Analysis of pulmonary surfactant by Fourier transform infrared spectroscopy after exposure to sevoflurane and isoflurane

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ABSTRACT

Pulmonary surfactant, consisting primarily of phospholipids and four surfactant-specific proteins, is among the first structures that is exposed to inhalation anesthetics. Consequently, changes of pulmonary surfactant due to this exposure could cause respiratory complications after long anesthetic procedures. Fourier transform infrared (FTIR) spectroscopy was used to explore the effects of two inhalation anesthetics, sevoflurane and isoflurane, on a commercially available pulmonary surfactant. The research was primarily focused on the effect of anesthetics on the lipid component of the surfactant. Four different concentrations of anesthetics were added, and the doses were higher from the low clinical doses typically used. Recorded spectra were analyzed using principal component analysis, and the Student's *t*-test was performed to confirm the results. The exposure to both anesthetics induced similar changes, consistent with the increase of the anesthetic concentration. The most pronounced effect was on the hydrophilic head group of phospholipids, which is in agreement with the disruption of the hydrogen bond, caused by the anesthetics. A change in the band intensities of CH_2 stretching vibrations, indicative of a disordering effect of anesthetics on the hydrophobic tails of phospholipids, was also observed. Changes induced by isoflurane appear to be more pronounced than those induced by sevoflurane. Furthermore, our results suggest that FTIR spectroscopy is a promising tool in studying anesthetic effects on pulmonary surfactant.

KEY WORDS: Pulmonary surfactant; isoflurane; sevoflurane; Fourier transform infrared; spectroscopy; principal component analysis
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INTRODUCTION

Isoflurane and sevoflurane are volatile anesthetics, widely used for the maintenance of general anesthesia. The mechanism and site of action of inhalational anesthetics have remained unknown despite years of research and numerous, lipids versus proteins, proposed theories. Their route of administration is by inhalation, making the lungs with a surface of up to 80 m^2 [1] a major site of exposure, and pulmonary surfactant one of the first structures to be exposed. Pulmonary surfactant is a complex mixture of several lipids, primarily phospholipids, and lesser amounts of triglycerides, cholesterol, and fatty acids. In addition, it contains four surfactant proteins labeled SP-A, SP-B, SP-C, and SP-D. Its main function is to reduce the surface tension, counteracting the tendency of alveoli to collapse at the end of expiration, thus facilitating gas transport [2]. Adequate surfactant production in the fetal lung is essential for the initiation of breathing at birth. In adults, its disfunction is found in a condition known as acute respiratory distress syndrome, which is caused by multiple factors that may include prolonged mechanical ventilation and anesthesia, leading to lung injury and surfactant deficiency.

Fourier transform infrared (FTIR) spectroscopy is a suitable tool in studying the molecular structure of lipids and their interaction with pharmacologically active substances. This technique is based on the fact that each molecule has a distinct pattern of absorption bands, which can be used as a fingerprint for identification of that particular molecule [3]. It also gives information about the concentrations of molecules in a mixture, making it a successful qualitative as well as semi-quantitative tool. To the best of our knowledge, studies on the effect of isoflurane and sevoflurane on pulmonary surfactant using FTIR spectroscopy have not been published yet.

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Studies on the effect of anesthetics on lipids were in most cases performed on model membranes, a structure that bears many similarities to pulmonary surfactant. The result were contradictory [4-10], and the research methods varied from nuclear magnetic resonance (NMR) [5,8,11-13], molecular dynamics simulations, [9,10] to vibrational spectroscopy [6,7,14]. In this work, we used FTIR spectroscopy to get a better insight into the interaction between isoflurane and sevoflurane and the lipid component of pulmonary surfactant.

MATERIALS AND METHODS

Curosurf® (poractant alpha) intratracheal suspension, a porcine pulmonary surfactant, is manufactured by Chiesi Farmaceutici and was obtained from Providens, Croatia. It was used as commercially supplied. Curosurf is a liposomal drug. The composition of Curosurf as provided in the manufacturer's brochure was as follows: Total phospholipid concentration 76 mg/ml (1,2-dipalmitoylphosphatidylcholine [DPPC] 30 mg/ml), SP-B 0.40 mg/ml, SP-C 5-11.6 μg protein/μmol L^{-1} phospholipid, while the excipients included sodium chloride, sodium bicarbonate, and water for injections. Organic solvent extraction and liquid-gel chromatography were performed (as per manufacturer's brochure). Curosurf also contained 11 mol% of neutral lipids, with 54 mol% of neutral lipids being free fatty acids, while cholesterol was not found [15].

