

Novel method to process cystic fibrosis sputum for determination of oxidative stress

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Supplementary data

There are no temperature driven effects on determination of GSH

To exclude temperature driven effects of DTT on GSH measurements four induced sputum samples of cystic fibrosis patients were processed a) by the presented method with solely D-PBS, b) with DTT at 37 °C, and c) DTT on ice. Detailed description of processing steps is given in the methods section of the manuscript. The effect of DTT on GSH measurements was similar for both temperature conditions studied. Statistical analyses were performed with the Kruskal-Wallis test with Dunn's multiple comparison post-hoc test.

Free GSH concentrations and ratio of free / total GSH as assessed by RP-HPLC was significantly higher in DTT treated samples compared to samples processed with the mechanical method ($p < 0.05$) (Supplementary Figure 1).

Intracellular GSH levels as measured by flow cytometry were higher in DTT treated samples compared to samples without DTT and was significant for epithelial cells ($p < 0.05$). For neutrophils, the difference did not reach statistical significance which is most likely due to small numbers of samples. Data for neutrophils and epithelial cells are presented. The number of lymphocytes was too low to be measured in all samples processed for these experiments. For both temperature conditions, intracellular GSH levels were similarly high in DTT treated samples (Supplementary Figure 2).

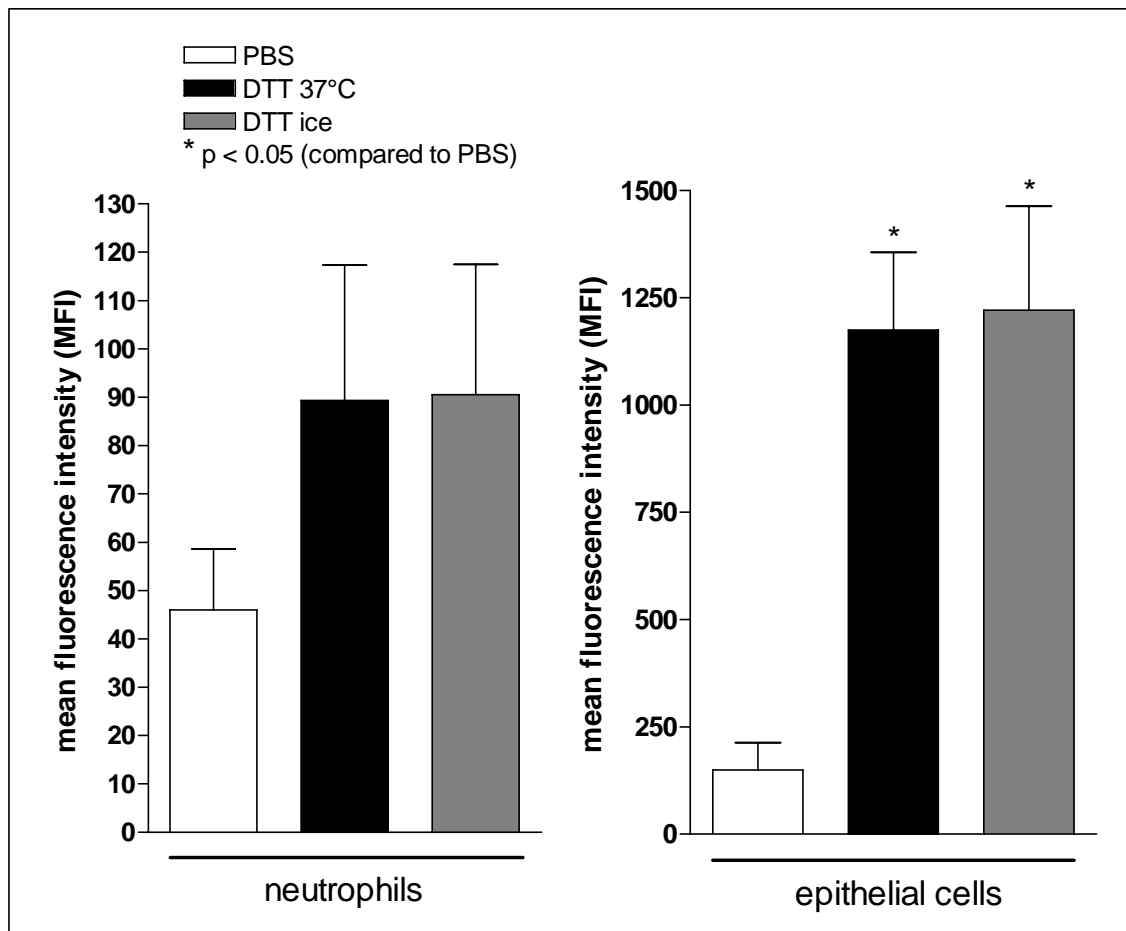
There is no effect of DTT on inflammatory markers

