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# Open-labeled pilot study of cysteine-rich whey protein isolate supplementation for nonalcoholic steatohepatitis patients

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## Abstract

**Background and Aims:** Glutathione (GSH) depletion contributes to liver injury and development of steatohepatitis. Undenatured cysteine-rich whey protein isolate has been clinically proven to raise GSH in several patient groups. The aim of this study was to evaluate the effect of oral supplementation with whey protein on patients with nonalcoholic steatohepatitis (NASH).

**Methods:** In an open-labeled clinical trial, 38 patients (18 male, 20 female; mean age 48 ± 14 years) with NASH confirmed by computed tomography measurements and liver biochemistries were given with a daily dose of 20 g whey protein isolate for 12 weeks.

**Results:** A significant reduction in alanine aminotransferase (ALT) (64 ± 72 *vs* 46 ± 36, $P = 0.016$) and aspartate aminotransferase (AST) (45 ± 49 *vs* 33 ± 18, $P = 0.047$) were observed. Plasma glutathione and total antioxidant capacity increased significantly at the end of study (53 ± 11 *vs* 68 ± 11, $P < 0.05$ and 1.26 ± 0.10 *vs* 2.03 ± 0.10, $P < 0.05$). Liver attenuation index improved from −13.4 ± 11.1 to −9.7 ± 13.1 ($P = 0.048$). Hepatic macrovesicular steatosis decreased significantly after 12 weeks of supplementation (33.82 ± 12.82 *vs* 30.66 ± 15.96, $P = 0.046$). Whey protein isolate was well tolerated. No serious adverse events were observed.

**Conclusions:** The results indicate that oral supplementation of cysteine-rich whey protein isolate leads to improvements in liver biochemistries, increased plasma GSH, total antioxidant capacity and reduced hepatic macrovesicular steatosis in NASH patients. The results support the role of oxidative stress in the pathogenesis of this disease.

## Introduction

Nonalcoholic fatty liver disease (NAFLD) is becoming a common cause of chronic liver disease in Thailand reflecting the increasing prevalence of obesity and diabetes. Nonalcoholic steatohepatitis (NASH) is part of a spectrum of NAFLD that ranges from pure fatty liver (steatosis) to steatohepatitis and cirrhosis.[1] Long-standing NASH with cirrhosis has been associated with the development of hepatocellular carcinoma.[2] The pathogenesis of NASH is not well defined. A 'two hit' hypothesis has been proposed, whereby steatosis (first hit) sensitizes the liver to a variety of metabolic injuries (second hit) that lead to necrosis, inflammation and fibrosis.[3,4] Mitochondrial dysfunction is thought to play a central role in disease progression from steatosis to cirrhosis. Increase production of reactive oxygen species and mitochondrial outer membrane permeabilization result in a cascade of events leading to inflammation, hepatocellular apoptosis, fibrogenesis and fibrosis.[5] Hepatocyte and plasma glutathione (GSH) decreased in nonalcoholic liver disease patients; whereas glutathione disulfide (GSSG) increased in these patients.[6–8] As a consequence, strategies to increase glutathione concentrations and to compensate for the oxidant-antioxidant-imbalance were brought into focus of clinical research.

There are no medical drugs approved for the treatment of NASH. However, the postulated links with factors of oxidative stress, together with the observation of glutathione depletion, provide a compelling rationale for the evaluation of treatment modalities that increase glutathione. Sufficient cysteine supply is essential for the maintenance of the glutathione pool.[9] An undenatured cysteine-rich whey protein isolate has been proven to raise glutathione levels by supplying the precursors required for intracellular glutathione synthesis. This has been demonstrated in several glutathione-deficient patient groups including those with advanced human immunodeficiency virus (HIV)-infection.[10]

Consequently, a pilot, prospective clinical study was performed to determine the potential benefits of supplementation with undenatured cysteine-rich whey protein isolate in untreated patients with NASH. Hepatic steatosis was evaluated by quantitative assessment of liver-spleen attenuation differences from computed tomography and specific biochemical parameters were monitored in plasma to investigate the potential role of glutathione in patients with NASH.