Isoflurane (Forane; 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) and sevoflurane (Sevorane; 1,1,1,3,3,3-hexafluoro-2-(fluorometoxy)propane) are manufactured by Abbott and were obtained from Abbott Laboratories, Croatia. Both anesthetics were used as commercially supplied. Both of these anesthetics are polar substances.

The samples were prepared by placing Curosurf in plastic 2 ml sealable vials, and each anesthetic was added to create 10%, 20%, 30%, and 40% volume concentration of anesthetic/Curosurf mixtures, respectively, accounting for eight treated samples. One sample was left untreated for control. Samples were sealed and refrigerated at 4°C overnight, and then left at room temperature for another hour before recording the spectra.

FTIR spectroscopy

For spectroscopic measurements, 10 μl of each sample was placed on an optical grade silicon wafer and dried in a vacuum for 60 minutes to eliminate free water. This was performed to avoid free water bands overlapping the spectra of surfactant. FTIR spectra were recorded in transmission mode with the PerkinElmer Spectrum GX spectrometer (PerkinElmer, Waltham, Massachusetts, USA) equipped with a liquid N_2-refrigerated MCT detector (Mercury Cadmium Telluride). Recording was performed in the 4000-700 cm^{-1} region. Before scanning the samples, the background was recorded by averaging 200 scans at a resolution of 4 cm^{-1}. Each sample was recorded by averaging 100 scans at a resolution of 4 cm^{-1}. All spectra were obtained at room temperature. Thirty consecutive replicate measurements were recorded from a single sample for statistical relevance. Mean wavelength calculation was performed to obtain mean spectra for each of the nine groups of samples. All samples were treated in the same way and recorded under same conditions, separately.

FTIR data analysis

To determine the difference between recorded groups of spectra, we used principal component analysis (PCA). PCA was performed using the Matlab add-on PLS Toolbox (Eigenvector Research, Inc., Wenatchee, Washington, USA). Each wavenumber is a variable, and PCA statistical analysis is an unsupervised statistical method that enables the reduction of variables by building linear combination of wavenumbers that vary together [16]. The first principal component (PC_1) accounts for most variance present in the dataset, and the second principal component (PC_2) is built with the residual variance and is uncorrelated to the first one [17]. Mean center preprocessing was applied to all spectra before performing PCA.

Statistics

The Student's *t*-test was performed to gain more insight into the differences between the groups of spectra. Recorded spectra were processed with a specific software Kinetics (provided by Prof. E. Goormaghtigh), which runs under Matlab [18]. The recorded spectra were first baseline corrected and then normalized to the maximum intensity of the C=O ester band. To obtain the difference spectra, a mean spectrum of each treated sample was subtracted from the mean spectrum of untreated sample. The positive and negative peaks in the difference spectra indicate differences between the two groups of spectra. The difference spectra obtained by this method were analyzed using the Student's *t*-test, which was performed for each point in the spectrum to mark spectral regions where statistically significant differences ($p < 0.01$) were observed between two groups of samples. The wavenumbers for which a significant difference was observed were indicated by asterisks.

RESULTS

Analysis of spectra

The infrared spectrum of Curosurf (Figure 1) reveals the positions of the major infrared bands that are in agreement with the corresponding phospholipid bands reported in the literature [19]. The band assignments for the major functional

