Histamine Release Associated with Intravenous Delivery of a Fluorocarbon-Based Sevoflurane Emulsion in Canines

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ABSTRACT: The purpose of this study was to evaluate the effectiveness of a novel fluorocarbon-based sevoflurane emulsion in dogs previously shown to produce short-term rodent anesthesia. On the basis of an unexpected allergic-type clinical reaction, we also tested the hypothesis that this type of formulation causes histamine release and complement activation. Physiological parameters, plasma histamine levels (radioimmunoassay), and complement activation (enzyme immunoassay) were quantified in response to emulsion components, including F13M5 (the emulsion's fluorocarbon-based polymer) and methoxy poly(ethylene glycol) 5000 (the polymer's hydrophilic block). Although the emulsion produced general anesthesia in dogs, they also experienced hypotension and clinical signs suggestive of an allergic-like response (i.e., vasodilation, urticaria, and pruritus upon recovery). Emulsions lacking sevoflurane failed to induce anesthesia but did elicit the allergic response. Plasma histamine levels were significantly increased following injection of micellar solutions of F13M5. Direct complement activation by the emulsion or its components was weak or absent. An allergic response leading to histamine release, likely initiated by the F13M5 component via an immunoglobulin pathway, is associated with an intravenous fluorocarbon-based emulsion of sevoflurane. Subsequently, its usefulness in medicine in its present formulation is limited. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:2685–2692, 2011

Keywords: anesthesia; emulsion; sevoflurane; complement; hypersensitivity; physicochemical properties; micelle; polymers; drug design

INTRODUCTION

General anesthesia induced by volatile inhalants (e.g., sevoflurane, isoflurane, and desflurane) and delivered via precision anesthetic vaporizers offers many distinct advantages over intravenously (i.v.) administered drugs, including the elimination of the inhalant mainly via the patient's lungs rather than metabolism and excretion. However, when anesthetic drugs are delivered by inhalation, there is a significant delay in the onset of anesthesia, as the concentrations in the anesthetic breathing system, lung (alveoli), blood, and brain rise slowly over time. To avoid this delay in anesthetic onset and action, i.v. delivered halogenated volatile anesthetics have been developed. Injection of pure liquid forms of halogenated anesthetics causes significant pulmonary damage and death in animals and humans.[1–3] In contrast, lipid emulsions based on Intralipid (a phospholipid-stabilized soybean oil emulsion, Baxter, Deerfield, IL) have been found to be safe and effective for the delivery of isoflurane and sevoflurane,[4–6] and may be beneficial in the treatment of local anesthetic toxicity.[7,8] Because fluorinated volatile anesthetics do not mix well with classical lipids,[9] the maximum concentration of sevoflurane in Intralipid emulsions is only 3.5%,[6] a level that is inadequate for the efficient induction and maintenance of general anesthesia.

Abbreviations used: CARPA, complement activation-related pseudoallergy; EIA, enzyme immunoassay; PEG, polyethylene glycol; RIA, radioimmunoassay; SAP, systolic arterial pressure; MAP, mean arterial pressure; DAP, diastolic arterial pressure; HR, heart rate; SpO_2, percent saturation of oxygen.

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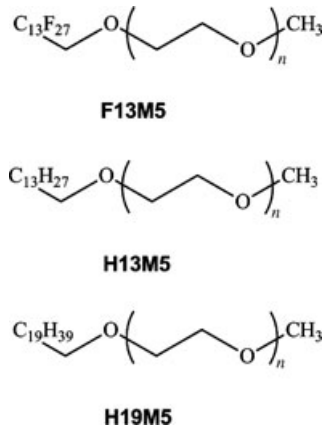

Figure 1. The structure of the flouropolymer used in the emulsion, F13M5. The nomenclature stands for a 13-chain fluorocarbon (F13) attached to a methoxy poly(ethylene glycol) of average molecular weight 5000 (M5) ($n\sim113$). Shown below are two similar hydrocarbon polymers, H13M5 (hydrocarbon-based structural analogue of F13M5) and H19M5 (equivalent critical micelle concentration to F13M5).

