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ORIGINAL ARTICLE

Oral supplementation with liposomal glutathione elevates body stores of glutathione and markers of immune function

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BACKGROUND/OBJECTIVES: Glutathione (GSH) is the most abundant endogenous antioxidant and a critical regulator of oxidative stress. Maintenance of optimal tissues for GSH levels may be an important strategy for the prevention of oxidative stress-related diseases. We investigated if oral administration of liposomal GSH is effective at enhancing GSH levels *in vivo*.
SUBJECTS/METHODS: A 1-month pilot clinical study of oral liposomal GSH administration at two doses (500 and 1000 mg of GSH per day) was conducted in healthy adults. GSH levels in whole blood, erythrocytes, plasma and peripheral blood mononuclear cells (PBMCs) were assessed in 12 subjects at the baseline and after 1, 2 and 4 weeks of GSH administration.
RESULTS: GSH levels were elevated after 1 week with maximum increases of 40% in whole blood, 25% in erythrocytes, 28% in plasma and 100% in PBMCs occurring after 2 weeks ($P < 0.05$). GSH increases were accompanied by reductions in oxidative stress biomarkers, including decreases of 35% in plasma 8-isoprostane and 20% in oxidized:reduced GSH ratios ($P < 0.05$). Enhancements in immune function markers were observed with liposomal GSH administration including Natural killer (NK) cell cytotoxicity, which was elevated by up to 400% by 2 weeks ($P < 0.05$), and lymphocyte proliferation, which was elevated by up to 60% after 2 weeks ($P < 0.05$). Overall, there were no differences observed between dose groups, but statistical power was limited due to the small sample size in this study.
CONCLUSIONS: Collectively, these preliminary findings support the effectiveness of daily liposomal GSH administration at elevating stores of GSH and impacting the immune function and levels of oxidative stress.

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INTRODUCTION

Glutathione (GSH) is the most abundant nonprotein thiol in cells and has an array of critical functions, which include detoxifying drugs, protecting macromolecules from oxidative damage and maintaining immune functions.[1–7] GSH is synthesized from cysteine (Cys), glutamic acid and glycine with Cys most often being the rate-limiting substrate.[8,9] As a result, GSH levels can be depleted when Cys levels are limited such as during periods of fasting.[10,11] GSH depletion has numerous detrimental effects, including impaired immune function[6] and increased susceptibility to xenobiotics[12] and oxidants.[13] Maintenance of optimal tissue levels of GSH is thought to be an important factor for maintaining health and low GSH levels have been associated with increased risks for diseases, including cancer, cardiovascular diseases, arthritis and diabetes.[14–16]

GSH enhancement represents a potentially important approach in the treatment and prevention of disorders associated with GSH depletion. Studies linking dietary GSH intake with increased blood levels and reduced risk for cancer[17,18] support the use of orally administered GSH for this purpose. Studies in laboratory animals have demonstrated that oral GSH is bioavailable and effective at enhancing blood and tissue GSH levels[19–24] and can be protected against aging-related impairments in immune function,[25] influenza infections[26] and cancer.[27–30] In a recent clinical trial, we demonstrated that daily oral supplementation of GSH was effective at enhancing GSH levels in oral buccal cells and a variety of intra- and extracellular blood compartments.[31]

Liposomes have been used as an effective means of drug delivery allowing for more efficient absorption and delivery of both hydrophilic and lipophilic substances and greater protection against oxidation and degradation. Since GSH is subject to destruction in the acid environment of the stomach, we proposed that oral liposomal GSH might be an effective means of GSH delivery *in vivo*. While liposomal GSH preparations are commercially available, there have been few clinical reports on their effectiveness and no data on their ability to enhance body GSH stores. Thus, our current objectives were to conduct a pilot study to determine the short-term (1 month) effects of daily oral supplementation with liposomal GSH on the levels of GSH in different intracellular and extracellular blood compartments in healthy adults. In addition, the effects on specific immune functions and biomarkers of oxidative stress were assessed.