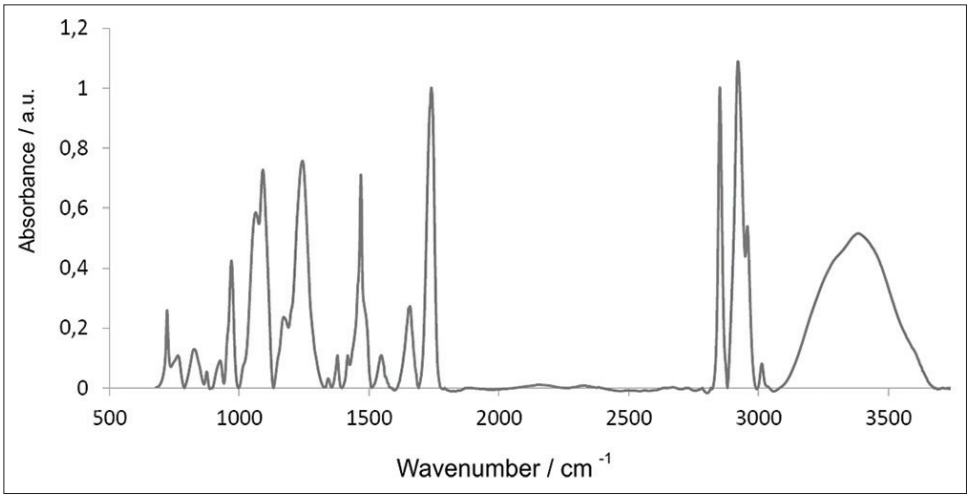

FIGURE 1. Infrared spectrum of Curosurf obtained by calculating average spectrum of all recorded spectra. The spectrum reveals the positions of the major infrared bands that are in agreement with the corresponding phospholipid bands reported in the literature [19]. The band assignments for the major functional groups are as follows: PO_2^- stretching at 1244 cm^{-1} for asymmetric and at 1091 cm^{-1} for symmetric stretching vibrations, $(CH_3)_3N^+$ stretching at 970 cm^{-1}, C=O stretching at 1740 cm^{-1}, CH_2 stretching at 2921 cm^{-1} for asymmetric and 2851 cm^{-1} for symmetric stretching vibrations, and CH_3 stretching at 2957 cm^{-1} for the asymmetric and 2873 cm^{-1} for symmetric stretching vibrations.

groups are as follows: PO_2^- stretching of the hydrophilic head at 1244 cm^{-1} for asymmetric and at 1091 cm^{-1} for symmetric stretching, $(CH_3)_3N^+$ stretching of the choline head at 970 cm^{-1}, C=O stretching of the fatty acid esters at 1740 cm^{-1}, CH_2 stretching of the hydrocarbon tails at 2921 cm^{-1} for asymmetric and 2851 cm^{-1} for symmetric stretching vibrations, and CH_3 stretching at 2873 cm^{-1} for symmetric and 2957 cm^{-1} for the asymmetric stretching vibrations.

To gain insight into the visible differences between spectra, we compared the mean spectra of untreated and treated samples. In the head group of pulmonary surfactant phospholipids, upon the addition of isoflurane (Figure 2A), the band at 1244 cm^{-1} assigned to asymmetric PO_2^- stretching vibration of the hydrophilic head was shifted to lower wavenumbers, while the intensity of the band increased with the isoflurane concentration. The band at 1091 cm^{-1}, assigned to symmetric PO_2^- stretching, showed no change in the position, but it showed an increase in the intensity that was consistent with the increase of isoflurane concentration. The band at 970 cm^{-1}, assigned to choline stretching vibration, showed no change in the frequency or intensity.

The spectra of the group treated with sevoflurane (Figure 2B) showed similar changes of surfactant compared to those treated with isoflurane. A band at 1244 cm^{-1} showed a shift to lower wavenumbers consistent with the increase of the anesthetic concentration. However, these changes appeared weaker than those observed in isoflurane-treated group of samples. A small, but consistent, increase in the band intensity was observed as well. A band at 1091 cm^{-1} showed a very weak shift to lower wavenumbers as well as an increase in the intensity, consistent with the increase of sevoflurane concentration. A choline band at 970 cm^{-1} showed no change in the position.

In the interfacial region of phospholipids, a common band of both untreated and all isoflurane-treated samples (Figure 2C) appeared at 1739 cm^{-1}, assigned to the carbonyl group stretching vibrations, involved in the ester bonds of the interfacial region. With the increase of isoflurane concentration, a weak shoulder at 1743 cm^{-1} became more pronounced as well as the additional band at 1733 cm^{-1}.

A common peak of both, untreated and all sevoflurane-treated, samples (Figure 2D) appeared at 1739 cm^{-1}, whereas the shoulder at 1743 cm^{-1} was less pronounced than the one observed for isoflurane-treated samples. The band at 1733 cm^{-1} appeared less pronounced in the sevoflurane-treated samples as well.

The most prominent bands corresponding to the hydrocarbon tail region of a phospholipid were, as expected, those assigned to CH_2 stretching vibrations. In isoflurane-treated samples (Figure 2E), a symmetric stretching CH_2 band at 2851 cm^{-1} showed a significant increase in the intensities, consistent with the increase in isoflurane concentration. The same result was observed for a band at 2921 cm^{-1} assigned to asymmetric CH_2 stretching. No apparent change of the bands assigned to symmetric (2873 cm^{-1}) and asymmetric (2957 cm^{-1}) CH_3 stretching vibrations was observed.

In sevoflurane-treated samples (Figure 2F), bands corresponding to symmetric and asymmetric stretching CH_2 vibrations showed inconsistent changes in the intensities and no change in the band position compared to the spectra of untreated sample. No change of the bands at 2873 cm^{-1} and 2957 cm^{-1} was observed.

