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Antioxidant-related Parameters in Patients Treated for Cancer Chemoprevention With *N*-Acetylcysteine

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***N*-acetylcysteine (NAC) is an antioxidant, possibly effective in the early steps of carcinogenesis, and is applied to prevent second primary tumours in the upper aerodigestive tract and the lungs. In this study, we evaluated the pharmacodynamic profile of 600 mg NAC treatment, given daily for 3 months. Treatment caused a significant increase of the non-protein-SH concentration in blood plasma (38%) and erythrocytes (31%). Glutathione levels in exfoliated buccal mucosa cells appeared not to be influenced by treatment. The total radical-trapping ability parameter (TRAP) of blood plasma showed no change. *In vitro*, the addition of glutathione, but not of NAC did increase the TRAP value. In addition, when peroxyl radicals were generated *in vitro*, NAC was shown to be consumed more rapidly than glutathione. This suggests that NAC prevents early damage, while glutathione functions over a longer time period.**

Key words: *N*-acetylcysteine, antioxidant, chemoprevention, glutathione, thiol
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INTRODUCTION

THE OCCURRENCE of second primary tumours (SPT) is the major cause of treatment failure in early stage, radically treated, head and neck squamous cell carcinoma (HNSCC) patients [1]. Therefore, the interruption of the carcinogenic process by chemopreventive drugs seems an attractive strategy to prevent the occurrence of SPTs.

N-acetylcysteine (NAC) is a thiol that is intracellularly deacetylated to cysteine that efficiently supports glutathione (GSH) biosynthesis [2, 3], and is currently being clinically tested as a chemopreventive agent. NAC and GSH function as antimutagenic and anti-carcinogenic agents. Properties of these compounds can be ascribed to multiple protective mechanisms, such as the scavenging of reactive oxygen species and the modulation of detoxification and DNA repair processes [4–6]. Prevention of carcinogenesis by NAC has been shown *in vitro* [4] and *in vivo* [5, 6]. Based on these preclinical data, NAC was chosen in the EUROSCAN trial, a prospective chemoprevention study in curatively treated patients with early stage oral, laryngeal and lung cancer [7].

A serious limitation of chemoprevention studies is the long follow-up time needed for the assessment of the efficacy of chemopreventive agents. If good biomarkers for the prediction and monitoring of therapy response were available, we could test many more chemopreventive agents, in less time, with fewer patients in future. For NAC, both systemic and site (the tissue where tumours may develop)-specific markers, related to antioxidant activity would be relevant. Moreover, solid data on the pharmacodynamic profile of NAC in humans are scarce.

PATIENTS AND METHODS

From 10 patients, blood and exfoliated cells were taken before and after 3 months of 600 mg NAC therapy daily on an empty stomach. NAC (Fluimucil®), was kindly donated by Zambon (Milan, Italy). Blood was obtained not more than 2 h after NAC intake using a heparinised tube. Exfoliated cells were collected by scraping the buccal mucosa with a Cytobrush (Medscand AB, Malmö, Sweden) and washed in phosphate-buffered saline. Both samples were stored in nitrogen at −70°C until use. The patients had been cured for early stage oral cavity ($n = 2$) and laryngeal ($n = 8$) cancer, classified as T_1 and T_2, N_0 and M_0 by the TNM classification from 1987 [8]. There were seven males and three females with a median age of 66 years (range 52–81). 3 patients had never smoked, and the others had smoked between 11 and 90 pack-years. A pack-year is defined as the number of years multiplied by the number of cigarette packs smoked daily, assuming that one pack contains 25 cigarettes.

Collected plasma samples were analysed with the total radical-trapping ability parameter (TRAP) assay [9]. This assay reflects the synergistic protection of various antioxidants against the biological damage caused by peroxyl radicals in blood plasma. Ascorbate was measured by HPLC [10] and uric acid was assayed enzymatically [11]. GSH in plasma was measured by HPLC

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using precolumn derivatisation with orthophtalaldehyde and fluorometric detection [12]. Non-protein thiol in erythrocytes was determined according to Beutler and colleagues [13]. The glutathione (reduced and oxidised GSH) determinations on exfoliated cells of the buccal mucosa were performed enzymatically using a sensitive flow injection analysing system [14].

Statistical analysis was performed by the paired Student's *t*-test.