## Methods

### Patients

Study participants were recruited from the Division of Gastroenterology and Hepatology at the Faculty of Medicine, Chiang Mai University, Thailand. Study inclusion criteria were defined by the diagnosis of NASH, and age between 15 and 60 years. The diagnosis of NASH was established in all patients based on the following criteria: (i) persistent elevation of aminotransferase at least 1.5 times the upper limits of normal for at least 3 months; (ii) unenhanced computed tomography showing low parenchymal liver attenuation diagnostic for hepatic macrovesicular steatosis. All patients did not respond to dietary program by their physicians before being enrolled in the study. Patients were excluded for the following reasons: (i) evidence of viral (hepatitis B virus [HBV], hepatitis C virus [HCV]) or autoimmune hepatitis; primary biliary cirrhosis; biliary obstruction; Wilson's disease; hemochromatosis and decompensated cirrhosis; (ii) presence of secondary causes of fatty liver, such as gastrointestinal bypass surgery or medications that induce steatosis; (iii) weekly ethanol consumption of more than 140 g as confirmed through an interview with the patients; (iv) pregnancy or lactation; (v) history of cow's milk protein allergy; (vi) on protein restricted diet.

All patients provided written informed consent. The protocol was reviewed and approved by the Chiang Mai University Review Board.

### Experimental design

Patients were instructed to take 20 g per day of undenatured cysteine-rich whey protein isolate for 12 weeks in two equal portions of 10 g mixed with water. No dose adjustments were made. A detailed clinical assessment was carried out in every patient. Patients were followed up at 3-week intervals for 12 weeks. At each contact point, adverse events, concurrent medication and protocol compliance were assessed. Body weight, height, waist and hip circumference, systolic and diastolic blood pressures were measured at baseline and repeated every 3-week interval during the study period. Blood samples were taken at the beginning and end of the study to allow measurement of biochemical parameters (aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, creatinine, fasting glucose, fasting lipids [triglyceride, high densit lipoprotein [HDL], low density lipoprotein [LDL] and total cholesterol], and fasting insulin), plasma glutathione level and total antioxidant capacity. Hepatic steatosis was quantitatively estimated by computed tomography at baseline and at the end of the study.[11]

### Objective assessment of NASH by computed tomography

Computed tomography (CT) was performed with a 16-slice multidetector (Aquilion16, Toshiba, Tokyo, Japan) scanner at baseline and 12 weeks after treatment. Contiguous transverse images were acquired through the liver with 7 mm collimation without intravenous contrast agent administration. Liver attenuation was measured by means of a random selection of 25 circular regions of interest (ROIs) on both lobes on five transverse sections at different hepatic levels (five ROIs per section). The ROI values were averaged to give a mean of liver attenuation. The mean splenic attenuation was measured as an internal control. The liver attenuation index (LAI) was derived from the difference between mean liver attenuation and mean splenic attenuation. A high degree of correlation between LAI and histologic steatosis was derived by using linear regression analysis. LAI was used as a parameter for prediction of the degree of hepatic macrovesicular steatosis.[11]

### Determination of glutathione content

Plasma glutathione level was determined by using dithiobisnitrobenzoic acid (DTNB) reagent.[12] A 400 μL sample of whole blood was precipitated with 3 mL of precipitating solution (0.2 g EDTA, 1.67 g meta-phosphoric acid and 30 g of sodium chloride in 100 mL of distilled water) and 1.6 mL of distilled water. After being centrifuged at 6,000 rpm for 3 minutes, 200 μL of clear filtrate was mixed with 400 μL of 0.3 M phosphate buffer (pH 8.0) and 400 μL of DTNB solution (40 mg in 100 mL of distilled water containing 1% sodium citrate). Within 5 min of incubation, color development was read at 412 nm with spectrophotometer (Libra S11, Bio-chrom, Cambridge, UK). The glutathione concentration was calculated by comparing with standard reduced glutathione (Sigma, St Louis, MO, USA). The data was presented in milligram per deciliter of plasma.

