# The effect of dietary supplementation of N–acetyl–L–cysteine on glutathione concentration and lipid peroxidation in cigarette smoke–exposed rats fed a low–protein diet

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## ABSTRACT

**Objectives:** The aim of the study is to investigate the modulatory effects of dietary supplementation of N-acetyl-L-cysteine (NAC) on glutathione (GSH) concentration in liver and lung, and lipid peroxidation in cigarette smoke-exposed rats fed a low-protein diet.

**Methods:** Rats were divided randomly into 4 dietary groups; 8 per group. The control group (Group 1) was fed a normal-protein diet and received room air as a sham-smoke exposure. Group 2 was fed a normal-protein diet, Group 3 was fed with a low-protein diet and Group 4 was fed with a low-protein diet supplemented with NAC, and exposed to the smoke of 10 cigarettes/hour/day until the end of the experiment (4 weeks) period. Glutathione in liver and lung and serum albumin level were measured. Also, thiobarbituric acid reactive substances (TBARS) were measured, as an indication of oxidative stress. The study was conducted in the College of Applied Medical Sciences, King Saud University, Riyadh, Kingdom of Saudi Arabia, in the year 2003.

**Results:** Smoke-exposed rats fed with the low-protein diet had significantly lower hepatic GSH concentration compared to other dietary groups. Moreover, NAC supplementation to the low-protein diet, in the smoke-exposed rats, significantly increased hepatic GSH concentration compared to the corresponding animals fed the same amount of protein, but without NAC supplementation. No reduction in lung GSH concentration occurred in cigarette-smoke rats fed a low-protein diet supplemented with NAC. Cigarette smoking significantly increased the level of TBARS in serum in all dietary groups compared to the control. However, the elevation in the TBARS level was higher in the low protein dietary group.

**Conclusion:** The results show no significant reduction in the lung and hepatic GSH concentration in cigarette smoke-exposed rats fed a normal-protein diet compared with the corresponding control rats fed a same level of protein. The study indicates the efficiency of NAC supplementation in scavenging free-radicals and enhancing GSH concentration in smoke-exposed rats fed a low-protein diet.

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It is well documented that cigarette smoking is a major risk factor for many chronic diseases, such as cancer, chronic bronchitis, chronic obstructive, pulmonary disease, and cardiovascular disease.[1-3]

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Smoke contains high levels of free radicals.[4,5] One way in which cigarette smoking may cause damage to the body is by producing oxidant molecules. Consequently, imbalance between oxidant and antioxidant redox occurs initiating damage to the lung, the main organ affected by smoking, and to the other tissues of the body. It is well documented that oxidant molecules cause cell injury by damaging cellular components, such as lipid membranes (such as initiating lipid peroxidation), proteins (such as decrease enzyme activity and inactivation of protease inhibitors), and DNA (such as causing DNA mutation, damage to DNA-repairing enzymes and polymerases).[6-10] Furthermore, in response to an inflammatory stimulus, phagocytes and neutrophils are activated, and produce superoxide, hydrogen peroxide, hydroxyl radical, and hypochlorous acid causing further injury to the body cells.[11,12]

Oxidant molecules have also been shown to enhance cytokine production via activation of the nuclear transcription factor (NFkB).[13,14] Excessive or overproduction of cytokines has been associated with morbidity and mortality in a wide range of diseases, such as malaria, asthma, cancer, meningitis, inflammatory bowel disease and rheumatoid arthritis.[15-18] Furthermore, excessive production of cytokines has been shown to have a role in the pathogenesis of multiple sclerosis, atherosclerosis, and Alzheimer's disease.[19-21]

Kim et al[22] have shown that serum thiobarbituric acid reactive substances concentrations, as an indication of oxidative stress, were higher in teenage-smoker girls compared to non-smokers. Malondialdehyde levels in plasma, a free-oxygen radical, increased dramatically, 151% higher in rats exposed to cigarette smoke compared to rats inhale clear air as a control.[23]

