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Applied nutritional investigation

Plasma glutathione of HIV$^+$ patients responded positively and differently to dietary supplementation with cysteine or glutamine

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ABSTRACT

Objective: Patients with positivity for the human immunodeficiency virus (HIV$^+$) present low concentrations of antioxidant nutrients, including total glutathione (GSH) and its precursors. We investigated the responses of the sulfur-containing amino acid pathway to cysteine and glutamine (Gln) dietary supplements in patients with HIV$^+$ compared with healthy controls.
Methods: Twelve treated patients (six men and six women, 22–45 y old) and 20 healthy controls (10 men and 10 women, 20–59 y old) were randomly assigned to 7-d dietary supplements containing *N*-acetylcysteine (NAC; 1 g/d) or Gln (20 g/d), with a 7-d washout period ingesting their usual diet. Blood samples were drawn after an overnight fast. High-performance liquid chromatographic plasma analysis of sulfur-containing amino acids (methionine, homocysteine, cysteine, and taurine), GSH, oxidized GSH, and serine, glycine, glutamic acid, and Gln was carried out moments before and after 7-d supplementations. Statistical comparisons were undertaken between groups and between dietary supplements ($P < 0.05$).
Results: Patients with HIV$^+$ showed higher oxidized GSH and lower concentrations of GSH and all amino acids except homocysteine. The HIV$^+$ group responded to the NAC by increased levels of sulfur-containing amino acids and GSH and equalized taurine and GSH levels in the control group. The Gln supplements also equalized the levels of GSH, Gln, and glycine in the control group.
Conclusion: An increase in GSH may be attained by NAC or Gln supplementation, with NAC acting by increasing cysteine levels and Gln likely acting by replenishing the glycine pool (trial registered at http://www.clinicaltrials.gov, identifier NCT00910442).
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Introduction

Several studies have shown that patients infected with the human immunodeficiency virus (HIV$^+$) present a deficiency of antioxidant nutrients, including total glutathione (GSH) and its precursors [1–4], thus contributing to disease progression.

The virus and lymphocytes use hydrogen peroxide (H_2O_2) to signal their survival and function. H_2O_2 is generated near the cell membrane, where lymphocytes establish contact with mitogens, viruses, and other activation agents [5]. The generated H_2O_2 is rapidly inactivated by local peroxidases, mostly by GSH peroxidase, resulting in oxidized GSH (GSSG), which is converted back to reduced GSH by the enzyme GSH reductase to maintain the reduced intracellular status [6].

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is the major intracellular hydrosoluble antioxidant agent responsible for important functions in biochemical processes and cell control, especially antioxidant defenses and immune responses. This agent is necessary for lymphocyte proliferation, antibody-dependent and cell-mediated cytotoxicity, and the protection of lymphocytes against superoxides produced to destroy invading pathogens [5]. The content of GSH in mammalian cells

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is dynamically maintained by the γ-glutamyl cycle, which provides a substrate for transpeptidases located on their external membranes. Tissues that present low transpeptidase activity (the liver, pancreas, and muscle) export GSH, which is carried by the blood to cells that have high transpeptidase activity, such as kidney cells [5,7]. Plasma GSH arises largely from the liver. It can also be used as a mechanism for cysteine storage and transport, with consequences for the regulation of signal transduction pathways and gene transcription [8]. Conversely, cysteine is known to be the rate-limiting substrate for GSH resynthesis [9, 10]. Cysteine is also the main source of taurine, a protective antioxidant that enables the antigen-presenting cells to promote the proliferation of T lymphocytes [11].

Improving cysteine availability for the biosynthesis of GSH is the most extensively studied approach for increasing the cell GSH pool. Among the agents tested are *N*-acetyl-L-cysteine (NAC), lipoic acid, cysteamine, and 2-oxothiazolidine 4-carboxylate. Other reducing agents, such as dithiocarbamate, β-mercaptoethanol, and dithiothreitol, can also improve GSH synthesis through the extracellular reduction of cystine to cysteine. NAC and α-lipoic acid have generated the most interest because of their proved clinical safety features and efficacy in vivo.