SUBJECTS AND METHODS

Study protocol

The study (ClinicalTrials.gov identifier: NCT02278822) was approved by the Institutional Review Board of the Penn State College of Medicine in accordance with the Helsinki Declaration of 1975, as revised in 1983. Subjects were recruited from the local Hershey/Harrisburg, PA, USA area using fliers, online announcements and word of mouth. Interested individuals were prescreened by telephone and eligible subjects were asked to visit the Clinical Research Center at the Penn State Cancer Institute, Hershey, PA, USA. After providing informed consent, the subjects were further screened for eligibility based on the following criteria: healthy

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nonsmokers, 50–80 years of age, had no antioxidant supplementation for ⩾1 month. Eligible subjects were randomly assigned to one of the two treatment groups with equal probability: low-dose (500 mg, *per os*) or high-dose (1000 mg *per os*) liposomal GSH (Tri-Fortify Orange [phosphatidylcholine liposome GSH] provided by Researched Nutritionals, Los Olivos, CA, USA). Liposomal GSH was provided in 8-oz tubes and individual daily doses were prepared by subjects using a 5.6-ml spoon (low dose, 1 spoonful; high dose, two spoonfuls). The tubes were returned and weighted at the completion of the study to assess compliance. Questionnaire data were collected on demographics, occupation, lifestyle habits, medical history and medication, supplements and alcohol use. Supplementation continued for 1 month and blood and urine were collected at the baseline and for 1, 2 and 4 weeks.

SUBJECTS

A total of 12 subjects were enrolled and received intervention from 11/13/2014 to 04/28/2015 and none of them withdrew or were withdrawn (Figure 1). There were no significant differences in study subject characteristics between treatment arms at the baseline (Table 1). Compliance was assessed by daily diary entries and by the difference in tube weights before and after the completion of the study.

Collection and processing of biological samples

Blood was collected between 0900 hours and 1300 hours into tubes containing sodium heparin and immediately placed on ice and processed in ⩽1 h. Plasma and erythrocytes were obtained by centrifugation and were further processed for GSH (see below) or stored at –80 °C. Peripheral blood mononuclear cells (PBMCs) were obtained from ~24 ml of whole blood by density-gradient centrifugation with Ficoll-Hypaque (Sigma, St Louis, MO, USA), as described previously.[31] Viable cells were counted using trypan blue and aliquots of 5×10^6 cells/ml in 95% FBS and 5% DMSO were frozen at − 80 °C and stored in liquid nitrogen.

For GSH and glutathione disulfide (GSSG) analyses, whole blood, red cells and PBMCs were deproteinized with metaphosphoric acid (MPA), as described previously.[31] Acid-insoluble pellets were stored at –80°C until analysis for GSSP. Plasma samples were first reduced with KBH_4 prior to deproteinization with MPA.[32]

Analytical procedures

Glutathione. GSH and GSSG levels were determined in MPA extracts using a 5,5′-dithio-bis(2-nitrobenzoic acid)/GSSG reductase enzymatic recycling procedure, as described previously.[33] For GSSP, MPA insoluble pellets from whole blood or erythrocytes were reduced with KBH_4 at neutral pH and re-acidification with MPA prior to analysis of released GSH, as described previously.[34] Protein concentrations were measured by the bicinchoninic acid procedure (Pierce, Rockford, IL, USA). Hemoglobin was determined spectrophotometrically using Drabkin's reagent.[35]

Table 1. Study subject characteristics

	Liposomal GSH (500 mg/d)	*Liposomal GSH (1000 mg/d)*	*All*
Number of subjects	6	6	12
Age (yr)			
Mean	60.8	59.7	60.2
s.d.	7.39	4.46	5.85
Range	51–72	55–67	51–72
Sex, n *(%)*			
Female	5 (83%)	6 (100%)	11 (92%)
Male	1 (17%)	0 (0%)	1 (8%)
BMI (kg/m^2)			
Mean	26.9	29.9	28.4
s.d.	4.07	3.93	4.12
Range	21.7–32.0	24.7–34.6	21.7–34.6