In a previous study [1], we reported that DTT had no significant effect on measurements of inflammatory markers. Induced sputum samples of cystic fibrosis were obtained with the same protocol as described in the methods section of the present study. Induced sputum samples were either homogenized 1:1 with DTT or 1:1 with D-PBS. Inflammatory markers were analyzed in supernatant by a sandwich ELISA according to the manufacturer's instructions (R & D Systems). The sputum processing method described in our previous study is not exactly the same as the method used in the present study. However, taken together with the extent review of Kelly MM et al [2] DTT is unlikely to have a significant effect on cytokines and chemokines included in the previous study.

GSH measurement by RP-HPLC

GSH levels were assessed in sputum supernatants by RP-HPLC. The addition of DTT for sputum processing resulted in about 4 times higher area under curve values compared to the mechanical method regardless of the temperature applied in DTT treated samples (Suppl. Fig. 4 A-C). Because it was previously reported that DTT produces unspecific byproducts in combination with SBD-F [3] we performed blank runs with and without DTT (Suppl. Fig. 4 D and E). Unspecific peaks appeared after retention times of about 6.5 - 6.8 and 7.8 - 8.5 minutes (Suppl. Fig. 4 E). The peak of GSH appeared after 8.6 - 8.9 minutes (Suppl. Fig. 4 A-C). Therefore, the by-products should not or only minimally interfere with our GSH measurements.