To increase the amount of sevoflurane that can be delivered IV, novel emulsions using semifluorinated surfactants have been developed and used successfully to produce sevoflurane anesthesia.[10] The fluoropolymer used in this emulsion, F13M5 (Fig. 1), surrounds nanodroplets composed of sevoflurane and the stabilizing agent perfluoroctyl bromide. This nanoemulsion, which is stable in solution but releases rapidly upon IV administration, can contain up to 30% of sevoflurane. This represents a powerful technology for inducing general anesthesia by IV administration of an "inhaled agent."

Although previous experiments conducted in rats demonstrated that this novel formulation is effective, extremely fast, and safe,[10] cardiorespiratory effects were not measured, and efficacy and safety were tested only in this one species. Therefore, we sought to test whether the i.v. fluoropolymer-based sevoflurane emulsion would produce dose-dependent general anesthesia, and if so, to measure the associated cardiovascular effects in dogs. However, an unexpected allergic-type reaction was observed in all dogs during these initial studies. Therefore, we further test whether injection of the fluoropolymer-based sevoflurane emulsion and its constituents was associated with histamine release and/or complement activation.

Experimental

Sevoflurane (20%, v/v) Emulsion Preparation

Two hundred ninety-eight milligrams of F13M5 [molecular weight (MW) = 5668 g/mol] was dissolved in 11.9 mL of normal saline. Perfluorooctyl bromide [PFOB (1.7 mL)] and sevoflurane (3.4 mL) were added. PFOB was supplied by SynQuest Laboratories, Inc. (Alachua, Florida), and sevoflurane was manufactured by Abbott Labs (Chicago, Illinois). This mixture was subjected to high-speed homogenization (Power Gen 500; Fisher Scientific, Hampton, New Hampshire) at 22,500 rpm for 1 min. A microfluidizer (model 110 S; Microfluidics Corp., Newton, Massachusetts) was used to make the final emulsion at 5000 psi for 1 min. Larger particles were eliminated with a 0.45-micrometer nylon syringe filter (Microliter Analytical Supplies, Inc., Suwanee, Georgia).

An emulsion without sevoflurane was also made. This solution contained all of the components of the original emulsion except sevoflurane, which was replaced with additional saline. The steps to create the emulsion were the same as above. On the day of injection, the emulsions were sized by dynamic light scattering.

Animals

All studies were approved by the University of Wisconsin Animal Care and Use Committee. Three healthy, adult male Beagles (Ridglan Laboratories, Mt. Horeb, WI) were fasted overnight before each study period.

Pilot Studies

In pilot studies directed at determining an effective dose, cephalic i.v. catheters were placed for drug delivery. Three doses of the fluoropolymer-based 20% sevoflurane emulsion were tested: 0.041, 0.123, and 0.41 mL/kg, based on previously published rat studies. Dog 1 received all three doses in ascending order. On the basis of the observations from Dog 1, Dog 2 received only the 0.041 and 0.41 mL/kg doses, and Dog 3 only received the 0.41 mL/kg dose. Injections within each dog were administered in a single session and were separated by 10–15 min when appropriate. Dogs were observed for sedation or loss of reflexes (e.g., righting, pedal, swallow, and corneal reflexes) and the unconsciousness indicative of general anesthesia, and for any unexpected side effects.

Part I: Cardiovascular Measurements

Ten days after the pilot studies, dogs were instrumented to investigate the cardiovascular effects of the fluoropolymer-based sevoflurane emulsion. Topical 4% lidocaine cream was placed over the cranial tibial artery for 20 min to facilitate catheter placement. Cephalic i.v. catheters were placed for drug delivery. Dogs were instrumented with electrocardiography (ECG) to monitor heart rhythm, pulse oximetry to monitor heart rate (HR), and hemoglobin saturation (SpO_2), and the cranial tibial arterial line was connected to a transducer to measure systolic (SAP), diastolic (DAP), and mean (MAP) arterial blood

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pressures (Cardiocap 5; Datex-Ohmeda, Madison, Wisconsin). Awake baseline measurements of HR, arterial blood pressures, and SpO_2 were made for 5 min. Dogs 1 and 3 then received the injectable sevoflurane emulsion at 0.41 mL/kg. All physiologic parameters were monitored every 10 s throughout the injection, during general anesthesia, and for 30 min into the recovery period (S/5™ Collect; Datex-Ohmeda–GE Healthcare, Helsinki, Finland). Because the first two dogs exhibited profound hypotension with clinical signs of hypersensitivity reactions at 0.41 mL/kg (see *Results* section), further doses were not tested and Dog 2 was not administered the fluoropolymer-based sevoflurane emulsion.

