

The Use of Whey Protein Concentrate in Management of Chronic Hepatitis C: A Pilot Study

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Abstract: Whey protein contains a wide range of biologically active ingredients that can prevent and attenuate disease. To clarify the effects of oral administration of whey protein concentrate (WPC) on viral load and host defense mechanisms, in particular, phagocytic function of neutrophils, selected immunomodulatory cytokines and serum inflammatory markers, in compensated chronic HCV patients. Twenty seven HCV patients recruited from the hepatology clinic of TBRI, and started the oral supplementation of WPC twice daily, only 12 patients (9 males, 3 females) had achieved the study period (8 weeks). Fifteen age and sex matched healthy participants were also included, as control group. Neutrophil phagocytic activity, serum intercellular adhesion molecule (sICAM), interleukin-2 (IL-2), nitric oxide (NO), as well as HCV-RNA levels and routine investigations were determined for patients, before and after WPC supplementation and once for controls. There was significant decrease in viral load and markers of active inflammation, namely; (sICAM, NO, and IL-2), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), while serum albumin, showed significant elevation accompanied by significant improvement in neutrophil phagocytic activity with a noticed increase in both total leucocytic and absolute neutrophil counts after WPC supplementation compared to pretreated levels. The oral WPC supplementation was well tolerated without any serious adverse events. Oral supplementation of WPC would have promising results as a new therapeutic strategy against HCV and its sequelae by decreasing the viral load and active inflammation as well as improving the synthetic capacity of the liver and the phagocytic function of neutrophils, in these patients.

Key words: Whey protein, Hepatitis C virus, Inflammatory markers, Phagocytic function of neutrophils.

INTRODUCTION

HCV is a viral pandemic. Nearly about 200 million individuals worldwide are currently infected with HCV (Dixit *et al*, 2004). Egypt has a high seroprevalence for HCV infection that reaches up to 20% in some countryside areas (Frank *et al.*, 2000).

It has been documented that HCV genome is genetically very unstable and mutates rapidly, leading to persistent viremia and chronicity in more than 80% of cases, despite the strong HCV- specific T- cell mediated immune response (El-Bassiouny, 2003). Chronically infected patients often develop progressive liver disease from compensated to decompensated state, cirrhosis, hepatic failure, and hepatocellular carcinoma, making HCV infection the 1st indication for liver transplantation (Gjertsen *et al.*, 2006). Chronic compensated hepatitis patients had relatively normal ammonia tolerance and liver function tests on the other hand, decompensation characterized by abnormal ammonia tolerance and liver function tests (Warren and Rebouças, 1966)

The treatment of chronic HCV with interferon alpha (IFN- α) was initially introduced in 1985 and marked a significant advance (Heathcote, 2007). However, the obtained response was not as high as expected initially

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and combination therapy with pegylated interferon and ribavirin has substantially enhanced its efficacy up to 55% (Watanabe *et al.*,2000). In spite of such progress, this bitherapy is in balance with numerous side effects such as neutropenia causing associated risk of bacterial infections and sepsis, psychiatric and flu-like symptoms, the existence of non-responders and relapses besides being relatively expensive(*Abi-Nassif et al.*,2003).

Earlier studies showed that, in Africa and Middle East, prevalence of genotype 4 of C virus, which having frequently poorer response to conventional interferon therapy than other genotypes (*Zekri et al.*,2005). Therefore, the need for the intervention of new therapeutic agents, that activates host immunity, is a challenge.

WPC demonstrates variable effects in HCV patients by correcting decreased lymphocytes glutathione, met in these patients, in order to activate the cell mediated immune function(*Walzem et al.*,2002). Its biological components, in particular bovine serum albumin, alpha-lactalbumin and lactoferrin, which are cysteine-rich proteins, contributing to its potent antioxidant activity, by aiding in the synthesis of glutathione (GSH), a potent intracellular antioxidant and demonstrate a range of immune-enhancing properties (*Nozaki et al.* ,2002).

This notion is supported, since 1980, by the observation that, normal mice fed on WPC as 20% of a formula diet exhibited a marked increase in antibody production in response to a T cell dependent antigen (*Ishii et al.*, 2003). Moreover, it has been reported that, the grade of activity of the liver disease is correlated with the level of GSH. Also, the effect of IFN was observed to be improved by administrating agents that restore the decrease of GSH levels (*Marshall* , 2004) .