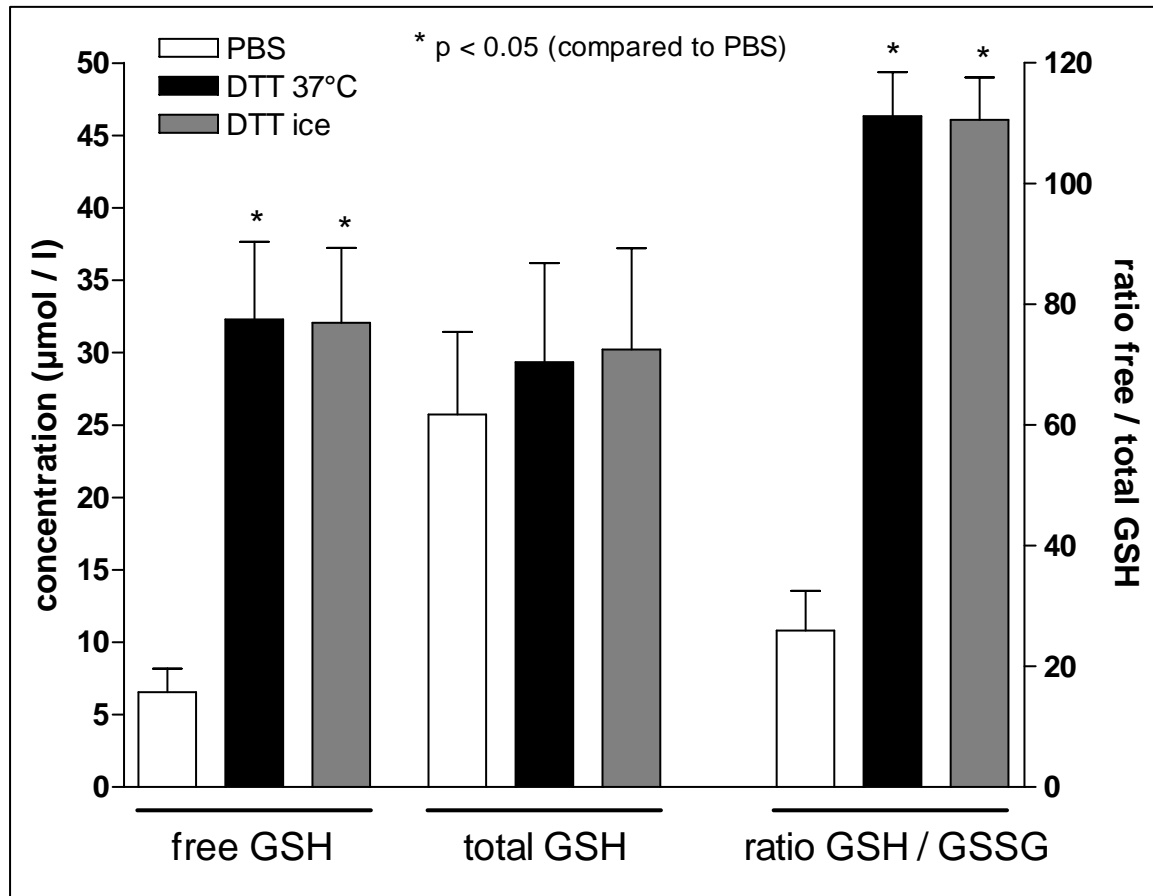
Supplementary Figures



Suppl. Fig. 1. Intracellular glutathione levels

Supplement Figure 1

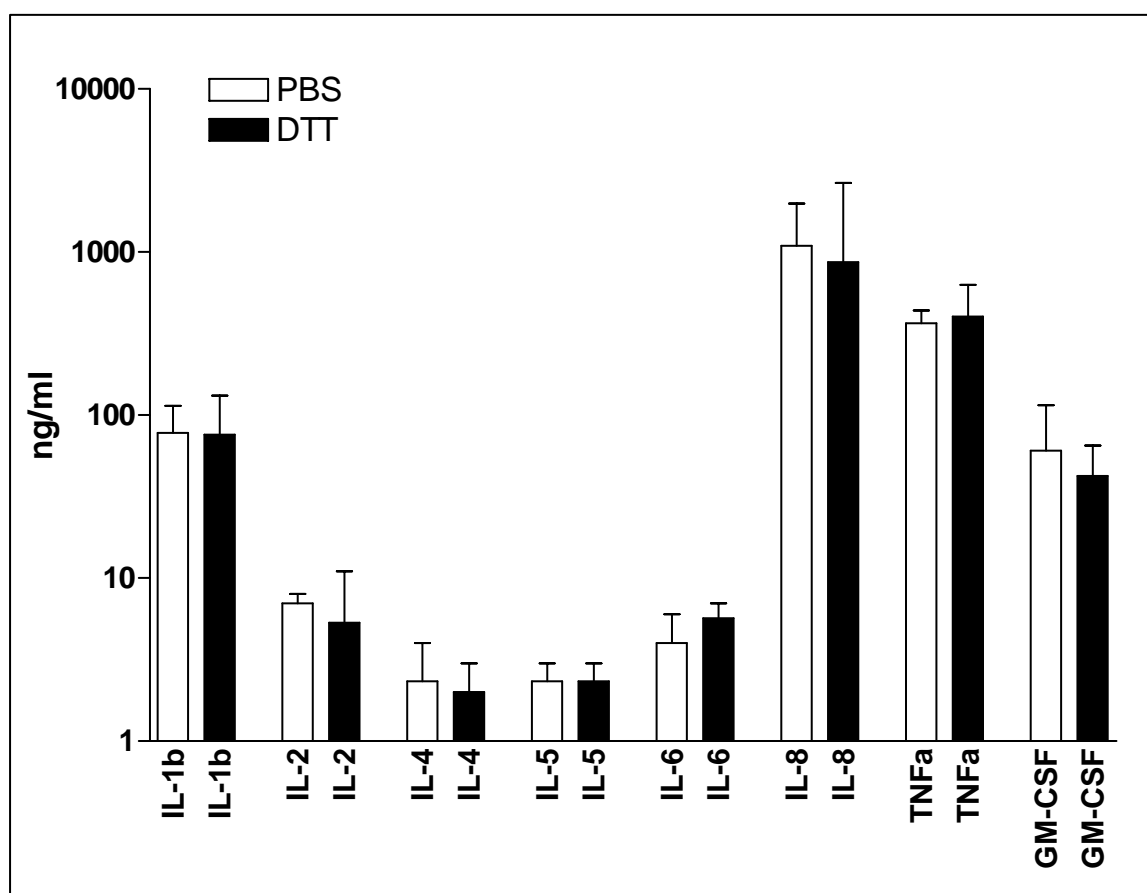
Intracellular glutathione levels. Mean fluorescence intensity (MFI) of intracellular GSH in neutrophils and epithelial cells are given for comparison of sputum processing methods a) with the mechanical method (white columns), b) with DTT at 37 °C (black columns), and c) with DTT on ice (grey columns). Intracellular GSH levels were higher in DTT treated samples compared to the mechanical method and was significant for epithelial cells ($p < 0.05$) ($n=4$).



