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Improved glutathione status in young adult patients with cystic fibrosis supplemented with whey protein

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Abstract

Background: The lung disease of cystic fibrosis is associated with a chronic inflammatory reaction and an over abundance of oxidants relative to antioxidants. Glutathione functions as a major frontline defense against the build-up of oxidants in the lung. This increased demand for glutathione (GSH) in cystic fibrosis may be limiting if nutritional status is compromised. We sought to increase glutathione levels in stable patients with cystic fibrosis by supplementation with a whey-based protein. **Methods:** Twenty-one patients who were in stable condition were randomly assigned to take a whey protein isolate (Immunocal, 10 g twice a day) or casein placebo for 3 months. Peripheral lymphocyte GSH was used as a marker of lung GSH. Values were compared with nutritional status and lung parameters. **Results:** At baseline there were no significant differences in age, height, weight, percent ideal body weight or percent body fat. Lymphocyte GSH was similar in the two groups. After supplementation, we observed a 46.6% increase from baseline ($P < 0.05$) in the lymphocyte GSH levels in the supplemented group. No other changes were observed. **Conclusion:** The results show that dietary supplementation with a whey-based product can increase glutathione levels in cystic fibrosis. This nutritional approach may be useful in maintaining optimal levels of GSH and counteract the deleterious effects of oxidative stress in the lung in cystic fibrosis.

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1. Introduction

The lung disease of cystic fibrosis (CF) is characterized by a chronic inflammatory reaction. Central to this response is an intense neutrophil recruitment with a release of inflammatory cytokines resulting in an overabundance of oxidants relative to anti-oxidants. It is believed that patients with the mutant CFTR have increased pro-inflammatory cytokines. While the exact mechanism for this has not been elucidated prolonged Nuclear Factor kappa-B (NFkB) activation has been implicated [1]. Treatments that block this activation could be useful in the management of cystic fibrosis. Reactive oxygen species stimulate NFkB activation and increasing the levels of anti-oxidants such as glutathione (GSH) may counteract this [2,3]. In the lung, glutathione

functions as a major front-line defense against oxidants. In a recent study [4] we showed that peripheral circulating lymphocyte glutathione concentration increases proportionately with nutritional status and at the same time inversely with lung function. The increasing demand for glutathione in the face of ongoing inflammation suggests that precursors of glutathione synthesis may be limiting if nutritional status is compromised. In this study we sought to increase lung glutathione levels in stable patients with cystic fibrosis by supplementation with a whey-based protein. We have previously shown in healthy young adults that supplementation with a whey-based protein increased lymphocyte glutathione levels [5]. Studies in rodents demonstrate that lymphocyte glutathione levels reflect lung levels [6,7]. There was no change in the lymphocyte glutathione in subjects given a casein-based diet. The whey-based supplement is richer in cysteine an important precursor of glutathione. Our results show a significant increase in lympho-

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cyte glutathione levels in patients with cystic fibrosis after supplementation.

2. Materials and methods

Twenty-four patients (12M, 12F) were enrolled (13 whey, 11 casein placebo) and twenty-one (9M, 12F) completed the trial (10 whey, 11 placebo). The diagnosis of CF was based upon a positive sweat chloride test (>60 mmol/l) and a compatible history. The patients were stable, i.e. not admitted nor had a course of oral corticosteroids within the previous 2 months. They had mild to moderate lung disease ($FEV_1 >40\%$ predicted), and were not severely malnourished (weight $>75\%$ ideal). CF patients were recruited from the two adult CF centres in Montreal (Montreal Chest Institute & Hôpital Hotel Dieu). After signing informed consents, subjects were randomly assigned to take either a whey protein isolate (Immunocal™, 10 g/dose twice daily) or an equivalent amount of casein placebo for 3 months. Randomization was done with alternating block-of-four and block-of-two design, using numbers from a computer-generated random number table. Both Immunocal™ and casein placebo were provided by Immunotec Research, Vaudreuil, Quebec. Subjects were supplied with a canister containing a 30-day supply. Both whey and casein were provided as a fine white powder with no discernable differences in colour, taste, or texture. Subjects returned each month for a refill of their treatment. The change in weight of the canister was used as an indicator of compliance. Both subjects and principal investigators were blinded as to which supplement was given until the subject completed the trial.

Subjects were studied in the morning, 3–4 h after a standardized breakfast (1 glass of low-fat milk, 2 slices of unbuttered toast with jam, and 1 glass of juice). Subjects underwent the same measures at the beginning and end of the trial.

Subjects in stocking feet had their height measured on a stadiometer, and their weight was recorded while they were lightly clothed and standing on an electronic balance. Weight was also expressed as a percentage of ideal weight for height and age [8]. Triceps and subscapular skinfold thickness were used to assess their percentage of body fat [9]. Expiratory spirometry was performed according to ATS standards, and results reported as percent of predicted values [10,11].