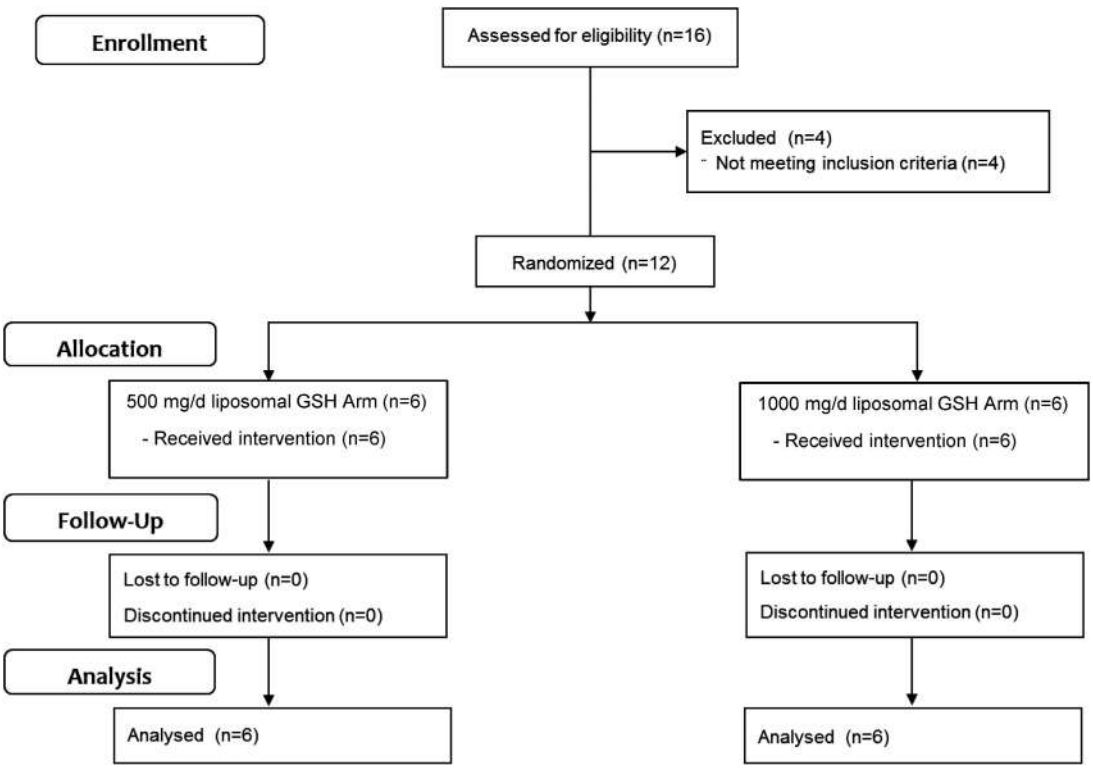

Figure 1. Subject flowchart summary.

