Effects of a whey protein supplementation on intrahepatocellular lipids in obese female patients

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Abstract: IHCL, intrahepatocellular lipids; WPS, whey protein supplement-ation; MR, Magnetic Resonance; 1H-MRS, 1H- Magnetic Resonance Spectros-copy; NAFLD, non-alcoholic fatty liver disease; OGTT, oral glucose tolerance test; HOMA, homeostasis assessment model; HOMA IS, HOMA of insulin sensitivity; NEFA, non esterified fatty acids; BOHB, beta-hydroxybutyrate.

Methods: Eleven obese women received a 60 g/day whey protein supplement (WPS) for 4-weeks, while otherwise nourished on a spontaneous diet. IHCL concentrations, visceral body fat, total liver volume (MR), fasting total-triglyceride and cholesterol concentrations, glucose tolerance (standard 75 g OGTT), insulin sensitivity (HOMA IS index), creatinine clearance, blood pressure and body composition (bio-impedance analysis) were assessed before and after 4-week WPS.

Results: IHCL were positively correlated with visceral fat and total liver volume at inclusion. WPS decreased significantly IHCL by 20.8 ± 3.7%, fasting total TG by 15 ± 6.8%, and total cholesterol by 7.3 ± 7.8%. WPS slightly increased fat free mass from 54.8 ± 2.2 kg to 56.7 ± 2.5 kg, p = 0.005. Visceral fat, total liver volume, glucose tolerance, creatinine clearance and insulin sensitivity were not changed.

Conclusions: WPS improves hepatic steatosis and plasma lipid profiles in obese non diabetic patients.

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hypothesis, we assessed the effects of a 4-week supplementation with 60 g/day whey protein (Whey Protein Supplement : WPS) in obese non diabetic female patients.

2. Research design and method

2.1. Participants

11 obese female patients, aged 38 ± 2 years, were recruited at the obesity clinics of the Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland. They had a mean body weight of 99.7 ± 5.3 kg, a mean height of 1.63 ± 0.02 m, and a mean BMI of 37.6 ± 1.8 kg/m². None had liver or renal disease, nor was on antidiabetic or antilipemic agents. They were sedentary (less than two sessions of physical activity per week). All reported a daily alcohol intake less than 20 g. The experimental protocol was approved by the Ethical Committee of Lausanne University School of Medicine and was registered at ClinicalTrials.gov (Trial Number: NCT00870077, ClinicalTrials.gov). Subjects gave their written informed consent before participating in the study.

2.2. Study design

After inclusion, subjects reported in the morning after an overnight fast to the Cardiomet Clinical Investigation Center (Cardiomet CIC) of the Lausanne University Hospital. Their body weight and blood pressure were measured and their body composition was assessed by bioelectrical impedance analysis. Thereafter, they underwent a standard 75 g oral glucose tolerance test (OGTT) with measurement of plasma glucose and insulin at time 0 and 120 min. Fasting plasma triglycerides, total cholesterol, HDL cholesterol, urea, creatinine, ASAT, ALAT and urinary urea and creatinine concentrations were assessed using a bench-top clinical chemistry analyzer (RX Monza, Randox Laboratories Ltd, Crumlin, UK). Plasma insulin and glucagon were measured by radio-immuno assays using kits from Millipore, St Charles, Missouri, USA). A 24 h urine collection was obtained for determination of urea and creatinine excretion. Total nitrogen excretion was calculated assuming that urea accounted for 85% of total urinary nitrogen and that extra-renal nitrogen losses were 2 g/day. Total energy expenditure and net substrate oxidation rates were measured during 45 min before and after the 120 min after oral glucose ingestion by indirect calorimetry (Deltatrack II, Datex Instruments, Helsinki, Finland).

On the following day, intrahepatic cellular lipids (IHCL), visceral fat volume, and total liver volume were measured by clinical Magnetic Resonance (MR) methods at the Department of Clinical Research of University Bern.

IHCL content was determined by 1H MR spectroscopy (MRS) on a clinical 3 T MR system (TIM Trio, Siemens Medical, Germany) using the whole body coil for excitation. A volume of interest (2.5 × 2.5 × 3 cm³) was localized in the liver using the body array surface coils for signal detection and a double echo localization sequence combined with Siemens’ 2D “prospective acquisition correction” (PACE) scheme, based on a 2D gradient echo image to monitor the position of the diaphragm for triggering in expiration (MRS echo time TE 30 ms, TR according to the breathing cycle between 2.5 and 6 s, 4000 Hz spectral width, 2048 data points). MRS was preceded by fast spin echo MRI (HASTE [Half Fourier Acquisition Single Shot Turbo Spin Echo], echo time 89 ms, repetition time 1030 ms, flip angle 150°, nominal resolution 1.7 × 1.3 × 5 mm³) in three planes using the same PACE triggering to visualize the liver and to reliably reproduce the placement of the ROI in follow-up examinations. The ROI was placed evading large vessels and proximity to extrahaepatic fat. The magnetic field distribution over the ROI was optimized in