To evaluate the clinical efficacy and safety of non-toxic dietary whey protein in compensated chronic HCV patients, the present study was designed to estimate the serum levels of ICAM-1, IL-2, and NO as well as neutrophil count and its phagocytic activity, liver functions and viral load, before and after oral WPC supplementation with special emphasis to the statistical comparison between the results of studied groups.

Subjects and Methods:

Subjects:

Twenty seven patients, with compensated HCV disease (20 males & 7 females), with an age range of 22-60 years and a mean of 42.04±8.25 years, were enrolled in the study. Patients were selected from the hepatology clinic of TBRI, Egypt, who came for evaluation of their chronic liver disease (CLD) status between April 2006 and June 2007.

Selection of patients at the time of recruitment into the study was based on; proper history taking, complete clinical examination, routine laboratory investigations, in particular, elevated ALT for a period more than 6 months to determine chronicity, the presence of anti-HCV antibodies (Abs) confirmed by HCV- RNA in serum and screening abdominal ultrasonography.

None of our patients had experienced signs of decompensation such as ascites, jaundice, gastrointestinal bleeding, flapping tremors or encephalopathy. Special groups including, children, patients with CLD other than HCV, patients either having renal dialysis or with transplants and patients who had received IFN treatment, are beyond the scope of this study. Concerning non-specific drugs for liver disease, mainly silymarin, or similar drugs, the continued administration was permitted if the dose was kept constant during the study period. Given that, all patients enrolled in the study were class A Child-Pugh according to Pugh *et al* classification in (1973) . A group of 15 healthy subjects selected from the medical staff and matched for age and sex, served as control group. All subjects were informed about the study and a written consent was obtained from each one. All procedures are according to Helsinki Declaration of (1975) as revised in (1983).

Study design:

The study was designed to supplement all patients with WPC twice daily (morning and evening) during meals or within 30 min after meals for 8 weeks. To evaluate the efficacy of whey protein, all investigations were performed before and after supplementation for patients and once for the controls. When treatment was discontinued because of patient request, prior data were included in the study as pretreated results.

Preparation of WPC:

Sweet whey (pH 6.0-6.2) was obtained from Ras cheese manufacture, Arab Dairy Co., Kaha, Kalubia, Egypt. Fat and fine curd were removed from the whey by a cream separator (Alfa-Laval Lund, Sweden). Particulated whey protein concentrates were prepared according to Paquin *et al.* ,(1992). and El-Sheikh *et al* ,(2001) by ultrafiltration of Ras cheese whey. The gross characteristics of the particulated WPC were determined; total solids (TS) according to AOAC,(1990); total protein, fat and pH according to Ling ,(1963); lactose according to Barnett and Tawab ,(1957). After preparation, WPC was packaged immediately in glass bottles and kept in the refrigerator. The ingredients of WPC are presented in table (I).

Assay Methods:

The following investigations were performed for all patients immediately before WPC supplementation and at the end of the supplement period (8weeks) and once for the controls. Reference ranges were provided by each manufacturer.

Routine Investigations:

All individuals enrolled in the study were subjected to urine and stool analysis; an automated haemogram using Celtac MEK 8118 (Nihon Kohden, Japan). Blood smears were stained with Lieshman – Giemsa, and differential leucocyte counts were performed by counting 300 cells and the absolute number of neutrophils was calculated. PT and PC were done using fibrin timer (Behring, Germany). Liver function tests were carried out using commercially available kits. HBsAg, anti-HBs Abs and total IgM and IgG Abs for (HBcAg), were determined using enzyme immunoassay (ELISA) kits (Abbott laboratories, North Chicago, IL), to exclude co-infection with HBV.

Anti-HCV Abs were detected using a third generation ELISA kit (Murex anti-HCV, Version III, Murex Diagnostics, Dartford, England). Serum HCV-RNA was determined by a quantitative reverse-transcription polymerase chain reaction (RT-PCR). HCV was extracted using acid guanidinium thiocyanate-phenol chloroform single step method (Chomczynski and Sachi, 1987). Two pairs of primers in the 5' untranslated region (UTR) have been used in the nested reaction as described previously by Matsumoto *et al.*, (1994). The sensitivity level of this assay is at 50 IU/ml.

