using a modified Staples' technique. The internal sex of the fetus was determined and recorded during the visceral examination.

Cephalic Examinations: Decapitated fetal heads were fixed in Bouin's solution for a minimum of one week and then transferred to 70% ethanol at least 24 hr prior to examination using a modified Wilson's Razor Blade Technique. Heads were examined from all control and high dose fetuses; examination of lower dose groups was not warranted since no adverse findings were observed in the high dose group.

Skeletal Examinations: Designated fetuses were processed for skeletal examination. Fetuses were fixed in ethyl alcohol, single-stained with Alizarin Red-S/potassium hydroxide solution and cleared. Fetal skeletons from the vehicle control and high dose groups were examined; examination of the lower dose groups, was not warranted since no adverse findings were observed in the high dose group.

Statistical Methods: Maternal body weight and food consumption data were collected using an on-line acquisition system (LabPractica). Litter data were manually collected, transferred to Excell® spreadsheets, and means and standard deviations were calculated for all parameters using Sytat® 10 software. Dam body weights, and food consumption were analyzed for homogeneity of variance using Levene's test. When the variances were homogeneous ($p > 0.001$), the data were further analyzed by one-way analysis of variance (ANOVA). All post-hoc comparisons were performed using Dunnett's test (two-tailed). A minimum statistical significance level of $p \leq 0.05$ was used in all cases.

Rabbits

DSPC was administered by intraperitoneal injection in a vehicle of corn oil at dose levels of 0 (vehicle control), 1, 25, and 50 mg/kg/day to 23 time-mated New Zealand White female rabbits/group (Covance Laboratories). The animals were approximately 4-6 months old and weighed approximately 3-4 kg at the time of receipt. Rabbits arrived at the testing facility before Gestation Day 5. Doses were administered once daily at a constant volume of 1 mL/kg during the period of major organogenesis (Gestation Days 6 through 18) and covered implantation until closure of the hard palate. In addition, each treated group had 3 additional satellite does designated for TK evaluations (TK does were dosed over Gestation Day 7-17 and blood samples were collected prior to and at designated times after the 1st and last dose). As such, maternal body weight, body weight gain and food consumption were measured throughout the gestation period. Dams were euthanized on the 29th day of gestation and subjected to a cesarean section and gross necropsy. The uteri were weighed, opened and inspected for implantation sites. In addition, all fetuses were subjected to a visceral examination and processed for skeletal evaluation (vehicle control and high dose groups were examined), while approximately one-third of the fetuses from each litter were designated for cephalic examination (vehicle control and high dose groups were examined). The 50 mg/kg/day dose was considered the maximum feasible and was based on a range-finding study (Data was not shown). The highest dose administered was intended to provide systemic exposure with little to no accumulation of DSPC in the peritoneal cavity.

Moribundity/Mortality and Clinical Observation: The method is the same as described for the fertility and early embryonic development study in rats.

Study Termination: Core dams were euthanized by anesthetic overdose on Gestation Day 29; TK does were euthanized after their last scheduled blood collection.

Body Weight: Each dam was weighted by the supplier on Gestation Day 0 (day mating was observed), at receipt and days 6, 7, 9, 12, 15, 18, 21, 24, 27, and 29 by the test facility.

Food Consumption: Food consumption measurement corresponded with body weight collection and was measured for each rabbit over Gestation Days 4-6 (pre-treatment), 6-7, 7-9, 9-12, 12-15, 15-18, 18-21, 21-24, 24-27, and 27-29.

Toxicokinetics: Blood specimens were collected from the central ear artery into tubes containing K_2EDTA as anticoagulant. Samples were collected prior to treatment initiation (for pre-dose; this sample was collected on Ges-

tation Day 6) and after the 1st dose and last dose at 0.5, 1, 2, and 4 hr post-dose on Gestation Day 6 and 17. Rabbits were bled prior to dosing and at the aforementioned time on gestation times on Gestation Days 7 and 17. Approximately 0.5 mL of whole blood/animal was collected at any one time.

Tissue Collection: Fetuses were removed from the dams by cesarean section on Gestation Day 29 (sperm-positive day/plug day was Gestation Day 0). Tissue masses and suspect lesions were dissected, if present, sliced into appropriate-sized sections, and fixed in 10% neutral buffered formalin.

Cesarean Sections and Gross External Evaluations: The method is the same as described for the Embryo-fetal development study in rats.

Visceral, Cephalic and Skeletal Examinations: The method is the same as described for the Embryo-fetal development study in rats.

Statistical Methods: Doe and litter body weights were analyzed by an analysis of variance (ANOVA). In the presence of a significant main effect, all post-hoc comparisons were performed using Dunnett's test (two-tail). Gross, visceral, cephalic and skeletal data were analyzed by Chi-Square/Fisher Exact test (fetal N) or a Log-Linear Model (which allows nesting of pups within doe) when the incidence in the treated rabbits was higher than control rabbits. Conventional or alternative statistical procedures based on data outcome were employed to characterize possible test material-related effects (or lack thereof). Test may have included those for linear trends, data transformation, and nonparametric procedures. A significance level of $p \leq 0.05$ was used for all comparisons. All statistical analysis performed using Systat® version 10 software.

Prenatal and postnatal development, including maternal function

Approximately 108 time-bred female Sprague-Dawley rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA) for use in this study. The animals were at least 8 weeks old and weighed approximately 190-246 g at the time of receipt. Rats were received at the testing facility on or before Gestation Day 4 (sperm-positive day/plug day is Gestation Day 0). DSPC was administered by intraperitoneal injection in a vehicle of corn oil at dose level of 0 (vehicle control), 1, 25, and 50 mg/kg/day to 24 sperm positive dams (F_0 Sprague-Dawley rats) per group for assessment of maternal reproductive function/parturition, prenatal and postnatal development including effects on growth, behavior and general development through sexual maturity including evaluation of reproductive competence of the offspring. F_0 dams were dosed by intraperitoneal injection from Gestation Day 6 through parturition and lactation until weaning (Postnatal Day 21). Doses were based on the most recent body weight and were administered at a constant volume of 1 mL/kg. The F_1 rats selected to produce the F_2 generation were not intentionally exposed to DSPC. Litters from the F_0 and F_1 dams were culled on Postnatal Day 4. Food consumption, body weights, body weight gain, reproductive performance and organ weights were evaluated during the study, along with offspring body weights (growth), survival and developmental landmarks (vaginal patency and preputial separation for the F_1 generation). Gross sensory function and reflex responses, as well as automated acoustical startle, motor activity and water maze (learning and memory paradigm) were evaluated in the F_1 pups selected to produce the F_2 generation. The 50 mg/kg/day dose was considered the maximum feasible and was based on a range-finding study (Data was not shown). The highest dose administered was intended to provide systemic exposure with little to no accumulation of DSPC in the peritoneal cavity.

Moribundity/Mortality Observation: The method is the same as described for the fertility and early embryonic development study in rats.

Study Termination: Study animals were euthanized by an appropriate method (*i.e.*, CO_2 asphyxiation) at scheduled necropsy. The termination schedule was as follows: F_0 dams after weaning of the F_1 generation, Unselected F_1 rats at weaning, Selected F_1 males after mating, Selected F_1 dams after F_2 rats had reached Postnatal Day 21, F_2 generation after Postnatal Day 21, Culled pups of either generation were euthanized by decapitation or hypothermia with barbiturate overdose.

Clinical Observations: A hand-held clinical observation was performed on all animals when they were weighed (at receipt for randomization and on Gestation Days 6, 7, 8, 9, 12, 15, 18, and 20, and Lactation Days 0, 4, 7, and weekly thereafter).

Body Weights: Each dam was weighed at receipt (randomization), and on Gestation Days 6, 7, 8, 9, 12, 15, 18, and 20. Dams and their litters were weighed on the day of observed parturition (Postnatal Day 0), and again on Postnatal Days 4, 7 and weekly thereafter until their scheduled termination.

Food Consumption: Food consumption measurement corresponded with body weight collection and was measured for each rat over Gestation Days 6-7, 7-8, 8-9, 9-12, 12-15, 15-18, 18-20, and Postnatal Days 0-4, 4-7, and weekly thereafter until their scheduled termination.

Mating: F_0 rats were mated by the supplier, while F_1 rats were mated overnight using a 1:1 (one male to one

female) ratio and the mating status of F_1 female rats was assessed via vaginal smears. The presence of sperm in the smear during the cohabitation period was indicative of a positive mating and was designated as Gestation Day 0.

Vaginal Smears: Vaginal smears were collected from the F_1 generation prior to cohabitation (2 days) and from 10 F_1 rats/group over 12 consecutive days prior to mating to examine cyclicity.

Offspring Evaluations: Each F_1 and F_2 pup was individually sexed, weighed and given a gross external morphological examination on the day of birth (Postnatal Day 0) and again on days 4, 7, 14, and 21; however, each pup was assigned an arbitrary identification (*e.g.,* p1, p2, p3, etc.) each time they were weighed/examined. Cannibalized pups that could not be sexed were arbitrarily recorded as male. Survival of offspring was evaluated daily and summarized on the following intervals: day 0-4, 4-7, 7-14, and 14-21. On Postnatal Days 0-4, dead or moribund pups were fixed in 70% ethanol. Pups were stored in 70% ethanol for possible future skeletal processing and evaluation. Skeletal processing and evaluation was not performed because survival of the offspring of the dams that underwent parturition was not affected by treatment.

