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Infection, Oxidative Stress and Changes in Circulating Regulatory T cells of Heart Failure Patients Supported by Continuous-Flow Ventricular Assist Devices

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Abstract

The objective of this study was to investigate the changes in oxidative stress (OS) and circulating regulatory T cells (Tregs) of the immune system in CF-VAD patients with or without infection. We recruited 16 CF-VAD patients (5 with infection and 11 without infection) and 7 healthy volunteers. Generation of reactive oxygen species (ROS) from lymphocytes, superoxide dismutase (SOD) in erythrocyte, total antioxidant capacity (TAC) and oxidized low density lipoprotein (oxLDL) in plasma were measured. Circulating Tregs were evaluated by flow cytometry. HF patients had elevated OS than healthy volunteers as evident from higher lymphocyte ROS, elevated oxLDL as well as depleted SOD and TAC levels. At baseline, HF patients had decreased % of Tregs (5.12 ± 1.5 vs. $8.14 \pm 3.01\%$, $p < 0.01$) when compared to healthy volunteers. Post-implant patients with infection illustrated 35% and 44% rise in ROS and oxLDL respectively, 31% decrease in TAC and marked rise in % of Tregs (14.27 ± 3.17 vs. $9.38 \pm 3.41\%$, $p < 0.01$) when compared to the patients without infection. Elevated OS and rise in Tregs were more prominent in CF-VAD patients with infection. In conclusion, OS and compromised immune system may be important indicators of systemic response of the body to CF-VAD among HF patients with infection.

Keywords

Heart failure; left ventricular assist devices; Infection; oxidative stress; Regulatory lymphocytes; weekend immune system

Introduction

The continued development of ventricular assist devices (VADs) has played a pivotal role in the current treatment of patients with end-stage heart failure (HF). The success of the VAD therapy is attributed to the emergence of continuous-flow VADs (CF-VADs), which are

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small in size, low weight and very durable. Superior clinical outcomes with use of these CF-VADs have been demonstrated in comparison with earlier pulsatile VADs. As studies continue to demonstrate the benefits and improving survival outcomes of CF-VAD implantation worldwide, efforts to reduce the associated complications are needed.¹ Infection remains a serious complication associated with significantly increased morbidity and mortality; and is the 2nd most common cause of death in HF patients with CF-VAD support.²

Since a CF-VAD is a foreign body, circulating blood is continuously experiencing non-physiological high shear stresses and in direct contact with biomaterials of device. These support devices depend upon a driveline exiting the body percutaneously to connect to a power source. Thus, the incidence of infectious complications remains substantial. These infections can be life threatening and can limit access to heart transplantation.^{3,4} Over the past two decades, significant evidence has suggested a close relation between pathophysiology of HF and increased oxidative stress, both systemically and in pericardial fluid.⁵⁻⁷ In this study, we examined generation of reactive oxygen species (ROS) from lymphocytes, superoxide dismutase (SOD) in erythrocyte, total antioxidant capacity (TAC), oxidized low density lipoprotein (oxLDL) and circulating regulatory T lymphocytes (Tregs) in serially collected blood samples from patients supported with CF-VAD and their link with infection.

Materials and Methods

Subjects

We studied 16 HF patients with NYHA class IV undergoing CF-VAD implantation as bridge to transplant or destination therapy and 7 healthy volunteers as the reference group. Among the 16 patients, 5 patients developed infection due to bacteremia within the first month after CF-VAD implantation. The infections were confirmed by the identification of *Pseudomonas*, *Staphylococcus*, *Clostridium* or other Gram positive bacteria at mediastinum, bloodstream, gastrointestinal tract, pulmonary or driveline of CF-VAD patients. None of the patients included in this study were treated with either extracorporeal life support, *continuous renal replacement therapy* or other possible causes of inflammation at the time of sample collection. The other 11 patients were free of any infection during that time frame. All procedures involving collection of human blood were approved by the Institutional Review Boards (IRB). All patients and volunteers gave their written informed consent and were informed about the aims of the study in accordance with the Declaration of Helsinki.