N-acetyl-L-cysteine is a well-characterized antioxidant that counteracts the effects of reactive oxygen intermediates in living cells, thus preventing the activation of nuclear factor-κB by H_2O_2 [12]. Oral NAC has been found to increase GSH in the plasma, muscle, and bronchoalveolar lavage fluid [13–15]. Oral L-glutamine (Gln) increases Gln levels in the plasma of healthy subjects [16] and has been shown to improve plasma GSH levels and significantly increase lean body mass in HIV infection [2]. The two precursors influence GSH biosynthesis through different pathways and provoke isolated and synergistic effects on patients' immunology and clinical improvements. Therefore, we investigated not only the status of GSH and its precursor in patients with HIV^+ supplemented with NAC or Gln and but also possible approaches for augmenting their GSH content.

Materials and methods

Subjects

The HIV^+ group was composed of 12 patients (6 men and 6 women, 22–45 y old) who had been diagnosed clinically and in the laboratory by viral load (enzyme-linked immunosorbent assay and western blotting detection methods) and $CD4^+$ and $CD8^+$ lymphocyte counts (flow cytometry). All patients had been under highly active antiretroviral treatment for at least 1 y, receiving one HIV protease inhibitor (indinavir 800 mg twice daily, $n = 10$; or ritonavir 600 mg twice daily, $n = 2$) in combination with two nucleoside analogs (zidovudine 250 mg plus lamivudine 150 mg twice daily, $n = 11$; or lamivudine 150 mg plus stavudine 40 mg twice daily, $n = 1$). The exclusion criteria were the presence of any renal or liver failure and the ingestion of GSH precursors or any form of vitamin B. The healthy control group consisted of 20 adults (10 men and 10 women, 20–59 y old) who were negative for HIV and clinically healthy. Written informed consent was obtained from all subjects, and the study was approved by the ethics committee of the Botucatu Medical School.

Design

At baseline, all selected subjects underwent an anthropometric assessment for body mass index (kilograms per meter squared) calculation and had fasting blood drawn (12 mL) to analyze markers for glomerular filtration (creatinine and urea) and hepatocyte injury marker (γ-glutamyl transpeptidase), nutritional variables (albumin, calcium, folate, and vitamin B12), glucose, lipids (triacylglycerols and cholesterol fractions), and uric acid. Folate and vitamin B12 were assayed by chemiluminescence (Immulite System®, Siemens Healthcare Diagnostics, Marburg, Germany), and the others by a dry chemistry method (Vitros System®, Ortho Clinical Diagnostics, Johnson & Johnson Company, Raritan, NJ, USA).

The two groups were randomly assigned to different dietary supplements (NAC 1 g/d or Gln 20 g/d) with their usual diet as the baseline and washout in a crossover design. All dietary supplements were administered throughout the consecutive 7-d period preceded and followed by fasting blood sampling. The high-performance liquid chromatographic (LC10AD System®, Shimadzu, Kyoto, Japan) analysis included plasma free amino acids (methionine, serine, taurine, glycine, and Gln), glutamic acid quantified by the method of Halawa et al. [17], and plasma homocysteine and cysteines quantified according to the method of Ubbink et al. [18]. For total GSH, aliquots of plasma were incubated with dithiothreitol, deproteinized by perchloric acid, and derivatized with *o*-phthalaldehyde. For GSSG, the blood, having been previously blocked by *n*-ethylmaleimide, was then processed for total GSH [19].

Statistical analysis

All statistical analyses were performed using SAS 8.01 (SAS Institute, Cary, NC, USA). Parametric and non-parametric variables were expressed, respectively, as mean (standard deviation) and median (interquartile range, 25th–75th percentiles). A general linear model procedure for repeated measures using type III sums of squares statistics was used to test the interaction between groups (HIV^+ and control groups) and/or dietary supplements (NAC, washout, and Gln). An adjusted γ-regression model was used for the non-parametric variables. The groups and/or dietary supplements with minimal significant differences were compared based on generalized estimating equations. A 5% significance level was set.

