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EFFECT OF A FERMENTED NUTRACEUTICAL ON THIOREDOXIN LEVEL AND TNF-α SIGNALLING IN CIRRHOTIC PATIENTS

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The aim of this study is to gain further insights into the possible nutraceutical effect on redox balance via thioredoxin (Trx) modulation and on the intrinsic susceptibility of monocytes to generate an inflammatory response. The study group consisted of thirty-two patients with compensated Child A-C, HCV-related cirrhosis. The patients were supplemented for 6 months with 6g/day of a certified fermented papaya preparation (FPP). Fifteen unsupplemented, age/gender-matched healthy subjects served as controls. The patients filled in a detailed diet-life style questionnaire, and blood samples were collected to test routine biochemistry, Trx, redox status (GSH, GSSG, GSH/GSSG ratio, 4-HNE and α-tocopherol). Moreover, isolated monocytes were tested for *ex-vivo* LPS-stimulated TNFα production and TNFα mRNA. As compared to control, patients with liver cirrhosis showed a significantly higher serum level of Trx. A significant correlation occurred with GSH/GSSG ratio in Child B and C patients. FPP supplementation brought about a significant reduction of Trx with levels comparable to the ones of healthy controls. Ten patients Child C (31.2%) showed borderline low levels of α-tocopherol while all cirrhotic patients, as a whole, showed a significantly abnormal redox balance. Supplementation with FPP did not modify α-tocopherol depletion but significantly improved redox balance parameters. Patients with liver cirrhosis showed a significantly upregulated TNF-α production in a time-dependent manner and this effect was more pronounced in more advanced stages of the disease and showed a significant correlation with α-tocopherol level. Supplementation with FPP significantly, although partially, downregulated TNF-α production from monocytes. Taken altogether, it would appear that the typical oxidative-inflammatory biochemical milieu of these patients is mirrored by a significant TNF-α upregulation at a monocyte level while a targeted nutraceutical might be a potentially amenable intervention to be part of validated scheduled treatments.

There is a solid evidence that reactive oxygen species (ROS) are key players in activating transcription factors such as nuclear factor-κB (NF-κB), nuclear factor–α interleukin (IL)-6 and activator protein-1, which in turn trigger several crucial inflammatory cytokine gene releases from

monocytes (1-2). Moreover, a number of stressors, such as reactive oxygen species and tumor necrosis factor-α (TNF-α) have been shown to upregulate thioredoxin production and release (3-4) which acts as a functional bridge between oxidant stress and initiation of an inflammatory signal. Thus, thioredoxin represents an ubiquitous stress-inducible system and together with glutaredoxin are small proteins containing an active site with a redox-active disulfide which are active in electron transfer via a reversible oxidation of two vicinalprotein-SH groups to a disulfide bridge (5-6). Increased circulating levels of thioredoxin have been found in patients with several acute and chronic diseases and in liver diseases ranging from non-alcohol steatohepatitis to chronic hepatitis and to overt liver cirrhosis (7-9). Indeed, extracellular thioredoxin has proinflammatory effects by potentiating cytokine release from fibroblasts as well as monocytes. DNA damage following ROS injury (10) can account for the genetic changes that take place along with the progression from cancer-predisponent abnormalities to precancerous lesions and, eventually, to anaplastic cancerous growth and metastasis. In the course of HCV-related liver disease, chronic inflammatory events and oxidative stress (11-13) can lead to DNA damage. Indeed, hepatocellular carcinoma frequently develops in patients with chronic hepatitis and liver cirrhosis and is considered as a part of the natural history and as an unavoidable event occurring at a rate of 10/10.000 cases. In this respect, monocytes from patients with advanced alcohol-induced cirrhosis have an increase in lipopolysaccharide (LPS)-induced TNF-alpha production and quite recently it has been shown that they express an upregulation of TNF-α signalling (14). Quite recently we have shown that a fermented papaya preparation which has significant protective antioxidant properties (15-16). is able to significantly lower the serum level of oxidative stress, soluble TNF-receptor and 8OHdG in leukocytes of patients with HCV-related liver cirrhosis (17). Thus, nutraceutical therapy might offer a worthwhile adjunctive tool, especially in long-term management of patients in whom several concurrent metabolic abnormalities take place (18). However, we wanted to gain further insights into the possible nutraceutical effect on redox balance via thioredoxin modulation and on the intrinsic susceptibility of monocytes to generate an inflammatory response.

