EFFECT OF ORAL SUPPLEMENTATION OF WHEY PROTEIN ISOLATE ON NON-ALCOHOLIC STEATOHEPATITIS PATIENTS

Prasong Tienboon MD, PhD.[1], Taned Chitapanarux MD.[2], Suwalee Pojchamarnwiputh MD.[3], Donrawee Leelarungrayub PhD.[4]

[1]Division of Nutrition, Department of Pediatrics, Faculty of Medicine,
[2]Division of Gastrohepatology, Department of Medicine, Faculty of Medicine,
[3]Division of Diagnostic Radiology, Department of Radiology, Faculty of Medicine,
[4]Department of Physical Therapy, Faculty of Associated Medical Sciences,
Chiang Mai University, Chiang Mai 50200, Thailand.

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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 to 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]. Mitochondrial dysfunction is thought to play a central role in disease progression. An increased production of reactive oxygen species, and mitochondrial outer membrane permeabilization, results in a cascade of events leading to inflammation, hepatocellular apoptosis, fibrogenesis and fibrosis[4]. Hepatocyte and plasma glutathione (GSH) decreased in nonalcoholic liver disease patients; whereas glutathione disulfide (GSSG) increased in these patients[5]. As a consequence, strategies to increase glutathione concentrations and to compensate for the oxidant-antioxidant-imbalance have become a focus of clinical research.

There are no medicinal 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[6]. HMS 90 R, 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 HIV-infection. Consequently, a pilot, prospective clinical study was performed to determine the potential benefits of supplementation with undenatured, cysteine rich whey protein isolate (HMS 90 ®*) 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.A brief description of the study design, methods used and a summary of the results is presented below. A full paper will be published shortly.

Methods

1. Patients - Study participants were recruited from the Division of Gastroenterology and Hepatology at the Faculty of Medicine, Chiang Mai University. Study inclusion criteria were defined by the diagnosis of NASH, age between 15 and 60 years. The diagnosis of NASH was established in

all patients based on the following criteria: (1) persistent elevation of aminotransferases at least 1.5 times the upper limits of normal for at least 3 months; (2) unenhanced computed tomography showing low parenchymal liver attenuation diagnostic for hepatic macrovesicular steatosis. All patients did not respond to a dietary program by their physicians before being enrolled in the study. Patients were excluded for the following reasons: (1) presence of other forms of liver disease; (2) presence of secondary causes of fatty liver, such as gastrointestinal bypass surgery or medications that induce steatosis; (3) weekly ethanol consumption of more than 40 g as confirmed through an interview with the patients; (4) pregnancy or lactation; (5) history of cow milk's protein allergy; (6) on protein restricted diet. All patients gave written informed consent. The protocol was reviewed and approved by the Chiang Mai University Review Board. HMS 90® is a registered trademark of Immunotec Inc., Canada

2. Experimental Design - Patients were instructed to take 20 g per day of undenatured, cysteine rich, whey protein isolate (HMS 90?) 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 every contact point, adverse events, concurrent medication and protocol compliance were assessed. Body weight, height, waist and hip circumference, systolic and diastolic blood pressure was measured at baseline and repeated at 3-week intervals during the study period. Blood samples were taken at the beginning and end of the study to allow measurement of biochemical parameters [(aminotransferases, alkaline phosphatase, gamma glutamyl transpeptidase, fasting glucose, fasting lipids [triglyceride, HDL, LDL and total cholesterol], plasma glutathione level, Total antioxidant activity (TAC), Malondialdehyde (MDA), and Protein hydroperoxide (PrOOH))]. NASH severity was estimated by computed tomography at baseline and at the end of the study.

3. Objective assessment of NASH by Computed Tomography - Computed tomography (CT) was performed with a 16-slice multi-detector (Aquilion16, Toshiba, Tochigi-Ken, Japan) scanner. 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 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. LAI was used as a parameter for prediction of the degree of hepatic macrovesicular steatosis[7].

