Original Paper

Cardiorenal Syndrome Type 5 in Sepsis: Role of Endotoxin in Cell Death Pathways and Inflammation

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Key Words

Endotoxin Activity • Cardiorenal Syndrome Type 5 • Renal Tubular Cells • Apoptosis • Cytokines • Sepsis

Abstract

Background/Aims: Cardiorenal Syndrome Type 5 (CRS Type 5) is characterized by concomitant cardiac and renal dysfunction in the setting of different systemic disorders, such as sepsis. In this study, we investigated the possible relationship between endotoxin levels, renal cell death and inflammation in septic patients with CRS Type 5. **Methods:** We enrolled 11 patients with CRS Type 5. CRS Type 5 was defined according to the current classification system. AKI was defined by Acute Kidney Injury Network (AKIN) criteria. Acute cardiac dysfunction was documented by echocardiography as acute left and/or right ventricular dysfunction leading to decreased ejection fraction. Endotoxin activity was measured by the Endotoxin Activity Assay (EAA). Plasma from CRS Type 5 patients was incubated with renal tubular cells (RTCs) and cell death levels were evaluated. Plasma cytokines levels were measured as well. **Results:** Accordingly to EAA levels, patients were divided into two groups: 45.4% of patients had low endotoxin activity level (negative EAA), while 54.5% of patients showed high endotoxin activity (positive EAA). RTCs incubated with plasma from EAA positive patients showed significantly higher apoptosis levels and higher caspase-3 activation compared to cells incubated with plasma from EAA negative patients, and a significant positive correlation was observed between EAA levels and RTC apoptosis levels. Furthermore, IL-6 and IFN- γ levels were significantly higher in CRS Type 5 patients with positive EAA. **Conclusion:** Our data suggest a possible relationship between endotoxin levels and renal cell death in septic patients with CRS Type 5. Furthermore,

this study highlights the presence of renal apoptosis, the immune deregulation and the strong inflammation in CRS Type 5 patients, especially in those with high endotoxin activity.

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Introduction

Cardiorenal Syndrome Type 5 (CRS Type 5) is characterized by concomitant cardiac and renal dysfunction in the setting of a broad spectrum of systemic disorders, such as sepsis, autoimmune disease, drug toxicity and diabetes mellitus [1-3]. Specifically, CRS Type 5 is characterized by the activation of inflammation, coagulation and fibrinolytic systems with cellular and molecular changes in the heart and kidneys with a time-specific pattern [4].

Inflammatory mediators, cell apoptosis, caspase cascade and oxidative stress are known to alter organ function, to cause abnormal cell signaling, cell cycle arrest and mitochondria dysfunction, thus leading to tissue and organ injury [3, 5]. In particular, direct pro-apoptotic and pro-inflammatory mediators affect cardiomyocytes and kidney resident cells [1, 3, 6]. Lipopolysaccharide (LPS) or endotoxin is the major constituent of the outer membrane of Gram-negative bacteria and it may lead to severe generalized inflammation, manifesting clinically as sepsis with multiple organ dysfunctions, such as myocardial depression and renal impairment [7-11]. In this study, we investigated the possible relationship between endotoxin levels, renal cellular death and inflammatory mediators release in septic patients with CRS Type 5.

Materials and Methods

Ethical statement

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional research committee at which the study were conducted and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The protocol and consent form were approved by the Ethics Committee of San Bortolo Hospital (46/11). Informed consent was obtained from all individual participants included in the study.

Subjects characteristics

We enrolled 11 septic patients with CRS Type 5, defined according to the current classification system [2], in Intensive Care Unit (ICU). Estimated Glomerular Filtration Rate (eGFR) was calculated with the MDRD study equations [12]. AKI was defined by Acute Kidney Injury Network (AKIN) criteria [13]. We considered as the baseline value, the sCr level of the 3 months before the admission. Patients exposed to contrast media in the 72 h preceding AKI were excluded. Acute cardiac dysfunction was documented by echocardiography as acute left and/or right ventricular dysfunction leading to decreased ejection fraction. Demographic and clinical data were collected at admission. SOFA (Sequential Organ Failure Assessment) score and ICU stay were calculated as well. Negative outcome was defined as death during the ICU stay.