The OH stretching vibrations appeared as a broad band with a maximum peak intensity at 3390 cm^{-1} in spectra of both treated and untreated samples, showing no shift in the

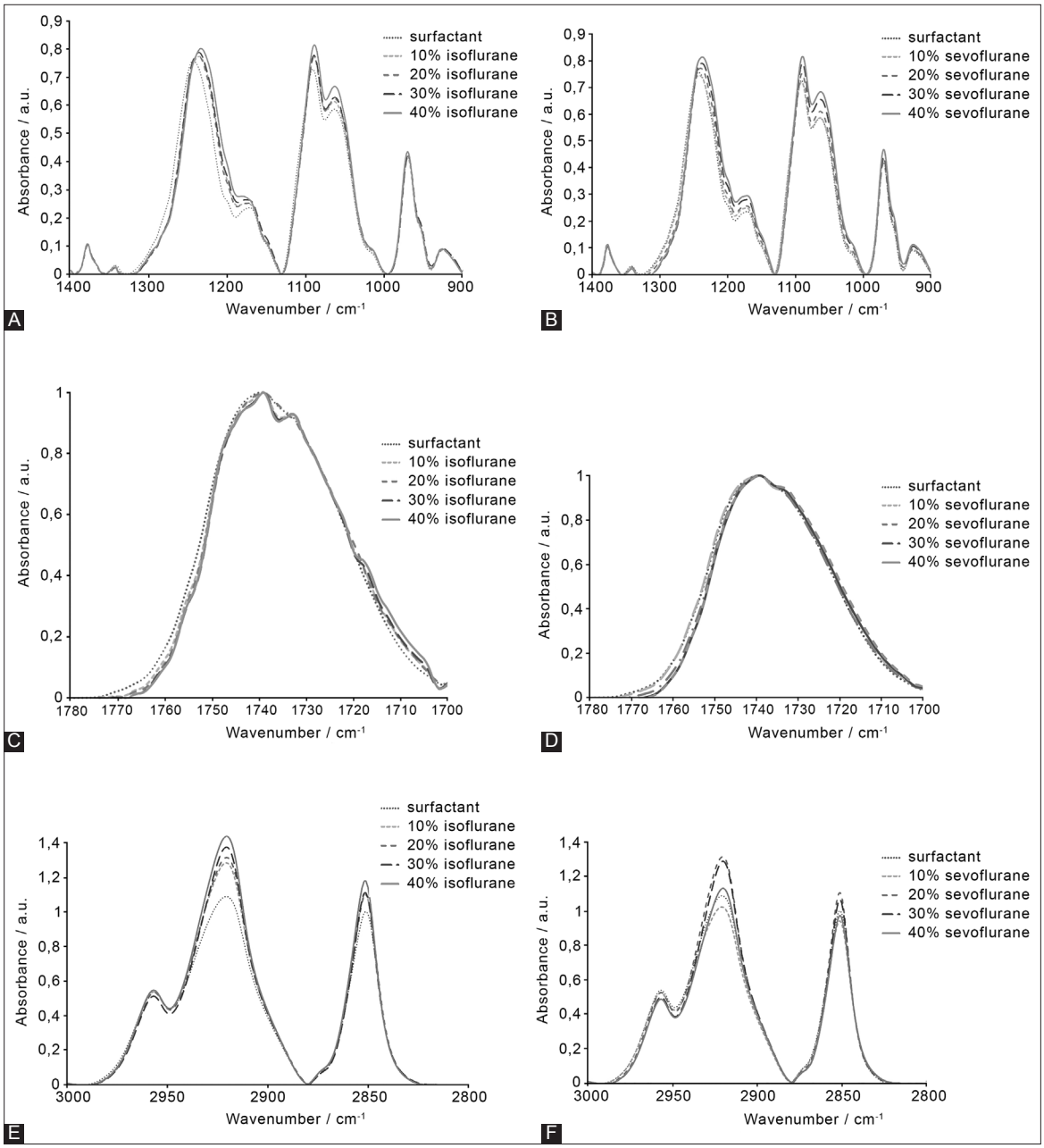

FIGURE 2. Infrared spectra of the phospholipid head group in the isoflurane (A) and sevoflurane (B) groups, infrared spectra of the phospholipid interfacial region in the isoflurane (C) and sevoflurane (D) groups, and infrared spectra of the phospholipid hydrocarbon tail in the isoflurane (E) and sevoflurane (F) groups.

frequency. The intensity of these bands was increased when the anesthetic was added. These changes appeared weaker for sevoflurane-treated samples than those observed in the isoflurane-treated group.

To determine whether the bands typical of isoflurane and sevoflurane spectra appear in the spectra of treated surfactant samples, we have recorded the spectra of both anesthetics and compared them to those of our samples. No bands typical of sevoflurane and isoflurane spectra were detected by FTIR in the spectra of treated samples, presumably due to the anesthetics' small volume and rapid evaporation once the sample was placed on the wafer and dried.

