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Brief Original Article

Alterations in neutrophil extracellular traps is associated with the degree of decompensation of liver cirrhosis

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Abstract
Introduction: Liver cirrhosis (LC) constitutes one of the main 10 causes of death worldwide. LC has a characteristic asymptomatic compensated phase followed by a progressive decompensated phase, in which diverse complications are presented. LC patients are highly prone to bacterial infections. The neutrophils in these patients present defects in the production of oxygen radicals, which are essential for bacteria elimination as in the activation of neutrophil extracellular traps (NETs). The main objective of this work was to determine the NETs and neutrophil activation markers in LC patients.
Methodology: Neutrophil purification was done with Ficoll Histopaque from a sample of the peripheral blood of patients with compensated and decompensated LC. Neutrophils were activated with Phorbol 12-myristate 13-acetate to evaluate the release of NETs by means of fluorescence microscopy and fluorometry, while expression of activation markers (CD69, CD80, perforin, and CAP-18) was evaluated by flow cytometry.
Results: A significant decrease in the release capability of NETs was observed as the level of LC in the patient increased. When comparing serum levels in inflammatory cytokines among the different study groups, significant differences were observed. No significant differences were detected on neutrophil activation markers; nevertheless, there was a correlation between diminution of CD69 and CD80 expression in decompensated patients.
Conclusions: We demonstrated that LC patients with neutrophil extracellular trap release deficiencies could have an increased rate of complications.

Key words: liver cirrhosis; neutrophils; neutrophil extracellular traps; NETs.

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Introduction

Liver cirrhosis (LC) constitutes a major health problem and is found among the top 10 causes of death worldwide [1]. This condition is defined as an advanced stage of progressive fibrosis of the liver that is identifiable by disruption of the lobular architecture and hepatic vasculature [2].

The clinical advancement of the disease is identifiable by a compensated asymptomatic phase followed by a decompensated progressive phase in which many complications can occur [3]. The most frequent complications are jaundice, rupture of varicose veins, encephalopathy, ascites, spontaneous bacterial peritonitis (SBP), and hepatorenal syndrome [4].

Patients with LC and ascites are highly prone to bacterial infections, mainly due to defects in their defense mechanisms; the most frequent and severe infectious complication is SBP [5]. Deficiencies in the immune system show that both in the innate and adaptive responses, the principal flaws are associated with bactericidal capability, phagocytosis, and opsonization, as well as deficiencies in chemotaxis in both mononuclear and polymorphonuclear cells (PMNs) [6]. PMNs are cells that constitute the first component of innate immune defense. They react rapidly in response to infection or damage, migrating to inflammation sites by a chemokines gradient, where they eliminate pathogens through diverse mechanisms, such as phagocytosis, degranulation, and NETosis [7].

NETosis is a microbicidal mechanism recently described. The structures formed and released by neutrophils are known as neutrophil extracellular traps (NETs), which entrap and eliminate the microorganisms. Their structures are mainly formed by nuclear chromatin, associated with histones and diverse granular proteins (elastase and myeloperoxides) [8].

It has been demonstrated that neutrophils in patients with hepatic diseases present defects in the production of oxygen radicals, mainly when producing superoxide anion and hydrogen peroxide [9], both essential in the elimination of bacteria and indispensable to the correct release of NETs. This can be determined in the high incidence of bacterial infections that is observed in these patients.

In an attempt to elucidate the mechanism by which patients with cirrhosis present a high incidence of susceptibility to infectious disease, we proposed this work, the main objective of which was to determine whether there is an association between the degree of decompensation of patients with cirrhosis and their capability for the release of NETs.

Methodology

Design and population of the research

A transversal study was carried out in 80 individuals, who were incorporated into four different groups: healthy subjects (HS; n = 20), patients with compensated cirrhosis (LC; n = 20), patients with decompensated cirrhosis who present ascitic fluid (AF) (LC with AF; n = 20), and patients with decompensated cirrhosis with SBP (LC with SBP; n = 20).

All of the patients were recruited from the Gastroenterology Service of the Hospital Civil Fray Antonio Alcalde. NETs release capability and concentration of inflammatory cytokines were determined and compared with the group of HS. Diagnosis of patients with LC was performed by clinical, laboratory, and radiological analysis, and/or liver biopsy. Diagnosis of SBP was conducted by polymorphonuclear cell (PMN) count in AF; a count of ≥ 250 PMN/μL was a confirmatory measure of the diagnosis of the SBP group.

