Oxidative Medicine and Cellular Longevity

Oxidative Stress in the Critically Ill Patients: Pathophysiology and Potential Interventions

Lead Guest Editor: Demosthenes Makris
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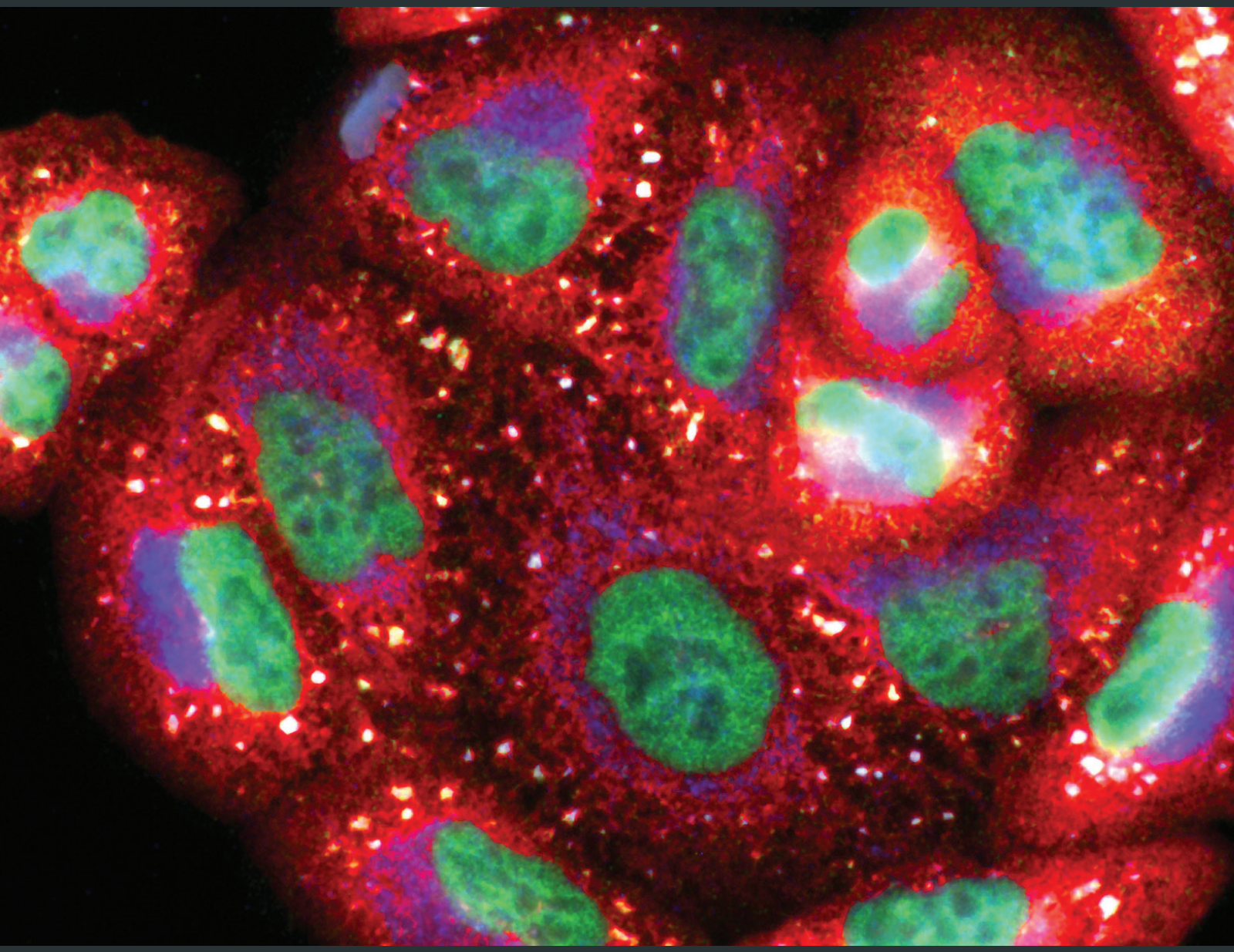

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Editorial Board

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Editorial

Editorial: Oxidative Stress in the Critically Ill Patients: Pathophysiology and Potential Interventions

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Oxidative stress constitutes a mechanism of injury seen in many disease processes [1]. Increased production of reactive oxygen and nitrogen species can induce the activation of inflammatory mediators and modification of proteins, lipids, and nucleic acids, thus contributing to cellular injury and dysfunction [2]. In this respect, oxidative stress can be associated with the dysfunction of major organs and systems and might be important in the outcome of critically ill patients. Notably, clinical data have shown that sepsis survivors had a greater antioxidant potential than nonsurvivors [3].

An increased oxidative burden can affect several cellular components. A potentially notable impact in critical illness is the alteration of mitochondrial function which may be pivotal in states of increased oxygen demand or hypoperfusion, that is, shock/sepsis. Indeed, there is evidence suggesting that the severity of mitochondrial dysfunction following increased oxidative stress correlates with the severity of sepsis and has been associated with adverse outcomes in septic patients [3]. The pathogenesis of mitochondrial dysfunction is rather complex. Enhanced production of reactive oxygen species may lead both to mitochondrial dysfunction and damage by the initiation of apoptotic events via the caspase pathway while nitrogen oxide metabolic products may exhibit inhibitory actions in the mitochondrial respiratory chain by interrupting ATP production reactive nitrogen leading to further mitochondrial dysfunction [4]. Thus, impaired O_2 utilization from dysfunctional mitochondria may represent the generator of fundamental abnormalities in critical illness.

Those abnormalities at the cellular level may have significant implications in the function of organs which are frequently impaired in critical care patients such as the heart or the kidneys. Increased oxidative stress can induce ultrastructural and functional changes in cardiomyocytes that may result in altered electrical properties and compromised contractility. Intriguingly, experimental treatment with antioxidant vitamins alleviated both the systemic and myocardial inflammatory cytokine responses and decreased myocardial apoptosis [5]. On the other hand, acute or chronic kidney disease represents extraordinary states of oxidative stress which may be further exaggerated by diagnostic and therapeutic challenges which are common scenarios in critical care such as iron infusions, contrast medium application, dialysis filter exposure, artificial nutrition, and the loss of antioxidants during renal replacement therapy. Deranged kidney function directly translates into skewed adjustments in electrolyte and body water homeostasis, acid-base composition,

calcium-phosphate metabolism, and erythropoiesis further resulting in accelerated atherosclerosis and disproportionally increased cardiovascular burden [6, 7].

This special issue may stimulate our effort to thoroughly understand the underlying mechanisms of oxidative stress and to reveal its impact on organ functions and systemic homeostasis. The increasing knowledge of its pathogenesis and impact on several aspects of critical illness may provide further insight in our perception of the development and management of critical illness and in the evaluation of novel concepts of treatment at the clinical level. As always, this constitutes a work in progress with exciting prospects.

Demosthenes Makris
Peter R. Mertens
Evangelia Dounousi
Gregory Giamouzis
Saad Nseir

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Research Article

Noninvasive Real-Time Characterization of Renal Clearance Kinetics in Diabetic Mice after Receiving Danshensu Treatment

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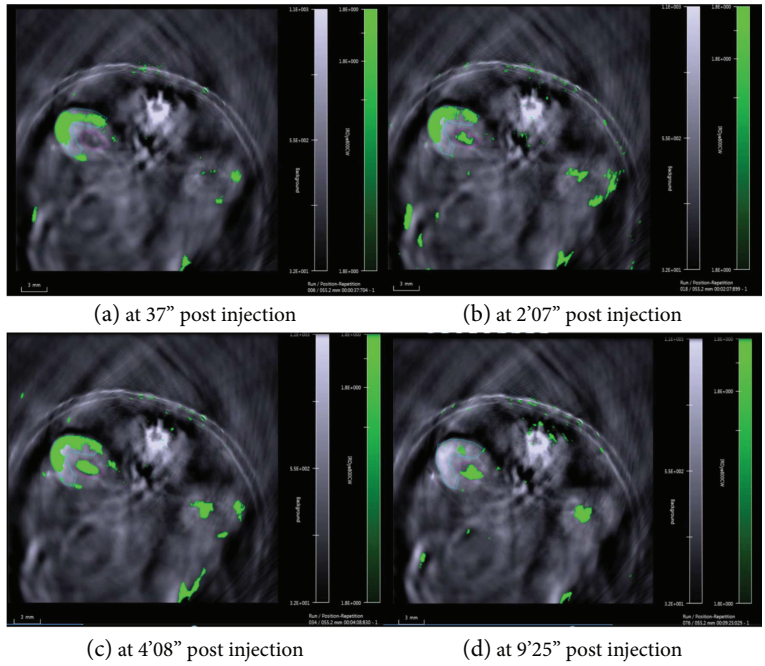
Danshensu (DSS) is an active ingredient extracted from the root of the Danshen that could ameliorate oxidative stress via upregulation of heme oxygenase- (HO-) 1. Little is known about the treatment effects of DSS on kidney function in diabetic mice. Therefore, the primary aim of the present study was to characterize the renal clearance kinetics of IRdye800CW in *db*/*db* mice after DSS treatment. The secondary aim was to measure several biomarkers of renal function and oxidative stress (urinary F2-isoprostane, HO-1 in kidney and serum bilirubin). Fourteen *db*/*db* diabetic mice were randomly assigned into two groups and received either DSS treatment (DM + DSS) or vehicle treatment (DM). A third group that comprised of *db*/+ nondiabetic mice (non-DM control) received no DSS treatment and served as the nondiabetic control. At the end of a 3-week intervention period, serum and urinary biomarkers of renal function and oxidative stress were assessed and the renal clearance of IRdye800CW dye in all mice was determined noninvasively using Multispectral Optoacoustic Tomography. The major finding from this study suggested that DSS treatment in *db*/*db* mice improved renal clearance. Increased expression of HO-1 after DSS treatment also suggested that DSS might represent a potential therapeutic avenue for clinical intervention in diabetic nephropathy.

1. Introduction

The hyperglycemic and hyperinsulinemic conditions in diabetes are major risk factors promoting lipid peroxidation [1–3] and impair kidney function [4–6]. Growing evidence indicates that heme oxygenase- (HO-) 1 and unconjugated bilirubin are potent antioxidants with therapeutic potential in diabetes [7–9]. Many bioactive compounds extracted from natural medicinal herbs/fruits, including Danshensu (DSS) and Paeonol, may hold beneficial antioxidant and anti-apoptotic effects, mediated via activation of factor-erythroid 2-related factor 2 (Nrf2)/HO-1 signaling [10]. DSS, an active ingredient extracted from the root of the Danshen (*Salvia miltiorrhiza*), has been used for the treatment of cardiovascular disease [11, 12]. Also, the renoprotective effect of DSS has previously been linked with the suppression of oxidative stress [13], inflammation, and fibrosis [14], in addition to a reduction in lipid peroxidation by scavenging free radicals and preventing thiol oxidation [15, 16]. Moreover, the combined prescription of DSS with *Rheum rhabarbarum* is a well-recognized, effective, and safe traditional Chinese medicinal regimen for treating chronic kidney disease [17] and suppressing oxidative stress [18, 19].

Insulin glomerular filtration rate currently represents the gold standard assessment method of renal function. However, with recent advances in photoacoustic imaging, assessment of renal function in small animals (including the assessment of IRdye800CW renal clearance) can now be determined noninvasively using Multispectral Optoacoustic Tomography (MSOT). MSOT is an emerging technique that captures photoacoustic signals from chromophoric spectra or molecules that are distributed within tissues [20]. With the development of new imaging probes [21], photoacoustic imaging has now been applied to visualize the anatomy,



(a) at 37" post injection (b) at 2'07" post injection

(c) at 4'08" post injection (d) at 9'25" post injection

Figure 1: The transition of IRdye800CW peak signal intensity (from the renal cortex and renal pelvis) in the right kidney over time in a *db*/*db* mouse with no DSS treatment.

function, and blood oxygenation in different organs [22, 23]. Yet, the assessment of DSS on renal clearance kinetics in a diabetic mice model has not been investigated to date.

Inadequate HO-1 expression has been demonstrated in obese diabetic mice [24], and the systemic induction of HO-1 can improve insulin sensitivity, decrease inflammatory cytokine expression, and increase circulating adiponectin [25, 26]. Also, the induction of HO-1 within renal structures normalized blood pressure, protected against oxidative injury, and consequently improved renal function in spontaneously hypertensive rats [27]. Bilirubin is generally considered as the by-product of heme catabolism. However, new evidence suggests that it may also possess physiological significance. Despite the uncertainty of its physiological importance, unconjugated bilirubin has demonstrated potent antioxidant capacity in vitro and ex vivo [28, 29]. An argument for a physiological role of bilirubin is further supported by reduced bilirubin concentrations in patients who had chronic kidney disease [30]. Similarly, individuals with elevated serum bilirubin have decreased prevalence of kidney complications in diabetes [9]. These findings, therefore, support that HO-1 and bilirubin might protect the kidney from oxidative stress by acting as an antioxidant [31–33]. The abrogation of Nrf2/HO-1-dependent signaling cascade has been largely implicated in chronic/acute kidney injury, cardiac/endothelial dysfunction, and cerebral ischemia [34]. Many researchers have demonstrated that DSS-mediated tissue protection against chronic kidney disease occurs via cytoprotective and prosurvival Nrf2/HO-1 and PI3K/Akt signaling pathways [10, 35]. However, whether overexpression of HO-1 is implicated in the DSS treatment effect in diabetic renal function remains unknown. In this regard, the present study aimed to (1) characterize the renal clearance kinetics of IRdye800CW dye in *db*/*db* mice after DSS treatment and (2) quantify the expression of several biomarkers for renal function and oxidative stress in *db*/*db* mice with and without DSS treatment.

2. Materials and Methods

2.1. Animals and Intervention. Female 10 wk old diabetic homozygous (*db*/*db*) mice and nondiabetic heterozygous (*db*/*+*) mice on a C57BLKS/J background were housed in the Central Animal facilities, Hong Kong Polytechnic University, in a 12 h light/dark cycle and under tight control of temperature and humidity. The *db*/*db* homozygotes exhibit persistent hyperphagia and obesity with spontaneously developed elevated leptin, glucose, and insulin concentrations [36]. All mice received regular laboratory chow and tap water ad libitum during the study. After 1 week of acclimation, all diabetic mice were randomly divided into two groups ($n = 7$/group): DM and DM + DSS, while heterozygote nondiabetic mice ($n = 6$) were assigned to a non-DM control group. During the intervention period of 3 weeks, all mice were treated according to the following schedule: the non-DM control group received no treatment, the DM group received i.p. vehicle treatment while the DM + DSS group received DSS (HPLC ≥ 98%, dissolved in water, Nanjing Zelang Pharmaceutical Technology Co. Ltd.) at a dose of 10 mg/kg i.p. daily. The kidney absorption level of DSS was found to be at around 69 μg/g of tissue via i.p. method [37]. Experimental protocols were performed in accordance with the approved license granted under the Department of Health and approved by the Animal Subjects Ethics Sub-Committee (ASESC) of Hong Kong Polytechnic University.

Table 1: A summary of all measured variables collected from serum, urine, and MSOT in the present study.

		Non-DM control	DM	DM + DSS
Baseline measurement				
Obesity	Body weight (g)	20.8 ± 0.69	51.79 ± 4.03*	50.21 ± 3.08*
Serum	Fasting glucose (mmol/l)	7.58 ± 0.60	23.83 ± 5.10*	19.60 ± 3.58*
After 3 weeks				
Obesity	Body weight (g)	18.78 ± 1.21	56.52 ± 6.07*	57.09 ± 4.03*
Serum	Fasting glucose (mmol/l)	8.17 ± 1.36	24.24 ± 6.67*	24.97 ± 6.32*
	Creatinine (μmol/l)	9.79 ± 1.37	14.90 ± 3.79*	14.80 ± 3.10*
	Total bilirubin (μmol/l)	1.40 ± 0.21	1.50 ± 0.51	1.69 ± 0.32
	Conjugated bilirubin (μmol/l)	0.35 ± 0.10	0.43 ± 0.11	0.41 ± 0.16
	Unconjugated bilirubin (μmol/l)	1.02 ± 0.23	1.10 ± 0.58	1.33 ± 0.37
Urine	F2-isoprostane (ng/mg of creatinine)	15.82 ± 8.73#	24.46 ± 6.59	18.00 ± 6.39
	Urinary albumin : creatinine ratio (mg/g of creatinine)	33.5 ± 14.86	115.7 ± 41.54*	118.2 ± 47.31*
MSOT	Tmax delay (s)	90.21 ± 14.93	142.97 ± 13.96*	81.96 ± 19.51

All data presented as mean ± SD. * denotes $p < 0.05$ when this group is compared to the non-DM control group. # denotes $p < 0.05$ when this group is compared to the DM group.

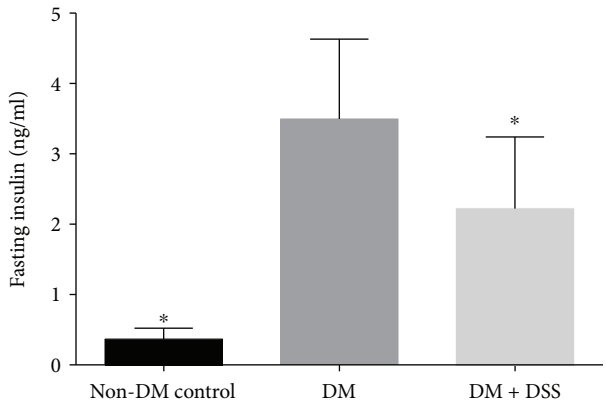


Figure 2: The fasting insulin level of mice at the end of the 3-week intervention period ($n = 6 - 7$/group). * denotes $p < 0.05$ when compared to the DM group.

2.2. Fasting Glucose, Body Weight, and Urinary Samples. At the start and the end of the study, fasting blood glucose was assessed using a glucometer (Bayer Contour TS), and the body weight of mice was assessed using an electronic scale. Daily urinary samples were collected, for four days before the end of the study using individual metabolic cages for the determination of F2-isoprostane (IsoPs), microalbumin, and creatinine excretion of each mouse. The 24 hr urinary concentration of IsoPs was determined by commercial ELISA method (item number 516351, Cayman Chemical, Ann Arbor, Michigan, USA), while the levels of urinary albumin and creatinine were determined using a clinical chemical analyzer (AU480; Beckman Coulter, Brea, CA, USA).

2.3. Serum and Kidney Samples. After overnight fasting, the mice were sacrificed and blood samples were collected via cardiac puncture at the end of the study. Serum concentrations of creatinine and bilirubin (total, conjugated, and unconjugated) were assessed using clinical chemistry (AU480; Beckman Coulter, Brea, CA, USA). The concentration of fasting serum insulin was assayed by commercial ELISA method (catalogue number 32270; Li Ka Shing Faculty of Medicine, the University of Hong Kong, Hong Kong). The cortex of the kidney was carefully dissected for the analysis of HO-1, p-Akt, and t-Akt expression using western blot. The total protein concentration was determined using a Bio-Rad Protein Assay Kit II (Bio-rad, catalog number 500-0002). The blots were incubated with primary antibodies overnight, including HO-1 Antibody (Cell Signaling Technology, Beverly, MA, USA), pan-Akt (Cell Signaling Technology, Beverly, MA, USA), and Phospho-Akt^{Thr308} (Cell Signaling Technology, Beverly, MA, USA). After washing, blots were incubated with horseradish peroxidase- (HRP-) conjugated secondary antibody (Santa Cruz Biotechnology). Finally, protein expression was determined by a microplate reader (Bio-Rad Laboratories, Richmond, CA) and quantified using ImageJ software (IJ 1.46r).

2.4. Measurement of Renal Clearance of IRdye800CW Dye Using MSOT. On the last day of the intervention period, all mice were anesthetized using isoflurane in oxygen [3-4% per liter of 100% oxygen for induction and 1.5% per liter of 100% oxygen during maintenance], with hair removed from the chest to lower abdomen as per previously published experimental protocols [38]. In brief, mice were put into a water chamber within the MSOT (inVision 128 MSOT imaging system, iThera Medical, Munich, Germany) in a prone position, and the kidney region was then scanned at a rate of 10 Hz continuously using a multispectral protocol for 10 minutes after injection of 200 μl (20 nmol in 0.9% of saline) of IRdye800CW (LI-COR, USA) via the tail vein (Figure 1). IRdye800CW is a small molecule that is rapidly excreted by the kidneys in unmetabolised form [39]. After multispectral decomposition of IRdye800CW signals over the anatomical background, the time points at the mean of peak signal

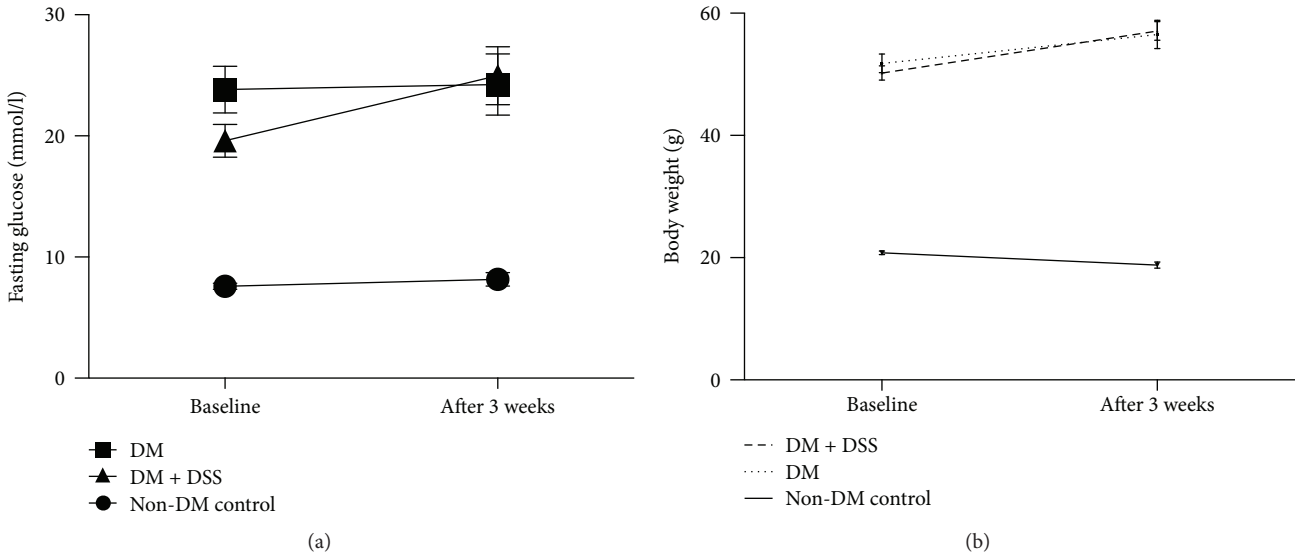


Figure 3: Changes in fasting blood glucose concentration (a) and body weight (b) of mice before and after 3 weeks of intervention.

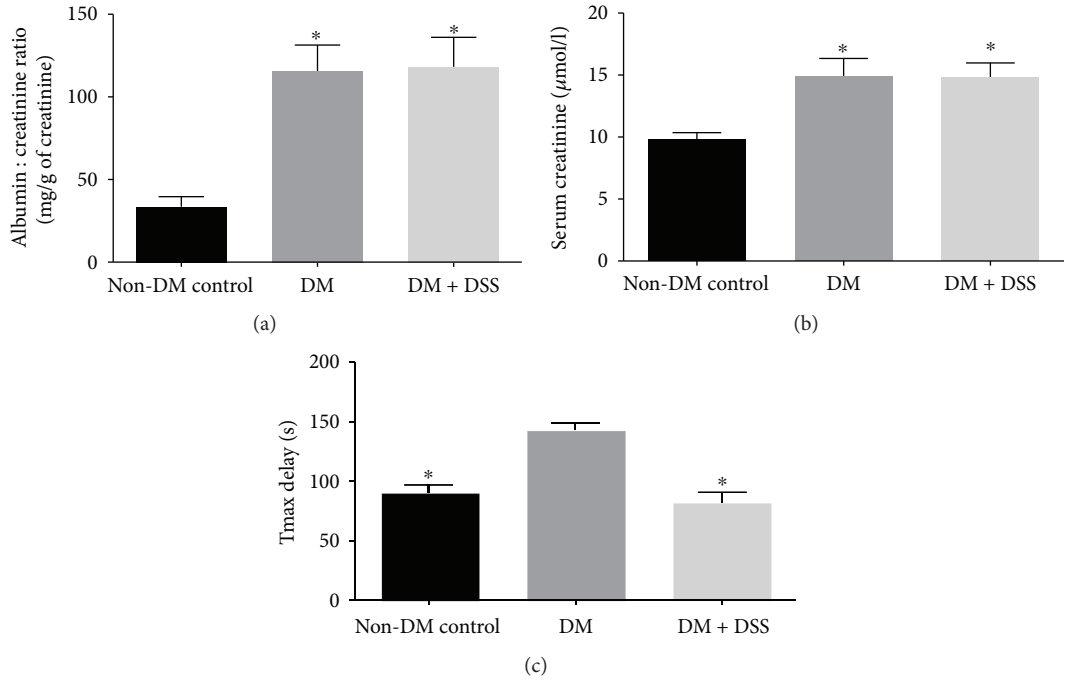


Figure 4: Kidney function markers: urinary albumin : creatinine ratio (a), serum creatinine (b), and Tmax delay (s) of mice after 3 weeks of intervention. $*$ denotes $p < 0.05$ when compared to the non-DM control group in (a) and (b) and when compared to the DM group in (c).

intensity (Tmax) over the renal cortex and renal pelvis regions of the right kidney were determined, and the time difference between Tmax-Pelvis and Tmax-Cortex was calculated as "Tmax delay," which represents the efficiency of IRdye200CW dye clearance [38].

2.5. Statistical Analyses. The assumptions of normality and homogeneity of variance were first assessed. ANOVA with multiple post hoc LSD adjustments or Kruskal-Wallis H test with multiple post hoc Dunn adjustments was used to compare the differences in the three groups where applicable. Paired *t*-tests were used to test for significant differences between the start and end fasting glucose concentrations in each group. All data were expressed as means ± SD. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 22 for Windows, and the significant level was set at $p < 0.05$.

3. Results

A summary of all measured variables collected from serum, urine, and MSOT in the present study can be found in Table 1. The results indicated that all *db*/*db* mice exhibited hyperglycemia and hyperinsulinemia and were more obese

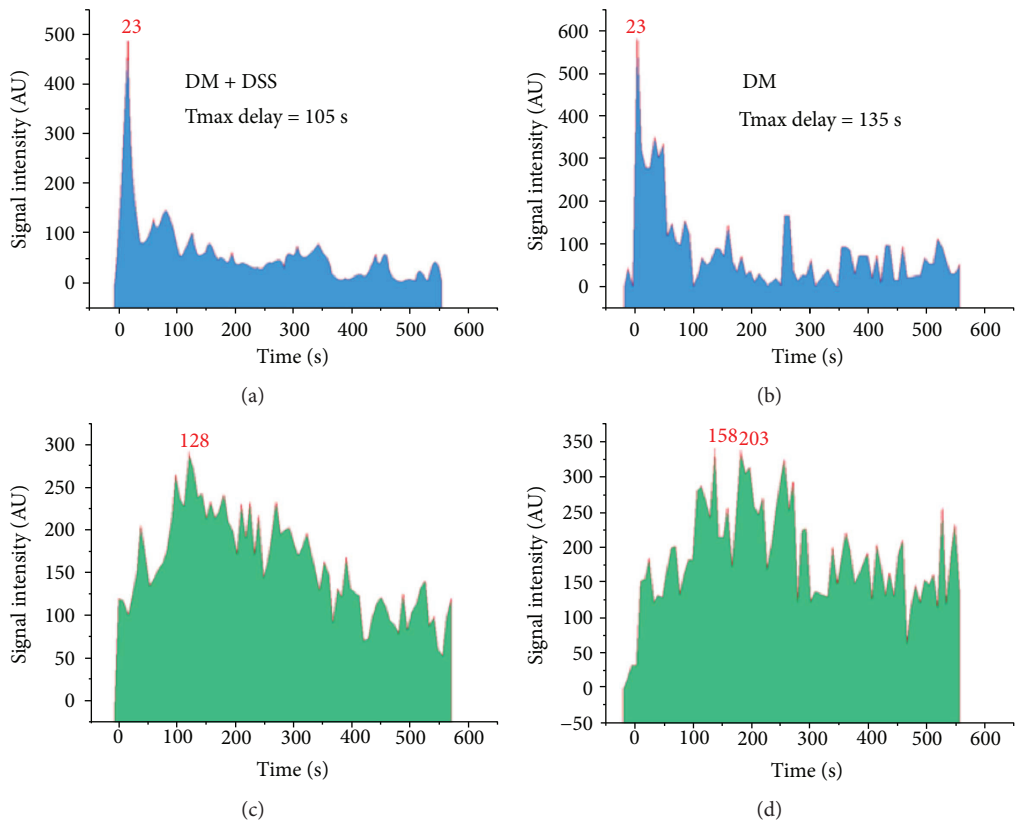


Figure 5: A plot of signal intensity over time over the renal cortex and renal pelvis of the kidney. (a, c) The left shows the spectrum collected from a mouse in the DM + DSS group [peak at 23 s and 128 s in the renal cortex (a) and renal pelvis (c), respectively; Tmax delay = 105 s]. (b, d) The right shows the spectra collected from a mouse in the DM group [peak at 23 s and 158 s in the renal cortex (b) and renal pelvis (d), respectively; Tmax delay = 135 s].