PCA statistical analysis

The results of the PCA statistical analysis for the isoflurane (Figure 3A) and sevoflurane (Figure 3B) groups are reported as a projection of the spectra in the first two principal components. The clear separation between all spectra is obtained in the principal components PC1-PC2 space, suggesting a clear difference between the FTIR spectra of all samples. The spectral range used for this analysis was 3700-700 cm^{-1}.

Student's *t*-test

With PCA, we showed that recorded spectra of all samples were different with regard to the whole transmitting

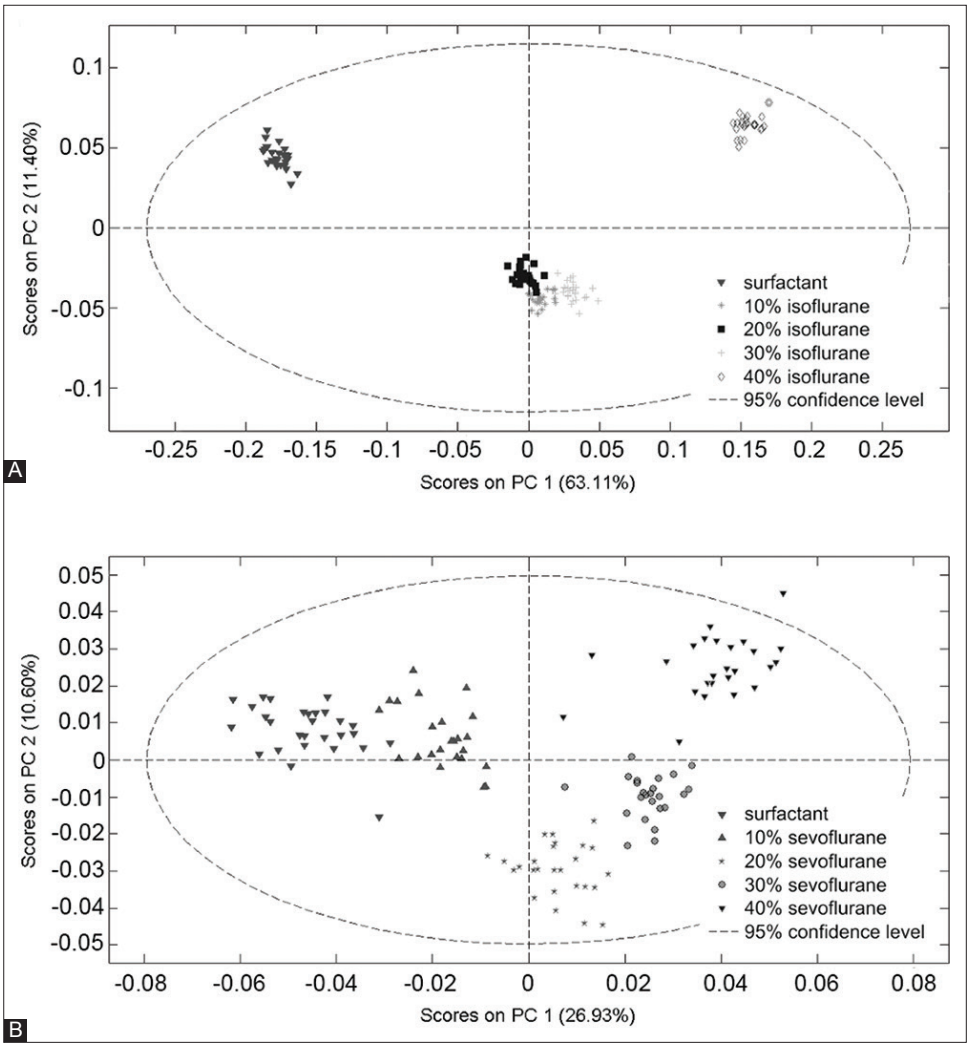

FIGURE 3. Scores plot of all recorded spectra in the isoflurane (A) and sevoflurane (B) groups. Each point of the plot is a projection of a spectrum in the principal component PC1-PC2 space. The spectral range used for this analysis was 3700-700 cm^{-1}.

region. To identify the wavenumbers at which statistically significant differences occur, Student's *t*-test was used to analyze the difference spectra obtained from subtracting the mean spectrum of each of the eight treated groups of samples from the mean spectrum of untreated sample at each wavenumber. In Figure 4, we present an example of a Student's *t*-test performed on untreated surfactant and surfactant treated with the lowest anesthetic concentration (10%). Regions marked with an asterisk indicate wavenumbers at which a statistically significant difference ($p < 0.01$) occurred between the spectrum of untreated sample and the spectrum of treated sample.