### Total antioxidant capacity by Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay was based on the reduction of the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation by antioxidants present in 80% methanolic extracts.[12] The ABTS radical cation was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture had to stand for 12–24 h until the reaction was complete and the absorbance was stable. For measurements, the ABTS solution was diluted with 80% methanol to the absorbance of $0.700 \pm 0.020$ at 734 nm. For the photometric assay, 1.48 mL of the ABTS solution and 20 μL of the extracts or Trolox standards were mixed and measured immediately at 30°C after 6 min at 734 nm using a spectrophotometer (UV-160 1PC, Shimadzu, Kyoto, Japan). Appropriate solvent blanks were run in each assay. The TEAC of 80% methanolic extracts was calculated on the basis of percentage inhibition of absorbance at 734 nm using a Trolox standard curve.

### Biochemical measurements

The biochemical assays were performed using standard laboratory techniques with minor adaptation for local laboratory constraints.

Insulin sensitivity indices were determined by homeostasis model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI). β-cell secretion was determined by homeostatic model assessment of pancreatic beta-cell function (HOMA-β) percentage.[13,14]

### Study endpoints

The primary endpoint of this study was improvement of the liver attenuation index by computed tomography at the end of the study compared to baseline. Improvement was defined as a decrease in the liver attenuation index ≥ 25% for each individual patient.[11] The secondary endpoints were changes in liver biochemistry parameters, glutathione level and total antioxidant capacity.

### Statistical analysis

Categorical data were presented as number (percentage). Continuous data were presented as mean ± standard deviation (SD), and median (range). Paired t-test was applied for pair-wise comparison of continuous variables. Spearman's correlation was used to assess the associated changes in metabolic outcomes, liver attenuation index, insulin sensitivity indices and change in aminotransferases. A two-tailed *P*-value of less than 0.05 was considered significant. All statistical analyses of study data were performed using SPSS statistical software package, version 10.01 (SPSS, Chicago, IL, USA).

## Results

### Patients enrolled

Fifty-six (56) patients suspected of having NASH were evaluated, and 38 patients were enrolled in the study. Reason for exclusion was normal liver attenuation at baseline unenhanced computed tomography ($n = 18$). The demographic features of the patient population at baseline were given in Table 1. The 38 enrolled patients comprised of 18 males and 20 females. The mean age and standard deviation of the patients were 48 years (SD 14.0 years). The mean BMI at study entry was 31 ± 5 kg/m$^2$ (range, 21–48 kg/m$^2$), with 14 (37%) patients being overweight (BMI 25–29 kg/m$^2$) and 21 (55%) being obese (BMI ≥ 30 kg/m$^2$). Six (16%) patients had impaired fasting glycemia (fasting glucose, 110–125 mg/dL). Sixteen (42%) patients had hypertriglyceridemia (fasting triglyceride ≥ 150 mg/dL), twenty-six (68%) patients had hypercholesterolemia (fasting cholesterol > 200 mg/dL), twenty-six (68%) patients had low HDL-cholesterol levels (fasting HDL-cholesterol < 50 mg/dL for female or < 40 mg/dL for male). Twenty-five (66%) patients had hypertension (blood pressure ≥ 130/≥ 85 mmHg). Twenty-one (55%) patients were diagnosed metabolic syndrome.[15]

**Table 1** Demographic features of participants at baseline (N = 38)

| Feature | N | % |
|---|---|---|
| Mean age (SD) | 48 | 14 |
| Gender | | |
| male | 18 | 47 |
| female | 20 | 53 |
| Overweight, BMI 25–29 kg/m$^2$ | 14 | 37 |
| Obese, BMI ≥ 30 kg/m$^2$ | 21 | 55 |
| Impaired fasting glucose, 110–125 mg/dL | 6 | 16 |
| Hypertriglyceridemia, ≥ 150 mg/dL | 16 | 42 |
| Hypercholesterolemia, > 200 mg/dL | 26 | 68 |
| Low HDL, female: < 50 mg/dL; male: < 40 mg/dL | 26 | 68 |
| Hypertension, ≥ 130/≥ 85 mmHg | 25 | 66 |
| Metabolic syndrome | 21 | 55 |

HDL, high density lipoprotein.