Special investigations:

Determination of nitric oxide:

Heparinized blood samples were centrifuged and the plasma collected then filtered through a 1.2 µm multiscreen filter plate (Millipore, Bedford, MA). Plasma NO concentrations were determined using the Griess reaction as described previously by Ding and Rana, (1998) with the following modification. Six µL of plasma were mixed with 135.44 µL of H₂O, 20 µL of 0.31 M phosphate buffer (pH 7.5) and 10 µL of 0.86 mM nicotine adenine dinucleotide phosphate (Boehringer Mannheim Corp., New York, NY), 10 µL of 0.11 mM flavinadenine dinucleotide (Sigma) and 10 µL of nitrate reductase (1.0 U/ml) (Boehringer Mannheim Corp.) in individual wells of a 96-well plate. Samples were allowed to incubate for 1 hr at room temperature in the dark. One hundred µL of Griess reagent [1:1 mixture of 1% sulfanilamide (Sigma) in 5% H₃PO₄ and 0.1% N-(1-naphthyl) ethyl-enediamine (Sigma)] was added to each well and plates were incubated for an additional 10 min at room temperature. Absorbance was measured at 540 nm using a microplate reader.

Determination of sICAM-1:

Serum ICAM-1 levels were assayed by sandwich ELISA technique (Immunotech, France) according to the manufacturer's instructions. Each sample was assayed in duplicate.

Determination of IL-2:

EDTA anticoagulated samples were used to avoid in vitro production of cytokines because EDTA acts as a chelating agent. IL-2 was performed using commercially available ELISA kit, (Predicta, Human Cytokine Assays).

Nitroblue-tetrazolium test (NBT):

Unstimulated NBT test was performed immediately or within 2 hours at 4°C using heparinized blood. Briefly, 0.1% NBT was prepared in PBS by adding 10 mg of NBT powder (Sigma, St Louis) to 100 ml PBS (ph 7.2) and stirred at room temperature for 1 hour. The NBT staining was done for neutrophils after their separation using dextran sedimentation (Johnson *et al* ,1980) as follows: (i) Equal volumes of 0.1% of NBT solution and cell suspension were mixed and incubated for 30 minutes at 37°C. (ii) The tubes were centrifuged at 250×g for 5 minutes. (iii) Smears were prepared from the pellet and stained after drying with Wright stain, and then scoring cells containing formazan deposits (reduced NBT) were performed by counting 200 neutrophils as described by Baehner and Nathan, (1968).

Statistical Analysis

Statistical analysis was performed using SPSS version 16 software. Results presented as mean ± standard deviation and percentages, were compared using one-way ANOVA analysis of variance, and Scheff's between groups was used. The data were considered significant when $p < 0.05$. Statistical analysis between pre and post WPC intake by Paired t test was used.

RESULTS AND DISCUSSION

Results:

Twelve patients out of 27, who had started receiving WPC oral supplementation, have continued the test period (8 weeks). The causes of discontinuation among the 15 subjects were: nausea and vomiting in 5 patients, diarrhea and abdominal discomfort in 2 patients and 8 patients preferred shifting to interferon-based therapy. Hence, after performance of the study, the enrolled subjects were classified into: pretreated group of patients (group A; n =27), post treated group (group B; n=12) in addition to the control group (n =15).

Routine investigations indicated significant reduction of PT, PC% and total leucocytic counts (Table 2), besides absolute neutrophil counts, (Table 3), in patients' groups compared to control subjects. Although, WPC treatment did not change these parameters, significant improvement of neutrophil phagocytic activity was noticed (Table 3).

HCV-RNA results, recorded in (Table 4), showed that a statistically significant reduction comparing group B with A. In addition, 2 patients had undetected viremia after WPC treatment. Results of screening liver function tests pointed to a significant rise of albumin and reduction of ALT and AST in group B. Although, albumin was normalized after 8 weeks treatment with WPC, both enzymes, were still significantly higher than normal. As regards to the results of parameters of active inflammation, a significant reduction of the 3 parameters (sICAM-1, IL-2 and NO) was recorded in group B. However, after WPC supplementation, all 3 parameters were still significantly higher than control subjects as shown in (Table 4). The results of studied parameters in A and B group were illustrated in Figures 1-6.

