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Original/*Otros*

Effect of antioxidant potential on severity of cirrhosis in humans

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Abstract

Background/Aims: **to examine the relationship between the antioxidant potential and severity parameters of cirrhosis in humans.**

Methods: **fifteen patients with hepatic cirrhosis (nine subjects – Child group B, and six subjects – Child group C) and nine control subjects were enrolled in the study. The main criteria taken into account to characterize the diagnosis of cirrhosis and its complications were the AST: ALT ratio, AST to platelet ratio index, Bonacini score, Meld score and Child classification. Those parameters were determined based on laboratory results and patient's clinical records. Se, Zn, ascorbic acid (AA) levels and oxidative stress parameters were measured in blood samples of cirrhotic patients.**

Results: **the analysis of plasma levels of Se and AA showed low concentrations in cirrhotic patients compared with control subjects ($P < 0.05$). Though, there was a positive correlation between plasma of Se and severity parameters of cirrhosis in patients of Child group B and C. In the activity of the antioxidant enzymes only catalase was lower in patients of Child group C compared with control group.**

Conclusion: **we found low plasma levels of Se and AA among cirrhotic patients. However, is not clear why selenium levels tend to increase with the severity of liver cirrhosis.**

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Key words: *Selenium. Ascorbic acid. Flow cytometry. Hepatic cirrhosis.*

EFECTO DEL POTENCIAL ANTIOXIDANTE PARA LAS COMPLICACIONES DE LA CIRROSIS EN LOS SERES HUMANOS

Resumen

Introducción/Objetivos: **examinar la relación entre los potenciales antioxidantes y los parámetros de gravedad de la cirrosis en los seres humanos.**

Métodos: **quince pacientes con cirrosis hepática (nueve sujetos - grupo Child B, y seis sujetos - grupo Child C) y nueve sujetos control fueron incluidos en el estudio. Los principales criterios que se tuvieron en cuenta para caracterizar el diagnóstico de la cirrosis y sus complicaciones fueron la AST: relación de ALT, AST índice de la relación de plaquetas, clasificación Bonacini, clasificación MELD y clasificación de Child. Estos parámetros fueron determinados con base en los resultados de laboratorio y los registros clínicos del paciente. Se midieron los niveles de Zn, ácido ascórbico (AA) y los parámetros de estrés oxidativo en muestras de sangre de pacientes cirróticos.**

Resultados: **el análisis de los niveles plasmáticos de Se y AA mostraron bajas concentraciones en los pacientes cirróticos en comparación con los sujetos control ($P < 0,05$); sin embargo, hubo una correlación positiva entre el plasma de Se y los parámetros de gravedad de la cirrosis en pacientes del grupo Child B y C. En la actividad de las enzimas antioxidantes catalasa solamente fue menor en los pacientes del grupo Child C, en comparación con el grupo control.**

Conclusión: **se encontraron niveles bajos en plasma de Se y AA en pacientes cirróticos. Sin embargo, no está claro por qué los niveles de selenio tienden a aumentar con la gravedad de la cirrosis hepática.**

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Palabras clave: *Selenio. Ácido ascórbico. Citometría de flujo. Cirrosis hepática.*

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Introduction

The metabolism of endogenous and exogenous substances, as well as the viral load, lead to the generation of reactive oxygen species (ROS) which cause the oxidative stress involved in the pathogenesis of some hepatic diseases[1-3] Some enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), are essential components of the antioxidant system and the inorganic elements such as zinc (Zn), copper (Cu) and selenium (Se) are required for their synthesis. These inorganic elements, in addition to ascorbic acid (AA) and glutathione, are essential to reduce the effects of the oxidative stress and they are defective in chronic hepatic disease[2,4-8]. For these reasons, the consumption of vitamins and minerals prevents oxidative stress in patients with alcoholic liver disease[9]. However, studies on the supplementation of those compounds among patients with chronic diseases have not shown any significant effect on all-cause mortality (RR 0.84, 95% CI 0.60–1.19, I^2= 0%)[3,10]. To better understand this process, it is necessary to evaluate how the interaction occurs between different micronutrients and hepatic cirrhosis. In the current study, we evaluate the relationship of the antioxidant potential with severity parameters of cirrhosis in humans.

Methodos

Patients

This study was approved by the Research Ethics Committee (protocol number 0033.0.243.000-10) of the Universidade Federal de Santa Maria (UFSM) and a written informed consent was obtained from all participants. The enrolled cirrhotic patients were from the Hospital Universitario de Santa Maria/UFSM.