Culling of Litters: F_1 and F_2 pups were sexed and randomly culled to 4 males and 4 females on Postnatal Day 4. When an even distribution could not be achieved because there were less than 4 rats of a sex in a litter, then the closest approximation was acceptable (*e.g.*, 3 males and 5 females). Litters with less than 8 pups were not culled. Terminal body weights were collected and necropsy was performed on those pups selected to produce the F_2 generation. The method of offspring selection was random.

Weaning: All surviving F_1 and F_2 pups were weaned on Postnatal Day 22. F_1 weanlings were group-housed and tattooed for the first 24-72 hr post-weaning for acclimation purposes.

Selection for F_1 Cross Mating: At least one male and one female from each F_0 litter (in order to achieve a target of 20 F_1 offspring) were randomly selected for a mating trial. Each male pup was randomly paired with a female pup from the same treatment group, but a different litter, for mating.

Developmental Landmarks: Vaginal opening (evaluated from Postnatal Day 28 to 45 or achievement, whichever came first) and preputial separation (evaluated over Postnatal Days 35 to 55 or until achievement) were determined for the F_1 pups selected to produce the F_2 generation. Vaginal opening and preputial separation are landmarks of hormonally-mediated sexual maturity. F_1 body weights were collected on the day of patency or when preputial separation was observed. In addition, eye opening was evaluated prior to weaning by litter on Postnatal Days 7-16 (pups were examined daily until the landmarks was achieved by the entire litter).

Sensory Function, Reflex response, Motor Activity, Acoustical Startle and Water Maze: Sensory function (pupil response and visual placing) and reflex response (aerial righting reflex, hind-limb extension, and negative geotaxis) were evaluated in the F_1 generation. Pupil response, visual placing, aerial righting and hind-limb extension were evaluated at or shortly after weaning in those pups selected to produce the F_2 generation. Negative geotaxis was evaluated prior to weaning by litter beginning on Postnatal Day 7 (the test was performed until the entire litter achieved the milestone). Approximately 1/2 of the selected pups (10 rats/sex/group) from F_1 generation were subjected to acoustical startle response and motor activity tests around Postnatal Days 36-37 and again around Postnatal Days 65-66. Spatial relationship, learning and memory were evaluated using a water maze in the remaining 1/2 of the F_1 selected pups (10 rats/sex/group), around Postnatal Days 35 and other 1/2 around Postnatal Day 65. Acoustical startle response (ASR) was evaluated using Coulbourn ASR System (Coulbourn Instruments, Holliston, MA, USA). Motor activity was evaluated using TruScan 99 (Coulbourn Instruments). Water Maze was evaluated using a 6 ft round pool with a plexi-glass platform submerged in one quadrant. Acquisition testing was followed 2 days later by three 45 sec trials (or less) to assess memory.

Statistical Methods: Parametric endpoints (body weight, food consumption, organ weights, motor activity, water maze, acoustical startle and developmental landmarks) and litter viability data were analyzed for homogeneity of variance using Levene's test. In the presence of a significant main effect, all post-hoc comparisons were performed using Dunnett's test (two-tail). A minimum statistical significance level of $p \leq 0.05$ was used in all cases. In addition, absolute pup survival (live/dead counts) were analyzed by Chi-Square/Fisher's Exact tests.

RESULTS

Inhalation 6 months repeated-dose studies

A summary of inhalation 6 months repeated-dose studies in rats and dogs are presented in Table 1.

There were no clinically meaningful differences observed in the vehicle (the porous particles excipient comprised of DSPC and $CaCl_2$) and air control groups in rats and dogs study. No porous particle treatment-related effects were observed during ophthalmic examination. There were no porous particles-related changes in electro-

Table 1. Porous Particle (composed of DSPC and $CaCl_2$): 6-month inhalation toxicity studies in rats and dogs.

Species	Rat				Dog			
Group	Air Control (Air only)		Porous Particles (12 mg/kg/day)		Air Control (Air only)		Porous Particles (0.9 mg/kg/day)	
Exposure Time (min.)	120				30			
Sex	male	female	male	female	male	female	male	female
No. of animals	15	15	15	15	4	4	4	4
Morality/Morbidity	0	0	0	0	0	0	0	0
Clinical Observation	No treatment-related effect				No treatment-related effect			
Body Weights (rat:g, dog:kg, mean)	483.39	294.70	490.29	294.28	10.225	9.220	10.150	9.175
Ophthalmology	No treatment-related effect				No treatment-related effect			
Electrocardiograms (leads I, II, III and AVR, AVL, AVF, Heart Rate)	NA[a]				No treatment-related effect			
Hematology (mean)								
Basophils Absolute (10^3/μL)	0.044	0.03	0.037	0.03	0.045	0.068	0.045	0.053
Eosinophils Absolute (10^3/μL)	0.081	0.06	0.065	0.07	0.323	0.300	0.400	0.275
Hematocrit (%)	49.940	46.64	49.267	46.67	47.25	46.80	45.48	46.65
Hemoglobin (g/dL)	15.633	14.81	15.107	14.69	16.13	16.38	15.60	15.95
Large unclassified cells (10^3/μL)	0.057	0.05	0.047	0.040	0.030	0.040	0.045	0.043
Lymphocytes (10^3/μL)	4.931	3.29	4.426	3.56	2.588	2.653	2.308	2.733
Mean cell hemoglobin (pg)	17.28	18.30	17.013	18.06	23.65	24.00	22.90	23.88
Mean cell volume (fL)	55.220	57.64	55.533	57.31	69.45	68.38	66.70	69.73
Mean corpuscular hemoglobin (g/dL)	31.30	31.77	30.64	31.51	34.05	35.10	34.28	34.25
Monocytes Absolute (10^3/μL)	0.163	0.12	0.151	0.12	0.395	0.400	0.375	0.313
Neutrophils Absolute (10^3/μL)	1.377	0.70	1.231	0.65	4.283	5.330	4.893	4.780
Platelet count (10^3/μL)	972.53	1013.67	957.53	941.86	304.0	324.7	255.5	309.3
Red Blood Cell (10^6/μL)	9.049	8.10	8.877	8.14	6.815	6.835	6.825	6.693
Reticulocyte relative (%)	1.633	1.77	1.767	1.61	0.68	0.70	0.83	0.80
White Blood Cell (10^3/μL)	6.653	4.24	5.957	4.47	7.668	8.793	8.068	8.193
Serum Chemistry (mean)								
Albumin (g/dL)	3.97	4.94	3.96	5.14	3.55	3.58	3.53	3.53
A/G[b] Ratio	1.87	2.48	1.72	2.59	1.98	1.95	1.85	2.03
Alkaline Phosphatase (IU/L)	77.27	27.87	93.60	25.13	43.3	42.5	50.8	89.3
Alanine Aminotransferase (IU/L)	55.73	57.33	50.27	45.67	46.8	41.5	52.8	44.5
Aspartate Aminotransferase (IU/L)	166.07	158.93	152.27	154.73	33.0	32.3	30.3	29.0
Blood Urea Nitrogen (mg/dL)	17.20	14.93	17.07	17.20	17.5	19.5	17.8	17.0
BUN/CRE[d] Ratio	44.6	36.7	39.5	42.3	29.0	30.8	31.0	31.0
Calcium (mg/dL)	9.93	10.48	10.30	11.047	10.30	10.25	10.48	10.30
Chloride (mmol/L)	102.93	103.13	102.60	101.80	112.5	112.5	114.8	114.8
Cholesterol (mg/dL)	63.33	95.40	66.13	92.93	154.3	176.0	153.0	183.0
Creatinine (mg/dL)	0.39	0.41	0.45	0.42	0.60	0.65	0.58	0.55
Globulin (g/dL)	2.17	2.01	2.34	2.00	1.80	1.83	1.88	1.73
Glucose (mg/dL)	187.73	159.73	192.53	154.87	71.8	76.3	84.5	84.3
Phosphate (mg/dL)	7.29	5.97	6.64	6.45	5.10	4.75	5.18	5.15
Potassium (mmol/L)	5.29	4.63	5.21	4.41	4.85	4.60	5.05	4.75
Sodium (mmol/L)	145.53	147.13	145.27	145.07	145.8	146.0	146.8	147.3
Total Protein (g/dL)	6.15	6.95	6.30	7.14	5.35	5.40	5.40	5.25
Total Bilirubin (mg/dL)	0.10	0.18	0.11	0.18	0.10	0.10	0.10	0.10
Triglyceride (mg/dL)	76.20	69.20	92.60	87.67	43.0	66.5	38.0	43.3
Urinalysis Parameters	No treatment-related effect				No treatment-related effect			
Gross Pathology								
Organ Weights (mean, g)								
Brain	2.26	2.06	2.21	2.03	77.02	72.92	76.54	72.69
Heart	1.35	0.98	1.46	0.96	84.00	65.93	88.20	70.77
Kidney(s)	2.65	1.75	2.65	1.65	52.98	39.45	54.40	43.32
Liver	10.96	6.80	12.15	6.90	335.97	278.97	325.04	300.83
Lung(s)	2.12	1.61	2.15	1.51	67.90	58.76	73.71	62.82
Spleen	0.66	0.53	0.70	0.51	114.90	121.58	112.14	122.12
Testis(es)	3.67	NA	3.58	NA	13.91	NA	12.36	NA
Ovary(ies)	NA	0.12	NA	0.12	NA	1.20	NA	0.96
Microscopic Lesion Incidence: Number of the change within group								
Larynx: Metaplasia, Squamous	0	0	0	3	0	0	0	0
Lung: Inflammation, Mixed (Alveolar)	0	0	0	0	0	3	3	1
Lung: Accumulatio, Alveolar macrophage	12	11	11	9	1	3	0	0
Lung: Aggregate, Alveolar macrophage (Focal, Dense)	0	0	0	0	0	2	0	3
Lung: Hypertrophy/hyperplasia Epithelial (Focal)	0	0	1	0	1	1	2	1
Nasal turbinates[c] 2: Hyaline; Respiratory Epithelium	1	1	0	1	0	0	0	0
Nasal turbinates 3: Hyaline; Olfactory Epithelium	4	3	6	5	0	0	0	0
Nasal turbinates 4: Hyaline; Olfactory Epithelium	6	7	6	7	0	0	0	0
No Observed Adverse Effect Level	12 mg/kg/day				0.9 mg/kg/day			