The study was carried out according to the Declaration of Helsinki and the Regulation of Studies in Humans in Health Matters of Mexico. The patients included in this study were informed of the purpose of the investigation and the importance of their participation. They signed a written authorization form providing their informed consent.

Blood samples

From all study groups, peripheral blood (PB) samples were collected to obtain neutrophils and serum.

Neutrophils collection

Neutrophils were obtained by Ficoll Histopaque 1119/1077 density gradient (Sigma-Aldrich, Saint Louis, Missouri, USA). The cellular ring was resuspended in RPMI-1640 medium (Sigma-Aldrich, San Luis, Missouri, USA). Cell count and viability were performed in a Neubauer chamber by means of trypan blue (Sigma-Aldrich, San Luis, Missouri, USA).

Induction of NETs in vitro

Induction of NETs *in vitro* was carried out with 50 nM Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., San Luis, Missouri, USA) put in polystyrene plates 5 $\times 10^5$ PMN and incubated at 37°C with 5% CO_2 for three hours and fixed with formaldehyde (Sigma Chemical Co., San Luis, Missouri, USA). Two main NETs components (DNA and elastase) were stained. DNA marking was conducted adding 1 μg of 4',6-diamidino-2-phenylindole (DAPI) (Sigma_Chemical Co., Saint Louis, Missouri, USA). Subsequently, elastase was detected through immunomarking. The cells were incubated with anti-elastase primary antibody (Ab21595; (Abcam Inc., Cambridge, USA)) and with the Alexa Fluor 488-conjugated secondary antibody (Ab150069; (Abcam Inc., Cambridge, USA)). The samples were observed in a Zeiss Axio Imager A2 fluorescence microscope (Zeiss, Oberkochen, Alemania), and random but representative images were taken of each sample with 10X and 40X magnification. NETs quantitation by microscopy was carried out according to the method previously described by Hosseinzadeh *et al*. [13]. The total number of cells was counted; NETs-forming cells were expressed as a percentage of the total of events.

NETs quantitation

DNA/NETs quantitation was performed by fluorometry. In this assay, the fluorescence intensity reflects the amount of DNA/NETs and is quantified in a fluorometer (Biorad, Hercules, USA), utilizing excitation/emission (485/538) filters. Data were reported as relative fluorescence units (RFU).

Neutrophil immunological phenotype

Immunological phenotypification of neutrophils was performed by flow cytometry.

First, 1×10^6 PMN of PB was incubated with 0.5 µg anti-CD16-PerCP antibodies (BioLegend, San Diego, USA), anti-CD11b-PE/Cy7 antibodies (BioLegend, San Diego, USA), and one of the antibodies against activation markers phycoerythrin (PE) conjugated as anti-CD69 (BioLegend, San Diego, USA), or anti-CD80 (BioLegend, San Diego, USA) was fixed with formaldehyde. Intracellular marking of CAP18 and perforin was carried out utilizing the IntraPrep kit (BioLegend, San Diego, USA), 0.5 µg of anti-CAP18-FITC antibodies (Santa Cruz Technology, Paso Robles, USA), or anti-perforin Alexa Fluor 488 (BioLegend, San Diego, USA). The samples were analyzed in the Guava easyCyte Flow Cytometer (Millipore, Billerica, USA). Analysis was focused on the granulocyte population, according to their characteristics of forward and side scatter (of light). A second electronic window was carried out in the CD16b+ CD11b+ population and evaluated by fluorescence percentage; intensity was evaluated by the expression of CD69, CD80, CAP-18, and perforin.

Cytokine determination

The inflammatory cytokine concentrations (IL-6, tumor necrosis factor alpha [TNF-α], IL-1β, IL-12, IL-8, IL-10, and interleukin-1 receptor antagonist [IL-1Ra]) were determined in serum based on the manufacturer's specifications of the Human Inflammatory Cytokine kit (BD, cytometric bead array [CBA], 551811 (Becton Dickinson, Franklin Lakes, USA)). The samples were analyzed in a BD FacsCanto II Flow Cytometer (Becton Dickinson, Franklin Lakes, USA), and FCAP Array (Becton Dickinson, Franklin Lakes, USA) software was used to analyze the data obtained.