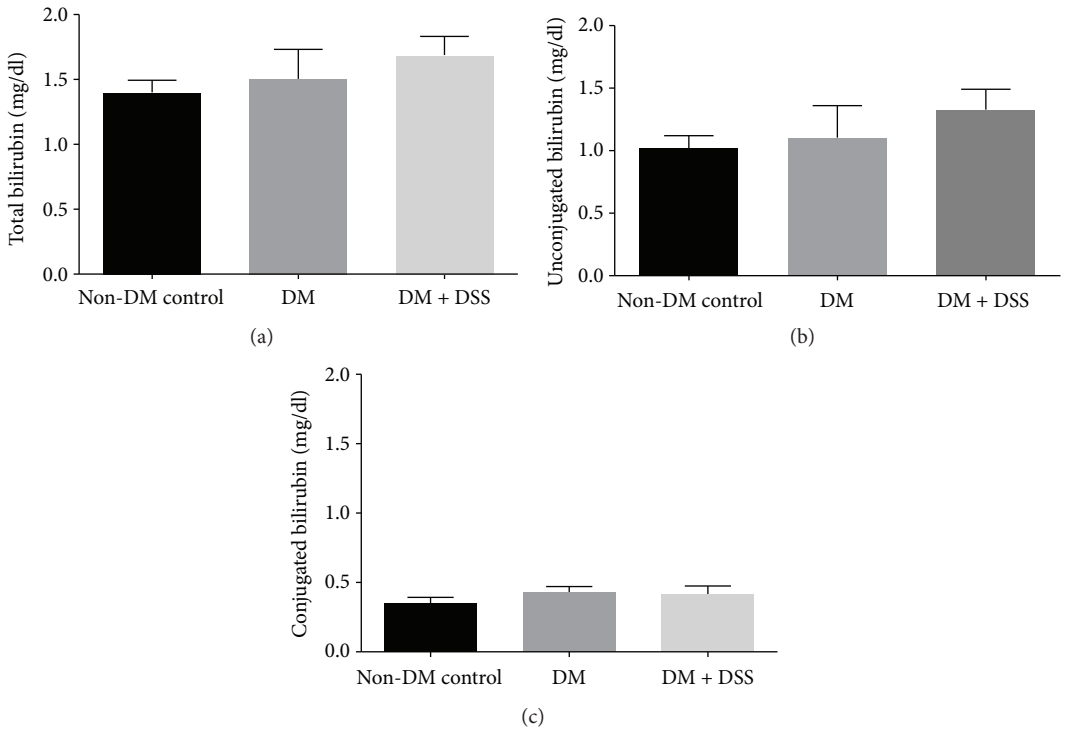


Figure 6: The serum total bilirubin (a), unconjugated bilirubin (b), and conjugated bilirubin (c) levels of mice after 3 weeks of intervention period ($n = 6 - 7$/group).

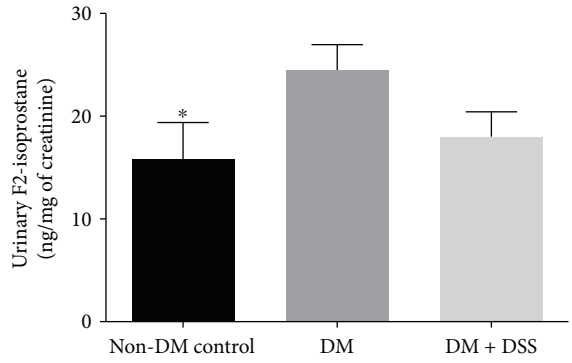


Figure 7: The urinary F2-isoprostane concentration of mice at after 3 weeks of intervention. * denotes $p < 0.05$ when compared to the DM group.

(Figure 2) when compared to *db*/+ mice at baseline and after 3 weeks. However, the fasting insulin concentration at the end of the study in the DM group (3.50 ± 1.14 nmol/l) was significantly greater when compared to those in the DM + DSS (2.22 ± 1.02 nmol/l, $p = 0.035$) and non-DM control (0.37 ± 0.16 nmol/l, $p = 0.007$) groups, suggesting that DSS treatment might improve insulin resistance in the *db*/*db* mice. On the contrary, there was no significant change in fasting glucose and body weight between baseline and after 3 weeks in all groups, except that the fasting blood glucose concentration tends to be increased in the DM + DSS group (from 19.60 ± 1.35 mmol/l at baseline to 24.97 ± 2.39 mmol/l after 3 weeks, $p = 0.098$) (Figure 3).

3.1. DSS Treatment Failed to Reduce ACR and Serum Creatinine Level but Improved the Tmax Delay (Renal Clearance) in db/db Mice. Both the DM and DM + DSS groups demonstrated increased urinary albumin : creatinine ratio (ACR) (Figure 4(a)) and serum creatinine (Figure 4(b)) when compared to the non-DM control group, which was consistent with a previous study [40]. From the graphs shown in Figure 5, Tmax delay determined by MSOT was longer in a *db*/*db* mouse without DSS treatment (Figures 5(a) and 5(c)) when compared to another *db*/*db* mouse with DSS treatment (Figures 5(b) and 5(d)). Collectively, the mean value of Tmax delay was significantly longer in the DM group when compared to the DM + DSS ($p = 0.001$) and non-DM control ($p < 0.001$; Figure 4(c)) groups, suggesting an improved renal clearance after DSS treatment in the DM + DSS group.

3.2. DSS Treatment Did Not Increase Serum Bilirubin or Significantly Reduce Urinary F2-Isoprostane Concentrations in db/db Mice. In the present study, the total bilirubin, unconjugated bilirubin, and conjugated bilirubin levels in the three groups (Figure 6) were similar, and the result was comparable to a previous reported study [41]. Although the DM + DSS group exhibited the highest concentrations of total bilirubin and unconjugated bilirubin when compared to the non-DM and DM groups, this difference did not reach statistical significance (all p values > 0.2). On the other hand, after the 3-week intervention period, the DM (24.46 ± 2.49 ng/mg) group demonstrated a greater urinary concentration of F2-isoprostane when compared to the non-DM control (18.01 ± 2.41 ng/mg, $p = 0.046$) group. With DSS treatment, urinary F2-isoprostane over 24 hours reduced to 15.81 ± 3.56 ng/mg in the DM + DSS group, although this reduction was not statistically significant ($p = 0.113$) when compared to the DM group (Figure 7).

3.3. Upregulation of HO-1 Expression in the Kidney of Diabetic Mice after 3 Weeks of DSS Treatment. Finally, we analyzed the renal cortex for expression of HO-1 and the p-Akt/t-Akt ratio. Significantly increased expression of HO-1 (~2-fold) was noted in the DM + DSS group when compared to the DM ($p = 0.029$) and non-DM control ($p = 0.016$) groups (Figure 8(a)). Although the p-Akt/t-Akt ratio was also significantly increased (~3-fold, $p = 0.011$) in the DM + DSS group when compared to the non-DM group, the mean difference of the p-Akt/t-Akt ratio between the DM + DSS and DM groups remained insignificant ($p = 0.125$; Figure 8(b)). The corresponding western blot data of HO-1 and AKT are presented in Figure 8(c).

4. Discussion

4.1. DSS Treatment and Diabetic Status. db/*db* mice spontaneously develop hyperinsulinemia due to mutation in the leptin receptor, which leads to impaired function of beta cells of the pancreatic islets. At 4 weeks of age, hyperglycemia, hyperinsulinemia, and insulin resistance are observed [42]. In the present study, although there was no observable change in the fasting glucose level in diabetic mice after DSS treatment, fasting insulin concentrations in the DSS treatment group was decreased when compared to non-treated diabetic group. This finding agreed with a previously published study [13], suggesting the possibility of improved insulin sensitivity mediated by DSS.

4.2. DSS Treatment and Renal Clearance. Significant reduction in renal function was evidenced in diabetic mice of the present study, as indicated by higher ACR and serum creatinine when compared to the nondiabetic group. However, the DSS antioxidant treatment failed to ameliorate the serum creatinine level, probably due to the difference in the injection approach and hence a lower daily effective dosage of DSS employed in the present study when compared to other published studies [43, 44]. ACR and serum creatinine are conventional and clinically relevant parameters for the assessment of kidney function and are significantly correlated with oxidative stress due to inactivation of NO [45]. However, proteinuria and changes in circulating creatinine concentrations or clearance have their limitations in regard to sensitivity and are typically modulated in moderate and late stages of renal disease. Therefore, we applied a novel, noninvasive measurement of renal clearance kinetics to determine the impact of DSS on renal function in diabetic animals, using the same methodology suggested by Scarfe's group [38]. This noninvasive examination technique provides a clear, sensitive, and specific optical signal from the target tissue with the utilization of IRDye800CW. Our results

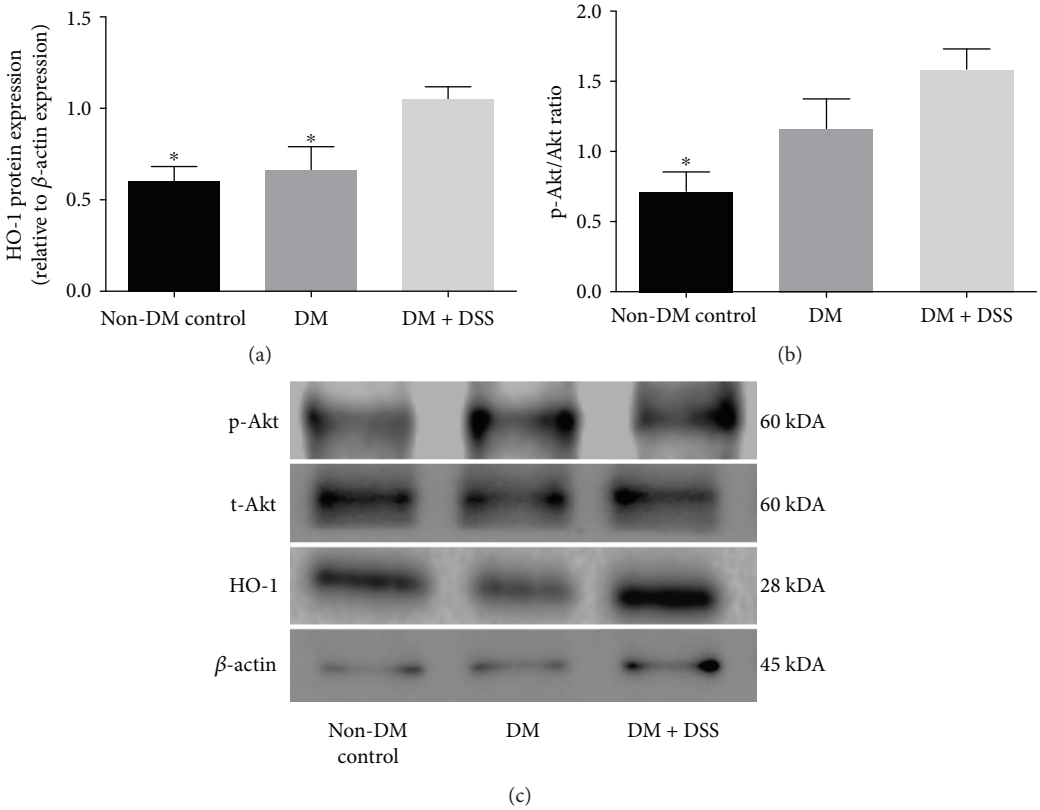


Figure 8: The expression of HO-1 (a) and p-AKT/t-Akt ratio (b) of mice at the end of the 3-week intervention period. $N = 4$ in each group, and the corresponding western blot data of HO-1 and Akt (c). $*$ denotes $p < 0.05$ when compared to the DM + DSS group.

of Tmax delay in our diabetic mouse model were similar to the previous work that studied the acute effect of adriamycin-induced nephropathy on Tmax delay [38]. However, our results on Tmax delay have the following limitations. Firstly, it should be noted that Tmax delay mainly assesses the hyperfiltration of IRdye800CW and does not account for tubular reabsorption of metabolites in the kidney and variations in hourly production of creatinine. Second, IRdye800CW could bind to plasma proteins and lead to underestimation of the true "Tmax delay" in the present study [38].

4.3. DSS Treatment and Lipid Peroxidation. DSS treatment was previously reported to ameliorate oxidative stress and lipid peroxidation via Akt/Nrf2/HO-1 [46, 47]. Lipid peroxidation is elevated in patients with diabetes, especially in those with increased HbA1c, LDL cholesterol, total cholesterol, and triglycerides [48]. In obese and diabetic patients, the accumulation of lipids and advanced glycation end products in plasma or organs represents an important source of lipid peroxidation, which further leads to DNA damage, protein/enzyme oxidation, and release of proinflammatory cytokines [49–51]. Many previous studies have shown that urinary IsoPs are a reliable biomarker of lipid peroxidation and could act as an indicator of oxidative stress [52, 53]. In the present study, diabetic mice exhibited higher levels of urinary IsoPs when compared to nondiabetic controls, which agrees with previous findings [54, 55]. However, the 3-week period of DSS treatment failed to significantly reduce the urinary IsoP concentration in *db*/*db* mice. At present, only a few studies have investigated the effect of *Salvia miltiorrhiza* (containing DSS) treatment on IsoPs [56, 57], with most results indicating that DSS-containing herbs could attenuate IsoPs in nondiabetic murine models. Therefore, our study is the first report to investigate the effect of DSS specifically on IsoP in *db*/*db* mice.

4.4. DSS Treatment and HO-1 Expression. We postulated that DSS is a potential druggable adjuvant in ameliorating diabetic nephropathy via induction of HO-1 synthesis. Previous studies have indicated that the HO system may act as a crucial mediator of cellular redox homeostasis by degrading heme, generating the antioxidant bilirubin, and releasing free iron (bound by ferritin) especially in the renovascular system [27, 58, 59]. Through activation of the nuclear factor-erythroid 2-related factor-2- (Nrf2-) targeting antioxidant response element (*ARE*)/heme oxygenase-1 (HO-1) signaling cascade, DSS has attenuated acute kidney injury [35]. The induction of HO-1 further activated adiponectin synthesis/release, which in turn improved cellular redox status, diminished apoptotic signaling kinase-1 expression, and protected from oxidative stress via activating p-Akt/Akt signaling [59–61]. In the present study, although DSS treatment was associated with increased expression of HO-1 in the kidney of *db*/*db* mice when compared to DSS-treated *db*/*db* mice, the levels of total and unconjugated bilirubin in the blood were only mildly elevated, suggesting an argument against HO-1-mediated protection via bilirubin in our

diabetic mice model. According to a previous study, downregulation of Akt could attenuate the antioxidant effects of HO-1 [62]; however, our data demonstrate that DSS could only mildly elevate the p-Akt : t-Akt ratio. Therefore, the failure of DSS-induced overexpression of bilirubin and Akt suggests other key players might be involved in mediating the beneficial effects of HO-1, such as carbon monoxide (CO) production or improved heme clearance. In this context, further studies on the effect of DSS treatment on CO production and heme clearance are warranted.

This study has several limitations. First, our team failed to collect enough blood for the baseline measurement of all selected biomarkers in the present study. Therefore, we only measured the serum fasting glucose at baseline, which required minimal blood volume. Second, the tail vein cannot be recovered within 3 weeks after the injection of dye; therefore, we could not complete the baseline measurement of renal clearance.

In summary, this study suggests that DSS might represent a potential viable preventative/treatment worthy of further investigation in patients with, or at risk of developing, diabetic nephropathy. Although HO-1 is known to ameliorate diabetic nephropathy [63], its effect in *db*/*db* mice remained poorly understood. In the present study, DSS treatment significantly improved renal clearance in *db*/*db* mice and was associated with upregulation of HO-1/Akt signaling pathways. However, the exact mechanism concerning how DSS mediates HO-1 activity and preserves renal physiological function remains unknown and requires further study.

Conflicts of Interest

All the authors declare no conflict of interest.

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Supplementary Materials

A video showing the signal intensity of IRdye800CW accumulated in the kidneys of a mouse after postinjection of IRdye800CW for 15 minutes. The region of interest in blue represents the renal cortex of the right kidney while the region of interest in purple represents the renal pelvis. The strength (in arbitrary units) of the photoacoustic signals from both the anatomical background and IRdye800CW dyes were denoted as different grey scale and green color on the right side of the panel. *(Supplementary Materials)*

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Review Article

Involvement of Mitochondrial Disorders in Septic Cardiomyopathy

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Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. It remains a leading cause of death worldwide, despite the development of various therapeutic strategies. Cardiac dysfunction, also referred to as septic cardiomyopathy, is a frequent and well-described complication of sepsis and associated with worse clinical outcomes. Recent research has increased our understanding of the role of mitochondrial dysfunction in the pathophysiology of septic cardiomyopathy. The purpose of this review is to present this evidence as a coherent whole and to highlight future research directions.

1. Introduction

Sepsis is defined by consensus as a life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. For clinical operationalization, patients with sepsis can be identified by the presence of an infection and an increase in the sepsis-related organ failure assessment score of 2 points or more. With short-term mortality over 10%, septic patients require hospitalization in intermediate or intensive care units [2]. Septic shock is defined as a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a hospital mortality rate of over 40% [1]. Patients with septic shock can be clinically identified by a vasopressor requirement to maintain a mean arterial pressure of 65 mm Hg or greater and serum lactate level greater than 2 mmol/L in the absence of hypovolemia. Both sepsis and septic shock are frequent in Western countries, with an incidence ranging from 270 to 437 cases per 100000 inhabitants per year [3]. The mortality attributable to sepsis is 5.3 million people per year worldwide, representing around 10% of deaths [4].

The incidence of cardiac dysfunction in septic shock (septic cardiomyopathy) is between 40 and 60%, as diagnosed within the first 3 days [5]. The presence of cardiac dysfunction is associated with increased mortality in patients with sepsis: 28-day mortality of patients hospitalized for sepsis was 16% in absence and 47% in the presence of myocardial dysfunction [6]. Sepsis-related cardiomyopathy is clearly multifactorial (Figure 1), and the general mechanisms of underlying this dysfunction have been reviewed elsewhere [7–10]. In summary, the potential extracellular candidates responsible for septic cardiomyopathy include pathogen-associated molecular patterns, cytokines, nitric oxide (NO), and damage-associated molecular patterns. Moreover, myocardial edema due to vascular leakage may also influence cardiac function in sepsis. At the cardiomyocyte level, mechanisms include the attenuation of the adrenergic response (i.e., downregulation of beta-adrenergic receptors and depressed postreceptor signaling pathways), alterations of intracellular calcium trafficking, blunted calcium sensitivity of contractile proteins, and mitochondrial dysfunction. Sepsis-induced myocardial dysfunction is not associated with significant

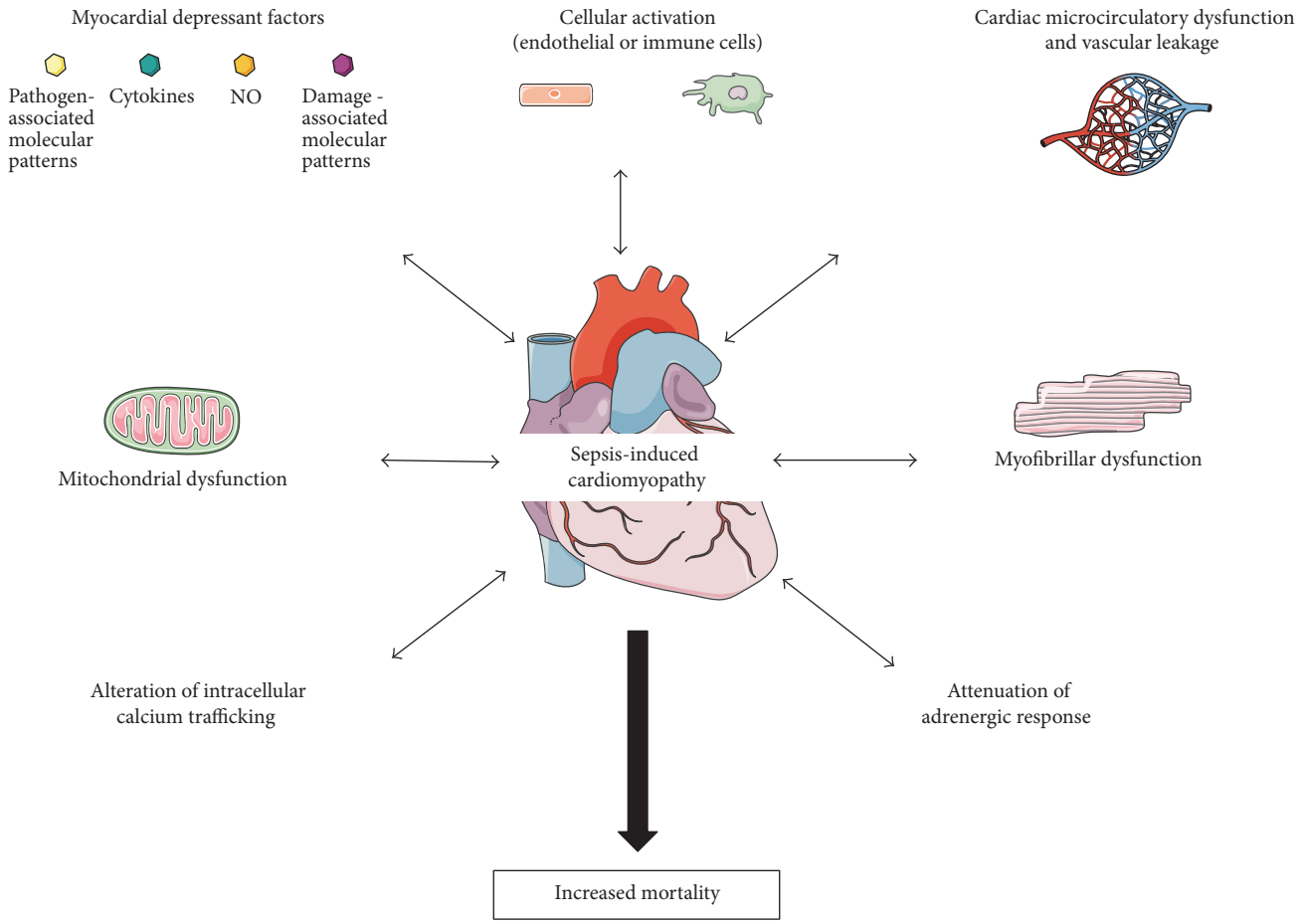


Figure 1: Main pathophysiological mechanisms of sepsis-induced cardiomyopathy. During sepsis, recognition of pathogen-associated molecular patterns by immune cells activates inflammation pathways and the release of myocardial depressant factors in the extracellular space. The subsequent activation of endothelial cells leads to alterations of microcirculatory perfusion and vascular leakage that are implicated in sepsis-induced myocardial dysfunction. Among intracellular mechanisms, myofibrillar dysfunction, alterations of calcium trafficking, attenuation of adrenergic response, and mitochondrial dysfunction seem to play important roles in sepsis-induced cardiomyocyte impairment. NO: nitric oxide.

cell death, and complete recovery occurs in survivors within two weeks [11].

Cardiomyocytes are characterized by a high density of mitochondria tightly packed between the sarcomeres and in the subsarcolemmal area [12]. Cardiac mitochondria are responsible for generating energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) and are crucial for cardiac function. Although mitochondrial impairment has been described in both human sepsis [13] and septic cardiomyopathy [14] for more than 15 years, mitochondria-targeted management is still absent from current clinical practice. A better understanding of mechanisms and any causative involvement of mitochondrial dysfunction in septic cardiomyopathy may guide future treatments in this field. Recent progress in sepsis research has increased our understanding of the underlying molecular alterations that contribute to cardiac dysfunction, including those related to mitochondrial dysfunction. In the following sections, we will review the evidence for the role of mitochondrial dysfunction in septic cardiomyopathy (Figure 2).

2. Ultrastructural Abnormalities of Myocardial Mitochondria in Sepsis

Mitochondria are intracellular organelles with a double membrane. This structure permits and is indeed essential for the mitochondrial production of the bulk of energy needed by the cell for normal function. Proton transfer from the matrix, across the inner membrane into the intermembrane space, is driven by the electron transfer chain and leads to phosphorylation of adenosine diphosphate (ADP) to ATP. This cascade of reactions requires mitochondria to be the most impermeable organelle in the cytoplasm and necessitates homeostasis of mitochondrial organization. When either inner or outer membrane permeability is disrupted, mitochondria change shape and this is associated with dysfunction and the release of content into the cytoplasm. Assessment of morphological characteristics such as swelling, disruption of cristae, and the length or density of mitochondria represents an easy way to identify mitochondrial dysfunction in various tissues.

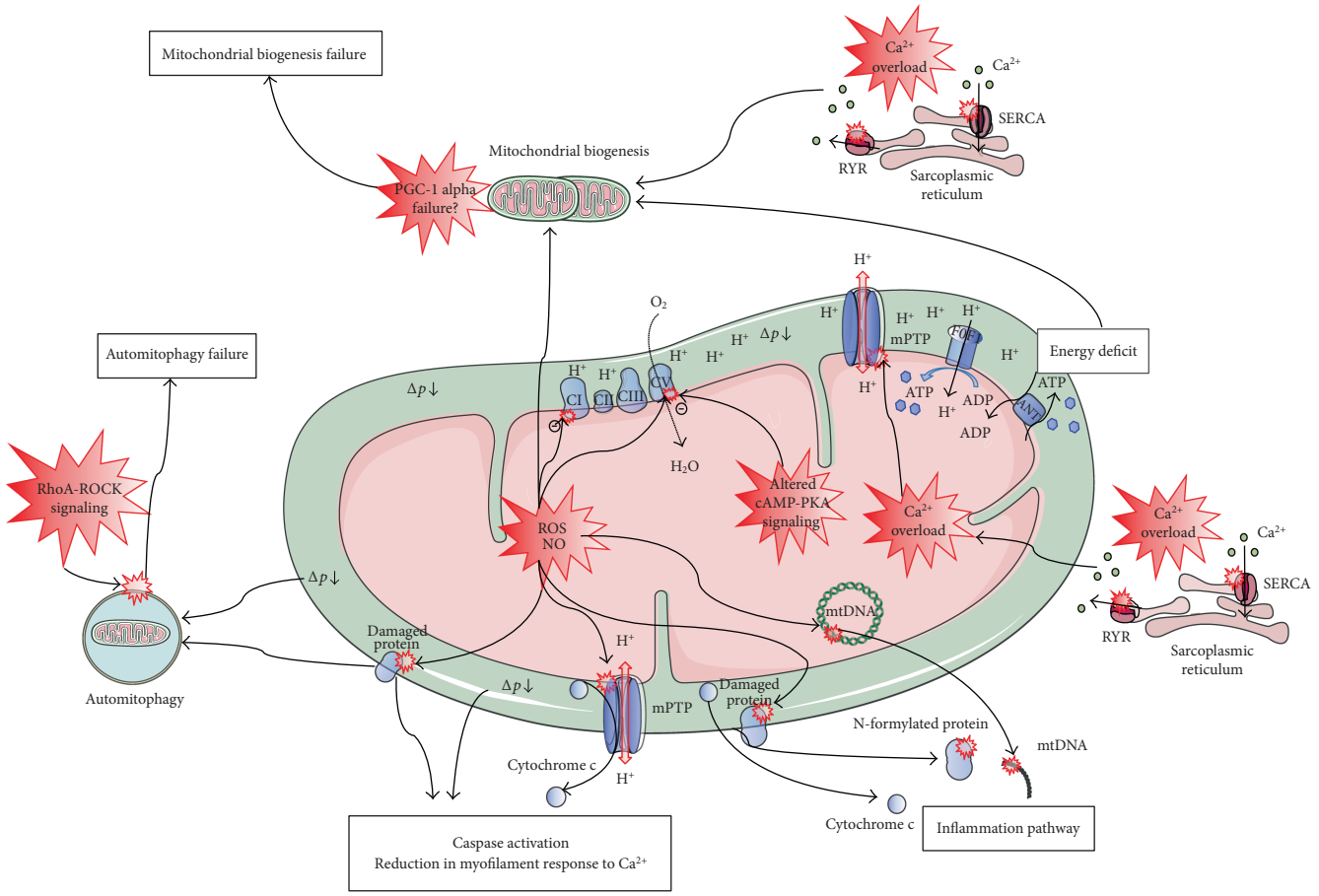
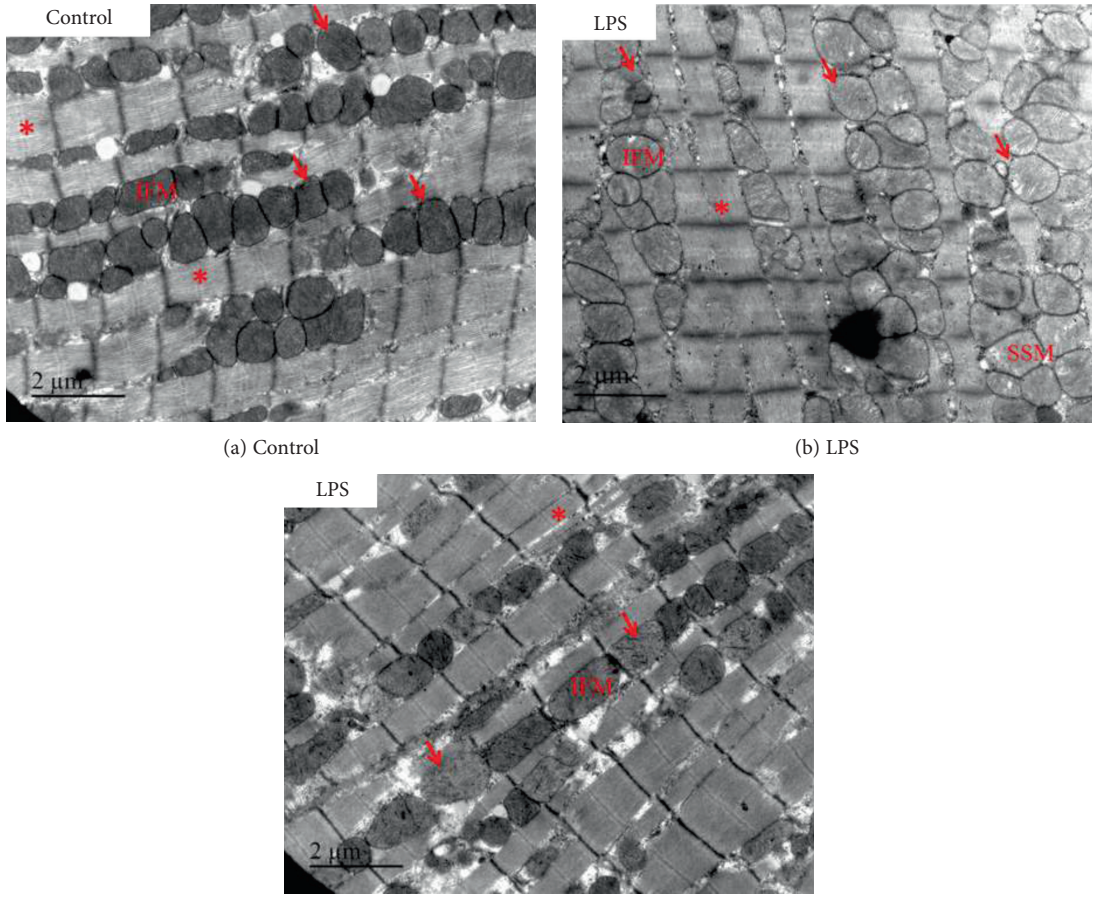


FIGURE 2: Mitochondrial disorders in septic cardiomyopathy. Increased reactive oxygen species (ROS) and nitric oxide (NO) production may cause direct oxidative or nitrosative damage and inhibition of oxidative phosphorylation (OXPHOS) complexes, leading to decreased O_2 consumption and proton-motive force between the intermembrane space and the matrix (Δp). Reduced calcium (Ca^{2+}) uptake by and increased Ca^{2+} leakage from the sarcoplasmic reticulum result in cytosolic and mitochondrial Ca^{2+} overload. Increased ROS/NO production, along with Ca^{2+} overload trigger the opening of the mitochondrial permeability transition pore (mPTP). This results in mitochondrial uncoupling of adenosine triphosphate (ATP) synthesis from O_2 consumption (i.e., OXPHOS uncoupling) and a decreased Δp. Altered mitochondrial cyclic adenosine monophosphate (cAMP) protein kinase A (PKA) signaling also promotes OXPHOS uncoupling and decreased Δp. Decreased Δp leads to ATP synthase (FOF1) inhibition and energy deficit. The mPTP opening and other mechanisms, as yet poorly described, induce externalization of mitochondrial components to the cytosol and the extracellular space that activates the inflammation pathway. Decreased Δp, the presence of oxidized proteins, and externalization of mitochondrial components in the cytosol activate intrinsic apoptosis, leading to reduction in myofilament response to Ca^{2+}. Although Δp and the presence of oxidized proteins activate auto-mitophagy, RhoA-ROCK activation results in automitophagy failure. As increased ROS production, cytosolic Ca^{2+} overload and energy deficit activate mitochondrial biogenesis, and peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1 alpha) disorders result in mitochondrial biogenesis failure. Overall, increased inflammation, energy deficit, reduced myofilament response to Ca^{2+}, impaired automitophagy, and failure in mitochondrial biogenesis are the features of mitochondrial disorders in septic cardiomyopathy. ADP: adenosine diphosphate; CI, CII, CIII, and CIV: the four complexes in the mitochondrial respiratory chain; mtDNA: mitochondrial DNA; RYR: ryanodine receptor; SERCA: sarcoendoplasmic reticulum Ca^{2+} pump.