Subjects had 10 ml of blood drawn from an antecubital vein into a heparinized syringe for analysis of lymphocyte GSH concentration on two different days in the same week before and after the study. The mean of the two measurements was used for pre- and post-supplementation comparison.

2.1. Lymphocyte preparation

Blood was diluted in an equal amount of RPMI-1640 medium, and the resultant mixture was placed in a tube

containing 4 ml of Ficoll-Hypaque and centrifuged at 400 g (1400 rpm, IEC-7) for 30 min. The cells at the interface (90% lymphocytes) were removed by pipette and resuspended in 10 ml of 4 °C RPMI-1640 and kept on ice. The suspension was then centrifuged at 450 g (1800 rpm) in a 4 °C centrifuge (IEC-PR6) for 10 min. After removal of the supernatant, the pellet was washed again in cold RPMI-1640. The pellet was resuspended in 4 ml of 1×PBS (pH 7.40), and a 0.2-ml aliquot was removed for automated cell counting (Coulter S-plus JR). The cell count was used to calculate the suspension volume required for a 2×10^6 lymphocyte aliquot.

Aliquots of appropriate volume were then centrifuged in prechilled tubes at 500 g (800 rpm, Eppendorf 5402) for 10 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 970 μ l of cold, distilled water. To this, 30 μ l of 30% 5-sulfosalicylic acid (SSA), were added to make a final concentration of 0.9% SSA, and the solution was incubated for 15 min on ice. The solution was then centrifuged at $5000 \times g$ (8000 rpm, Eppendorf 5402) for 10 min at 4 °C. The supernatant was removed and stored at -70 °C for later analysis of GSH.

2.2. GSH analysis

Total GSH in the 0.9% SSA extract was determined by the glutathione reductase recycling method of Tietze [12] and adapted for the Cobas Mira spectrophotometer (Roche Diagnostics) [13]. Briefly, the Cobas Mira pipettes 210 μ l NADPH (0.3 mmol/l), 30 μ l 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (6.0 mmol/l), and 95 μ l of sample, standard, or 0.9% SSA into cuvettes. After a 4-min incubation at 37 °C, 15 μ l glutathione reductase (1.0 U/100 μ l) are added, and the reaction is monitored every 24 s for 12 min. Under these conditions, the method is linear for GSH concentrations between 0 and 6 μ mol/l. The instrument constructs a calibration curve by assaying known GSH standards, and from this the GSH concentration of the unknown is evaluated. Reproducibility for GSH at these concentrations is $<2\%$ (intra-assay coefficient of variation). Laboratory control mean value ($n=9$) is 1.55 nmol/ 10^6 lymphocytes, with a range of 1.17–2.17 nmol/ 10^6 lymphocytes.

2.3. Data analysis

Statistical analysis was performed by using Statistica 5.1 for Windows (Statsoft, Tulsa, OK). Data were expressed as means \pm S.D. The groups' baseline data were compared by unpaired *t*-testing. Changes from baseline within each group were assessed by paired *t*-testing, and the changes between the groups by unpaired *t*-testing. The study was powered (80%) to detect a 30% increase in lymphocyte GSH levels in the whey-supple-

Table 1
Baseline characteristics of patients

	Supplemented	Placebo
Age (years)	25.5±6.35	24.27±3.95
Height (cm)	165±9.04	168±8.24
% Body fat (%)	17.7±6.03	17.3±7.23
% IBW (%)	91.3±12.85	86.6±9.45
Weight (kg)	57.3±11.77	58.2±7.72
FEV ₁ (% predicted)	65.9±19.41	70.5±21.17
Lymphocyte GSH (nmol/10 ⁶ cells)	1.62±0.32	1.91±0.47

Values are means ±S.D.; supplemented and placebo groups were administered Immunocal 20 g/day or casein, respectively.

mented group. A *P* value <0.05 was considered as significant.

3. Results

Results for the 21 patients (10 whey, 11 placebo) completing the trial are reported. The three whey patients were dropped from the analysis because of loss to follow-up (1), non-compliance (1), and infection and pneumothorax requiring in-hospital intravenous antibiotics (1). At baseline there were no significant differences in age, height, weight, percent ideal body weight, or percent body fat (Table 1). Lymphocyte GSH levels was also similar in the two groups. Mean values for GSH at baseline for the two baseline days were 1.60±0.29 and 1.63±0.38 (average 1.62±0.32) nmol/10⁶ cells for the whey group, and 1.82±0.46 and 2.00±0.65 (average 1.91±0.47) nmol/10⁶ cells for the casein group. The results in Table 2 show a significant increase (46.6%) in the lymphocyte GSH levels (2.14±0.37 and 2.32±0.88, average 2.23±0.55 nmol/10⁶ cells). No such changes were seen with the placebo group (2.02±0.90 and 1.92±0.83, average 1.97±0.61 nmol/10⁶ cells). There was no significant change in %body fat, weight or lung function in either of the groups