breath-hold using the manufacturers automated gradient shim routine. For choice of proper flip angle, a B1 mapping scan was recorded in expiration prior to MRS. MR spectra were recorded with water presaturation to determine the lipid and metabolite spectra (32 acquisitions, 60 Hz suppression bandwidth, center frequency at 3 ppm) and without water suppression to acquire the water signal as internal standard (16 scans, center frequency at 4.7 ppm). Automatic fitting of the MR spectra was performed with the home-written software FTAID allowing for the use of Voigt lines and implementation of prior knowledge restraints. The lipid spectrum was modeled using 9 Voigt lines to describe all spectral components and initial model optimizations based on an average spectrum from several subjects, further 5 lines were used to cover the metabolites and residual water. Absolute quantification was performed in analogy to Bortolotti et al and was based on the peak areas of the methylene protons that are not neighbors of an allylic or carboxylic carbon, basic assumptions on lipid composition, the water peak area from non-water-suppressed scans, an assumed liver water content and relaxation corrections based on literature values. Results were expressed as volume percentage of lipid.

Volumes of the liver and visceral adipose tissue (VAT) were determined using T1-weighted images of the abdomen, recorded in breath-hold (multi-slice turbo echo technique, echo train length 7, echo spacing 7.6 ms, repetition time 452 ms, echo time 38 ms, flip angle 130°). 30 axial slices in 6 slabs covering the pelvis at the lower end and the diaphragm at the upper end, slice thickness of 10 mm, gap between slices 10 mm, 5 slices per breath-hold sequence, acquisition matrix 256 × 147 with a resolution of 2 mm/pixel, body coil was used for excitation and signal acquisition). Volumetry was performed using a semi-automatic implementation of the point counting method, which represents a sparse sampling scheme whereby an operator accepts or rejects points from a regular grid that covers the targeted anatomic structure in a random orientation. Visceral fat was counted on images between pelvis and the upper end of the diaphragm.

After these initial measurements, WPS was provided as bags containing 20 g of commercialized whey protein (WheyProtein94, Sponser, Wollerau, Switzerland), with instructions to consume the content of one bag diluted into 300 ml water 30 min before breakfast, lunch and dinner. Total WPS supplementation amounted to 60 g/day. Their food and drink intake was otherwise left ad libitum. The study was performed as an open label, unblinded, uncontrolled study.

1, 2 and 3 weeks after the beginning of WPS, volunteers returned to the cardiomet CIC and fasting blood sample was obtained for the measurement of plasma triglycerides, total cholesterol, HDL cholesterol, urea, creatinine, ASAT, ALAT and glycemia. 24-h urine collections were also obtained to measure urea and creatinine excretion. Compliance to WPS was assessed by collecting the empty supplementation bags.

After 4-weeks WPS, all measurements performed at inclusion, OGTT and MR determination of IHCL, visceral fat volume, and liver volume were repeated.

2.3. Analytic procedures

After collection, blood and urine samples were sent to the Central Laboratory of CHUV for measurements of fasting plasma total- triglycerides, total cholesterol, HDL cholesterol, urea, creatinine, ASAT, ALAT and 24 h urea and creatinine excretion. For the other blood parameters, blood was centrifuged at 4 °C for 10 min, at 3600 rpm, and plasma were stored at −20 °C/−80 °C until further analysis. Glucose concentrations were measured by the glucose oxidase method with a Beckman Glucose Analyzer II (Beckmann Glucose Analyzer II, Beckmann Instruments, Fullerton, CA). Plasma
radio-immunoassays, plasma non esteri- 
MO, USA) and glucagon (RIA kit from LincoMillipore, St Charles-

insulin (RIA kit from LincoMillipore, St Charles-

Billerica, Missou-

Germany) were measured enzymatically.

concentrations (kit from Boehringer Mannheim, Mannheim, 

Neuss, Germany), and plasma beta-hydroxybutyrate (BOHB) 

from Boehringer MannheimWako Chemical GmbH, Man-

heim, Germany) were measured enzymatically.

characteristics of subjects before and after WPS. 

Table 1

Characteristics of subjects before and after one month of WPS.