Table 1: Composition of the Whey protein concentrate

Ingredients	Content
Total Solids %	18.24
Total Protein%	15.08
Lactose%	1.22
Fat%	1.05
pH	7.08

Table 2: Demographic and clinical, characteristics of different studied groups.

Parameter	Group	Control Group	Patients Groups	
			Group A	Group B
Number of Patients		15	27	12
Gender: Male/Female Ratio		3/2	2/1	3/1
Age (years)		43.1 ± 9.7	41.2 ± 7.3	42.4 ± 9.3
Child Pugh Class (A) percentage		0	100	100
PT (sec)		11.7 ± 0.22	15.1 ± 2.87*	14.7 ± 1.98*
PC %		96.2 ± 3.01	63.9 ± 17.5*	62.7 ± 16.9*
Hb (g/dl)		13.52 ± 1.42	11.05 ± 1.12	11.2 ± 2.11
RBCs (×10 ¹² /L)		4.73 ± 0.67	3.87 ± 0.52	3.86 ± 0.63
Platelet count (×10 ⁹ /L)		268.42 ± 55.05	165.25 ± 58.65	166.1 ± 38.9
Total leucocytic count (x10 ⁹ /L)		7.7 ± 1.78	5.22 ± 1.98*	6.08 ± 1.1*

Data were expressed as mean ± SD or as percent value (%).

* Statistically significant from the control group.

Group A: Pretreated group.

Group B: Post-treated group.

PT:prothrombin time, PC: prothrombin concentration, Hb: heamoglobin concentration, RBCs : red blood cells.

Table 3: Total leucocytic and absolute neutrophil counts and phagocytic activity in the studied groups.

Parameter	Group	Control Group (n=15)	Patients Groups	
			Group A (n=27)	Group B (n=12)
Total leucocytic count (×10 ⁹ /L)		7.7 ± 1.78	5.22 ± 1.98*	6.08 ± 1.1*
Absolute neutrophil count(×10 ⁹ /L)		3.9 ± 0.9	1.8 ± 1.98*	2.1 ± 0.86*
Percentage of unstimulated NBT +ve neutrophils		8.7 %	6.7%	8.3%
Absolute unstimulated NBT+ve neutrophils (×10 ⁹ /L)		0.33±0.017	0.12± 0.012 *	0.17±0.0094*#

Group A: Pretreated Group B: Post-treated group

NBT +ve: Nitroblue tetrazolium positive.

* Statistically significant from the control group.

Statistically significant from the pretreated group.

Table 4: Results of viral load, biochemical and inflammatory markers in studied groups.

Parameter	Group (n = 15)	Patients Groups		
		Control Group (n = 15)	Group A (n = 27)	Group B (n = 12)
HCV-RNA (10 ⁴ Copy/ml)	undetected		18.7 x 10 ⁴	3.75 x 10 ^{4#}
Albumin (g/dl)	4.4 ± 0.52		3.1 ± 0.38 [*]	4.20 ± 0.32 [#]
ALT (IU/L)	10.1 ± 1.81		47.5 ± 7.01 [*]	30.5 ± 3.34 [#]
AST (IU/L)	10.13 ± 1.08		36 ± 6.87 [*]	28.9 ± 5.7 [#]
sICAM-1 (ng/ml)	230.0 ± 11.00		877.3 ± 335 [*]	555.8 ± 218.8 [#]
IL-2 (pg/ml)	6.9 ± 1.10		34.6 ± 1.8 [*]	26.5 ± 2.6 [#]
NO (µmol/L)	32.17 ± 2.19		76.36 ± 8.11 [*]	54.34 ± 11.95 [#]

Data were expressed as mean ± SD or as (copyⁿ/ml).

^{*} Statistically significant from the control group.

[#] Statistically significant from the pretreated group.

P-values refer to paired t-test (before & after vs control).

Group A: Pretreated group. Group B: Post-treated group.