Sample Collection

The procedures were in accordance with the Helsinki Declaration. Peripheral venous EDTA-blood samples were collected within 4 hours after CRS Type 5 diagnosis. Plasma was immediately separated and stored at -80°C until use.

Endotoxin Activity Assay (EAA)

Serum endotoxin activity was determined by the EAA (Endotoxin Activity Assay) (Estor Spa, Milan, Italy). Endotoxin activity levels are expressed as units on a scale ranging from 0 to 1. We defined low endotoxin activity level (EAA^{low}: 0.00–0.39) as negative EAA and high endotoxin activity level (EAA^{high} ≥ 0.60) as positive EAA.

RTC Culture

An immortalized human proximal renal tubular epithelial cells (RTCs) line was generated by infection with a hybrid Adeno5/SV40 virus [14]. RTCs were cultured grown in RPMI 1640 medium (International PBI, Milan, Italy) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Sigma Chemical Co, St. Louis, MO, USA). These cells were maintained in a standard condition.

Induction of Apoptosis

RTCS were plated at 1.2×10^4 cells per well in 48-well plates, and incubated with 90% RPMI 1640 medium and 10% EDTA plasma from CRS Type 5 patients in standard conditions. Each incubation was performed in triplicate.

Evaluation of Apoptosis: Annexin V and Propidium Iodide Detection Assay

Cell viability, apoptosis and necrosis were assessed using the Annexin V-FITC kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol. Analysis was performed using a Navios flow cytometer (Beckman Coulter, Brea, CA., USA). We used untreated RTCs as negative controls.

Determination of caspase-3,8,9 Activity

Caspase-3,8 and 9 concentrations were measured by Human instant enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, Calif., USA) with a fluorometric assay. RTCs incubated for 24h with plasma of CRS Type 5 patients were processed according to the manufacturer's instructions and finally caspase-3,8 and 9 levels were measured in cell lysates by VICTOR™ X4 Multilabel Plate Reader (PerkinElmer Life Sciences, Waltham, Mass., USA).

Cytokine Enzyme-Linked Immunosorbent Assay

The quantitative determination of IL-6, IL-1β, IL-8, IL-10, IL-18 and IFN-γ in the plasma of CRS Type 5 patients was performed by the Human Instant ELISA kits (eBioscience, San Diego, CA, USA). Cytokine determinations were performed according to the manufacturer's protocol and instructions. Optical density was read at 450 nm. The amount of cytokines was calculated from the standard curve. All tests were performed in duplicate.

Statistical Analysis

Statistical analysis was performed using the SPSS (version 15; SPSS Inc., Chicago, Ill., USA). The Mann-Whitney U test or T test were used for comparison between two groups, as appropriate. Spearman's rho correlations were calculated to verify the correlation between variables. A p-value <0.05 was considered significant.

Results

CRS Type 5 Patients Characteristics

Demographic and laboratory data were collected from all the patients (Table1). The mean age of 11 septic patients with CRS Type 5 was 69.4 ± 10.8 years and 72.8% of these patients were male. 54.5% CRS Type 5 patients had diabetes mellitus and 45.4% had hypertension. The median baseline sCr of CRS Type 5 patients was 1.06 mg/dl (IQR 0.95-1.41), the median baseline eGFR was 62 ml/min/1.73m² (IQR 50-75); in particular, 18.1% of patients had an eGFR lower than 45 and 81.8% higher than 45 ml/min/1.73m². The median sCr of CRS Type 5 patients at admission was 1.85 mg/dl (IQR 0.77-3.64), the median eGFR at admission was 36 ml/min/1.73m² (IQR 17.5-86). The median SOFA score of CRS Type 5 patients was 7.5 (IQR 4.5-10.5) at admission. The median ICU stay was 8 days (IQR 4-18). Furthermore, 3/11 had a negative outcome during ICU stay. No patients needed mechanical ventilation, while 4 patients required Continuous Renal Replacement Therapy (CRRT) (Table1). Sepsis was caused by Gram-positive infections in 27.2% of patients, by Gram-negative infections in 36.3% of patients and by fungal infections in 18.2% of patients. In 18.2% patients the cause of sepsis was unknown.