Thus, the presence of antioxidant defenses, such as glutathione (GSH), which is one of the major antioxidant compounds within the cell, could play a major role in protecting the body from the toxicity of oxidant molecules. Maintaining adequate amounts of intracellular, GSH could help in protecting the body from the damaging effect of oxidant molecules in 2 ways. Firstly, by restoring the balance of the antioxidant and oxidant redox (such scavenging reactive oxygen species and convert them to non-harmful substances). Secondly, by reducing the ability of oxidant molecules to enhance the activity of the NFkB, thus it helps to regulate the cytokine production. The liver is a major site for GSH synthesis and distribution to other organs.[24] Glutathione consists of 3 amino acids, namely cysteine, glycine and glutamate, of which cysteine is the most limiting amino acid for GSH synthesis.[25] Because GSH is mostly degraded in the extracellular compartment, due to the presence of the enzyme g-glutamyl transpeptidase, GSH cannot be taken up by cells directly.[26] Thus, several compounds have been used as precursors for GSH synthesis. N-acetyl-L-cysteine (NAC), a cysteine-delivery agent, is considered to be one of the best precursors for GSH synthesis.[27]

The aim of the study is to investigate the modulatory effects of dietary supplementation of NAC on GSH concentration in liver and lung, and lipid peroxidation in rats fed a low-protein diet and exposed to cigarette smoke.

Methods. All diets were prepared by mixing dry ingredients in a mixer; this was followed by addition of maize oil. Water was added to these diets to make it into small biscuits, which were dried in oven at 80$^0$C for up to 48-72 hours. All groups were fed casein as the sole dietary protein source. The casein powder contained 88% protein. The amount of sulfur amino acids in casein was 2.76 gms methionine and 0.43 g cystine/100 gms casein as determined by high pressure liquid chromatography.

Animal procedures were conducted in accordance with legislation laid down by the College of Applied Medical Sciences, King Saud University, Riyadh, Kingdom of Saudi Arabia (KSA). Thirty-two male-Wistar rats were obtained from the Experimental Animal Care of King Saud University. Animals were housed individually in cages and maintained at 22 ± 1$^0$C with a 12-12 hour light-dark cycle (lights on at 7:30 am), and were fed a standard laboratory food (rat chow) for 4 days. After 4-days adaptation period, rats were divided randomly into 4 dietary groups of 8 animals, each was based on a different dietary regime. Experimental animals were transferred daily to a ventilated glass chamber and exposed to the smoke of 10 cigarettes (one cigarette contains 6 mg tar and 0.05 mg nicotine) per day for one hour until the end of the experiment (4 weeks period). The control group received room air as a sham-smoke exposure and kept in a similar chamber for an equal period of time.

The 4 dietary groups were fed the experimental diets for 4 weeks as follows: 1) The first group is the control group, and was fed a normal-protein diet (NP) (18% protein diet + 0.03% L-methionine). 2) The second group was fed a NP, similar to the diet consumed by the control group. It was called the rats fed NP diet and exposed to cigarette smoke (NP+S) group. 3) The third group was fed a low-protein diet (LP) (6% protein diet). It was called the rats fed LP diet and exposed to cigarette smoke (LP+S) group. 4) The fourth group was fed a diet, similar to that consumed by the LP+S group, but it was supplemented with NAC to bring the amount of total sulfur amino acids to 65 mmol sulfur amino acids/kg diet, which is equivalent to the amount of sulfur amino acids present in the control and the NP+S groups. The fourth group was

Table 1 - Treatment and dietary composition (g/kg) of the 4 animal groups

| Dietary groups | 180 g protein/kg NP (Control) | 180 g protein/kg NP + S | 60 g protein/kg LP + S | 60 g protein/kg LP + NAC + S |
|---|---|---|---|---|
| Smoke exposed (10 cigarettes/ hour/day) | No | Yes | Yes | Yes |
| Casein | 204 | 204 | 68 | 68 |
| L-Methionine | 3 | 3 | 0 | 0 |
| N-acetyl-L-cysteine | 0 | 0 | 0 | 8.16 |
| Cellulose | 100 | 100 | 100 | 100 |
| Sucrose | 319 | 319 | 388 | 385 |
| Maize starch | 319 | 319 | 389 | 384 |
| Maize oil | 30 | 30 | 30 | 30 |
| Vitamins mix* | 5 | 5 | 5 | 5 |
| Minerals mix* | 20 | 20 | 20 | 20 |

NP (Control) group - rats fed normal-protein diet;
NP+S group - rats fed NP diet and exposed to cigarette S
LP+S group - rats fed LP diet and exposed to cigarette S
LP+NAC+S group - rats fed LP diet supplemented with NAC and exposed to cigarette S
*Vitamin and mineral mix (AIN-76, American Institute of Nutrition 1977).
NP - normal-protein diet, S - smoke, LP - low-protein, NAC - N-acetyl-L-cysteine

called the rats fed LP diet supplemented with NAC and exposed to cigarette smoke (LP+NAC+S) group. **Table 1** shows the treatment and dietary composition of the various groups. At the end of the experiment, blood was collected in a clean centrifuge tube by cardiac puncture under light ether anesthesia. The blood was allowed to clot, and then centrifuged at 2000 rpm/minute for 15 minutes at 4$^0$C. The serum was collected, and stored at -20$^0$C for the analysis of lipid peroxidation and albumin. The liver and lung from each rat were rapidly dissected out, washed with normal saline and weighed. Small samples were taken from liver and lung for immediate analyses of GSH content.