RESULTS

The GSH concentration in plasma after a 3-month treatment period of 600 mg NAC increased significantly ($P < 0.001$) by 38%. The non-protein SH levels in erythrocytes also increased significantly ($P < 0.001$) by 31% (Table 1). In erythrocytes, an upper limit of approximately 2 mM seemed to be attained. The TRAP value of plasma, uric acid levels and ascorbate levels showed no significant changes after this treatment period (Table 1). The GSH concentrations in exfoliated cells of the buccal mucosa were around the detection limit. In six of 20 samples, the levels were below the detection limit (50 pmol/cell pellet). The available data did not indicate an effect of NAC treatment. In most cases, a change in GSH levels was observed. The day-to-day variation, measured in a series of control persons, showed a similar variability (Table 2). Oxidised GSH could not be detected in any of the samples.

To explain the lack of effect of NAC treatment on TRAP values, we extended our experiments by adding relatively high concentrations of NAC and GSH *in vitro* to plasma of untreated patients. The mean TRAP value of untreated plasma was 786 μM ($n = 2$), 795 μM and 794 μM after addition of 100 and 200 μM NAC, respectively ($n = 5$). Despite repeated measurements, no marked alterations were observed after NAC addition, but the TRAP increased to 825 and 886 μM after adding 100 and 200 μM GSH, respectively ($n = 2$), as was shown in earlier studies [9]. *In vitro* incubations of the dithiols, dihydrolipoic acid [TRAP value with 100 μM: 798 μM ($n = 2$), with 200 μM: 782 μM ($n = 2$)] and dithiotreitol [TRAP value with 100 μM: 779 μM ($n = 2$), with 200 μM: 788 μM ($n = 2$)] to blood plasma did not increase the TRAP value. The assays were performed in the same individuals. However, an explanation for this phenomenon was found after addition of GSH to heated plasma. Plasma heated for 2 min at 90°C prevented increase of the TRAP value after GSH addition. The TRAP value of untreated plasma was 546 μM ($n = 2$), and 531 and 548 μM when 100 and 200 μM GSH were added, respectively ($n = 2$). To determine which enzymatic pathway was activated by GSH, we subsequently added catalase to heated plasma.

Table 2. Glutathione values during follow-up in exfoliated buccal mucosa cells of cancer patients and healthy individuals

	GSH concentration in exfoliated cells (pmol/mg protein)	
	Day 1	Day 10
Control no.		
1	457 ± 157*	208 ± 11*
2	187 ± 68	208 ± 78
3	47 ± 12	80 ± 38
4	134 ± 43	153 ± 27
5	151 ± 28	103 ± 9
Patient no.		
1	113 ± 16	48 ± 15
2	u.d.l.	u.d.l.
3	53 ± 0	u.d.l.
4	93 ± 6	147 ± 31
5	u.d.l.	u.d.l.
6	154 ± 29	412 ± 35
7	52 ± 6	69 ± 3
8	u.d.l.	112 ± 20
9	69 ± 18	51 ± 26
10	62 ± 67	150 ± 14

*Mean value of two samples with S.D.
u.d.l., under detection limit.

Interestingly, catalase did not increase the TRAP value (546 versus 533 μM).

Further studies on thiol concentrations during *in vitro* incubation of plasma with 2,2'-azobis(2-amidinopropane). HCl (ABAP), to generate free radicals and NAC or GSH showed that NAC was detectable within 15–20 min, whereas GSH could be measured over more than 1 h, covering the whole period of the TRAP assay (Figure 1).

Spontaneous oxidation of non-protein-SH groups in the plasma samples with buffer, NAC or GSH, in non-ABAP-exposed control samples was nil.

DISCUSSION

One of the mechanisms that supports the choice of NAC as a cancer-preventing agent is its ability to scavenge free oxygen radicals [6]. The TRAP assay has yielded useful information on the protective role of the synergistic action of various endogenous antioxidants, particularly against peroxyl radicals. The TRAP values of this patient population after radical treatment for their

Table 1. Important antioxidant-related parameters before and after 3 months of treatment with N-acetylcysteine

	Erythrocytes NP-SH* (mM)		Plasma							
			TRAP* (μM)		GSH* (μM)		Ascorbic acid (μM)		Urate (μM)	
	Pretreatment	3 months†	Pretreatment	3 months	Pretreatment	3 months	Pretreatment	3 months	Pretreatment	3 months
Mean‡	1.40	1.83	1068	1032	3.2	4.4	13	16	410	413
S.D.‡	0.33	0.19	436	400	0.9	1.6	7	10	56	78

NP-SH, non-protein-bound SH groups.*Samples determined in duplicate. †Samples after 3 months NAC treatment. ‡Based on measurements in 10 patients.