4. Discussion

We have demonstrated that ingestion of a native whey supplement at a dose of 10 g twice daily resulted in a 46.6% increase in circulating lymphocyte GSH concentration. Lymphocyte GSH was used as a marker of tissue GSH as evidence from animal studies suggested that this could track tissue levels in response to both L-2-oxothiazolidine-4-carboxylic acid, a cysteine precursor [6], and buthionine sulfoxamine, an inhibitor of the first, rate-limiting step in GSH synthesis, γ -glutamylcysteine synthase [7].

The lung inflammation of cystic fibrosis is associated with an increased oxidative stress and this places demands on the use of GSH. In a previous study [4] we demonstrated that lymphocyte GSH levels varied

inversely with lung function and proportionately with nutritional status. This suggested that in response to oxidative stress the respiratory epithelium upregulates GSH production, both de novo synthesis and recycling, with a resultant increase in intracellular levels of GSH. The de novo synthesis of GSH demands an increased supply of precursors, most notably cysteine, which is considered to be rate limiting [14]. When nutritional status is compromised, less cysteine may be available for GSH synthesis. Here we demonstrate that GSH levels can be increased with the supplementation of a cysteine rich whey protein.

Patients with cystic fibrosis have decreased levels of glutathione in the extracellular lining fluid. CFTR has recently been implicated in GSH efflux [15]. Cells containing defective CFTR secrete less GSH than cells with wild-type CFTR. Although the CFTR defect results in lower efflux of glutathione, the extracellular lining fluid (ELF) of CF patients does contain glutathione suggesting that this may not be the only mechanism of export [16]. Glutathione can also be metabolized in the ELF by increased γ -glutamyl transpeptidase activity [17]. While inhibition of γ -glutamyl transpeptidase increased the GSH in the apical fluid, it did not completely restore the levels to that of the cells transfected with CFTR [16]. Although we did not measure glutathione in the sputum, increased level of lung tissue glutathione in the CF patients after whey supplementation may serve to increase the ELF levels.

Recently, Velsor et al. [18] have shown that both uninfected CFTR-KO mutant and wild-type mice had similar levels of glutathione in lung tissue but that the CFTR mutant had lower glutathione levels in ELF fluid. Unlike CF patients these animals lack spontaneous lung inflammation. Nonetheless there was increased tissue lipid oxidation and oxidation of DNA bases in the lung tissue of the CFTR-KO mice implying that the CFTR gene defect contributes to the oxidative stress [18]. This is reminiscent of the clinical situation wherein there is evidence that the CF lung epithelium may be pro-inflammatory [19].

In this study we did not observe any difference in effect on lung function in the two groups of patients. This suggests that the increased lung tissue GSH levels did not reverse the effects. Most likely it arrests the further damage but a study of longer duration is needed

Table 2
Percent change from baseline

	Supplemented	Placebo
% Body fat	5.34±7.07	1.4±7.23
Weight	0.03±3.2	0.2±2.84
FEV ₁	-7.0±9.23	4.0±15.33
Lymphocyte GSH	46.6±55.1*	5.0±28.9

* Significantly different from baseline, *P*<0.05.

to assess this. These results are similar to the experience with high-dose ibuprofen [20] whereby adults with well established disease did not see any changes in lung function, while children had a dramatic slowing of their disease progression. Preliminary work in a murine model suggests that use of whey prior to significant lung injury may be particularly beneficial.

The study was limited by the sample size, which was chosen to demonstrate a 30% increase in lymphocyte GSH in the whey-supplemented group. This sample size did not allow us to look at other factors that could change with glutathione augmentation, such as inflammatory markers and body composition. However, it was important to show that, like healthy young adults, adult patients with CF can have their glutathione stores increased, using nutritional supplementation. We still need to examine how such increases in glutathione would be beneficial in CF. With the use of cell lines, we are now examining the effect of novel whey proteins on downregulating the inflammatory response in lung epithelia with either wild-type or mutant CFTR. We will also continue our work with a murine model of CF lung disease.

In conclusion, we have shown that dietary supplementation with a whey-based product can increase lymphocyte glutathione levels in patients with cystic fibrosis. Since augmenting GSH levels by systemic administration of GSH is limited because of rapid clearance and short plasma half-life [21,22] this nutritional approach can be useful in maintaining optimal levels of GSH in these patients and potentially counteract the deleterious effects of oxidative stress in cystic fibrosis.

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