Glutathione in liver and lung was measured by a colorimetric reaction. Simply, the reduced form of GSH is oxidized by the aromatic disulfide compound, 5,5,-dithiobis-2-nitrobenzoic acid (DTNB), to form the oxidized form of GSH and the aromatic thiol, 5,thio-2-nitrobenzoic acid.[28] The yellow color formed, due to the reduction of DTNB, was measured at 412 nm, and is proportional to the amount of GSH present in the sample. Glutathione-chemical reagents were obtained from Sigma Chemical Company (Poole, Dorset, England).

Albumin concentration was measured in 10 ml serum using albumin kit from Human GmbH (Wiesbaden, Germany). The assay kit is based on the bromocresol green dye method.[29]

Serum levels of lipid peroxides, as an indication of oxidative stress, were measured as reaction products of malondialdehyde (a compound generated as a result of lipid oxidation) with thiobarbituric acid. These products are called thiobarbituric acid reactive substances (TBARS). The formation of TBARS was measured at 532nm using a standard spectrophotometer.[30] The TBARS Assay Kit was purchased from ZeptoMetrix Corporation (Buffalo, New York, USA).

Results were expressed as mean values±SEM. Statistical analysis were compared using one-way analysis of variance with a significant level of $p<0.05$, as indicated in tables and figures. Where significant effects were found, differences between groups were examined using unpaired Student's t-test with a significant level of $p<0.05$.

Results. No significant difference was found in the initial weights among various groups. The initial weights in grams were 200 ± 3.6 for Group 1, 197 ± 2.9 for Group 2, 195 ± 3.2 for Group 3 and 196 ± 1.4 for Group 4. **Table 2** shows that cigarette smoking significantly reduced the final body weight of rats in all dietary groups (the NP+S, LP+S and

Table 2 - Final body weight of control and smoke-exposed rats fed normal-protein diet, low-protein diet or low-protein diet supplemented with N-acetyl-L-cysteine.

| Dietary groups | NP (Control) | NP + S | LP + S | LP + NAC + S | One-way ANOVA *p* value | One-way ANOVA F ratio |
|---|---|---|---|---|---|---|
| Final body weight (g) | 261.5 ± 7.30[a] | 236.9 ± 6.9[b] | 169.2 ± 6.20[c] | 211.2 ± 5.60[d] | 0.000 | 36.6 |

NP = normal-protein diet. NP + S = normal-protein diet + exposed to cigarette smoke, LP + S = low-protein diet + exposed to cigarette smoke, LP + NAC + S = low-protein diet + N-acetyl-L-cysteine + exposed to cigarette smoke.
Values are means ± SEM, n=8 per group. The results were compared using one-way analysis of variance (ANOVA).
Values having different letters differ significantly ($p<0.05$).

Table 3 - Organ weights of control and smoke-exposed rats fed normal-protein diet, low-protein diet or low-protein diet supplemented with N-acetyl-L-cysteine

| Dietary groups | NP (Control) | NP + S | LP + S | LP + NAC + S | One-way ANOVA *p* value | One-way ANOVA F ratio |
|---|---|---|---|---|---|---|
| Liver weight (g) | 9.81 ± 0.45[a] | 8.94 ± 0.40[a] | 6.68 ± 0.29[b] | 11.05 ± 0.48[c] | 0.000 | 20.2 |
| Relative liver weight (g/kg body weight) | 37.6 ± 1.8[a] | 37.6 ± 0.82[a] | 39.5 ± 1.2[a] | 52.3 ± 1.9[b] | 0.000 | 22.1 |
| Lung weight (g) | 2.04 ± 0.09[a] | 1.59 ± 0.07[b] | 1.48 ± 0.06[b] | 1.56 ± 0.07[b] | 0.000 | 10.3 |
| Relative lung weight (g/kg body weight) | 7.53 ± 0.40[a] | 6.88 ± 0.18[a] | 9.01 ± 0.59[b] | 7.45 ± 0.48[a] | 0.000 | 4.2 |

NP = normal-protein diet, NP + S = normal-protein diet + exposed to cigarette smoke, LP + S = low-protein diet + exposed to cigarette smoke, LP + NAC + S = low-protein diet + N-acetyl-L-cysteine + exposed to cigarette smoke.
Values are means ± SEM, n=8 per group. The results were compared using one-way analysis of variance (one-way ANOVA).
Values having different letters differ significantly ($p<0.05$).