The first description of morphological impairments of cardiomyocyte mitochondria was made in an animal model of septic cardiomyopathy in 1994 [14]. Recently, in a rat model of endotoxin-induced peritonitis, Vanasco et al. [15] reported both a dysfunction of OXPHOS by determining respiration and electron transfer in isolated cardiac mitochondria and an alteration of cardiac mitochondrial ultrastructure by transmission electron microscopy in left ventricle myocardium. A half-maximal effect on respiration was observed about 4 h after LPS administration. Simultaneously, the percentage of damaged mitochondria increased in 6 h from 1.4% to 6.3%. It is worth recalling that impaired biochemical function seems to precede the structural damage. The effects on heart mitochondrial structure of LPS administration are illustrated in Figure 3. After 6 h to 18 h of LPS injection, cardiac mitochondria display abnormalities such as swelling, loss of cristae, cleared matrix, internal vesicles and rupture of the inner and outer membranes, and alterations that persisted up to 24 h [15].

Cardiac tissue remains difficult to study in humans, requiring invasive procedures, while skeletal muscle is less



(a) Control (b) LPS

(c) LPS

Figure 3: Altered cardiac mitochondrial morphology in lipopolysaccharide- (LPS-) treated mice. Representative, longitudinal electron microscopy micrographs of the left ventricle from control (a) and LPS-treated (b, c) mice. Six hours after LPS administration, cardiac mitochondria displayed abnormalities such as swelling, loss of cristae, and cleared matrix. Representative areas of mitochondrial clustering (arrow) and myofibrils (asterisk) are indicated. IFM: interfibrillar mitochondria are arranged along the myofibrils; SSM: subsarcolemmal mitochondria.

problematic in this regard. Using an established scoring system for mitochondrial structure damage, ranging from 0 (no abnormalities) to 4 (severe abnormalities), Fredriksson et al. studied a cohort of 10 patients with sepsis-induced multiple organ failure. In intercostal and leg muscle, they observed a trend of increased abnormalities in the septic group compared with controls. The same results were evident in both subsarcolemmal and intermyofibrillar mitochondria [16]. Only one study has evaluated sepsis-related changes in human cardiac mitochondrial ultrastructure [17], focusing on the mechanistic basis of cardiac and renal dysfunction in patients who died from septic shock. Electron microscopy on 17 septic hearts revealed a mitochondrial injury score of 3 in 6 patients (35%), whereas abnormalities were absent in the control group comprising cardiac donor hearts for transplant [17].

Ultrastructural abnormalities of mitochondria are associated with mitochondrial dysfunction and, through disruption of OXPHOS, a decreased capacity for ATP production—the primary role of mitochondria.

3. Oxidative Phosphorylation Disorders of Myocardial Mitochondria in Sepsis

Mitochondrial OXPHOS is responsible for over 90% of total oxygen consumption and ATP generation in the body. The mitochondrial respiratory chain is organized into four individual enzyme complexes in the inner membrane (complex I, complex II, complex III, and complex IV) that create an electrochemical gradient of protons between the matrix and the intermembrane space [18, 19]. Beta-oxidation of fatty acids and the Krebs cycle provide reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) to the respiratory chain. NADH and FADH2 provide electrons to complex I and complex II, respectively. Electron transport from complex I and/or complex II to complex III then on to complex IV leads to proton translocation from the matrix to the intermembrane space and reduction of O_2 to H_2O. The oxidative activity of the respiratory chain thus leads to an accumulation of protons in the intermembrane space and creates a proton

motive force (Δp) between the intermembrane space and the matrix [19, 20]. The ATP synthase (F0F1), located in the inner mitochondrial membrane, is driven to produce ATP by this Δp and the passage of H^+ from the intermembrane space to the matrix. Thus, F0F1 couples oxidative activity and O_2 consumption of the respiratory chain to ATP regeneration. The ADP/ATP ratio is the physiologic activator of OXPHOS [21]; moreover, numerous posttranslational changes in respiratory complexes can subtly regulate OXPHOS and ATP production [22].

Alterations of organ function during sepsis are associated with OXPHOS dysfunction. Brealey et al. reported an association between shock severity, OXPHOS dysfunction (decreased activity of Complex I), ATP depletion, intracellular antioxidant depletion (reduced glutathione), and NO production in skeletal muscle tissue of patients with septic shock [13]. Mitochondrial dysfunction and reduced Δp have also been described in septic patients and were associated with clinical disease severity in peripheral blood monocytes [23] and platelets [24]. Reduced activity and quantity of platelet complex IV were independent predictors of 6-month mortality in septic patients [25]. Reduced ADP-stimulated mitochondrial respiration (state 3) in peripheral blood mononuclear cells has been associated with increased prevalence of organ failure and mortality [26].

Mitochondrial dysfunction in septic cardiomyopathy is also characterized by decreased rates of state 3 respiration in mice and rats [27–29]. Moreover, increased rates of ADP-independent mitochondrial respiration (state 4) have been demonstrated in septic hearts from murine models [27, 28, 30], indicative of proton leakage across the inner mitochondrial membrane bypassing F0F1 (i.e., OXPHOS uncoupling). Thus, the respiratory control ratio (RCR—i.e., the state 3/state 4 ratio) is decreased in murine models of septic cardiomyopathy. This decrease in RCR reflects a reduced capacity of the respiratory chain to maintain a constant gradient of protons and is also associated with decreased Δp [27, 28, 31].

The principal mechanisms involved in OXPHOS disorders seen in septic cardiomyopathy include overproduction of reactive oxygen and nitrogen species, calcium overload, and altered cAMP-PKA signaling. Increased production of superoxide anions and NO can cause direct oxidative or nitrosative damage and inhibition of OXPHOS complexes, leading to reduced O_2 consumption and decreased Δp. Firstly, reactive oxygen species (ROS) and nitrogen species are produced in substantial excess during sepsis [32], partly by mitochondria [33]. Mitochondrial complex I and complex IV can be inhibited by ROS, NO, and other nitrogen species. While it has been established that complex IV inhibition is always reversible by NO [34], prolonged exposure to NO results in a gradual and persistent inhibition of complex I [35]. Mitochondrial production of ROS and NO contributes to mitochondrial dysfunction during sepsis in various tissues, including the heart [15, 30]. Secondly, the antioxidant systems seem to be inhibited: inhibition of mitochondrial complex I by NO was facilitated, *in vitro*, by a depletion of reduced glutathione [36]. LPS-related depletion of reduced glutathione occurred within 6 h in the cardiac mitochondria of rats [37]. The activities of manganese superoxide dismutase and glutathione peroxidase enzymes in cardiac mitochondrial fractions from septic rats decreased significantly within 4 h after bacterial challenge [38]. Thirdly, when superoxide anions and NO are produced simultaneously, they rapidly react together to yield the peroxynitrite anion, which may result in significant inactivation of F0F1-ATP synthase [39]. Furthermore, complex I inhibition appears to result from *S*-nitrosylation of critical thiols in the enzymatic complex [35]. The increased activity of the inducible mitochondrial NO synthase is responsible for increased mitochondrial peroxynitrite levels [40]. In a mouse model of cecal ligation and puncture, Escames et al. showed that increased oxidative stress and mitochondrial dysfunction were restored by genetic deletion of inducible NO synthase (which includes the mitochondrial isoform), as well as by melatonin treatment, a known inhibitor of inducible NO synthase [41]. Together, these results suggest a significant role for peroxynitrite in myocardial mitochondrial dysfunction in sepsis. Finally, ROS species can lead to a diverse array of both reversible and irreversible toxic modifications of proteins, lipids, or nucleic acids. This oxidative stress alters respiratory capacity by decreasing proteomic expression of several mitochondrial-encoded subunits of complex I, complex IV, and F0F1 [37]. In addition, activation of mitochondrial calpain-1 can disrupt F0F1, leading to increased mitochondrial ROS generation, further alteration of mitochondrial components and promotion of a proinflammatory response during endotoxemia [42].

Under pathological conditions, some protons return into the matrix while bypassing F0F1 via uncoupling proteins (UCPs) located in the inner mitochondrial membrane [43]. This results in OXPHOS uncoupling—the dissociation of ATP synthesis from O_2 consumption in the mitochondria. The role of UCPs in sepsis remains controversial. In a rat model of cecal ligation and puncture, Roshon and colleagues found an increase in UCP_2 mRNA levels concurrent with decreased cardiac work and mechanical efficiency [44], but no UCP_2 protein was detected in the hearts 12 h after insult. Significant increases in levels of UCP_2 and UCP_3 mRNA were observed in the hearts of LPS-treated rats [37], while both mRNA and protein levels of UCP_2 were increased in the myocardium in a canine model of endotoxin-induced shock [45]. In this model, Wang et al. found an association between elevated UCP_2 expression and decreased ATP generation [45]. Conversely, increased UCP_2 expression in cardiac mitochondria may produce only mild uncoupling that prevents ROS production without altering ATP generation. Indeed, Zheng et al. reported that H9C2 cells cultured with LPS and peptidoglycan showed increased mRNA expression of UCP_2 and that this was associated with mitochondrial dysfunction [46]. These effects were worsened by silencing UCP_2, suggesting that UCP_2 may play a protective role in cardiomyocytes under septic conditions.

Permeability of the inner mitochondrial membrane is mediated by a voltage- and calcium dependent, cyclosporine A-sensitive, high-conductance channel called the mitochondrial permeability transition pore (mPTP). Mitochondrial calcium overload is the primary trigger of mPTP opening, but its sensitivity to this challenge is dependent on prevailing

conditions [47]. In a rat model, Hassoun et al. reported that an increased mitochondrial calcium content (induced by LPS) was associated with reduced calcium uptake by and increased calcium leakage from the sarcoplasmic reticulum and it was also associated with decreased Δp, mitochondrial uncoupling, altered state 3 respiration, and impaired RCR. Prevention of calcium leakage from sarcoplasmic reticulum by dantrolene prevented mitochondrial and cardiac contractile dysfunction, suggesting that mitochondrial calcium overload could contribute to myocardial mitochondrial dysfunction in sepsis [28]. In a mouse model of acute peritonitis, Larche et al. observed that improvement of mitochondrial function via inhibition of mPTP opening (with cyclosporine A or overexpression of the antiapoptotic protein Bcl-2) prevents mortality and heart dysfunction [27]. Inhibition of mPTP opening also prevented a sepsis-related decrease in Δp and RCR in the septic heart. Overall, mitochondrial calcium overload and mPTP opening seem to be the main mechanisms of mitochondria uncoupling in septic cardiomyopathy.

Posttranslational changes in respiratory complexes may also be involved in sepsis-induced mitochondrial and contractile dysfunction in the heart. Cyclic adenosine monophosphate (cAMP) is produced within mitochondria by HCO_3/CO_2-responsive adenylyl cyclase, which couples CO_2 generation in the Krebs cycle with OXPHOS activity [48–50]. Increased mitochondrial cAMP induces mitochondrial protein kinase A (PKA) activation. Thus, allosteric ATP inhibition of mitochondrial complex IV is prevented by reversible PKA-mediated phosphorylation of serine 58 of complex IV subunit IV-1. The mitochondrial cAMP-PKA pathway couples the Krebs cycle and OXPHOS activity to generate ATP. In a mouse model of acute peritonitis, Neviere et al. observed that sepsis-related cardiomyopathy was associated with impaired cAMP-PKA signaling, decreased complex IV phosphorylation of serine 58, decreased mitochondrial RCR, and left ventricle contractile dysfunction [51]. Ex vivo inhibition of phosphodiesterase 2A by mitochondria-permeant Bay 607550 prevented mitochondrial cAMP depletion, complex IV phosphorylation, OXPHOS uncoupling, and contractile dysfunction. *In vivo* administration of Bay 607550 did not prevent contractile dysfunction but restored energetic efficiency of the left ventricle in septic mice.

While the mitochondrial capacity for generating ATP is decreased in sepsis, mitochondrial dysfunction is not associated with significant myocardial necrosis in human septic shock [11]. Nonetheless, respiratory chain dysfunction is associated with contractile dysfunction and prevention of OXPHOS disorders prevents cardiac dysfunction in murine models of sepsis [27–31]. The importance of mitochondria in cellular homeostasis could provide a mechanistic explanation for the link between mitochondrial dysfunction and heart failure in septic cardiomyopathy [52].

4. Alteration of Mitochondrial Signaling in Septic Cardiomyopathy

Mitochondrial ROS production [53, 54], or externalization of other mitochondrial components in the cytosol (e.g., cytochrome c, SMAC/diablo, PGAM5, and mitochondrial DNA (mtDNA)) [55–57], may be considered to be a part of cellular signaling. These components are involved in both the apoptotic pathway and inflammation, key features in the pathophysiology of septic cardiomyopathy.

Mitochondria are critical players in the regulation of programmed cell death [58]. They modulate apoptosis during the initiation and regulation of the intrinsic apoptotic pathway, also known as mitochondria-mediated apoptosis. Caspase 9-dependent activation of executioner caspases such as caspase 3, 6, and 7 is a characteristic of the intrinsic pathway. Oxidative stress, OXPHOS dysfunction, and mPTP opening are the main triggers for mitochondria-mediated apoptosis. Initially, ROS-induced protein alterations lead to the accumulation of Nix and Bnip3 proteins on the outer mitochondrial membrane. Thus, Nix and Bnip3 recruit proapoptotic proteins from the cytosol (such as Bax and Bak) on the surface of mitochondria. Thereafter, Bax and Bak permeabilize the outer membrane to molecules such as cytochrome c which triggers the apoptotic pathway [57, 58]. Secondly, OXPHOS dysfunction and a decrease in the Δp can lead to the cleavage of a mitochondrial phosphatase, PGAM5. Cleaved PGAM5 translocates to the cytosol where it activates the apoptotic pathway [56, 57]. Thirdly, the mPTP opening directly permeabilizes mitochondria and can release cytochrome c to the cytosol, activating the intrinsic apoptotic pathway. Finally, the extrinsic apoptotic pathway may also interfere with mitochondria-induced apoptosis. The extrinsic pathway can activate Bax and Bak, which directly permeabilize the outer mitochondrial membrane and trigger the intrinsic apoptotic pathway [59].

Nevière et al. first demonstrated the importance of apoptosis regulation in an experimental cardiomyopathy model of endotoxin-treated rat. Caspase 9 and 3 activities and the apoptosis pattern (DNA fragmentation or cytochrome c release) were enhanced in this experimental model. Cotreatment with a nonspecific caspase inhibitor not only reduced caspase activity and nuclear apoptosis but was also associated with a complete correction of endotoxin-induced myocardial dysfunction [60]. The same authors also demonstrated that specific inhibition of caspases 9 and 3 prevented reduction in myofilament responses to calcium, troponin T cleavage, and sarcomere destruction in endotoxin or septic serum-treated rats [61]. Mitochondrial activation of intrinsic apoptosis was also implicated in rats with post cecal ligation puncture-induced septic cardiomyopathy. In this model, prevention of cardiomyocyte dysfunction was also prevented by inhibition of the intrinsic apoptotic pathway using cyclosporine A or Bcl-2 overexpression [27].

All these experimental data converge to implicate mitochondrial upregulation of the intrinsic apoptotic pathway in the genesis of septic cardiomyopathy. However, while the mitochondrial apoptotic pathway has not been evaluated in human sepsis, the importance of its activation seems overestimated by experimental models as apoptosis-related death of cardiomyocytes does not significantly occur in human sepsis [11, 17]. Whether caspase activation and/or caspase-induced cleavage of troponin T occurs in the cardiomyocytes of patients with septic shock remains unknown.

Lovett et al. [62] suggested that myocardial cell depression may be due to circulating myocardial depressant factors. Parrillo et al. later confirmed the existence of depressant factors in human sepsis, showing that serum obtained from septic shock patients and incubated *in vitro* with cardiomyocytes decreased the extent and velocity of myocyte shortening. Removal of the preparation by washing with serum obtained from nonseptic patients rapidly restored the contractile force of cardiomyocytes [63]. Proinflammatory cytokines (TNF-α and IL1-β) and NO were the first myocardial depressant factors described [64]. Damage-associated molecular patterns (DAMPs) are endogenous molecules released in cytoplasm or the circulatory torrent in response to cellular stress, whereupon they can react to pattern recognition receptors also called host defense receptors of innate immunity. From the "Danger model" developed by Matzinger, DAMPs can trigger the immune system and inflammation pathway [65, 66], engaging a defensive reaction to cellular damage. A potential role has been proposed for DAMPs in sepsis-induced organ dysfunction such as that seen in septic cardiomyopathy. For instance, HMGB1, a nuclear DAMP, can induce cardiac dysfunction via TLR4 interaction and consequently enhance oxidative stress and impairment of cardiac excitation-contraction coupling [9, 67]. Mitochondria can also react to infectious or inflammatory aggression by liberating numerous components, collectively known as mitochondrial DAMPs (mtDAMPs), into cytoplasm and the circulatory torrent. Once there, mtDAMPs can interact with pattern recognition receptors and induce a proinflammatory response to damage, thereby implicating mitochondria in the regulation of danger signaling [68]. The structural homology of mtDNA (containing CpG-nonmethylated DNA and formylated proteins) underpins the proposed bacterial origin of mitochondria (the endosymbiotic theory) and partially explains its recognition by pattern recognition receptors. The mtDNA is the most studied mtDAMPS in experimental or human sepsis, but other mtDAMPs have been described such as cytochrome c or N-formyl proteins [69, 70]. Although conflicting data exist, owing largely to technical issues around the detection of free plasmatic mtDNA, most experimental and human results report elevated free plasmatic circulating mtDNA in sepsis [71–73]. In addition, quantitative expression seems to correlate with severity and mortality in sepsis in a way similar to a large number of diseases encountered in critical care [74, 75]. However, the mechanism of mtDNA externalization in septic subjects remains unclear. Mechanism does not seem as simple as in trauma patients and may implicate necrosis, necroptosis, apoptosis, or autophagy process [76–78]. Mitoptosis, a recently described caspase-independent mechanism by which mitochondria undergo extensive fragmentation, is another potent mechanism for the release of mitochondrial content to the cytoplasm or plasma [79].

It is nowadays clear that circulating mtDNA can contribute to systemic inflammation [80], neutrophil activation [81], or even postseptic organ dysfunction of the immune system [82], lung [83–85], or kidney [86]. No data are yet available on the putative induction role of mtDNA in septic cardiomyopathy, but Oka et al. described mtDNA as a candidate inducer of cardiac dysfunction in an experimental mouse model of heart failure [87]. Likewise, cytochrome c and N-formyl protein have been shown capable of injuring cardiomyocytes and provoking cardiac dysfunction in various models [69, 70]. While mtDAMPs may be a myocardial depressant factor, new experimental data are necessary to better understand their role in septic cardiomyopathy.

5. Mitochondrial Biogenesis and Mitophagy in Septic Cardiomyopathy

The quality and quantity of mitochondria largely depend upon both mitochondrial biogenesis and the autophagy/mitophagy system. Mitochondrial biogenesis is mainly activated in cases of low ATP production, oxidative stress, and calcium overload. In the presence of biogenesis activators, peroxisome proliferator-activated receptor γ coactivator (PGC-1 alpha) and nuclear transcription factors such as nuclear respiratory factors (NRFs), estrogen-related receptors (ERRs), and peroxisome proliferator-activated receptors (PPARs) are overexpressed and activated [88]. The transcription of nuclear genes coding for metabolic enzymes, respiratory chain proteins, mitochondrial transcription factors (mTFs), and other mitochondrial proteins is thus activated. In humans, over 97% of mitochondrial proteins are translated from nuclear mRNA and subsequently translocated to mitochondria. The mTFs A, B1, and B2 activate the replication of mtDNA and transcription of the 37 mitochondrial genes, essential for mitochondrial activity, and permit adequate interplay between nuclear and mitochondrial genomes during mitochondrial biogenesis. While insufficient biogenesis limits ATP production and induces cell necrosis [89], an excessive production of mitochondria leads to disruption of myofibrils and cardiomyocyte dysfunction [90]. Mitochondria undergo continuous oxidative stress due to oxidative activity of the mitochondrial respiratory chain. Production of superoxide anion, hydrogen peroxide, and hydroxyl radical leads to impairment of protein structure, peroxidation of membrane lipid, and alteration of mtDNA [19]. Nonspecific autophagy and mitochondria-specific autophagy (i.e., mitophagy) remove damaged and dysfunctional mitochondria [91]. Mitophagy mainly involves activation of PINK1/Parkin, PGAM5/FUNDC1, or the Nix/Bnip3 pathways. A decreased Δp leads to accumulation of PINK1/Parkin and PGAM5/FUNDC1 on the outer mitochondrial membrane. The oxidation of mitochondrial proteins drives to Nix/Bnip3 accumulation on the cytosolic surface of mitochondria. Thus, PINK/Parkin, PGAM5/FUNDC1, and Nix/Bnip3 activate mitophagy and removal of dysfunctional mitochondria [88]. Autophagy and mitophagy can limit accumulation of depolarized mitochondria, generation of superoxide anion, hydrogen peroxide, and hydroxyl radical, and the release of mitochondrial content into cytosol or the extracellular space. There exists significant interplay between mitophagy and mitochondrial biogenesis. On the one hand, biogenesis activation by mitophagy permits renewal of the degraded organelles [92]; on the other hand, mitophagy activation by mitochondrial biogenesis creates space for new functional organelles [93].

In the septic heart, there is an early (H6 to H24) decrease in mitochondrial mass with in parallel, early activation of biogenesis pathways [15, 37, 94, 95]. In rat cardiomyocytes, endotoxins induced significant increases in biogenesis markers such as PGC-1 alpha, NRF-1, and mTFs [96]. Autophagy was also stimulated in this model, as indicated by an increased expression of microtubule-associated light chain 3 (LC3). In humans, survival was associated with activation of mitochondrial biogenesis (assessed by PGC-1 alpha, NRF-1, and mTFA) in the skeletal muscle of septic patients [97]. Importantly, manganese superoxide dismutase expression (representing antioxidant system activity) was associated with an increase in these markers of biogenesis. Unsurprisingly, patients who died from sepsis had significantly decreased expression of nuclear genes coding for Krebs cycle enzymes and respiratory chain proteins [98]. The exact mechanisms by which transcription of nuclear genes coding for mitochondrial proteins is altered in patients dying from sepsis remain unclear. Nevertheless, ROS-induced damage of mitochondrial DNA might be responsible for a relative insufficiency of mitochondrial transcription and mitochondrial biogenesis in septic subjects [37].

Carbon monoxide can cause cardiac ischemia with an inhibition of the respiratory chain together with an increase in cardiac contractility [99]. Interestingly, as exposure to elevated doses of carbon monoxide increased mortality in a mouse model of sepsis, while exposure to low doses activated mitochondrial biogenesis in the heart and prevented Δp alterations in cardiac mitochondria, as well as acute mortality [31]. Thus, minor inhibition of the respiratory chain may, to some extent, play a beneficial role in septic models [100, 101].

Nevertheless, partial restoration of cardiac mitochondrial mass is not always accompanied by improvement of mitochondrial function in acute endotoxemia [15]. Overexpression of PGC-1 alpha in the heart could be deleterious. Transgenic mice overexpressing PGC-1 alpha displayed a reversible cardiomyopathy: this cardiomyopathy was characterized by an increase in ventricular mass (eccentric hypertrophy) and chamber dilation (echocardiographic data) [102]. In the neonatal stages of this model, overexpression of PGC-1 alpha increased the number and size of mitochondria; in adult mice, this overexpression only modestly increased the number of mitochondria, leading to changes in mitochondrial ultrastructure (e.g., apparition of vacuoles and granular inclusions) and cardiomyopathy.

A relative insufficiency of autophagy/mitophagy may impede clearance of dysfunctional mitochondria despite appropriate biogenesis in the septic heart. In a LPS injection model, autophagy was activated in the mouse heart [29]. In this model, inhibition of Drp1 phosphorylation by fasudil (a ROCK inhibitor) further activated autophagy and prevented cardiac and mitochondrial dysfunction. Similarly, autophagy stimulation in a model of cecal ligation and puncture could restore both cardiac function and cardiac ATP production [103], whereas inhibition of autophagy in a murine peritonitis model increased apoptosis and hepatic injury [104]. Carbon monoxide exposure may regulate both mitochondrial biogenesis and autophagy. Indeed, low doses of carbon monoxide prevented acute mortality in a murine peritonitis model via the systemic enhancement of autophagy and phagocytosis [105]. Mice deficient for the autophagic protein Becn1 (Becn1+/−) were more likely to die from sepsis and be unresponsive to carbon monoxide therapy. The role of cardiac mitophagy per se in sepsis has been evaluated in only one animal study [106]. Sublethal doses of endotoxin led to early mitophagy activation, transitory cardiac dysfunction (assessed by isolated heart preparation), and reversible alteration of mitochondrial respiration in wild-type mice. Conversely, Parkin−/− mice displayed only partial recovery of mitochondrial and cardiac function, despite residual mitophagy activation. Therefore, early and complete activation of mitophagy pathways seem essential for recovery of mitochondria and cardiac function during septic cardiomyopathy.

Overall, the relative and/or absolute insufficiency of mitochondrial biogenesis and auto-/mitophagy may be important for contractile and mitochondrial dysfunction in the septic heart. Nonetheless, the precise mechanisms of mitochondrial biogenesis alteration and mitophagy insufficiency are still poorly understood and require further investigation.

6. Mitochondrial Genetic and Sepsis

According to the endosymbiotic theory, mitochondria originated from aerobic free-living bacteria-like organisms (alpha-proteobacteria). At some point in evolution, they were engulfed by primitive, nucleated anaerobic cells to form symbiotic, eukaryotic cells [107]. The organizational features supporting endosymbiotic theory are the presence of a double membrane and its own mtDNA. The mtDNA is a small, circular, double-stranded molecule only 16.5 kb in length. The 37 genes of the human mitochondrial genome encode 13 essential components of the OXPHOS system (i.e., complex I, complex III, complex IV, and FOF1), 22 transfer RNA, and 2 ribosomal RNA [108]. Nevertheless, nuclear genes code for the majority of mitochondrial proteins subsequently translocated into mitochondrion from cytosolic ribosomes. The mtDNA is almost exclusively inherited from the maternal line. A high random mutation rate of mtDNA can be due to the lack of protective histones, inefficient DNA repair mechanisms, and mutagenic effects of mitochondria-generated ROS [109]. Consequently, a large number of single-nucleotide polymorphisms of mtDNA have accumulated among maternal lineages and have diverged as human populations dispersed more widely to different geographical regions of the world. These specific single-nucleotide polymorphisms are known as mtDNA haplogroups [110]. Nine haplogroups have been successively described (H, J, T, U, K, V, W, I, and X), the majority of the European population belonging to haplogroup H (44%) [111].

A longitudinal clinical and genetic study of 150 patients with septic shock revealed that haplogroup H patients presented proportionally better survival than other haplogroups at 28 days, upon hospital discharge and at a six-month follow-up [112]. Furthermore, the Spanish sepsis group of researchers reported a protective effect of haplogroup H on sepsis incidence in a study of 240 patients with postoperative sepsis [113]. Haplogroup H is the most recent addition to the

group of European mtDNA but, perhaps paradoxically, is the most common: indeed, increased survival after septic shock may provide one explanation for this. The hypothesis of a direct, functional consequence of improved mitochondrial efficiency arising from this clinical report was subsequently tested by Amo et al. [114]. Using a transmitochondrial cytoplasmic hybrid cell, they found no difference in mitochondrial bioenergetic capacities or coupling efficiencies when comparing mitochondria from haplogroup H with those from haplogroup T. Haplogroup H survival protection remains poorly understood, and haplogroup effects on mitochondrial proliferation or signaling processes have not yet been fully explored. Other research groups have described the potential consequences of belonging to other haplogroups: for example, haplogroup JT was associated with increased survival in a prospective cohort of 96 patients with severe sepsis and an increased complex IV activity in patients from this haplogroup relative to others [115, 116].

Together, these data indicate a potential effect of genetic haplogroup variants on survival in sepsis cases. The association with functional mitochondrial activity remains unclear; however, no data yet exist on the consequences for cardiac mitochondria during sepsis.

