

Influence of secretor and non secretor phenotypes on the solubilization of pulmonary mucus by three common medicines in cystic fibrosis patients assessed using photoacoustic analysis

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Work carried out in the Immunogenetics Laboratory of the Molecular Biology Department, FAMERP and Research and Development Institute – UNIVAP.

Partially supported by: BAP–FAMERP 2007/2008

MAIB Is a Doctorate student of the Postgraduation Course in Health Sciences of FAMERP.

Introduction

Cystic fibrosis is a monogenic, autosomal recessive inherited disease that results in alterations of the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) [1]. The defect of the *CFTR* gene (7q31.2) contributes to an accentuated increase in the viscoelasticity of pulmonary secretions provoking an obstruction of the airways by thick and viscous mucus [2]. Thus, cystic fibrosis patients have a difficulty to eliminate pulmonary secretions and require therapeutic aerosols to solubilize and facilitate expectoration [3, 4].

The secretor and non secretor phenotypes are genetic trait controlled by the α -2-L-fucosyltransferase enzyme (FUTII) coded by the *FUT2* gene (19q13.3) which influences the expression of ABH glycoconjugates (glycoproteins and glycolipids) in exocrine secretions [5]. ABH glycoconjugates are structurally related to antigens which define ABO blood groups and the presence or absence of these molecules alter the biochemical properties of exocrine secretions as they reflect the nature of part of the carbohydrates in secretions [6, 7].

Published studies have determined the frequency of secretor and non secretor phenotypes in cystic fibrosis patients and normal controls. The statistical analysis was unable to prove any association between this genetic trait and the disease [9-10]. The viscoelasticity and the expression of ABH glycoconjugates are two distinct processes that affect the exocrine secretions [2, 5], but the influence of the secretor and non secretor phenotypes on the solubilization of the pulmonary mucus of cystic fibrotic patients treated with therapeutic aerosols in order to reduce the viscoelasticity is unknown.

The photoacoustic technique has being employed to study the interaction between therapeutic aerosols and different secretions. It enables the determination of the typical interaction time of solubilization (t_0) between the aerosol and the sample of secretion and the solubilization interval (Δt) in minutes. The photoacoustic effect consists in the production of a sound due to the absorption of modulated light. Light energy absorbed by a sample is converted into heat, causing temperature modulation 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 samples, which may vary with time. When a sample undergoes changes in its composition or structure, the propagation of heat produced inside is modified thereby altering the photoacoustic signal [11].

The photoacoustic technique has proved to be useful to monitoring the absorption of isotonic saline by human mucus [12] and revealed the differences in the absorption times of isotonic saline solution in the sputum of cystic fibrosis patients with

and without pneumonia [13]. Additionally, this technique proved to be a precise instrument to monitor the typical interaction time solubilization of sputum from cystic fibrosis patients using 3% and 5% hypertonic saline solutions and N-acetylcysteine [14].

The aim of our 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 cystic fibrosis patients nebulized with therapeutic aerosols.

Material and Method

Patient selection

To perform this in vitro experimental study, ten cystic fibrosis patients were selected. Of these, six are regularly treated in the Cystic Fibrosis Reference Center of the Regional Foundation of Medicine School in São José do Rio Preto (FUNFARME) and four in the Cystic Fibrosis Reference Center of the State University in Campinas (UNICAMP). Cystic fibrosis was confirmed for all patients by the positive sweat test, which is considered the gold standard, and also by measuring the fecal fat. Of the ten patients, seven had the $\Delta F508$ mutation and one had the G542X mutation of the *CFTR* gene. The disease had not been confirmed by molecular analysis for two patients. All the five men and five women were Caucasians. Their mean age was 16.9 years old (range: 10 to 29 years). This study was approved by the Research Ethics Committee of FAMERP (366/2006) and the informed consents were obtained after the parents and or guardians being informed about the study protocol.

Blood sampling and extraction of genomic DNA

Five mL of whole blood was drawn from each patient and placed in vacuum tubes with EDTA. Leucocytes were utilized for the extraction of genomic DNA according to the protocol of Miller and colleagues [15].