Suppl. Fig. 2. Extracellular glutathione levels

Supplement Figure 2

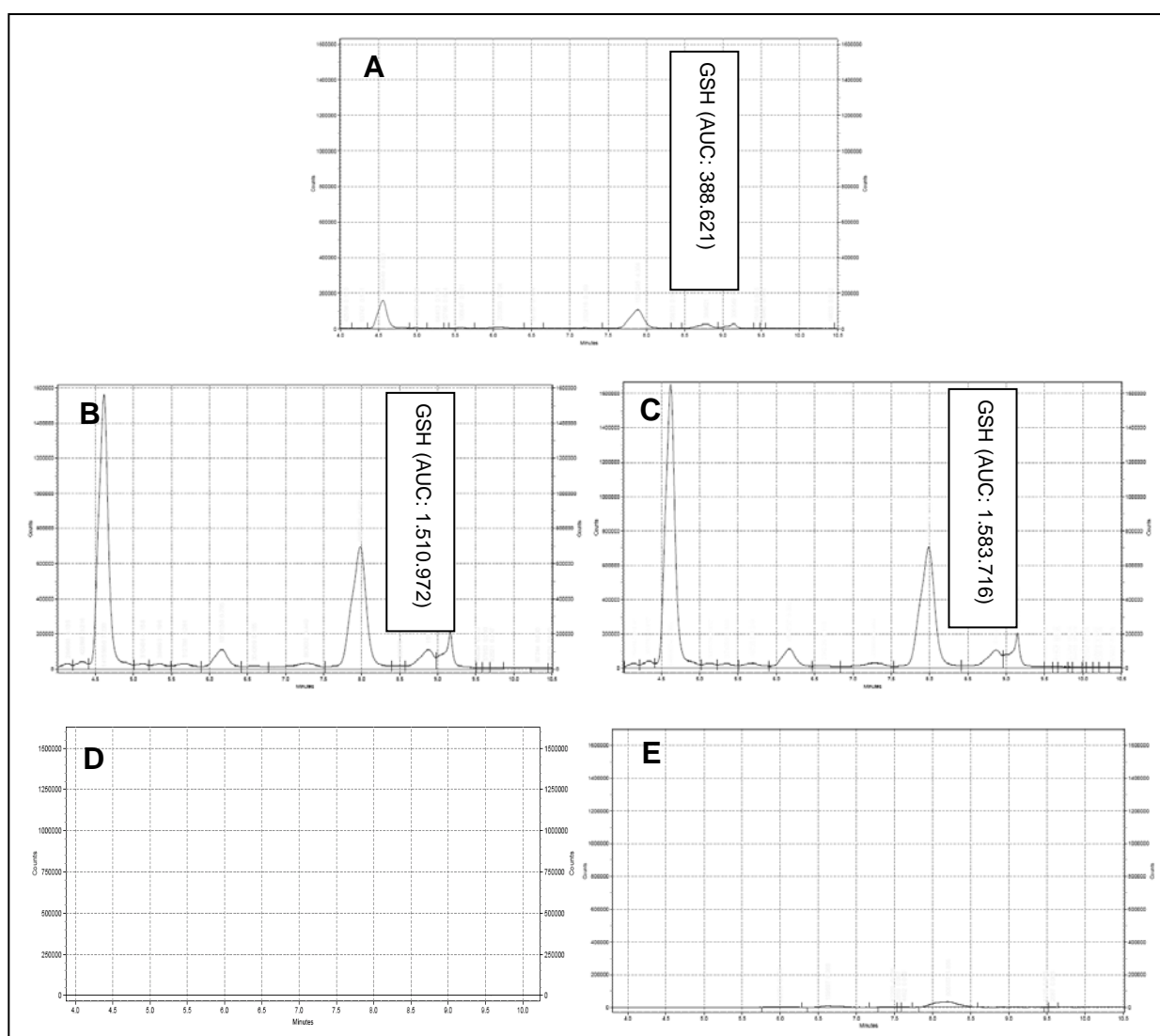
Extracellular glutathione levels. Extracellular GSH levels are given in $\mu\text{mol} / \text{l}$ for induced sputum samples processed a) with the mechanical method (white columns), b) with DTT at 37°C (black columns), and c) with DTT on ice (grey columns). Free glutathione and ratio of free / total glutathione was significantly higher in DTT treated samples regardless of the temperature conditions compared to samples processed with the mechanical method ($p < 0.05$). For both temperature conditions, extracellular GSH levels were similarly high in DTT treated samples ($n=4$).