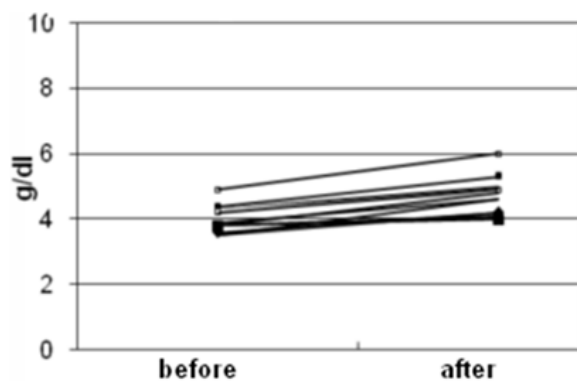


Fig. 1: Serum Albumin level before and after whey protein intake

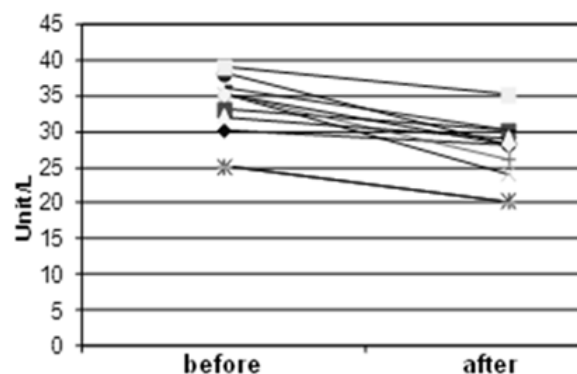


Fig. 2: ALT level before and after whey protein intake

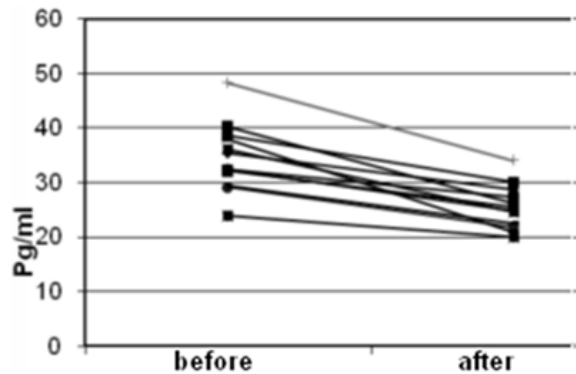


Fig. 3: IL2 level before and after whey protein intake

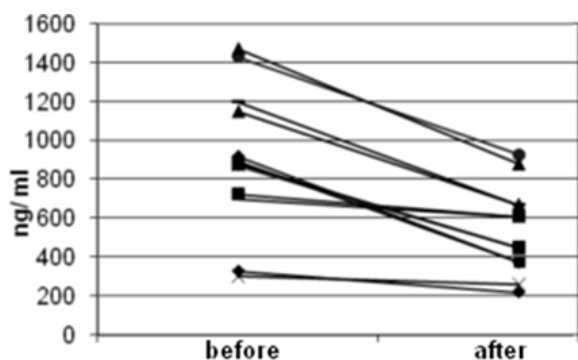


Fig. 4: Serum ICAM level before and after whey protein intake

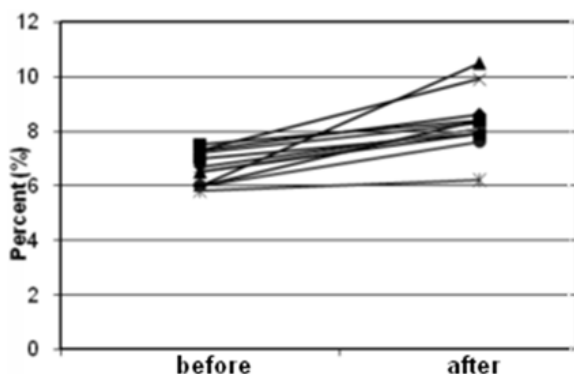


Fig. 5: Phagocytic cells (%) before and after whey protein intake

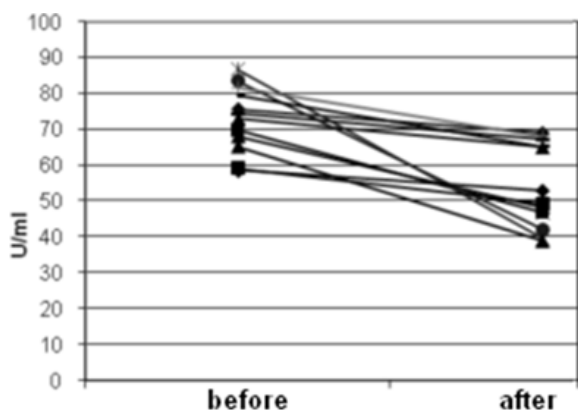


Fig. 6: Nitric Oxide level before and after whey protein intake

Discussion:

Conventional hepatitis C therapy focuses on reducing the viral load, while it does nothing to address the ongoing liver damage caused by the virus (Thevenot *et al*, 2006).