Patients with a confirmed diagnostic of hepatic cirrhosis, with positive serology for HCV and history of alcoholism were evaluated. Exclusion criteria included acute hepatitis, HIV/AIDS, diabetes mellitus, neoplastic diseases and innate errors of metabolism, since these are conditions known to be associated with oxidative stress. The healthy subjects were recruited from members of the staff of the Hospital Universitario de Santa Maria/UFSM, reportedly being healthy, not showing any signs of liver disorders, observed by clinical examination and not reported frequent alcohol consumption.

Cirrhosis diagnostic criteria

The diagnosis of cirrhosis was based on disease etiology, clinical data, biochemical tests, imaging and Child-Pugh classification. Besides that, data on age, gender, prior or current history of heavy alcohol consumption (≥ 40-80g per day) were also collected. Hepatitis B and C viruses' co-infection was ruled out by routine serology. In addition to the Child-Pugh classification[11], other criteria such as the ALT/AST ratio[12], aspartate aminotransferase to platelet ratio index (APRI)[13], Bonacini score[14], Lok index[15] and Model for End-Stage Liver Disease (Meld score)[16] were evaluated to confirm hepatic cirrhosis and its complications.

Blood samples collection and analysis

Fasting venous blood samples were obtained as aliquots of the blood collected for routine tests. Peripheral blood mononuclear cells (PBMC) were used to determine intracellular ROS formation through DCF-DA fluorescence detection by flow cytometry immediately after blood collection. Red blood cells (RBC) were used to determine CAT, SOD and GPx activities. The samples used for measuring enzyme activities and micronutrient levels were stored at -80 ºC for 4 weeks. The levels of ascorbic acid, Se, Zn and Cu were determined in plasma.

Determination of ROS by Flow Cytometry

Intracellular H_2O_2 was determined using DCF-DA (Sigma Chemical Co.) as described by Walrand *et al.*[17] with modifications. Leukocyte DCFDA fluorescence was measured by flow cytometry using the FACScalibur Analyzer (BD Biosciences). Leukocytes (granulocytes, monocytes and lymphocytes) were isolated by mixing total blood with Lysing Solution (BD Facs™) as indicated by the manufacturer. The cells (10^6/ml) were washed twice with ice-cold PBS (pH 7.4), centrifuged at 1,800 rpm for 5 min and resuspended in ice-cold PBS. Cells were then incubated with DCFDA (2 μM) for 30 min at 37 ºC. Excess extracellular DCF-DA was then removed by washing the cells once with PBS. At least 50,000 events were counted for each blood sample.

Determination of Se, Zn, Cu, Fe, Mg and AA

Homogenized samples (about 250 mg) were transferred to PTFE-TFM vessels of a pressurized microwave digestion system (Model Multiwave 3000, Anton Paar, Austria), concentrated nitric acid (6 ml) was added, vessels were closed and heated to 210 ºC and maximum pressure of 30 bar. Cu, Se and Zn were determined using inductively coupled plasma mass spectrometry (ICP-MS, Model ELAN DRC II, Perkin Elmer). Mass to charge ratios (m/z) of 63, 82 and 68 were used for Cu, Se and Zn, respectively. Fe and Mg were determined using inductively coupled plasma optical emission spectrometry (ICP-OES, Model Opti-

ma 4300DV, Perkin Elmer). Wavelengths of 317.933, 259.939 and 285.213 nm were used for Fe and Mg determination, respectively. Ascorbic acid determination was performed as described by Jacques-Silva *et al*.[18]

Other parameters

CAT, SOD and GPx activities were measured as described elsewhere (Aebi[19], Boveris and Cadenas[20] and Pagalia and Valentine[21], respectively). Aspartate and alanine aminotransferases (AST and ALT, respectively), bilirubin, Gamma GT, albumin were obtained on Cobas Micros system (Hematology Analyzer, Roche Diagnostics®). Hemogram was performed on PENTRA equipament. International normalized ratio (INR) was performed to evaluate blood coagulation.

Statistical analysis

The statistical analysis was carried out using the Statistic 6.0 software package. The analysis was performed using the nonparametric Mann-Whitney test and Sperman Correlation. Data are expressed as mean ± standard deviation (S.D.). Results were considered significant when $p<0.05$.