4. Biochemical measurements - The biochemical assays performed used standard laboratory techniques with minor adaptation for local laboratory constraints.

5. Study Endpoints - The primary endpoint of this study was improvement of the liver attenuation index at the end of the study compared to baseline. The secondary endpoints were changes in biochemical parameters.

6. Statistical Analysis - 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. The mean age and standard deviation (SD) of the subjects were 48 years (SD 14 years). The demographic features of the patient population at baseline are given in Table 1.

Table 1. Demographic Features of Participants at Baseline (N = 38)

Feature		N	(%)
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-cholesterol (female: < 50 mg/dl, male: < 40 mg/dl)		26	68
Hypertension, >130/>85 mmHg		25	66
Metabolic syndrome		21	55

Anthropometric Results - The results of the anthropometric measurements made during the study are summarized in Table 2.

Measurement of anti-oxidants and markers of oxidative stress - The results of measurements of plasma glutathione, total anti-oxidants capacity, malondialdehyde (MDA), protein hydroperoxide (PrOOH) and Haemolysis are presented in Table 3. After supplementation with HMS 90(®) protein, the results showed that glutathione (GSH), total antioxidant activity (TAC) improved significantly, whereas the malondialdehyde (MDA), protein hydroperoxide (PrOOH), and percentage of haemolysis decreased significantly.

Biochemical Responses - The baseline levels and changes in serum biochemistries after 12 weeks supplementation with undenatured, cysteine rich, whey protein isolate are summarized in Table 4.

Table 2. Anthropometric data at baseline and after 12 weeks supplementation

	Baseline Mean + SD	End of study Mean + SD	P-value
Body weight (kg)	**78.1 + 16.7**	**77.0 + 16.3**	**0.024**
Body mass index (kg/m^2)	**30.9 + 5.2**	**30.5 + 5.2**	**0.031**
Waist circumference (cm)	**99.7 + 14.1**	**95.7 + 11.4**	**0.001**
Waist-hip ratio	0.9 + 0.1	0.9 + 0.1	0.431
Systolic blood pressure (mmHg)	136.0 + 18.1	137.8 + 17.1	0.305
Diastolic blood pressure (mmHg)	87.9 + 14.6	89.8 + 14.6	0.207
Liver attenuation index	**-13.4 + 11.1**	**-9.7 + 13.1**	**0.048**
Predicted histologic steatosis (%)	**33.8 + 12.8**	**30.6 + 16.0**	**0.046**

Table 3. Biochemical parameters; GSH, TAC, MDA, PrOOH, and Haemolysis at baseline and after 12 weeks supplementation

Parameters	Baseline Mean + S.E. (range)	End of study Mean + S.E. (range)
Glutathione (mg/dl)	52.95 + 1.82 (32-75)	67.80 + 1.75* (41-85)
Total antioxidant activity (TAC) (mmol/ml Trolox)	1.26 + 0.097 (0.50-2.50)	2.03 + 0.092 * (0.75-3.50)
Malondialdehyde (MDA) (micromol/L)	22.52 + 0.78 (10-35)	18.10 + 0.69* (0-28)
Protein hydroperoxide (PrOOH) (micromol/L)	2.28 + 0.16 (0.75-10.00)	1.68 + 0.25 * (.50-7.50)
Haemolysis (%)	26.47 + 0.75 (18-35)	24.78 + 0.61 * (9-35)

(* $p < 0.05$ compared to baseline data for each parameter)

Table 4. Clinical and metabolic characteristics at baseline and after 12 weeks supplementation.