MATERIALS AND METHODS

This study, previously approved by the internal review board, was carried out according to the principles of the Helsinki Declaration and informed consent was obtained from all patients. The study group consisted initially of forty patients who, after early drop out and protocol violation, finally amounted to thirty-two patients with compensated Child A-C, HCV-related cirrhosis without having a history of any ethanol consumption for the past 10 years. Clinical characteristics are shown in Table I. Exclusion criteria were: hemocromatosis, Wilson's disease, alpha-1-antitrypsin deficiency, autoimmune diseases, hepatocellular carcinoma, recent variceal haemorrhage or scheduled endoscopic session of variceal banding, any other prior malignancy, chronic illness requiring steroids, immunosuppressive agents, allopurinol treatment, antiviral or NSAIDs. Patients were supplemented for 6 months with 6g/day of a certified fermented papaya preparation (Immun-Age®, ORI Europe, Grenoble, France; FPP) made under ISO 9001 (production quality) and ISO 14001 (environmental protection) from a patented biofermentation process of non-GMO carica papaya. Fifteen unsupplemented, age/gender-matched healthy subjects served as controls.

Blood collection and storage

After an overnight fast, venous blood samples were taken and put into a dry tube for serum, a citrate-containing tube, while an EDTA-containing tube for plasma was used for clinical chemistry analyses. On the examination day, blood samples were taken for routine testing (glucose, transaminases, haemoglobin, urea, creatinine, electrolytes) by automated standardized procedures (Hitachi 911 using commercial kits) and for further studies as described below. Blood samples were taken at entry, at 3 and at 6 months.

Diet and lifestyle questionnaire

A detailed lifestyle questionnaire was given to all subjects, with particular care to stress factor and physical activity. Patients were instructed to refrain from physical exercise, and subjects under overt psychological and physical distress (overnight and night-shifter workers, habitual users of hypnotics or antidepressants etc.) were excluded at the time of selection, as well as smokers. Moreover, the web-based version of the National Institutes of Health Diet History Questionnaire (NIH DHQ) was used to assess diet history over the past month and during the study period. The NIH DHQ is a food frequency questionnaire (FFQ) consisting of 124 food

items also including portion sizes. Data indicate that this instrument provides reasonable nutrient estimates and sufficient reliability and validity (19).

The patients were advised not to use any multivitamin supplement or fortified food while maintaining their usual diet. BMI and body mass composition were assessed by a multi-channel bioelectrical impedance analyzer.

Serum level of Thioredoxin

Plasma Trx-1 was measured by SELDI-TOF mass spectrometry and each prepared sample was analyzed by four types of ProteinChip (Ciphergen Biosystems): CM10 (Weak Cation Exchange), Q10 (Strong Anion Exchange), IMAC30 (Immobilized Metal Affinity Capture coupled with copper) or H50 (Hydrophobic) to check their ability to bind Trx-1. The binding/washing buffers for the ProteinChips were 50 mmol/l Tris-HCl, pH 8.0 and 50 mmol/l sodium acetate, pH 5.0 (for Q10), 100 mmol/l sodium acetate, pH 4.0 (for CM10), 50 mmol/l HEPES, pH 7.0 (for H50) or 100 mmol/l sodium phosphate-500 mmol/l NaCl, pH 7.0 (for CopperII-IMAC30).