Inclusions and exclusions criteria

The inclusion criteria for the selection of HF patients were: (i) ages between 18 to 70 years, (ii) undergoing CF-VAD implantation and (iii) able to provide informed consent for the study. The inclusion criteria for the control subjects were (i) no clinical evidence of HF or other cardiovascular diseases, (ii) no history of malignancy and (iii) no inflammatory disease on careful examination and routine laboratory tests. Pregnant or breastfeeding women or women using oral contraceptives were excluded from the study.

Collection and preparation of blood sample

Blood samples (EDTA-anticoagulated) were collected from all the HF patients at baseline prior to CF-VAD implantation (as pre-implant sample) and after one month of device support (as post-implant sample) for the measurement of oxidative stress and regulatory T lymphocytes in the circulation. Patients who developed infection within one month were included in the infection group in the current study. Blood samples from the healthy donors were collected once. All the blood samples from the HF patients and the healthy volunteers were aliquoted and processed immediately according to the study protocol.

Flow cytometric measurement of ROS generation in blood lymphocytes

Generation of ROS was measured in blood lymphocytes by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCF-DA) following the procedure of Rothe and Valet (1990) with slight modification.⁸ In brief, an aliquot of blood was centrifuged at 200×g at 4°C for 10 min and the buffy coat containing leukocytes and supernatant plasma were collected separately. An aliquot of 200 µl buffy coat was mixed with 300 µl of freshly collected plasma. Then 10 µl of the mixture was diluted with 1 ml of HBSS containing 0.15 M NaCl and 5 mM HEPES, pH7.35. Thereafter, 20 µl of 0.5 mM DCF-DA solution in dimethyl formamide was added to the cell suspension and incubated at 37°C for 30 min in darkness. After washing in ice-cold PBS, 10,000 events were acquired immediately in a flow cytometer using Cell Quest software (BD Bioscience, San Jose, CA). The lymphocytes were gated on the basis of their characteristic forward and side scatters on dot plot. Generation of ROS by the lymphocytes resulted in green fluorescence that was recorded in fluorescence channel-1 and was expressed as mean fluorescence intensity (MFI) in arbitrary unit.

Assessment of antioxidant status

The activity of the antioxidant enzyme SOD was assayed in blood erythrocyte lysate spectrophotometrically using a commercially available kit (Cell Technology Inc. Mountain View, CA, USA) following the manufacturer's instruction. The absorbance (OD) was measured at 450 nm using the SpectraMax® M3 multi-mode microplate reader (Molecular Devices, California, USA). The SOD activity was calculated using SoftMax Pro software (Molecular Devices, California, USA) and expressed as units per milliliter (U/mL). TAC in blood plasma was also measured using a commercially available kit (Randox Laboratories, Antrim, UK) according to the manufacturer's instruction. The OD was measured at 600 nm and the result was expressed as mmol/L.

Assessment of oxidized low density lipoprotein (oxLDL)

The concentration of oxLDL in plasma was measured by ELISA using a commercially available kit (MercodiaInc, Winston Salem, NC) following the manufacturer's instruction. Each sample was assayed in duplicate. The lowest detection limit of the kit was 1.0 mU/L.

Flow cytometric analysis of Treg cells

To identify the percentage of Tregs in peripheral circulation, the flow cytometric procedure of Fujimoto et al. was followed with slight modification.⁹ In brief, a 25 µl aliquot of whole blood was diluted with 75 µl of phosphate buffered saline (PBS, pH 7.4) and the diluted

blood samples were incubated with 10 μ l each of FITC anti-human CD4 and PE anti-human CD25 monoclonal antibodies (BioLegend, San Diego, CA, USA) and isotype-matched negative controls for 30 min in the dark at room temperature. The erythrocytes were then lysed by incubating the samples with 2 ml of RBC lysing solution (BD biosciences, CA, USA) for 5 min at room temperature. Thereafter, the cells were fixed with 0.5% paraformaldehyde and 15,000 events were acquired and analyzed in a flow cytometer (FACS Calibur with sorter, BD biosciences, San Jose, CA, USA). Cells were identified from their characteristic forward and side scatter profile on dot plots and gated. Data acquisition and analysis of FL-1 (FITC) and FL-2 (PE) were done using Cell Quest software (BD biosciences, USA). The relative proportion of Tregs was calculated from the statistical package of the Cell Quest software from quadrant gate setting.