Principal component analysis (PCA)

The PCA, a type of multivariate analysis was used to evaluate the relationship among variables and Child-Pugh index score. Initially, data were transformed by ranking on a scale ranging from 1 to 10. The average value of the evaluated parameters corresponded to 5 on the scale with 1 being the lowest assessed value and 10 being the highest assessed value. The average data were analyzed using CANOCO® statistical software (version 4.5, Fa. Biometris). The data matrix was submitted to PCA analysis to compound variables.

Results and discussion

Study subjects laboratory results are shown in table I. Fifteen cirrhotic patients fulfilling the criteria for the diagnosis of cirrhosis were enrolled in the study, nine patients with Child B (average age 52 ± 13 years old) and six patients with Child C (average age 56 ± 12 years old). Nine control subjects with an average age 57 ± 5 years old participated in the study. Among them, 13 (87%) exhibited ascites, 8 (53%) exhibited spider nevi and hepatomegaly and 6 (40%) presented jaundice.

Udell *et al*.[22] proposed a set of criteria to confirm or to exclude cirrhosis in adults with known or suspected liver disease; for instance, presence of ascites, platelet count, spider nevi, and combination of simple laboratory tests with the Bonacini score and Lok index. They concluded that Lok index <0.2, a platelet count >160 $x10^3/\mu L$ and the absence of hepatomegaly were associated with lowered likelihood of cirrhosis.

Wai *et al*.[23] developed an index based on the ratio between serum AST level and platelet count (APRI), to stratify patients with chronic hepatitis C. They showed that this simple index based on widely available laboratory results can identify patients with significant fibrosis and cirrhosis with high accuracy.

In our study, about 40% of the patients reached the APRI criteria of cirrhosis. Whereas 60% of cirrhotic patients reached AST: ALT ratio ≥ 2; 87% reached Bonacini > 7; and 87% reached Lok Index > 0.5 (see supplementary material). Sperman correlation analysis indicated significant positive correlations between theses parameters: AST/ALT ratio x APRI (R= 0.69; p= 0.001) and Bonacini (R= 0.51; p= 0.03), APRI x

Table I
Routine laboratory test results of cirrhotics and healthy subjetcs

Routine laboratory	*Control*	*Cirrhotics Child B*	*Cirrhotics Child C*
ALT (UL)	36.4 ± 3.0	40.1 ± 2.1	54 ± 82**
AST (UL)	19 ± 4.8	63.1 ± 15.8	92.3 ± 29.1*
Total Bilrubin (mg/dl)	0.4 ± 0.04	1.2 ± 0.2	5.3 ± 1.6**
Gamma GT (UL)	44.3 ± 12.3	276.4 ± 140.5	180.2 ± 61.6
Alkaline Phosphatase (UL)	56.6 ± 7.0	172.5 ± 20.1*	180.3 ± 34.3*
Platelet (x $10^3/mm^3$)	244.5 ± 12.7	80.9 ± 11.7*	81.0 ± 15.9*
Hemoglobin (gdL)	13.9 ± 0.30	11.6 ± 0.8*	12.1 ± 0.6
Urea (mg/dL)	32.8 ± 1.5	32.9 ± 3.3	41.7 ± 9.2
Creatinine (mg/dL)	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1

Data are expressed as means ± S.E.; *p<0.05 in relation a control group; **p<0.05 in relation a Child group B. One-way ANOVA-Duncan.

Table II
Blood levels of inorganic elements, ascorbid acid and enzymatic activity in cirrhotics and healthy subjects

Markers	*Control*	*Cirrhotics Child B*	*Cirrhotics Child C*
Cu (mg/L)	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
Fe (mg/L)	2.1 ± 0.2	2.1 ± 0.3	2.0 ± 0.3
Mg (mg/L)	181 ± 7	170 ± 18	168 ± 11
Se (µg/L)	90.5 ± 14	42.9 ± 43*	52.8 ± 5.5*
Zn (mg/L)	0.9 ± 0.08	0.7 ± 0.2	0.5 ± 0.01
Ca (mg/L)	115.2 ± 7.7	127.6 ± 26	111.1 ± 12
AA (mg/L)	6.7 ± 0.4	4.9 ± 0.9	2.95 ± 1.5*
Catalase	57:7 ± 2.5	59.8 ± 4.2	45.5 ± 2.9*
GPx	6.4 ± 0.3	62 ± 0.3	5.9 ± 0.3
SOD	52 ± 2.9	51.4 ± 3.8	44.1 ± 2.0

Data are expressed as means ± S.E.; *p<0.05 in relation a control group. One-way ANOVA-Duncan.