Statistical analysis

Data are presented as means ± standard deviations. The statistical analysis was performed utilizing SPSS version 20 statistical software package (IBM, Armonk, USA). Comparisons were performed by non-parametric tests using the Friedman and/or the Mann-Whitney *U* test. These were considered significant when $p < 0.05$.

Results

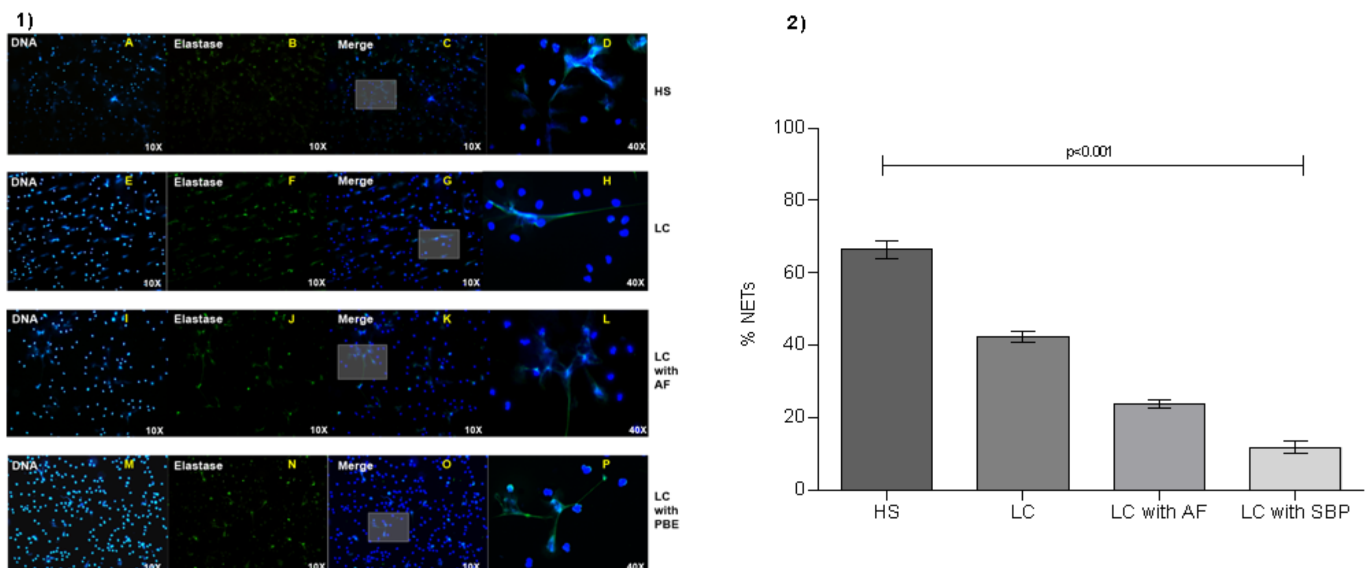
Patients' characteristics

Sixty patients with LC were included in the study. The etiology of the three groups of patients with cirrhosis was alcohol, 70% (n = 42); hepatitis C virus (HCV), 10% (n = 6); and other, 20% (n = 12). At the time of sample taking, none of the patients were receiving antibiotic treatment nor had they had antibiotic treatment in the previous month.

NETs release capability

After stimulus with PMA, the neutrophils that were observed on microscopy and images were obtained by fluorescence microscopy. The results showed a decrease in the capability of PMN to release extracellular traps according to complications of the

Figure 1. NETs expression in study groups. 1) Neutrophils stimulated with PMA and stained with DAPI (blue) and antielastase antibodies (green). A–D: Neutrophils of healthy subjects (HS). E–H: Neutrophils of patients with compensated liver cirrhosis (LC). I–L: Neutrophils of patient with liver cirrhosis with ascitic fluid (LC with AF). M–P: Neutrophils of patient with liver cirrhosis with spontaneous bacterial peritonitis (LC with SBP). 2) Quantification of NETs by immunofluorescence. The percentage of NETs is shown for the four study groups; HS, LC, LC with AF, and LC with SBP ($p < 0.001$).



disease of patients with LC; thus, neutrophils of patients with decompensated LC present smaller capability for release of NETs than the neutrophils of patients with compensated LC (Figure 1).