In the isoflurane group of samples (Figure 4A), in the region corresponding to the phospholipid head group, a statistically significant difference was observed for all anesthetic concentrations, for both asymmetric and symmetric PO_2^- stretching. Two bands corresponding to the asymmetric PO_2^- stretching region showed a transition from a negative peak at 1260 cm^{-1} to a positive peak at 1216 cm^{-1} and from a negative peak at 1111 cm^{-1} to a positive peak at 1087 cm^{-1} for a symmetric PO_2^- stretching region. These transitions occur when there is a shift in wavenumber between the observed groups of spectra. A band corresponding to the interfacial region displayed a statistically significant negative peak for all anesthetic concentrations. In the hydrocarbon chain region, two positive peaks are clearly displayed for all anesthetic concentrations, corresponding to symmetric and asymmetric CH_2 stretching at 2851 cm^{-1} and 2921 cm^{-1}, respectively. Statistically significant difference occurred at the 40% anesthetic concentration for the symmetric and at 30% and 40% concentration for asymmetric stretching band. The band corresponding to OH stretching showed a statistically significant positive peak for all concentrations.

In the sevoflurane group of samples (Figure 4B), in the region corresponding to the phospholipid head group, a statistically significant difference was observed for anesthetic concentrations of 20% and higher for both asymmetric and symmetric PO_2^- stretching. Two bands corresponding to the asymmetric PO_2^- stretching region showed a transition from a negative peak at 1265 cm^{-1} to a positive peak at 1217 cm^{-1} and from a negative peak at 1112 cm^{-1} to a positive peak at 1084 cm^{-1} for the symmetric PO_2^- stretching region. In the

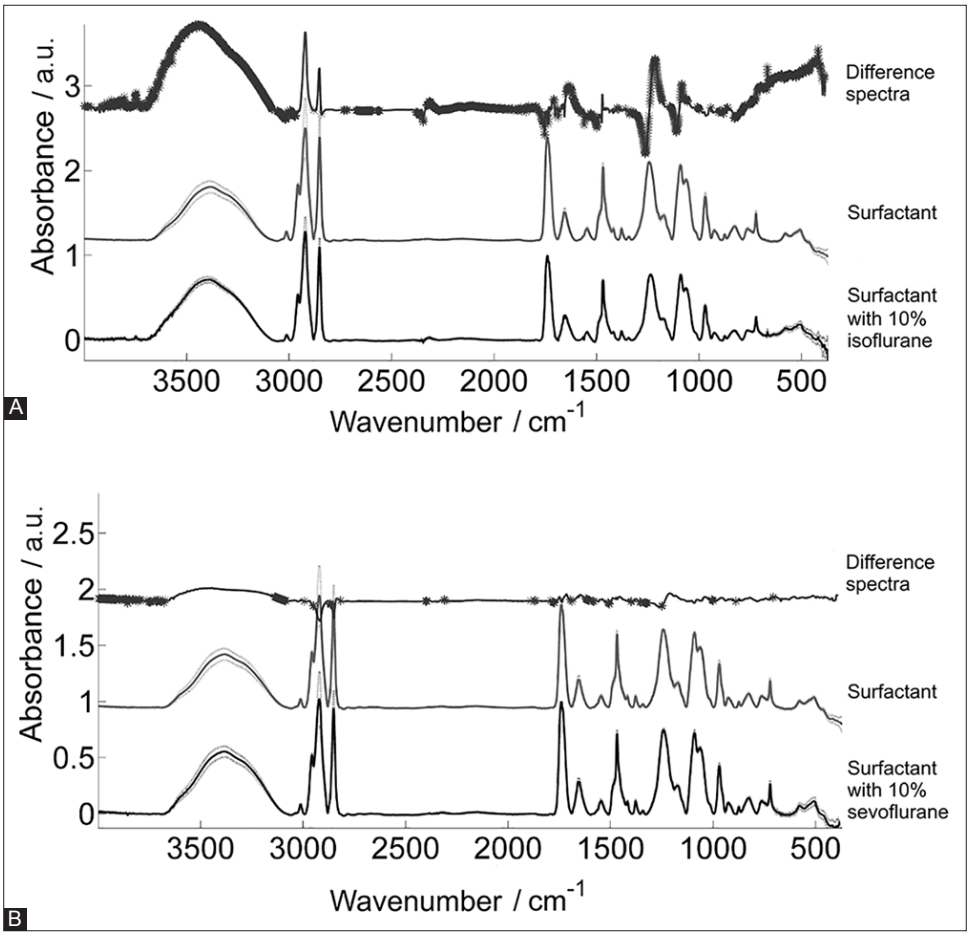

FIGURE 4. Difference spectra obtained by subtracting the mean spectrum of surfactant with 10% anesthetic from the mean spectrum of surfactant in the isoflurane (A) and sevoflurane (B) groups. The Student's *t*-test was computed at every wavenumber with a significance level of $p<0.01$ (Student's *t*-test). A statistically significant difference between the means was marked with asterisks. Thinner lines represent standard error.