LP+NAC+S groups) compared to the control group. The final body weight of rats in the NP+S group was higher than the LP+S and the NP+NAC+S groups. The addition of NAC to the low-protein diet significantly increased the final body weight compared to the corresponding animal fed the same diet, but without NAC supplementation. The organ weights in **Table 3** were presented as total organ weights and as relative organ weights (percentage of body weight) to allow differences due to body weight. The addition of NAC to a low-protein diet significantly increased the total liver weight and the relative liver weight compared to other dietary groups, including the control group. No significant difference in the relative liver weight was found among the control, NP+S and the LP+S groups. Smoking exerts a significant reduction in the total lung weight in all dietary groups. The relative lung weight of the LP+S group was higher than other dietary groups.

**Table 4** shows that albumin concentration was not significantly affected by changing dietary protein level or by smoking. There was a slight decrease, not statistically significant, in the albumin level in the smoke-exposed rats fed a low-protein diet, but not in rats fed a low-protein diet supplemented with NAC, compared with other dietary groups.

Hepatic GSH concentration was influenced by dietary protein content and by NAC supplementation (**Figure 1)**. The results show that the NP+S group had hepatic GSH concentration similar to the control group fed with the same diet. Smoke-exposed rats fed the LP diet had a significantly lower hepatic GSH concentration compared to all dietary groups. Addition of NAC to the low-protein diet, in the smoke-exposed rats, significantly increased hepatic GSH concentration compared to the corresponding animals fed the same amount of protein, but without NAC supplementation. The increase in hepatic GSH

Table 4 - Serum albumin concentration of control and smoke-exposed rats fed normal-protein diet, low-protein diet or low-protein diet supplemented with N-acetyl-L-cysteine.

| Dietary groups | NP (Control) | NP + S | LP + S | LP + NAC + S | One-way ANOVA *p* value | One-way ANOVA F ratio |
|---|---|---|---|---|---|---|
| Albumin (g/l) | 30.8 ± 1.5[a] | 30.7 ± 1.6[a] | 28.6 ± 2.0[a] | 32.1 ± 1.9[a] | 0.558 | 0.70 |

NP = normal-protein diet, NP + S = normal-protein diet + exposed to cigarette smoke, LP + S = low-protein diet + exposed to cigarette smoke, LP + NAC + S = low-protein diet + N-acetyl-L-cysteine + exposed to cigarette smoke.
Values are means ± SEM, n=8 per group. The results were compared using one-way analysis of variance (ANOVA).
Values having different letters differ significantly ($p<0.05$).

Table 5 - Serum thiobarbituric acid reactive substances concentration of control and smoke-exposed rats fed normal-protein diet, low-protein diet or low-protein diet supplemented with N-acetyl-L-cysteine.

| Dietary groups | NP (Control) | NP + S | LP + S | LP + NAC + S | One-way ANOVA *p* value | One-way ANOVA F ratio |
|---|---|---|---|---|---|---|
| TBARS (nmol/mL) | 11.4 ± 0.32a | 12.7 ± 0.49b | 14.6 ± 0.56c | 12.9 ± 0.57b | 0.001 | 7.09 |

NP = normal-protein diet, NP + S = normal-protein diet + exposed to cigarette smoke, LP + S = low-protein diet + exposed to cigarette smoke; LP + NAC + S = low-protein diet + N-acetyl-L-cysteine + exposed to cigarette smoke.
Values are means ± SEM, n=8 per group. The results were compared using one-way analysis of variance (ANOVA).
Values having different letters differ significantly ($p<0.05$).

concentration, which was found after addition of NAC to the low-protein diet, reached the level found in the control and in the NP+M+S groups. Lung GSH concentration was reduced in the LP+S group compared to the control and the NP+S groups (**Figure 2**). This contrasted with the effect of NAC supplementation to the low-protein diet, where no fall in lung GSH concentration occurred.