Results

Characteristics of the subjects before the dietary supplementation are presented in Table 1. Thirty-two subjects completed the intervention program.

Compared with the usual diet, the NAC and Gln supplements increased GSH, taurine, methionine, and Gln and decreased serine in patients with HIV^+ ($P < 0.05$). In addition, NAC supplementation led to significant increases in cysteine and homocysteine, whereas Gln supplementation increased glycine concentrations (Table 2).

Among the three GSH precursors, plasma glutamic acid and glycine concentrations increased significantly after Gln supplementation, whereas cysteine was augmented by NAC; however, only glycine reached the control values, whereas glutamic acid and cysteine remained decreased (Table 2).

The NAC and Gln supplements showed similar effects on plasma levels of methionine, taurine, glutamic acid, and Gln (Table 2). Nevertheless, NAC supplementation produced stronger effects than Gln by increasing GSH, cysteine, and homocysteine levels, whereas Gln produced a specific effect on glycine (Table 2).

Table 1
Subjects' demographic, anthropometric, and biochemical data at baseline

Variables	Controls	Patients with HIV^+	*P*
Age (y)	24 (23–28)	27 (25–36)	0.070
BMI (kg/m^2)	25 (3.1)	25 (3.0)	0.960
γ-GT (IU/L)	24.9 (14.7)	52.7 (21.7)	<0.001
Urea (mg/dL)	29.0 (7.4)	34.1 (6.4)	0.061
Creatinine (mg/dL)	0.9 (0.83–1.1)	0.9 (0.8–1.05)	0.726
Albumin (g/dL)	4.1 (0.3)	3.9 (0.4)	0.112
Folate (ng/mL)	7.5 (6.3–9.0)	1.9 (1.4–6.6)	0.017
Vitamin B12 (pg/mL)	288 (130)	367 (139)	0.115
Calcium (mg/dL)	9.2 (9.0–9.4)	8.7 (7.8–9.6)	0.425
Glucose (mg/dL)	87 (83.5–91)	103 (93.5–117)	0.001
TG (mg/dL)	88 (66.5–117)	191 (111–247)	0.002
TC (mg/dL)	165.6 (26.7)	222 (45.6)	<0.001
HDL-C (mg/dL)	52.5 (19.9)	38.3 (17.9)	0.052
LDL-C (mg/dL)	90.6 (32.9)	146.7 (31.6)	<0.001

BMI, body mass index; γ-GT, γ-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; HIV^+, positive for the human immunodeficiency virus; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triacylglycerols
Data are presented as median (25th–75th percentiles) or mean (SD).

Table 2
Plasma amino acid and glutathione concentrations in different dietary conditions*