10% concentration sample, a statistically significant negative peak was displayed at 1256 cm^{-1} followed by a positive peak at 1214 cm^{-1}. No significant bands for symmetric stretching were displayed for this concentration sample. The band corresponding to the interfacial region displayed a statistically significant negative peak for anesthetic concentrations of 20% and higher. In the hydrocarbon chain region, two bands were clearly displayed for all anesthetic concentrations, corresponding to symmetric and asymmetric CH_2 stretching at 2851 cm^{-1} and 2921 cm^{-1}, respectively, yet their direction differed inconsistently with the increase of the anesthetic concentration. The band corresponding to OH stretching showed a statistically significant positive peak only in the 40% anesthetic concentration sample.

DISCUSSION

The exact site of action and effect of inhalational anesthetics remain controversial. Some authors support the view that the preferred residence of anesthetics is the water/membrane interface [4-7]. In a FTIR spectroscopy study performed by Tsai et al., volatile anesthetics decreased the hydration in a water-in-oil reverse micelle system by apparently forming competitive proton donor-acceptor complex with the phosphate moiety, releasing the hydrogen-bonded water molecules [7]. Hydrogen bond breaking activity of anesthetics was proposed by Eyring and Jhon [20] and documented by several consequent studies [21-23]. Studies performed using NMR showed that anesthetics remained at the lipid/water interface [5] and distributed preferentially to the regions of the membrane that permit easy contact with water [24].

In contrast to these findings, a study using deuterium NMR and nuclear overhauser effect measurements suggests that the anesthetics are located primarily in the hydrocarbon tail region [8]. These findings are in agreement with studies performed using molecular dynamics simulations showing that halothane molecules are found to preferentially segregate in the upper part of the hydrocarbon tails, with the maximum probability near the C5 methylene group. This leads to increased disorder of the lipid tails, except for the region constrained by the anesthetic [9,10].

We have studied the effect of the two most commonly used anesthetics on a commercial porcine pulmonary surfactant. The concentrations of the anesthetics used were

well above the concentrations used clinically, to augment the observed effect. To confirm whether the spectra of untreated and treated samples differ regarding the whole transmitted region, we performed a PCA that showed a clear difference between the groups of spectra. To further examine the bands involved, we compared the mean spectra of untreated sample with treated samples and additionally performed the Student's *t*-test to highlight statistically significant differences. The results of the Student's *t*-test are in concordance with the changes observed comparing the mean spectra of all groups of samples.

Hydrophilic head

The phosphate moiety of phospholipids has long been recognized as the main water binding site [25]. Curosurf is a liposomal preparation with water added to aid injecting the drug into the lungs. We presume that the water from the preparation has also bonded with the phosphate moiety of the phospholipids of Curosurf. Our results mostly correspond to the studies showing a dehydrating effect of the anesthetics on the phospholipids of pulmonary surfactant [7,21-23]. The most obvious effect of both, isoflurane and sevoflurane, was on the phosphate moiety. A PO_2^- asymmetric stretching vibration wavenumber has shifted to the lower wavenumber consistently with the increase of anesthetic concentration in both groups of spectra, suggesting the interaction between the anesthetic and the phosphate moiety that resulted from the release of bound water. A transition from a negative to a positive peak in the difference spectra is in concordance with this observation. The choline head appears not to be affected by the presence of anesthetics, despite the presence of the positive electrostatic charge. Shielding of the charge with hydrophobic CH_3 groups has been proposed as an explanation for this group's weak role in hydration [6], presumably resulting in the lack of interaction with anesthetics as well.

Interfacial region

Phospholipid molecules contain two ester groups linked to the two lipid tails. A band at 1742 cm^{-1} has been assigned to a non-hydrogen-bonded free *sn-1* C=O group and a band at 1727 cm^{-1} to a hydrogen-bonded *sn-2* C=O group [26,27]. Both of these bands corresponded to a fully hydrated DPPC molecules. In a study performed by Tsai et al., using FTIR attenuated total reflection method, three peaks appeared in this area when anesthetic was added: At 1744, 1735 and 1728 cm^{-1} for a halothane sample and at 1740, 1734 and 1729 cm^{-1} for the enflurane sample [7]. Wong and Mantsch suggested that the peak at 1744.3 cm^{-1} corresponds to the C=O stretching mode of the free *sn-1* carbonyl group, and the peaks at 1737.5 and 1728.2 cm^{-1} are the C=O stretching modes of the free and hydrogen-bonded *sn-2* carbonyl moieties, respectively [27]. Based on these findings, it has been suggested that the perturbation by anesthetics resulted in the formation of a free *sn-2* carbonyl component after breaking hydrogen bonds at the glycerol skeleton [7].