Secretor and non secretor genotypes identification

Definition of the secretor and non secretor phenotypes was achieved by genotyping the *FUT2* gene using PCR-RFLP according to the protocol of Svensson and colleagues [16]. Briefly, a fragment with 1033 base pairs of the exon 2 of the *FUT2* gene was amplified using the 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. Each PCR mix was performed in a final volume of 25 μ L containing 10 mM TRIS-HCL, 50 mM KCl, 1.5 mM MgCl₂, 20 mM of each dNTP [dATP, dTTP, dCTP, dGTP], 10 pM of each primer, 0.5 U of Taq and 5

ng do genomic DNA. The amplification conditions involved pre-desnaturation (94°C for 5 minutes) followed by 35 cycles (94°C for 1 minute, 63°C for 1 minute and 72°C for 1 minute) and an additional extension at 72°C for 5 minutes. The amplified fragments were digested by *Ava II* enzyme given 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 stated as secretors and *AA* individuals as non-secretors of ABH glycoconjugates.

Sputum sample collection

Three sputum samples containing 5 mL were collected from each selected patient in three consecutive days using spontaneous expectoration according to the protocol of Bossi [17]. The sputum was expectorated onto a universal collector and covered with sterile gauze to absorb any excess of saliva. Later it was placed in a polystyrene tube lubricated with liquid Vaseline to avoid dehydration and stored at -20°C until photoacoustic analysis. All the samples were evaluated by routine laboratorial based on culture of microorganism tests to discard pulmonary infection.

Utilization of tobramycin, alpha dornase, and N-acetylcysteine

Tobramycin, alpha dornase, and N-acetylcysteine were utilized at doses as recommended by the fabricants for clinical use.

Preparation of the sputum samples for photoacoustic analysis

Each sample was naturally thawed at room temperature and subsequently submersed in xylol for five seconds to remove the liquid Vaseline. Following this each sample was divided in three portions with volumes of 0.1 mL for a double blind fashion photoacoustic analysis.

Determination of t_0 and Δt by photoacoustic analysis

Photoacoustic analyses were performed according to the protocol by Coelho and colleagues [13]. Before applying each therapeutic aerosol, each sputum sample was evaluated for a period of five minutes to measure the baseline photoacoustic signal. This measurement assessed the stability of the photoacoustic analyses signal as increases or decreases of this signal over time would compromise later analysis of the solubilization process. Subsequently, each sputum sample was individually nebulized using each of the three therapeutic aerosols and the solubilization was evaluated by means of monitoring the amplitude of photoacoustic analyses over time.

The evolution over time of photoacoustic analyses 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 analyses signal at time t, A₁ and A₂ are the baseline and final amplitudes of the photoacoustic analyses signal, respectively, t₀ is the time to reach the maximum rate of change in the process and Δt the effective time interval corresponding to the solubilization process. The figure 1 shows the standard curve for the adjustment of the photoacoustic signal of the t₀ and Δt parameters. The data were input on a computer and adjustment curves produced by the Origin 7.5[®] computer program (Microcal Software Inc.). The t-test, using the GraphPad InStat computer program, calculated the mean and standard deviation for each adjustment parameter. The photoacoustic analysis was carried out by an examiner which had no previous knowledge about the secretor or secretor negative phenotypes of the sputum samples.

Results

The patients selected for this study were divided in two groups according to secretor (60%; 6/10) and non secretor (40%; 4/10) phenotypes. The mean values in minutes and their respective standard deviations for the t₀ and Δt parameters are shown in table 1. Some of the differences between the t₀ values and the Δt values were statistically significant depending on the aerosol tested.

Nebulization using tobramycin

The mean value of t₀ obtained by nebulization using tobramycin was higher for non secretor compared to secretor phenotype carriers (p-value = 0.03). Additionally, non secretors presented a smaller standard deviation indicating greater homogeneity in the values obtained for each sample analyzed. The mean values of Δt and their standard deviations were similar for both secretor and non secretor phenotypes carriers (p-value = 0.88).