Amino acid and group	Usual diet	*N*-acetylcysteine	Glutamine	Washout
Methionine (μmol/L)				
Control	30.8 ± 8.8	30.3 ± 9.6	30.3 ± 9.6	31.9 ± 8.6
HIV+	20 ± 6.6‡	24.7 ± 7.4†	25.5 ± 11	24.3 ± 7.7†,‡
Homocysteine (μmol/L)				
Control	13.9 ± 5.5	13.7 ± 2.5	11.3 ± 2.7†	13.8 ± 5.3
HIV+	9.8 ± 1.6‡	12.4 ± 3†	10.2 ± 1.3	9.8 ± 1.5‡
Serine (μmol/L)				
Control	108 ± 7.8	110 ± 12	112 ± 14	113 ± 11†
HIV+	86.9 ± 20‡	61 ± 17.2†,‡	60.6 ± 17†,‡	63.8 ± 19.3†,‡
Cysteine (μmol/L)				
Control	413 ± 175	534 ± 54†	420 ± 177	409 ± 180
HIV+	158 ± 10‡	251 ± 32†,‡	175 ± 26†,‡	169 ± 13†,‡
Taurine (μmol/L)				
Control	63.2 ± 3.9	68.8 ± 9.6†	63.2 ± 3.9	65.4 ± 10.5
HIV+	48.1 ± 3.7‡	57.2 ± 4.9†,‡	57.2 ± 5.6†,‡	66.4 ± 10.4†,‡
Glutamate (μmol/L)				
Control	94 ± 18.5	133 ± 27†	144 ± 29†	113 ± 23†
HIV+	56.4 ± 11.3‡	63.9 ± 14†,‡	64.1 ± 14†,‡	63.6 ± 14.7†,‡
Glycine (μmol/L)				
Control	485 ± 68.5	481 ± 76	480 ± 76	483 ± 73
HIV+	371 ± 20.2‡	347 ± 63‡	472 ± 60†	329 ± 75†,‡
Glutamine (μmol/L)				
Control	476 ± 84	565 ± 85.3	565 ± 85.4	477 ± 100
HIV+	285 ± 59‡	341 ± 56.9‡	341 ± 57‡	287 ± 64‡
GSH (μmol/L)				
Control	9.2 ± 2.1	8.5 ± 0.9	8.3 ± 0.8†	8.7 ± 1.4
HIV+	4.9 ± 0.7†,‡	9.2 ± 0.9†,‡	8.4 ± 0.4†,‡	5.8 ± 0.7†,‡
GSSG (μmol/L)				
Control	0.77 ± 0.86	0.58 ± 0.31	0.48 ± 0.23	0.44 ± 0.26
HIV+	1.44 ± 0.4‡	1.76 ± 0.5‡	1.81 ± 0.17‡	1.64 ± 0.14‡
GSSG/GSH (μmol/L)				
Control	0.08 ± 0.07	0.07 ± 0.04	0.06 ± 0.03	0.05 ± 0.04
HIV+	0.29 ± 0.06‡	0.19 ± 0.04†,‡	0.2 ± 0.02†,‡	0.28 ± 0.02‡

GSH, reduced glutathione; GSSG, oxidized glutathione; HIV+, positive for human immunodeficiency virus

* All values are presented as mean ± SD. Data were analyzed using repeated-measures analysis of variance. A significant group-by-treatment interaction was observed for all amino acids except glutamine and GSSG ($P < 0.05$).

† Significantly different from the habitual diet (baseline; $P < 0.05$).

‡ Significantly different from control at the same time point ($P < 0.05$).

Discussion

Although highly active antiretroviral treatment has been demonstrated to increase antioxidant defenses [20], the data from the present work showed that the antioxidant deficit persisted as indicated by the higher levels of oxidative stress markers, such as GSSG.

Therefore, patients with HIV+ have coexisting infection, oxidative stress, and antiretroviral therapy. In this sense, it is necessary to determine the emergent role of micronutrients, particularly those with antioxidant actions (e.g., selenium and zinc) and immune function to minimize the effects of such metabolic disorders as lipodystrophy, insulin resistance, and dyslipidemia, which contribute to the morbidities of this coinfection [21,22].

The decreased GSH concentrations observed in this study have been found previously in patients with HIV+ [20–23]. GSH decreases in response to low-protein ingestion or specific deficits of its component amino acids [24]. In the present work, the two groups had their usual diet as the baseline and washout, in addition to similarly presenting with overweight and normal albuminemia, thus excluding a low-protein intake situation. Lower plasma GSH in the HIV+ group followed the lack of its components, cysteine, glutamic acid, and glycine, which in turn paralleled the lower levels of their precursors methionine and serine (for cysteine) and Gln (for glutamic acid). Therefore, the lower GSH concentration could be due to an increased usage associated with low synthesis secondary to the decrease in the availability of substrates [21,22]. It is known that, in pathologic conditions, the three amino acid precursors of GSH synthesis (Gln, glycine, and cysteine) become essential [25].

Decreases in cysteine and sulfate levels are provoked by liver GSH synthesis and muscle cysteine catabolism, respectively. Moreover, the consistently increased profile of cytokines (especially interleukin-1, interleukin-6, and tumor necrosis factor-α) can aggravate muscle catabolism in these patients, increase the demand for amino acids, and compromise the adaptive response in HIV infection [20,26].