Data analyses

The data are presented as mean $\pm$ SD (standard deviation) or median with *interquartile* range (IQR) and statistically analyzed using the SPSS statistical software (Statistical Package for Social Sciences for windows, release 18.0; SPSS Inc., Chicago, IL, USA). Statistical differences were determined by using Chi-square test, Student's t-test and Mann-Whitney *U* test as applicable. Statistical significance was assigned at $P < 0.05$.

Results

Demography and baseline characteristics

Among the 16 HF patients, 5 experienced the challenge of infection within one month after CF-VAD support. Comparative analyses of demographic and clinical characteristics of the patients with infection and those who did not experience infection ($n=11$) prior to CF-VAD implantation were summarized in Table 1.

Severity of oxidative stress

The flow cytometric analysis of lymphocyte ROS generation in terms of MFI of DCF-DA indicated that ROS generation was significantly higher in all the HF patients prior to CF-VAD implantation when compared to that of the healthy volunteer (387.9 ± 101.8 vs. 170.7 ± 31.6 , $p < 0.0001$ in Student's *t*-test). On the other hand, significantly lower levels of antioxidant enzyme SOD (202.4 ± 23.0 vs. 384.1 ± 7.1 , $p < 0.0001$) and total antioxidant capacity (1.1 ± 0.1 vs. 1.5 ± 0.2 , $p < 0.0001$) were noticed in the baseline samples of all the HF patients compared to that in the healthy volunteers. These findings suggest oxidative stress is a preexisting condition among all the HF patients. Further, we also compared the severity of oxidative stress in those patients with infection and without infection after CF-VAD implantation (Fig. 1). There was no significant difference in lymphocyte ROS ($p=0.89$) between pre- and post-VAD blood samples in the patients without infection. The VAD patients with infection showed significantly higher lymphocyte ROS ($p=0.04$) in the post VAD blood compared to that in the pre-VAD blood (Fig. 1A). Post-VAD severity of ROS generation was found to be 35% higher in the patients with infection than the patients without infection ($p=0.03$). While looking at the levels of antioxidant enzyme SOD and TAC, both the groups showed significant reduction after CF-VAD implantation when compared to the baseline levels (Fig. 1B,C). The TAC was found to be 31% reduced in the

patients with infection compared to the patients without infection ($p=0.01$) (Fig. 1C). Higher levels of oxLDL, an important biomarker of oxidative stress, was also observed in the HF patients compared to the healthy controls (95.6 ± 19.8 vs. 44.6 ± 7.6 , $p<0.0001$) even before VAD implantation. The levels of oxLDL in all the patients increased significantly after CF-VAD implantation when compared to their corresponding baseline values. Similar to ROS production, higher levels of oxLDL was also noticed in the post-implant blood of the patients with infection than the patients without infection ($p=0.002$) (Fig. 1D). Taken together, the severity of oxidative stress was more pronounced in those CF-VAD patients with infection compared to the CF-VAD patients without infection.

Abundance of regulatory T lymphocytes

At baseline, as evident from flow cytometry analysis, the HF patients had a significantly lower percentage of Tregs when compared to the healthy volunteers (Fig. 2A). Interestingly, the percentage of Tregs in patients with and without infection showed a significant increase after CF-VAD implantation when compared to their corresponding baseline values (Fig. 2B). Moreover, the patients with infection had a 1.8-fold rise in % of Tregs when compared to the patients without infection ($p=0.03$).