### Biochemical responses

The baseline levels and changes in serum biochemistries after 12 weeks of supplementation with undenatured cysteine-rich whey protein isolate were summarized in Table 2. The mean pretreatment alanine aminotransferase (ALT) for all the patients was 64 ± 72 U/L. This substantially decreased at the end of study to 46 ± 36 U/L ($P = 0.016$). Similarly, the mean pretreatment aspartate aminotransferase (AST) was 45 ± 49 U/L, which was significantly reduced to 33 ± 18 U/L post-treatment ($P = 0.047$). Serum gamma glutamyl transpeptidase (GGT) levels fell from an average of 58 U/L at baseline to 49 U/L at 12 weeks ($P = 0.006$). There were no significant changes in plasma glucose, total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, alkaline phosphatase and fasting insulin levels from the beginning to the end of study. The mean pretreatment glutathione level and total antioxidant capacity significantly increased after 12 weeks of whey protein supplementation (53 ± 11 *vs* 68 ± 11, $P < 0.05$ and 1.26 ± 0.10 *vs* 2.03 ± 0.10, $P < 0.05$, Fig. 1).

**Table 2** Clinical and metabolic characteristics at baseline and after 12 weeks of whey protein isolate supplementation

| | Baseline | End of study | *P*-value |
|---|---|---|---|
| FPG (mg/dL) | 97.37 ± 23.02 | 98.08 ± 21.18 | 0.708 |
| Cholesterol (mg/dL): Total | 225.05 ± 46.61 | 221.24 ± 48.98 | 0.357 |
| LDL | 147.66 ± 42.12 | 147.03 ± 39.58 | 0.876 |
| HDL | 43.95 ± 7.70 | 44.45 ± 7.32 | 0.569 |
| Triglycerides (mg/dL) | 183.13 ± 155.01 | 168.58 ± 108.34 | 0.177 |
| AST (U/L) | 44.58 ± 49.03 | 33.13 ± 17.78 | 0.047 |
| ALT (U/L) | 64.18 ± 72.07 | 45.89 ± 35.99 | 0.016 |
| ALP (U/L) | 81.82 ± 50.12 | 79.24 ± 41.19 | 0.260 |
| GGT (U/L) | 57.71 ± 38.23 | 48.92 ± 31.69 | 0.006 |
| Fasting insulin (μU/ml) | 12.88 ± 8.77 | 15.78 ± 11.25 | 0.102 |
| HOMA-IR | 3.15 ± 2.34 | 3.77 ± 2.61 | 0.164 |
| QUICKI | 0.338 ± 0.039 | 0.327 ± 0.029 | 0.055 |
| HOMA-β | 192.32 ± 190.71 | 217.64 ± 206.79 | 0.049 |

NOTE. Data are presented as mean ± SD.
AST, Aspartate aminotransferase; ALT, Alanine aminotransferase, ALP, Alkaline phosphatase; FPG, fasting plasma glucose; GGT, gamma glutamyl transpeptidase; HDL, high-density lipoprotein; HOMA-β, homeostasis model assessment of β cell function; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; QUICKI, quantitative insulin-sensitivity check index.