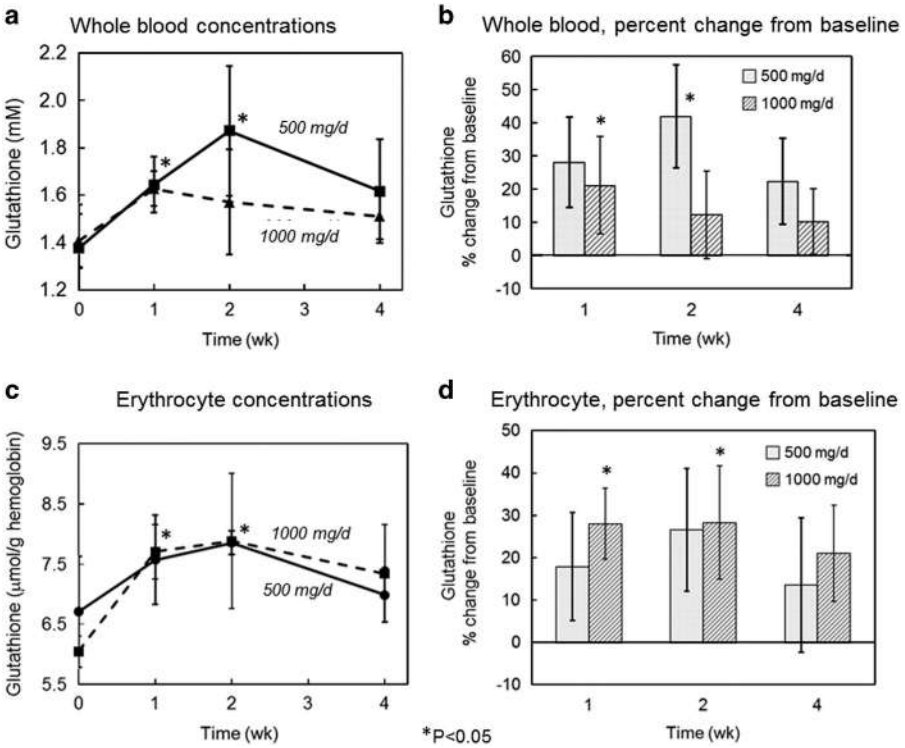

Figure 2. Effect of liposomal glutathione supplementation on whole blood glutathione concentrations. The subjects were randomized to 500 or 1000 mg/d liposomal GSH for 4 weeks. Blood was collected at the baseline and after 1, 2 and 4 weeks, and free and protein-bound GSH was determined in whole blood (**a**,**b**) and in erythrocytes (**c**,**d**), as described in the text. Whole-blood levels are expressed as mmol/l (**a**) or percent change from the baseline (**b**). Erythrocyte GSH levels are expressed as μmol/g hemoglobin (**c**) or percent change from the baseline (**d**). Bars are mean ± s.e. *Significantly different from the baseline by repeated measures of ANOVA, $P < 0.05$.

8-Isoprostane. Plasma 8-isoprostane levels were measured as a marker of oxidative stress by competitive ELISA (Cayman Chemical, Ann Arbor, MI, USA).

Immune function assays

Lymphocyte proliferation. Lymphocyte proliferation was assessed by measuring the incorporation of ^{3}H-thymidine, as described previously.[31] In brief, lymphocytes were thawed, washed 3× prior to counting and determination of viability. Cells were plated at three dilutions (2×10^5, 1×10^5 and 5×10^4 cells per well) and incubated in RPMI-1640 with 10% FBS for 48 h at 37 °C. After addition of 0 or 2 μg/ml phytohemagglutinin (PHA), cells were incubated for 72 h prior to addition of ^{3}H-thymidine. Radioactivity was assessed by liquid scintillation counting after 6 h. All assays were run in triplicate.

NK cell cytotoxicity. NK cell cytotoxicity was assessed using a standard ^{51}Chromium release assay as described previously.[31,36] In brief, lymphocytes were thawed, washed 3× prior to counting and determination of viability. The cells (1×10^4 cells per well) were incubated in triplicate in complete RPMI-1640 with 10% FBS for 48 h at 37 °C, and then combined with human K562 cells labeled overnight with 200 μCi sodium ^{51}chromate at a 10:1 effector:target cell ratio. After 4 h at 37 °C, the cells were analyzed for radioactivity by gamma counting. The results are expressed as percent of target cells lysed calculated as follows: (counts per min (CPM) experimental-CPM spontaneous release)/(CPM maximum–spontaneous) × 100.

Statistics. Descriptive statistics were provided as means and standard errors of the mean. The normality of data distribution was assessed by using the Kolmogorov–Smirnov goodness-of-fit test. Dosage group differences at the baseline were assessed by ANOVA followed by Tukey's *post hoc* test or χ^2 where appropriate. Comparisons over time were assessed by repeated measures of ANOVA followed by *post hoc* testing where appropriate. Correlations of the changes in outcomes with levels at the baseline or between measures were evaluated using Pearson (*r*) correlations.