Following a second 10-day washout period, dogs were instrumented and monitored as above. Dog 1 was subsequently injected with an emulsion that did not contain sevoflurane (0.41 mL/kg; 10%, v/v, PFOB and 25 mg/mL F13M5). Dogs 2 and 3 received F13M5 alone (25 mg/mL) in 0.9% saline.

Part II: Component Analysis

On the basis of results obtained in Part I, the same three dogs were used to investigate the effects of individual chemical components of the emulsion. A cephalic venous catheter was again placed for drug delivery and blood sampling. Dogs were instrumented with ECG and pulse oximetry to measure HR, rhythm, and SpO_2, and an oscillometric blood pressure monitor was placed on the front limb above the carpus with a cuff approximately 40% of the limb diameter (Cardiocap 5; Datex-Ohmeda). Dogs received in random order 0.41 mL/kg of (1) 0.9% saline (negative control); (2) 2.64 mM methoxy poly(ethylene glycol) (mPEG; MW = 5000 g/mol) in saline; (3) 2.64 mM F13M5, the fluorocarbon polymer used in the emulsion; (4) 2.64 mM H13M5, a hydrocarbon-based structural analog of F13M5; and (5) 2.64 mM H19M5, a hydrocarbon-based polymer with an equivalent critical micelle concentration to F13M5. Injections of Cremophor EL (25 mg/mL, BASF Corp., Florham Park, NJ) were used as a positive control. Each dog received each treatment with at least an intervening 4-day washout period. HR, respiratory rate, SpO_2, SAP, DAP, and MAP were recorded 5 min before the injection (baseline) and every minute thereafter for 30 min. At that time, treatment for hypersensitivity reactions was administered to dogs when necessary (diphenhydramine 0.5 mg/kg i.v., dexamethasone SP 0.025 mg/kg i.v.). Blood samples were taken for the analysis of histamine levels during the baseline period and 10–15 min following injection.

Histamine Analysis

Five milliliters of whole blood was taken from each dog just prior to and 10–15 min following i.v. administration of 0.41 mL/kg of (1) 0.9% saline (negative control), (2) mPEG in saline, (3) F13M5, (4) H13M5, (5) H19M5, and (6) Cremophor EL (positive control). Samples were placed in tubes containing 50 μg/mL of lepirudin and 10 mM EDTA, and cold centrifuged at 2053 g for 15 min. Plasma was removed for subsequent histamine analysis by a commercial laboratory (Antech Diagnostics GLP, Morrisville, North Carolina) via radioimmunoassay (Immunotech Laboratories, Glendale, California).

Complement Analysis

Pooled complement-preserved human serum (Innovative Research, Novi, Michigan) was combined with components of the emulsion to test for complement activation *in vitro*. Complement activation was analyzed by generation of C3a and C5a via enzyme immunoassay (EIA) kits (Quidel, Santa Clara, California). Serum was pooled from 15 donors to account for possible differences in complement protein concentrations and reactivities. The emulsion (with and without sevoflurane), F13M5 (2.64 mM), and mPEG 5000 (Sigma–Aldrich, St Louis, Missouri) (2.64 mM) were tested for their ability to activate complement. Each substance was tested in duplicate wells and in multiple EIAs. Dextran sulfate (0.1 mg/mL, MW >500,000) (Sigma–Aldrich) was used as a positive control for complement activation and 0.9% saline was the negative control. One hundred microlitres of each reagent was added to 100 μL of serum and heated in 37°C water bath. Incubation times were 15 min for C3a and 15–30 min for C5a. Each combination was transferred to ice and subsequently diluted in ice-cold, EIA-kit-specific buffer to stop any remaining complement activity. Generation of C3a and C5a products were then analyzed according to manufacturer's protocol.

The emulsions used for complement analysis were sized by a NICOMP 380 ZLS Particle Sizer (Particle Sizing Systems, Santa Barbara, California) and the micellar solutions of F13M5 were sized by a Zetasizer Nano ZS (Malvern Instruments, Westborough, Massachusetts). Complement studies were completed within 7 days of micellar solution preparation.