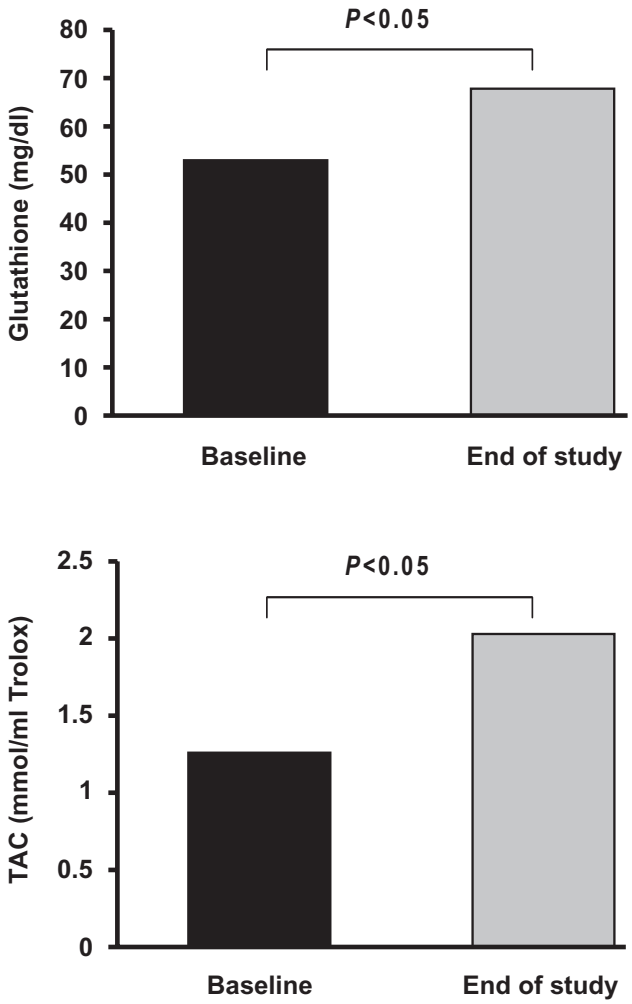


**Figure 1** Reduced glutathione and total antioxidant capacity (TAC) are significantly increased after 12 weeks supplementation with cysteine rich whey protein isolate compared to baseline.

Insulin sensitivity indices were determined by homeostasis model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI). Sixteen (42%) of patients were determined as insulin resistant (HOMA-IR > 2.6) at the beginning of the study. HOMA-IR did not change from baseline (HOMA-IR, 3.15 ± 2.34 *vs* 3.77 ± 2.61, $P = 0.164$), nor did QUICKI (0.338 ± 0.039 *vs* 0.327 ± 0.029, $P = 0.055$). β-cell secretion as determined by homeostatic model assessment of pancreatic beta-cell function (HOMA-β) percentage decreased by 25.3 ± 18.6% ($P = 0.049$).

### Anthropometric results

A majority of patients (56%) lost weight during whey protein isolate supplementation. The average reduction in weight was 1.15 (range, −11 to +3) kilograms, which represented a 1.5% decrease from baseline (range, −11.96 to +5.21%). The results of the anthropometric measurements made during the study were summarized in Table 3. Mean body mass index decreased by 0.44 ± 1.21 kg/m$^2$ ($P = 0.308$) at the end of study. However, reduction in BMI did not correlate with improvement in either AST ($P = 0.129$) or ALT ($P = 0.116$). Waist-hip ratios did not change, but waist circumferences decreased significantly ($P = 0.001$). Improvement in liver attenuation index (LAI) ≥ 25% was observed in 24 of 38 patients (63%). Mean LAI increased from −13.4 ± 11.1 at baseline to −9.7 ± 13.1 after treatment ($P = 0.048$). Improvement in LAI by unenhanced computed tomography correlated with improvement in either AST ($r = 0.310$, $P = 0.029$) or ALT ($r = 0.280$, $P = 0.045$). Improvement in LAI also correlated with improvement in BMI ($r = 0.343$, $P = 0.018$). The degree of hepatic steatosis estimated by unenhanced computed tomography decreased significantly after 12 weeks of whey protein isolate supplementation (33.82 ± 12.82 *vs* 30.66 ± 15.96, $P = 0.046$). No significant difference in systolic blood pressure (136 ± 18 *vs* 138 ± 17 mmHg, $P = 0.305$) or diastolic blood pressure (88 ± 15 *vs* 90 ± 15 mmHg, $P = 0.207$) was observed before and after supplementation.

**Table 3** Anthropometric data at baseline and after 12 weeks of whey protein isolate supplementation

| | Baseline | End of study | *P*-value |
|---|---|---|---|
| Body weight (kg) | 78.12 ± 16.69 | 76.97 ± 16.31 | 0.024 |
| Body mass index (kg/m$^2$) | 30.93 ± 5.23 | 30.49 ± 5.19 | 0.031 |
| Waist circumference (cm) | 99.75 ± 14.10 | 95.74 ± 11.42 | 0.001 |
| Waist-hip ratio | 0.93 ± 0.08 | 0.92 ± 0.07 | 0.431 |
| Systolic BP (mmHg) | 136.08 ± 18.13 | 137.79 ± 17.06 | 0.305 |
| Diastolic BP (mmHg) | 87.92 ± 14.55 | 89.82 ± 14.61 | 0.207 |
| Liver attenuation index | −13.4 ± 11.1 | −9.7 ± 13.1 | 0.048 |
| Predicted hepatic steatosis (%) | 33.82 ± 12.82 | 30.66 ± 15.96 | 0.046 |

Note. Data are presented as mean ± SD.

### Side effects and adverse events

Undenatured cysteine-rich whey protein isolate was generally well tolerated. No serious adverse events were recorded. Gastrointestinal disturbance was most troublesome on initiation of treatment although often improved after a few days. No change in renal function was observed during the study.