RESULTS

Compliance and adverse effects

Overall, compliance appeared to be high based on diary entries with < 1.5% of scheduled doses missed. Based on returned tube weights, mean ± s.d. compliance was 109 ± 9.5% in all subjects (108 ± 11.1% for the low-dose group, 111 ± 8.4% for the high-dose group). Values >100% could be due to slightly larger doses in some individuals resulting from overfilling of the spoon during dosing.

No serious adverse effects were reported by the study participants in either dose group. All potential adverse events were minor and none of them were attributed to either treatment group.

Effects of oral liposomal GSH supplementation on body GSH stores

In whole blood, a trend of higher GSH was observed in both groups with liposomal GSH administration (Figure 2a). The largest increase (>40%) occurred in the 500 mg/d dose group at 2 weeks (Figure 2b) ($P < 0.05$). GSH levels in blood primarily reflect those in erythrocytes due to the relatively low GSH levels in plasma and low abundance of other cell types. Thus, as expected, GSH profiles in erythrocytes were similar to those observed in whole blood with a trend of higher levels in both groups with liposomal GSH administration (Figure 2c). The largest increase (~28%) occurred in

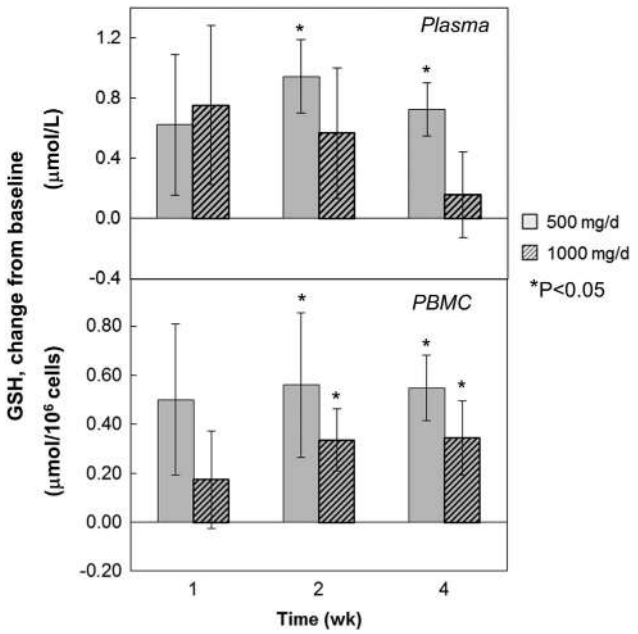

Figure 3. Effect of liposomal glutathione supplementation on plasma and PBMC glutathione concentrations. The subjects were randomized to 500 or 1000 mg/d liposomal GSH for 4 weeks. Blood was collected at the baseline and after 1, 2 and 4 weeks and PBMCs were isolated and analyzed for GSH, as described in the text. The results are expressed as % changes from the baseline in μmol/l of plasma (top panel) or μmol/10^6 PBMCs (bottom panel). For plasma, the baseline values ranged from 2.2 to 10.9 μmol/l (mean ± s.e.: 4.57 ± 0.62) for all subjects (low-dose group: 2.2–10.9 (mean ± s.e.: 4.63 ± 1.29); high-dose group: 3.94–5.15 (mean ± s.e.: 4.51 ± 0.17)). For PBMCs, the baseline values ranged from 0.23 to 1.34 μmol/10^6 cells (mean ± s.e.: 0.89 ± 0.11) for all subjects (low-dose group: 0.23–1.34 (mean ± s.e.: 0.77 ± 0.19); high-dose group: 0.67–1.34 (mean ± s.e.: 1.01 ± 0.11)). Bars are mean ± s.e. *Significantly different from the baseline by repeated measures of ANOVA, $P < 0.05$.