In this study we tried to elucidate the efficacy of WPC in treatment of HCV infection. To achieve this goal, the present work monitored the liver status and viral load, in addition to selected immunological and haematological parameters, in compensated chronic HCV patients on comparing pre with the post-treated results.

Based on published clinical studies, it has been demonstrated that monitoring viral load during the early phases of treatment may provide early information on the likelihood of a response to antiviral therapy (Obeid and Bauvois , 2006) In the present study when comparing the pre and post-treated groups (A and B), a virologic response (a 50% or greater decrease in HCV RNA level) was achieved in 8 out of the 12 patients belonging to group B. From these 8 patients, 2 subjects had undetectable levels of HCV RNA at the end of the test period. It has to be noted that the 4 patients that their viral load did not decline appropriately after

the 8-weeks oral course of WPC, had higher pretreated level than the other patients. Probably, it appears that whey protein administration may be more rapidly effective in case of low pretreated viral load. Higher viral loads would need longer courses.

Conversely, WPC intake for 8 weeks did not have favorable effect on the serum ALT level, as only 4 out of 12 studied subjects (about 33%) reached the level of biochemical response (a 50% or greater decrease in the serum ALT level). This may be referred to the fact that, our studied subjects had an ALT baseline only about 4 fold the control level. Subsequently, the ALT decline seemed to approach the normal level to a certain extent without fulfilling the biochemical response. Eventually, however, on comparing pre to post-treated group, there was a significant reduction in the viral load and the ALT level.

In agreement with our results, Tanaka *et al*, 1999) has demonstrated in his pilot study among 11 patients having low pretreated viral loads of HCV, and receiving daily bovine lactoferrin, for 8 weeks, a decrease in HCV-RNA and ALT than those having higher pretreated HCV loads. Conversely, a study conducted among 17 HCV patients received 12 g whey (Immunocal) twice daily for 12 weeks, preceded and followed by 12 g casein protein daily for two weeks and four weeks respectively, no significant changes were noted (Watanabe *et al*, 2000). These conflicting results may be attributed to the selected criteria of the studied patients, particularly viral load baseline and the HCV genotype prevalence.

Post treated results revealed that, albumin was significantly increased, while, for PT, insignificant improvement was noticed, which may need longer oral course duration. The improvement in albumin level, reaching the level of normal control subjects, indicates positive nitrogen balance which is a good achievement, as malnutrition is a cause of many of the complications of chronic liver disease. Assuming that, hypoalbuminemia met in our patients, was due to decreased hepatic synthesis, not a consequence of losing capacity, as none of our patients suffered from diarrhea, proteinuria or nutritional disorders.

WPC provides all essential amino acids and is rich in branched-chain amino acids (BCAAs) that are important factors in tissue growth and repair (Watanabe *et al*, 2000). Leucine, in particular, has been identified as a key amino acid in protein metabolism during the translation-initiation of protein synthesis (Anthony *et al*. 2001) . Moreover, whey proteins, "fast proteins," do not coagulate under acidic conditions. They reach the jejunum quickly after entering the gastrointestinal tract where the hydrolysis of whey is slow, allowing for greater absorption over the length of the small intestine (Marshall ,2004).

Taken together, the above-mentioned properties of whey protein, with correction of malnutrition frequently met in CLD patients improves the clinical outcome of these patients.

Earlier studies showed that sICAM-1 is correlated with degree of active inflammation and histopathological activity (Granot *et al*, 2001). Subsequently, it can be considered as a useful marker of hepatocellular necrosis and inflammatory activity in chronic hepatitis and has a prognostic significance in cirrhotic patients (Capra *et al*, 2000).

The resulted data revealed a significant elevation of sICAM-1, IL- 2 and NO levels in diseased groups A and B compared to the control group, while a significant decrease was observed on comparing group B to A. In addition, correlation studies showed that sICAM-1 was directly correlated with the viral load in group A and inversely correlated with serum albumin in group B. Therefore, a reducing effect of WPC on sICAM-1 and viral load, accompanied by improvement of the liver status of HCV patients was recorded, as indicated by increasing serum albumin being considered as a monitor of the synthetic capacity of the liver.