EAA levels

The median EAA level of the 11 patients with CRS Type 5 was 0.67 (IQR 0.39-0.76). In particular, 45.4% of patients had low endotoxin activity level (0.34; IQR 0.24-0.37), no patients had intermediate endotoxin activity level, and 54.5% of patients had high endotoxin activity level (0.72, IQR 0.67-0.85).

Effects of Plasma on RTC Viability

The RTCs incubated with plasma from EAA positive patients showed significantly higher apoptosis (31.3%; IQR 28.8-39.0) compared to cells incubated with plasma from EAA negative patients (14.2%; IQR 13.8-15.3) ($p=0.02$). Cells incubated with plasma from EAA positive patients did not show significantly different levels of necrosis and viability when compared with those incubated with EAA negative plasma (viability EAA positive 63.5, IQR 55.1-68.3; EAA negative 78.8, IQR 57.4-80.1, $p=0.30$; necrosis EAA positive 5.2, IQR 1.3-10.1; EAA negative 4.6, IQR 3.8-10.8, $p=0.60$) (Figure 1). However, a negative trend with higher necrosis and lower viability was evident in EAA positive patients.

A significant positive correlation was observed between EAA levels and RTC apoptosis percentage (Spearman's $\rho=0.6$, $p<0.007$). In concordance with the apoptosis rate, the RTCs incubated with plasma from EAA positive patients showed significantly higher caspase-3 activation (4.53 ng/mL, IQR 3.29-4.59) compared to cells incubated with plasma from EAA negative patients (3.30 ng/mL, IQR 2.95-3.48) ($p=0.01$) (Figure 2). Moreover, significantly higher levels of caspase-8 were observed in cells incubated with plasma from EAA positive patients (0.96, IQR 0.91-1.4) compared to EAA negative patients (0.63, IQR 0.53-0.99) ($p=0.04$). On the contrary, there were no differences in the levels of caspase-9 between EAA positive and negative patients (Figure 3).

Table 1. Clinical and biochemistry parameter about CRS Type 5 patients enrolled

	CRS Type 5 patients	Normal range
Male (n)	8/11	
Age (years)	69.4±10.8	
Creatinine baseline (mg/dl)	1.06 (0.95-1.41)	0.7-1.3
eGFR baseline (ml/min/1.73 m ²)	62 (50-75)	90-120
Creatinine admission (mg/dl)	1.85 (0.77-3.64)	0.7-1.3
eGFR admission (ml/min/1.73 m ²)	36 (17.5-86)	90-120
Temperature (°C)	38.3 (36.1-38.9)	36.5-37.5
Urine output (ml/day)	3040 (1542-3225)	800-2000
Urea (mg/dl)	91 (59-192)	42-126
Na (mEq/l)	138 (135-144)	136-145
K (mEq/l)	3.9 (3.7-4.1)	3.4-4.5
White Blood Cells (/mm ³)	10.8 (7.25-14.2)	4.4-11.0
Platelets (/mm ³)	133 (109-149)	110-330
PaO ₂ /FiO ₂	241.5 (194.5-273.5)	>500
pH	7.45 (7.39-7.49)	7.35-7.45
Procalcitonin (ng/mL)	10.8 (0.98-32.1)	<0.5
SOFA at admission	7.5 (4.5-10.5)	
ICU Stay (days)	8 (4-18)

Fig. 1. Cell viability, necrosis and apoptosis. Apoptosis percentage in RTCs is significantly higher after incubation with plasma from EAA positive compared to EAA negative patients. There is no significant difference in terms of viability and necrosis in RTCs when incubated with plasma from EAA positive and negative patients.