	Baseline Mean + SD	End of study Mean + SD	P-value
FPG (mg/dl)	97.4 + 23.0	98.0 + 21.2	0.708
Cholesterol (mg/dl)			
Total	225.0 + 46.6	221.2 + 49.0	0.357
LDL	147.6 + 42.1	147.0 + 39.6	0.876
HDL	43.9 + 7.7	44.4 + 7.3	0.568
Triglycerides (mg/dl)	183.1 + 155.0	168.5 + 108.3	0.177
AST (U/L)	**44.5 + 49.0**	**33.1 + 17.8**	**0.047**
ALT (U/L)	**64.1 + 72.0**	**45.8 + 36.0**	**0.016**
GGT (U/L)	**57.7 + 38.2**	**48.9 + 31.7**	**0.006**
ALP (U/L)	81.8 + 50.1	79.2 + 41.2	0.260

FPG, fasting plasma glucose; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase, ALP, Alkaline phosphatase; GGT, gamma glutamyl transpeptidase.

CT Results - The mean attenuation values of liver and spleen at baseline and at the end of the study are shown on Table 5.

When the baseline LAI values are compared to those obtained at the end of the study, the primary efficacy criteria for the study, a statistically significant improvement was observed (Wilcoxon Signed Ranks test, P = 0.048).

Table 5. The mean CT attenuation of liver and spleen and LAI at baseline and after 12 weeks supplementation.

	Baseline (HU)			End of study (HU)		
	Liver	Spleen	LAI	Liver	Spleen	LAI
Mean CT Att^n	32.11	45.49	-13.39	35.12	44.88	-9.76
(range)	(8.17-51.55)	(39.88-55.16)	(-37.13)-(1.73)	(-2.87-56.56)	(39.22-54.76)	(-42.23)-(13.84)

Side Effects and Adverse Events - Undenatured cysteine rich whey protein isolate (HMS 90(®)) 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, conducted to evaluate the benefit of supplementing NASH patients with undenatured, cysteine rich, whey protein isolate (HMS 90 ®), both the primary and secondary objectives of the study were attained. Firstly, an objective assessment of the degree of severity of NASH determined by non-invasive methods (unenhanced CT scans) demonstrated a statistically significant improvement ($p < 0.05$) of the condition. Alternatively, if we consider that an LAI increase of >2 at the end of the study is a success, LAI + 2 from baseline is "no change" and an LAI reduction of <-2 as a failure then:

LAI	Success	No change	Failure
No of patients	24	3	11
No of Patients (%)	63	8	29

This is accompanied by a statistically significant reduction of the enzymes AST, ALT and GGT.

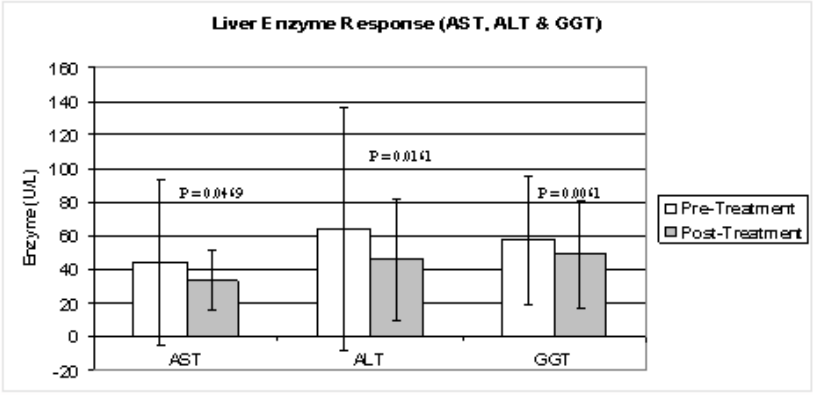

Figure 1.AST, ALT and GGT are significantly decrased after 12 weeks supplementation with Cysteine Rich Whey Protein Isolate (HMS 90®) compared to baseline.

Furthermore, a statistically significant improvement in antioxidant status, as measured by serum glutathione level and Total Anti-oxidant Capacity was also observed.