7. Conclusions

Cardiac dysfunction is common in patients suffering from sepsis and septic shock. Mitochondrial dysfunction takes part in the pathophysiology of septic cardiomyopathy and is associated with patient outcome. Respiratory chain disorders, the role of dysfunctional mitochondria in cellular homeostasis, and insufficient renewal of mitochondria are deleterious mechanisms in both development and persistence of cardiac dysfunction in septic subjects. The weight of evidence for the involvement of mitochondrial dysfunction in septic cardiomyopathy makes it a potential target for future treatment of sepsis. Nevertheless, the precise mechanisms and any causative role for mitochondrial impairments in human cardiomyopathy are still poorly understood and require further investigation before clinical application.

Conflicts of Interest

The authors declare no conflicts of interests.

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Review Article

Oxidative Stress and Acute Kidney Injury in Critical Illness: Pathophysiologic Mechanisms—Biomarkers—Interventions, and Future Perspectives

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Acute kidney injury (AKI) is a multifactorial entity that occurs in a variety of clinical settings. Although AKI is not a usual reason for intensive care unit (ICU) admission, it often complicates critically ill patients' clinical course requiring renal replacement therapy progressing sometimes to end-stage renal disease and increasing mortality. The causes of AKI in the group of ICU patients are further complicated from damaged metabolic state, systemic inflammation, sepsis, and hemodynamic dysregulations, leading to an imbalance that generates oxidative stress response. Abundant experimental and to a less extent clinical data support the important role of oxidative stress-related mechanisms in the injury phase of AKI. The purpose of this article is to present the main pathophysiologic mechanisms of AKI in ICU patients focusing on the different aspects of oxidative stress generation, the available evidence of interventional measures for AKI prevention, biomarkers used in a clinical setting, and future perspectives in oxidative stress regulation.

1. Introduction

Acute kidney injury (AKI) is a multifactorial clinical entity that presents with primary and secondary nonspecific manifestations due to a variety of causes (Table 1). Until the beginning of the 21st century, the incidence of AKI was not accurately reported due to the fact that AKI definition was highly dependent on clinician's opinion and widely varied among different centers [1]. The definition and diagnosis of AKI based on standard criteria were first developed in 2004 by the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group which introduced the RIFLE (Risk, Injury, Failure, Loss, End-stage kidney disease) criteria [2] (Table 1). The different stages in RIFLE classification are delineated according to changes in serum creatinine levels and/or glomerular filtration rate (GFR) or urine output [2]. In 2007, the Acute Kidney Injury Network (AKIN) published a report that established AKI is the term to be used in order to describe the whole spectrum of acute kidney failure and proposed a modified RIFLE classification without including separately renal replacement therapy (RRT) [3]. Most recently, in 2012, Kidney Disease: Improving Global Outcomes (KDIGO) working group proposed that for accuracy purposes, serum creatinine measurements should be used instead of GFR estimation when staging AKI [4] and a guideline report

Table 1: Common causes and susceptibilities for AKI.

Sepsis
Circulatory compromise (shock)
Burns/trauma
Cardiac surgery (especially with cardiopulmonary bypass)
Major (noncardiac) surgery
Nephrotoxic drugs
Radiocontrast agents
Poisonous plants/animals
Volume depletion
Advanced age
Female gender
Black race
Chronic kidney disease
Diabetes mellitus
Cancer
Anemia

was endorsed by the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-KDOQI) as well [5] (Table 2).

AKI represents a major public health problem with a reported incidence of 0.25% in the general population and 18% in the hospitalized patients [5]. Although AKI is not a usual cause for admission to intensive care unit (ICU), it often complicates critically ill patients' clinical course. Epidemiologic evidence of all-cause AKI incidence in ICU patients widely varies due to the remarkable polyphony previously used in diagnostic criteria and ranges from 5.7% [6] to 36% [7–9]. The severity of AKI defined by RIFLE classification has been reported to be 36.1% and seems to be an independent risk factor for patients' outcome and mortality [7, 8]. Moreover, in a critical care setting, AKI is connected with prolongation of hospitalization and need for RRT and occasional progress to chronic kidney disease. Sepsis is the leading cause of AKI in severely ill patients in ICU, accounting for nearly 50% of cases [10], while common concurrent diseases further complicate the outcome of these patients including congestive heart failure, liver disease, malignancies, and chronic obstructive pulmonary disease [11] as well as preventable causes that are derived from surgical procedures and prolonged hospitalization [12].

Kidney is a highly vulnerable organ, and the etiology of AKI is of multiple origins. Nevertheless, in the majority of situations, renal parenchyma integrity is disrupted either in terms of hypoperfusion that ends up in renal tubular dysfunction or by direct damage from "toxins" that further injure kidney's interstitial tissue and cellular functions [13]. Oxidative stress gives rise to a chain-like response through direct production of reactive oxygen species (ROS) and metabolic products that act as ligands for receptor types (i.e., toll-like receptors) whose activation is the "alarm" for an ongoing harmful process in AKI. Those circulating "toxins" are inflammatory products that mediate the expansion of injury and hemodynamic imbalance [14]. Critical illness is interwoven with acute inflammation and the consequent production of ROS that feed oxidative stress response. Albeit etiology (hemodynamic dysregulations, infections, rhabdomyolysis, cardiorenal syndrome, uremia, inadequate clearance of metabolism products, etc.), AKI and oxidative stress preserve a bidirectional relationship in critically ill patients. The purpose of this article is to present the main pathophysiologic mechanisms of AKI in ICU patients focusing on the different aspects of oxidative stress generation, the available evidence of interventional measures for AKI prevention, biomarkers used in a clinical setting, and future perspectives in oxidative stress regulation.

1.1. Oxidative Stress and Its Pathogenetic Role in AKI. Oxidative metabolism constitutes a fundamental process for aerobic organisms in order to cover energy needs and respond to emergency metabolic situations [15, 16]. Under normal circumstances, the balance between oxidants and antioxidant production is retained in favor of homeostasis. Oxidative stress was introduced for the first time by Stahland and Sies in 1985 [17] and is briefly defined as the metabolic disturbances, such as increased production of oxidants that leads to the depletion of endogenous antioxidants with inadequate decompensation and ends up in cellular damage [15], dysfunction of proteins, and damage of DNA, lipids, and enzymes [17]. The quantification of oxidative stress can be approached only indirectly, by measuring by-products such as isoprostanes [18], malondialdehyde levels [19], and other protein damage markers [20] with techniques and results that have been questioned. On the other hand, endogenous antioxidant systems are self-defense mechanisms with a crucial participation in the maintenance of immune system integrity that are activated when oxidative stress cannot be counterbalanced [21]. When organisms sense a possible threat, they have the ability to delay metabolic processes and even enter cell cycle arrest in order to avoid further oxidative damage.

The pathophysiology of AKI constitutes a complex interplay among vascular, tubular, and inflammatory factors which is followed by a repair process that can either restore epithelial cells and physiological function or result in progressive fibrosis and chronic kidney damage. Abundant experimental and to a less extent clinical data support the important role of oxidative stress-related mechanics in the injury phase of AKI (Figure 1). The more extensively explored and better-established mechanisms of oxidative stress involved in the pathogenesis of AKI will be reviewed.

1.1.1. Reactive Oxygen Species (ROS) and Nitric Oxide (NO). Mitochondrion is the primary energy factory of the human body and is abundant in proximal renal tubule making renal cortex a crucial field of oxygen use for energy production. Moreover, in AKI, mitochondrial injury precedes other renal manifestations even the increase of serum creatinine levels [22]. The main source of ROS generation is the reduction of oxygen by cytochrome oxidase in mitochondrial electron chain transport (ETC) that results in the production of hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-), and hydroxyl radical (HO) [15]. There is no specific target

Table 2: Acute kidney injury stratification criteria.

AKIN		KDIGO		
Serum creatinine	Stage	Stage	Serum creatinine	Urine output
≥0.3 mg/dL increase or increase ×1.5–2 from baseline	**1**	**1**	×1.5–1.9 from baseline or ≥0.3 mg/dL increase	<0.5 mL/min/kg ×6–12 h
Increase ×2-3 from baseline	**2**	**2**	×2–2.9 from baseline	<0.5 mL/min/kg for ≥12 h
Increase > ×3 from baseline or sCreatinine ≥4 mg/dL with acute increase of at least 0.5 mg/dL	**3**	**3**	×3 from baseline or sCreatinine ≥4 mg/dL or renal replacement therapy or eGFR <35 mL/min/1.73m^2 in patients <18 yo	<0.3 mL/min/kg for ≥24 h or anuria for ≥12 h

AKIN: Acute Kidney Injury Network; KDIGO: Kidney Disease: Improving Global Outcomes; GFR: glomerular filtration rate; ESKD: end-stage kidney disease.

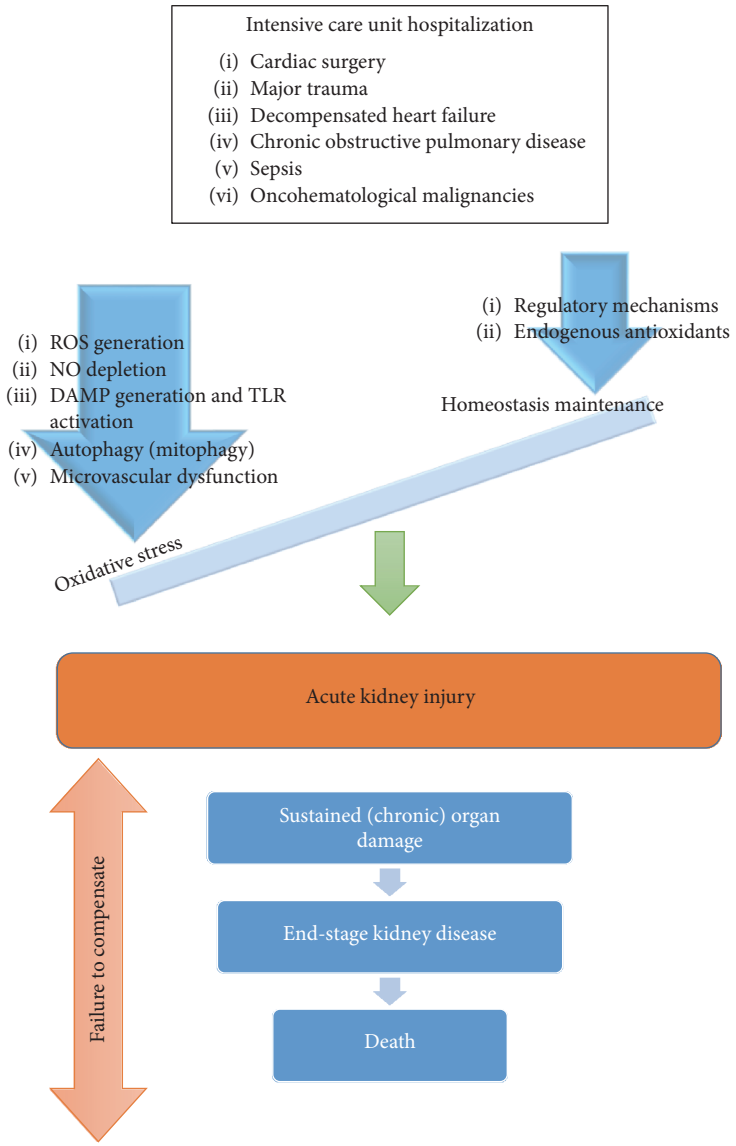

Figure 1: Progress of acute kidney injury in critical illness-associated oxidative stress. Critically ill patients in intensive care units suffer from multifactorial disorders that are added up against the potentiality of regulatory mechanisms to maintain homeostasis, leading to further imbalance in favor of oxidative stress generation through multiple pathogenetic pathways. Once this cataract leads to renal damage with the form of acute kidney injury, the prolonged exposure to oxidative stress environment leads to an uneventful outcome that ranges from chronic kidney disease to death. ROS: reactive oxygen species; NO: nitric oxide; DAMPs: danger-associated molecular patterns.

for ROS, but the attack on lipids, proteins, and amino acids results in the formation of unstable molecules that act as radicals and finally convert into compounds with multiple metabolic effects [16]. Consequently, lipid, protein, and nucleic acid peroxides belong in ROS family. Minor ROS generators (about 10% in total) are xanthine oxidase, NADPH oxidase complex (Nox), and adrenaline/epinephrine as well [15, 23].

Kidney receives about 25% of total blood supply and is rich in mitochondria that render it susceptible to damage from ROS and subsequent development of AKI [24]. Cellular apoptosis, lipid peroxidation, and imbalanced calcium concentration are few of the induced mechanisms by ROS [25]. Two characteristic representatives of AKI are ischemia reperfusion injury (IRI) and sepsis [24]. Aggressive fluid resuscitation for retaining hemodynamic balance may have adverse effects on renal function due to hemodilution and diminished oxygenation [25] but can be prevented through individualization and continuous therapy reassessment [26]. On the contrary, excessive oxygenation that leads to hyperoxia has been linked with further enhancement of ROS production and oxidative stress in patients with acute lung injury [27] and systemic inflammatory response (SIRS) [28].

The endothelial isoform of nitric oxide synthase (eNOS) is the main source of NO production from arginine and oxygen that is essential for the normal endothelial function and vascular tone, the prevention of platelet aggregation, and presenting anti-inflammatory properties [16, 25]. The "uncoupling" phenomenon is met when eNOS is deprived of its cofactors (i.e., calmodulin and tetrahydrobiopterin) and results in the oxidation of oxygen and the release of superoxide [29] that acts as a free radical adding on to oxidative stress. The above phenomenon takes place in inflammatory situations (such as sepsis) where there is an incremental cellular NO release (and oxygen consumption) and is mediated by the action of inducible nitric oxide synthase (iNOS). According to a theory, the heterogeneous iNOS expression in AKI that leads to focal increase of NO levels and is further enhanced by microcirculatory dysfunction results in the perpetuation of regional oxygen deprivation [30]. Thus, kidney damage not only is maintained but also expands. The iNOS-dependent inhibition of eNOS deteriorates endothelial function further shaping a triangle among ROS, NO, and oxygen [29, 30] in the pathophysiology of AKI and oxidative stress.

1.1.2. Toll-Like Receptors (TLRs) and Damage-Associated Molecular Pattern (DAMPs). TLRs are transmembrane, pattern recognition receptors, and currently, there have been about 10 recognized subtypes in humans [31]. DAMPs are endogenous molecules that may either initiate immune response or act as proinflammatory mediators (the latest are occasionally called alarmins) [32]. They are presented to the immune system after cellular lysis, scheduled exocytosis, or after the release of enzymes' matrix [33]. Apart from DAMPs, TLRs recognize pathogen-associated molecular pattern (PAMPs) (peptidoglycan and lipopolysaccharide from pathogens). Macrophages, endothelial cells, dendritic cells, and lymphocytes express TLRs. Kidney mesangial and tubular epithelial cells express TLR1, TLR2, TLR3, TLR4, and TLR6. Once a ligand is bind on the receptor, with the complicity of factors such as myeloid differentiation factor 88 (MyD88) and toll-receptor activator of interferon (TRIF), endogenous pathways are activated (nuclear factor kappa-B and mitogen-activated protein kinase pathway) and result in inflammation and interferon production [31, 34].

In 1994, Matzinger introduced the theory of "danger" that is sensed by the immune system, it does not necessarily originate from pathogens, and it has the ability to enhance or fire innate immune response so that the threat is sufficiently defeated [35]. DAMPs are the triggering factors for this process and come from endogenous, damaged cells, usually including proteins. In AKI, heat shock proteins (HSPs) and high-mobility group box-1 (HMGB-1) protein are the most common but several others have been suggested as well [33, 36]. According to accumulating evidence during oxidative stress, TLR activation from DAMPs further enhances the incremental release of the latest as it was shown with HSp70 and TLR2/TLR4 in an animal model during IRI [37]. On the contrary, the origin of HMGB-1 is not that clear. Evidence from in vitro studies in hypoxic hepatocytes is in favor of ROS regulation on HMGB-1 release with prerequisite functional TLRs [38]. As derived from the aforementioned evidence, there is an ambiguous relationship between DAMPs and TLR activation in oxidative stress. Also, the release of DAMPs is partly determined by TLRs who are the main regulators of overall immune answer in oxidative stress [33]. The magnitude of inflammation-oxidative stress complexity is yet to be revealed and translated.

1.1.3. Autophagy in AKI. Autophagy is a continuous, catabolic process conserved through evolution that takes place at a cellular level [39]. It is generally described as a "housekeeping" process and aims at the removal of damaged and dysfunctional molecules as well as at the enhanced response to acute situations such as nutrient deficiency, ensuring the recycling of components for protein and energy synthesis and the elimination of toxic material [40]. Fundamental for the initiation of autophagy is the expression of the autophagy-related genes (ATG) that were first discovered in yeast, with the produced proteins being subjected to multiple posttranslational modifications that regulate the final outcome [41]. According to evidence from animal models and clinical trials, the ATG proteins increase in AKI. In particular, ATG proteins that augment in AKI with tubular dysfunction are microtubule-associated protein light chain 3 (LC3) and Beclin-1 [42, 43]. The first step is the formation of an intracellular, double-membrane organelle called phagophore that after the sequestration of the target turns into autophagosome and with the subsequent lysosomal fusion becomes the autolysosome that with the intermediary action of lysosomal enzymes will lead to the degradation of the contained cytoplasmic components in order to provide matrix for "recycling" [44]. The process is complete after lysosomal reformation and the inhibitory effect on autophagy of mammalian target of rapamycin receptor (mTOR) [39, 40]. Nevertheless, there are pending issues regarding the further

clarification of the complicated signaling pathways in autophagy, their selectivity, and regulation [39].

In AKI, hypoxic damage in tubular epithelial cells is a potent stimulus for autophagy [45] that is generally considered beneficial and nephroprotective, preventing further structural compromise, especially at the S3 segment of the proximal tubule that is vulnerable to oxygen deprivation [46]. In AKI, apart from hypoxia, the increased ROS production due to inflammation and oxidative stress causes mitochondrial depolarization and dysfunction that through the PINK1/Parkin (PTEN-induced putative kinase protein 1) and the BNIP3/NIX/FUNDC1 pathway lead to selective mitochondrial autophagy ("mitophagy") [24, 44]. Contradictory opinions exist and claim that autophagy can be deleterious promoting cellular apoptosis, adding on to the renal injury [47–49].

1.1.4. Microvascular Dysfunction. Under normal circumstances, outer medulla is perfused with about half blood flow compared to cortex and the consequent partial oxygen pressure is 10–20 mmHg and 50 mmHg [50]. Thus, outer medulla is an especially vulnerable zone to circulatory disturbances and hypoxia. During AKI, the sustained renal perfusion through normal blood flow from renal artery does not secure the unhampered function of the complex renal microvasculature. Evidence data prove the existence of focal hypoxemic renal tissue in AKI [51] that add on to our comprehension regarding the pathophysiology of AKI [52]. In oxygen deprivation, anaerobic glycolysis is enhanced, lactic acid is accumulated, mitochondrial dysfunction is enhanced, and production of ROS and superoxide is upregulated [53]. The injury expands after reperfusion that is characterized by inflammatory response with leukocyte and complements activation that progresses to an oxidant environment that cannot be counterbalanced by antioxidant mechanisms [54] and uneventfully leads to excessive cell death [53].

Endothelium holds a crucial role regarding the expansion of inflammation, through expression of adhesion molecules such as selectins [55], the intracellular adhesion molecule-1 (ICAM-1) [56], and CX3CL1 (fractalkine) [57] that regulate inflammatory cell recruitment. The effect on vascular wall, along with the partly specified changes on glycocalyx [58], is increased permeability that in AKI, is expressed as proteinuria [58, 59].

1.2. Prediction of AKI by Oxidative Stress Biomarkers in Critically Ill Patients. A number of obstacles have hampered the investigation of the role of oxidant injury in multiple organ failure and AKI in critically ill patients. Among them is the fact that oxidative stress might be a focal, instant response resulting to the lack of stable, specific oxidative stress biomarkers that can be measured accurately and noninvasively in these patients [16]. Nevertheless, prevention of AKI requires among others the recognition of high-risk patients and early diagnosis based on accurate predictive tools. After the recognition of serum creatinine inadequacy in the prediction of AKI due to the variability of measured levels (based on age, gender, race, and muscle mass) with low sensitivity and specificity [60], novel plasma and urine biomarkers have been introduced. In the meantime, along with the enhanced comprehension of novel biomarker characteristics (for details refer to [61–65]), there is accumulating evidence concerning the predictive value and the clinical applicability of these molecules.

Oxidative stress can be assessed by indirect methods which can measure the stable by-products of ROS activity on biomolecules. In the setting of critical illness, the most commonly measured markers of oxidative stress are isoprostanes, hydroxynonenal and lipid peroxides, chlorinated compounds, oxidized glutathione, nitrated and oxidized proteins, and malondialdehyde detected as thiobarbituric acid reactants (TBARs) [15]. Among them, some biomarkers have been investigated in order to predict the occurrence of AKI in severely ill patients with different results. In an observational cohort study in ICU patients with severe sepsis, Ware et al. found that plasma levels of F2-isoprostanes and isofurans were associated with acute hepatic, coagulation, and renal failure [66]. Liver-type fatty acid-binding protein (L-FABP) has been considered as an important cellular antioxidant during oxidative stress by maintaining low levels of free fatty acids in the cytoplasm of tubular cells through facilitation of intracellular metabolism and excretion in urine. In a number of studies, urine L-FABP has been able to reliably predict the occurrence of AKI and death in ICU patients [64]. Recently, Costa et al. found that erythrocyte superoxide dismutase (SOD1) activity could play a role as an early marker of septic AKI and could be seen as a new research avenue in the field of biomarker in AKI [67].

According to robust evidence, an AKI-specific biomarker is the neutrophil gelatinase-associated lipocalin (NGAL) that can be measured in both plasma and urine [68, 69]. NGAL is a multifaceted protein that is rapidly induced and released from the injured distal nephron—among others. It has the ability to scavenge iron whose role is crucial for bacterial survival and is an important component to free radical generation. Thus, NGAL levels have been implicated in various types of organ injury, including myocardial infarction, cancer, sepsis, and AKI [70]. Apart from bacteriostatic effects [71], the protection against oxidative stress damage has been suggested [71, 72], while the exact antioxidative mechanisms of NGAL are still under question. There are data in favor of the upregulation of endogenous antioxidants such as SOD1 and SOD2 as well as HO1 levels [73, 74]. According to a 2009 meta-analysis in 8500 critically ill patients, the area under the curve (AUC) for the prediction of AKI (12 hours earlier) reached 0.85 for plasma NGAL and 0.86 for urine NGAL, superior to the predictive value of serum creatinine levels and eGFR, with sensitivity of 81–96% and specificity of 51–68% [75]. Urinary kidney injury molecule-1 (KIM-1) and interleukin-18 (IL-18) are suggested as good markers for the prediction of progressive AKI [75]. The high diagnostic value of IL-18 in AKI (odds ratio (OR): 5.11, AUC: 0.77) [76] is not corroborated by equal prognostic significance in critically ill patients [77], and the careful interpretation of urine IL-18 levels is highly recommended. KIM-1 was attributed with a good prognostic value of AKI development after cardiac surgery with high sensitivity 92–100% and AUC 0.78–0.91 [78], while the persistent elevation of urine KIM-

1 levels might correlate with poor prognosis [79]. What should be mentioned is that NGAL, IL-18, and KIM-1 are inflammatory mediators that increase in inflammatory situations regardless of the presence of AKI and are indivisible parameters concerning their assessment in the prediction of AKI [65].

Recently, tissue inhibitor of metalloproteinase 2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP-7) have been investigated as predictive urine biomarkers of AKI in high-risk patients [80, 81]. Both TIMP-2 and IGFBP-7 are cell cycle arrest biomarkers as they have been implicated in the G1 cell cycle arrest phase during the very early stages of cellular stress. It has been shown that renal tubular cells go through this G1 cell cycle arrest phase following stress due to a number of different causes. Specifically, the SAPPHIRE study assessed the urine product of TIMP-2 and IGFBP-7 and concluded that it is superior in the prediction of KDIGO stage 2-3 AKI compared with the rest of the biomarkers of the study, even NGAL and KIM-1 ($p < 0.002$) in critically ill patients [80]. Further analysis in the SAPPHIRE and OPAL study cohorts has set cut-off values for risk stratification of AKI with high-risk patients when TIMP-2 × IGFBP-7 is over 0.3 and the highest risk for patients with product value is over 2 [81]. Nevertheless, in persisting AKI that is equal with the ongoing damage, the levels of TIMP-2 × IGFBP-7 product remain elevated indicating the maintenance of cell cycle arrest (in G1 phase) that may uneventfully lead to failure of recovery and renal fibrosis [82]. Thus, the potential selective intervention in the activation and disruption of cell cycle might be beneficial for renal protection.

1.3. Clinical Evidence in AKI Prevention by Targeting Oxidative Stress. Albeit evidence for the role of oxidative stress in the pathogenesis of AKI originating mainly from experimental models and distinctive pathways remains obscure, the idea that controlling oxidative stress in patients with AKI may prevent or attenuate the severity of cellular injury has been explored in the clinical setting. Existing clinical evidence in this field, regarding critically ill and ICU patients, comes from small cohorts and studies. Nevertheless, the scavenging of free radicals in order to avoid the provocation of chain reactions that will lead to regional or generalized oxidative stress demonstrates great interest.

Anesthetics have been suggested as potential oxidative stress scavengers and in particular SOD mimetics (sodium pentothal and propofol) and lidocaine, when used in critical care practice [83]. N-acetylcysteine (NAC) as shown by in vitro studies acts as a direct scavenger of OH^- mainly, but when administered orally, the bioavailability is low and even untraceable. The antioxidant action of NAC is mediated by the induction of glutathione synthesis [84]. Data from trials in humans imply that NAC reduces the incidence of AKI after contrast media administration ($p = 0.02$) [85], but the direct intravenous administration of glutathione has been shown to be superior as regards renal protection against contrast-induced nephropathy compared to NAC per os [86].

Apart from the first-line treatment in lipid-lowering therapy, HMG-CoA reductase inhibitors, globally known as statins, participate further in vascular endothelium function preservation through upregulation of eNOS, thus increasing the available NO and contribute to the restriction of free radical generation from lipids' oxidation [87, 88]. In this direction, results from cohort studies concerning severe illness are in favor of the benefits of statins in the protection of renal function after percutaneous coronary angiography [89], acute coronary syndrome [90], and IRI [91]. On the contrary, a Cochrane database meta-analysis on the prevention of AKI with statin administration prior to major surgery failed to show reduction in AKI incidence for critically ill patients undergoing surgery with cardiac bypass [92].

Ischemic preconditioning (IPR) was introduced in 1986 by Murry et al. in an animal model that sustained brief ischemic episodes before a major ischemic event and resulted in a beneficial outcome for the organ [93]. In 1993, Przyklenk et al. described a slightly different model of ischemic preconditioning (remote and rIPR) [94] that has been further modified and is currently followed, when the direct approach to the involved organ is not feasible. The underlying mechanisms are notably complex and not totally unraveled. In brief, after the main stimuli (ischemia) is withdrawn, a series of responses take place (neural, humoral pathway, and systemic anti-inflammatory response) with the final receiver being the mitochondrion [95]. The subsequent opening of the ATP-dependent mitochondrial potassium channel prevents the opening of the mitochondrial permeability transition pores (MPTP) that enhances the stability of its membrane [96] and the survival after IRI [97].

Generally speaking, rIPR concerns clinical practice and especially critical care when it comes to scheduled procedures that carry a significant burden for homeostasis and are closely related with the induction of systematic inflammation and oxidative stress, such as cardiac surgery procedures. The highly vulnerable to hemodynamic imbalance renal cortex and its complex microvasculature are affected by rIPR. According to a recent review (2016) by Ho et al., who included 17 clinical trials that examined the renal outcome in different rIPR cases, a notable renal protection is shown in 12/17 of the trials with no significant deviations in the rest of the trials [98].

1.4. Therapeutic Interventions and Future Perspectives. In the current clinical practice, there is a lack of standardized preventive measures against AKI in severely ill patients apart from general suggestions for maintenance of fluid and electrolyte balance, avoidance of unnecessary exposure to potentially nephrotoxic agents, and continuous clinical monitoring [99, 100]. Earlier efforts to show benefit in renal outcome in critical care setting through administration of low-dose dopamine in continuous infusion have shown a temporary benefit in urine output [101], but with no significant protection against the development of AKI, the prevention of RRT, and mortality according to meta-analyses [102–104]. In the same patient group, fenoldopam seems to be superior compared to dopamine in the improvement of serum creatinine levels when renal dysfunction is present [105] and according to a meta-analysis in 1290 patients,

fenoldopam administration reduced the need for RRT support and intensive care unit hospitalization [106].

As regards interventional measures in order to control oxidative stress response in critically ill patients, the interest has been focused on macro- and micronutrients and the correlation of their levels with patients' general clinical course and outcome and not just with the prevention or therapy of AKI. The early recommencement of enteral versus parenteral feeding in ICU patients (even before 48 hours of hospitalization) that contributes on the maintenance of normal intestinal microflora has been correlated with better survival and less infections [107]. Nevertheless, the optimal dose that permits autophagy and provides the highest benefit for ICU patients has not been quantified yet [107, 108]. The supplementation of trace elements in critically ill patients has employed investigators and in particular the administration of thiamine, vitamin C and E, and selenium separately has been found to improve survival and reduce infectious complications [109]. On the contrary, no clear benefit on critically ill patients' survival was shown in a meta-analysis of 4 trials with zinc administration, neither a benefit in the duration of ICU stay [110]. In a meta-analysis of 21 randomized control trials, it was shown that the supplementation of trace elements correlated with reduction in the number of days with need for mechanical ventilation, but the establishment of clear conclusions regarding the best possible route of administration (enteral versus intravenous) was not feasible due to significant heterogeneity of the available data [111]. Even if this replenishment concerns relatively short periods, toxicity [109, 110] is to be kept in mind and appropriate measures should be applied in order to avoid it. In general, the substitution of more than 66% of the recommended daily allowance of vitamins A, C, and E has been shown to improve antioxidant capacity [112]. REDOXS (Reducing Deaths due to Oxidative Stress) study is a blinded randomized trial in 1223 critically ill patients that failed in meeting its original rationale and concluded that the administration of antioxidants and glutamine increased mortality [113]. Among the possible reasons are the doses chosen of the implemented therapeutic strategy and the potential toxicity that may have defined the final outcome [114]. Enteral administration of melatonin [115] and parenteral administrations of NAC plus deferoxamine [116] have been correlated with better total antioxidant capacity (TAC) in serum. As derived from the presented data, we are not yet capable to reach safe conclusions with clinical applicability as regards the initiation, dose, route, and duration of therapy for the aforementioned strategies.