The chip surface was washed twice with 5 μl 0.1M sodium acetate, pH 4.5, twice with 5 μl double distilled H_2O and allowed to air dry at room temperature before two additions of 1 μl 50% 3,5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile with 0.5% trifluoroacetic acid, allowing air drying between applications. The ProteinChip arrays were analyzed using a Ciphergen PBS IIc ProteinChip Reader, which were calibrated externally using molecular mass standards (Ciphergen Biosystems). Each plasma sample was analyzed in triplicate.

Detection and quantitation of Trx-1 in plasma

Immunodepletion studies were performed using recombinant purified human and mouse Trx-1 and mouse monoclonal anti-humanTrx-1 antibody (5A3G5) cross-linked to cyanogen bromide-activated-Sepharose 4B beads. Trx-1 in 2 μl supernatant was assayed by SELDI-TOF mass spectroscopy. A standard curve for Trx-1 was generated by the addition of recombinant human purified Trx-1 to normal plasma. The limit of detection of the assay for Trx-1 was 10ng/ml with saturable binding occurring at 500ng/ml. The Trx-1 concentration in a plasma sample was determined by comparison of the relative signal intensity of the test sample with that for recombinant Trx-1 added to plasma as a standard.

Assessment of Redox Status, 4-hydroxynonenal and alpha-tocopherol

The plasma concentration of glutathione (GSH) and oxidized glutathione (GSSG) was assayed as follows. In this system, glutathione is oxidized by 5,5'-dithiobis-2-nitrobenzoic acid (DNTB 10 mmol/l; Merck, Darmstadt, Germany), and then 2-nitro-5-thiobenzoic acid is formed, which can be detected spectrophotometrically by a change of absorption at 412 nm. Standard curves were constructed using reduced glutathione (0.25-10 mmol/l; Sigma, St Louis, MO, USA). Three standard solutions containing normal, medium and high GSH concentrations were analysed on the same day and on 10 different days with 20 replicates. The intra- and interassay coefficients of variation were 1.1% and 1.7%, respectively. The related GSH/GSSG ratio was calculated accordingly while glutathione peroxidase (GSH-Px) was assayed by using haemoglobin catalysed oxidation of 10-N-methylcarmoyl-3,7-dimethylamino-10-H-phenothiazione after treatment with phospholipase. Values were read by a fluorescence detector. 4-hydroxynonenal (4-HNE) were determined following the method of Esterbauer and Cheeseman (20) and the results were expressed as μmol/l. In brief, preformed malondialdehyde and 4-HNE were reacted with N-methyl-2-phenylindole and methanesulfonic acid. Total cholesterol was measured enzymically using a commercially-available kit. Total plasma protein sulphydryls and protein carbonyls were assayed spectrophotometrically. Alpha-tocopherol was determined by high-performance liquid chromatography. Briefly, aliquots of plasma (1 ml) were mixed with 1 ml of 100 mM sodium dodecylphosphate solution in water, 2 ml of absolute ethanol and 1 ml of n-heptane and shaken for one minute. After 15 minutes extraction in the dark, the heptane phase was separated by centrifugation, and 50 μl aliquots were used for HPLC assay. Values were read by a fluorescence detector set at 296 nm excitation and 325 nm emission.

Monocyte isolation and viability study

Twenty milliliters of peripheral venous blood was collected into heparinized containers and monocytes were isolated by density-gradient centrifugation over endotoxin-free Histopaque 1077 followed either by positive selection using a MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's protocol, or by plastic adherence. For monocyte isolation by plastic adherence, 5×10^6 PBMC per well were distributed into 12-well plates and allowed to adhere in a 5% CO_2 incubator at 37° for 2 h in 1 ml of RPMI-1640 containing 0.3 g/L l-glutamine (Sigma) supplemented with 5% (v/v) fetal calf serum (FCS, heat inactivated; Sigma), 100 U/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (referred to as complete medium, 5% CM). Non-adherent cells were removed and the adherent cells were washed carefully, twice, with prewarmed 5% CM.Viability of monocytes was assessed by either trypan blue exclusion and by the MTT (3-[4,5-2-yl]-2,5-diphenyl tetrazolium bromide) assay and it was found to

be >90%. Approximately 95 ± 5% of isolated monocytes were adhered after 2 h of cultivation and cell viability was checked by MTT assay which showed in both controls and patients that the absorbance was directly proportional to the number of monocytes.