Nebulization using alpha dornase

The mean value of t₀ obtained by nebulization using alpha dornase was higher for secretors compared to non secretors, but the difference was not significant (p-value

= 0.35). However, secretors demonstrated a larger standard deviation which indicates greater heterogeneity in the values of t_0 obtained for these samples. The mean values of Δt and their standard deviations presented statistically significant differences between secretor and non secretor phenotypes (p-value = 0.04).

Nebulization using N-acetylcysteine

The mean value of t_0 obtained by nebulization using N-acetylcysteine was higher for non secretors but the differences were not statistically significant (p-value = 0.35). Additionally, the mean values for Δt and their standard deviations were similar between secretor and non secretors (p-value = 0.99).

Discussion

The aim of this study was to determine, using the photoacoustic technique, the influence of the secretor and non secretor phenotypes in the solubilization of sputum from cystic fibrosis patients using therapeutic aerosols. Hence the typical interaction time of solubilization (t_0) and the solubilization interval (Δt) of sputum were measured after nebulization with solutions of tobramycin, alpha dornase, and N-acetylcysteine. From what the authors know this is the first study that analyzes the influence of the secretor status and the interaction of therapeutic aerosols with sputum of cystic fibrosis patients.

Functional alterations in the cystic fibrosis conductance factor (*CFTR*) resulting from mutations that affect the *CFTR* gene are responsible for the increased viscoelasticity of pulmonary mucus making it difficult to eliminate particles that enter the small and large airways and increase the risk of morbidity and mortality [3, 4]. Therefore, cystic fibrosis patients require therapeutic aerosols to solubilize the sputum and facilitate expectorations [3, 18].

The results of this study suggest that tobramycin is more effective in the solubilization of sputum in secretors. Carriers of this phenotype reached the typical time of solubilization (t_0) faster than non secretor. The difference statistically significant might be resulting from many factors.

Tobramycin is an aminoglycoside antibiotic commonly utilized in cystic fibrosis patients for the treatment of infections by *Pseudomonas aeruginosa* and, as well as its pharmacodynamic effect contributes to solubilization of the pulmonary mucus [19, 20]. One study that analyzed the binding of antibiotics to the sputum of cystic fibrosis patients reported that the degree of binding of tobramycin is dependent on the concentration of macromolecules in the secretion [21]. Analyses of the inhibition of sputum of cystic fibrosis patients by tobramycin demonstrated that the MUC5B mucin

influences the binding of this aminoglycoside to the mucus [22]. More recently, it was observed that the pattern of glycosylation of MUC5B is dependent on the ABO blood group and secretor phenotype [23].

The exocrine secretions of secretor and non secretor phenotype carriers, including pulmonary mucus, are characterized by the present or absence of ABH glycoconjugates, respectively [24]. The ABH glycoconjugates profile produced by carriers of the secretor phenotype is glycosylated by glycosyltransferases coded by the *FUT2* and *ABO* genes [6]. Also, the joint action of these genes create differences in the chemical composition and in the structure of oligosaccharide chains and alters the properties of secretions as they reflect part of the nature of carbohydrates present [6, 7]. Thus, it is possible that ABH oligosaccharides of secretors contribute, at least in part, to the interaction of tobramycin with sputum. Although no pharmacodynamical analysis of this interaction was made in this study, the results indicate a reduction in the typical interaction time of solubilization for carriers of secretor phenotype, but with maintenance of the solubilization interval similar to non secretor carriers.

Alpha dornase is a human recombinant deoxyribonuclease that reduces the viscoelasticity of the sputum by means of fragmentation of high molecular weight DNA molecules released by infiltrating neutrophils [25]. This inhalation drug, used daily by cystic fibrosis patients to facilitate mucous clearance due to ciliary activity, has hydrosoluble properties.

According the results of this study, the distinct action of alpha dornase based on the typical interaction time with the sputum does not seem to be influenced by the secretor or non secretor phenotypes. It is possible that the structural and chemical variability in the ABH glycoconjugates profile do not exert any influence on the solubilization of sputum based in this parameter. However, the solubilization interval of the sputum showed a statistically significant difference between secretors and non secretor phenotype carriers, suggesting that this drug remain more time acting on the sputum from non secretor phenotypes.