Statistical Analysis

In Part I, HR, SpO_2, SAP, DAP, and MAP were averaged over 5 min bins and reported with their respective standard errors for each dog following the fluoropolymer-based sevoflurane emulsion injection in the figures. In Part II, histamine levels are reported as means ± SE. A two-way analysis of variance with repeated measures using time point (before or after treatment) and treatment (saline, mPEG, F13M5, H13M5, H19M5, and Cremophor EL) as factors was used to determine differences between groups with a Student–Newman–Keuls post-hoc test. A p value

of less than 0.05 was considered significant. Complement activation was also analyzed in Part II. Generation of complement split products are reported as a fold difference in concentration of C3a or C5a compared with the negative control (saline). Ninety five percent confidence limits using the Student's *t* distribution are shown in Figure 2 as the error bars for each test substance.

RESULTS

Emulsion Sizing

For the pilot and cardiovascular studies, the 20% sevoflurane emulsion was found to be 191.0 ± 29.2 nm; the emulsion without sevoflurane was 195.9 ± 41.3 nm. The emulsions used for the complement analyses with sevoflurane ranged in size from 158.9 ±

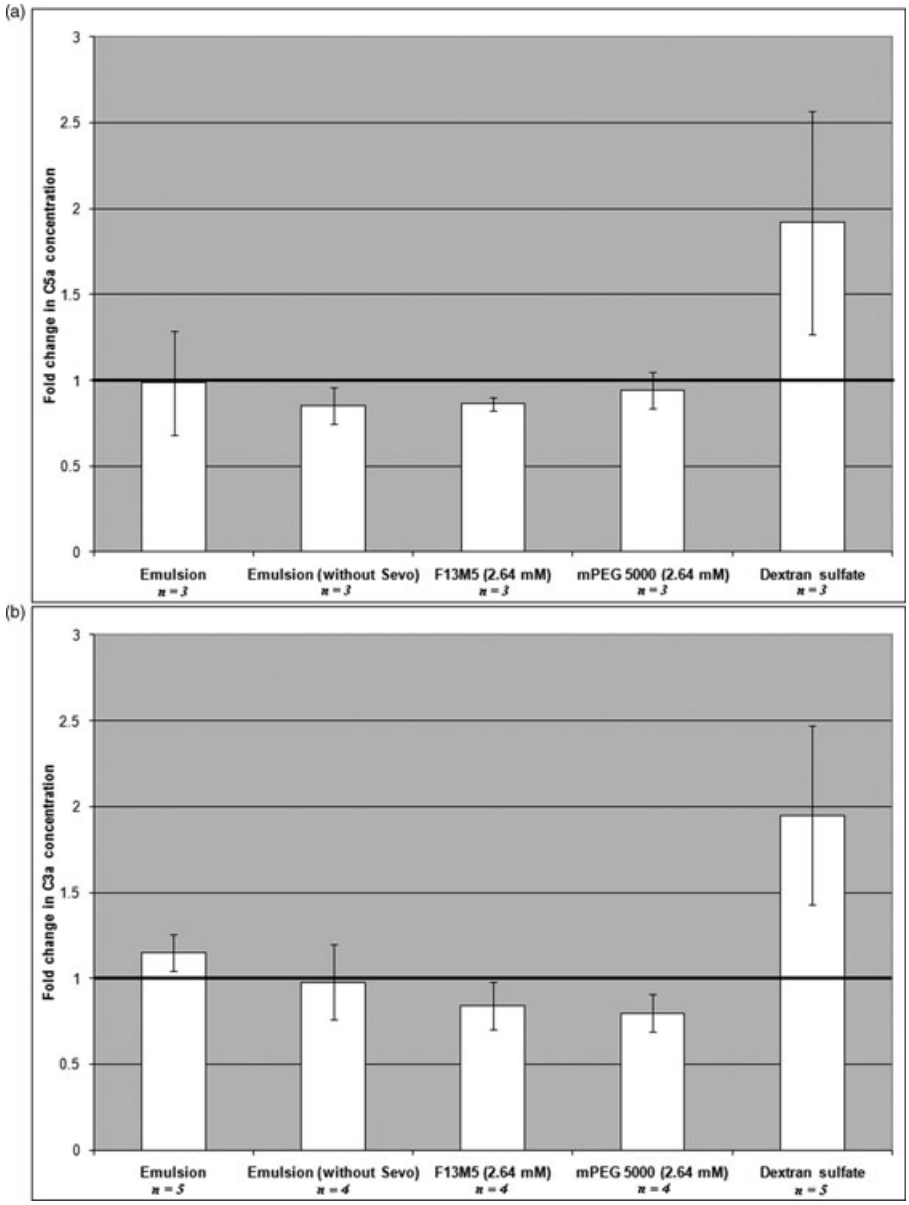

Figure 2. Enzyme immunoassay (EIAs) tested the generation of complement split products C5a (a) and C3a (b). Mean fold difference was used to compare the emulsion and its separate components to controls. The bold line at "1" represents the baseline, when saline is added to serum. Dextran sulfate (0.1 mg/mL) is a potent activator of complement and the positive control. "*n*" designates the number of EIAs used in the average of each component. Each EIA tested the components in duplicate. The error bars are displayed as 95% confidence intervals based on the Student's *t* distribution. Only dextran sulfate shows strong complement activation. It should be noted that the values for dextran sulfate sometimes fell out of the range of the standard curve generated in the EIA. Therefore, the values for dextran sulfate are extrapolated.

48.4 to 325.7 ± 35.4 nm; the emulsions without sevoflurane ranged from 246.9 ± 96.2 to 311 ± 86.0 nm. All emulsions were sized within 1 day of complement analysis and used within 11 days of preparation. The micellar solutions of F13M5 used in complement analysis contained particles of approximately 19 nm.

Pilot Studies