<table>
<thead>
<tr>
<th>Anthropometric variables</th>
<th>BASELINE</th>
<th>WPS</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>99.7 ± 5.3</td>
<td>100.2 ± 5.4</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>37.6 ± 1.8</td>
<td>37.8 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>44.8 ± 3.4</td>
<td>43.5 ± 3.2</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Blood parameters

- Glucose (mmol/L): 5.2 ± 0.2 vs. 5.2 ± 0.3 (NS)
- Insulin (µU/mL): 16.8 ± 2.1 vs. 17.2 ± 2.9 (NS)
- HOMA IS: 4.0 ± 0.6 vs. 4.3 ± 1.0 (NS)
- 2h-Glucose (mmol/L): 7.6 ± 0.9 vs. 7.4 ± 0.8 (NS)
- 2h- Insulin (µU/mL): 111.1 ± 20.6 vs. 97.1 ± 19.1 (NS)
- Glucagon (ng/L): 37 ± 2 vs. 40 ± 4 (NS)
- NEFA (µmol/L): 615 ± 57 vs. 616 ± 60 (NS)
- BOHB (µmol/L): 58 ± 25 vs. 41 ± 9 (NS)
- Triglycerides (mmol/L): 1.65 ± 0.22 vs. 1.34 ± 0.17 (0.020)
- Cholesterol (mmol/L): 5.65 ± 0.32 vs. 5.25 ± 0.35 (0.024)
- HDL Cholesterol (mmol/L): 1.13 ± 0.07 vs. 1.13 ± 0.05 (NS)
- ASAT (U/L): 21 ± 1 vs. 21 ± 1 (NS)

Substrate oxidation

- Energy Expenditure (kcal/FFM/min): 0.019 ± 0.001 vs. 0.018 ± 0.001 (NS)
- Carbohydrate oxidation (kcal/FFM/min): 1.39 ± 0.31 vs. 1.33 ± 0.35 (NS)
- Lipid oxidation (kcal/FFM/min): 0.85 ± 0.12 vs. 0.63 ± 0.20 (NS)

MR

- IHCL (vol%): 7.8 ± 2.2 vs. 6.3 ± 2.1 (0.017)
- Liver volume (cm³): 1761 ± 138 vs. 1756 ± 169 (NS)
- Visceral mass (cm³): 3213 ± 245 vs. 3184 ± 229 (NS)

All values are expressed as mean ± SEMs. Differences between pre and post whey protein supplementation (WPS) were assessed by the paired parametric t-test. p-value < 0.05 was considered significant NS, not significantly different.

2.4. Statistical analysis

All data were expressed as mean ± SEMs. Parameters measured every week throughout the WPS were analyzed by a one way ANOVA for repeated time. An average value (WPS mean) was calculated for the whole WPS period, when time effect was not significant. Values were compared between pre and post WPS by paired t-tests. The distribution of IHCL concentrations was mark-
edly skewed, and data were log-transformed for statistical analysis. Correlations between IHCL and other parameters were assessed by the Spearman’s rank correlation coefficient test.

3. Results

Characteristics of subjects before and after WPS are shown in Table 1. Obese patients had a BMI ranging between 30.9 and 52.4 kg/m², and IHCL concentrations ranging between 1.9% and 20.5% of liver volume. 5 subjects had NAFLD using a cut-off values of IHCL of 5%. 3 subjects had 2 h plasma glucose concentrations >140 mg/dl (ca 7.8 mmol/l), indicating impaired glucose tolerance. Average HOMA index was >2.77 indicating that this group of obese women had significant insulin resistance.

Positive correlations were observed between IHCL and liver volume (p = 0.63, p = 0.036, Fig. 1), visceral fat volume (p = 0.86, p = 0.001, Fig. 1), ALAT (p = 0.73, p = 0.010), and HOMA IS index (p = 0.62, p = 0.041). No correlation was observed between IHCL and BMI or total fat mass.

WPS led to a sustained increase in calculated daily nitrogen excretion (Table 2). Plasma urea concentration also increased while plasma creatinine, daily urinary creatinine excretion and creatinine clearance did not change. Body weight remained unchanged over the 4-week supplementation while body fat mass was slightly reduced and fat free mass was slightly increased. Visceral fat volume and liver volume were not changed (Table 1).

After 4-week WPS, IHCL concentrations had decreased by 20.8 ± 7.7% (p = 0.017), fasting plasma triglyceride had decreased by 15.0 ± 6.9% (p = 0.020) and total plasma cholesterol concentration had decreased by 7.3 ± 2.7% (p = 0.024). Fasting and 2h plasma glucose and insulin concentrations were not changed (Table 1).