The ABH glycoconjugates present in the sputum of secretor phenotype carriers are believed to be derived from the type 1 precursor oligosaccharides, which are fucosylated by FUTII enzyme, as detected on the analysis of the respiratory mucins and different cell types of the human respiratory epithelium [24, 26, 27]. The action of this enzyme creates restrictions in the length of the type 1 precursor but allows diversification by the action of glycosyltransferases coded by the *ABO* gene, at least in gastro intestinal tract [6]. On the other hand, carriers of non secretor phenotypes do not express the same diversity of ABH glycoconjugates as they do not have the functional FUTII enzyme due to the inactivating mutations occurring in exon 2 of the *FUT2* gene

[16]. If the same genetic mechanism controlling the expression of the ABH glycoconjugates in the gastrointestinal tract [28] operates at respiratory tract level, the type 1 precursor oligosaccharidic chains of non secretor individuals tend to present with longer and non-diversified structures as occur in secretors.

The structural differences in the ABH glycoconjugates resulting from the joining action of *FUT2* and *A*, and *B* genes may cause variations in the polarity and hydrosolubility of the sputum from secretors and non secretor and influence the solubilization interval with alpha dornase. Actually, analysis of ABH glycolipids extracted from small intestine secretions revealed the presence of different fractions of these glycoconjugates resulting from the diversity of oligosaccharidic chain lengths [28, 29]. Therefore, it is possible that the structural and chemical variability of the oligosaccharidic chains of non secretor patients contributes to the greater interaction of alpha dornase with the sputum due to the hydro soluble properties of this drug.

The photoacoustic analysis of the sputum of cystic fibrosis patients did not reveal differences statistically significant among the mean values for the typical interaction time and the solubilization obtained from the nebulization with N-acetylcysteine. N-acetylcysteine is a drug derived from the cysteine amino acid; it has a thiol reducing property which favors cleavage of the polypeptidic chains that constitute the mucins of sputum [30]. This process does not seem to be influenced by the presence oligosaccharidic portions of the ABH glycoconjugates in the mucins as a result of glycosylation controlled by the *FUT2* and *ABO* genes. The proteic portions of the mucins seem do not differ between positive and negative secretors and probably would be expected that the typical interaction time and the solubilization interval of N-acetylcysteine with the sputum to be similar. Therefore, it is possible that the structural and chemical variability of the ABH glycoconjugates of secretors and non secretor phenotype carriers did not influence the action of N-acetylcysteine in the solubilization of the sputum. These results are in agreement, at least in part, with those reported by Junqueira and colleagues, which did not find statistically significant differences in the mean values of the same parameters in sputum of cystic fibrosis patients nebulized with hypertonic saline solutions and N-acetylcysteine [14].

The results of this study show that the secretor and non secretor phenotypes influence the typical interaction time of solubilization for tobramycin and the solubilization time for alpha dornase. If these observations could be confirmed in future studies, would be expected to improve the design of therapeutic aerosols toward to adequate the spending time by cystic fibrosis patients using these drugs according their secretor and non secretor phenotypes.

Conclusions

The results of this study show that the secretor and non secretor phenotypes influence the *in vitro* solubilization of the sputum from cystic fibrosis patients nebulized with tobramycin and alpha dornase but not with N-acetylcysteine, when evaluated using photoacoustic technique.

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Table 1. Mean values in minutes and respective standard deviations t_0 and Δt obtained for each aerosol on the sputum samples of secretor and non secretor phenotype cystic fibrosis patients.

	Secretors (N=6)	Non secretors (N=4)	p-value
Tobramycin			
t_0	8.8 ± 5.2	13.7 ± 1.4	0.03
Δt	4.8 ± 2.4	4.9 ± 1.2	0.88
Alpha dornase			
t_0	10.6 ± 5.2	8.1 ± 2.6	0.35
Δt	2.7 ± 1.6	4.8 ± 1.5	0.04
N-acetylcysteine			
t_0	8.4 ± 4.3	12.4 ± 6.6	0.35
Δt	2.4 ± 2.0	2.4 ± 2.1	0.99