Three i.v. doses of the fluoropolymer-based 20% sevoflurane emulsion were used to determine efficacy [0.041, 0.123, and 0.41 mL/kg, delivered in ascending doses to each dog were appropriate (see *Methods* section)]. Only the highest volume produced general anesthesia with unconsciousness, areflexia, and no purposeful movement in the first two dogs and was subsequently administered to the third dog (data not shown). Thus, this dose was used in Part I and Part II of these investigations. At 0.41 mL/kg, dogs had subjectively weak femoral pulses (based on palpation) and hyperemic, vasodilated mucous membranes and conjunctiva (although these clinical signs were not subjectively as profound as those seen in subsequent studies; see *Part I: Cardiovascular Effects* section). However, cardiovascular parameters were not quantified during these studies.

Part I: Cardiovascular Effects

To assess the cardiovascular effects of the sevoflurane emulsion, blood pressure by arterial catheter, ECG, and pulse oximetry were monitored, and sevoflurane was injected IV. Injection of 0.41 mL/kg sevoflurane emulsion in the first two study dogs did not measurably alter SpO_2 or HR from baseline levels (data not shown). However, within 10 min following i.v. sevoflurane administration, SAP, DAP, and MAP had decreased substantially from average baseline values of 121, 80, and 97 mmHg, respectively, to minimum values of 36, 33, and 34 mmHg, respectively (Figs. 3a–3c). In Dog 1, the MAP remained below the levels defined as hypotensive (<60 mmHg) for 20 min, whereas Dog 2 remained hypotensive until the study was terminated (30 min; Fig. 3c). HR changes were variable (Fig. 3d). Both dogs exhibited signs of hypersensitivity, including erythema of the skin, urticaria, vasodilation of the capillary beds in the conjunctiva and pinna, and pruritus upon anesthetic recovery. Because the hypotension and associated physical examination findings were so profound (i.e., MAP of 23 mmHg in one dog), Part I was discontinued using the sevoflurane emulsion.

Because this allergic-type response was seen when dogs received the full sevoflurane emulsion, Dog 1 was subsequently administered emulsion without sevoflurane to evaluate the effects of the sevoflurane itself versus the other components of the emulsion, and Dogs 2 and 3 received F13M5 in saline alone. As expected, none of these three dogs showed signs of anesthesia (because no sevoflurane was injected), but all three experienced extreme pruritus and it was exceedingly difficult to maintain proper instrumentation and data collection. Therefore, specific quantification of data during these injections is not reported. These dogs were quickly treated with diphenhydramine, as described in *Methods* section, with rapid resolution of symptoms.