Monocyte culture

Freshly isolated monocytes were approximately 95% viable (assessed by their ability to exclude trypan blue dye). Peripheral blood mononuclear cells were cultured at a concentration of 0.5 x 10^6 cells/ml in culture medium (RPMI-1640 medium without phenol red, supplemented with 2mM L-glutamine, penicillin (100 U/ml), streptomycin (100 pg/ml) and 10% FCS). The RPMI-1640 and FCS contained less than 0.03 ng endotoxin per ml. The cells were incubated with/without rGM-CSF in different concentrations in 12 x 75 mm polystyrene or polyethylene tubes. There were no differences in the results obtained with the different types of tubes. After 2 h, non-adherent cells were removed by washing and fresh medium was added. They were then cultured in fresh medium for various times (0-24 h) with 100 ng/mL LPS (Escherichia coli serotype 0111:B4). Supernatants from cultured monocytes were collected and frozen immediately at -70°C until analysis of TNF-α levels.

Determination of TNF-alpha concentrations

Monocytes were plated in triplicate in 96-well pyrogen free culture dishes (Nunc A/S, Roskilde, Denmark), in complete medium (RPMI-FCS 10%). After the adherence period, monocytes were cultured with or without LPS. Twenty-four hours after treatment with stimulus or without stimulus treatment, cell culture supernatants were collected and stored at −20°C until assayed for TNF-α production by a commercially available solid-phase sandwich enzyme-linked immunosorbent assay following the manufacturers' instructions (CytokineNet, Shanghai, China). The intensity of the color was measured in a microplate reader.

RT-PCR for detection of TNF-α mRNA

Total RNA was prepared from monocytes activated by the incubation with LPS and it was analysed by reverse transcription-polymerase chain reaction (RT-PCR) for expressing TNF-α mRNA. The PCR was performed with complementary desoxyribonucleic acid (cDNA) derived from 100 ng RNA, 1 unit Taq polymerase and reaction kits in a volume of 20 μl. The PCR conditions included denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 2 min. Thirty cycles of PCR were performed on each sample, after which the products were separated on 1.3% agarose gels. The PCR primer used for amplification was 5'-GAGTGACAAG CCTGTAGCCCATGTTGTATGA-3' (sense strand), 5'-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3' (anti-sense strand).

The PCR products were subjected to electrophoresis on 1.4% agarose gel and quantified by using Gelmatrix Scan software (BioMatrixscan Inc, Shanghai, China).

Statistics

Group data were distributed non-parametrically and comparisons between groups were made using the Kruskal-Wallis test for multiple group comparisons followed by the Mann-Whitney U test for comparisons between two groups. Correlations were calculated using Spearman's rank test. $p < 0.05$ was considered statistically significant.

RESULTS

All patients completed the study with thorough compliance with the dietary, lifestyle, and treatment requirements. No significant weight change was observed. As a whole, routine blood tests were not affected by FPP supplementation (data not shown).

Serum level of Thioredoxin

As compared to control, the patients with liver cirrhosis showed a significantly higher serum level of Trx ($p<0.01$, Fig. 1). There was no correlation between Trx level and routine biochemical parameters, α-tocopherol included, 4-HNE, duration and stage of liver disease or clinical status (data not shown). Nonetheless, a positive but not significant trend was observed between Trx level and a more catabolic profile of patients (when examining independently the following variables: BMI, lean body mass. WBC counts, albumin, data not shown). A significant correlation occurred with GSH/GSSG ratio but only in Child B and C patients (r: +0.71, $p<0.01$). Moreover, no overt correlation appeared between Trx and the dietary questionnaire profile (when examining the following variables independently: protein-calorie intake, fruit-vegetables intake, their estimated antioxidant capacity, carbohydrate and fat intake, data not shown). UDCA-treated patients did not show any significant difference to the others for Trx level. FPP supplementation brought about a significant reduction of Trx ($p<0.05$ *vs* baseline) with levels comparable to those of healthy controls. The decrease of Trx did not show a time-related fashion and all peak decreases occurred at the first 3-month

