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Regional Citrate Anticoagulation Limits Sepsis-Associated Tissue Injury Through The Decreased Release Of Microvesicles From Activated Leukocytes And Platelets

[1]Vincenzo Cantaluppi, [1]Federico Figliolini, [1]Davide Medica, [1]Alessandro D. Quercia, [1]Sergio Dellepiane, [1]Claudia Cavallari, [1]Ilaria Deambrosis, [2]Alfonso Pacitti, [3]Ciro Tetta, [1]Giovanni Camussi

[1]Nephrology, Dialysis and Kidney Transplantation Unit, University of Turin, Italy, [2]Nephrology and Dialysis Unit, Cuneo Hospital, Italy, [3]Fresenius Medical Care, Bad Homburg, Germany

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BACKGROUND

Sepsis represents the leading cause of acute kidney injury (AKI), often in a clinical scenario of multiple organ failures (MOF), immunoparalysis and increased mortality rate.

Recent studies suggested that during extracorporeal blood purification for sepsis-associated AKI, regional citrate anticoagulation (RCA) (Fig. 1) may inhibit the inflammatory response leading to a decrease of mortality.

Microvesicles (MVs) are small particles released from different activated cell types playing a role in cell-to-cell communication through the transfer of proteins, bioactive lipids and genetic information (mRNAs, microRNAs).

Our working hypothesis is that during sepsis, MVs may be released from activated leukocytes and platelets causing tissue injury in association with inflammatory cytokines and hypoperfusion-related hypoxia.

AIMS OF STUDY

The aims of this study were:

1) **to characterize MVs from plasma of septic patients and to correlate their concentration with outcome;**
2) **to define a potential role of plasma MVs in the mechanisms of sepsis-associated AKI and immunoparalysis;**
3) **to evaluate the clearance of plasma MVs during conventional CVVH/CVVHD and to define the role of RCA in the inhibition of MV-mediated cellular injury.**

METHODS

Plasma samples were collected from septic patients (n=20) to analyze MVs (FACS, Nanosight and RNA profiling). RIFLE/SOFA scores were calculated. CVVH or CVVHD with heparin or citrate (CiCa Multifiltrate, Fresenius Medical Care) were performed.

During *in vitro* CVVH/CVVHD, whole blood or separated leukocytes and platelets were activated by LPS and cytokines in presence or absence of heparin or citrate to evaluate MV release.

The biological effects of septic plasma MVs were also evaluated *in vitro* on cultured human kidney-derived endothelial and tubular epithelial cells or lymphocytes.

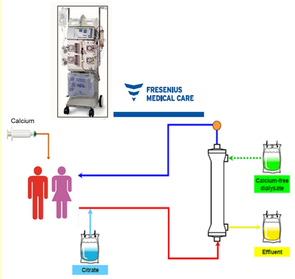

Figure 1. CRRT circuit with Regional Citrate Anticoagulation (RCA)

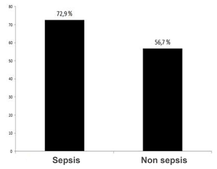

Figure 2. Mortality in septic vs. non septic patients (day 28)

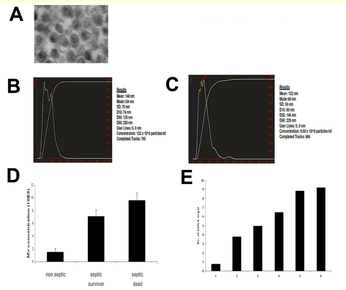

Figure 3. Electron microscopy (A) and Nanosight analysis of MVs derived from plasma of healthy (B) and septic (C) subjects. MV plasma concentration (D) and correlation with organ failures (E)

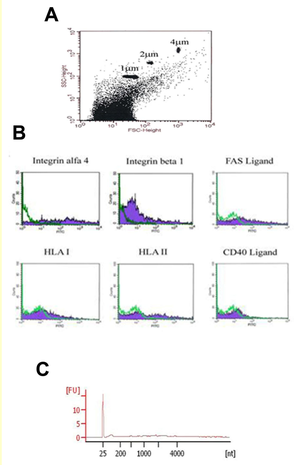

Figure 4. FACS (A, B) and bioanalyzer (C) analysis of septic plasma MVs

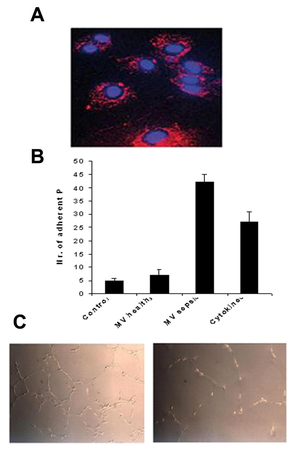

Figure 5. Internalization of septic MVs (A) and their effect on leukocyte adhesion (B) and on angiogenesis (C) in kidney-derived endothelial cells

