Hypertonic saline solutions do not influence the solubility of sputum from secretor and non-secretor cystic fibrosis patients

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Abstract

Introduction: Functional alterations of the cystic fibrosis transmembrane conductance regulator gene (CFTR) increase the viscoelasticity of pulmonary secretions of cystic fibrosis (CF) patients and require the use of therapeutic aerosols. The biochemical properties of exocrine secretions are influenced by the expression of the *FUT2* gene which determine the secretor and non-secretor phenotypes of the ABH glycoconjugates. The aim of this study was to determine the influence of secretor and non-secretor phenotypes by means of photoacoustic analysis, both the typical interaction time (t_0) and the solubilization interval (Δt) of the sputum of secretor and non-secretor CF patients nebulized by hypertonic saline solutions at different concentrations.

Material and methods: Sputum samples were obtained by spontaneous expectoration from 6 secretor and 4 non-secretor patients with CF. Each sample was nebulized with 3%, 6%, and 7% hypertonic saline solutions in a photoacoustic cell. The values of t_0 and Δt were determined using the Origin 7.5® computer program (Microcal Software Inc.). The *t*-test was employed using the GraphPad Instat 3.0® computer program to calculate the mean and standard deviation for each parameter.

Results: For all hypertonic saline solutions tested, the mean values of t_0 and Δt do not show statistically significant differences between secretor and non-secretor patients.

Conclusions: The secretor and non-secretor phenotypes do not influence the *in vitro* solubilization of the sputum nebulized by hypertonic saline solutions at different concentrations when analysed by photoacoustic technique.

Key words: photoacoustic technique, secretor phenotype, cystic fibrosis, hypertonic saline solutions.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease resulting from cystic fibrosis transmembrane conductance factor (*CFTR*) gene alterations

affecting the CFTR protein function contributing to increased viscoelasticity of pulmonary secretions, which represents the main risk factor for the development of irreversible pulmonary disease [1]. Additionally, dehydration of the pulmonary secretions, bacterial infections and infiltration of neutrophils also contribute to an increase in the viscoelasticity of the pulmonary mucus [2]. As a consequence, CF patients have difficulty to eliminate pulmonary secretions and are required to use therapeutic aerosols to solubilize them and facilitate expectoration [3].

The secretor and non-secretor phenotypes are genetic traits related to the ABO histo-blood group system under control of the *FUT2* gene (19q13.3). Carriers of secretor phenotype produce ABH glycoconjugates (glycoproteins and glycolipids) in the exocrine secretions according to their ABO blood types. On the other hand, carriers of non-secretor phenotype are unable to secrete these glycoconjugates [4]. Therefore, the presence or absence of ABH glycoconjugates alters the biochemical properties of exocrine secretions as they reflect the nature of part of the carbohydrates in secretions [5, 6].

Previous papers failed to demonstrate an association between CF, the ABO histo-blood group system, and secretor and non-secretor phenotypes based on statistical analysis, although a protective effect of the secretor phenotype on epithelialized organs has been proposed [7-10].

The photoacoustic technique is useful to evaluate the interaction between therapeutic aerosols and exocrine secretions. It allows the determination of the typical interaction time (t_0) between the aerosol and the secretion sample and the solubilization interval (Δt) in minutes. The photoacoustic effect consists in the production of acoustic waves due to the absorption of modulated light by the secretion sample. Light energy absorbed is converted into heat, modulating the temperature which produces the mechanical effect of periodic expansion and contraction, originating sound waves that can be detected by a microphone. The photoacoustic signal depends on the optical and thermal properties of the sample, which may vary with time. When a sample undergoes changes in its biochemical composition or structure, the propagation of heat produced inside is modified thereby altering the photoacoustic signal [11].

Our recent demonstration that the secretor and non-secretor phenotypes influence the solubilization of pulmonary mucus from CF patients by nebulization with tobramycin and α dornase using the photoacoustic technique [12] prompted us to investigate whether other therapeutic aerosols used by CF patients can be influenced by those genetic traits under control of the *FUT2* gene.