One of the proposed mechanisms for this massive loss is the controlled protein catabolism that excretes cysteine to spare protein nitrogen and restrain the catabolism [27]. Cysteine catabolism produces sulfate and protons, thus favoring Gln synthesis and preserving nitrogen from the amino acid pool in the form of Gln. However, in patients with HIV+, the adaptive response is not achieved, and they lose thiol homeostasis [26].

The present data showed that the NAC and Gln supplements increased GSH availability. In a study carried out by Jahoor et al. [21], only an increase of the cysteine supply modified the GSH synthesis in individuals with HIV+, because the levels of the other two constituent amino acids (glutamic acid and glycine) were normal [21]. In the present study, all three amino acid levels were lower than those of controls at baseline and below the reference values for the healthy population [17]. These results agree with findings in prior studies showing deficiencies of cysteine and Gln in patients with HIV+ [26,28].

The NAC supplementation almost doubled the level of GSH, significantly surpassing the control group. Although cysteine and taurine levels increased, the resulting values were still below those of controls. NAC supplementation has been reported to increase plasma and muscular GSH and cysteine levels in a dose-dependent response [10]. The present use of 1.0 g/d did not result in cysteine normalization in the plasma. This was shown by an increase of the cysteine concentration, although it did not reach the level in healthy subjects. In the fasting state, cysteine is maintained mostly by the GSH turnover [4], whereas after methionine loading, most cysteine might originate from the transsulfuration pathway [29].

Available estimates have suggested that the endogenous formation of glycine is 5- to 10-fold higher than the dietary intake [30]. Most of the glycine pool arises from glutamic acid metabolism, but glycine and serine are readily interchangeable by the enzymatic step catalyzed by serine hydroxyl methyl transferase. Choline, by dimethylglycine, also contributes to glycine for GSH synthesis in humans [31]. Total glycine flow is determined mainly by the direct conversion to serine synthesis by serine hydroxyl methyl transferase (41%) and glycine decarboxylation for the generation of 1-carbon units and CO_2 production (39%) [32].

Serine decreased significantly with NAC and Gln supplementations. Serine is consumed by the direct formation of glycine (through the serine hydroxyl methyl transferase

pathway) or by cysteine formation through the transsulfuration pathway from homocysteine to cystathionine. Only glycine reached the control levels and that occurred with glutamic acid supplementation.

In the case of cysteine concentrations, it appears that NAC supplementation led to some plasma increase of cysteine, which slowed the transsulfuration pathway, sparing homocysteine (which increased significantly) and serine. The existing serine was then diverted from the cysteine to the glycine pathway, which was consumed with the spared cysteine (from NAC) to normalize GSH levels.

The increase of plasma homocysteine concentrations (>10 μmol/L) with NAC may be due to the decreased consumption of homocysteine by remethylation to methionine (reduced folate in this case) or, more likely, by transsulfuration to cysteine (spared by NAC).

The Gln-induced GSH increase and normalization were probably achieved by generating glycine (from glutamic acid) and sparing serine to form cysteine (from homocysteine), and all three (glutamic acid, glycine, and cysteine) together generated GSH.

The NAC and Gln, through different mechanisms, were able to supply substrates to increase GSH levels. Normalized GSH was unable to restore cysteine or glutamic acid concentrations to the normal control level, and neither of the two supplements had significant effects on the GSSG/GSH ratio, indicating a need for an additional supplement (perhaps riboflavin).

Thus, a GSH increase can be achieved by NAC or Gln supplementation, with the former acting by sparing cysteine and the latter increasing GSH possibly by replenishing the glycine pool. Only glycine reached control values; therefore, the rate-limiting step for GSH synthesis may be not completely dependent on cysteine availability and vice versa.

Conclusion

Acting through replenishment of GSH and by improving cysteine and taurine, each dietary supplement, NAC and Gln, was effective, but it would be even better to test the ability of their combination to strengthen the antioxidative immune competence of patients with HIV^+.

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