Our samples, both treated and untreated, showed a common band at 1739 cm^{-1} and 1740 cm^{-1} for isoflurane and sevoflurane groups respectively, which would correspond to a free *sn-1* carbonyl group, as it is present in both untreated and treated samples. In the isoflurane group of samples, a weak band at 1743 cm^{-1} appears as a shoulder on a much stronger band at 1739 cm^{-1} showing a decrease in the intensity which is consistent with the increase of isoflurane concentration. In addition, this finding is in concordance with the results of the Student's *t*-test, showing a decrease in the abundance of this moiety.

Upon addition of isoflurane, we observed a formation of a band at 1733 cm^{-1} most likely corresponding to a free *sn-2* carbonyl group which, in the spectrum of the untreated sample, appears only as a weak shoulder of a band at 1739 cm^{-1}. An exception is the 20% isoflurane-treated sample showing unexplained behavior. Formation of this band might suggest a perturbation in this region. No bands with lower frequencies that would correspond to a hydrogen-bonded *sn-2* carbonyl group appeared in our spectra.

In sevoflurane-treated group of samples, a change at 1743 cm^{-1} appeared as a weak narrowing of a band at 1740 cm^{-1}, consistent with the increase of the anesthetic concentration. However, the results of the Student's *t*-test support the decrease in the abundance of this moiety, in the samples treated with 20% sevoflurane concentration and higher. No significant change to the free *sn-2* C=O group appeared in this group of samples, indicating less if any perturbation of this region caused by the addition of sevoflurane.

Hydrophobic tail

Apart from the interaction between anesthetics and the polar head, we also observed an effect on the methylene groups of the hydrophobic hydrocarbon tail in isoflurane-treated group. These changes presented as an increase in the intensities of the bands consistent with the increase of the anesthetic concentration and were in concordance with the results of the Student's *t*-test. This increase in the intensities of both asymmetric and symmetric CH_2 stretching vibrations might reflect an increase in the intermolecular chain disorder [28], which is consistent with research performed using molecular dynamics simulations [9,10].

Sevoflurane-treated group, however, showed inconsistent behavior as the increase in the peak intensity did not follow

the increase in the anesthetic concentration but rather formed a random pattern.

Membranes are supported by the hydrogen-bonded water matrix and when the supporting force is weakened, the whole membrane becomes disordered [25]. Our results show an interaction between a water binding phosphate moiety and anesthetics, and a dehydrating effect of both isoflurane and sevoflurane as well as a disordering effect of isoflurane on the hydrocarbon tail. These findings suggest that anesthetics might weaken the membrane-water interaction and destabilize the membrane structure. It should be emphasized that in humans, pulmonary surfactant exists as a lipid monolayer between the air and the fluid lining of alveoli, unlike in Curosurf where it is in the form of a liposome. The extent to which the destabilizing effect of the anesthetics could be applied to a lipid monolayer is a subject for future studies.

It is interesting that the effect of sevoflurane appeared to be weaker and less consistent than the effect of isoflurane. However, a more thorough investigation using clinical concentrations and under clinical conditions should be performed. It would certainly be interesting, but also a great challenge, to perform a FTIR study on a surfactant collected at different times during general anesthesia from patients exposed to different clinical concentrations of these two anesthetics and to investigate the changes of the protein component of pulmonary surfactant. This could be a next step in understanding the extent and reversibility of changes to lung surfactant during and after anesthetic procedures.

CONCLUSION

The FTIR technology has proven to be a promising tool in the research of anesthetic effect on lipids. It has also shown to be quite useful in the present basic study of the molecular mechanisms involved in the effect of inhalational anesthetics isoflurane and sevoflurane on the lipids of pulmonary surfactant. When combined with statistical methods, it provided an insight into which parts of the surfactant phospholipid molecule are the most affected by the anesthetics and their changing concentrations, and in what way. Our findings suggest a dehydrating effect of the anesthetics on the phospholipid head group as well as a disordering effect on the phospholipid tails, both of which might act to destabilize the structure of the phospholipids. We suggest that the application of this technique might be a further step in the research of the anesthetic effect on pulmonary surfactant in clinical conditions.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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