**Table 5** shows that smoking significantly increased the level of TBARS in all dietary groups compared to the control. However, the elevation in the TBARS level was higher in the LP+S group.

Discussion. The study shows that, in all dietary groups, the body weight of rats was affected by exposing rats to cigarette smoke. Addition of NAC to the low-protein diet significantly increased the body weight compared with the corresponding animals fed the low-protein diet, but without NAC supplementation. Cysteine, which is considered as one of the semi-essential amino acids, can be synthesized from methionine. However, several studies indicate that there is a requirement for the amino acid cysteine in the diet of growing rats. When methionine was provided at the minimum requirement needed to support growth (0.17%), the addition of cysteine to such a diet improved the growth performance of growing rats.[31,32] Cho et al[33] have observed that the mean daily weight gain of rats fed diets providing adequate or excess amounts of sulfur amino acids, in the form of methionine or cystine, was significantly higher than rats fed diet providing only the absolute minimum methionine requirement of growing rats.

The results show no significant reduction in the lung and hepatic GSH concentration in cigarette smoke-exposed rats fed the normal-protein diet, and exposed to cigarette smoke, compared with the corresponding control rats fed the same level of protein. The results were consistent with the results of the study conducted by Wright et al,[34] in which they found no effect of cigarette smoke on lung GSH levels either immediately or after 24 hours compared to control rats. Park et al[35] have shown a significant reduction in GSH concentration in both the liver and lung of rats subjected to inhalation of cigarette smoke for 30 days, 3 times a day compared to the control rats. However, the GSH results were expressed per mg of tissue protein. In general, the data in the literature conflict in respect to the effect of smoking on GSH levels in

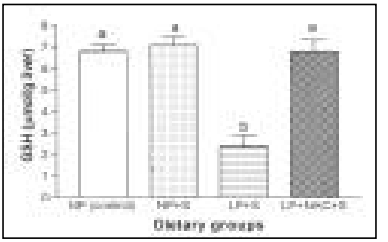

Figure 1 - Glutathione (GSH) concentration in liver of control and smoke-exposed (S) rats fed normal-protein (NP) diet, low-protein (LP) diet or low-protein diet supplemented with N-acetyl-L-cysteine (NAC). Results are means ± SEM, n=8 per group. The results were compared using one-way analysis of variance (ANOVA). Groups with a different letter differ significantly ($p$<0.05).

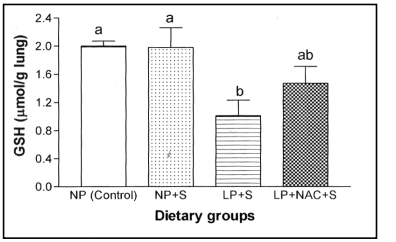


Figure 2 - Glutathione (GSH) concentration in lungs of control and smoke-exposed (S) rats fed normal-protein (NP) diet, low-protein (LP) diet or low-protein diet supplemented with N-acetyl-L-cysteine (NAC). Results are means ± SEM, n=8 per group. The results were compared using one-way analysis of variance (ANOVA). Groups with a different letter differ significantly ($p$< 0.05).

tissues.[36-38] In human, Moriarty et al[39] have shown that plasma GSH concentration decreased in smokers compared to non-smokers. Cysteine is unstable in its reduced form, and has been reported to be toxic in-vivo and in-vitro studies,[40-43] and because GSH is mostly degraded in the extracellular compartment, due to the presence of the enzyme g-glutamyl cysteine transferase, several compounds have been used as a cysteine-delivery agents. 2-oxothiazolidine-4- carboxylate, and NAC, are the most common compounds used as cysteine-delivery agents.[44] Our study indicates the efficiency of NAC supplementation in enhancing GSH concentration in smoke-exposed rats fed the low-protein diet. The magnitude of the increase in GSH concentration was greater in the liver.

The low GSH concentrations in liver and lung, found in the low-protein group, after the exposure to cigarette smoke, could be an indication of the reduction in the ability to scavenge free radicals. This was supported by the large elevation of serum TBARS in this group compared to other smoke-exposed groups. Moreover, the inability to enhance GSH concentrations in liver and lung in the above group is an indication of a low availability of cysteine in these organs. Both cysteine and GSH have been shown to be important in optimizing T-cells and macrophage immune function.[45-48] Glutathione is also an important compound needed to maintain the function of other antioxidant compounds. Glutathione is used by several transhydrogenase to maintain other antioxidant compounds in their reduced form, such as vitamins C and α-tocopherol.[49,50]

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