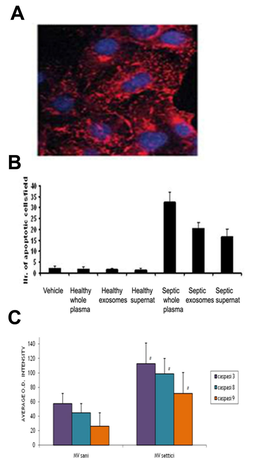

Figure 6. Internalization of septic MVs (A) and their effect on apoptosis (B) and caspase-3, -8, -9 (C) in kidney-derived tubular epithelial cells

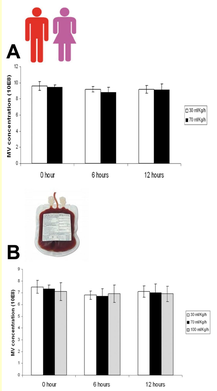

Figure 7. MV plasma concentrations during CVVH in septic patients (A) or *in vitro* using LPS-activated blood (B)

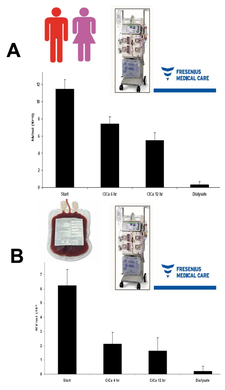

Figure 8. MV plasma concentrations during CVVHD-CiCa in septic patients (A) or *in vitro* using LPS-activated blood (B)

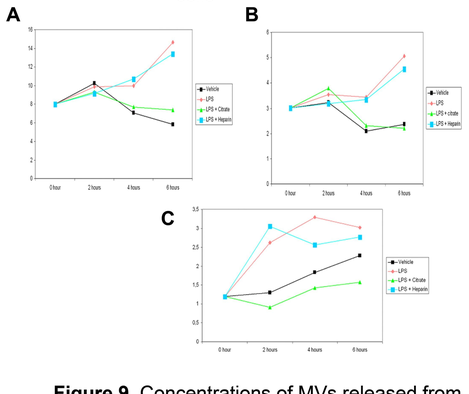

Figure 9. Concentrations of MVs released from PMNs (A), PBMCs (B) and platelets (C) activated in vitro by LPS in presence of heparin or citrate at different time points

RESULTS

We enclosed in the study 20 patients with sepsis and 10 patients admitted to ICU without sepsis. According to data collected in the whole AKI population of our hospital, at day 28 the mortality in the septic group was greater than in the non septic group ($p<0.05$) (Fig. 2). Septic patients were all included in the Failure group of RIFLE criteria with a median SOFA score of 12.6 1.4.

MVs isolated from plasma showed a spheroid shape and sized 50-150 nm, as detected by electron microscopy (Fig. 3A) and Nanosight analysis (Fig. 3B-C). Plasma MV concentration was higher in septic than healthy or non septic subjects (Fig. 3D) and correlated with mortality (Fig. 3D) and with severity of illness (Fig. 3E).

By FACS analysis, we observed that MVs from septic patients were immunologically active (class I and II HLA antigens) and expressed proteins (Fas-L/CD40-L) involved in inflammation and apoptosis (Fig. 4A-B). Moreover, MVs carried different subsets of mRNAs and microRNAs able to modulate the biological behaviour of target cells (Fig. 4C). Septic plasma MVs were internalized in kidney-derived endothelial (Fig. 5A), tubular epithelial (Fig. 6A) cells and lymphocytes (not shown) inducing functional alterations and apoptosis. In particular, in endothelial cells MVs induced an increase of leukocyte adhesion (Fig. 5B) and a decrease of angiogenesis (Fig. 5C). In tubular cells, MVs induced an increase of apoptosis through the up-regulation of caspase-3,-8,-9 (Fig. 6B-C).

Conventional CVVHD or CVVH also using a very high convective dose did not efficiently remove MVs from septic plasma and only 0.2-1.5% of MVs were found in the ultrafiltration fluid (Fig. 7A). Of interest, RCA but not heparin anticoagulation was associated with lower levels of circulating plasma MVs a few hours after the start of the procedure (Fig. 8A). Similar findings were observed in vitro during CVVH or CVVHD with LPS-activated blood (Fig. 7B and Fig. 8B). Last, citrate significantly reduced the release of MVs from cultured granulocytes (Fig. 9A), monocytes (Fig. 9B) and platelets (Fig. 9C) activated *in vitro* by LPS.

CONCLUSIONS

- During sepsis MVs are released by activated leukocytes and platelets in correlation with severity of illness and mortality.

- MVs play a potential role in the pathogenetic mechanisms of AKI and immunoparalysis after internalization into target cells.

- RCA may exert a protective effect by blocking intracellular calcium essential for MV release by activated leukocytes, platelets and endothelial cells

- RCA may contribute to limit inflammation, mitochondrial dysfunction and apoptotic cell death during sepsis-associated AKI and immunoparalysis.