Part II: Component Analysis

Dogs were injected separately with F13M5, mPEG 5000, H13M5, and H19M5 (two similar hydrocarbon-based polymers, Fig. 1), and a positive control, Cremophor EL. To correlate possible histamine release with clinical signs, plasma histamine levels were measured before and after IV administration of these components as described in *Methods* section. Preinjection histamine levels were not significantly different between treatment groups (all $p > 0.05$, Fig. 4). However, histamine levels were significantly increased from baseline levels following injection of Cremophor EL and F13M5 (all $p < 0.05$, Fig. 4).

Complement Analysis

During Part II, serum was not collected from the dogs to check for complement activity *ex vivo*. Still, complement activation by a component of the emulsion could have played a role in the mechanisms underlying the hypersensitivity response. To check for this possibility, human serum was mixed with components of the emulsion *in vitro* and analyzed for C3a and C5a via EIA. Human serum was used instead of canine serum because no suitable antibodies for canine complement split products were available. C3a and C5a were monitored because these two split products anaphylatoxins would be the complement proteins most likely to lead to mast cell degeneration and a pseudoallergic response. Neither the emulsion, nor its constituents, activated complement to any substantial extent (Fig. 2). Rather, F13M5, mPEG 5000, or emulsion without sevoflurane led to a smaller generation of complement split products than saline alone. The emulsion itself led to a statistically significant, but nevertheless slight, increase in C3a. Only the positive control, dextran sulfate, showed a strong change in C3a and C5a generation compared with saline.

DISCUSSION

Intravenous volatile anesthetics have the potential to considerably change the way general anesthesia is performed in human and veterinary medicine because they allow for rapid alterations in anesthetic planes without prolonged hemodynamic changes as the inhalants are eliminated rapidly from the body through the lungs. In addition, more rapid titration of drug levels via i.v. delivery of inhalant agents would