The aim of this study was to determine the influence of secretor and non-secretor phenotypes by means of photoacoustic analysis, both the typical interaction time (t_0) and the solubilization interval (Δt) of the sputum of secretor and non-secretor CF patients nebulized by hypertonic saline solutions at different concentrations.

Material and methods

Ethical considerations

This study was approved by the Research Ethics Committee of the FAMERP (case 366/2006). Written informed consent was obtained after the parents and/or guardians of the patients were informed about the protocol study.

Patient selection

To perform this *in vitro* experimental study, ten Caucasians CF patients, 5 men and 5 women, with mean age 16.9 years old (range: 10 years old to 29 years old), able to expectorate spontaneously, were selected. Six of them are regularly treated in the Cystic Fibrosis Reference Center of the Medical School in São José do Rio Preto (FUNFARME) and four in the Cystic Fibrosis Reference Center of the State University in Campinas (UNICAMP). Seven patients had the ΔF508 mutation and one had the G542X mutation in the *CFTR* gene. Two patients had not had the disease confirmed by molecular analysis but all of them had CF diagnosis confirmed by the positive sweat test and also by measuring the faecal fat, which are the gold standard.

Although a small number of patients was selected, it was sufficient to demonstrate the difference for the secretor and non-secretor phenotypes influencing both the typical interaction time (t_0) and the solubilization interval (Δt) as reported in our previous paper [12].

Blood sampling and extraction of genomic DNA

A sample of 5 ml of whole blood was drawn from each patient and placed in vacuum tubes with EDTA. White blood cells were used for the extraction of genomic DNA according to the protocol of Miller *et al.* [13].

Secretor and non-secretor phenotype identification

The secretor and non-secretor phenotypes were identified by *FUT2* genotyping using PCR-RFLP according to the protocol of Svensson *et al.* [14]. Briefly, a fragment containing 1033 base pairs from exon 2 of the *FUT2* gene with primers sense (5'-CGC TCC TTC AGC TGG GCA CTG GA-3') and antisense (5'-CGG CCT CTC AGG TGA ACC AAG AAG

CT-3') to differentiate the *G* and *A* alleles at the 428 position was amplified. Each PCR mix was performed in a final volume of 25 µl containing 10 mM TRIS-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 20 mM of each dNTP (dATP, dTTP, dCTP, dGTP), 10 pM of each primer, 0.5 U of Taq and 5 ng of genomic DNA. The amplification conditions involved pre-denaturation (94°C for 5 min) followed by 35 cycles (94°C for 1 min, 63°C for 1 min and 72°C for 1 min) and an additional extension at 72°C for 5 min. The amplified fragments were digested by *Ava II* enzyme given a variable number of fragments (459, 295, 149 and 130 base pairs for the *G* allele; 459, 425 and 149 base pairs for the *A* allele) which were separated by electrophoresis in 2% agarose gel stained with ethidium bromide under UV light. Thus, *GG* and *GA* individuals were identified as secretors and *AA* individuals as non-secretors of ABH glycoconjugates.

Sputum sample collection

By spontaneous expectoration, 5 ml of pulmonary mucus were collected onto a universal collector and covered with sterile gauze to absorb any excess saliva, according to the protocol of Bossi [15]. Subsequently, it was placed in polystyrene tubes lubricated with liquid Vaseline to avoid dehydration and stored at –20°C until photoacoustic analysis. Routine laboratory tests were performed to rule out the presence of pulmonary infection.

Preparation of 3%, 6% and 7% hypertonic saline solutions

The hypertonic saline solutions at 3%, 6% and 7% were prepared by diluting a 20% NaCl stock solution using distilled water and following the formula below:

$$V_1 \times C_1 = V_2 \times C_2$$

where V_1 indicates the volume of 20% NaCl solution to be diluted, C_1 the concentration of 20%, V_2 the final volume to be obtained and C_2 the final desired concentration.