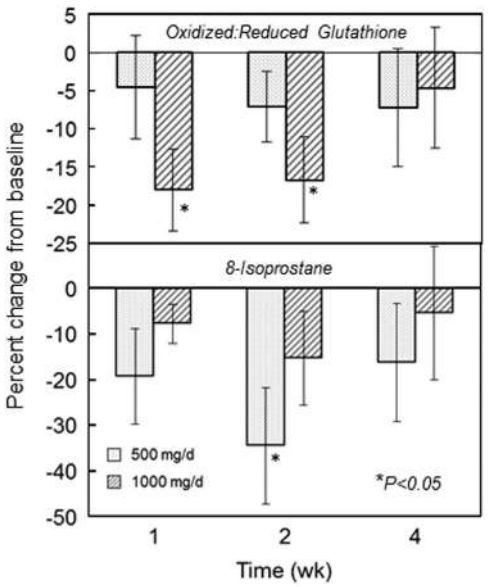

Figure 4. Effect of liposomal glutathione supplementation on blood biomarkers of oxidative stress. The subjects were randomized to 500 or 1000 mg/d liposomal GSH for 4 weeks. Blood was collected at the baseline and after 1, 2 and 4 weeks. Top panel: GSH and its major oxidized forms GSSG and GSSP were determined in whole blood, as described in the text. The baseline ratios ranged from 0.24 to 0.42 (mean ± s.e.: 0.31 ± 0.02) for all subjects (low-dose group: 0.24 to 0.34 (mean ± s.e.: 0.30 ± 0.02); high-dose group: 0.25 to 0.42 (mean ± s.e.: 0.32 ± 0.02)). Bottom panel: plasma 8-isoprostane levels were measured by ELISA, as described in the text. The results are expressed as % changes in pg/ml from the baseline. The baseline values ranged from 63.1 to 1170 pg/ml (mean ± s.e.: 214 ± 90.6) for all subjects (low-dose group: 78.6–1170 (mean ± s.e.: 331 ± 123); high-dose group: 63.1–181 (mean ± s.e.: 97.7 ± 12.8)). The results are expressed as % of the baseline and symbols and bars are expressed as mean ± s.e. *Significantly different from baseline by repeated measures of ANOVA, $P < 0.05$.

the high-dose group after 1 and 2 weeks and in the low-dose group after 2 weeks ($P < 0.05$) (Figure 2d).

In plasma, GSH levels showed an increased trend above the baseline in both groups after liposomal GSH administration; however, the results were only significant after 2 and 4 weeks for the 500-mg dose group (Figure 3, upper panel). The largest increase (~25%) occurred in the low-dose group after 2 weeks.

In PBMCs, GSH levels tended to be increased above the baseline in both groups after liposomal GSH administration; however, the results were significant only after 2 and 4 weeks for both dose groups (Figure 3, lower panel). The largest increase (nearly twofold) occurred in the low-dose group after 2 weeks.

Effects of oral liposomal GSH supplementation on oxidative stress biomarkers

In general, the ratios of oxidized (GSSG+GSSP) to reduced GSH were lower after liposomal GSH administration (Figure 4, top panel; Supplementary Figure 1) with the largest decreases (18–20%) observed in the high-dose group after 1 and 2 weeks ($P < 0.05$). When the dose groups were combined, significant reductions of ~14% were observed after 1 and 2 weeks, respectively ($P < 0.05$).

Plasma 8-isoprostane levels tended to decrease after liposomal glutathione administration (Figure 4, bottom panel). The largest decrease (35%) was observed in the 500 mg/d group after 2 weeks ($P < 0.05$).

In order to determine if the effects differed by baseline GSH, changes in GSH after 1, 2 and 4 weeks were correlated with GSH levels at the baseline. Baseline GSH levels in red cells ranged from 4.66 to 10.9 μmol/g hemoglobin (mean ± s.e.: 6.38 ± 0.47). There were strong inverse correlations between baseline GSH and the changes in GSH levels at different time points ($r = 0.6$–0.8).

Effects of oral GSH on immune function markers in blood

Lymphocyte proliferation. Proliferative capacity tended to be increased at 1–2 weeks after liposomal GSH administration in both groups; however, this was only significant in the high-dose group after 2 weeks where an increase of 60% was observed ($P < 0.05$) (Figure 5, top panel).