As regards future perspectives, antioxidants targeted to mitochondria have been developed and the main axis of their action is through the electrical potential and the pH gradient of the mitochondrion that leads to the selective accumulation of these cationic molecules. Mito-vitE, MitoQ, MitoPBN, and MitoPeroxidase have the ability to prevent ROS generation and enhance mitochondrial survival [117–119]. The optimization of understanding the mechanisms of action has gained a lot of interest, as well as the enhancement of their chemical synthesis [120]. Unfortunately, current literature lacks in vitro or in vivo studies investigating the administration of antioxidant targeted molecules.

2. Conclusions

Acute kidney injury is a multifactorial clinical entity representing a major health problem. In critical care, AKI remains highly prevalent, complicating the clinical course of patients, extending the need for ICU hospitalization, requiring RRT, and carrying high mortality. Pathogenesis of AKI is complex and remains incompletely elucidated. Oxidative stress is involved in the pathogenesis of AKI and is characterized by complex, codependent mechanisms that progress to organ response and damage. More extensively, main experimentally explored mechanisms of oxidative stress involved in AKI summarize to ROS generation, NO depletion, DAMP generation and TLR activation, autophagy, and microvascular dysfunction. These mechanisms prevail over endogenous antioxidants and regulatory mechanisms so that physiological homeostasis is abolished and AKI is finally installed.

Prevention of AKI is essential and requires among others the recognition of high-risk patients and early diagnosis based on accurate predictive tools. In the setting of critical illness, the most commonly measured markers of oxidative stress are isoprostanes, hydroxynonenal and lipid peroxides, chlorinated compounds, oxidized glutathione, nitrated and oxidized proteins, and TBARs. Novel AKI-specific biomarkers available are NGAL, KIM-1, and levels of TIMP-2 × IGFBP-7 with accumulating evidence being in favor of their diagnostic and prognostic value. Further progress that will encompass in daily practice techniques allowing more accurate assessment of oxidative stress will further improve the prevention of AKI in critical care. Therapeutic interventions trying to control oxidative stress response in critically ill patients have been focused on macro- and micronutrients. Currently, there are encouraging results from the inhibition of oxidative stress via exogenous administration of antioxidants and methods as ischemic preconditioning, but no standardized therapeutic protocols exist. The role of antioxidant therapy requires further elucidation and attention in the care of critically ill patients and in AKI. The meticulous study and interpretation of available observational data and expansion of existing knowledge through well-designed interventional studies in the setting of critical illness are necessary.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Sepsis-Induced Cardiomyopathy: Oxidative Implications in the Initiation and Resolution of the Damage

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Cardiac dysfunction may complicate the course of severe sepsis and septic shock with significant implications for patient's survival. The basic pathophysiologic mechanisms leading to septic cardiomyopathy have not been fully clarified until now. Disease-specific treatment is lacking, and care is still based on supportive modalities. Septic state causes destruction of redox balance in many cell types, cardiomyocytes included. The production of reactive oxygen and nitrogen species is increased, and natural antioxidant systems fail to counterbalance the overwhelming generation of free radicals. Reactive species interfere with many basic cell functions, mainly through destruction of protein, lipid, and nucleic acid integrity, compromising enzyme function, mitochondrial structure and performance, and intracellular signaling, all leading to cardiac contractile failure. Takotsubo cardiomyopathy may result from oxidative imbalance. This review will address the multiple aspects of cardiomyocyte bioenergetic failure in sepsis and discuss potential therapeutic interventions.

1. Introduction

Myocardial depression may develop in patients with severe sepsis and septic shock, complicating the course of their disease. There are reports that it may develop in nearly 60% of septic patients [1]. Parker et al. were the first to describe this entity in 1984 [2]. Sepsis-induced cardiomyopathy is characterized by the presence of left ventricular dilation with normal or low filling pressures and decreased ejection fraction. Characteristically, the syndrome has a reversible character, beginning to normalize within 7–10 days of onset [3]. Importantly, sepsis-induced cardiac dysfunction has a negative impact on patient's survival [4].

The exact pathophysiologic mechanisms, ultimately leading to cardiac dysfunction, are not well clarified. Endotoxins and inflammatory cytokines seem to play a key role in the genesis of myocardial depression. Moreover, hypoxia and acidosis, hypotension and hypovolemia, metabolic disturbances, coagulation abnormalities, and increased production of reactive oxygen and nitrogen species (ROS and RNS) have been proposed to participate in myocardial depression during sepsis [5]. On the other hand, hypotension/hypoperfusion does not appear to be the key mechanism in the genesis of myocardial dysfunction. Measurements of coronary artery-coronary sinus oxygen content difference revealed a reduced value, pointing out the oxygen utilization problem rather than oxygen delivery [6]. In the late phases of sepsis, tissue oxygen tension is increased emphasizing that the major problem is oxygen utilization, a condition known as cytopathic hypoxia [7]. The majority of body oxygen is taken up by the mitochondria and is used for energy production (in the form of adenosine triphosphate (ATP)). Thus, it appears that these organelles may play a pivotal role in the pathogenesis of sepsis-induced organ dysfunction. Oxidative stress has well been implicated in sepsis in humans, having been found to correlate with the severity of the disease and mortality [8, 9]. Reactive oxygen and nitrogen species are produced in excess and have been implicated in the genesis of sepsis-induced myocardial dysfunction [10–16]. Imbalance in oxidative status leading to overproduction of reactive

oxygen species (ROS) on the one hand and nitric oxide (NO) and its toxic derivative, peroxynitrite, on the other are major contributors to myocardial injury [15, 17].

In the present review, we will focus on the role of reactive oxygen and nitrogen species in the generation of myocardial dysfunction in sepsis. Moreover, we will review current treatment options targeting oxidative stress imbalance, responsible for cardiomyopathy in sepsis.

1.1. Search Strategy. Data for this review were collected by searching PubMed and from references of the related articles. We attempted a comprehensive research in PubMed, through April 2017, using the terms "septic cardiomyopathy and reactive oxygen species," "septic cardiomyopathy and mitochondrial dysfunction," "reactive oxygen species and heart and sepsis," "redox and heart and sepsis," "mitochondria and heart and sepsis," and "energy metabolism and heart and sepsis." The search was limited to publications in English. In addition, we searched the online registry of randomized controlled trials of the US National Institutes of Health (http://www.clinicaltrials.gov) and the Current Controlled Trials website (http://www.isrctn.com) for ongoing investigations regarding this subject using the aforementioned terms. Seven hundred and ninety studies were initially found: 667 of them were excluded after abstract review because they were irrelevant. We focused on the rest 127 clinical studies which evaluated the relationship between oxidative stress and the myocardial dysfunction in sepsis.

2. Reactive Species

There is evidence that during sepsis, there is increased production of free radicals from cardiomyocyte mitochondria which depresses myocardial function. A free radical is a molecule characterized by the presence of one or more free electrons in the outer orbit. The presence of these electrons gives the molecule great instability, making it highly reactive and toxic. The reactivity of different free radicals varies, but some can cause severe damage to biological molecules, especially to DNA, lipids, and proteins [18]. Oxygen containing free radical molecules and their precursors formed in biological systems are collectively termed reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH). On the other hand, NO produced from nitric oxide synthases (NOS) may react with free radical of oxygen forming peroxynitrite ($ONOO^-$), a molecule supposed to be the NO toxicity mediator, exhibiting multiple inhibitory actions in the mitochondrial respiratory chain [19, 20]. In a redox balance, reactive species play an important role in the life cycle of cells, the induction of cell signaling pathways, the activation of intra- and intercellular secondary messengers, and immune cell defense mechanisms. RNS are also involved in the regulation of blood pressure and vascular tone, activation of NF-kB, release of inflammatory cytokines, and expression of adhesion molecules [18, 21–23]. Oxidative stress occurs when the production of ROS and RNS lays beyond antioxidant protection mechanisms, leading to mitochondrial failure.

Mitochondria, which are placed in the cytoplasm of the cardiomyocyte, are the main sources of energy supply through oxidative phosphorylation. Sepsis diminishes the total capacity of the respiratory chain leading to energy imbalance. During oxidative phosphorylation, a small amount of O_2^- (superoxide anion) is produced, which is scavenged to generate H_2O_2 by Mn-superoxide dismutase (MnSOD), one of the major antioxidant systems in cells. On the other hand, mitochondria are one of the major organelles that initiate and sustain energy imbalance in the cardiomyocyte during sepsis, leading to myocardial dysfunction [24].

2.1. ROS Production in Septic Hearts. Endotoxins, produced during sepsis, are capable of inducing ROS production by the mitochondria [25, 26]. In cardiomyocytes, endotoxin has been shown to induce the production of superoxide, hydrogen peroxide, and hydroxyl radical through xanthine oxidase, NADH/NADPH oxidases, and mitochondria [27–29]. ROS generation by the mitochondria further stimulates ROS production in endothelial cells, triggering a vicious cycle of free radical production resulting in a wide variety of reversible and irreversible toxic modifications on biomolecules [30–32]. ROS production leads to ultrastructural and functional changes in mitochondria, some of them being reversible during the recovery phase of sepsis, others causing irreversible mitochondrial failure leading to multiple organ dysfunctions (mechanisms summarized in Figure 1) [33–35]. NADPH oxidases consist of a membrane-bound catalytic subunit (NOX) and a number of cytosolic regulatory subunits, which have been found to increase their activity in response to sepsis (lipopolysaccharides, LPS) [28, 36, 37]. Enhanced NO and superoxide production and thus peroxynitrite occur in dysfunctional hearts from rats, while increased levels of the lipid peroxidation product, malondialdeyde (MDA), have also been found indicating the role of underlying oxidative stress in septic hearts [35, 38]. Moreover, using an animal model, it was shown that ROS production correlated with the increase of a NADPH subunit (NOX_1) mRNA, leading to increased cardiomyocyte apoptosis, while animals deficient of this subunit did not present increased rates of apoptosis. Furthermore, the same study showed that after LPS infusion, there was a significant reduction in heart performance (indicated by the fraction shortening (%FS)), as well as an increase in left ventricular end systolic diameter, indicating myocardial contractile dysfunction [39]. Noteworthy, there is evidence suggesting that the severity of mitochondrial dysfunction correlates with the severity of sepsis [37].

2.1.1. ROS Production Affects Proteins, Lipids, and DNA

(1) Enzyme Dysfunction. ROS and RNS lead to lipid peroxidation, protein oxidation, and nitration and DNA fragmentation. Therefore, oxidative stress imbalance may compromise the integrity of cell membranes and may affect enzyme function and gene expression. ROS and RNP overproduction by the mitochondria has been found to inhibit oxidative phosphorylation, thus resulting in decreased production of ATP [40]. The state where mitochondria cannot

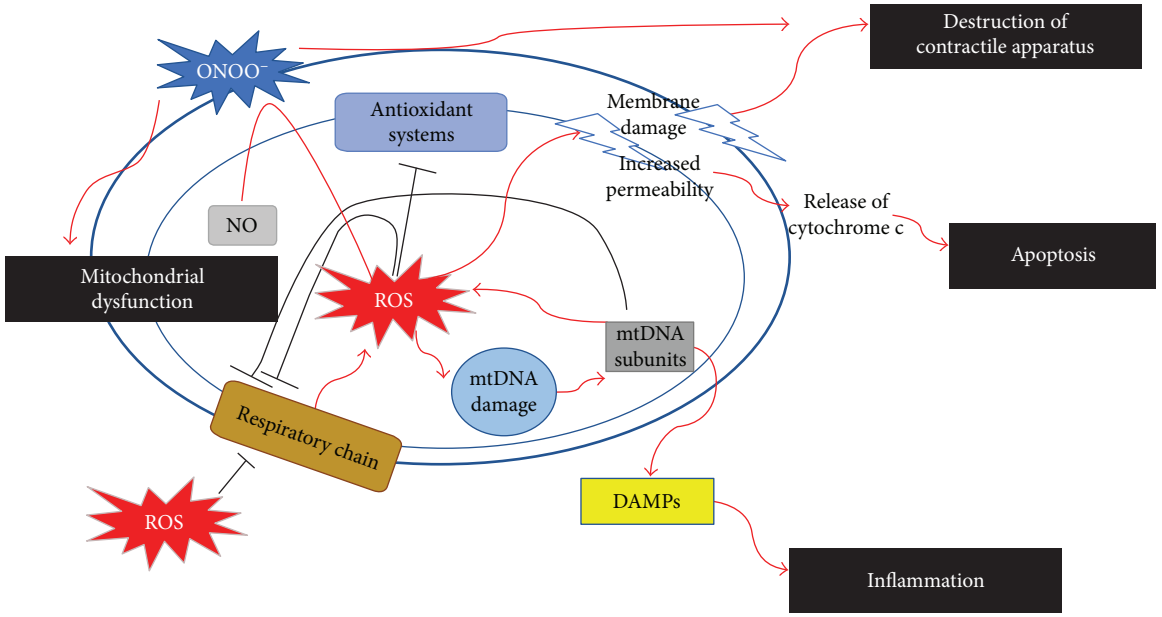

Figure 1: Oxidative damage in septic cardiomyocytes. In the presence of ROS, electron flow through the respiratory chain is impaired leading to further production of ROS. Mitochondrial ROS production leads to oxidative damage to proteins, lipids, and DNA subsequently leading to further mitochondrial dysfunction, apoptosis, destruction of the contractile apparatus, and promoting inflammation. NO reacts with ROS to generate $ONOO^-$, the cytotoxic product of NO. ROS: reactive oxygen species; mtDNA: mitochondrial DNA; DAMPs: danger-associated molecular patterns; NO: nitric oxide; $ONOO^-$: peroxynitrite.

utilize the delivered oxygen is known as "cytopathic hypoxia." This condition is verified by the decreased oxygen content difference between the coronary arteries and coronary sinus, previously found (indicating reduced cell utilization of oxygen in the abundant presence of oxygen molecules). "Cytopathic hypoxia" is the most important step in the development of multiorgan failure in sepsis [6, 41, 42]. Endotoxin administration in animals results in reduction in mitochondrial state 3 respiration rate and reduces ATP production, while oxygen consumption decreases. All these result in reduced cardiac pressure-generating capacity [38]. Animal models with sepsis presented inhibited electron flow through complexes I, II, and III of the electron transport chain and inhibition of oxidative phosphorylation and ATP generation [35]. Moreover, mitochondrial dysfunction alters myocyte contractility and its electrical properties and leads eventually to cell death [43]. Smeding et al., reviewing literature on cardiac structural alterations during sepsis, concluded that the impairment in cardiac mitochondrial function correlated with decreased cardiac contractility [44]. Matkovich et al. showed that there is wide downregulation (up to 50%) of cardiac mitochondrial genes during sepsis, with the majority of genes coding for members of the electron transport chain and almost every step of the Krebs tricarboxylic acid. Interestingly, they also found decreased expression levels of genes encoding major proteins of the cardiac sarcomere and the excitation-contraction coupling process in septic cardiomyopathy [45].

Oxidative and nitrative stress can lead to activation of the nuclear enzyme poly (adenosine 5′-diphosphate [ADP]-ribose) polymerase (PARP) with subsequent loss of left ventricular systolic work index [46].

(2) Lipid and Protein Oxidation. ROS overproduction induces lipid oxidation, further compromising the integrity of membranes. In a time-course study in a rat model of pneumonia-related sepsis, Zang et al. found that sepsis produces progressive oxidative mitochondrial damage in the heart, as confirmed by mitochondrial outer membrane damage and release of cytochrome c [47]. They also found greater lipid and protein oxidation, following downregulation of the activities of antioxidant enzyme SOD and GPx in mitochondria [47]. Cytochrome c release from mitochondria is initiated by ROS-mediated peroxidation of cardiolipin, a phospholipid component of the mitochondrial inner membrane [48]. Cardiolipin oxidation leads to mitochondrial transition pore opening and dissociation of cytochrome c. Cytochrome c is released to the cytosol, activating caspase 9 and subsequently caspase 3 and 7, being responsible for the biochemical and morphological change characteristic of apoptosis [49–51].

Oxidative damage to lipids and proteins is responsible for structural myocardial changes and responsible for the clinical presentation of septic cardiomyopathy. These changes seem to precede phenotypic changes that characterize septic cardiomyopathy. There is evidence of sarcolemma damage leading to increased plasma membrane permeability as an early event in cecal ligation and puncture- (CLP-) induced severe sepsis in mice [14]. Increased sarcolemma permeability indicates functional impairment of the dystrophin glycoprotein complex (DGC) in severe sepsis. Of the DGC proteins, dystrophin forms a strong mechanical link between the sarcolemma and costameric cytoskeleton in the cardiac muscle cells, providing structural stability to the cell membrane and the sarcolemma against stresses generated during muscle

contraction [52]. Furthermore, sepsis mitochondrial damage is probably the triggering factor leading to myocardic cell vacuolation indicating apoptosis in the myocardium [53]. Other studies have confirmed loss of mitochondrial structure integrity, as a response to septic stimuli, such as derangements of mitochondrial cristae and mitochondrial matrix edema [46, 54].

(3) ROS Production Initiates Inflammatory Responses. Mitochondrial damage resulting from imbalanced production of ROS (mtROS) impairs mitochondrial structure on the one hand and mitochondrial biogenesis via oxidative modifications on macromolecules, such as mitochondrial DNA (mtDNA), on the other. Mitochondrial structures (mtROS, mtDNA, cytochrome c, ATP, and cardiolipin) are increasingly recognized as regulators/promoters of inflammation, as they function as danger-associated molecular patterns (DAMPs) (mechanisms summarized in Figure 1) [55–61]. In animal models, it was seen that after the induction of sepsis, almost half of mtDNA loses its integrity, being dependent on mtROS signaling. MtROS mediate reduction of mitochondria-located mtDNA repair enzymes, also inducing mitochondrial functional deficiency and structural impairment in the heart. In the same study, mtROS-mediated mtDNA damage increased the expression of MYD88 and RAGE, both being implicated in promoting cytokine production through a cytosolic DNA-TLR9-dependent signaling pathway, inducing downstream inflammatory responses [62].

Mitochondria's implication in the regulation of inflammation is assumed by the activation of nuclear factor-kappa B (NF-kB), a crucial mediator of apoptosis. Activation of NF-kB is associated with its translocation from the cytosol to the nucleus. Septic challenge decreases cytosolic with a simultaneous increase of nucleic NF-kB, indicating activation of this molecule [47, 53]. The mitochondrial matrix proteins MAVS and DOC-4 were identified as signaling factors that regulate NF-kB activation, and changes in mitochondrial Ca^{2+} have been suggested to modulate cytokine production in cardiomyocytes [63–65]. Inflammatory reactions in the septic heart increase progressively but not before the changes in cardiac mitochondria. This finding suggests that sepsis produces a cascade of myocardial mitochondrial damage, namely, mitochondrial release of cytochrome c, damage to the mitochondrial outer membrane, increase in lipid and protein oxidation, and decrease in mitochondrial ROS defenses, followed by progressive myocardial inflammation and late cardiac dysfunction [47].

(4) Intracellular Signaling. Many extracellular stimuli recognized by cells involve a complex intracellular signaling network; the most important of which being mitogen-activated protein kinases (MAPK). MAPK are sensitive to reactive oxygen species, being activated by NADPH oxidase [66]. The most extensively studied members of the MAPK are extracellular signal-regulated kinase 1/2 (ERK 1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK). Activation of these members has been found to be implicated in the genesis of circulatory shock, while, via activation of other inflammatory enzymes (such as COX-2), overproduction of prostanoids may be involved in the myocardial dysfunction associated with sepsis [67–69].

2.2. Role of NO and Peroxynitrite. The role of high NO concentration in the septic heart is still, as yet, controversial. NO is an important bioactive substance which plays an important role in the regulation of normal body function and disease occurrence. It is thought of as a signaling molecule with a multitude of biological actions and targets. It has a half-life of a few seconds and is produced in many cell types within the heart [70]. NO synthesis is activated by one of the three isoforms of NOS that catalyze NADPH-dependent oxidation of l-arginine to NO and l-citrulline: NOS_1 (neuronal or nNOS), NOS_2 (inducible or iNOS), and NOS_3 (endothelial or eNOS) [71]. All three isoforms are found in cardiomyocytes. NO plays multiple roles in cardiac physiology in health and disease [70]. It results in vasodilation (including coronary arteries), suppresses mitochondrial respiration (regulatory control), and regulates the release of proinflammatory cytokines. It regulates adhesion and aggregation of platelets and smooth muscle cell proliferation, thus functioning as a cardioprotective substance [72–74]. Apart from coronary vasodilation, NO may increase ventricular compliance, resulting in increased cardiac preload and myocardial blood supply [75]. Furthermore, NO may serve to restore myocardial function by promoting de novo synthesis of mitochondrial proteins. Additionally, by reducing oxygen consumption, NO preserves calcium sensitivity and contractile function, contributing to hibernation in response to myocardial ischemia [76, 77]. However, excessive formation of NO plays a central role in septic shock and has been found to contribute to contractile dysfunction [4, 78]. Increased oxidative stress, impairment in oxidative phosphorylation function, and a decrease in ATP production were restored by genetic deletion of iNOS (iNOS −/− mice). Moreover, inhibition of iNOS by melatonin prevented the impairment of mitochondrial homeostasis after sepsis and, finally, improved survival [79, 80].

Studies of animals subjected to endotoxemia have demonstrated that NO production, production of O_2^- and H_2O_2, global protein nitration, nitrotyrosine content, protein carbonylation, and lipid peroxidation are increased in cardiac mitochondria [81–83]. Nitric oxide (NO) and superoxide (O_2^-) rapidly react to form the toxic product peroxynitrite anion ($ONOO^-$) [84]. $ONOO^-$ is a crucial pathophysiological event which occurs during sepsis, since it represents a critical cytotoxic factor in oxidative stress-mediated tissue damage, supposed to be the NO toxicity mediator, inhibiting in multiple ways the mitochondrial respiratory chain [19, 20].

Peroxynitrite is able to enter the cell membrane and consequently oxidize multiple target molecules, either directly or through the generation of reactive radicals, resulting in structural modification and dysfunction of lipids, proteins, and nucleic acids. They can disrupt DNA integrity, impair the activity of ion channels, break down the mitochondrial respiratory chain, and induce cell death [85]. Several animal models have demonstrated the implication of peroxynitrite

in sepsis and in septic cardiomyopathy as well. It has been shown that LPS-treated mice are under oxidative stress and reactive oxidative species, such as superoxide, and peroxynitrite is mainly involved in the oxidative stress formation [86]. Endotoxemic shock is accompanied by a marked increase in mtNOS activity in the heart, leading to increased production of NO, O_2^-, H_2O_2, and $ONOO^-$, causing mitochondrial dysfunction and contractile failure [87]. Many of these oxidative radicals such as superoxide, nitric oxide, and peroxynitrite have been demonstrated in septic hearts in animal models, whereas the latter may be responsible for cardiovascular alterations met in septic shock, such as vascular hyporeactivity, myocardial impairment, and energetic failure [88]. Moreover, increased expression of peroxynitrite participates in the fall of blood pressure, endothelial injury, multiple organ dysfunction, and subsequent death, as was depicted in rats treated with LPS [82, 83, 89]. Endogenous formation of peroxynitrite induces cytotoxic effects in myocardic cells, which, in turn, decreases the ability of the heart to convert ATP into mechanical work, leading to myocardial contractile dysfunction [90] (mechanisms summarized in Figure 1).

Recently, it was shown that animals with preexisting cardiac disease (atherosclerosis) presented impaired ventricular dilation (the relaxation time constant τ decreased while dp/dt_{max} increased) and preserved systolic function (unchanged ejection fraction), after the induction of faecal peritonitis. Cardiac nitrotyrosine formation, a well-established marker for both augmented oxidative and nitrosative stress, increased [91].

In humans, there is evidence for significant presence of peroxynitrite in myocardial specimens from septic patients who have died, and it has been shown that septic hearts demonstrate peroxynitrite-induced protein nitration and activation of the proteolytic ubiquitin-proteasome pathway [92–94]. The most abundant proteins for nitration modification within cardiac myocyte are actin and myosin. The observation of scattered foci of actin and myosin filament disruption in septic hearts supports the idea that tyrosine nitration could potentially decrease myocardial contractility by directly modifying the contractile apparatus [93]. In another series of biopsies obtained from septic patients, it was shown that peroxynitrite is overproduced in the heart of septic but not control patients and the inducible isoform of NOS (NOS-2) is overexpressed in the left as well as the right ventricle and both atria. This study also demonstrated that peroxynitrite-induced tyrosine nitration, which has been shown to alter contractile protein function, leads to contraction and relaxation alterations in septic hearts [92].

2.3. Takotsubo Cardiomyopathy. Takotsubo cardiomyopathy, also known as stress-induced cardiomyopathy, is an acute syndrome characterized by reversible wall-motion abnormalities, triggered by an emotional or physical stressor, occurring in acute medical illness, such as sepsis, trauma, intracerebral haemorrhage or even postpartum [95–98]. The exact pathophysiological mechanisms leading to this entity are not well clarified, with catecholamines being the most appealing explanation leading to myocardial stunning [99]. Oxidative stress is a rising, not thoroughly evaluated, pathogenetic mechanism, implicated in the pathophysiology of the syndrome. Upregulation of HO-1 was observed in an animal model of stress-induced takotsubo cardiomyopathy. Cardiac-specific induction of OH-1 is cytoprotective against oxidative stress and has been found to restore ventricular function, protecting tissue from ischemia/reperfusion injury and postmyocardial infarct remodelling [100–102]. In takotsubo cardiomyopathy, isolated hearts show impaired contractile-metabolic coupling, while there is an altered mitochondrial oxidative metabolic state, increased mitochondrial fragility, and oxidative stress. Interestingly, there was a noted decrease in the activities of respiratory chain complexes I and II (as high as 65 and 82%, respectively, in state 3) [103].

2.4. Antioxidant Reserve. The term antioxidant is vaguely defined in the literature and, according to its use, can refer to an array of compounds with varying mechanisms of action [104]. One proposed definition emphasizes that "an antioxidant is any substance that, when present at concentrations lower than those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" [105]. Mitochondria are protected from damage caused by ROS, through several antioxidant systems. When ROS production exceeds antioxidant protection mechanisms, oxidative stress damages nitric acids, proteins, and lipids in mitochondria, ultimately leading to impairment of ATP production through loss of enzyme function in the energy transport chain (ETC) [106]. Antioxidants can be damaged through protein oxidation and peroxidation of cardiolipin (leading to the dissociation of cytochrome c and further generation of ROS) [107]. Antioxidant systems are classified as enzymatic and nonenzymatic as well as endogenous and exogenous. Enzymatic molecules include those that scavenge ROS (superoxide dismutase SOD, glutathione peroxidase (GPx), catalase, and thioredoxin). Among nonenzymatic molecules usually ingested in the diet are vitamins (A, C, and E), amino acids, and metals (copper and selenium) [108]. These mechanisms act synergistically to balance redox overproduction [104].

Intramitochondrial production of NO causes glutathione depletion [19]. Sepsis has been found to increase the activity of enzymes related to the metabolism of glutathione. Tissues are able to increase glutathione levels through de novo synthesis in response to infection, whereas there are other factors that decrease its synthesis during sepsis such as anti-inflammatory cytokines, malnutrition, hyperglycemia, and the administration of erythropoietin, glucocorticoids, and catecholamines [109]. Low glutathione levels are associated with higher mortality in sepsis experimental models, as glutathione is the main mechanism protecting cells from oxidative damage [105].

Cardiac mitochondrial SOD and GPx decrease after sepsis, with SOD activities being reduced 4–8 h after sepsis challenge and GPx activity falling to 70% after 12–24 h [47]. Moreover, glutathione peroxidase, degrading hydrogen peroxide, H_2O_2 is found reduced 16 hours after sepsis, with the reduction in the levels coinciding with reduced cardiac contractility [11]. LPS-induced myocardial depression (measured as peak tension generated by myocardial contraction)

coincides with decreased activity of GPx, the most abundant antioxidant enzyme in myocardium, and decreased levels of GSH, the most important thiol in combating oxidative stress [11]. Moreover, strain echocardiography identified septic cardiomyopathy which correlates with reduced expression of key mitochondrial ROS scavengers [110]. In animals, it has been found that both superoxide dismutase and glutathione peroxidase activities, in cardiac mitochondria, decrease (as much as 40% and 70% compared to animals without sepsis) early after sepsis induction and remain at lower levels throughout the first 24 hours after LPS challenge. These findings suggest that sepsis depletes mitochondria of their defense mechanisms against ROS [53].

2.5. Treatment Implications and Future Directions. Since oxidative damage to mitochondria is central to the pathology of sepsis, antioxidants could be potential therapies in resuscitating mitochondrial function further implementing organ resuscitation. Antioxidants have been used to improve cardiac function in other medical conditions as well [111–113].

2.5.1. Conventional Treatments. Preparation of septic animal models with antioxidants prevents the increase in cardiac mitochondrial generation of reactive oxygen species and, most importantly, prevents reductions in systolic pressure-generating capacity of the septic hearts [38]. Treatment with antioxidant vitamins has been found to improve myocardial contraction and relaxation defects in septic animals as a consequence of alleviated inflammatory response and apoptosis [114]. Naringin, an antioxidant, anti-inflammatory, and anti-apoptotic flavanone glycoside found in grapefruits and oranges, when given orally in septic mouse models, regulated the expression and release of superoxide dismutase (SOD) and malondialdehyde (MDA) to inhibit the subsequent myocardial oxidative stress, suppressed myocardial cell apoptosis, and ameliorated heart morphological changes, all these leading to improved mouse survival [115].

Another experimental sepsis model showed that treatment with antioxidant vitamins alleviated both the systemic and myocardial inflammatory cytokine response and that it inhibited NF-kB nuclear translocation, decreasing caspase-3 and caspase-8 myocardial activity, thus decreasing myocardial apoptosis [114]. Other studies have found, in vivo, that antioxidant treatment significantly attenuated the loss of sarcolemma dystrophin expression and the increased plasma membrane permeability [14]. Cardiomyocytes lacking dystrophin are abnormally vulnerable to mechanical stress-induced injury, with loss of sarcolemma integrity and increased fragility and permeability [116, 117].