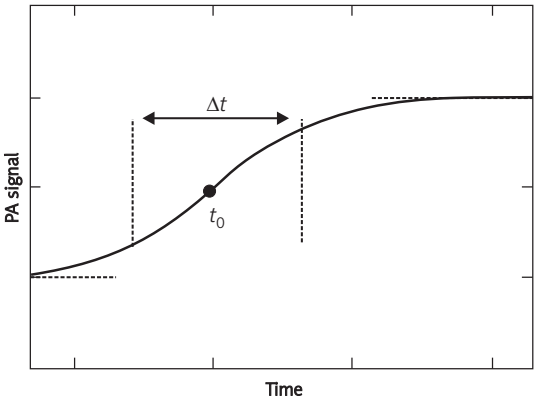

Figure 1. Standard curve for the adjustment of the photoacoustic signal (PA) for the typical interaction time (t_0) and solubilization interval (Δt) parameters

Preparation of the sputum samples for photoacoustic analysis

The samples were naturally thawed at room temperature and subsequently submersed in xylol for 5 s to remove the liquid Vaseline. Each sample was divided into three portions with volumes of 0.1 ml for a double blind fashion photoacoustic analysis.

Determination of t_0 and Δt by photoacoustic analysis

The protocol used to perform the photoacoustic analyses was based on our previous study [11]. Each sputum sample was evaluated for a period of 5 min to measure the baseline photoacoustic signal before applying each hypertonic saline solution to assess the stability of the photoacoustic signal. Increases or decreases of this signal over time would compromise later analysis of the solubilization process. Next, each sputum sample was individually nebulized with each hypertonic saline solution concentration and its solubilization was evaluated by monitoring the amplitude of the photoacoustic signal over time. The evolution over time of the photoacoustic signal was adjusted using the Boltzmann equation given by:

$$PA(t) = \frac{A_1 - A_2}{1 + e^{\frac{t - t_0}{\Delta t}}} + A_2$$

where PA(t) is the amplitude of the photoacoustic signal at time t, A_1 and A_2 are the baseline and final amplitudes of the photoacoustic signal, respectively, t_0 is the maximum solubilization and Δt the effective time interval corresponding to the solubilization process. Figure 1 shows the standard curve for the adjustment of the photoacoustic signal of the t_0 and Δt parameters. The data were transferred to a computer and adjustment curves were produced by the Origin 7.5® computer program (Microcal Software Inc.). The *t*-test, using the GraphPad Instat 3.0® computer program, calculated the mean and standard deviation for each adjustment parameter.

Results

According the results of *FUT2* genotyping, the patients were classified as secretor (60%; 6/10) and non-secretor (40%; 4/10) phenotypes.

The mean values of t_0 and Δt for each of the hypertonic saline solution concentrations tested are shown in Table I. Although the solubilization of the pulmonary mucus from secretors nebulized with 3% presents a higher value for t_0 as compared with

non-secretors, the difference was not statistically significant. Similar mean values for t_0 and Δt were observed for solubilization with hypertonic saline solution at 6% and 7% for both secretor and non-secretor phenotype carriers as well.

Discussion

Our study was aimed at determining, by using the photoacoustic technique, the influence of the secretor and non-secretor phenotypes in the solubilization of the pulmonary sputum from cystic fibrosis patients treated regularly with hypertonic saline solutions. The typical interaction time of solubilization (t_0) and the solubilization interval (Δt) were measured after the nebulization of the sputum by hypertonic saline solutions at 3%, 6% and 7%.

The utility of the photoacoustic technique to evaluate the interaction of human mucous with therapeutic aerosols has been proved [11, 12]. The results reported by Dumas *et al.* supported the empirical practice of the use of isotonic saline solutions to clear the airways of healthy individuals and patients presenting pulmonary symptoms as well [11]. However, this study did not investigate the influence of the secretor and non-secretor phenotypes on the solubilization of the sputum.

Our recent previous study demonstrating the influence of secretor and non-secretor phenotypes on the solubilization of the CF sputum nebulized *in vitro* by tobramycin and α dornase shed some light on the importance of genetic traits and drug interactions [12]. Therefore this approach could be used to validate the utility of phenotyping and genotyping before the prescription of drugs including therapeutic aerosols used by CF patients. The results of this investigation are in disagreement with those reported in our previous study [12] since the differences of t_0 and Δt for secretor and non-secretor phenotype carriers were not statistically significant for all the hypertonic saline solutions tested. The homogeneity of the results may be due to various factors.