DNA of NETs

After conducting neutrophil stimulation with PMA, the DNA released by means of fluorometry was quantified. The results corroborate the obtained by fluorescence microscopy analysis (data not shown).

Expression of CD69, CD80, perforin, and CAP-18

After analyzing the data obtained by FC, there were no significant differences in the percentage of expression and mean fluorescence intensity of the different activation markers in the neutrophils of the four study groups (data not shown).

Inflammatory cytokines in serum

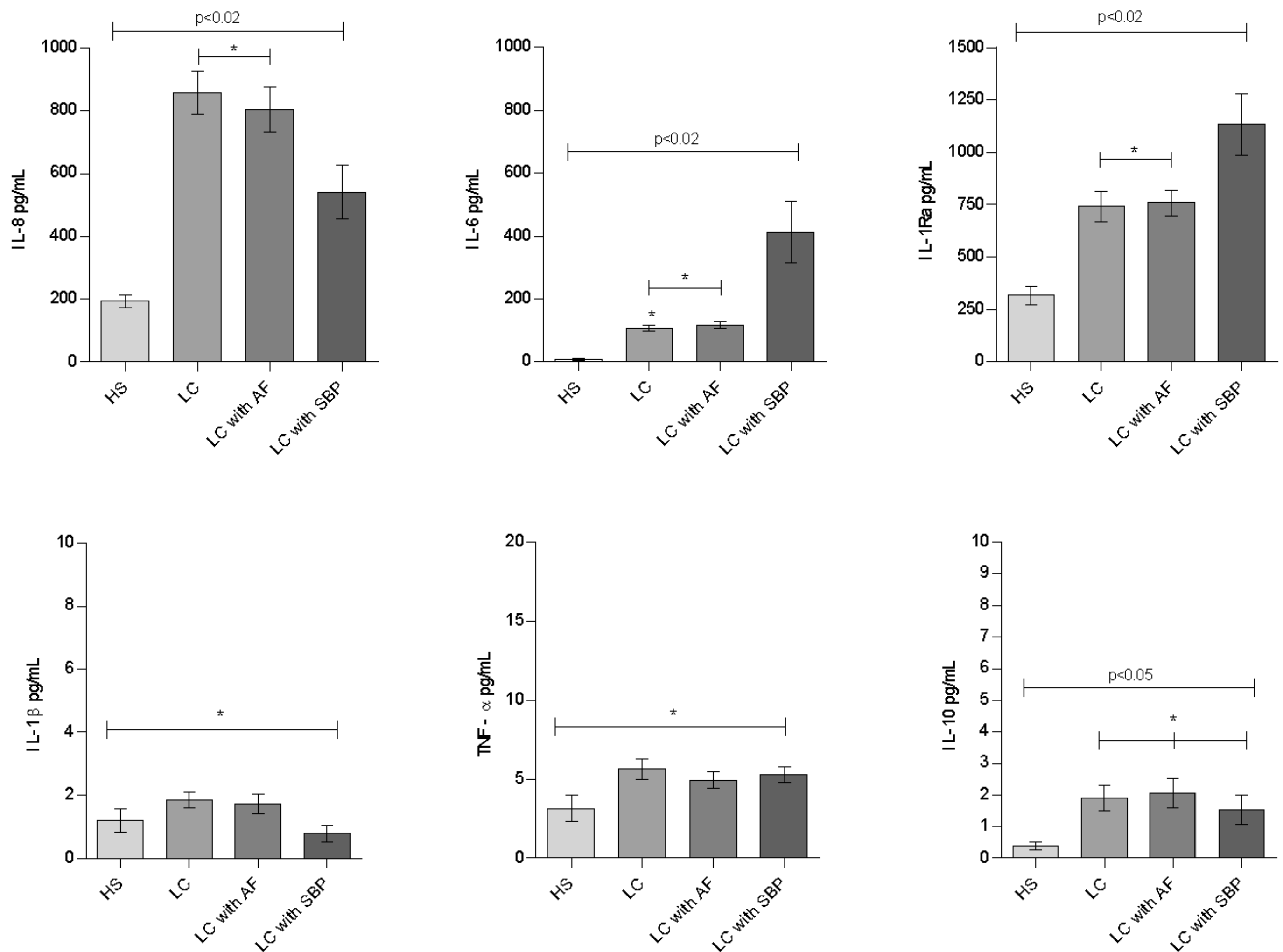
The levels of IL-6, TNF-α, IL-1β, IL-12, IL-8, IL-10, and IL-1Ra in the serum samples of the four study groups were quantified. Significant differences were observed in the serum levels in inflammatory cytokines among the different study groups ($p < 0.02$) (Figure 2).