a: NA = Not Applicable

b: A/G = Albumin/Globulin Ratio

c: Four transverse sections of the nasal cavity were produced and evaluated. Sections were taken from approximate areas as follows: 1; caudal surface of upper incisor, 2; between the incisive papilla and the first palatal ridge, 3; between last palatal ridge and mild-point of first molar, 4; 2nd molar

d: BUN/CRE = Blood Urea Nitrogen/ Creatinine Ratio

cardiography parameters, and all the electrocardiograms evaluated in this study were qualitatively and quantitatively considered normal for the canine (Only Dogs study). And also DSPC/$CaCl_2$ demonstrated no significant gross pathology or histopathologic changes in respiratory tissue. The NOAELs were identified as the highest doses administered, 12 mg/kg/day in rats and 0.9 mg/kg/day in dogs (DSPC/$CaCl_2$).

Genotoxicity studies

The results of the mutation assays indicate that the DSPC, at up to 5000 μg/plate, did not induce any significant increase in the number of revertant colonies for any of the tester strains in the presence or absence of induced rat liver S-9 (Table 2). Under the conditions of this study, DSPC was negative in the *Salmonella typhimurium / Escherichia coli* plate incorporation mutation assay with and without metabolic activation.

The results of the chromosome aberration assay indicate that DSPC, at up to 1000 μg/mL, did not induce a statistically significant increase ($p \leq 0.05$) in the percentage of cells with aberrations at any of the concentrations tested, both with and without metabolic activation when compared to the solvent controls (Table 3). Under the conditions of this study and according to the criteria set for evaluating the test results, DSPC was negative in the *in vitro* Chromosome Aberration Assay in CHO cells when tested with and without an exogenous metabolic activation system, and therefore, is not considered to be a clastogenic agent.

The results of this micronucleus assay in mice indicate that, under the conditions of this test and according to the criteria set for evaluating the test results, DSPC, at intra-peritoneal doses up to 25 mg/kg, was negative in the Micronucleus Assay (Table 4). It was concluded that DSPC did not cause chromosome damage *in vivo* and, therefore is not considered to be a clastogenic agent.

Carcinogenicity studies

A summary of carcinogenicity studies in mouse and rat are presented Tables 5 and 6, respectively. The toxicology evaluation of the following parameters: mortality, survival rate, clinical observation, body weights, ophthalmology, gross pathology, organ weights, revealed no difference between the air control group and the excipient control group. Histopathological examinations revealed, no significant differences between the vehicle control and the air control groups. DSPC/$CaCl_2$ administered at mean estimated total inhaled doses of 18 mg/kg/day in mice and 10 mg/kg/day in rats for up to 104 weeks, showed no treatment related proliferative (*i.e.,* hyperplastic, pre-neoplastic, or neoplastic) changes and there was no increase in the type, site or severity of any tumour, compared to concurrent untreated air control animals, or to historical background incidence data.

Reproductive toxicity studies

In the reproductive toxicology studies, limited plasma analysis was conducted, intended as proof of exposure data only. Since DSPC is an endogenous compound, variable background levels were detected in all animals, including controls (Data was not shown). However, although variable, proof of exposure was observed across all dosed groups.

A summary of fertility and early embryonic development in rat is presented in Table 7. In this study, no treatment-related deaths and no clinical signs of toxicity were seen during the study. No treatment-related changes were seen in body weight and/or body weight gain. Gestation body weight and body weight gain were similar across groups and corrected body weights were unaffected by treatment. Mating performance (act of copulations as indicated by the presence of sperm) of the treated rats was unaffected by treatment with DSPC. The percentage of successful pregnancies in the treated groups ranged from 96-100%. Litter viability was also similar across groups and no biologically relevant differences in the average number of corpora lutea, total implants, resorptions, pre- or post- implantation loss were seen between the treated and control groups. Reproductive organ weights of the treated rats, compared to controls, were unaffected by treatment. Overall mating performance (percent sperm positive successful mating outcome, oestrus cyclicity, sperm motility and morphology) was unaffected by treatment. Based on the results of this study, the NOAEL of DSPC on reproductive function is 50 mg/kg/day for males and females, and the NOAEL for systemic toxicity was also 50 mg/kg/day.

A summary of embryo-fetal development in rats is presented in Table 8. In this study, no treatment related clinical signs were seen during the study. No changes in food consumption, body weight, body weight gain or corrected body weight/body weight gain were seen in the treated dams compared to the controls. No evidence of maternal toxicity was seen in the monitored parameters. Overall, litter viability (live, non-live, total implants, pre- and post implantation loss) were unaffected by intraperitoneal dosing with DSPC at doses up to 50 mg/kg/day. No effect on fetal growth was noted as fetal body weights were similar between the treated groups and controls. Fetal examinations (gross external, skeletal, visceral and cephalic) did not reveal any frank pattern of teratogenicity. Based

Table 2. Mutation assay without/with S-9 activation of DSPC.

S. typhimurium / E. coli		Average No. of Revertants/Plate						
		Positive[a] Control	Solvent Control	Concentration/plate (µg/plate) 313	625	1250	2500	5000
Without S-9 activation								
TA 98	Revertains (STD.DEV.)	752 (49)	32 (6)	31 (9)	31 (5)	26 (3)	31 (3)	27 (4)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
TA100	Revertains (STD.DEV.)	367 (89)	49 (8)	70 (9)	66 (10)	59 (6)	64 (6)	60 (8)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
TA1535	Revertains (STD.DEV.)	323 (38)	13 (3)	12 (3)	11 (5)	12 (1)	14 (3)	8 (3)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
TA1537	Revertains (STD.DEV.)	191 (26)	9 (3)	8 (3)	8 (4)	7 (4)	6 (3)	5 (3)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
WP2 *uvr*A	Revertains (STD.DEV.)	372 (23)	14 (4)	12 (2)	16 (4)	10 (6)	14 (1)	8 (3)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
With S-9 activation								
TA 98	Revertains (STD.DEV.)	871 (69)	39 (2)	49 (6)	41 (7)	42 (3)	38 (5)	37 (6)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
TA100	Revertains (STD.DEV.)	640 (93)	50 (7)	59 (4)	55 (6)	57 (7)	56 (8)	44 (5)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
TA1535	Revertains (STD.DEV.)	160 (13)	14 (1)	10 (4)	13 (4)	14 (2)	15 (1)	9 (4)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
TA1537	Revertains (STD.DEV.)	81 (7)	12 (1)	8 (1)	10 (2)	9 (4)	7 (1)	4 (2)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP
WP2 *uvr*A	Revertains (STD.DEV.)	106 (8)	13 (3)	11 (3)	13 (3)	12 (2)	12 (4)	10 (1)
	Lawn	NL	NL	NL	NL	NL	NL	NL
	Precipitate	NP	NP	NP	NP	NP	SP	MP

a: Positive Control: TA98 (-/+S-9) = 2-Nitrofluorence/2-Aminoanthracene, TA100 (-/+S-9) and TA1535 (-/+S-9) = Sodium Azide/2-Aminoanthracene, TA1537 (-/+S-9) = 9-Aminoacridine/2-Aminoanthracene, WP2 uvrA (-/+S-9) = Methyl Methanesulfonate/2-AminoanthraceneNL: Normal, healthy microcolony lawn. NP: No precipitate.SP: Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting. MP: Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration.

Table 3. Chromosome aberrations in cultured Chinese Hamster Ovary cells without/with S-9 activation of DSPC.