Table I. *Patients' characteristics.*

Male/female	20/12	Ongoing treatment	
Mean age (Age range)	66 (57-73)	Furosemide	14 (43%)
BMI, mean (range)	22 (17-25)	Lactulose	16 (50%)
Lean body mass (%)	64.1 ± 9.6	K^+-canrenoate	19 (59%)
Child A-B-C	10-10-12	UDCA	11 (34%)
HCV RNA (10^3 copies/ml)	20 ± 120	insulin	8 (25%)
ALT (IU)	48 ± 55	prior IFN + ribavarin tx	13 (40%)

Modified Child-Pugh classification of severity of liver disease according to the degree of ascites, the plasma concentrations of bilirubin and albumin, the prothrombin time, and the degree of encephalopathy. A: well-compensated disease; B: significant functional compromise; C: descompensated disease. (Child CG, Turcotte JG. Surgery and portal hypertension. In: The liver and portal hypertension. Edited by CG Child. Philadelphia: Saunders 1964:50-64)

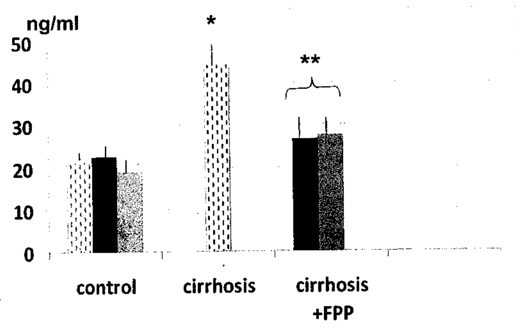

Fig. 1. *Serum level of thioredoxin in cirrhotics: effect of nutraceutical supplementation.*
*Dotted bar: baseline level; black bars: 3-month level: grey bars: 6-month level. At baseline observation, serum level of thioredoxin in cirrhotic patients was over two times higher than in healthy control, * p< 0.01 vs healthy control. Nutraceutical supplementation reverted to normal such value, ** p<0.05 vs unsupplemented cirrhotics.*

observation and maintained the plateau thereafter.

Assessment of Redox Status, 4-hydroxynonenal and α-tocopherol concentration

Results are shown in Table II. Ten patients (31.2%) showed borderline low levels of α-tocopherol and, of these, 8 (25% of the whole study group) had a level which was significantly below the normal range (serum levels below 12.5μmol/L as a 5% percentile of healthy controls; p<0.05 *vs* controls). When plotting the levels of α-tocopherol against the stage of the disease, such depletion seemed to affect only those patients with more advanced disease (Child C patients), but the numerical limitation of data suggesting such trend did not allow to make a statistical analysis. However, there was a significant correlation between α-tocopherol with 4-HNE in Child C patients (r: 0.66, p<0.05, data not shown). Irrespective of the stage of the disease and of any ongoing treatment, cirrhotic patients, as a whole, showed a significantly abnormal redox balance with GSH depletion and low GSH/GSSG ratio and increased 4-HNE (p<0.01 *vs* controls). Supplementation with FPP did not modify α-tocopherol depletion but significantly improved redox balance parameters and, partially, also 4-HNE (p<0.05 *vs* untreated patients).