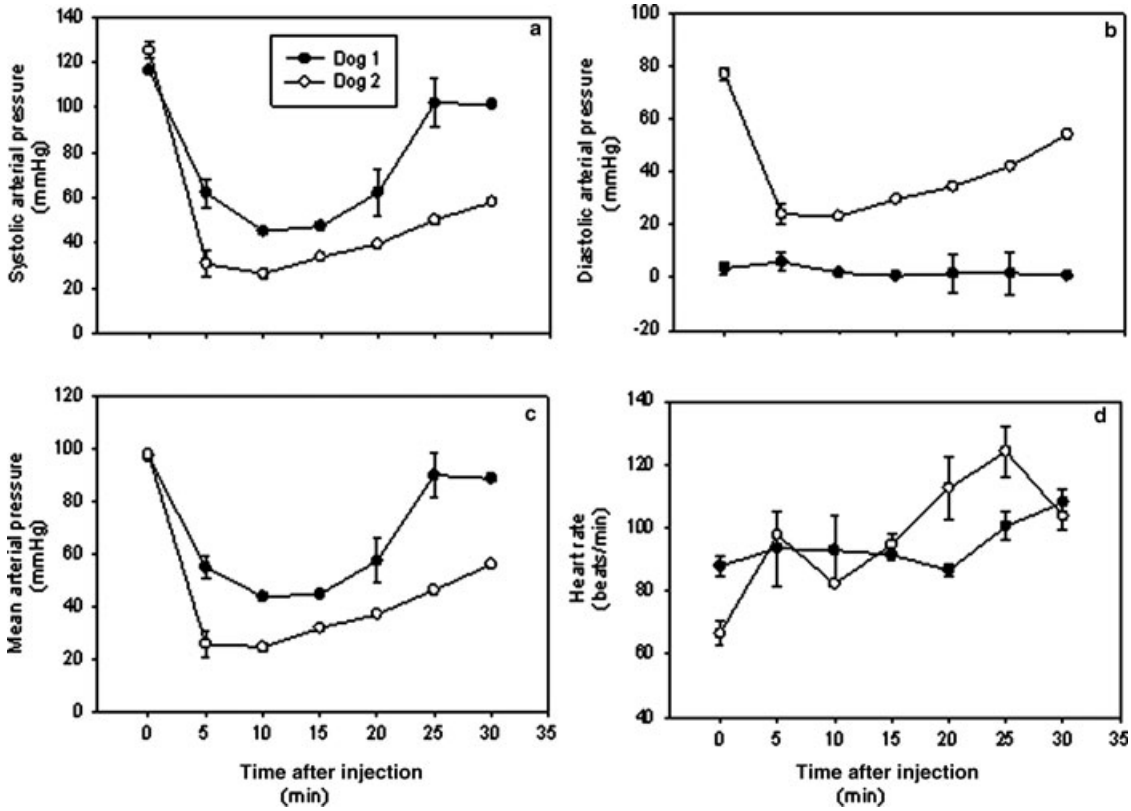

Figure 3. Individual arterial blood pressure and heart rate values from Dog 1 and Dog 2 before and after intravenous administration of the novel sevoflurane emulsion. The emulsion was injected at time 0. Systolic, diastolic, and mean arterial pressures were markedly decreased from baseline values following injection for at least 20 min. However, heart rate trends did not show a predictable pattern following injection in Dog 1 and Dog 2.

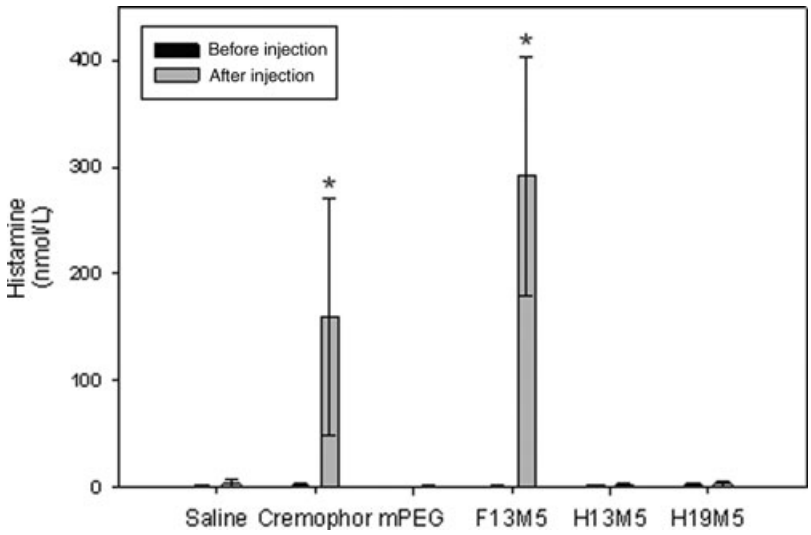

Figure 4. Plasma histamine levels at baseline and following intravenous administration of the emulsion components. There were no significantly different histamine levels during baseline (all $p > 0.05$). However, following injection of Cremophor EL (positive control) and F13M5, plasma histamine levels were significantly elevated from baseline and were significantly different from saline injection (*both $p < 0.05$). When not visible, error bars are within data set.

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allow for the use of volatile anesthetics where delivery via inhalation is difficult, such as in the MRI suite where magnetized metal is not feasible, in the field or at the farm for veterinary procedures, or in humane societies, or rural areas where the cost of anesthesia machines prohibits their use in veterinary patients.

We show here that a sevoflurane emulsion that was previously found to produce general anesthesia in rats, also induces general anesthesia in dogs. However, it also results in profound hypotension and histamine release. The cardiovascular effects of the emulsion were only tested in two dogs due to the profound hypotension seen. Although no statistical analysis could be performed on only two dogs, there was a clear, clinically significant decrease in blood pressure, with variable effects on HR. Indeed, a mean blood pressure of less than 60 mmHg in dogs is defined as hypotension, and the mean blood pressure fell from an average of 97 mmHg to a minimum of 34 mmHg 10 min following injection. Blood pressure is a complex cardiovascular parameter and is determined by the systemic vascular resistance and cardiac output. Cardiac output is determined by the product of HR and cardiac stroke volume. Because histamine release is associated with decreases in systemic vascular resistance and vasodilation, this is the most likely cause for the hypotension seen with emulsion injection. Although it does not appear that sevoflurane injection meaningfully altered HR, we cannot rule out hypotensive effects of the sevoflurane emulsion through direct effects on cardiac output via changes in stroke volume.