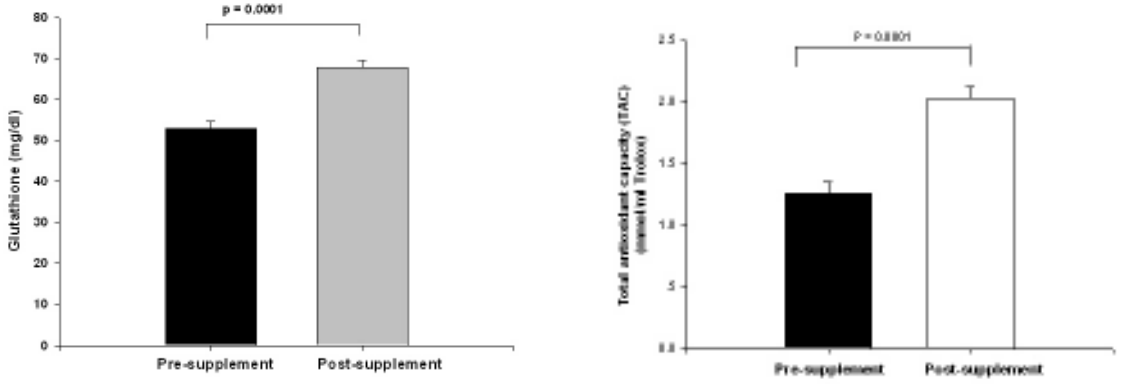

Figure 2.Reduced Glutathione and Total antioxidant capacity (TAC) are significantly increased after 12 weeks supplementation with Cysteine Rich Whey Protein Isolate (HMS 90®) compared to baseline

This was associated with a corresponding decrease of MDA and PrOOH, markers of oxidative stress. The latter was also statistically significant.

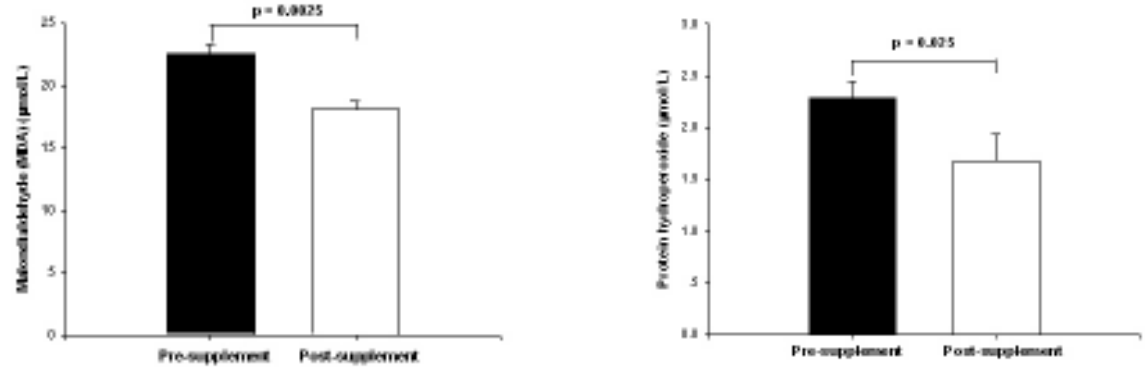

Figure 3. Malondialdehyde (MDA) and Protein hydroperoxide (PrOOH) were significantly reduced after 12 weeks supplementation with Cysteine Rich Whey Protein Isolate (HMS 90®).

Undenatured cystine rich whey protein isolate produces a sustained delivery of cysteine to cells via normal metabolic pathways. By providing abundant cysteine, this whey protein allows cells to synthesize and replenish glutathione (L-gamma- glutamyl -L-cysteinyl-glycine; GSH) levels with out adverse or toxic effects. The significant increase of glutathione plasma concentrations in the present study was associated with improvement in liver enzymes and with a reduction of the degree of macrovesicular steatosis. The results of this study support the postulate that oxidative stress is a key mechanism responsible for liver damage and disease progression in NAFLD.

In conclusion, undenatured cystine rich whey protein isolate (HMS 90®) supplementation of NASH patients leads to a significant reduction of hepatic steatosis resolution determined by computed tomography, a significant reduction of AST and ALT levels and a significant increase of glutathione and total anti-oxidant capacity. Supplementation with this protein might well find other applications for patients where oxidative stress and pathology of GSH metabolism are implicated.

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