	Without S-9					With S-9				
Test Article Concentration (µg/mL)	Mean No. of Cells (x10^6)	RCG[c] (%)	MI[d]	RMI[e] (%)	% Cells with Aberration	Mean No. of Cells (x10^6)	RCG (%)	MI	RMI (%)	% Cells with Aberration
Untreated	1.57	159	10.6	67	0.0	1.25	136	11.1	148	0.0
Solvent	0.99	100	15.9	100	0.5	0.92	100	7.5	100	1.0
300	0.96	97	15.9	100	NI[g]	1.10	120	9.1	121	NI
400	1.42	143	13.8	87	1.0	1.09	118	6.9	92	1.0
500	0.97	98	12.2	77	0.5	0.89	97	8.2	109	2.5
1000	0.43	43	7.5	47	1.0	1.05	114	6.8	91	2.0
MMC[a] 0.4	NA[f]	NA	9.6	91	28.0*	NA	NA	NA	NA	NA
MMC 0.8	NA	NA	11.1	105	NI	NA	NA	NA	NA	NA
CP[b] 7.5	NA	NA	NA	NA	NA	NA	NA	4.3	39	27.5*
CP 12.5	NA	NA	NA	NA	NA	NA	NA	1.7	15	NI

a: Mitomycin-C, b: Cyclophosphamide, c: Relative Cell Growth = (No. of Cells in the Test Flask/No. of Cells in the Solvent Flask) x 100,d: Mean Mitotic Index = No. of dividing cells scored from 1000 cells/10, e: Relative Mitotic Index = (Test Concentration MI/Solvent Control MI) x 100, f: Not Applicable, g: Not Implemented: Based on the RMI results, the chromosome aberrations were scored from the three highest concentration of 400, 500 and 1000 µg/mL in both the non-activated and activated systems. *: Statistically significant response using Chi-square test.

on findings from this study, the NOAEL for maternal and developmental toxicity of DSPC was 50 mg/kg/day.

A summary of embryo-fetal development in rabbits is presented in Table 9. In this study, none of the rabbits died during the study and no clinical signs of toxicity were seen. No evidence of maternal toxicity was seen in food consumption, body weight/body weight gain or gravid uterus weight. Total weight gain corrected for uterine weight was similar across groups and unaffected by treatment. No statistically significant difference in litter viability (corpora lutea, pre- and post- implantation loss, live, non-live and total implants) were seen between the treated and controls. Overall, fetal viability was unaffected by treatment and no increase in fetal loss was observed

Table 4. Micronucleated polychromatic erythrocytes in mouse bone marrow cells of DSPC.

Dose (mg/kg)	Male					Female				
	Cell counts		PCE(%)	Change in %PCE[c]	MPCE[d]/ 2000 PCE	Cell counts		PCE(%)	Change in %PCE	MPCE/ 2000 PCE
	PCE[a]	NCE[b]				PCE	NCE			
24 hr after treatment										
DMSO	104	96	52.1	-	0.8	96	104	48.0	-	0.4
6.25	106	94	52.9	1.5%	0.4	109	91	54.7	14.0%	0.6
12.5	119	81	59.7	14.6%	0.0	100	100	50.0	4.2%	0.0
25	107	93	53.3	2.3%	0.8	103	97	51.4	7.1%	0.0
CP[e]	93	107	46.5	-10.7%	38.4*	98	102	48.8	1.7%	31.2*
48 hr after treatment										
DMSO	109	91	54.4	-	0.2	116	84	57.9	-	1.0
6.25	111	89	55.7	2.4%	0.6	117	83	58.7	1.4%	0.0
12.5	110	90	55.0	1.1%	0.6	119	79	60.3	4.1%	1.0
25	116	84	58.1	6.8%	0.0	114	86	57.1	-1.4%	0.0

a: polychromatic erythrocytes, b: normochromatic erythrocytes, c: Change of Percent PCE in comparison with concurrent DMSO, calculated by the following formula: [(Percent PCE for Test Dose – Percent PCE for DMSO)/Percent PCE for DMSO] x 100, d: micronucleated polychromatic erythrocytes, e: Cyclophosphamide, CP was used as positive control and was dosed at 80 mg/kg by oral gavage. *: Statistically significant response using a one-tailed Student's t-test.

(post implantation loss). Fetal weights were unaffected by treatment at doses up to 50 mg/kg/day. No frank morphological changes were seen in the gross external, visceral, cephalic or skeletal examinations of the treated fetuses. Findings observed were low in incidence and within published historical control range. Based on the findings from this study, the NOAEL for maternal and developmental toxicity was 50 mg/kg/day.

A summary of pre- and postnatal development, including maternal function in rats is presented in Table 10. In this study, no treatment-related deaths occurred in adult F_0 animals. No treatment-related clinical signs of toxicity were seen during the study. The average length of gestation was 22 days and the percentage of F_0 dams undergoing successful parturition (delivered/pregnant x 100) was 100% for the controls, 92% in the 1 and 50 mg/kg treated groups and 96% in the 25 mg/kg treated group. The number of implantation sites and average F_1 litter size (total born for combined sexes) were similar in the control and treated groups and no difference was seen in post-implantation loss. Overall survival ranged from 86 to 96% on Postnatal Day 0 and 98 to 100% over Postnatal Day 4-21. Developmental landmarks (eye opening and negative geotaxis of F_1- and F_2-generations, preputial separation and vaginal patency of F_1-generation) were not affected by treatment. There were no apparent treatment-related differences in the evaluation of sensory function (pupil response, tactile placing), reflex response (aerial righting reflex and hind-limb extension), acoustical startle response, motor activity or learning and memory (water maze) in the F_1-generation. Mating performance and fertility of the F_1-generation was similar across groups. Preweaning body weights of the F_2-generation were also similar across groups. No overt maternal toxicity was seen in F_0 dams treated with 1, 25, or 50 mg/kg DSPC. No treatment-related alterations in body weights, growth, survival or development were seen in the F_1- or F_2- generation. Based on the findings from this study, the NOAEL for systemic exposure of DSPC in the F_0 generation was 50 mg/kg/day. The NOAEL for reproductive toxicity of the F_0 generation was 50 mg/kg/day and the reproductive NOAEL was also 50 mg/kg/day for males and females of the F_1 generation. Finally, the NOAEL for developmental toxicity, was 50 mg/kg/day.

DISCUSSION

DSPC has the Chemical Abstracts Registry (CAS) number 816-94-4 and is a long-chain fully saturated PC, with stearic acid as its fatty acid component. The DSPC material used in all experiments described was a synthetic, non-animal derived material. PCs are natural components of cell membranes, and are the primary lipid constituent of lung surfactant. Pulmonary surfactant is composed of roughly 90% lipids: 80-85% of which are phospholipid, 5-10% are neutral lipids, and 10% are proteins (Glasser and Mallampalli, 2012). The predominant phospholipid in surfactant is DPPC, a saturated 16-carbon fatty acid (Palmitic acid), representing 68% of the total PC. DSPC, a saturated 18-carbon fatty acid (Stearic acid), representing 4.5% of the total PC (Hallman *et al.*, 1982, Rooney, 1992). The roles of surfactant, and the phospholipid component in particular, include stabilization of the alveolar surface, maintenance of osmotic homeostasis of the alveolar space, and host defense (Glasser and Mallampalli, 2012). The amount of saturated (containing no ethylenic bonds) PCs in human lung is 3.9 mg/g of lung tissue and of unsaturated (containing one or more ethylenic bonds) PCs is 3.2 mg/g of human lung tissue (Clements *et al.*, 1970). Lipid such as PC, DSPC and DPPC are considered as GRAS (generally recognized as safe) because they are endogenous to the lungs and local-

Table 5. Porous Particle (composed of DSPC and $CaCl_2$): Carcinogenicity study (inhalation) in mice.