*Evaluation of
plasma immune-
mediated molec-
ules*

Plasma pro- and anti-inflammatory cytokine levels were measured by ELISA in CRS Type 5 patients. IL-6 and IFN- γ levels were significantly elevated in EAA positive patients compared with EAA negative patients. There were no differences in the levels of IL-10, IL-8 and IL-1 β

Fig. 2. Caspase-3 levels. Caspase-3 levels are significantly higher in RTCs incubated with plasma from EAA positive compared to EAA negative patients.

Fig. 3. Caspase-8 and 9 levels. Caspase-8 levels are higher in RTCs incubated with plasma from EAA positive patients compared to EAA negative ones. No significant difference has been observed in terms of caspase-9 levels between these two groups.

in EAA positive patients compared with negative EAA patients (Table 2). A significant positive correlation was observed between EAA and IL-6 levels (Spearman's $\rho=0.58$, $p<0.008$).

Discussion

A growing body of research indicates that apoptotic mechanisms and inflammation may play a role in the pathogenesis of CRS Type 5, especially in patients with sepsis [3, 5]. In this study, we examined the complex pathophysiology of CRS Type 5 in septic patients, in terms of apoptotic mechanisms and inflammation. In particular, we investigated the role of endotoxin in CRS Type 5 and its possible relationship with RTC death and cytokine levels. We analysed the *in vitro* cytotoxic effects induced by CRS Type 5 plasma incubated with RTCs in patients with high and low levels of endotoxin. The RTCs incubated with plasma from EAA positive patients showed significantly higher apoptosis compared to cells incubated with plasma from EAA negative patients. Moreover, a significant positive correlation was observed between EAA levels and RTC apoptosis percentage. In concordance with the apoptosis rate, the

RTCs incubated with plasma from EAA positive patients showed significantly higher caspase-3 and -8 activation compared to cells incubated with plasma from EAA negative patients. Even though no

significant difference in terms of necrosis, viability and caspase-9 levels was found between the two groups, a negative trend with higher necrosis, lower viability and higher caspase-9 activation was evident in cells incubated with plasma from EAA positive patients.

Apoptotic mechanisms have been demonstrated to play a pivotal role in the setting of CRS and sepsis. Our data are supported by several previous studies in the context of CRS. In fact, decreased viability and increased apoptosis rates were reported by Virzi et al. in RTCs incubated with plasma of CRS Type 1 patients [1]. In particular, both intrinsic and extrinsic apoptotic pathways have been found to be implicated in CRS Type 1 pathogenesis [15]. Our data about caspase-8 levels suggest a possible involvement of the extrinsic pathway in the apoptotic process. Probably, caspase-9 levels did not differ between the two groups due to the small analyzed sample. Furthermore, in a CRS Type 2 animal model, the Angelini's group observed significantly increased levels of both pro-inflammatory cytokines and apoptosis in the heart, kidney, lung, skeletal muscle, as well as in the vascular smooth muscle [16, 17]. In particular, cellular death occurred almost exclusively in renal tubular cells while glomerular cells were spared [16, 18, 19]. Furthermore, pro-apoptotic mechanisms and activation of caspase pathway have been observed in RTCs incubated with plasma from septic patients as well [4, 20].

In the setting of endotoxemia, LPS was reported to increase the levels of Fas mRNA and Fas protein in the kidneys, producing evidence of nephrons apoptosis. In fact, TNF- α and Fas-ligand bind directly to tubular cell receptors, promoting the activation of caspase-8 and inducing apoptosis [21]. Furthermore, in recent experimental animal models the exposure to LPS was associated to a significant increase in vacuolar degeneration, renal cell apoptosis and neutrophils and macrophages infiltration [22-25]. In particular, Langford et al. observed *in vitro* multiple caspase activation in rabbit kidney cells treated with LPS, suggesting the stimulation of the extrinsic, intrinsic and endoplasmatic reticulum/stress apoptotic pathways [26]. Moreover, caspase inhibition has been shown to prevent apoptotic proximal, distal, and peri-tubular cell death in LPS animal models of septic acute kidney injury [11].