The utility of these emulsions in veterinary practice is predicated on elimination of any allergic-like responses associated with their use. Therefore, a clear understanding of the hypersensitivity seen in these dog studies is needed. We began investigations into the underlying mechanisms via injection of the individual components of the emulsion in order to determine which components were responsible for this hypersensitivity. The emulsion (with and without sevoflurane) and the fluorocarbon polymer F13M5 were all associated with a similar allergic-type response upon injection, and F13M5 injection was associated with histamine release, most likely from mast cell/basophil degranulation. Presumably, micelles composed of either H13M5 or H19M5 did not induce any immune response due to their quick dissociation in blood. We speculate that the ability of fluoropolymers to form micellar structures that can be stable in blood for up to several days (unpublished observations), rather than the characteristics of the monomers, is related to their immunogenicity.

Two possible mechanisms for this histamine release are complement activation-related pseudoallergy (CARPA) and classical Immunoglobulin E (IgE)-mediated hypersensitivity. CARPA is an IgE-independent mechanism of allergy that is associated with other PEGylated solvent systems and nanoparticles, including Cremophor EL and pluronics, which appear to activate complement and cause a pseudoallergic response.[11] Mild clinical signs (weak pulses, vasodilation of capillary beds) that may have been indicative of an allergic-type response were observed during our pilot studies directed at finding an effective dose to produce anesthesia in the three dogs. However, these effects were not as profound as those seen during subsequent studies, and they were not quantified because dogs were not instrumented for cardiovascular monitoring. These signs were observed upon first injection of the emulsion, without prior sensitization. Because of this, and the similarity of F13M5 to other particles associated with CARPA, direct complement activation was the initial focus of our studies.

However, Figure 2 suggests that, at least in the case of human serum, no direct complement activation by the emulsion or its components is apparent. This would tend to rule out CARPA as the cause for the hypersensitivity. Nevertheless, it is possible that differences in the complement proteins between humans and canines could explain the lack of effect seen in these EIAs. An additional consideration for this EIA is the pooled human serum itself. One of the 15 patients could have a high concentration of complement-inhibiting proteins, or some other variation, that would inhibit complement activation by the polymer in the serum. Variation in donor serum reactivity could mean that other serum samples could show complement activation, especially if activation by this emulsion is rare in humans.

An alternative explanation for the hypersensitivity reaction is that an antibody-mediated response occurred. This possibility is supported by the observation that the allergic-type response was enhanced upon repeat injection of the emulsion. In this scenario, an increase in antibody titer would have occurred as a result of antigen exposure each time the emulsion was injected. A traditional explanation for the observed immune response would be an IgE-mediated reaction. To our knowledge, IgE has yet to be linked to PEGylated particles. Nevertheless, this remains a possibility because IgE is the antibody involved in a typical type I immediate hypersensitivity.

Alternatively, IgM or IgG may have played a role in this hypersensitivity. Anti-PEG IgM and IgG have been discovered in instances of repeat administration of PEGylated particles to various animals.[12–14] This leads to faster clearance of the particle from the body, a process called "accelerated blood clearance phenomenon." Another group of studies showed that anti-PEG antibodies (IgM and IgG) were found in up to 25% of healthy blood donors.[15–18] Thus, it is possible that the dogs had endogenous anti-PEG

antibodies that reacted with F13M5 nanoparticles, potentially due to incidental exposure to these or cross-reactivity to similar polymer antigens at some time during the animals' lifetimes. If these dogs had endogenous IgM, a complement-mediated response could still have occurred through classical pathway activation that would not be detected by our experimental methods. Alternatively, endogenous IgG could have led to degranulation of basophils and release of platelet-activating factor.[19] Although it is unknown whether this pathway of anaphylaxis exists in dogs, this mechanism has been suspected in at least one case of allergy to PEGylated liposomes in mice.[14] Current studies in our laboratory are directed toward evaluating the possible roles of the different types of antibodies in the hypersensitivity seen in dogs exposed to the emulsion.

CONCLUSION

In conclusion, our data suggest that injectable sevoflurane emulsions have the potential to be useful in human and veterinary medicine because they do produce general anesthesia. However, our formulation was also associated with profound hypotension and histamine release in dogs, by a mechanism that likely involves immunoglobulin-mediated hypersensitivity. Understanding the basis of this allergic-type response requires further investigation.

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