It has been documented that neutralization of peroxynitrite can reduce its accumulation and improve myocardial contractile dysfunction and inflammation in septic animal models [118, 119]. Peroxynitrite neutralizers can prevent left ventricular systolic function alterations of endotoxin-treated hearts, left ventricular developed pressure, and its maximal first derivatives (i.e., dp/dt). Moreover, they can prevent I-kappa-B degradation and reduce plasma TNF-alpha levels in endotoxin-treated rats, leading to reduced leucocyte infiltration and endothelium-leucocyte activation [119].

2.5.2. Mitochondria-Targeted Antioxidants. Mitochondria-targeted delivery of antioxidants provides mitochondrion-specific antioxidant defense, protects mitochondria from oxidative damage, prevents mitochondrial membrane damage, improves mitochondrial respiratory function in the heart with sepsis, and improves cardiac function in septic animals.

Mitochondria-targeted vitamin E prevented NF-kB activation, suppressed myocardial injury denoted by serum troponin-I (cTnI) levels, and prevented myocardial apoptosis, ameliorating sepsis-induced disorganization and DNA fragmentation. Taken together, these data suggest that targeted suppression of mtROS suppresses cardiac inflammation and improves cardiac performance in sepsis [62]. Mitochondria-targeted vitamin E increased antioxidant capacity in a rat pneumonia-related sepsis model, reduced the leakage of cytochrome c from mitochondria to cytosol, and suppressed sepsis-induced myocardial inflammation, all of them preventing sepsis-induced left ventricular decompensation. Rats receiving mito-Vit E preserved their % EF (ejection fraction) and % FS in contrast to controls [120]. Furthermore, this study provides clear evidence that mitochondria-targeted antioxidant therapy could be more effective in ameliorating oxidative damage and improving organ function in sepsis, than conventional antioxidant therapies, and this is because antioxidants are distributing throughout the body and not accumulating in the mitochondria, where they are mostly needed. Conventional antioxidants may have failed to present significant efficacy due to their low penetrance to the mitochondria interior, where ROS are mainly produced. Mitochondria-targeting antioxidants have been effective in counterbalancing ROS production in other disease states, such as kidney ischemia/reperfusion injury [121].

2.5.3. Other Potential Treatments. Cytochrome oxidase (CcOX), the terminal oxidase of the respiratory chain, uses electrons donated by cytochrome c to reduce oxygen to H_2O [122]. CcOX inhibition is competitive and reversible early after the induction of sepsis in experimental models, becoming irreversible and noncompetitive during the late phase of sepsis, which is associated with deterioration in myocardial function and survival [123]. It has been shown that exogenous administration of cytochrome c could gain access to cardiomyocyte mitochondria and replete mitochondria with supranormal levels of substrate, thus overcoming competitive inhibition of CcOX. Exogenous administration of cytochrome c improved myocardial contractility and relaxation, as depicted by an increase in left ventricular systolic pressure and a 45% increase in dP/dtmax and dP/dtmin. Importantly, these improvements occurred without significant increases in heart rate, LV end-diastolic pressure, or tau (the LV isovolumic relaxation constant) [124].

Sepsis triggers intracellular signaling cascades, regulated, mainly, by intracellular kinases, phosphorylating downstream targets. Among these, small GTPases of the ras homologous (Rho) family and one of their effectors, RhoA-associated coiled-coli-containing protein kinases (ROCK) are known to act in regulating actin cytoskeleton organization and cell migration. RhoA/ROCK activation plays an

essential role in vascular physiology and pathophysiology [125, 126]. Recently, a study trying to evaluate whether activation of RhoA/ROCK pathway could be involved in mitochondrial dysfunction induced by endotoxemia demonstrated that RhoA/ROCK inhibition normalized mitochondrial respiration in LPS heart and reduced proinflammatory and oxidative stress responses, cytoskeleton disorganization, and mitochondrial ultrastructural damage. Additionally, the study revealed that sepsis caused LV contractile dysfunction, while administration of the ROCK inhibitor improved parameters of LV contractile function (LV tension and maximal positive and negative first derivatives of developed tension (dF/dtmax, dF/dtmin)) [127]. Targeting the ROCK pathway in sepsis could have further therapeutic implications in reducing oxidative stress and inflammation via a NO-dependent mechanism [128].

The peroxisome proliferator-activated receptor- (PPAR-) γ coactivator-1α (PGC-1α) and coactivator-1β (PGC-1β) modulate members of the PPARs, which further regulate mitochondrial energy metabolism and the production of mitochondrial ROS in the heart. Both pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) downregulate PGC-1α and PGC-1β and cause impaired cardiac energy metabolism [129, 130]. A newly synthetic antimicrobial peptide 19-2.5 (Pep2.5), acting against PAMPs, has been shown to counterbalance mitochondria dysfunction in cardiomyocytes during sepsis. Martin and coworkers showed that Pep2.5 enhances mitochondrial respiration, increases ATP levels, and downregulates the production of mtROS in cardiomyocytes during sepsis, by attenuating the suppression of PPARs and PGC-1α/β [131].

3. Conclusion

Mitochondrial injury and dysfunction are two of the major determinants of a clinical spectrum of phenomena seen in septic patients, called septic cardiomyopathy. Oxidative and nitrosative stress, generated in mitochondria, impairs cardiac contractility during sepsis. Oxidative stress leads to energetic (and thus functional) and structural failure of the cardiomyocyte. On the other hand, during sepsis, there are various mechanisms through which the organism tries to protect itself against energy dysfunction, including a reduction in the basal functions (and therefore the energy requirements) of cells and metabolic pathways, an increase in the consumption of energy reserves, and the activation of damage repair mechanisms. This phenomenon is known as cell hibernation and is an effort to avoid cytopathic hypoxia. There is evidence that cells can and do change their energy metabolism [132, 133].

It appears that inhibition of oxidative stress diminishes myocardial damage. However, once mitochondrial damage has occurred, recovery depends on the efficiency of biogenesis (removal and replacement) of the damaged mitochondria. Current research focuses on mitochondrial dysfunction, and mitochondria-targeted therapies are expected to gain wide acceptance. Mitochondria-targeted antioxidants represent an attractive therapeutic approach for diseases complicated by mitochondrial oxidative damage. In the future, managing energetic failure may be a more efficacious treatment modality, rather than treatments focusing on multiple organ failures.

Conflicts of Interest

Vasiliki Tsolaki has received funds from Luoxis Diagnostics Inc. for measuring oxidative stress with the RedoxSYS™ Diagnostic system; the results of the study are not being presented in this review. None of the rest of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Review Article

Oxidative Stress in Hemodialysis Patients: A Review of the Literature

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Hemodialysis (HD) patients are at high risk for all-cause mortality and cardiovascular events. In addition to traditional risk factors, excessive oxidative stress (OS) and chronic inflammation emerge as novel and major contributors to accelerated atherosclerosis and elevated mortality. OS is defined as the imbalance between antioxidant defense mechanisms and oxidant products, the latter overwhelming the former. OS appears in early stages of chronic kidney disease (CKD), advances along with worsening of renal failure, and is further exacerbated by the HD process per se. HD patients manifest excessive OS status due to retention of a plethora of toxins, subsidized under uremia, nutrition lacking antioxidants and turn-over of antioxidants, loss of antioxidants during renal replacement therapy, and leukocyte activation that leads to accumulation of oxidative products. Duration of dialysis therapy, iron infusion, anemia, presence of central venous catheter, and bioincompatible dialyzers are several factors triggering the development of OS. Antioxidant supplementation may take an overall protective role, even at early stages of CKD, to halt the deterioration of kidney function and antagonize systemic inflammation. Unfortunately, clinical studies have not yielded unequivocal positive outcomes when antioxidants have been administered to hemodialysis patients, likely due to their heterogeneous clinical conditions and underlying risk profile.

1. Introduction

Oxidative stress (OS) is defined as a disruption of the balance between oxidative products and antioxidant defense mechanisms, with the former overwhelming the latter [1]. Oxidative molecules include reactive oxygen species (ROS) and nitrogen species, such as nitric oxide (NO), while antioxidants can be endogenous or dietary/exogenously administrated molecules. OS triggers the oxidation and subsequent damage of molecules, such as proteins, lipids, nucleic acids (DNA), and carbohydrates. OS is implicated in the pathologic pathways of various conditions, such as diabetes mellitus (DM), atherosclerosis, inflammation, and progression of chronic kidney disease (CKD) to end-stage renal disease (ESRD). OS is very common in CKD; it is present even in the early stages, gradually increases along with renal impairment, and is further exacerbated by hemodialysis (HD) procedures. CKD is accompanied by pronounced oxidation of proteins, carbohydrates, and lipids, leading to lipid peroxidation and the accumulation of advanced glycation end products (AGEs) which cause severe tissue damage. Also, OS has been associated with renal anemia, malnutrition, β2-microglobulin amyloidosis, and cardiovascular disease (CVD) and is an independent predictor of mortality and morbidity in this group of patients [1, 2].

2. CKD and OS

There is a growing body of evidence showing that CKD patients are characterized by enhanced OS, even in early

stages [3, 4]. OS increases in later stages of CKD and becomes more severe in end-stage renal disease patients undergoing maintenance patients [5]. It has been shown that the presence of OS may cause dramatic modifications in the normal kidney, similar to those seen in CKD. Oxidant molecules contribute to progressive kidney damage by promoting renal ischemia, by inciting glomerular injury, cell death, and apoptosis, and finally by stimulating a severe inflammatory process [6, 7]. Moreover, OS is a major contributor to several conditions which predispose to CKD, such as diabetes, hypertension, and atherosclerosis promoting indirectly the progression of renal damage [6]. In kidney failure, accumulation of ROS or reduction in antioxidant systems can be observed [6]. Specifically, accumulation of ROS, especially O_2^-, leads to NO inactivation and deficiency, which is a critical antioxidant that protects kidney function by increasing renal blood flow, enhancing pressure natriuresis, regulating tubuloglomerular function, and preserving fluid and electrolyte homeostasis. NO deficiency and high levels of plasma O_2^- are considered critical promoters of OS. Several *in vivo* studies highlighted that CKD is a state of NO deficiency: hypertensive animal models showed increased levels of O_2^- in the endothelium and kidneys; animals with NO deficiency developed salt retention, hypertension, albuminuria, and glomerulosclerosis; and oral intake of the NO precursor molecule L-arginine in nephrectomized rats increased estimated glomerular filtration rates (eGFR) and improved glomerular function [6, 8, 9]. Chen et al. showed that plasma levels of O_2^- were significantly increased in maintenance hemodialysis patients compared to healthy controls [10].

Many studies concluded that NO deficiency due to inactivation by O_2^- or reduced renal NO production contributes to CKD progression [6]. Yilmaz et al. showed that red blood cells (RBCs) from patients in early stages of CKD (1-2) present enhanced OS status compared to healthy subjects [11]. Furthermore, OS markers were strongly inversely associated with eGFR and were progressively augmented, along with deterioration of renal function [11]. Similar results were reported by Terawaki et al. In a cohort of 55 nondialysis patients with various degrees of kidney function (mean eGFR 50 mL/min), plasma levels of oxidized albumin were augmented progressively along with CKD deterioration [12]. In a multivariate regression model, the plasma concentration of oxidized albumin was independently and strongly inversely correlated with eGFR. In another, cross-sectional study OS biomarkers such as plasma levels of 8-isoprostanes and serum total antioxidant capacity (TAC) increased gradually along with deterioration of renal function in patients with CKD stages 1–4 and eGFR was a strong and independent predictor of 8-isoprostanes levels, after adjustment for several confounding factors [13]. However, these results are not in line with the findings by Oberg et al. who reported no correlation between F2-isoprostane levels and eGFR in a cohort of 60 patients with moderate-to-advanced CKD (stages 3–5). The relatively small number of subjects and the distribution in each stage (60% were in stages 4-5) could be identified as a serious limitation of the latter study [14]. Another prospective cohort study assessed changes of several markers of OS and inflammation prior and post renal transplantation in 19 patients. Plasma levels of C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), F2-isoprostanes, and protein carbonyls were significantly elevated compared to healthy controls before transplantation and were dramatically decreased after renal transplantation. Therefore, the authors suggested that restoration of kidney function by renal transplantation could play a beneficial role in the suppression of chronic inflammation and OS due to ESRD and maintenance renal replacement therapy [15].

OS accompanies CKD even in the early stages, progresses along with deterioration of kidney function, and becomes more severe in ESRD. OS, CKD, and inflammation are tightly interrelated and may lead to further CKD progression.

3. Hemodialysis and OS

Altered dietary restrictions and preferences may exaggerate the depletion of antioxidant defense mechanisms, such as low levels of vitamins C and E (mainly because of dietary restrictions of vegetables and fruits, malnutrition, and loss of vitamins during HD procedure), reduced selenium levels, and reduced function of the GSH-scavenging mechanism [16, 17]. Moreover, several factors accelerating prooxidant activity in this group of patients have been demonstrated, including the chronic excessive inflammatory status of CKD, the uremic environment, other commonly encountered characteristics of HD patients (such as hypertension, diabetes, obesity, dyslipidemia, advanced age, and enhanced vascular calcification), and factors related to the HD procedure per se [1].

There is a growing body of evidence suggesting that HD is characterized by excessive OS status, which results from loss of antioxidants during dialysis procedures and accumulation of oxidative products (Table 1). Chen et al. suggested that the HD procedure promotes the formation of O_2^-, a powerful prooxidant reactive oxygen molecule [10], and another study showed a direct increase in ROS plasma levels after each HD session [18]. During HD, blood exposure to dialyzer membranes and dialysate trigger activation of complement factors, platelets and polymorphonuclear white blood cells (PMNs), and subsequently ROS production, within minutes after initiation of HD sessions [16, 18–24]. PMN stimulation was reported as a significant OS biomarker that is progressively increased along with the stages of CKD and is more pronounced in HD [25]. Maher et al. reported that within 30 minutes of HD initiation, lipid peroxidation products increase and hypothesized that complement factor activation or production of free fatty acids induced by heparin might be the pathophysiologic mechanisms underlying these effects [19]. Loughrey et al. recruited 15 patients on regular HD and 15 ESRD patients with eGFR < 15 ml/min that were managed with supportive care without renal replacement therapy. Compared to the ESRD group, HD patients presented significantly higher levels of lipid peroxidation markers (MDA) and reduced plasma concentrations of antioxidants (vitamin C, GSH-Px, and selenium), indicating that OS is further exacerbated by the HD procedure [24]. Furthermore, Nguyen-Khoa showed that the duration

Table 1: Effect of HD session on OS.

Study (ref.)	Year	OS biomarker	Patients	Results
Data regarding effect of HD on OS				
Sela et al. [25]	2005	MPO activity in PMNLs SOD release rate in PMNLs	30 CKD (stages 2–5) 30 CAPD 30 HD 30 healthy controls	HD > CAPD > CKD > controls
Maher et al. [19]	1987	Free radicals	51 HD 33 healthy controls	Baseline: same HD = control After 15′ of HD: ↑ After 30′ of HD: peak
Descamps-Latscha et al. [20]	1991	Complement activation and stimulation of PMNLs	20 HD	After 15′ of HD: ↑, peak
Himmelfarb et al. [21]	1993	ROS	10 HD	After 15′ of HD: ↑ (×6.5 times) After 30′ of HD: peak
Yang et al. [28]	2006	ROS Plasma vitamin C level	80 HD	After HD: ↑ (×14 times)
Clermont et al. [22]	2001	Plasma vitamin C level ascorbyl free radical/vitamin C ratio PMNL activation	16 HD	After HD: ↑↑
Chen et al. [10]	1998	SOD MPO	104 HD 98 healthy controls	Baseline: HD > control After HD: HD >>> control
Nguyen et al. [18]	1985	ROS	35 HD 44 healthy controls	Baseline: HD < control After 15′ of HD: ↑ HD >> control End of HD: ↓ HD < control

CKD: chronic kidney disease; HD: hemodialysis; OS: oxidative stress; MDA: malondialdehyde; ADMA: asymmetric dimethylarginine; SOD: superoxide dismutase; Zn: zinc; Cu: copper; Se: selenium; GSH-Px: glutathione peroxidase; TAS: total antioxidant status; ESRD: end-stage renal disease; CAPD: continuous ambulatory peritoneal dialysis; MPO: myeloperoxidase; PMNL: polymorphonuclear neutrophil; ROS: reactive oxygen species.

of HD treatments is a significant independent factor of OS [26]. This observation may be counterintuitive, given that prolonged dialysis session, such as extended overnight, reveals beneficial outcomes.

HD patients are characterized by increased inflammation and lipid peroxidation [27]. The HD process promotes the formation and accumulation of oxidative products through activation of platelets, complement, and PMNs. After HD, serum ROS were 14 times higher than before the start of the HD session in a cohort of 80 chronic stable maintenance HD patients [28]. Two studies showed that ROS plasma levels were significantly increased in HD patients compared to healthy controls [29, 30]. To explain this finding, Granata et al. suggested that dysfunction of the mitochondrial respiratory system, which is prominent in CKD patients and further impaired in HD patients, might be the cause of ROS generation [30]. Handelman et al. showed that plasma levels of F2-isoprostanes were significantly higher in HD patients than in controls with normal renal function. Moreover, F2-isoprostanes were strongly associated with CRP values in the HD group, suggesting thus a tight link between OS and inflammation in patients undergoing maintenance HD [31].

4. Modifiable Factors Aggravating OS in HD Patients

4.1. Not Dialysis-Related (Lifestyle) Factors. In CKD and HD patients, lifestyle and dietary habits play a crucial role on OS status, independently from renal failure and HD-related oxidative burst.

4.1.1. Diet. In a study by Bergesio et al., patients with advanced CKD on vegan diet not only had better lipid profile parameters but also had decreased OS and inflammation status compared to patients with the same level of renal function on conventional diet. The authors concluded that vegan diet might have a beneficial protective role against CV events in this particular group of patients [32]. Several studies showed that Mediterranean diet has a significant protective effect on lipid profile, inflammation, and lipid peroxidation in patients with advanced CKD, while dietary glycemic load triggered the activation of inflammatory and OS mediators in HD patients [33, 34]. A recent meta-analysis of several cohort studies showed that in CKD patients—especially ESRD undergoing HD—healthy dietary interventions, including Mediterranean, vegan, low carbohydrate diets, may have a beneficial effect on quality of life, blood pressure, and lipid profile but their impact on OS, mortality, and CV events is yet uncertain [35, 36]. Moreover, oral supplementation of fermentable dietary fiber, flaxseed, or virgin argan oil improved oxidative and systemic inflammation status in HD patients [37–40]. Therefore, healthy dietary interventions that include a balanced Mediterranean, low-carbohydrate diet with supplementation of fibers and virgin oil might be suitable for HD patients, since it showed promising and protective results against OS and inflammation. In this group of patients, larger cohort studies are required to

investigate the direct effects of healthy eating patterns on OS and clinical outcomes in HD patients.

4.1.2. Smoking. There are numerous studies supporting that cigarette smoking causes an increase of blood neutrophils and generates OS and inflammation in the general population [41]. Moreover, it was repeatedly shown that tobacco smoking has an additive and negative effect on serum lipid peroxidation products, as well as oxidative injury mediated by ROS in HD patients and patients with overt nephropathy [42, 43]. Due to the decreased serum levels of antioxidants such as vitamin C or total glutathione that are common in smokers [44, 45], smoking HD patients are more prone to oxidative tissue injury than nonsmoking HD subjects or even healthy individuals [26, 42, 46]. Smoking was significantly and negatively correlated with serum levels of the antioxidant total GSH in a cohort of chronic HD patients [45]. In conclusion, OS is tremendously enhanced by tobacco smoking, independently of dialysis modality, providing another reason for which smoking cessation is strongly recommended in HD patients.

4.1.3. Uric Acid. During the past decades, uric acid is becoming a novel and interesting player involved in CKD and OS. Data from epidemiological studies suggest a strong association between hyperuricemia and hypertension, CV events, progression of CKD, and mortality, where OS plays a key role [47]. Furthermore, allopurinol—an inhibitor of xanthine oxidase that lowers plasma uric acid levels—was shown to act as an antioxidant in general population and CKD patients [48–50]. Regarding the possible association between hyperuricemia, OS, and poor outcomes in HD patients, the evidence is still debatable. Reducing serum uric acid levels after allopurinol administration in HD patients with metabolic syndrome resulted in significant improvement of lipid parameters and therefore might protect from future CV risk [51]. A recent study on a large cohort of 27,229 HD subjects showed that low and not high serum uric acid levels predicted all-cause and CV mortality [52]. In conclusion, the data regarding the association of hyperuricemia with OS and hard CV endpoints in HD patients is contraindicatory and scarce. Therefore, no strong recommendations regarding the lowering of uric acid in this group of patients can be made.

4.1.4. Sodium and Fluid Overload. Increased sodium intake has been tightly linked with excessive OS and endothelial damage in both animal models and human studies acting through the pathway of ROS, SOD, NADPH, and NO [53–55]. In HD patients, high sodium intake and subsequent excessive fluid retention between HD sessions were significant independent predictors of all-cause and CV mortality [56], while low sodium diet was associated with prolonged survival [57, 58] and lower degree of left ventricular hypertrophy [59]. Furthermore, reduction of dialysate sodium from 140 to 137 mEq/L was accompanied by significant improvement of endothelial damage, hemodynamics, and OS status [60, 61]. In conclusion, high dietary salt intake and subsequent fluid retention in HD patients are associated with OS, endothelial injury, and poor outcomes. Therefore, strict volume control and low-salt diet are mandatory in this group of patients.

4.2. Dialysis-Related Factors. It has been reported that OS in HD patients is complicated by several factors, the most studied are type of dialyzer, type and dosage of heparin, medications administered, HD solution, presence of central venous catheter, and duration of HD treatment.

4.2.1. Type of Dialyzer Membranes. Several investigators have reported that the type of dialysis membrane used in HD patients may play a significant role in OS production. Dasgupta et al. found that the use of the polysulphone dialyzer was accompanied by lower levels of lipid peroxidation products, compared to cuprophane [62]. Another study reported that compared with cuprophane, regenerated cellulose membrane was associated with lower production of OS markers [63]. These effects of the two types of membranes on OS production have been attributed to increased H_20_2 production and water generation, caused by regenerated cellulose membranes, through catalase and GPx activities. In agreement with these findings, other investigators have shown that patients undergoing HD with cuprophane membranes exhibit significantly higher levels of ROS in monocytes and PMNs, when compared to those who dialyze with synthetic polysulphone membranes [20, 64]. Besides ROS production, several investigators studied the impact of different HD membranes on lipid peroxidation. Sevillano et al. found that HD with cuprophane membrane caused an increase in red blood cell MDA concentrations whereas the levels decreased during a HD session with the cellulose-acetate membrane [65]. Kosch et al. conducted a randomized, single-blind, crossover study to determine the impact of different HD membranes on OS and endothelial dysfunction. Twelve stable MHD patients were randomized to either cuprophane or polysulphone membrane dialysis. Flow-mediated dilatation of brachial artery and plasma levels of alpha-tocopherol (AT) and ox-LDL were assessed pre- and postdialysis. In contrast to polysulphone, HD with cuprophane was accompanied by a significant reduction in both brachial artery flow-mediated dilatation and AT serum levels, suggesting that the type of HD membrane is a significant determinant of endothelial dysfunction and OS. However, ox-LDL levels remained unaffected by either treatment [66]. On the contrary, some investigators found no difference in the effects of dialysis with cuprophane versus dialysis with polysulphone membranes on ROS production [67], while others reported that polysulphone induced an OS lower than cuprophane [63]. Another study compared cellulose membranes coated with the antioxidant vitamin E and polysulphone membranes. The cellulose-diacetate membranes resulted in increased OS-induced DNA damage in leukocytes compared to polysulphone membranes, whereas DNA damage was approximately the same for vitamin E and polysulphone dialyzers [68]. Similarly, polysulphone membranes resulted in higher plasma levels of MDA and reduced GSH-Px activity and selenium plasma levels, compared with modified cellulose (hemophan) membrane in maintenance HD patients [69]. Another study showed that

regenerated cellulose HD dialyzers caused a significant increase of serum MPO, AOPP, and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels, compared with polysulphone membranes in a cohort of maintenance HD patients. The authors reached the conclusion that the biocompatibility of the HD membrane has critical effects on the development of HD-derived OS [70]. Dialysis with cuprophane dialyzer resulted in greater increase of MDA and a more severe decrease of antioxidants (vitamin E, catalase) compared to HD with polysulphone membrane [71]. However, studies examining end-points such as overall survival and major cardiovascular events have not been performed in dependency of the abovementioned different dialysis membranes.

Thus, the type of dialyzer membrane used in HD is a significant determinant of OS status and may play a role in the development of endothelial dysfunction. Although several investigators studied the effects of different dialyzer membrane types on OS production, the results remain contradictory.

4.2.2. Anticoagulation. Artificial surfaces contacted by blood components in HD procedures trigger activation of platelets and PMN, which release several bioactive molecules, including MPO. MPO favors the generation of ROS, leading thus to irreversible oxidation of DNA and proteins and modification of carbohydrates and lipids. During this chain reaction, there is a significant increase in accumulation, adhesion, and degranulation of PMN cells. Accumulation and adhesion of PMNs are the early key steps for complement activation, and PMN degranulation seems to be a continuous process, independent of the complement activation pathway. However, the degranulation process is highly linked with the presence of divalent calcium cations. While the effect of the type of dialyzer on OS status in HD patients has been thoroughly studied, there is limited data on the impact of anticoagulation on the development of OS and specifically the activation of PMNs. Bos et al. were the first to investigate the effect of citrate administration as alternative anticoagulation and substitute of heparin in HD on OS granule products (MPO and lactoferrin) and PMN degranulation. Heparin and citrate administration were compared in 10 stable patients undergoing maintenance HD with cellulose-triacetate membranes. Citrate abrogated MPO release to a significant degree, whereas lactoferrin release was abolished in a lesser degree. Citrate's effect was mainly explained due to its impact on calcium divalent cations. The authors also reported that degranulation started at the beginning of HD procedures and was not associated with complement activation or neutropenia [72]. Sela et al. explored the effect of heparin on OS induced during HD in 22 stable maintenance HD patients. All patients received HD treatment with and without heparin and both pre- and postdialysis plasma levels of oxidized glutathione (GSSG) and superoxide release rate from activated neutrophils were measured. Heparin administration resulted in less superoxide release and reduced plasma levels of GSSG. These results indicate that heparin may suppress the OS derived by the HD process [73]. Another randomized crossover trial compared the effect of heparin, citrate, and dalteparin on OS and PMN degranulation in 8 maintenance HD patients and showed that degranulation of PMN and platelets are taking place early on and are highly dependent on divalent calcium cations. HD with heparin and dalteparin was accompanied with severe degranulation immediately after the start of HD, and citrate completely abolished the release of MPO and platelet factor 4, that are well-known granule molecules. Furthermore, after 7 days of citrate administration, plasma levels of ox-LDL were significantly decreased compared to HD with heparin and dalteparin [74].

4.2.3. Ultrapure Dialysate. Besides dialyzer bioincompatibility, it is widely accepted that white blood cell and platelet activation leading to oxidative response are significantly enhanced by microbial contaminants of HD fluid. Even slight amounts of dialysate contamination undermine the biocompatibility of HD treatment and may aggravate HD-derived amyloidosis, inflammation, atherosclerosis, and subsequent accumulation of oxidative products [75, 76]. There is a growing body of evidence that the use of ultrapure dialysate in HD patients reduces serum levels of inflammatory mediators, β2-microglobulin, carbonyl, and OS biomarkers and improves anemia status [77–83]. Multiple hit theory, including blood exposure to dialyzer membranes and dialysate, endotoxin amplification, administration of intravenous anticoagulation, and finally loss of antioxidants, provides currently the best explanation concerning the oxidative burst during HD sessions, explaining thus, at least partially, the discrepancies between different studies.

4.2.4. Anemia—Erythropoietin and i.v. Iron Administration. In ESRD patients under HD, anemia and OS are interrelated factors associated with poor outcomes. However, their relationship is still not clear. Renal anemia has been shown to trigger accumulation of oxidative products in HD patients, while correction of anemia may improve OS status [46]. Several observational studies highlighted that increased lipid peroxidation status might be associated with renal anemia severity in chronic HD patients [84–86]. Improvement of anemia by erythropoietin (ESA) has been accompanied by significant inhibition of the oxidation process, suggesting that anemia itself might aggravate OS [84, 87]. Both ESA and intravenous iron administration are involved in the oxidative response. Several studies supported the antioxidant effects of ESA treatment [84, 87–89], while others found no positive impact of ESA on OS status [90].

Iron metabolism is a major contributor to excessive OS in HD patients. It has been repeatedly reported that intravenous (i.v.) administration of iron sucrose causes excessive production of OS [91, 92] both *in vitro* (cell cultures) and *in vivo* [31]. Intravenous iron infusion in patients with advanced CKD (stages 3 and 4) has been shown to cause OS very rapidly and independently of transferrin saturation. Within 15 to 30 minutes after iron administration, plasma and urine levels of MDA were significantly increased. Administration of NAC (N-acetylcysteine) reduced significantly OS production, but did not show any protective effect on proteinuria and renal tubular damage [91]. Similarly, intravenous iron sucrose in maintenance HD patients was accompanied by a significant increase in free iron, total peroxide, and biological markers of lipid peroxidation, which accumulated almost

immediately after the start of iron administration and reached maximum levels in 30 minutes [93]. In agreement with these studies, Muller et al. found that patients receiving i.v. iron during HD session presented significantly increased production of plasma MDA and specific OS-induced DNA damage, 30 minutes after the start of iron infusion [68]. Tovbin et al. sought to determine the effect of i.v. iron on protein oxidation and inflammation in HD patients. Intravenous administration of 100 mg of iron after 3.5 hours of high-flux HD caused a 37% elevation in protein oxidation, as assessed by AOPP levels [94]. Furthermore, in maintenance HD patients treated with intravenous iron, formation and accumulation of ox-LDL have been shown to be closely associated with iron load and enhanced erythrocyte lipid peroxidation [95]. In contrast, another study found no association between plasma levels of several OS biomarkers (GPx, SOD, TAC, vitamin C, and MDA) and ferritin levels, transferrin saturation values, and intravenous iron administration in 34 chronic HD patients receiving erythropoiesis-stimulating agent (ESA) treatment [96]. These results ignited the development of less "toxic" and "aggressive" iron preparations, which are applied as glycosylated complexes through i.v. lines, promoting biocompatibility and bioavailability studies [97]. The way of intravenous administration of iron compounds in a HD session affects directly the oxidative state. Rapid or bolus intravenous administration aggravates OS in HD patients mainly due to oversaturation of transferrin and prolonged duration of iron overload [98, 99]. On the contrary, slow i.v. iron infusion may eliminate these effects, since the native antioxidant system has more time to respond to the gradual accumulation of oxidative products [100]. In agreement with this theory, Malindretos et al. reported that slow i.v. intradialytic administration of iron sucrose or iron dextran did not trigger an oxidative or inflammatory response [101]. Moreover, administration of the antioxidant NAC significantly reduced the OS that was caused by intravenous iron therapy during a HD session [102], and therefore, it might be used as an antioxidant "shield" whenever i.v. iron infusion is necessary. Although it is widely accepted that i.v. iron is strongly linked with oxidative response during HD sessions, the lack of evidence to support a direct relationship between i.v. iron and poor clinical outcomes and the undisputed beneficial role of renal anemia correction on OS, inflammation, and survival leads to a strong recommendation for cautious and properly prescribed iron treatment for anemia management in HD patients.