4. Discussion

Previous studies have shown that a high protein diet reduced hepatic lipid concentrations in high-fat or high-sucrose fed rodents or humans, suggesting that a high protein intake may, directly or indirectly, improve hepatic steatosis.3-5 We therefore assessed, in obese glucose intolerant young women, whether a 4-week
supplementation with whey protein, with an otherwise spontaneous, uncontrolled food intake, would reduce hepatic steatosis and concomitantly improve hyperlipidemia and insulin sensitivity. IHCL concentrations showed a large interindividual variability in obese subjects, ranging from 1.3% to 20.5%. IHCL concentrations were correlated with total liver volume and with visceral fat volume, but not with total body fat or BMI, corroborating several reports showing that hepatic fat deposition is tightly linked to visceral obesity. IC1,2 IHCL were correlated with HOMA IS, corroborating the well known association of NAFLD with insulin resistance. IC1,3 IHCL were also weakly correlated with ALT levels, which are known to be a marker of hepatic fat.1

After 4-week WPS, IHCL were significantly decreased by 21%, and fasting plasma triglycerides and cholesterol concentrations were decreased by 15% and 8% respectively. This reduction of IHCL concentrations was not related to changes in visceral fat volume or total liver volume, nor with important changes in body weight or body fat mass. This therefore indicates that the improved IHCL and plasma triglyceride profiles were not to be attributed to an effect of protein rather than to changes in body composition.

We can only speculate about the possible mechanisms underlying the reduction in IHCL and plasma lipids induced by WPS. Although WPS provided a 250 kcal/day, body weight did not change significantly while total body fat decreased significantly although to a very slight extent. This suggests that WPS led to a spontaneous decrease of food intake. This hypothesis appears corroborated by the evolution of daily urinary nitrogen excretion over time. Pre-WPS daily nitrogen excretion amounted to ca 15 g/day, which corresponds, assuming 16% nitrogen content in proteins, to an average of 94 g/day protein intake. After WPS, daily nitrogen excretion increased significantly to 21 g/day, which corresponded to a total daily protein intake of 129 g/day. This means that, if subjects were hundred percent compliant and consumed the totality of the prescribed 60 g protein supplementation, WPS led to a reduction of spontaneous protein intake from other foods by 27%. This can be readily explained by the well known satietogenic effect of dietary protein.4 It is likely that the satiating effect of WPS decreased the intake not only of dietary proteins, but of dietary carbohydrate and fat as well. It is therefore possible that a decreased carbohydrate and lipid load was responsible for the decreased IHCL and plasma triglyceride concentrations observed after WPS. In support of this hypothesis, it has indeed been demonstrated, in overfed rats with hepatic steatosis, that increasing the dietary protein content of the diet reduced intrahepatic lipids through a decreased carbohydrate intake.3

Besides a reduction in spontaneous carbohydrate and fat intake, it is possible that a high protein intake also reduced hepatic fat through more direct effects. A high protein diet is known to enhance postprandial thermogenesis, an effect which is linked, at least in part, to the high energy cost of urea synthesis and amino acid conversion into glucose.15,16 Since these two processes take place in the liver, one can expect that the increased energy requirement of the hepatocytes was met, at least in part, by an increased intrahepatic lipid oxidation. Although not documented in this study, where only fasting concentrations were monitored, feeding high protein meals is also known to increase postprandial glucagon concentrations.17 The ensuing high glucagon : insulin ratio may therefore have stimulated hepatic lipid oxidation and ketogenesis while inhibiting hepatic de novo lipogenesis.18 Finally, other, direct effects of specific amino acids on intrahepatic lipid metabolism may be speculated.

Dietary protein metabolism also has complex interaction with glucose metabolism. On one hand, an amino-acid infusion enhances hepatic glucose production19 and decreases whole body insulin mediated glucose disposal.20 On the other hand, co-ingestion of protein and glucose have been shown to decrease postprandial glycemia, an effect which can be attributed to a delayed gastric emptying.21,22 WPS however failed to significantly alter fasting and 2 h plasma glucose and insulin concentrations. Insulin sensitivity was also not grossly altered, as indicated by HOMA IS index.

In summary, this study demonstrates that a 4-week supplementation with 3 times 20 g whey protein per day significantly reduced intrahepatic and fasting plasma triglyceride in obese subjects consuming an otherwise spontaneous diet. A satiating effect of the protein supplementation, leading to a lower carbohydrate or fat intake, an increased liver energy expenditure, and/or a higher glucagon:insulin ratio, may all be involved in these effects. This preliminary, uncontrolled study therefore suggests that a high protein diet may, in the long term, reduce the risk of non-alcoholic steatohepatitis and of cardio-vascular disease in obese patients. While a high protein diet may also have adverse effects, the present study did not hint at adverse renal effects. Further studies will however be needed to evaluate the optimal amount and sources of dietary proteins intake.

**Statement of authorship**

MB, KAL, CB and LT designed and developed the protocol; MB, EM, MC, EVD, GC and VG recruited subjects, carried out the clinical trial, and analyzed data, AB, DGQC, TB, RK and CB developed and performed MRS protocols, MB, LT and PhS drafted the manuscript, all reviewed and edited the manuscript.

**Conflict of interest**

LT has received research support from the Nestlé company, Vevey, Switzerland for other studies. Other authors have no conflict of interest to disclose.
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