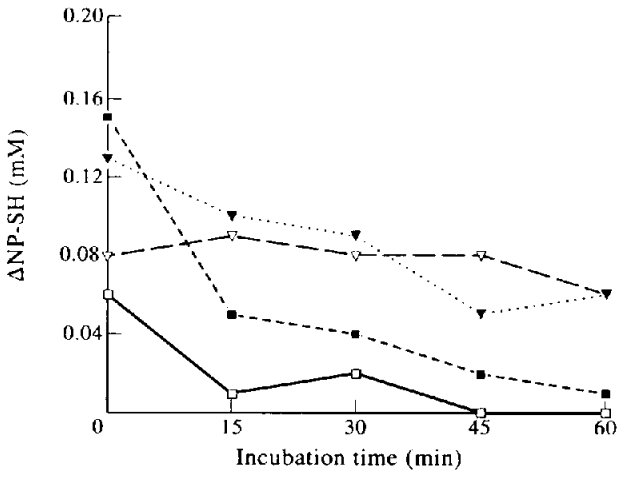

Figure 1. *In vitro* exposure of a blood plasma sample to 2,2'-azobis(2-aminopropane). HCl (ABAP) with addition of 100 μM *N*-acetylcysteine (NAC) (□), 100 μM glutathione (GSH) (▽), 200 μM NAC (■) or 200 μM GSH (▼). NP-SH was measured every 15 min for 1 h. Δ non-protein-bound SH reflects the difference between the samples with NAC or GSH addition and the control sample, without drug. The coefficient of variation was less than 6%; determinations were performed in duplicate.

HNSCC showed a broad range (1068 ± 436 μM), but did not differ significantly from a population of healthy volunteers described previously [9]. In the present study, NAC treatment did not lead to significant changes in the TRAP values. Because of the large contribution of uric acid and ascorbate to the TRAP [11], we could exclude possible interference of changes of these metabolites by measuring their concentrations in the plasma samples (Table 2). A possible explanation for the lack of effect of NAC on the TRAP is that the relative contribution of GSH and NAC to the TRAP is too small to lead to a change (1 mole GSH can trap 0.3 mole peroxyl radicals) [9]. Apparently, NAC traps peroxyl radicals with an efficiency like GSH, and this system does not allow the detection of the relatively small increase in antioxidants in human blood plasma, as observed in this study. However, the GSH reservoir in erythrocytes increased in most cases (Table 1), which may also physiologically contribute to antioxidant delivery into blood plasma.

To test the ability of NAC to act as a precursor of GSH, GSH levels before and after NAC treatment were determined. GSH is an important intracellular defence system against reactive oxygen species. The GSH concentration in plasma after NAC therapy increased significantly. Our measurements of non-protein-bound thiols in erythrocytes, which gives a reflection of the intracellular whole body situation, also showed a significant increase, and supports the theory that most of the non-protein-bound thiols are stored in the erythrocytes. In exfoliated cells, changes by NAC treatment were seen, but these were insignificant because in controls, there was wide intra-individual variation so no definitive conclusion could be drawn (Table 2). However, it can be concluded that the available data did not show a consistent effect of NAC treatment.

To explain the lack of effect of NAC treatment on TRAP values, we extended our TRAP experiments by adding relatively high concentrations of NAC and GSH to plasma of untreated patients in the test tube. Despite repeated measurements, no marked alterations were observed after NAC addition, but the TRAP increased after GSH addition. *In vitro* addition of the dithiols dihydrolipoic acid and dithiothreitol to blood plasma did not increase the TRAP value. An explanation for this phenomenon was found after addition of GSH to heated plasma. Heating of the plasma prevented the increase of the TRAP value following GSH addition, suggesting that the GSH effects on TRAP may be enzyme mediated. Two glutathione-dependent peroxidases could be involved, i.e. the Se-dependent and Se-independent forms. Both organic and inorganic hydroperoxides can be reduced in this way. Interestingly, catalase did not increase the TRAP value. This suggests that the GSH-dependent increase of the TRAP value occurs via enzymatic reduction of organic hydroperoxides.

Our further *in vitro* experiments showed that thiols added to a blood plasma sample are rapidly consumed after peroxidation exposure (Figure 1). In these *in vitro* studies, NAC is more rapidly consumed than GSH, which suggests that NAC prevents early damage while GSH functions over a longer period.

The present study in a relatively small group of patients shows a possible beneficial effect of NAC. No relevant changes in GSH levels could be detected in the target tissue, the exfoliated cells of the buccal mucosa. Therefore, the GSH levels in exfoliated cells are not a useful parameter for monitoring chemoprevention efficacy. However, the thiol status in blood plasma and erythrocytes for the efficacy of thiol-containing chemopreventive agents increased and may have a beneficial effect on the oxidative stress status. The next step will be to use these parameters as biomarkers to predict the occurrence of SPTs. But this can only be assessed after evaluation of the ongoing clinical trial.

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