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Profile of oxidative stress markers is dependent on vitamin D levels in patients with chronic hepatitis C

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ABSTRACT

Objectives: Although vitamin D deficiency can change liver injury progression in patients with hepatitis C virus (HCV), the main molecular mechanisms involved are largely unknown. The first aim of this study was to evaluate the association between oxidative stress and hypovitaminosis D in patients with HCV. The second aim was to verify whether oxidative stress is involved in the molecular mechanisms related to liver injury.

Methods: The study included 147 participants: 89 controls and 58 patients with HCV (vitamin D < 30, n $= 32$; vitamin D > 30, n $= 26$).

Results: Patients with HCV and hypovitaminosis D presented significantly higher aminotransferase-to-platelet ratio index (APRI; $P = 0.0464$) and viral load ($P = 0.0426$) levels than patients with HCV without hypovitaminosis D. Regarding oxidative stress, HCV patients with hypovitaminosis D had higher advanced oxidation protein products ($P = 0.0409$), nitric oxide metabolites ($P = 0.0206$) levels, and oxidative stress index ($P = 0.0196$), whereas total radical-trapping antioxidant parameter ($P = 0.0446$) levels were significantly lower than HCV patients without hypovitaminosis D. Vitamin D in patients with HCV showed inverse correlations with levels of iron ($r = -0.407$, $P = 0.0285$), ferritin ($r = -0.383$, $P = 0.0444$), APRI ($r = -0.453$, $P = 0.0154$) and plasma lipid hydroperoxides levels ($r = -0.426$, $P = 0.0189$).

Conclusion: Vitamin D insufficiency contributes to the inflammatory process and oxidative stress imbalance in patients with HCV. The profile of oxidative stress markers in these patients depends on vitamin D levels, which probably change intracellular signalling pathways and increase inflammation and liver injury.

Introduction

Hepatitis C virus (HCV) infection is a main cause of chronic liver disease worldwide, and it has reached a pandemic spread [1]. HCV is a noncytopathic virus that enters the liver cell and undergoes replication causing cell necrosis by several mechanisms including immune-mediated cytolysis. Additionally, it provokes various other phenomena, such as hepatic steatosis, oxidative stress, and insulin resistance [2].

There is a marked increase in oxidative stress in HCV infection due to the direct action of structural and nonstructural components of the virus [3]. Additionally, patients with HCV present iron overload and increased ferritin [4], which can further increase oxidative stress and liver injury [5].

Vitamin D deficiency is one of the most prevalent micronutrient deficiencies worldwide, and the rate of this deficiency is estimated to be 30% to 50% [6]. A growing number of studies

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reveal pleiotropic roles of 1,25-dihydroxyvitamin D_3 beyond bone and calcium metabolism, including the induction of antimicrobial genes and the reduction of inflammation and fibrogenesis [6]. Vitamin D has been investigated in the setting of chronic liver disease and several immunopathologic processes have been reported, such as modulation of transcription of >200 genes involved in immune response, inflammation, cell differentiation, and fibrogenesis [7]. Experimental and clinical studies suggest that vitamin D deficiency is a risk factor in HCV-infected patients and that its supplementation might protect against liver disease progression and improve response to treatment [8,9].

A growing body of evidence suggests that vitamin D may have a protective role against oxidative stress in the liver [10]. Although there are still limited data on antioxidant properties of vitamin D, some studies have considered its antioxidant potential to be even stronger than vitamin E and melatonin [11,12]. However, to our knowledge, few studies to date have demonstrated the effect of vitamin D deficiency on oxidative stress [13,14].

Although vitamin D deficiency can change liver injury progression in patients with HCV, the main molecular mechanisms involved are largely unknown. Furthermore, we are not aware of any study, to date, that has verified the association between hypovitaminosis D and oxidative stress in patients with HCV. To evaluate the redox state of a determined pathologic condition, it is necessary to use a robust and complete methodological approach. In this context, an interpretation of the several methods will allow a better understanding of the oxidative phenomena that occur [15]. Therefore, the first aim of this study was to evaluate the association between oxidative stress and hypovitaminosis D in patients with HCV. The second objective was to verify if oxidative stress is involved in the molecular mechanisms related to liver injury.

Methods

Patients

The Institutional Research Ethics Committees of the University of Londrina approved the protocol. All invited individuals were informed in detail about the research and a voluntary written consent form was obtained from 58 patients with HCV and 89 healthy volunteers enrolled consecutively in the study. The two groups were matched for age, sex, ethnicity, smoking, and body mass index (BMI). After that, the HCV group was divided into groups of patients with hypovitaminosis D (vitamin D < 30, n = 32) and without hypovitaminosis D (vitamin D > 30, n = 26). Healthy individuals from the same geographic area comprised the control group. All patients with HCV were seropositive for HCV antibodies and HCV RNA viral load. We excluded patients with concurrent hepatitis B virus or HIV infections or autoimmune hepatitis evidence. None of the participants in the study presented clinical or laboratory characteristics of malabsorption syndrome; autoimmune disease; chronic infection; renal, heart, or oncologic disease; or iron deficiency anemia (IDA), and none were receiving corticosteroids, vitamin D, antioxidant supplements, or drugs for IDA. None of the individuals enrolled in the study drank alcohol or practiced physical activity regularly.