4.2.5. HD Modality—Hemodiafiltration. Compared with standard hemodialysis, treatment with hemodiafiltration in patients undergoing maintenance HD has a significant positive effect on OS markers and inflammation status and therefore may play a protective role against atherosclerosis and CVD [103]. The beneficial protective effects of HDF against OS are due to several factors: the ultrapure dialysate, the biocompatible membranes, the hemodynamic stability and the better anemia control of the patients, and the improved clearance of middle and large molecular weight uremic molecules such as inflammatory cytokines, homocysteine and polyamines, and β2-microglobulin [103–106]. After a 6-month period of standard HD, Filiopoulos et al. treated 9 stable patients with postdilution hemodiafiltration for a period of 9 months and showed that at the end of the study, there was a significant increase of TAC and a significant reduction of OS markers, such as SOD and ROS. Furthermore, hemodiafiltration suppressed inflammation, as assessed by high sensitive CRP (hs-CRP) and IL-6 [107].

4.3. Dialysis Access. It has been suggested that extended use of central venous catheters for vascular access might be a significant prooxidative factor that favors development of inflammation and atherogenesis [108], while Weiss et al. found an increased expression of OS biomarkers and hyperplasia factors in failed arteriovenous fistulae and grafts [109]. It may be concluded that besides the HD process itself, several conditions that are common in ESRD are involved in the pathogenesis of OS in end-stage renal disease patients.

5. Residual Renal Function and OS

Impaired residual renal function (RRF) in ESRD patients was associated with high inflammatory activity. Moreover, eGFR was a significant independent determinant of increased inflammatory state in ESRD patients close to the initiation of renal replacement [110]. Preserved residual kidney function was linked with decreased plasma levels of lipid peroxidation products and other markers of carbonyl and OS in ESRD patients undergoing PD [111]. The authors hypothesized that this relation might be a possible explanation for the prolonged survival of PD patients with preserved RRF [112]. Although the possible association of several HD-related factors with OS have been thoroughly studied, the role of RRF regarding OS status has not yet been elucidated. Future studies are needed in order to clarify this topic.

6. Diabetes and OS

OS has been suggested to play a key role in the progression of micro-/macrovascular complications of diabetes mellitus (DM). DM and CKD share common underlying pathogenetic processes which lead to accumulation of free radical products. The high glucose environment in DM triggers protein glycation and oxidation [113]. The glycated proteins are further modified and oxidized and release free radical products, the advanced glycation end products (AGEs) [114]. AGE plasma levels are significantly elevated in ESRD patients and favor OS in these patients [108]. Moreover, OS-induced accumulation of AGEs is increased in HD patients, independent from blood glucose concentrations [115].

Giugliano et al. suggested that the production and accumulation of ROS in DM is the pathogenic pathway linking impaired glucose metabolism with tissue damage and therefore may lead to DM-derived vascular complications [116]. Specifically, when endothelial cells are exposed to excessive glucose levels, they are modified and produce O_2^-, which inactivates NO, a well-known endothelium-relaxing factor that regulates homeostasis of the vasculature, leading thus to early, subclinical atherosclerosis. Moreover, increased plasma levels of triglycerides have been reported as common

features in DM and CKD leading to accelerated free radical production [117]. Ceriello et al. [118] suggested that the impaired glycemic environment caused by high glycosylated hemoglobin increases superoxide anion generation and subsequently alters NO activity in diabetes. Dursun et al. [119] sought to investigate the effects of HD and diabetes on OS and found that both, diabetes and ESRD, induce oxidative activity. Furthermore, both conditions combined yielded synergic deleterious effects with the highest OS status, as observed in diabetics under maintenance HD. Ceriello et al. hypothesized that OS is the common pathogenic pathway linking insulin resistance with structural and functional modifications in endothelial and beta cells and subsequently leading to accelerated atherosclerosis, CVD, and DM [120]. The inter-relationship between AGEs, OS, and CVD was highlighted in a study of 225 HD patients: lipid peroxidation and glycoxidation were strongly associated with accelerated coronary vascular calcification in maintenance HD patients [121].

7. CVD and OS

There is a growing body of evidence that OS along with inflammation are key elements in the development and progression of vascular calcification, all-cause, and CVD mortality in patients with renal failure [108, 122, 123]. The first step of vascular calcification is the development of endothelial dysfunction. Ghiadoni et al. investigated the possible relationship between OS, CKD severity, and endothelial dysfunction in 40 CKD patients stages 3–5, 20 maintenance HD patients, and 20 healthy controls and found that flow-mediated dilatation of brachial artery was significantly reduced in CKD patients compared to controls and was further decreased in the HD group. Moreover, flow-mediated dilatation was significantly and positively related to eGFR. After oral administration of 2 g vitamin C in HD patients, flow-mediated dilatation of brachial artery was significantly elevated and OS biomarkers were reduced and therefore the authors suggested that OS and endothelial dysfunction are tightly associated in advanced CKD [124]. HD itself has been shown to trigger the accumulation of numerous oxidative factors and therefore contributes to the development of endothelial dysfunction and CVD [108], while proatherogenic molecules such as vascular endothelial growth factor and the OS marker Cu/Zn SOD were strongly associated with duration of HD treatment [125]. Similarly, duration of HD (in years) tended to be positively associated with coronary artery calcification score (CAC) in a cohort of 225 maintenance HD patients. In a multivariable model, lipid peroxides were strong predictors of CAC, independently of several traditional risk factors for atherosclerosis [121].

Although several investigators tried to explore the strong linkage between OS and progression of atherosclerosis, the exact pathophysiologic mechanisms are yet unclear. It has been reported that OS results in decrease in NO availability and subsequently causes endothelial dysfunction [126]. This affects directly the vascular tone. LDL cholesterol enters into the intima layer, where it undergoes an oxidization process and is converted into ox-LDL, a profoundly atherogenic molecule that favors the development and progression of vascular inflammation [123]. Oxidation of LDL cholesterol results in the release of MDA, a short-chain aldehyde that stimulates the expression of white blood cell adhesion and other inflammation molecules which accumulate into the subendothelial area. Tissue macrophages take up the oxidized LDL molecules in the arterial wall and form foamy cells, a key first step in the development of atherosclerosis [123]. Due to these qualitative changes in LDL, high levels of ox-LDL and increased titers of antioxidized LDL antibodies [127], along with increased concentrations of lipid peroxidation markers such as MDA [128] have been found in HD patients. Morena et al. showed that high density lipoprotein (HDL cholesterol) loses the protective ability to abrogate the LDL oxidation in HD patients and therefore may be a promoter of HD-mediated atherosclerosis [129] while Usberti et al. showed that the degree of CVD was tightly linked with the severity of lipid peroxidation in maintenance HD patients [130]. In a cohort of 32 maintenance HD and 39 continuous ambulatory peritoneal dialysis patients, MDA was negatively correlated with cardiac function—assessed by ejection fraction—and the antioxidant SOD was significantly negatively associated with systolic and diastolic blood pressure, suggesting thus that HD-induced OS may play a role in the development of left ventricular hypertrophy [131].

The primary endogenous inhibitor of NO synthase that is involved in endothelial dysfunction is asymmetric dimethylarginine (ADMA) which is increased in ESRD, possibly due to its renal secretion. High intracellular ADMA levels result in significant reduction of NO regeneration and have been repeatedly linked to endothelial dysfunction and atherosclerotic risk in both the general population and HD patients [132–134]. OS attenuates the function of the specific enzyme converting ADMA into citrulline resulting in high intracellular ADMA levels. As expected, ADMA was negatively associated with eGFR, ranking as the third risk-predicting factor after proteinuria and hemoglobin in a cohort of 131 patients with mild to severe CKD (eGFR 8 to 77 ml/min/1.73 m^2), [135]. Moreover, plasma levels of ADMA have been found to be six times higher in HD patients compared to healthy controls [136] and five times higher in peritoneal dialysis patients than in healthy subjects [137]. Another study showed that plasma ADMA levels were higher only in HD and renal transplant recipients compared to healthy controls and not in patients with early and advanced stages of CKD [132]. Numerous studies showed the strong link between ADMA levels and early atherosclerosis and CVD complications in HD patients. Zoccali et al. showed that ADMA levels are strong predictors of cIMT (carotid intima-media thickness) and atherosclerosis progression, independently of several well-established CVD risk factors in ESRD patients [134]. Yilmaz et al. demonstrated that ADMA plasma levels were inversely correlated with eGFR and were independent, strong predictors of endothelial dysfunction in patients with CKD stages 1–5 [11]. Another study reported that increased plasma levels of ADMA are tightly linked with left ventricular hypertrophy in a cohort of 198 stable maintenance HD patients [138]. Plasma concentration of ADMA was also a strong independent predictor

of cardiovascular events, all-cause, and cardiovascular mortality in a cohort of 225 ESRD patients [135, 139]. Moreover, in mild-to-severe CKD, ADMA plasma levels predicted progression to HD and all-cause mortality independent of several traditional risk factors including eGFR, hemoglobin, proteinuria, and serum CRP [135]. Therefore, there is a growing body of evidence suggesting that ADMA might be a novel risk factor for mortality and cardiovascular events in HD patients [135, 139, 140].

Besides lipid peroxidation, ADMA, and MDA, several other biomarkers of OS have been linked with development of atherosclerosis in dialysis patients. Chronic accumulation of advanced oxidation protein products (AOPPs) have been associated with high values of cIMT [141] and CVD [142]. More interestingly, Liu et al. reported a strong causal relationship between elevated plasma AOPP levels, inflammation, and atherosclerosis in animal models [143]. Dursun et al. reported that in HD patients, OS status assessed by high levels of oxidative markers (plasma TBARS) and low levels of antioxidants (catalase and plasma sulfhydryl activity) was a strong and significant independent predictor of cIMT [144]. OS-mediated DNA damage was also reported as a significant independent predictor of all-cause mortality in a cohort of 220 stable maintenance HD patients, potentially due to its effect on vascular calcification [145].

OS is tightly associated with the development and progression of atherosclerosis in CKD and HD patients. A biomarker of OS, ADMA is an inhibitor of NO synthase. ADMA is a strong predictor of atherosclerosis, mortality, and major adverse cardiac events in CKD and HD patients.

8. Inflammation and OS

Both enhanced OS and inflammation status are well-known interrelated factors in ESRD with common underlying mechanisms including endothelial dysfunction and common complications, such as CVD and death. It has been hypothesized by several investigators that OS causes inflammation, and on the other hand, chronic inflammation might also stimulate an oxidative response. The inflammatory status and HD duration were reported as determinants of OS in stable maintenance HD patients [26, 31], and F2-isoprostanes, well-known markers of OS, were strongly and independently associated with CRP in HD patients. Moreover, it has been shown that acute phase proteins were significantly associated with OS status in ESRD patients [146]. The exact pathophysiologic mechanisms underlying the link between OS, inflammation, and endothelial dysfunction in ESRD patients are yet unclear, although activation of neutrophils, myeloperoxidase secretion, and dysregulation of the NO system have been hypothesized as players linking these disorders [147, 148].

9. Hypoalbuminemia, β2-Amyloid Arthropathy, and Sleep Disorders

Several studies have shown that low serum albumin levels reflect poor nutritional status and are strong predictors of all-cause and CVD mortality in maintenance HD patients [149]. Danielski et al. investigated the possible association between hypoalbuminemia, chronic inflammation, and OS biomarkers in a cohort of patients undergoing HD. Plasma levels of IL-6, CRP, protein carbonyl formation, and protein thiol oxidation were significantly increased in severely hypoalbuminemic HD patients compared to normoalbuminemic. Therefore, the authors proposed that the combined additive effect of hypoalbuminemia, OS, and inflammation might result in increased CVD morbidity and mortality in maintenance HD patients [150]. Chronic accumulation of oxidative products leads to β2-microglobulin amyloid arthropathy. Enhanced OS and accumulation of AGEs in DM and ESRD is a key factor for the formation of amyloid fibrils [115]. OS has been associated with sleep disorders in HD patients. A recent study in 37 patients undergoing HD reported that severe sleep apnea syndrome is associated with accelerated OS status reflected by increased plasma levels of MPO and ox-LDL [151]. Chen et al. reported that improvement of sleep quality, anxiety, and fatigue in HD patients was accompanied by a significant reduction in inflammation and OS markers [152].

10. Antioxidants and OS in CKD and HD

The HD procedure per se is characterized by a significant depletion of antioxidants. Bayes et al. found reduced serum levels of vitamin E in HD patients [27]. Morena et al. found that patients undergoing chronic hemodiafiltration had significantly lower plasma levels of vitamin C, compared to healthy controls. Serum concentration of vitamin E did not differ significantly among these groups. Furthermore, Morena et al. quantified the exact loss of vitamin C during a hemodiafiltration session and showed that vitamin C deficiency was associated with increased levels of several oxidants (MDA, AOPP) and reduced activity of the antioxidant GSH-Px [44]. Therefore, it has been speculated that the administration of antioxidants such as vitamins E and C might be of benefit in HD patients. *In vitro*, vitamin E is the most powerful lipid-soluble antioxidant molecule in cell membranes. It not only preserves the stability of biological membranes and protects them from injury induced by ROS and lipid peroxides but it also modifies the cell reaction to OS via regulation of signal-transmission molecular pathways [153]. Moreover, in salt-sensitive hypertensive rats, combined therapy of vitamins E and C ameliorated the accumulation of oxidative products, improved kidney hemodynamics, and subsequently protected the kidney from further damage [154]. However, the clinical data regarding the use of oral vitamin supplements for antioxidant protection in HD remains controversial and does not preclude vitamin E supplementation for HD patients (Table 2).

10.1. Vitamin C Supplementation and OS. Several investigators have reported no effect of oral or intravenous administration of vitamin C on various markers of OS (plasma levels of TBARS and isoprostanes, Cu/Zn activity, and LDL susceptibility to oxidation), in patients undergoing maintenance HD [155–158]. Fumeron et al. conducted a prospective, randomized open-label trial to investigate the possible effects of oral vitamin C administration on inflammation

Table 2: Effect of antioxidant supplementation on OS in HD patients.

Study (ref.)	Patients	OS biomarker	Antioxidant	Study period	Result
Vitamin C					
Yang et al. [28]	40 on Vit. C 40 controls	ROS	i.v. 1 g	4 hours	↓ OS
Ghiadoni et al. [124]	20 on Vit. C	MDA Lipoperoxides Ferric-reducing plasma ability	p.o 2 g	4 hours	↓ OS
Fumeron et al. [159]	33 on Vit. C	Serum carbonyls, RBC concentrations of reduced and oxidized glutathione	p.o 0.25 g ×3/week	8 weeks	Same OS
Candan et al. [160]	17 on Vit. C 17 on placebo	MDA RBC osmotic fragility	p.o 0.25 g	12 weeks	↓ OS
Abdollahzad et al. [161]	21 on Vit. C 21 on placebo	MDA	p.o 0.25 g	12 weeks	↓ OS
Eiselt et al. [155]	20 on Fe i.v. 5 on Fe i.v. + Vit. C	TBARS	i.v. continuous 2 mg/min	4 weeks	↑ OS
Chan et al. [156]	10 on 250 mg p.o 11 on i.v.	F2-isoprostanes	p.o/iv	12 weeks	Same OS
Ramos et al. [157]	17 on Vit. C 17 on placebo	TBARS Lipoperoxides	p.o 1 g/d	1 year	Same OS
Washio et al. [158]	16 on Vit. C	Cu/Zn-SOD	p.o 0.2 to 1 g	3 weeks	Same OS
Vitamin E					
Diepeveen et al. [162]	12 on Vit. E 11 on placebo	Ox-LDL	p.o 800 IU/d	12 weeks	Same OS
Lu et al. [163]	14 on Vit. E 13 on placebo	Oxidative protein modifications Lipoperoxides	p.o 800 IU/d	24 weeks	Same OS
Kamgar et al. [164]	20 on multivitamin (including Vit. E) 17 on placebo	F2-isoprostane protein carbonyl	p.o 800 IU/d	8 weeks	Same OS
O'Byrne et al. [165]	16 on Vit. E	Ox-LDL antibodies	p.o 800 IU/d	12 weeks	Same OS
Sanaka et al. [166]	11 on Vit. E 11 on placebo	PCOOH	p.o 500 mg/d	—	Same OS
Smith et al. [167]	11 on Vit. E	F2-isoprostanes	p.o 400 IU/d	8 weeks	Same OS
Antoniadi et al. [168]	27 on Vit. E 20 on placebo	TAS RBC SOD activity GSH-Px	p.o 500 mg/d	1 year	↑ OS
Inal et al. [170]	36 on EPO (100 U/kg) 36 on 50% decreased EPO dosage + Vit. E	MDA SOD activity CAT activity	p.o 300 mg/d	12 weeks	↓ OS
Badiou et al. [173]	14 on Vit. E	Cu-induced LDL oxidation TBARS	p.o 500 mg/d	24 weeks	↓ OS
Galli et al. [174]	7 on Vit. E	GSH TBARS NO	p.o 800 mg/d	3 weeks	↓ OS
Giray et al. [175]	36 on Vit. E	GSH-Px SOD + CAT TBARS	p.o 600 mg/d	14 weeks	↓ OS
Domenici et al. [176]	29 on Vit. E	8-OHdG	p.o 300 mg/d ×3/week	4 weeks	↓ OS
Ono [177]	30 on Vit. E	RBC osmotic fragility	p.o 600 mg/d	4 weeks	↓ OS

Table 2: Continued.

Study (ref.)	Patients	OS biomarker	Antioxidant	Study period	Result
Cristol et al. [178]	7 on ESA + Vit. E 30 control	MDA RBC SOD RBS GSH RBC Vit. E	p.o 500 mg/d	24 weeks	↓ OS ↑ Hb
Nemeth et al. [179]	10 children on ESA for 2 weeks, then ESA + Vit. E for 2 weeks	GSSG/GSH	p.o 15 mg/kg/d	4 weeks	↓ OS ↑ Hb
Uzum et al. [180]	19 on Vit. E 15 controls	MDA RBC osmotic fragility	p.o 300 mg/d	20 weeks	↓ OS
Hodkova et al. [181]	7 on i.v. iron + Vit. E	AOPPs PMNLs burst	p.o 200 mg/d	7 days	Same OS
NAC					
Swarnalatha et al. [102]	14 on NAC 14 on placebo	MDA	p.o 600 mg ×2/day Prior to i.v. iron	10 days	↓ OS
Garcia-Fernandez et al. [191]	10 iron 50 10 iron 50 + NAC 10 iron 100 10 iron 100 + NAC	MDA	i.v. 2 g Prior to i.v. iron	10 days	↓ OS
Trimarchi et al. [188]	12 on NAC 12 control	MDA	p.o 600 mg ×2/day	30 days	↓ OS
Witko-Sarsat et al. [189]	16 HD Cells incubated with NAC (in vitro study)	Serum albumin AOPPs	2 mg/mL	30′	↓ OS
Thaha et al. [190]	20 on NAC 20 on placebo	ADMA	i.v. 5 g	4 hours	↓ OS
Statins					
Diepeveen et al. [162]	12 on atorvastatin 11 on placebo	Ox-LDL	p.o 40 mg/d	12 weeks	↓ OS 30–43%
Ando et al. [192]	11 on EPA 11 on placebo	Ox-LDL	p.o 1.8 g/d	12 weeks	↓ OS 38%
Nishikawa et al. [193]	38 on simvastatin	MDA	p.o 5 mg/d	24 weeks	↓ OS

RBC: red blood cell; TBARS: thiobarbituric acid-reactive substances; Ox-LDL: oxidized low-density lipoprotein; PCOOH: phosphatidylcholine hydroperoxide; EPO: erythropoietin; CAT: catalase; NO: nitric oxide; 8-OHdG: 8-hydroxy 2′-deoxyguanosine; AOPPs: advanced oxidation protein products; GSSG: oxidized glutathione; EPA: eicosapentaenoic acid.

and OS biomarkers in a cohort of maintenance HD patients. Oral vitamin C (250 mg, thrice weekly) was given in 33 stable HD patients for two months. Although serum levels of vitamin C and ascorbate were normalized by oral supplementation, serum levels of CRP, albumin, carbonyls, or concentrations of reduced and oxidized glutathione in RBCs remained unchanged [159]. The investigators proposed the short period of treatment (2 months) and the route of administration (oral instead of intravenous) as possible explanations for their findings. In disagreement with the previous studies, Ghiadoni et al. reported that oral administration of 2 g vitamin C reduced OS biomarkers such as plasma MDA, lipoperoxides, and increased plasma antioxidant capacity (assessed by ferric reducing ability of plasma) in both HD and CKD stage 3 and 4 patients [124]. Two other randomized, placebo-controlled studies showed that 250 mg/day oral intake of vitamin C in HD patients reduced plasma and RBC MDA levels, although one study showed marginally nonsignificant effects [160, 161], while another study reported that intravenous administration of vitamin C during HD sessions significantly decreased HD-mediated OS in a study of 80 HD patients [28].

10.2. Vitamin E Supplementation and OS. Three randomized controlled trials showed no positive effect of vitamin E intake on OS development. Firstly, Diepeveen et al. administered 800 IU/day alpha-tocopherol for 12 weeks in a cohort of 23 HD and 21 PD patients and found that vitamin E did not alter plasma ox-LDL levels [162]. Secondly, Lu et al. showed that oral therapy with 800 IU of vitamin E every day for 6 months had no effect on plasma oxidative protein levels in stable HD patients compared to HD patients that received placebo [163]. Similarly, Kamgar et al. reported that after 8 weeks of daily oral treatment with a combination of vitamins (250 mg vitamin C, 800 IU vitamin E, 100 mg vitamin B_6, 250 μg vitamin B_{12}, and 10 mg folic acid), the plasma levels of protein carbonyls, F2-isoprostanes, IL-6, and CRP remained unchanged and suggested that oral antioxidant

multivitamin therapy does not improve OS, inflammatory, nutritional, and erythropoiesis status in maintenance HD patients [164]. In agreement, three open-label studies performed with small numbers of HD patients with and without DM2 failed to show any beneficial effect of oral vitamin E intake on plasma levels of isoprostanes, autoantibodies against ox-LDL and phosphatidylcholine hydroperoxide (PCOOH), [165–167]. One study in a relatively small cohort of subjects showed that prolonged (for one-year period), daily oral supplementation of 500 mg vitamin E in 27 HD patients resulted in a decrease in serum concentrations of some antioxidants (SOD, TAC) compared to the placebo HD group [168].

Several investigators suggested that vitamin E administration might have a protective role against OS in HD patients. Vitamin E intake has been shown to decrease membrane lipid peroxidation of platelets and red and white blood cells in HD patients [169]. Two small studies reported a beneficial effect of oral vitamin E intake on erythrocyte MDA levels and lipid peroxidation status in HD patients [170, 171]. Another study found that high daily oral intake of vitamin E for 2 months caused a decrease in serum ADMA levels in CKD predialysis patients [172]. Three open-label studies in HD patients reported that daily oral intake of AT (500–800 mg/day) resulted in significant reduction of plasma TBARS and induced antioxidant plasma levels of GSH, GPx, and SOD [173–175]. Domenici et al. reported that supplementation of vitamin E in both PD and HD patients resulted in decrease of protein oxidation and reduction of OS-induced DNA damage [176]. Several studies have suggested that vitamin E intake improves erythropoiesis factors by suppressing OS development in HD patients. The beneficial effect of oral vitamin E intake on erythrocyte fragility was first reported by Ono. Oral administration of 600 mg vitamin E every day for a month resulted in significant improvement of anemia, RBC fragility, and both serum and RBC vitamin E concentrations in a cohort of stable HD patients [177]. Oral administration of vitamin E improved renal anemia and lowered requirements of ESA [178] and had a protective role against OS induced by intravenous iron administration [93] in HD patients. Another study showed that combined treatment of ESA with oral intake of vitamin E (15 mg/kg daily) resulted in a significant reduction of the OS marker GSSG/GSH ratio and a considerable improvement in erythropoiesis compared to ESA therapy alone in children receiving chronic HD [179]. Uzum et al. conducted a randomized controlled trial and showed that treatment with 300 mg vitamin E daily for a period of 20 weeks resulted in a significant decrease of erythrocyte osmotic fragility and lipid peroxidation—as assessed by plasma MDA—in HD patients [180]. Hodkova et al. [181] reported that combination of oral AT and intravenous iron administration reduced PMN respiratory burst after iron intake in a cohort of HD patients. Therefore, vitamin E might be a beneficial supplement that suppresses the immunologic preoxidative activity induced by HD and iron infusion. However, it has to be cautioned, given that in other randomized studies, vitamin E supplementation yielded a negative result, that is, the supplementation led to no difference in cardiovascular events when supplemented over 4 years [182].

Although there is accumulating data suggesting that supplementation of various antioxidants such as vitamins C + E and NAC might reduce OS state in HD, the studies available are not consistent. The inconsistency between interventional studies aiming OS reduction by antioxidant supplementation in HD patients is due to several factors: firstly, OS status is assessed by numerous different biomarkers in different timelines and in heterogonous cohorts of patients; secondly, the dosage and the supplementation pathway of antioxidants differs between the trials; thirdly, the number of patients studied is relatively small to support strong conclusions; and finally, the exact pathophysiologic mechanism and the degree of OS abrogation by antioxidants is yet unclear. Although common antioxidants such as vitamins C + E seem to exert significant antioxidant effect on HD patients in small dosages, when they are administered in high dosages not only they lose their protective effect but it has been reported that they might actually act as prooxidants [168]. For all these reasons, there is a discrepancy between interventional studies regarding antioxidant supplementation and OS inhibition as well as patient adverse outcomes in HD patients. Therefore, antioxidant intake has not yet been adopted in guidelines or everyday clinical practice.

10.3. N-Acetylcysteine (NAC) and OS. NAC is a well-known thiol-containing free radical scavenger that induces cysteine and glutathione production. NAC exerts significant anti-inflammatory actions and is widely used as a pharmacologic antioxidant. NAC has the ability to scavenge ROS directly leading to production of cysteine, which triggers the release of glutathione, a powerful antioxidant [183]. NAC has been used for the treatment of several conditions related to OS, such as bronchiolitis and paracetamol overdose, whereas it has been shown to exert protective effects in the preservation of renal function in acute kidney injury and CKD [184–187]. Moreover, Trimarchi et al. reported that oral administration of NAC results in a significant reduction of MDA levels possibly through elevation of glutathione concentrations [188] and Witko-Sarsat et al. showed that NAC successfully decreased AOPP-derived responses of both normal and uremic neutrophils [189]. Another randomized placebo-controlled, double-blinded study showed that intravenous administration of high dose NAC (5 g) resulted in significant reduction of serum ADMA levels, compared to the placebo group [190]. Swarnalatha et al. [102] conducted a randomized placebo-controlled study and divided 28 HD patients that were treated with iron infusion in two groups: 14 were given NAC and 14 placebo. The NAC group showed reduced plasma levels of MDA compared to the placebo group. In a very similar study, Garcia-Fernandez et al. treated 80 HD patients with intravenous administration of 2 g of NAC and divided them in two groups: 40 received 50 mg of iron sucrose and the remainder 40 patients received 100 mg during HD. NAC resulted in significant increase in TAC in both groups, whereas MDA serum levels were only reduced in the low iron dose group [191].

Several studies showed that oral or i.v. administration of NAC—a powerful antioxidant scavenger—result in significant reduction of OS status in HD patients.

10.4. Statins and OS. Several investigators sought to determine the possible protective effects of statins and eicosapentaenoic acid (EPA)—a polyunsaturated omega-3 fatty acid—on the formation and accumulation of oxidized, atherogenic lipoproteins in patients undergoing renal replacement therapy. Ando et al. randomized 22 HD patients to either 1.8 g of EPA or placebo for 3 months and found that EPA supplementation significantly decreased plasma levels of ox-LDL and atherogenic remnant lipoproteins [192]. Nishikawa et al. showed that daily treatment with 5 mg of simvastatin for 6 months improved several lipid profile parameters and reduced the levels of MDA in 38 HD patients [193]. In contrast, Martinez-Castelao and coworkers [194] showed no effect of fluvastatin treatment on LDL susceptibility to oxidization in renal transplanted patients with dyslipidemia. The first prospective randomized, double blind placebo-controlled trial of treatment with statin and vitamin E in HD patients showed that the use of statins improved significantly the lipid profile and ox-LDL levels and might prevent CVD complications in these patients. Additional administration of vitamin E did not influence any lipid parameters but significantly reduced *in vitro* LDL oxizability, likely acting synergistically with the statin treatment [162]. Data from the LORD study suggest that administration of atorvastatin 10 mg/day for 3 years had no effect on plasma levels of F2-isoprostanes, protein carbonyls, glutathione peroxidase activity, and total antioxidant capacity in CKD patients [195].

Taken together, the possible antioxidant effects of statins in HD patients are not unequivocally shown.