Natural killer cell cytotoxicity. The increase in mean % lysis values were observed in both dose groups after 2 and 4 weeks with the largest increases of 400% and 210% occurring after 2 weeks in the low-dose and high-dose groups, respectively ($P < 0.05$) (Figure 5, bottom panel).

DISCUSSION

The results of this pilot study demonstrate for the first time increased body stores of GSH after oral administration of liposomal GSH in humans. Liposomal GSH appeared to be effective at two doses (500 and 1000 mg/d) and the effects were seen as early as 1 week. In addition, liposomal GSH had positive effects on several GSH-related parameters, including decreases in biomarkers of oxidative stress and enhancements in immune functions. Finally, liposomal GSH was highly tolerated and its

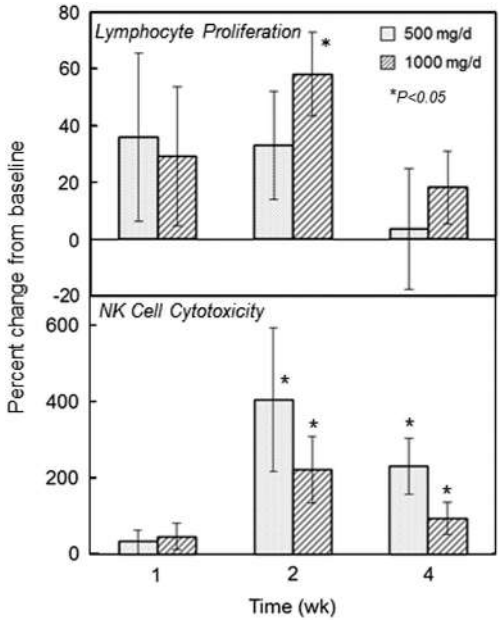

Figure 5. Effect of liposomal glutathione supplementation on lymphocyte proliferation and on NK cell cytotoxicity. The subjects were randomized to 500 or 1000 mg/d liposomal GSH for 4 weeks. Blood was collected at the baseline and after 1, 2 and 4 weeks and PBMCs were isolated. Top panel: Lymphocyte proliferation was assessed by measuring ^{3}H-thymidine incorporation after incubation with PHA, as described in the text. The results are expressed as % changes in CPM from the baseline. The baseline values ranged from 20 021 to 88 197 CPM (mean ± s.e.: 42 105 ± 6736) for all subjects (low-dose group: 22 270–40 295 (mean ± s.e.: 32 596 ± 1945); high-dose group: 20 021–88 197 (mean ± s.e.: 51 613 ± 8830)). Bottom panel: NK cytotoxicity was assessed using ^{51}Cr-labeled human K562 cells as the target and measuring the percent of target cells lysed after incubation with lymphocytes for 4 h at 37 °C. The results are expressed as % changes in the extent of cell lysis from the baseline. Baseline values ranged from 0.41 to 9.08% lysis (mean ± s.e.: 4.04 ± 0.82) for all subjects (low-dose group: 0.41–7.73 (mean ± s.e.: 3.89 ± 0.89); high-dose group: 1.41–9.08 (mean ± s.e.: 4.19 ± 0.83)). Bars are mean ± s.e. *Significantly different from the baseline by repeated measures of ANOVA, $P < 0.05$.

administration was not associated with any signs of adverse effects. Although small in size, the results from this study provide support for the potential use of oral liposomal GSH as an intervention strategy for enhancing the tissue GSH levels for use in disease therapy or prevention. In addition, they provide a rationale for additional larger placebo-controlled trials in both healthy and diseased individuals aimed at assessing the potential therapeutic efficacy of liposomal GSH. The results are consistent with previous findings where oral supplementation with non-liposomal glutathione was effective at enhancing the body stores of GSH in laboratory animals and humans.[19–24,31] While a direct comparison between the forms of GSH has not been made, liposomal GSH effects were often greater than those previously observed for non-liposomal GSH.[30] However, future side-by-side comparison studies will be required to establish the relative effectiveness of these GSH forms.