Lok index (R= 0.60; p= 0.01) and Meld (R= 0.50; p= 0.03) and between Meld x Child (R= 0.69; p= 0.001) and Bonacini (R= 0.63; p= 0.006).

Analysis indicated a decrease in the ascorbic acid and Se levels in cirrhotic patients when compared with control subjects (Table II). However, when patients were separated by Child classification (B and C), we observed that ascorbic acid levels of cirrhotics with Child group C was significantly lower than in control group (P=0.01; Table II). Selenium levels were lower in cirrhotic patients (Child B, P=0.01; and Child C, P=0.02) when compared with control group (Table II). The other microelements (Cu, Fe, Mg, Ca and Zn) did not differ between groups (P>0.05). In relation to the activity of the antioxidant enzymes SOD, CAT and GPx, only catalase was lower in patients with Child group C compared with control group (P=0.03; Table II).

Interestingly, there was an unexpected positive correlation between plasma of Se and parameters of disease severity in cirrhotic patients. For instance, Se correlated with Lok Index (R=0.56; P=0.02), Child score (R=0.66; P=0.007) and MELD score (R=0.55; P=0.03).

The PCA analysis (multivariate analysis) showed a different response between the control and cirrhotic individuals plotted separately for the variables analyzed (Fig. 1A, B and C). The application of PCA revealed almost 92% of total variance (Fig. 1A). The compound of PC1, which accounted for 76.8% variance, reflects a marked correlation among Ca, Zn, Fe, Cu and Se (Fig. 1A). PC2 contributed with 15.2% of variance to the data, not having variables with positive correlation. Moreover, we observed a high affinity among control subjects and Se levels, both an inverse relationship between Se levels and cirrhotic patients.

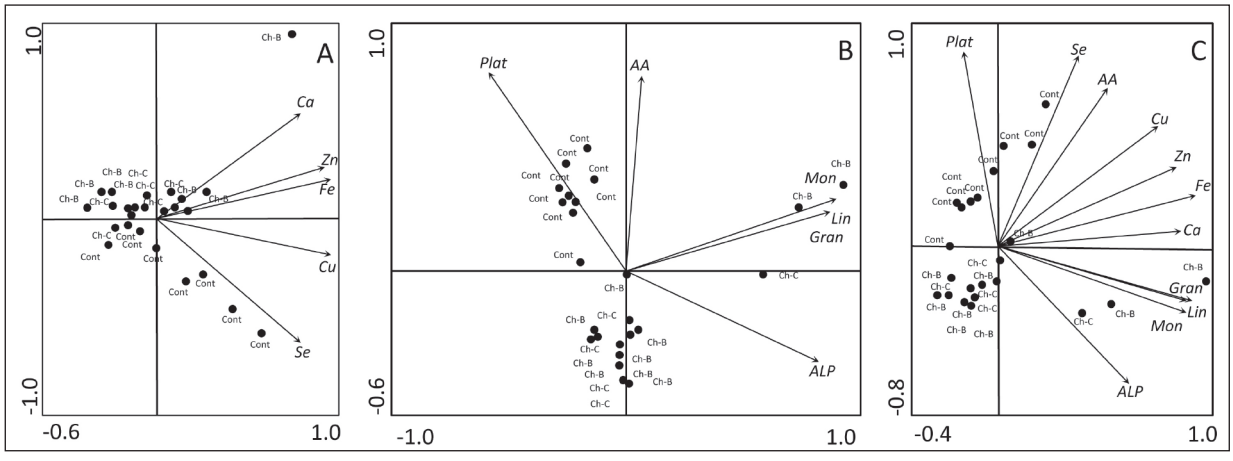

Fig. 1.—Biplot graphic of sources and weights (loadings) for the first two principal components (PC1 and PC2) for inorganic elements (Se, Ca, Cu, Fe and Zn), AA, oxidation of DCFDA parameters and Child index. Cont= healthy controls; Ch-B= cirrhotic patients, score B; Ch-C= cirrhotic patients, score C, Lin= lymphocytes, Mon= monocytes, Gran= granulocytes, AA= ascorbic acid, ALP= alkaline phosphatase, Plat= platelets.