Discussion

The present study found that elevation of lymphocyte ROS, oxLDL and depletion of SOD and TAC were a pre-existing condition among all the HF patients prior to CF-VAD implantation compared to the healthy volunteers – indicating oxidative stress. Levels of TAC and SOD were lower in all the 16 HF patients after CF-VAD implantation. Moreover, TAC was lowered in the patients with infection than those without infection after CF-VAD implantation. Elevated oxidative stress and rise in circulatory Tregs were two common observable phenomena among the HF patients who experienced infection after CF-VAD implantation when compared to the patients without infection. Previous reports indicated that oxidative stress is a common noticeable observation in patients with severe HF conditions.¹⁰ Studies on animal models revealed that the development of HF is accompanied by changes in the antioxidant defense mechanisms of the myocardium as well as elevated myocardial injury due to oxidative stress. Thus, the balance between oxidant and antioxidant status regulates intracellular redox phenomenon, and their imbalance causes oxidative stress, leading to cellular damage in cardiovascular systems.¹¹ Increased oxidative stress and reduction in antioxidant enzymes SOD have been suggested to be involved in the pathophysiology of congestive heart failure.¹² The status of oxidative stress cannot be explained only by investigating antioxidant enzyme SOD; as oxidative stress indicates an imbalance between antioxidant defenses (SOD, TAC etc.) and the production of ROS, which at high levels cause cell damage but at lower levels induce subtle changes in intracellular signaling pathways. We also noticed elevated ROS & oxLDL and concomitant depletion of antioxidant enzyme SOD and TAC among the 16 HF patients prior to CF-VAD implantation, suggesting that the body's antioxidant capacity might not be strong enough to minimize the deleterious effect of ROS. This imbalance may impose severity of oxidative stress as a preexisting condition. Earlier study reported that infections are likely associated with oxidative stress in a number of ways.¹³ In our study we found the severity of oxidative stress

was more prominent among the CF-VAD patients with infection indicating the active involvement of oxidative stress in this process.

Moreover, the role of redox regulation of T-lymphocyte function cannot be underestimated. The cellular redox state of a T cell can also upset T-cell receptor signaling, tilting the immune response.¹⁴ Low concentrations of ROS in T-lymphocyte are a prerequisite for cell survival, but elevated ROS can lead to various pathophysiological conditions including immune suppression. Animal studies indicated that oxidative stress inhibits the development of immune organs and finally impaired the immune function.¹⁵ Our study revealed that the HF patients had decreased % of Tregs when compared to the healthy volunteers. But interestingly, after CF-VAD implantation, we noticed a significant increase of circulating Tregs in all the HF patients when compared to their corresponding baseline values. Moreover, the number of Tregs was found to be much higher in those patients who had infection in comparison to the patients without infection after CF-VAD implantation. Earlier studies indicating that both innate and adaptive immune responses play a role in HF.^{16–18} Different subsets of lymphocytes and the cytokines, produced by the immune system, participate in the pathogenesis of cardiovascular disease. However, Tregs play an essential role in the modulation of immune responses and the control of potentially harmful immune activations due to their immunoregulatory and immunosuppressive characteristics.^{19,20} Among the several types of T cell subsets with suppressive functions reported till now, naturally occurring Tregs are the most important, because accumulating evidence points towards their crucial role in the maintenance of immunological self-tolerance and negative control of pathological as well as physiological immune responses.^{21,22}

The decreased % of circulatory Tregs among the HF patients prior to CF-VAD implantation may be understandable as the mounting evidence is now pointing towards a key role played by Tregs in chronic HF. Previous experiments have conclusively revealed the defects of Tregs may be an underlying mechanism of immune activation in HF patients.^{23,24} Multiple other studies on infection have demonstrated that, while the presence of Tregs does not contribute to the pathogen's lethality, depletion of Tregs leads to increased effector responses, supporting pathogen clearance. The exact mechanism of how the elevated Tregs in combination with oxidative stress promote infection in CF-VAD patients still needs further attention. In our study, elevated Tregs might be responsible for the suppression of effector cells, preventing the immune system from mounting an effective immune response, which could lead to pathogen persistence.

Shin et al., 2012 showed that progressive failure of the systemic immune function allows life-threatening opportunistic infections and may be characterized by increased *oxidative stress*.²⁵ The role of oxidative stress in the pathogenesis of *infections* and weakened immune system are the area of research that has recently gained momentum. So it is reasonable to assume that presence of a driveline, long-term exposure to high shear stress flow environment as well as artificial blood contacting materials in CF-VADs may have an additive role in accelerating oxidative stress and weakened immune system and infection.

In conclusion, oxidative stress and compromised immune system may be important indicators of systemic response of the body to CF-VAD among HF patients with infection.