Group	Air Control (Air only)		Porous Particles (20 mg/kg/day)	
Exposure Time (min.)	120			
Sex	male	female	male	female
No. of animals	60	60	60	60
Morality/Morbidity	14	19	20	19
Terminal sacrifice	46	41	40	41
Survival (%)	76.7	68.3	66.7	68.3
Clinical Observation	No treatment-related effect			
Body Weights (g, mean ± SD, Week 102)	38.9 ± 4.6	38.7 ± 5.9	39.2 ± 4.9	38.5 ± 5.1
Ophthalmology	No treatment-related effect			
Gross Pathology				
Organ Weights (g, mean ± SD)				
Adrenal	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01*	0.02 ± 0.02
Brain	0.57 ± 0.12	0.57 ± 0.10	0.53 ± 0.07	0.52 ± 0.04*
Epididymides	0.11 ± 0.03	NA[a]	0.11 ± 0.02	NA
Heart	0.24 ± 0.08	0.18 ± 0.08	0.22 ± 0.04	0.16 ± 0.03
Kidney	0.77 ± 0.14	0.45 ± 0.15	0.76 ± 0.10	0.42 ± 0.07
Liver	2.01 ± 1.10	1.44 ± 0.25	1.59 ± 0.34*	1.53 ± 0.39
Lung	0.40 ± 0.46	0.31 ± 0.12	0.33 ± 0.14	0.27 ± 0.07
Ovary	NA	0.04 ± 0.09	NA	0.04 ± 0.04
Spleen	0.14 ± 0.07	0.19 ± 0.10	0.10 ± 0.03	0.34 ± 0.44*
Testis	0.21 ± 0.04	NA	0.21 ± 0.03	NA
Thymus	0.02 ± 0.02	0.03 ± 0.02	0.02 ± 0.03	0.03 ± 0.02
Histopathology: Number of animals with neoplastic lesions				
Systemic				
Haemangiosarcoma	5	1	1	2
Histiocytic sarcoma	2	3	0	1
Lymphoma	1	19	1	17
Harderian glands				
Adenocarcinoma	0	1	3	1
Adenoma	9	1	4	5
Liver				
Hepatocellular Adenoma	20	4	17	8
Hepatocellular Carcinoma	19	1	18	2
Lung, Left				
Alveolar-bronchiolar Adenoma	2	3	3	2
Alveolar-bronchiolar Carcinoma	2	1	2	1
Carcinoma, metastatic	0	0	0	0
Hepatocellular Carcinoma, metastatic	6	0	2	0
Lung, Right				
Alveolar-bronchiolar Adenoma	6	0	6	0
Alveolar-bronchiolar Carcinoma	2	2	7	1
Carcinoma, metastatic	0	0	0	0
Hepatocellular Carcinoma, metastatic	6	0	2	0
Pituitary glands: Adenoma, Pars Distalis	0	6	1	4
Skin: Fibrosarcoma	1	1	0	1
Small Intestine-duodenum: Adenoma	1	0	0	0
Thyroid glands: Adenoma, follicular cell	8	12	19	27
Histopathology: Number of animals with non-neoplastic lesions				
Nasal turbinates 1[b]				
Inflammation, acute	1	1	0	1
Eosinophilic material, airway	0	0	0	0
Degeneration, hyaline, respiratory epithelium	0	3	27	36
Nasal turbinates 2				
Inflammation, acute	0	1	0	0
Eosinophilic material, airway	0	0	0	0
Degeneration, hyaline, respiratory epithelium	0	1	47	57
Degeneration, hyaline, olfactory epithelium	0	0	1	2
Nasal turbinates 3				
Inflammation, acute	0	1	1	0
Eosinophilic material, airway	0	0	0	1
Degeneration, hyaline, respiratory epithelium	0	0	42	14
Degeneration, hyaline, olfactory epithelium	0	0	38	53
Nasal turbinates 4				
Inflammation, acute	0	1	3	1
Eosinophilic material, airway	1	0	9	2
Degeneration, hyaline, respiratory epithelium	0	0	0	0
Degeneration, hyaline, olfactory epithelium	0	0	57	47
Stomach: Hyperplasia, mucosa, glandular	12	2	24	6
Thyroid: Hyperplasia, follicular cell	53	53	55	56
No Observed Adverse Effect Level	20 mg/kg/day			

*: $p \leq 0.05$ against Air control

a: NA = Not Applicable

b: Four transverse sections of the nasal cavity were produced and evaluated. Sections were taken from approximate areas as follows: 1; caudal surface of upper incisor, 2; between the incisive papilla and the first palatal ridge, 3; between last palatal ridge and mild-point of first molar, 4; 2nd molar

Table 6. Porous Particle (composed of DSPC and $CaCl_2$): Carcinogenicity study (inhalation) in rats.

Group	Air Control (Air only)		Porous Particles (10 mg/kg/day)	
Exposure Time (min.)	120			
Sex	male	female	male	female
No. of animals	60	60	60	60
Morality/Morbidity	22	38	33	34
Terminal sacrifice	38	22	27	26
Survival (%)	63.3	36.7	45.0	43.3
Clinical Observation	No treatment-related effect			
Body Weights (g, mean ± SD, Week 81)	764.8 ± 145.5	520.5 ± 111.6	765.9 ± 118.4	459.8 ± 65.3
Ophthalmology				
Gross Pathology	No treatment-related effect			
Organ Weights (g, mean ± SD)				
Adrenal	0.06 ± 0.01	0.09 ± 0.05	0.07 ± 0.03	0.12 ± 0.02
Brain	2.39 ± 0.69	2.05 ± 0.07	2.29 ± 0.09	2.04 ± 0.28
Epididymis	1.43 ± 0.19	NA[a]	1.49 ± 0.50	NA
Heart	1.73 ± 0.23	1.34 ± 0.14	1.80 ± 0.31	1.31 ± 0.19
Kidney	3.36 ± 0.48	2.44 ± 0.29	3.58 ± 0.82	2.29 ± 0.39
Liver	16 ± 3	12 ± 3	18 ± 7	14 ± 3
Lung	2.55 ± 0.34	2.04 ± 0.22	2.57 ± 0.71	1.99 ± 0.30
Ovary	NA	0.11 ± 0.04	NA	0.11 ± 0.04
Spleen	1.18 ± 0.30	0.79 ± 0.11	1.18 ± 0.03	0.87 ± 0.26
Testis	3.55 ± 0.61	NA	3.74 ± 0.42	NA
Thymus	0.57 ± 0.18	0.50 ± 0.28	0.61 ± 0.27	0.44 ± 0.15
Histopathology: Number of animals with neoplastic lesions				
Adrenal glands				
Adenoma, cortex	0	0	0	1
Carcinoma, cortex	0	0	0	1
Pheochromocytoma, benign	6	1	7	0
Pheochromocytoma, malignant	2	0	2	0
Mammary glands				
Adenocarcinoma	0	11	0	14
Adenoma	0	0	0	3
Fibroadenoma	0	11	0	14
Pituitary gland				
Adenoma, Pars distalis	29	40	29	41
Adenoma, Par intermedia	0	2	0	0
Thyroid glands				
Adenoma, C-cell	1	5	3	0
Adenoma, follicular cell	1	0	1	1
Carcinoma, C-cell	1	0	0	2
Histopathology: Number of animals with non-neoplastic lesions				
Larynx: Metaplasia, squamous	5	2	15	10
Lung: Aggregates, alveolar macrophage	47	30	30	31
Nasal turbinates 1[b]				
Degeneration, hyaline, respiratory epithelium	1	1	0	1
Inflammation, neutrophilic	1	0	4	2
Nasal turbinates 2				
Degeneration, hyaline, respiratory epithelium	2	2	1	3
Inflammation, neutrophilic	0	0	3	1
Nasal turbinates 3				
Degeneration, hyaline, olfactory epithelium	12	9	18	11
Nasal turbinates 4				
Degeneration, hyaline, olfactory epithelium	29	15	33	21
No Observed Adverse Effect Level	10 mg/kg/day			

a: NA = Not Applicable

b: Four transverse sections of the nasal cavity were produced and evaluated. Sections were taken from approximate areas as follows: 1; caudal surface of upper incisor, 2; between the incisive papilla and the first palatal ridge, 3; between last palatal ridge and mild-point of first molar, 4; 2nd molar

ly present in large quantities (Pilcer and Amighi, 2010).

According to FDA's public database of inactive ingredients, DSPC is present in an approved intravenous solution containing liposomes (FDA, Inactive Ingredients Database, 2018). In fact, most liposomal drug formulations are available for intravenous and intramuscular application (Bulbake *et al.,* 2017). On the other hand, TOBI®Podhaler™ is approved for inhaled administration, containing the excipient DSPC and $CaCl_2$. To support this approval, the repeat dose toxicity studies in rats and dogs that have vehicle control groups and Tobramycin Inhalation Powder (TIP) groups where DSPC and $CaCl_2$ is present, were submitted (TOBI Podhaler, 2013, FDA, NDA 201-688, Novartis Pharmaceuticals Corporation). In the repeat dose toxicity studies with TIP, dogs received the DSPC/$CaCl_2$ vehicle daily for 4 weeks and rats received it daily for 26 weeks (at inhaled doses of about 12 mg/kg/day for both species) without apparent toxicity. Dogs

Table 7. Fertility and early embryonic development of DSPC in rats.