The loadings plot (Fig. 1B) showed that, PC1 is dominated by AA levels and platelets, accounting for 60% of the total variance and it shows high affinity with control subjects. PC2 dominated by DCFDA oxidation in leukocytes and alkaline phosphatase, accounts for 26.2% of the total variance, being in association with cirrhotic patients. The PC1 and PC2 explained 86.2% of the total variances within the data (Fig. 1B).

Figure 1C shows the score plot with parameters of most relevance to the separation of groups used for the PCA that showed 82.2% of total variance. The compound of PC1 contributed with 51.9% of variance and PC2 with 30.3% of variance to the data. When evaluated together, there is a negative correlation between cirrhotic patients with microelements and AA levels. Furthermore, the platelets and Se levels have high affinity with control group. On the other hand, DCFDA oxidation in granulocytes, monocytes and lymphocytes is associated with cirrhotic patients. Interestingly we observe that Se levels have a strong association with the control subjects (Fig. 1A e 1C).

The production of intracellular ROS was measured by DCFDA oxidation (Fig. 2). The fluorescence intensity means in peripheral blood leukocytes in the control group, Child group B and Child group C were, respectively: granulocytes (118±20.7; 477.9±341.9; 591±482), monocytes (31±3.7; 281.9±226.4; 416.7±379) and lymphocytes (13.8±5.2; 54.2±39.5; 74.9±63.7). The production of intracellular ROS by DCFDA oxidation was higher in group C compared with the other groups, however, it was not significant.