In our study, plasma pro- and anti-inflammatory cytokine levels were evaluated to examine the potential role of these mediators in the pathogenesis of CRS Type 5 in the setting of sepsis. In particular, IL-6 and IFN- γ resulted significantly higher in CRS Type 5 patients with positive EAA compared with EAA negative patients. In addition, a significant positive correlation was observed between EAA and IL-6 levels. Similarly, Wan and co-workers showed that tissue injury mechanisms in case of systemic disorders, such as sepsis, seem to be directly related to circulating mediators with both pro- and anti-inflammatory properties [27]. Furthermore, LPS has been demonstrated to cause the release of several cytokines and to interact with the complement pathway and the coagulation system. Circulating inflammatory cytokines directly affect the renal parenchyma, and some of these mediators, such as TNF- α , IL-6, CD40-ligand and Fas-ligand, can directly interact with specific counter-receptors located on tubular epithelial cells, causing loss of function and apoptosis [28]. On the other hand, tubular cells directly contribute to systemic inflammation carrying out immune functions, as well as cytokine release and leukocyte recruitment [28]. Based on these preliminary results, we may speculate that an uncontrolled and dysregulated inflammatory response to LPS release might play a role in the pathogenesis of CRS Type 5 secondary to sepsis.

Our data suggest a possible association between endotoxin and apoptosis and inflammatory mediators levels. Anyway, this study has some limitations which should be

Table 2. Differences in immune-mediated molecules in patients with EAA negative and positive

	EAA negative	EAA positive	P
IL-6 (pg/ml)	62.5, IQR 59.1-68.9	190.9, IQR 93.3-279.2	0.001
IL-10 (pg/ml)	37.8, IQR 32.4-40.8	78.9, IQR 38.6-227.9	0.589
IL-8 (ng/ml)	218.0, IQR 207.3-220.3	224.6, IQR 211.1-420.6	0.127
IL-1 β (pg/ml)	74.9, IQR 64.9-171.6	184.0, IQR 99.7-328.3	0.521
IFN- γ (pg/ml)	18.1, IQR 17.5-21.9	32.8, IQR 21.0-67.9	0.049

taken into account when interpreting the results. Indeed, it is limited by the restricted number of patients and by the *in vitro* experimental design, which was performed to simplify experimental variables and to isolate different components in order to analyze them in well-controlled and reproducible conditions. Because of these limitations, we cannot make conclusion about causality, but we could suppose only a merely association between endotoxin and apoptosis and inflammatory mediators release in CRS Type 5 patients with sepsis. Therefore, our preliminary results can be considered as hypothesis-generating, and provide a further rational for ongoing clinical studies of novel pathophysiological mechanisms in CRS Type 5 secondary to Gram-negative sepsis.

Conclusion

The pathophysiology of CRS Type 5 during sepsis is very complex, but still poorly understood. Certainly, it involves a multitude of different factors and mechanisms which are strictly interrelated and dependent on each other. In this context, our data support a high degree of pro-apoptotic activation and immune deregulation with a strong enhanced inflammation in CRS Type 5 patients, especially in those with high endotoxin activity. Anyway, understanding the underlying pathophysiology of CRS Type 5 requires further research in order to develop therapeutical approaches aimed to antagonize target mediators implicated in the pathogenesis of this clinical condition.

Disclosure Statement

No authors have reported a conflict of interest. This manuscript was seen and approved by all authors listed. It is not under consideration for publication elsewhere in a similar form, in any language, except in abstract form. All persons listed have contributed sufficiently to the project to be included as authors, and all those who are qualified to be authors are listed in the author byline.

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