Anthropometric measurements

Body weight was measured to the nearest 0.1 kg using an electronic scale, with individuals wearing light-weight clothing, and barefoot, in the morning; height was measured to the nearest 0.1 cm using a stadiometer. BMI was calculated as weight (kg) divided by height (m) squared.

Biochemical and immunologic biomarkers

After a 12-h fast, the patients underwent the following laboratory blood analysis: iron, aspartate aminotransferase (AST), alanine aminotrasferase (ALT), γ-glutamyltransferase (GGT), and uric acid. Evaluations were conducted with a biochemical auto-analyzer (Dimension Dade AR Dade Behring, Deerfield, IL, USA), using Dade Behring® kits. Platelets were counted using Cell-Dyn 3700 (Abbott Laboratory, Abbott Park, IL, USA). Ferritin and 25-hydroxyvitamin-D_3 [25(OH)D] levels were determined by chemiluminescence microparticle immunoassay (Architect, Abbott Laboratory). RNA HCV viral load of patients was carried out using quantitative transcriptase reverse polymerase chain reaction (RT-PCR) with COBAS AMPLICOR (Roche Diagnostics, Branchburg, NJ, USA). HCV genotyping was assayed using PCR by amplifying the viral core genome region.

Aminotransferase-to-platelet ratio index

Aminotransferase-to-platelet ratio index (APRI) is a simple index consisting of two readily available laboratory results, AST level and platelet count, that can predict significant fibrosis (with area under receiver operating characteristic curve [AUC] = 0.88) and cirrhosis (AUC = 0.94) in treatment-naïve patients with chronic hepatitis C with a very high degree of accuracy. APRI was calculated as (AST/upper limit of normal range)/platelet count (10^9/L) × 100. Eighty-five percent of patients with APRI ≤0.50 would not have significant fibrosis; 88% of patients with APRI >1.50 would have significant fibrosis. Almost all (98%) patients with APRI ≤1 would not have cirrhosis and more than half (57%) of patients with APRI > 2 had significant cirrhosis [16].

Oxidative stress biomarkers

For oxidative stress evaluation, peripheral blood samples were collected with EDTA as anticoagulant. All samples were immediately centrifuged at 3000*g* for 15 min and plasma and serum aliquots were stored in a freezer at −80°C until use.

Analysis of tert-butyl-hydroperoxide-initiated chemiluminescence

Analysis of tert-butyl-hydroperoxide-initiated chemiluminescence (CL-LOOH) in plasma was evaluated as previously described and reported [17,18]. CL-LOOH is used to measure lipid hydroperoxides originating from phospholipids, cholesterol esters, protein, and free fatty acid oxidation and decreased antioxidants levels, brought about by previous free radical action mainly on plasma lipoprotein particles. This test is based on the assumption that an increase in CL is related to the oxidative stress previously suffered by the tissue, inducing the consumption of antioxidant defenses such as vitamin E and the formation of lipoperoxides resulting in an increase in photon emission. Differences in the curve inclination reflect differences in the amount of antioxidants in a sample, whereas higher photon emission levels reflect higher amounts in hydroperoxides previously produced in a sample [19]. Thus, the results were expressed by two different manners:

1. A mean of the readings performed after 1-h reaction, which reflects lipid hydroperoxide levels preformed in a sample and expressed as relative light unit [18]; and
2. A curve, which demonstrates the kinetic reaction at the same period [19,20].

Determination of advanced oxidation protein products

Advanced oxidation protein products (AOPP) was determined in the plasma using a semiautomated method described previously [21]. AOPP results in the oxidation of amino acid residues such as tyrosine, leading to the formation of dityrosine-containing protein cross-linked products detected by spectrophotometry [18,21,22]. AOPP concentrations were expressed as micromoles per liter (umol/L) of chloramine-T equivalents.

Determination of carbonyl protein

Carbonyl content was measured as estimative of oxidative injury to plasmatic proteins [23]. Plasma aliquots were incubated on ice with dinitrophenylhydrazine and deproteinized in trichloric acetic acid. After centrifugation, the pellets were treated with an ethanol/water solution (1:1). The final precipitates were dissolved in guanidine, incubated over 24 h at 37°C and the carbonyl content calculated by obtaining sample spectra at 355 to 390 nm of samples. The obtained peaks were employed to calculate carbonyl concentration using a molar extinction coefficient of 22 M-1 cm-1. Results were expressed in nmol/L/mg total protein content [24].

Determination of nitric oxide metabolites

Serum nitric oxide (NO_x) metabolites were assessed by nitrite (NO_2^-) and nitrate (NO_3^-) concentration according to the Griess reaction supplemented with the reduction of nitrate to nitrite with cadmium [25]. Results were expressed in mM.

Total radical-trapping antioxidant parameter

Total radical-trapping antioxidant parameter (TRAP) was determined as reported previously [26]. Briefly, this method detects hydrosoluble or lyposoluble