Dose (mg/kg/day)	Vehicle (Corn oil)		1		25		50	
Sex	Male	Female	Male	Female	Male	Female	Male	Female
No. of Animals	25	25	25	25	25	25	25	25
Mortality	0	0	0	0	0	0	0	0
Clinical Observation	No treatment related clinical signs							
Food consumption (g, mean ± SD)								
Male Day 7	217 ± 22		207 ± 19		210 ± 21		210 ± 18	
Male Day 14	210 ± 22		203 ± 19		204 ± 23		204 ± 20	
Male Day 28	238 ± 23		223 ± 23		232 ± 27		238 ± 32	
Female Day 14 (pre-mating)		183 ± 16		189 ± 13		184 ± 16		186 ± 20
Female Day 9 (gestation)		73 ± 9		73 ± 8		69 ± 12		75 ± 14
Female Day 12 (gestation)		78 ± 10		79 ± 10		73 ± 10		76 ± 10
Body Weights (g, mean ± SD)								
Male Day 7	323 ± 25		316 ± 21		320 ± 29		321 ± 21	
Male Day 14	367 ± 32		358 ± 25		364 ± 35		363 ± 26	
Male Day 28	436 ± 41		417 ± 34		430 ± 45		433 ± 37	
Female Day 14 (pre-mating)		258 ± 19		262 ± 20		265 ± 22		261 ± 23
Female Day 9 (gestation)		297 ± 20		305 ± 21		300 ± 24		304 ± 19
Female Day 12 (gestation)		318 ± 23		323 ± 23		315 ± 23		322 ± 20
Mating Performance								
Mating Pairs		25		25		25		25
Successful Mating (sperm-positive)		24		24		23		24
Misdiagnosed Smear		0		1		2		1
Percent Mated (%)		96		100		100		100
Successful Mating Outcomes (gravid)		24		24		25		25
% Successful Outcomes (+/pregnant)		100		96		100		100
Latency to Mate (days, mean ± SD)		2.4 ± 1.3		3.1 ± 2.0		2.4 ± 1.9		3.1 ± 2.5
Estrus frequency (days, mean ± SD)		3.0 ± 0.5		3.0 ± 0.5		2.7 ± 0.7		2.9 ± 0.3
Litter Viability (mean ± SD)								
Corpora Lutea/animal		18 ± 3		18 ± 4		16 ± 3		16 ± 3
Total Implants/animal		18 ± 4		18 ± 4		16 ± 3		15 ± 4*
Resorptions/animal		4.4 ± 3.7		5.0 ± 3.5		3.4 ± 2.9		1.7 ± 1.2*
Pre-Implantation Loss (%)		4.8 ± 11.8		2.6 ± 4.6		2.8 ± 9.1		9.0 ± 21.6
Post-Implantation Loss (%)		25.9 ± 23.8		28.6 ± 22.7		23.0 ± 23.2		14.5 ± 19.4
Sperm Analysis (n = 10, mean ± SD)								
Motile (%)	79.9 ± 5.0		81.1 ± 6.3		78.9 ± 8.1		82.4 ± 3.9	
Progressive (%)	22.1 ± 4.1		21.4 ± 4.5		20.3 ± 5.5		22.8 ± 4.7	
Sperm Count (/g x10^8)	9.7 ± 3.8		11.0 ± 3.7		11.2 ± 1.4		11.8 ± 2.4	
Sperm Morphology (Normal/200 sperm)	194 ± 4.5		NA		NA		195 ± 1.8	
Detached/Isolated Head	6 ± 4.5		NA		NA		5 ± 1.8	
Abnormal (%)	3 ± 2.3		NA		NA		2 ± 0.9	
Organ Weights (g, mean ± SD)								
Testis	3.49 ± 0.31		3.38 ± 0.33		3.52 ± 0.32		3.47 ± 0.33	
Epididymis	1.37 ± 0.18		1.35 ± 0.14		1.36 ± 0.17		1.38 ± 0.14	
Ovary		0.15 ± 0.02		0.15 ± 0.03		0.15 ± 0.02		0.15 ± 0.02
Uterus Weight		9.51 ± 2.31		9.64 ± 2.65		9.07 ± 2.55		9.69 ± 2.54
Gross Pathology	No related article							
NOAEL	50 mg/kg/day (male and female reproductive performance)							

NA: Not Applicable, *: Significantly different from vehicle, $p \leq 0.05$.

that received the vehicle weekly for 26 weeks (about 80 mg/kg inhaled dose on the first day, and about 14 mg/kg for the rest of the study) demonstrated no histopathologic changes in respiratory tissue and no changes in clinical chemistry parameters. The results of TOBI®Podhaler™ were confirmed in our repeated dose toxicity studies, and no difference was observed between the DSPC administration group (vehicle control) and the air control group in any of the studies using mice, rats and dogs, and no significant toxicological finding was observed.

In our 6-month MDI inhalation toxicity study in rats, minimal laryngeal squamous metaplasia was noted in the excipient control (3 females only). This is a common response in inhalation studies in the rat (Renne *et al.,* 2009). Minimal to mild hyaline degeneration of respiratory and olfactory epithelium was present in nasal mucosa in the air control group and the excipient control group. However, there was no overall difference in the incidence. Minor changes of this type are common in aging rats and are often seen to increase in incidence with inhalation exposure (Harkema *et al.,* 2006; Monticello *et al.,* 1990; Nagano *et al.,* 1997; Chan *et al*., 1998; Katagiri *et al.,* 2000). These types of minimal changes are commonly seen in inhalation studies and are considered adaptive in nature and not a toxic response. In our 104-Week MDI inhalation carcinogenicity study in mice, non-neoplastic lesions were identified in the nasal cavity. In the nasal passages, an increased incidence of hyaline degenera-

Table 8. Embryo-fetal development of DSPC in rats.

Dose (mg/kg/day)	Vehicle (Corn oil)	1	25	50
No. of animals	23	23	23	23
Food Consumption (g, mean ± SD, Gestation Day 21)	80 ± 14	85 ± 9	82 ± 12	86 ± 9
Body Weight (g, mean ± SD, Gestation Day 21)	377 ± 35	383 ± 28	379 ± 29	388 ± 28
Body Weight Gain (g, mean ± SD, Gestation Day 21)	47 ± 10	48 ± 12	49 ± 13	52 ± 10
Uterus Weight (g, mean ± SD, Gestation Day 21)	97 ± 16	102 ± 15	97 ± 8	98 ± 12
Litter Viability				
Initial Group Size (sperm-positive)	23	23	23	23
Actual Group Size (gravid)	22	23	21	23
Early Death	0	0	0	0
Viable Litters (≥ 1 live)[a]	22	23	21	23
Pre-implantation Loss[b] (%, mean[c]±SD)	1.2 ± 2.7	3.9 ± 9.7	0.7 ± 2.1	3.3 ± 5.9
Post-implantation Loss[d] (%, mean ± SD)	9.8 ± 9.1	4.3 ± 4.6*	3.6 ± 5.2*	4.2 ± 7.2*
Corpora Lutea (/animal, mean ± SD)	13.8 ± 1.7	14.0 ± 2.0*	12.8 ± 1.0*	13.1 ± 1.8*
Total Implants (/animal, mean ± SD)	13.7 ± 1.6	13.6 ± 2.4	12.7 ± 1.0	12.6 ± 1.5
Total Resorptions (/animal, mean ± SD)	1.1 ± 1.2	0.6 ± 0.7*	0.5 ± 0.7*	0.5 ± 0.9*
Total Deaths (/animal, mean ± SD) (Sum)	0.2 ± 0.4 (4)	0.0 ± 0.0* (0)	0.0 ± 0.0* (0)	0.0 ± 0.2* (1)
Total Non-live Resorption & Death (/animal, mean ± SD) (Sum)	1.3 ± 1.2 (29)	0.6 ± 0.7* (14)	0.5 ± 0.7* (10)	0.5 ± 0.9* (12)
Live Fetuses/Total Implants %	90.4	95.5	96.3	95.9
Total Resorptions/Total Implants %	8.3	4.5	3.7	3.8
Total Non-Live/Total Implants %	9.6	4.5	3.7	4.1
Malformed Fetuses/Live Implants %	1.5	0.3	0.8	1.1
Litters with (% Litter with)				
Resorptions	14 (64)	12 (52)	8 (38)	7 (30)
Deaths	4 (18)	0 (0)	0 (0)	1 (4)
Non-Live	16 (73)	12 (52)	8 (38)	8 (35)
Malformed	4 (18)	1 (4)	2 (10)	3 (13)
Parameter				
Litter Weight (g, male+female, mean ± SD)	5.72 ± 0.62	5.97 ± 0.36	5.84 ± 0.38	6.09 ± 0.26*
Number of Males (/animal, mean ± SD)	6.5 ± 2.6	6.3 ± 1.7	5.9 ± 1.9	6.6 ± 2.1
Number of Females (/animal, mean ± SD)	5.9 ± 2.2	6.7 ± 1.8	6.3 ± 1.7	5.5 ± 1.7
Male: Female Ratio	1.1:1	0.9:1	0.9:1	1.2:1
Gross External Morphology				
Number Examined (Number of Normal) Fetal/Litter	272/22 (269/22)	298/23 (297/23)	257/21 (255/21)	278/23 (275/23)
Adactyly (M)[f]	0/0	1/1	1/1	1/1[e]
Syndactyly (M)	2/1	0/0	1/1 [e]	2/2
Micromelia (M)	2/1	0/0	1/1 [e]	0/0
Cleft Palate (M)	1/1	0/0	0/0	0/0
Kinked/Short Tail (M)	0/0	0/0	1/1 [e]	1/1 [e]
Abnormal Body Curvature (M)	1/1	0/0	1/1 [e]	0/0
Visceral Observation				
Number Examined (Number of Normal) Fetal/Litter	135/22 (133/22)	149/23 (136/23)	130/21 (129/21)	138/23 (129/23)
Hydroureter/Hydronephrosis (V)	0/0	9/4	1/1	9/6
Hypoplastic Lungs (V)	1/1	0/0	0/0	0/0
Innominate Short/Absent (V)	1/1	3/2	0/0	0/0
Carotid artery mispositioned (V)	0/0	1/1	0/0	0/0
Cephalic Observations				
Number Examined (Number of Normal) Fetal/Litter	135/22 (133/22)	Not Examined		138/23 (138/23)
Cleft pale	2/2			0/0
Skeletal Examination				
Number Examined (Number of Normal) Fetal/Litter	137/22 (100/22)			140/23 (111/23)
Skull Incomplete Ossification (V)[f]: Score 1	11/8			4/3
Score 2	8/5			4/4
Score 3	0/0			1/1
Score 4	3/1			2/2
Sternebrae Incomplete Ossification (V): Score 1	2/2	Not Examined		0/0
Ribs Rudimentary 13 th (V): Score 1	0/0			0/0
Ribs Rudimentary 14 th (V): Score 2	11/8			12/9
Rudimentary other (V): Score 2	1/1			0/0
Incomplete Ossification, only (V)	0/0			1/1
Wavy/Bulbous (V)	2/1			5/4
Centrae Dumbell/bipartite	8/5			5/4
Pelvic Girdle Pubis Incomplete Ossification	2/2			2/2
No Observed Adverse Effect Level	50 mg/kg/day (maternal and developmental toxicity of DSPC)			