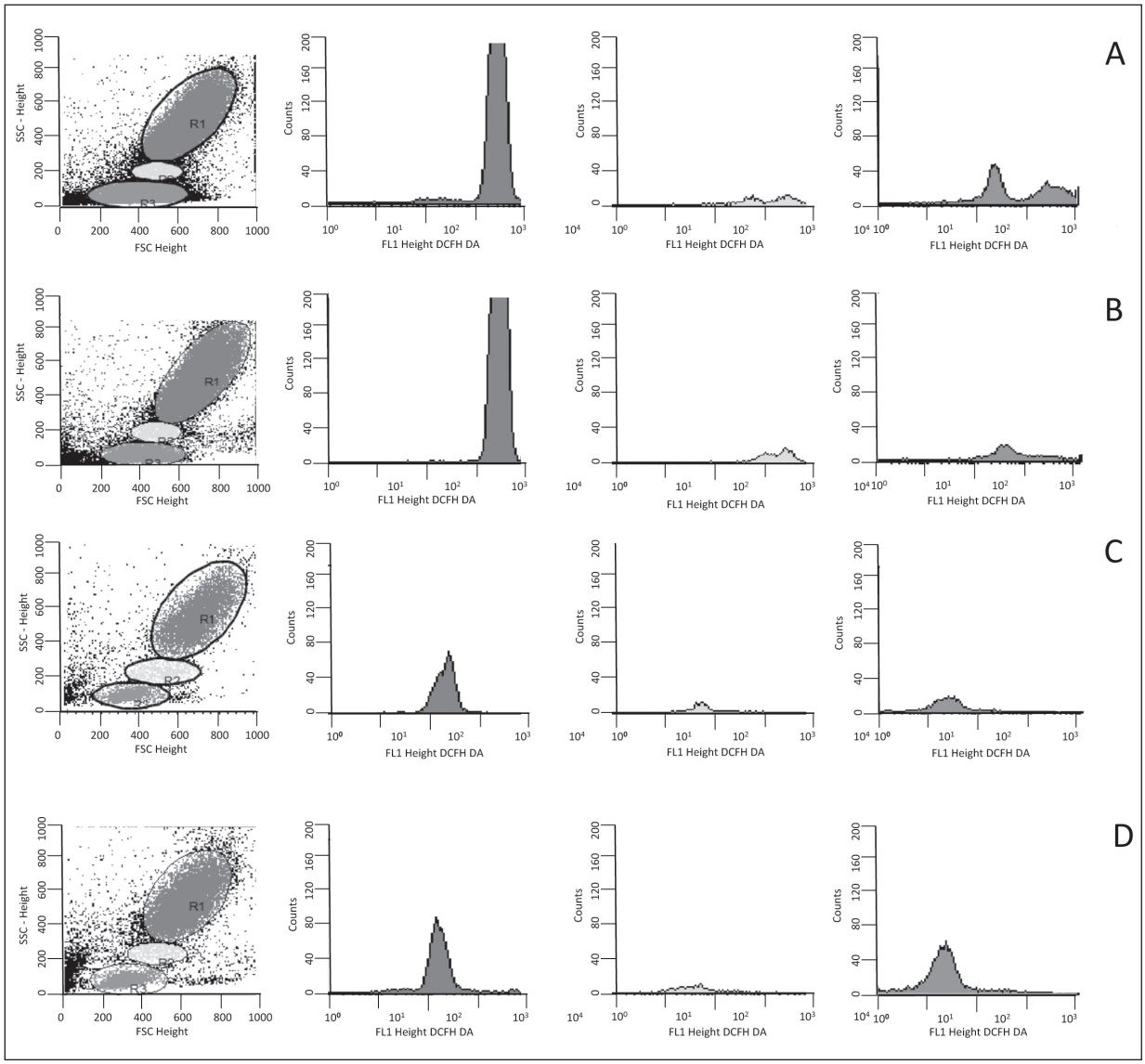

Fig. 2.—Flow cytometric analysis for intracellular H_2O_2 was determined using DCF-DA in peripheral blood leukocytes. A= Example of flow cytometry, with strong fluorescence DCFH-DA, of the cirrhotic patients with Child score B; B= cirrhotic patients with Child score B; C= cirrhotic patients with Child score C; D= healthy control, with mild fluorescence.

Appendice
Parameters of disease severity of cirrhotic subjects

Parameters	*Cirrhotic subjects*	*Frequency*	*Classification to Cirrhosis*	*Evaluated Parameters*
AST: ALT ratio	1.53 ± 0.8	9/15	=1	AST,ALT
AST: platelet ratio index (APRI)	2.75 ± 2.6	6/15	>2	(AST/upper limit of normal AST) x (100:platelet count[x 103/μL])
Bonacini cirrhosis discriminant score (CDS)	8.4 ± 0.8	13/15	>7	Platelet score + ALT:AST ratio score + INR score
Lost Index	0.7 ± 0.2	13/15	>0.5	exp (logodds) / [1 + exp (logodds)] logodds = – 5.56 – (0.0089 x Ptatelet count [x 103/mL]) + (1.26 x AST:ALT ratio) + (5.27 x INR)
Child Pugh	9.1 ± 1.9	9/15	07-09: Child B-	Ascites, Bilirubin, albumin, INR and encephalopathy
		6/15	>9: Child C	
MELD	13.9 ± 4.9	12/15	10-19: 27% mortality in 3 months	0.957 + loge (creatinina mg/dL) + 0.378 x loge (bilirrubinas mg/dL) + 1.120 x loge (RNI) + 0.643
		3/15	>20-29: 76% mortality in 3 months	

Data are expressed as means ± SD.

The micronutrients Se and ascorbic acid are important for the functioning of immune system and selenium is necessary for the synthesis of antioxidant selenoproteins and selenoenzymes[24-26]. Consequently, in cirrhotic patients, these physiological functions may be compromised. However, it is not possible to now whether low Se levels were involved in the progression or whether they were the consequence of the hepatic disease[27].

The liver plays a central role in trace elements' metabolism; therefore, the alterations of its structure and function typical of cirrhosis may alter the hepatic utilization of trace elements, as well as their release in the blood[28].

The effects of Se in cirrhotic patients have been little studied. Burk *et al*.[29] have demonstrated that Se in plasma decreases as the severity cirrhosis increases. However, the Se in glutathione peroxidase compartment raised in cirrhotic Child group C compared with Child group A and B. The correlation obtained here between Se and severity of cirrhosis (Lok index, Child score and Meld score) indicated an increase in Se levels with the worsening of cirrhosis. The discrepancies between the studies may be due to the small number of patients evaluated in both studies. Consequently, more studies are needed to clarify these controversies.

In contrast to Se, negative correlations between the levels of Zn and severity parameters of cirrhosis (Lok Index R=-0.63; P=0.007, Bonacini R=-0.50; P=0.05 and APRI R=-0.50; P=0.05) were observed, which are in accordance with the literature[30-32].

In the current study, we found low plasma levels of Se and AA among cirrhotic patients and an important association with control subjects. There are indications that selenium metabolism is altered in cirrhosis[29], Although the behavior of selenium in the severity of liver cirrhosis is not yet clear, it is not a good marker to assess the severity of cirrhosis in this study. Regardless of the few studies, AA seems to be a good marker to help with the study on the severity of cirrhosis, but further researches are necessary. Additionally, future research will be needed to elucidate the behavior of these micronutrients in humans.

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