Suppl. Fig. 3. Inflammatory markers

Supplement Figure 3

Inflammatory markers. Data are from our previous study [1]. Induced sputum samples of cystic fibrosis were obtained with the same protocol as described in the methods section of the present study. Induced sputum samples were either homogenized 1:1 with DTT or 1:1 with D-PBS. Inflammatory markers were analyzed in supernatant by a sandwich ELISA according to the manufacturer's instructions (R & D Systems). For measured cytokines and chemokines there was no significant difference between sputum samples treated with DTT or D-PBS.



Suppl. Fig. 4. GSH measurement by RP-HPLC

Supplementary Figure 4

GSH measurement by RP-HPLC. Representative RP-HPLC runs of CF induced sputum samples processed with the novel mechanical method (A), with DTT at 37 °C (B) and DTT on ice (C) are shown. Area under curve values were about 4 times higher in DTT treated samples compared to the PBS method (A-C). Because it was previously reported that DTT produces unspecific byproducts in combination with SBD-F [3] we performed blank runs with

(E) and without (D) DTT. Unspecific peaks appeared after retention times of about 6.5 - 6.8 and 7.8 - 8.5 minutes (E). The peak of GSH appeared after 8.6 - 8.9 minutes (A-C).

Reference List

1. Griesse, M., Latzin, P., Kappler, M., Weckerle, K., Heinzlmaier, T., Bernhardt, T., and Hartl, D. Alpha1-Antitrypsin Inhalation Reduces Airway Inflammation in Cystic Fibrosis Patients. *Eur.Respir.J.* 2007;29(2):240-50.
2. Kelly, M. M., Keatings, V., Leigh, R., Peterson, C., Shute, J., Venge, P., and Djukanovic, R. Analysis of Fluid-Phase Mediators. *Eur.Respir.J.* 2002;20(Suppl.37):24s-39s.
3. Kuhn, K. S., Krasselt, A. I., and Furst, P. Glutathione and Glutathione Metabolites in Small Tissue Samples and Mucosal Biopsies. *Clin.Chem.* 2000;46(7):1003-5.