a: Viable litters refer to the number of dams with at least 1 live implant on gestation day 21. b: [(Total Corpora Lutea – total Implants) / Total Corpora Lutea] x 100. When the number of implants is greater than the number of corpora lita, a zero was used to indicate 0% pre-implantation loss. c: The mean reported utilizes the litter as the unit or observation ± standard deviation (SD); the N used is the number gravid unless the parameter requires an intact fetus for evaluation (e.g., malformed), in which case the N utilized is viable litters. d: [(Total Implants – Live) / Total Implants] x 100. e: Single fetus with multiple malformations in each group. f: M = Malformation, V = Variation (Score 1 = within normal limits, 2 = slight, 3 = moderate, 4 = severe) *: Significantly different from Vehicle, $p \leq 0.05$

Table 9. Embryo-fetal development of DSPC in rabbits.

Dose (mg/kg/day)	Vehicle (Corn oil)	1	25	50
No. of animals	23	23	23	23
Food Consumption (g, mean ± SD, Gestation Day 29)	281 ± 80	302 ± 54	349 ± 255	289 ± 62
Body Weight (g, mean ± SD, Gestation Day 29)	3552 ± 222	3620 ± 186	3600 ± 224	3593 ± 274
Body Weight Gain (g, mean ± SD, Gestation Day 29)	20 ± 48	56 ± 30	58 ± 71	16 ± 103
Uterus Weight (g, mean ± SD, Gestation Day 29)	483 ± 88	521 ± 97	499 ± 84	489 ± 79
Litter Viability				
Initial Group Size (sperm-positive)	23	23	23	23
Not Pregnant	1	2	0	1
Gravid: pregnant N (% Pregnant)	22 (96)	21 (91)	23 (100)	22 (96)
Non-viable (no live/aborted)[a]	1	0	1	1
Viable Litters (≥ 1 live)[b]	21	21	22	21
Pre-implantation Loss[c] (%, mean[d]±SD)	11.8 ± 15.6	8.4 ± 11.5	5.3 ± 7.1	5.4 ± 7.9
Post-implantation Loss[c] (%, mean ± SD)	3.1 ± 6.4	3.7 ± 9.6	3.8 ± 16.0	3.3 ± 5.4
Corpora Lutea (/animal, mean ± SD)	10.0 ± 1.4	10.9 ± 2.2	10.0 ± 1.7	9.5 ± 1.4
Total Implants (/animal, mean ± SD)	8.9 ± 2.1	10.0 ± 2.6	9.5 ± 1.9	9.0 ± 1.5
Total Resorptions (/animal, mean ± SD)	0.1 ± 0.4	0.2 ± 0.7	0.1 ± 0.3	0.2 ± 0.4
Total Deaths (/animal, mean ± SD) (Sum)	0.1 ± 0.3 (2)	0.1 ± 0.3 (2)	0.4 ± 1.7 (8)	0.1 ± 0.3 (2)
Total Non-live Resorption & Death (/animal, mean ± SD) (Sum)	0.2 ± 0.4 (5)	0.3 ± 0.7 (7)	0.5 ± 1.9 (10)	0.3 ± 0.5 (6)
Live Fetuses/Total Implants %	97.3	96.7	95.2	96.8
Total Resorptions/Total Implants %	1.6	2.4	1.0	2.1
Total Non-Live/Total Implants %	2.7	3.3	4.8	3.2
Malformed Fetuses/Live Implants %	0.5	0.5	1.0	1.1
Litters with (% Litter with)				
Resorptions	3 (14)	3 (14)	2 (9)	4 (19)
Deaths	2 (10)	2 (10)	1 (5)	2 (10)
Non-Live	5 (24)	5 (24)	2 (9)	6 (29)
Malformed	1 (5)	1 (5)	2 (9)	2 (10)
Parameter				
Litter Weight (g, male+female, mean ± SD)	40.88 ± 6.57	39.35 ± 5.27	39.30 ± 4.21	40.63 ± 6.33
Number of Males (/animal, mean ± SD)	3.9 ± 1.6	4.5 ± 1.5	4.2 ± 1.8	4.1 ± 1.6
Number of Females (/animal, mean ± SD)	4.8 ± 2.0	5.1 ± 2.2	4.8 ± 1.8	4.6 ± 1.7
Male: Female Ratio	0.8:1	0.9:1	0.9:1	0.9:1
Gross External Morphology				
Number Examined (Number of Normal) Fetal/Litter	181/21 (180/21)	203/21 (202/21)	198/22 (197/22)	183/21 (183/21)
Distended Abdomen (M)[f]	1/1	0/0	1/1	0/0
Clubbed Feet (M)	0/0	1/1	0/0	0/0
Late Death (COXL)[g,h]	1/1	1/1	3/1	1/1
Visceral Observation				
Number Examined (Number of Normal) Fetal/Litter	181/21 (173/21)	203/21 (191/21)	198/22 (178/22)	183/21 (168/21)
Hydronephrosis (V)	4/4	10/6	14/5	7/4
Liver, ectopic lobe (V)	1/1	1/1	1/1	1/1
Heart Vessels/Common Truncus with abdominal fluid (M)	0/0	0/0	1/1	0/0
Heart Vessels/Pulmonary constricted (V)	1/1	0/0	0/0	0/0
Heart Vessels/Pulmonary dilated (V)	0/0	0/0	1/1	3/2
Heart Semi-Lunar Valve, small (V)	0/0	0/0	1/1	0/0
Herniated Diaphragm with Hypoplastic/Absent lungs (M)	0/0	0/0	1/1	0/0
Gallbladder, small (V)	0/0	0/0	1/1	0/0
Gallbladder, absent (M)	0/0	1/1	0/0	2/2
Spleen and gallbladder absent (M)	0/0	0/0	0/0	1/1
Stomach, enlarged (V)	0/0	0/0	0/0	1/1
Adhesions, intestines (M)	1/1	0/0	0/0	0/0
Cephalic Observations				
Number Examined (Number of Normal) Fetal/Litter	58/21 (57/21)	Not Examined		58/21 (58/21)
Hypoplasia (left ventricle and brain stem, V)	1/1			0/0
Skeletal Examination				
Number Examined (Number of Normal) Fetal/Litter	181/21(155/21)			183/21 (127/21)
Skull Incomplete Ossification (V)[f]: Score 1	0/0			4/3
Sternebrae Incomplete Ossification (V): Score 1	25/8			49/13
Score 2	0/0	Not Examined		2/2
Score 4	0/0			1/1
Ribs Rudimentary/Reduced/Unilateral 13/14 th (V)	74/21			44/18
Cervical Centra/Vertebrae Misaligned (M)	0/0			1/1
Pelvic Girdle Pubis Incomplete Ossification (V): Score 2	1/1			1/1
No Observed Adverse Effect Level	50 mg/kg/day (maternal and developmental toxicity of DSPC)			

a: Nonviable litters refers to the number of dams that had no live implants, but were pregnant on gestation day 29. b: Viable litters refer to the number of dams with at least 1 live implant on gestation day 29. c: [(Total Corpora Lutea – total Implants) / Total Corpora Lutea] x 100. When the number of implants is greater than the number of corpora lutea, a zero was used to indicate 0% pre-implantation loss. d: The mean reported utilizes the litter as the unit or observation ± standard deviation (SD); the N used is the number gravid unless the parameter requires an intact fetus for evaluation (e.g., malformed), in which case the N utilized is viable litters. e: [(Total Implants – Live) / Total Implants] x 100. f: M = Malformation, V = Variation (Score 1 = within normal limits, 2 = slight, 3 = moderate, 4 = severe) g: COXL = Common Observation for Late death fetus of macerated which is consistent with in utero death; diagnosed as non-live at C-section and classified as a late death**.** h: Excluded from live examined

Table 10. Pre- and postnatal development, including maternal function of DSPC in rats.