To gain a more comprehensive assessment of supplementation on body GSH stores, we measured GSH levels in different blood compartments. For most measures, GSH increases were time-dependent with maximal increases of up to 40% in whole blood, 25% in red cells, 28% in plasma and 200% in PBMCs occurring within 2 weeks. The strong inverse correlation of GSH changes with baseline levels suggests that supplementation has its greatest effect in subjects with the lowest baseline GSH levels. Increases occurred in all compartments, suggesting a systemic effect and that other less-accessible tissues may be impacted in a similar manner.

While there was a trend of increasing GSH at all time points and doses and in all cell types examined, not all changes were statistically significant, which is likely a result of the small sample size ($n = 6$ per group) in this pilot study. The lack of significant differences between dose groups may also be attributed, in part, to the limited power. While unlikely, an alternative explanation for the lack of a dose response could be a saturation effect, leading to optimal effects at the lower 500-mg dose. Additional larger-scale studies will be necessary to investigate these possibilities and establish the optimal dose of liposomal GSH.

For several parameters, the impact of liposomal GSH administration appeared to be lower at 4 weeks and then at earlier time points. A decrease in effectiveness is not expected based on previous studies. One possible explanation may be a drop in the self-administered dose occurring toward the end of the study. The test substance is a viscous liquid, which was measured out by participants in a deep-welled spoon (~1 teaspoon). In the laboratory, this dosing procedure was found to be precise (coefficient of variation = 8.2%). However, it remains possible that subjects could have had a tendency to deliver lower doses when reaching the end of the study. Unfortunately, there was no way to determine if this may have occurred in the current study.

While the majority of glutathione in cells is in the reduced form, GSH oxidation can occur, resulting in the formation of GSSG or GSSP. The levels of both GSSG and GSSP are greater during periods of oxidative stress and the ratio of GSSG and/or GSSP to GSH has often been used as biomarkers of oxidative stress.[34] Thus, the decreases observed in oxidized:reduced GSH as a result of oral liposomal GSH supplementation are likely to be indicative of a general systemic reduction in oxidative stress levels. This is supported by a concomitant decrease in the levels of the lipid peroxidation biomarker 8-isporostane. These differences were observed even though all subjects were healthy nonsmokers and, hence, were not likely experiencing high levels of oxidative stress in general. Even greater effects might be expected in individuals exposed to higher levels of oxidants such as tobacco smokers.

GSH plays an important role in the maintenance of numerous immune functions, including lymphocyte proliferation and NK cell activity.[37–39] We found that liposomal GSH administration resulted in increases in both of these activities; lymphocyte proliferation was enhanced up to 60% and as early as 1 week, and NK cell cytotoxicity was increased up to 400% and as early as 2 weeks. Further, the time course of these effects on immune function coincided with the observed increases in the levels of PBMC GSH. While the mechanisms are not known, these findings are consistent with previous *in vitro* studies of GSH on lymphocyte proliferation and NK cell activity.[40–43] These results are also consistent with earlier clinical findings with non-liposomal GSH[31] and the observed correlations of GSH content with NK cell activity.[44]

Overall, these results provide a promising rationale for the potential use of liposomal GSH to enhance antioxidant capacity and immune functions. There have been few previous reports of clinical trials with other antioxidants in healthy individuals. In fact, most of them have shown minimal or no effects on oxidative stress biomarkers and/or immune functions.[45–51] As noted above, the small size of this pilot study limits the overall representativeness of the findings to other healthy or diseased populations. Additionally, the study design did not use a placebo control. While, based on our previous study, we would not expect significant changes to occur in the measured study outcomes,[31] future placebo-controlled randomized trials with liposomal GSH will be required to confirm the specificity of its intervention effects.

CONFLICT OF INTEREST

RS and JPR received research funding for this study from Researched Nutritionals, LLC. Researched Nutritionals, LLC is a nutraceutical company that provides liposomal glutathione (Tri-Fortify Orange) to health care professionals. Other than providing research funding and liposomal GSH, Researched Nutritionals, LLC did not play a role in the design of the study, collection and analysis of the data and the decision to publish. There were no personal financial interests between any of the authors with Researched Nutritionals, LLC.

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