Limitation

We acknowledge that there are some limitations in this prospective observational study. The sample size was relatively small. Not all CF-VAD patients were enrolled for this study. Medication (aceI/ARBs, aldosterone antagonist, statins or BB blockers) might have some impact on oxidative stress status. Recent changes in medications should be addressed in further studies to rule out drug-drug interactions or a new side effect. A larger cohort of CF-VAD patients with infection and other associated clinical complications is needed to confirm our initial findings. It may be further important to investigate serial assessment of Tregs to identify temporal changes in those patients developed infection after CF-VAD implantation. This may ascertain if higher Tregs predict the possibility of the infection or if their activation is due to the attempt to self regulate the immunological response.

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Figure 1.

Box-whisker plot showing differences in generation of reactive oxygen species (ROS) from leukocytes (A), superoxide dismutase (SOD) in erythrocyte (B), total antioxidant capacity (TAC) (C) and levels of oxidized low-density lipoprotein (oxLDL) (D) before and after CF-VAD implantation in patients with infection and without infection groups. The lines across each box plot represent the median value. The lines that extend from the top and the bottom of each box represent the lowest and highest observations still inside the lower and upper limit of confidence. Dotted lines represent the interquartile range of measured parameters in healthy volunteers. * $P < 0.05$ is considered significant in Mann-Whitney U -test.

Figure 2.

Box-whisker plot showing differences in percentages of circulating Treg cells between healthy volunteers and heart failure patients (A). Pre- and post-VAD comparison of Treg cells among HF patients with infection and without infection (B). The lines across each box plot represent the median value. The lines that extend from the top and the bottom of each box represent the lowest and highest observations still inside the lower and upper limit of confidence. * $P < 0.05$ is considered significant in Mann–Whitney U -test.

Table 1

Demographic and baseline clinical characteristics of HF patients prior to CF-VAD implantation.

Characteristics	Pre-implant HF patients (n = 16)	
	Without Infection (n = 11)	With Infection (n = 5)
Demography		
Age in years, median (IQR)	60 (48 – 71)	66 (61 – 70)
Sex, n (% male)	11 (100 %)	4 (80.0 %)
Race		
Caucasian white, n (%)	5 (45.5 %)	3 (60.0 %)
Black, n (%)	6 (54.5 %)	2 (40.0 %)
Height in meter, median (IQR)	1.8 (1.7 – 1.9)	1.7 (1.6 – 1.8)
Weight in kilograms, median (IQR)	107.6 (80.3 – 127.9)	80.5 (68.9 – 106.5)
Body mass index (kg/m ²), median (IQR)	31.7 (24.8 – 38.2)	31.2 (23.0 – 31.8)
Body surface area (m ²), median (IQR)	2.4 (1.9 – 2.5)	1.9 (1.8 – 2.3)
History of smoking, n (%)	3 (27.3 %)	1 (20.0 %)
History of substance abuse		
Ethyl alcohol abuse, n (%)	2 (18.2 %)	1 (20.0 %)
Drug abuse, n (%)	4 (36.4 %)	2 (40.0 %)
Vital signs		
Systolic blood pressure (mmHg), mean $\pm$ SD	101.4 $\pm$ 7.4	91.5 $\pm$ 4.9
Diastolic blood pressure (mmHg), mean $\pm$ SD	57.2 $\pm$ 6.7	62.5 $\pm$ 3.5
Etiology of heart disease		
Ischemic cardiomyopathy, n (%)	4 (36.4 %)	2 (40.0 %)
Non-ischemic cardiomyopathy, n (%)	4 (36.4 %)	2 (40.0 %)
Idiopathic Cardiomyopathy, n (%)	3 (27.3 %)	1 (20.0 %)
Echocardiographic parameters		
Left ventricular end diastolic diameter (mm), mean $\pm$ SD	62.6 $\pm$ 3.7	64.0 $\pm$ 13.2
Left ventricular ejection fraction, (%), mean $\pm$ SD	14.3 $\pm$ 3.5	16.8 $\pm$ 6.3

Demographic and clinical parameters of without infection versus with infection groups of HF patients were statistically compared by Mann-Whitney 'U' test (for median values with IQR), χ^2 -test (for results presented as percentages) and Student's t-test (for results presented as mean $\pm$ SD) as applicable, $p < 0.05$ were considered significant. There were no statistically discernible differences between any of the demographic parameters for without infection and with infection groups.