Dose (mg/kg/day)	Vehicle (Corn oil)	1	25	50
No. of sperm positive dams (F_0)	24	24	24	24
Found Dead	0	1	0	1
Terminal Sacrifice[a]	0	1	1	1
Clinical Observations		No treatment-related effect		
Food Consumption (g, mean ± SD, Gestation Day 20)	57 ± 7	50 ± 9*	53 ± 6	52 ± 7
Lactation Day 21	578 ± 64	643 ± 130*	552 ± 90	559 ± 45
Body Weights (g, mean ± SD, Gestation Day 20)	369 ± 30	349 ± 20	355 ± 28	358 ± 30
Lactation Day 21	326 ± 24	317 ± 18	320 ± 18	319 ± 22
No. of successful deliveries (%)	24 (100)	22 (92)	23 (96)	22 (92)
F_0 Length of gestation (days, mean ± SD)	22 ± 0.6	22 ± 0.6	21 ± 0.5	22 ± 0.5
Total Born (/animal, mean ± SD)	10.8 ± 2.6	9.3 ± 3.0	12.3 ± 2.8	12.0 ± 1.9
No. of pups born (F_1)	215	158	212	235
Number alive on day 0	206	136	198	214
Number alive on day 4 (%)	202 (98.1)	136 (100.0)	192 (97.0)	205 (95.8)
Total surviving day 4-21 (%)	152 (100)	104 (98)	136 (99)	145 (99)
Male born: Female born ratio	1.1:1	0.9:1	1:1	1:1
Age at preputial separation (days, mean ± SD)	42.1 ± 1.6	42.6 ± 2.3	42.8 ± 1.4	41.9 ± 2.1
Age at vaginal patency (days, mean ± SD)	32.8 ± 2.8	33.1 ± 2.5	32.5 ± 2.1	33.0 ± 2.2
Body weight at vaginal patency (g, mean ± SD)	125 ± 19	124 ± 13	121 ± 15	125 ± 13
Organ Weight, Ovaries (g, mean ± SD)	0.149 ± 0.031	0.157 ± 0.028	0.151 ± 0.020	0.147 ± 0.029
F_1 Generation				
Total Born (male+female, mean ± SD)	11.3 ± 1.8	9.9 ± 2.9	11.8 ± 2.9	12.4 ± 1.5
Alive Day 21 [b] (male+female, mean ± SD)	8.0 ± 0.0	6.5 ± 2.7*	7.6 ± 1.1	7.6 ± 1.0
Litter body weights (Preweaning),				
Day 21 (male+female, mean ± SD)	58.9 ± 4.5	56.9 ± 6.0	56.4 ± 4.7	60.0 ± 5.4
Mean Food Consumption F_1 Males (post Weaning Selected Male Offspring)				
Day 49 (g, mean ± SD)	222 ± 23	210 ± 19	211 ± 20	222 ± 21
Mean Food Consumption F_1 females (post Weaning Selected Male Offspring)				
Day 49 (g, mean ± SD)	173 ± 22	169 ± 17	169 ± 19	168 ± 20
Mean Food Consumption F_1 females (Gestation Day: GD)				
GD 20 (g, mean ± SD)	58 ± 8	55 ± 8	57 ± 8	50 ± 14
Mean Food Consumption F_1 females (Lactation Day: LD)				
LD 21 (g, mean ± SD)	601 ± 104	528 ± 116	538 ± 134	572 ± 116
Mean Body Weights (Selected Offspring, Day 0=Equivalent to postnatal day 28)				
F_1 Males: Day 93 (g, mean ± SD)	673 ± 81	627 ± 52	619 ± 48*	656 ± 76
F_1 Females: Day 49 (g, mean ± SD)	278 ± 27	276 ± 20	272 ± 22	266 ± 25
Mean Body Weights (F_1 Females, g, mean ± SD)				
Gestation Day 20	424 ± 36	415 ± 26	413 ± 32	396 ± 44
Lactation Day 21	349 ± 17	346 ± 22	345 ± 26	336 ± 25
F_1 Sensory Function & Reflex Response, water Maze		No treatment-related effect		
Organ Weights F_1 Males (g, mean ± SD, n = 20):				
Testes	3.89 ± 0.58	3.86 ± 0.24	3.43 ± 0.58*	3.96 ± 0.38
Epididymides	1.36 ± 0.13	1.40 ± 0.08	1.31 ± 0.21	1.39 ± 0.12
Prostate	0.884 ± 0.219	0.810 ± 0.258	0.827 ± 0.272	0.861 ± 0.415
Seminal Vesicle	2.369 ± 0.470	2.308 ± 0.392	2.249 ± 0.384	2.350 ± 0.289
Organ Weights F_1 Females (g, mean ± SD, n = 20): Ovaries	0145 ± 0.035	0.139 ± 0.018	0.144 ± 0.025	0.142 ± 0.018
F_1 Dams Gross necropsy Observations				
Number Examined	20	20	20	20
Mating Pairs	20	20	20	19
Did not deliver/no implants	2	1	0	1
No Gross Lesions	19	20	18	19
Actual Group Size (gravid)	18	19	20	18
Number of successful deliveries (%)	18 (90)	19 (95)	20 (100)	18 (95)
Average length of Cohabitation (mean ± SD)	4.3 ± 6.2	2.2 ± 1.2	2.4 ± 1.1	2.2 ± 1.0
Length of gestation (days, mean ± SD)	21.9 ± 0.3	21.8 ± 0.4	22.0 ± 0.5	21.9 ± 0.5
Number of implantation sites (mean ± SD)	15.4 ± 1.7	15.4 ± 1.7	14.9 ± 2.3	14.5 ± 1.9
Post-implantation loss[c] (mean ± SD)	0.7 ± 0.8	0.9 ± 1.3	0.7 ± 0.8	0.8 ± 1.4
Estrus frequency (mean ± SD)	3.0 ± 0.5	2.7 ± 0.8	2.4 ± 0.5	2.4 ± 0.7
Number of pups born	265	276	284	247
Number of alive on day 0 (%)	262 (98.9)	269 (97.5)	275 (96.8)	236 (95.5)
Number alive on day 4 (%)	255 (97.3)	257 (95.5)	266 (96.7)	230 (97.5)
Number alive post culling	144	149	152	136
Total surviving day 4-21 (%)	144 (100)	146 (98)	151 (99)	136 (100)
Number of males born: females born ratio	1:1	1:0.9	0.9:1	1:1
Age at eye opening/litter (days, mean ± SD)	14.0 ± 0.5	14.1 ± 0.7	13.8 ± 0.5	13.9 ± 0.4
Total Born (mean ± SD, Combined Sexes,)	14.7 ± 1.9	14.5 ± 2.0	14.2 ± 2.4	13.7 ± 1.9
Alive: Day 21[d] (mean ± SD, Combined Sexes)	8.0 ± 0.0	7.7 ± 0.9	7.6 ± 1.8	7.6 ± 1.9
Litter Body Weights: Day 21, F_2 Generation Pre-Weaning (mean ± SD, Combined Sexes)	58.6 ± 4.4	55.1 ± 8.3	56.4 ± 6.8	58.7 ± 5.0
No Observed Adverse Effect Level	50 mg/kg/day (systemic toxicity & reproductive toxicity of F_0 generation, reproductive toxicity of F_1 generation, developmental toxicity)			

a: Animals did not undergo parturition and remained in gestation phase. b: Litters were culled on postnatal day 4. c: Implantation Sites - Total Born. d: Litters were culled on postnatal day 4. *: Significantly different from vehicle, $p \leq 0.05$.

tion of the respiratory and olfactory nasal epithelium was observed in the vehicle control, indicating its relationship with the inhalation of exogenous material (Nagano *et al.,* 1997; Harkema *et al.,* 2006; Monticello *et al.,* 1990; Renne *et al.,* 2009; Chan *et al.,* 1998; Katagiri *et al.,* 2000). This effect is thus considered to be adaptive and not to indicate any potential for toxicity. There were no note-worthy findings from either the 6-month MDI inhalation toxicity study in dogs, or in the 104-week MDI inhalation carcinogenicity study in rats, comparing vehicle to air control groups.

Based on the above inhalation toxicity studies results, there was no toxicological difference between the vehicle control group including DSPC and the air control group. There is no difference not only in the systemic toxicity but also the local effects (respiratory organ). This suggests that DSPC is safe to administer to humans.

A large dose of exogenous surfactant did not perturb lung PC metabolism to any detectable extent, and the clearance was rapid (no evidence for accumulation of material in the lungs). These results were consistent with no harmful effects from the instillation of large doses of surfactant into the lung (Pettenazzo *et al.,* 1988). DSPC was not genotoxic in a battery of genotoxicity studies. In the inhalation carcinogenicity studies in mice and rats, chronic administration of DSPC/$CaCl_2$ was not carcinogenic. In addition, we conducted reproductive toxicology studies by subcutaneous administration (DSPC, up to 50 mg/kg/day), which indicated no significant effects on fertility, embryofetal development or peri- and post-natal development. In conclusion, DSPC is considered to have been shown to be safe for use in pharmaceutical metered-dose inhalers.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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