Study of monocyte TNF-α signaling

Fig. 2 shows the results of the *ex vivo* test of TNF-α stimulation. Cells from patients with liver cirrhosis showed a significantly upregulated TNF-α production in a time-dependent manner (p<0.001 *vs* healthy control). This data clearly appeared on PCR-electrophoresis analysis. This parameter was unrelated to oxidative stress parameters and to Trx level. However, this effect was more pronounced in more advanced stages of the disease (Chid C > Child A, p<0.05, data not shown. No significant difference appeared between Child B and Child A) where it showed a significant correlation with α-tocopherol level (r: +0.66, p<0.05, data not shown). Supplementation with FPP significantly, although

Table II. *Redox status and α-tocopherol level in cirrhotics at 6 months observation (3-month values in brackets).*

	GSH μmol/l	GSH/GSSG	HNE μmol/l	α –tocopherol μmol/L
Healthy control (15)	9.2 ±3.2 (9.4 ±2.6)	14.5 ± 2.4 (15.4 ± 3.4)	7.6 ± 4.8 (6.8 ± 3.1)	24.5 ± 3.9 (26.9 ± 4.4)
Untreated cirrhotics (16)	4.2 ± 2.9* (4.6 ± 2.4*)	9.8± 3.5* (8.9± 3.9*)	19.8 ± 5.3* (19.9 ± 7.2*)	15.7 ± 5.1* (16.6 ± 4.3*)
Supplemented cirrhotics (16)	8.9 ± 2.3** (8.1 ± 3.1**)	13.8 ± 4.4** (11.9±5.1**)	14.4 ± 6.8** (13.3 ± 5.5**)	14.8 ± 6.3* (15.9 ± 2.8*)

** p< 0.01 vs healthy control, ** p<0.05 vs unsupplemented cirrhotics*

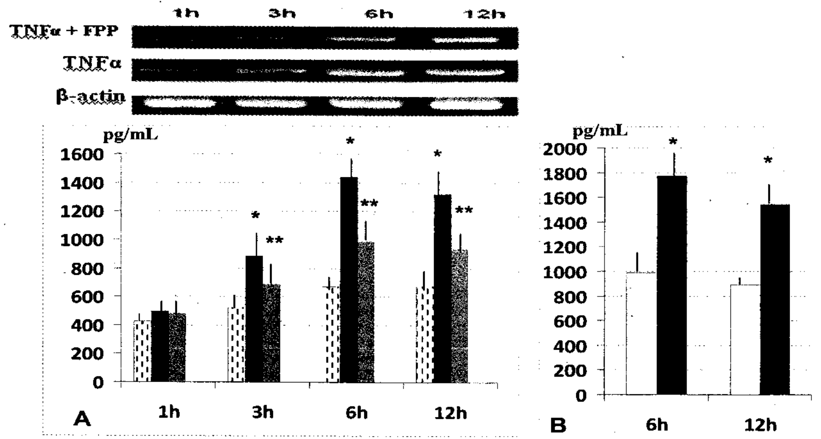

Fig. 2. *Ex-vivo LPS-stimulation test of TNF-α production from monocytes and PCR-electrophoresis: nutraceutical modulation. Data were obtained at 3 months observation. A) dotted bars: healthy control: black bars: unsupplemented cirrhotics; grey bars: FPP-supplemented cirrhotics. FPP: fermented papaya preparation. Stimulated monocytes from unsupplemented cirrhotic showed a significant time-course increase of TNFα production, * p< 0.01 vs healthy control. Nutraceutical supplementation partly but significantly decreased such phenomenon, ** p<0.05 vs unsupplemented cirrhotics. Top part of figure A shows the PCR electrophoresis of TNFα expression in LPS-stimulated cells from FPP-supplemented cirrhotic patients (TNFα + FPP) and in unsupplemented patients (TNFα).* ***B)*** *white bars: Child A; black bars: Child C, *p<0.05 vs Child A*

partially, downregulated TNF-α production from monocytes irrespective of alpha-tocopherol status (studies performed at 3 months observation, p<0.05 *vs* baseline level. Values were confirmed at the same extent at 6 months observation, data not shown).