Our findings are consistent with those of (Capra *et al*, 2000) and (Montalto *et al*, 2006) as regards sICAM-1 after IFN treatment of HCV patients and for IL-2 in other studies (Fan *et al* 1998) & (Priimägi *et al*, 2005) On the contrary to our IL-2 results, Zekri *et al* (2005) reported insignificant changes in serum IL-2 measurements in chronically HCV patients. The authors attributed this finding, to the presence of soluble receptor (sIL-2R) binding to IL-2.

The NO increase during the course of HCV infection is explained by (Machida *et al*. 2004).They reported that, the viral core and NS3 proteins can stimulate the production of NO in hepatocytes and B cells through activation of inducible NO synthase (iNOS) gene. NO causes DNA breaks and enhances DNA mutation and subsequently is correlated with the severity of the disease (Kandemir *et al*, 2002). A recent study conducted on rats injected with whey protein (300 mg/kg), revealed that alpha-lactalbumin, has a marked suppressive effect on pro-inflammatory cytokine release, particularly IL-6, through NO-mediated mechanism (Yamaguchi *et al*, 2007).

Increased susceptibility to recurrent and serious bacterial infections is a common association in CLD (Abdel Fattah *et al*, 1991). which in turn is due to impairment of phagocytic function of neutrophils and natural killer cell activity of lymphocytes among these patients (Nakamura *et al*, 2007). Data obtained from the present study revealed significant decrease in total leucocyte, absolute neutrophil and NBT +ve neutrophil counts in diseased groups compared to the control one. On comparing pre and post-treated groups, a significant increase in

number of absolute NBT +ve neutrophils was found in the post-treated group. Considering NBT as an indicator of the phagocytic activity of neutrophils, the present results agree with several studies (Omran *et al*, 1982 & Ahmad *et al*, 2004). Nakamura *et al* (2007). concluded that phagocytic function of neutrophils was significantly improved by 3 month-BCAA supplementation, although changes of albumin level were insignificant.

Studies on whey protein, in particular its contents of lactoferrin, and alpha-lactalbumin, have demonstrated its ability to activate natural killer (NK) cells and neutrophils by intracellular replenishment of depleted glutathione (Marshall *et al*, 2004). Also, lactoferrin has antiviral properties, through its iron chelating properties. Several studies have revealed that hepatitis C inflicts most of its damage by latching onto molecules of iron, resulting in free-radical damage to liver cells. Despite substantial scientific evidence, few physicians implement iron-depletion therapy when treating hepatitis C (Konishi *et al*, 2006).

Meanwhile, neutrophils have been shown by RT-PCR to be among the extra-hepatic sites of HCV replication (Crovato *et al*, 2000). Hence, the significant improvement of NBT +ve cells in the post-treated group and the increased absolute neutrophil count but not to a significant level, recorded in this work, indicates a beneficial effect of WPC mainly on qualitative and to a lesser extent on the quantitative status of neutrophils among HCV patients.

Finally, our present trial left many unanswered questions for future studies, including optimum dose, duration, and the potential effects of combining WPC supplementation with conventional treatment. In addition, the effects of whey protein administration and its long-term use on prognosis of patients with high pretreated viral loads, advanced stages of liver disease and in hepatocarcinogenesis, remain to be investigated in the future.

Conclusion:

WPC supplementation showed promising results among compensated hepatitis C virus patients. It has a viral load reducing effect, accompanied by normalization of serum albumin and improvement of phagocytic function of neutrophils, at the end of 8 weeks oral course. These responses were paralleled with a significant decrease in serum levels of ALT, ICAM-1, IL-2 and NO. Presumably, WPC administration provides nutrition which could positively affect the prognosis of the patients. Also, it is well tolerated and shows no side effects like that of conventional therapy. Herein, we recommend to try this supplementation as an adjuvant to interferon therapy or to be an alternative for non responders. Prospective studies are needed to enlighten these postulations.

Recommendations:

Although, we tried to elucidate the efficacy of WPC in treatment of HCV infection, yet still, the sample size, in this pilot study, is albeit small, 12 patients, large scale researches, with significant number of participants, including different grading of chronic HCV liver disease with longer follow up, are recommended.

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