## Discussion

In this pilot study, we demonstrated that oral undenatured cysteine-rich whey protein isolate supplementation for 12 weeks was associated with significant biochemical and metabolic improvement in patients with NASH. The improvement in steatosis was documented by hepatic imaging as unenhanced computed tomography of liver showed marked decrease in liver fat. A statistically significant improvement in antioxidant status, as measured by serum glutathione level and total antioxidant capacity was also observed. All patients tolerated whey protein isolate well, with no serious adverse effects.

NASH is now considered to be a common manifestation or even predictor of metabolic syndrome.[16,17] Our patients had a high frequency of metabolic risk factors for NAFLD and the majority of patients were diagnosed with metabolic syndrome. NASH could progress to cirrhosis in up to 20–30% of these patients.[1] No effective therapy for preventing disease progression or amelioration of this disease currently exists. After 12 weeks of whey protein isolate supplementation, sixty-six percent of the patients had liver enzyme improvement. Sixty-three percent of the patients had improvement in hepatic steatosis. The improvement in liver enzymes in the study was associated with the degree of hepatic steatosis resolution of NASH. Short-term oral supplementation with cysteine-rich whey protein isolate thus could prove a useful supplement for the management of NASH in addition to improving hepatic steatosis.

Undenatured cysteine-rich whey protein isolate produces a sustained delivery of cysteine to the cells via normal metabolic pathways. By providing abundant cysteine, this whey protein allows the cells to synthesize and replenish glutathione (L-g-glutamyl-L-cysteinyl-glycine; GSH) levels without adverse or toxic effects. This protein might well find applications in diseases where oxidative stress and pathology of GSH metabolism are largely implicated. Decreased GSH and/or increased GSSG contributed to liver injury susceptibility and toxic risk by altering fundamental cell functions such as protein synthesis, enzyme activities, transport processes, microtubular and other structural support, and secretion mechanisms. Oxidative stress characterized by an imbalance between pro-oxidant and antioxidant mechanisms in favor of the former, has long been recognized as a key mechanism responsible for liver damage and disease progression in NAFLD.[18] Enhanced oxidative stress occurs in the liver of patients with NASH, as well as in animal models of NASH. Because of the importance of oxidative stress in the pathogenesis of NASH, we used plasma glutathione and total antioxidant capacity as markers of systemic oxidative stress to reflect the level of oxidative stress in the liver. Cysteine-rich whey protein isolate supplementation significantly increased plasma glutathione levels and total antioxidant capacity in all patients. It, thus, seems reasonable to conclude that the results of this study support the postulate that oxidative stress is a key mechanism responsible for liver damage and disease progression in NAFLD.

Modest, but statistically significant, reduction in body weight, body mass index, and waist circumference were observed after supplementation of oral whey protein isolate for 12 weeks. Improvement in metabolic outcomes correlated with improvement in hepatic steatosis. However, reduction in body mass index did not correlate with improvement in aminotransferase. The improvement of hepatic steatosis was not associated with weight reduction. Because patients included in the study were not prescribed any specific diet or enrolled in a weight-reducing program, we believed that the reduction in weight, body mass index and waist circumference may be a secondary effect of whey protein isolate supplementation.

An association with insulin resistance and NASH has been shown in various studies.[19,20] However, the results of the study were inconclusive with the measurements of insulin sensitivity indices, except β-cell secretion determined by HOMA-β percentage. These indirect measurements may be too insensitive to reflect changes in insulin resistance. Alternatively, the main therapeutic mechanisms of whey protein may be by preventing of the second hit such as glutathione generation by oxidative stress pathway.

In conclusion, undenatured cysteine-rich whey protein isolate supplementation of NASH patients lead to a significant reduction of hepatic steatosis as determined by liver attenuation index from computed tomography, a significant reduction of AST and ALT levels, a significant increase of plasma glutathione level and total antioxidant capacity as well as improvement in metabolic outcomes. Supplementation with whey protein might well find other applications for patients where oxidative stress and pathology of glutathione deficiency are implicated. Randomized, long-term controlled trials including histologic assessment with cysteine-rich whey protein isolate are warranted.

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