Discussion

We have demonstrated that the degree of decompensation presented by patients with liver cirrhosis arbitrates an impact on the microbicidal activity of NETs-mediated neutrophils.

In patients with LC, deficiencies were observed in both the innate and adaptive immune responses [10]. Diverse studies have demonstrated defects in bactericidal and opsonization capability; low levels have been reported of protein C3 of the complement, as well as deficiencies in the chemotaxis mechanisms

Figure 2. Serum concentration of inflammatory cytokines. The serum concentration of inflammatory cytokines (pg/mL) is shown for the study groups. The mean values were statistically significant on comparison the HS, LC, LC with AF, and LC with SBP groups ($p < 0.02$).



* Data not statically significant.

[11,12]. To our knowledge, there are no studies to date that have evaluated the capability of neutrophils to release NETs in patients with LC.

There is a great variety of pro-inflammatory stimuli that induce the release of NETs. It is known that this microbicidal mechanism is reactive oxygen species (ROS) dependent [13]; it has been demonstrated that neutrophils in patients with liver diseases present defects in the production of ROS, mainly in the production of superoxide anion and hydrogen peroxide [14]. This could be the cause of the deficient capability of NETs release in patients with LC, and it could also be involved in the high incidence of bacterial infections. Some studies have shown that patients with chronic granulomatous disease and with defects in ROS production are incapable of forming NETs [15]. Besides this and by means of a refractory therapy, it is possible to reestablish nicotinamide adenine dinucleotide phosphate oxidase functionality, the subsequent ROS production, NETs release, and combating of infections [16].

In order to conduct correct activation of the defense mechanisms against infection, the participation of cytokines with mainly pro-inflammatory functions is necessary. However, to avoid damage to the organism due to an exacerbated inflammatory response, the synthesis and secretion of anti-inflammatory molecules such as IL-1Ra and IL-10 is required.

Some works have demonstrated a correlation between the serum concentration of IL-1β, IL-6, and IL-18 and the diverse clinical complications presented in patients with cirrhosis [17]. In addition, it has been observed that there is an increased expression of inflammatory cytokine genes, such as IL-1β, IL-6, IL-8, TGF-β, and IL-10 in patients with chronic liver disease, which is associated with the etiological agent of the disease [18].

In our work, we found elevated levels of various cytokines, principally IL-6, IL-8, IL-1Ra, and IL-10, in serum. The increase in the concentration of pro-inflammatory cytokines (IL-6 and IL-8) suggests an association between the degrees of decompensation of patients with the inflammatory process that they develop later. On the other hand, the raised levels of anti-inflammatory cytokines of IL-1Ra and IL-10 suggest an effort of the organism to attempt to counteract the inflammatory process, in an attempt to avoid damage to tissues and to the organs themselves.

Some works have shown that prolonged exposure to antimicrobial peptides and/or cytokines inhibits the spontaneous apoptosis of neutrophils [19]. Suppression of the apoptosis of PMN increases their half-life and can result beneficial to the host against bacterial invasion. However, the increase in PMN survival can cause uncontrolled release of cytotoxic metabolites and proinflammatory substances that lead to amplification of the inflammation system, tissue damage, and organ failure [20]. Inappropriate induction of PMN apoptosis can affect the number of these cells and can cause deficiencies in the development of their functions, which will in turn impede host defense and will favor bacterial invasion and persistence.

Conclusions

We demonstrated that the degree of decompensation of liver cirrhosis patients is associated with their capability to release NETs, which increases the risk of developing complications such as recurrent infections.

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