DISCUSSION

The liver is one of the most susceptible organs to ROS damage and DNA mutagenesis, and oxidative stress has been implicated as a causative factor in liver disease (21). Recently, thioredoxin was identified as a unique chemoattractant for human neutrophils, monocytes, and T cells and can be released by activated lymphocytes (22). However, unlike typical chemokines, thioredoxin does not enhance their intracellular calcium content and its modality of action is G-protein-independent (23) and may trigger a signal transduction for chemotaxis by oxidizing and cross-linking some cell surface

molecules due to its a protein disulfide-reducing enzymatic activity (22). Once secreted, even very low concentrations of thioredoxin (1 3 nM) are able to strongly stimulate cytokines in several cell lines (22). On the other hand, intracellularly, it helps maintaining a cell reduction/oxidation status by a disulfide bond within the conserved active site, Cys-Gly-Pro-Cys (24). Confirming our prior findings (17), also in this study we found a significantly reduced GSH, GSH/GSSG ratio and glutathione peroxidase in cirrhotic patients ($p<0.05$ *vs* healthy controls) and this applied to all subjects, irrespective of the stage of the disease, while all benefited from FPP supplementation. All patients showed a significantly elevated Trx level which paralleled the stage of the disease and significantly correlated with GSH/GSSG ratio in those with more advanced liver impairment. About one-third of patients exhibited a borderline low plasmatic level of α-tocopherol and 40% of this subset had overtly low level. Yodoi's group (25) had already shown that vitamin E supplementation was able to normalize thioredoxin concentration and improving transaminases level. In our prior study we indeed observed that α-tocopherol restoration after its supplementation could revert to normal the plasmatic redox status but was not as effective as FPP in improving TNF-α profile. This observation warrants some interest when considering the negative results of a recent hepatocellular carcinoma prevention clinical trial with α-tocopherol from Takagi et al. (26). Moreover, although Saeki et al. has shown that the expression of ROS-related molecules is upregulated in relation to serum aminotransferase levels (27), the presence of a strict association between the serum TRX level and the TRX concentration in the liver and the correlation of the latter to disease activity have been seriously questioned by a recent study of Hamano et al. (28). Nonetheless, in our study FPP supplementation significantly lowered Trx plasma level and this event positively correlated with the improvement of redox status. This is in agreement with the concept that Trx-1 acts as an antioxidant through its ability to reduce thioredoxin peroxidases that scavenge H_2O_2 and organic hydroperoxides and exerts redox control over a number of transcription factors by increasing their binding to DNA and regulating also gene transcription such as that of NF-κB (29). Thus, these findings on Trx interpretation maintain their biochemical, and possibly also clinical relevance, as already clarified by researchers studying HCC transformation (30). Moreover, most recently, elevated Trx level have been found to be a definite cofactor in the development of insulin resistance in patients with chronic hepatitis C (31). Although our limited diabetes data do not allow us any inference, it is worth mentioning that this nutraceutical has been found to lower plasma glucose level in type-2 diabetics (32). On the other hand, unlike what was found in a large study by prior researchers (33), UDCA treatment was of no benefit in lowering Trx plasma level, although our data have to be taken with caution because of the limited cohort of patients under such treatment.

Quite recently, a fine study from Tazi et al. (16) h proved that decreased Akt activity and a lack of IRAK-M induction are likely to be involved in the process of cirrhotic monocyte sensitization to produce TNF-alpha. While their study addressed only Child C ascitic cirrhotics, over 80% of them due to alcohol (70% active drinkers at the time of enrollment), in our investigation we wanted to ascertain whether HCV-related cirrhosis would harbor similar biochemical abnormality. From our data we suggest that, irrespective of the stage of the disease, monocytes of patients with HCV-related cirrhosis show a similar feature which is significantly mitigated by FPP administration. Taken altogether, it would appear that the typical oxidative-inflammatory biochemical milieu of these patients is mirrored by a significant TNF-α upregulation at a monocyte level. On the other hand, a targeted nutraceutical might be a potential amenable intervention as part of validated scheduled treatments while, from the methodological point of view, a placebo group and longer observation would be desirable.

The authors declare that no conflicts of interest were involved in the design and conduct of the present manuscript. Osato Research Institute laboratory personnel provided their unbiased expertise, as requested by the leading author.

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