Unlike tobramycin and dornase α, hypertonic saline solutions are not conventional medicines but also contribute to the solubilization of secretions [16-19]. Due to differences in osmolarity these hypertonic solutions act as hydrating secretions by removing water from the epithelial cells that line the airways [17, 18]. Moreover, they exert a mucolytic effect by breaking the ionic links between the glycoproteins of the mucus, thereby considerably reducing the viscoelasticity of the pulmonary secretions and facilitating the expectorations [16, 19].

Biochemical properties of the exocrine secretions are influenced, at least in part, by the presence of the ABH glycoconjugates. These

Table I. Mean values in minutes and respective standard deviations of t_0 and Δt using different concentrations of hypertonic saline solution in sputum of positive and negative secretors

	Positive secretor (n = 6)	Negative secretor (n = 4)	Value of p
3% NaCl			
t_0	14.3 ±4.7	9.6 ±5.8	0.23
Δt	3.0 ±1.3	3.0 ±2.5	0.99
6% NaCl			
t_0	13.8 ±7.3	13.8 ±4.0	0.98
Δt	2.7 ±1.7	2.9 ±2.5	0.89
7% NaCl			
t_0	11.6 ±3.8	11.2 ±1.0	0.83
Δt	3.7 ±3.0	3.0 ±2.8	0.70

glycosylated molecules related to the ABO blood group system are expressed under control of the *FUT2* gene and reflect the nature of the carbohydrates present in exocrine secretions [5, 6]. Secretors differ from non-secretor carriers as they express glycoproteins and glycolipids with distinct ABH antigenic specificities. The oligosaccharidic structures of these glycoconjugates are constituted of glucose, N-acetylglucosamine, fucose, galactose and N-acetylgalactosamine [4]. The control of glycosylation, responsible for the creation of ABH glycoconjugates, is performed by the α-2-L-fucosyltransferase enzyme coded by the *FUT2* gene, which determines the dynamics of glycosylation of the proteins and lipids in exocrine secretions, including in pulmonary mucus [20, 21].

Majima *et al.* reported that the high level of fucose present in nasal mucus correlates with an increase in its viscoelasticity [22]. This monosaccharide, present in purified respiratory mucus glycoproteins produced by goblet cells and submucosal gland cells and ABH glycoconjugates as well, largely contributes to the viscoelasticity of the nasal mucus in chronic sinusitis [23]. However, the solubilization of the sputum nebulized by hypertonic saline solutions seems not to be dependent on fucose.

The presence of the *FUT2* gene, apart from defining the secretor phenotype, contributes to a reduction in the length of the oligosaccharide chains of ABH glycoconjugates [5, 24]. On the other hand, the substitution of *G428A* in exon 2 of this gene, apart from abolishing the α-2-L-fucosyltransferase enzyme expression, determines the non-secretor phenotype, the absence of ABH glycoconjugates and the presence of long precursor oligosaccharide chains [24, 25]. These structural differences may cause variation in the polarity and the hydrosolubility of the sputum from secretor and

non-secretors but they could not be enough to influence the solubilization of the sputum when nebulized by hypertonic saline solutions.

The differences resulting from the presence or absence of the ABH glycoconjugates in exocrine secretions play an important biological role in the innate immunity of different microorganisms, including those that infect the airways causing pulmonary damage [6, 10]. However, the solubilization of sputum of secretor and non-secretor CF patients by hypertonic saline solutions is apparently independent of differences determined by the *FUT2* gene.

Since monogenetic traits have a predictable influence on therapeutic response to a large number of commonly prescribed medicines [26], it would be expected that secretor and non-secretor phenotypes could be useful to determine the adequate time for CF patients to spend in therapy, as we suggested in our previous paper [12]. However, the homogeneity of the data for t_0 and Δt seems to indicate that the secretor and non-secretor phenotypes have no effect on the solubilization of sputum from CF patients when nebulized by hypertonic saline solutions.

In conclusion, the secretor and non-secretor phenotypes do not influence the *in vitro* solubilization of the sputum nebulized by hypertonic saline solutions at different concentrations when analysed by photoacoustic measurements.

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