10.5. Antioxidants and CVD. Although many investigators published promising results about the potential beneficial effects of oral/intravenous antioxidants on OS and inflammation status, the data regarding the impact of antioxidants on mortality and CVD events is scarce. Ghiadoni et al. reported that oral administration of 2 g of vitamin C seemed to improve significantly the endothelial dysfunction status—assessed by brachial artery endothelium-dependent vasodilation to reactive hyperemia and the response to sublingual glyceryl trinitrate—in HD but not in CKD patients [124]. Ono found no effect of vitamin C administration on morbidity and mortality in a cohort of 61 HD patients, after a period of 2 years [196]. In disagreement, Boaz et al. conducted a randomized double-blinded, placebo-controlled study in HD patients with previous history of CVD. The patients were randomized into two groups: 97 received 800 IU per day of natural vitamin E and 99 received placebo. After a follow-up period of 519 days, the authors reported that the vitamin E group presented a significant 70% reduction in myocardial infarction and 40% in composite CVD endpoints [197]. Another large randomized double-blinded, placebo-controlled study showed significant decrease in composite cardiovascular end-points in HD patients that were treated with NAC compared to the control group of HD subjects that received placebo [198]. Himmelfarb et al. conducted a large prospective, double-blinded, placebo-controlled randomized trial (provision of antioxidant therapy in hemodialysis (PATH) trial) to investigate the effects of oral antioxidant treatment administered for 6 months on biomarkers of OS, inflammation, and erythropoiesis. The study included 353 patients undergoing maintenance HD that were randomized to treatment with a cocktail of tocopherols (666 IU per day) and α-lipoic acid (600 mg per day) or placebo. Plasma levels of hs-CRP, IL-6, F2-isoprostanes, and isofurans were assessed at baseline (similar concentration for all biomarkers measured) and at 3 and 6 months. The authors failed to show any significant differences for circulating levels of OS biomarkers between the two groups. Furthermore, during the 6-month period, the hospitalization and all-cause mortality rates were similar between the two groups [199]. A recent systematic review and meta-analysis included 10 randomized controlled studies that explored the possible effects of antioxidant agents (vitamins A, C, and E and NAC) on mortality, CVD, and CKD progression in 1979 patients with CKD stages 3–5, renal transplant recipients, and on HD (June 2012, 2013). Although the use of antioxidants failed to prevent all-cause/CVD mortality and CVD morbidity (coronary heart disease, cerebrovascular disease, and peripheral artery disease) in CKD, antioxidant treatment in HD patients exhibited significant protection against CVD events. Moreover, in the nondialysis group (404 patients with CKD 3 + 4 and renal transplant recipients), administration of antioxidants delayed the progression of ESRD and was associated with preservation and even increase of eGFR [200, 201].

Thus, antioxidant administration may play a significant protective role against death and major adverse cardiac events in HD patients and prevents progression of ESRD in CKD patients.

11. Vitamin E-Coated Membranes and OS

Vitamin E has been repeatedly shown to be a scavenger of lipid hydroperoxides involved in the regulation of lipid oxidation *in vitro* [202] and a significant antioxidant and antiatherogenic molecule *in vivo*. Based on the fact that the interaction of blood with the dialyzer triggers the production of prooxidants, the use of vitamin E as adjunct in the membranes to additively provide a scavenger seemed an interesting approach. Galli et al. were the first to report that coating the blood surface of HD filters with the antioxidant vitamin E retained blood antioxidants (especially vitamin E) and prohibited the lipoperoxidation process both *in vitro* and *in vivo* and therefore could be considered a profound biocompatible material [203]. Moreover, the same group found that vitamin E-coated membranes (VECM) prevented efficiently the production of free radicals (and particularly ROS) by phagocytic neutrophils *in vitro* and *in vivo* [23]. They suggested that administration of vitamin E abrogates lipid peroxidation, protects serum GSH and thiols from the oxidation process, and seems to play a pivotal role in the modification of the NO metabolism [174, 204]. The beneficial effects of VECM besides their antioxidant

activity lie on their ability to inhibit complement activation, a common side-effect of the less biocompatible generated cellulose membranes. VECM cellulose showed significantly reduced activation of both mononuclear cells and the complement pathway compared to cellulose acetate dialyzers [205]. Another crossover study showed that compared to polyamide dialyzers, VECM presented similar effects on lymphocyte function, but additionally, it suppressed the formation of proinflammatory cytokines [206]. This may be due to the fact that vitamin E abrogates directly the release of proinflammatory cytokines by white blood cells, especially monocytes [207]. Membranes coated with vitamin E showed a significant protective role in alleviating HD-mediated OS, especially when combined with intravenous administration of vitamin C in a cohort of 80 stable HD patients [28]. Another study showed that VECM resulted in a higher degree of fatty acid unsaturation in erythrocyte cell membrane [208]. Similarly, it was reported that a 3-month treatment with VECM resulted in significant improvement of serum and HDL vitamin E content that was linked with lower susceptibility of HDL to oxidation in 12 HD patients [209]. VECM was also found to suppress the peroxidation status in serum lipids and erythrocytes, to reduce the circulating levels of AGEs and to pacify the immune activity [206, 210]. Furthermore, they have been reported to play a protective role against hemodialysis-induced endothelial dysfunction and increased production of ox-LDL [184]. In another study, 10 nondiabetic and 8 diabetic patients underwent regular HD with polysulphone membrane for 1 month and then changed to treatment with VECM for 6 months. MDA, AGEs, and 8-OHdG were assessed as markers of lipid peroxidation, glycoxidation, and DNA damage, respectively. Plasma levels of MDA, AGE and 8-OHdG were significantly elevated after a polysulphone HD, while a single dialysis session with VECM completely abrogated this increase. After use of VECM for a period of 6 months, there was a significant decrease in the plasma levels of AGEs and 8-OHdG in both diabetics and nondiabetics, while plasma MDA was decreased only in diabetic patients after three months of treatment. Furthermore, the improvement of OS status was more pronounced in the diabetic group [211]. Another randomized crossover study reported that maintenance HD patients dialyzed repeatedly with vitamin E-coated membranes for 12 weeks presented significant improvement in lipid parameters, oxidative stress, and polymorphonuclear function, compared with patients treated with control dialyzer membranes, that were identical to VECM, except for AT binding [212]. The long-term beneficial effects of VECM on OS and inflammation status were evaluated by Takouli et al. who switched 9 HD patients to VECM for a period of 12 weeks and then back to the original polysulphone dialyzer for 24 weeks. At the end of the study, ROS were significantly reduced and TAC and SOD were elevated. Plasma levels of the inflammatory markers hs-CRP and IL-6 were dramatically decreased [213]. A meta-analysis of 14 studies suggested that treatment with VECM resulted in significant improvement of lipid peroxidation markers, such as TBARS and MDA [214].

Modification of the dialyzer with vitamin E might increase its biocompatibility [215]. After 2 years follow-up of 50 stable maintenance HD patients, the group that was dialyzed with VECM presented significantly lower levels of LDL malondialdehyde and ox-LDL, compared to the group that received treatment with standard cellulose membrane dialyzers. Additionally, the use of these modified dialyzers resulted in slower progression of atherosclerosis, assessed by the aortic calcification index [215]. In agreement with these results, Morimoto et al. found that long-term (6 months) dialysis treatment with VECM was accompanied by significant decrease in ADMA, ox-LDL, and MDA-LDL plasma levels compared to dialysis with polysulphone dialyzers. After the 6-month period of VECM exposure, the patients were switched to HD with polysulphone membranes for 1 year. ADMA, ox-LDL, and MDA-LDL serum levels were increased back to the baseline. Since VECM seem to exert a powerful antioxidant effect and decrease the levels of circulating ADMA (a well-known independent predictor of all-cause mortality and CVD in HD patients), it is interesting to hypothesize that VECM might prevent all-cause and CVD mortality in these patients [216]. A recent study suggested that HD treatment with VECM may play a significant protective role against OS, anemia, and vitamin E deficiency. The use of VECM was accompanied by a significant reduction in the levels of DNA damage induced by OS [217].

A very recent meta-analysis of 60 studies aimed to summarize the available data on the effects of VECM over standard HD membranes on inflammation, anemia, and OS. The data suggest that the new membranes exert positive effects on the erythropoietin resistance index. Regarding inflammation and OS status, VECM resulted in reduction of IL-6 levels, circulating MDA, and levels of TBARS, while plasma and RBC vitamin E levels were significantly increased [218]. Lipid parameters and dialysis adequacy were not altered by the use of the new membranes.

Compared to standard HD membranes, dialysis treatment with vitamin E-coated membranes may possibly confer a protective role against inflammation, OS, and erythropoietin resistance.

12. Conclusions

OS is a universal challenge in life and induces a counterresponse by exposed cell. The enhanced OS status that characterizes HD patients is mainly due to poor dietary intake of exogenous antioxidants, accumulation of oxidative products, and loss of antioxidant molecules during HD and is highly linked with development of atherosclerosis, chronic inflammation, and all-cause and CVD mortality in these patients. Although the administration of antioxidants seems to play a beneficial role against OS development in maintenance HD patients, it has not yet been adopted in the everyday clinical practice. Large, prospective studies are urgently needed to elucidate the possible protective role of antioxidant administration against cellular stress that hold the promise to ameliorate the cardiovascular risk profile in CKD and end-stage renal disease. It seems that

OS is an undisputed component of the uremic environment and since uremia is a well-established nontraditional risk factor for CV events and all-cause/CV mortality, it is tightly linked with early atheromatosis and CV disease. Therefore, OS should be incorporated in a "uremic milieu" abnormality approach and might constitute a novel but quite important therapeutic target in chronic HD patients. Based on the available data, the best renal replacement therapy for reducing OS is HDF with ultrapure dialysate and synthetic membranes. It is not justified or safe to derive strong recommendations for either oral or intravenous antioxidant supplementation in HD patients, due to the fact that the interventional studies regarding this topic have failed to produce concrete results. However, assessing OS status in HD patients is a matter under discussion and probably in the light of more solid evidence could be incorporated in future routine clinical practice or even guidelines.

Abbreviations

8-OHdG: 8-Hydroxy-2′-deoxyguanosine
ADMA: Asymmetric dimethylarginine
AGEs: Advanced glycation end products
AOPPs: Advanced oxidation protein products
AT: Alpha-tocopherol
CAC: Coronary artery calcification score
CIMT: Carotid intima-media thickness
CKD: Chronic kidney disease
CVD: Cardiovascular disease
DM: Diabetes mellitus
EGFR: Estimated glomerular filtration rate
EPA: Eicosapentaenoic acid
ESA: Erythropoiesis-stimulating agent
ESRD: End-stage renal disease
GDPs: Glycose degradation products
GSH-Px: Glutathione peroxidase
GSH: Reduced glutathione
GSSG: Oxidized glutathione
H_2O_2: Hydrogen peroxide
HD: Hemodialysis
HDL: High-density lipoprotein
Hs-CRP: High-sensitive C-reactive protein
IL-6: Interleukin-6
i.v.: Intravenous
LDL: Low-density lipoprotein
MDA: Malondialdehyde
MPO: Myeloperoxidase
NAC: n-Acetylcysteine
NO: Nitric oxide
O_2^-: Superoxide anion radical
$ONOO^-$: Peroxynitrite
OS: Oxidative stress
Ox-LDL: Oxidized LDL
PCOOH: Phosphatidylcholine hydroperoxide
PD: Peritoneal dialysis
PMNs: Polymorphonuclear white blood cells
RBC: Red blood cell
ROS: Reactive oxygen species
RRF: Residual renal function
SOD: Superoxide dismutase
TAC: Total antioxidant capacity
TBARS: Thiobarbituric acid-reactive substances
TNF: Tumor necrosis factor
VECM: Vitamin E-coated membrane.

Conflicts of Interest

All authors declare no conflict of interest.

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Review Article

Role of Oxidative Stress and Mitochondrial Dysfunction in Sepsis and Potential Therapies

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Sepsis is one of the most important causes of death in intensive care units. Despite the fact that sepsis pathogenesis remains obscure, there is increasing evidence that oxidants and antioxidants play a key role. The imbalance of the abovementioned substances in favor of oxidants is called oxidative stress, and it contributes to sepsis process. The most important consequences are vascular permeability impairment, decreased cardiac performance, and mitochondrial malfunction leading to impaired respiration. Nitric oxide is perhaps the most important and well-studied oxidant. Selenium, vitamin C, and 3N-acetylcysteine among others are potential therapies for the restoration of redox balance in sepsis. Results from recent studies are promising, but there is a need for more human studies in a clinical setting for safety and efficiency evaluation.

1. Introduction

Sepsis is the leading cause of mortality in the intensive care units [1, 2]. Recent publications regarding the definition [3] and management of sepsis [4] underline the keen interest of clinicians. Despite the research, sepsis pathogenesis remains obscure. In the past, the widely accepted theory reported that sepsis was an uncontrolled inflammatory response to a pathogen that was rather a bystander than the real insult [5]. The failure of numerous studies using anti-inflammatory agents questioned the hypothesis of hyperinflammation [6–9].

Therapies focused until recently on macrocirculatory failure such as decreased mean arterial pressure and cardiac output. Immunohistohemical analysis revealed that cell death is minor suggesting that mechanisms other than cell death are responsible for mortality [10]. A growing body of evidence suggests that the inability of the cell to consume oxygen may play a crucial role for sepsis pathogenesis. For example, studies where supranormal oxygen delivery was targeted failed to improve patients' outcomes [11]. Furthermore, in animal studies, mucosal acidosis persisted despite the fact that mucosal perfusion did not change [12]. Since mitochondrial O_2 consumption is 90% of the total body consumption, impaired O_2 utilization and dysfunctional mitochondria may explain sepsis' specific characteristics. Sepsis is also characterized by excessive production of oxidants. Therefore, they may represent the generator of the abovementioned abnormalities that lead to increased mortality. In this context, redox homeostasis may play a key role, and consequently, therapies targeted to redox abnormalities may be useful for better management of septic patients.

Despite the increasing evidence that oxidative stress is a cornerstone on sepsis pathogenesis, the role of oxidative stress in sepsis may be underestimated. For example, in recent sepsis guidelines, its significance has not been highlighted. In this respect, clinicians may not be aware of the potentially pivotal role of oxidative stress in sepsis evolution. The aim of this literature review article is to point out current aspects about the topic and the evaluation of potential therapies.

2. Oxidants and Antioxidants

Redox reactions represent the basis for numerous biochemical mechanisms imperative for physiological cell function like cell signaling [13, 14]. Oxidants and antioxidants play a key role in the abovementioned mechanisms. The term antioxidant refers to a substance which donates electrons,

whereas an oxidant is a substance that accepts electrons [15]. Oxidants are involved in the formation of deoxyribonucleotides, prostaglandin production, oxidation, and carboxylation and hydroxylation reactions that are essential for normal cell function. Free radicals also participate in the host defense against bacterial infections [16], the regulation of vascular tone, and cell adhesion reactions and act as a sensor for oxygen concentration [17]. Important reactive oxygen species (ROS) in sepsis pathogenesis include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO). O_2^- and HO are free radicals since they have unpaired electrons in their molecule. Reactive nitrogen species (RNS) include the free radical nitric oxide (NO) and the nonradical peroxynitrite ($ONOO^-$). There are several procedures involved in the genesis of oxidant molecules in health [18] and sepsis. Cells that represent the innate immune system, like neutrophils and macrophages, are responsible for the oxidative burst that takes place early in sepsis process [19, 20]. The generated ROS and RNS are important for host defense as it was demonstrated by studies with mice deficient to produce O_2^-, a fact associated with decreased bacterial clearance [21]. The expression of nitric oxide synthase (NOS) is enhanced by lipopolysaccharide (LPS) treatment and nuclear factor kB (NF-kB) activation, and consequently, NO concentration produced by L-arginine is increased. Thereafter, NO can be combined with O_2^- to form $ONOO^-$ [22]. Increased NO levels generate H_2O_2 in mitochondria by cytochrome c oxidase inhibition [23]. In addition, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cyclooxygenase, and electron transfer chain in mitochondria are sources for the increased intracellular O_2^- [24–26].

For the protection of cellular homeostasis, there are several enzymes or other small molecules that act as oxidant scavengers and comprise the antioxidant defense system. The main antioxidant enzymes are (i) glutathione peroxidase (GPx) which catalyzes the conversion of H_2O_2 into water, (ii) superoxide dismutase (SOD) which converts O_2^- to O_2 or to the less reactive H_2O_2, and (iii) catalase (CAT) which also catalyzes the breakdown of H_2O_2. There are also other low molecular weight substances like ascorbic acid (AA), glutathione (GSH), and α-tocopherol. Among these, GSH is one of the most important redox buffers for the cells, since it can be found in all cell compartments. GSH/GSSG (glutathione disulfide, the oxidized form of GSH) is a good marker of oxidative stress. GSH can act as a cofactor for several enzymes, help in DNA repair, scavenge HO, H_2O_2, and lipid peroxides, and regenerate other antioxidants such as AA and tocopherols (Table 1).

Under physiological conditions, there is a balance between the formation of oxidant substances and their removal by antioxidant scavenging compounds [27]. Oxidative stress is the imbalance between antioxidant defense and generation of oxidants leading to enhanced oxidant concentration and constitutes a mechanism of injury for many disease processes [28]. The role of oxidative stress in the pathophysiology of several common conditions like diabetes mellitus, chronic heart failure, cancer, and degenerative, neurological, and autoimmune diseases is outside the scope of this review. Oxidative imbalance has been demonstrated in several sepsis studies. Takeda et al., in an early study, found an increased thiobarbituric acid reactive substance level in septic patients suggesting increased lipid peroxidation [29]. Decreased levels of antioxidants were also detected [30].

Table 1: Summary of antioxidants and their effects.

Antioxidant	Mechanism of action
GPx	H_2O_2 to H_2O
SOD	O_2^- to O_2
CAT	H_2O_2 to H_2O and O_2
GSH	Antioxidant scavenger, DNA repair, cofactor for enzymes
AA	Acts against oxidation of lipids, proteins, and DNA
α-Tocopherol	Scavenger for lipid peroxidation products

GPx: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase; GSH: glutathione; AA: ascorbic acid.

The clinical significance of oxidative stress in sepsis is demonstrated by several studies. Cowley et al. found that sepsis survivors had greater antioxidant potential than nonsurvivors and also that it was rapidly raised to normal or supranormal levels [31]. In two other prospective observational studies, total antioxidant capacity was correlated with Acute Physiology and Chronic Health Evaluation II (APACHE II) score [32] and the presence of a greater antioxidant deficiency correlated with mortality [33]. This deficiency was strongly indicated by two markers, GSH levels and CAT activity in erythrocytes, and persisted in time. Lower plasma vitamin C levels were detected in patients with multiorgan failure [34]. Harmful mechanisms of increased oxidants level in sepsis include modification of proteins, lipids, and nucleic acids contributing to cellular injury and endothelial dysfunction. In addition, the impairment of glycocalyx and the cellular junctions between endothelial cells lead to increased vascular permeability, a cornerstone of sepsis development [35].

3. NO and Cardiovascular Dysfunction

NO is produced from L-arginine by NOS [36], an enzyme with different isoenzymes (neuronal NOS or nNOS, inducible NOS or iNOS, endothelial NOS or eNOS, and mitochondrial NOS or mtNOS). iNOS produces NO in micromolar range as opposed to the other isoforms that produce NO in nanomolar range [37]. In sepsis, NO can be produced by several cells like activated macrophages, neutrophils, lymphocytes, and others [38–40]. Various molecules such as interferon γ (IFNγ), tumor necrosis factor a (TNFa), and interleukin 1β (IL-1β) involved in the septic inflammatory process are incriminated in the induction of NO production via iNOS hyperactivity. When the cell interacts with one of these molecules, IkB in the cytoplasm is degraded, NF-kB is permitted to move to the nucleus, and therefore expression of iNOS-associated genes is enhanced [41–43].

The effects of NO can be divided into effects on cardiac performance and effects on microcirculation. NO plays a pivotal role in vasodilation and vascular hyporeactivity to

Table 2: Summary of NO effects in sepsis.

Positive effects of NO
Preventive of cardiac contractility in low concentrations
Mitochondrial proliferation
Scavenger of oxygen free radicals
Inhibition of oxygen free radical production
Low pulmonary vascular tone
Negative effects of NO
Vasodilation/hyporeactivity to vasopressors
Negative inotropic effect in high concentrations
Inhibition of mitochondrial respiration
Protein nitration/nitrosylation
Methemoglobinemia
Activation of NF-kB

NO: nitric oxide; NF-kB: nuclear factor kB.

vasopressors. There are several studies that indicate this relationship. The injection of LPS in iNOS-deficient mice and the wild type as control provided the evidence that iNOS mediates impairment of vascular contraction [44]. Moreover, the inhibition of NO synthesis reversed shock in dogs induced by bacterial endotoxin [45] and also in septic rats by cecal ligation [46]. NO mediates negative inotropic effects to the cardiac function. LPS treatment of failing myocardium decreased maximum inotropic response to isoprenaline. The depression of cardiac contractility was attributed to enhanced iNOS activity and release [47]. In another study by the same investigators, the negative inotropic effect on human atrial and ventricular myocardium seemed to be mediated via generation of cyclic guanosine monophosphate (cGMP) [48]. On the other hand, other studies revealed that NO has no effect on the myocardium [49] or even that low concentrations of NO are preventive of cardiac performance. In a rodent heart model, coronary flow and ventricular function were reduced by LPS, effects that were partially prevented by supplementation of NO substrate, L-arginine. These improvements were partially blocked by the administration of selective iNOS inhibitors [50]. These data lead to the assumption that NO effects on cardiac performance are concentration dependent.

Other deleterious effects include protein nitrosylation and nitration, convertion of haemoglobin (Hb) to methaemoglobin (MetHb) which leads to red blood cell lysis and thus iron availability to the pathogens, and enhance the production of IL-6 and IL-8 and activation of NF-kB [26, 51] (Table 2).

4. Mitochondria and Apoptosis

Mitochondria play a key role in redox dysregulation being at the same time sources and targets of oxidants. Oxidative phosphorylation takes place in the inner mitochondrial membrane where electron transport chain lies, consisting of five respiratory complexes (I–V). Electrons are transferred from one to another (I–IV) leading to adenosine triphosphate (ATP) generation in complex V (ATP synthase). Molecular oxygen is the final receptor of the electrons, and thus, an assessment of mitochondrial function can be performed through the measurement of oxygen consumption. The association between mitochondrial dysfunction and sepsis severity is addressed in several studies. In a fundamental one [52], skeletal muscle biopsies on 28 septic patients showed that nonsurvivors had lower ATP concentrations. Furthermore, vasopressor requirements were proportional to NO production as it was gauged by nitrite/nitrate concentrations and inversely correlated to complex I activity. Decreased ATP concentration and mitochondrial activity were also found in other human or animal studies [53, 54]. The pathogenesis of mitochondrial dysfunction is probably complex. NO seems to play a pivotal role by inhibiting the normal function of the respiratory complex IV. By binding to the specific complex, NO interrupts the normal transport of electrons and thus ATP production while at the same time the production of O_2^- is enhanced. The generated O_2^- reacts with NO leading to further mitochondrial dysfunction especially by complex I inhibition [55, 56] (Figure 1). The abovementioned mechanisms potentially explain the inability of the cells to utilize oxygen despite the adequate tissue oxygen tension. The term "cytopathic hypoxia" [57] refers to this phenomenon that eventually leads to multiorgan failure and worse outcomes. On the other hand, lower NO concentration seems to promote mitochondrial proliferation suggesting that NO effect on mitochondrial function may be concentration dependent [58].

Other potential mechanisms involve protein production and apoptosis. The decreased ATP synthase gene expression and subsequently impaired protein production were demonstrated by the administration of LPS in humans [59]. Apoptosis is the programmed cell death and is involved in sepsis pathogenesis. It can be triggered in a cell through either extrinsic or intrinsic stimuli. Mitochondria play a role in both pathways but especially in the intrinsic one. Mitochondrial damage by ROS can release cytochrome c, the mediator in electron flow between complexes III and IV, to cytosol. The next step is the formation of the "apoptosome" which reacts with caspases initiating the apoptotic pathway via deoxyribonucleic acid (DNA) fragmentation and chromatin condensation [60–63] (Figure 2).

5. Potential Therapies

The mainstay of sepsis management is source control, antibiotic administration, and haemodynamic support, but the relationship between antioxidant status and sepsis outcomes sets also the rationale for the use of antioxidant substances for the treatment of sepsis. Several molecules and different strategies were used in a plethora of studies in the past years with sometimes conflicting results.

5.1. Selenium. Selenium is essential for the synthesis of antioxidant enzymes, like GPx, and is involved in redox signaling and other immune responses [64]. The rationale for selenium supplementation derives from the correlation between low levels of selenium and disease severity and worse clinical

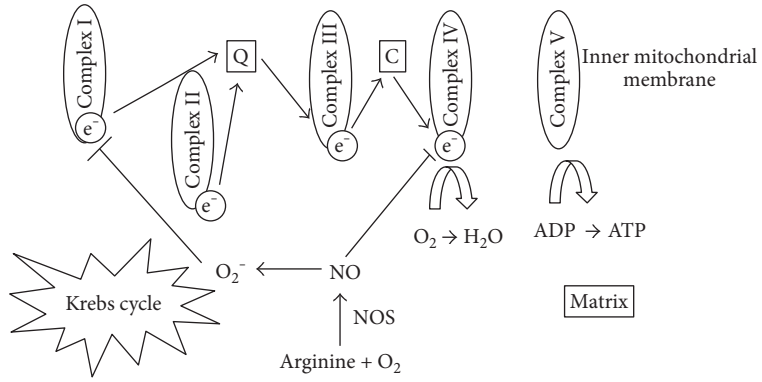

Figure 1: The mechanism of cytopathic hypoxia. The production of NO inhibits normal function of the respiratory complex IV interrupting the normal transport of electrons. O_2^- production is enhanced and reacts with NO inhibiting complex I normal function. NO, nitric oxide; NOS, nitric oxide synthase; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

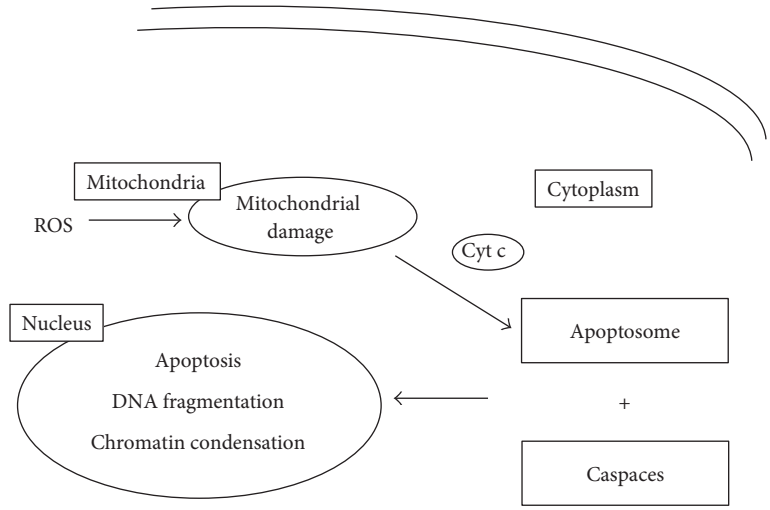

Figure 2: Mechanism of apoptosis. Mitochondrial damage by ROS releases cytochrome C, which contributes to the formation of apoptosome. The reaction of apoptosome with caspaces initiates cell apoptosis via DNA fragmentation and chromatin condensation. ROS, reactive oxygen species; Cyt C, cytochrome C; DNA, deoxyribonucleic acid.

outcomes in critically ill patients [65]. In a single-center clinical trial conducted on 54 septic patients, high-dose selenium administration did not result in reduction of 28-day mortality but increased the activity of GPx. No effect on the level of inflammatory cytokines was noted. However, selenium administration was associated with reduced incidence of ventilator-associated pneumonia (VAP) [66]. Moreover, in a recent multicenter randomized controlled trial (RCT), high-dose intravenous administration of sodium selenite was combined with procalcitonin-guided antimicrobial therapy in order to improve sepsis outcome. Both interventions failed to improve 28-day mortality [67]. In the most recent meta-analysis [68] after the review of 21 RCTs, the investigators concluded that parenteral supplementation of selenium in critically ill patients as a single agent or combined with other antioxidants had no effect on mortality, infections, length of stay, or ventilator days. The only significant effect was the reduction of infections in patients that were nonseptic at the initiation of therapy. In conclusion, even if there is a rationale for selenium administration, clinical trials failed to demonstrate benefits. Further research may reveal new insights in the role of selenium in sepsis pathophysiology.

5.2. Vitamin C. AA is the redox form of vitamin C and acts as a natural antioxidant. Plasma AA in patients with multiorgan failure was significantly lower [34], whereas low concentrations were inversely correlated with increased lipid peroxides [69] a marker of increased oxidative stress. Results from animal models demonstrated that AA ameliorates edema and hypotension and improves arteriolar responsiveness and capillary blood flow [70–73]. Experiments in healthy volunteers after induction of systemic inflammation by low doses of *E. coli* endotoxemia revealed that the hyporeactivity can be corrected by high doses of vitamin C, suggesting that oxidative stress may represent an important target for inflammation-induced impaired vascular function [74]. In a phase I safety trial of intravenous AA in patients with severe sepsis, infusion was safe and well tolerated [75].

In a retrospective analysis of the combination of hydrocortisone, vitamin C, and thiamine for the treatment of severe sepsis and septic shock, hospital mortality was 8.5% in the treatment group compared to 40.4% in the control group ($p < 0.001$). The propensity-adjusted odds of mortality in the patients treated with the vitamin C protocol was 0.13 (95% CI 0.04–0.48, $p = 0.02$). The sequential organ failure assessment score (SOFA score) decreased in all patients in the treatment group with none developing progressive organ failure. The duration of vasopressors was also smaller for the treatment group [76]. The very promising results of this study render the need for prospective randomized trials imperative for the determination of the role of vitamin C in sepsis treatment.

5.3. N-Acetylcysteine (NAC). GSH is an important molecule recognized not only as an antioxidant but also as a mediator of immune and inflammatory pathways. GSH function is potentially enhanced by the administration of NAC, which has also itself an antioxidant and immunomodulatory activity [77–82]. Studies in humans demonstrated that the administration of NAC can significantly increase hepatosplanchnic blood flow attributed to the increase of cardiac index [83] and can augment neutrophil phagocytosis in patients diagnosed with sepsis, systemic inflammatory response syndrome (SIRS), or multiple trauma [84]. On the other hand, there are studies that demonstrate no influence on outcomes and the level of cytokines [85]. Sometimes, sepsis-induced organ failure was even aggravated [86]. The conflicting results may be due to a limited number of patients. Findings need to be confirmed in larger clinical trials.

5.4. Mitochondria-Targeted Antioxidants. Several strategies were used in order to reduce oxidative stress generated in mitochondria. The ability of lipophilic cations to accumulate in the mitochondria makes them good candidates for clinical studies. MitoQ (ubiquinone attached to a triphenylphosphonium cation) has been shown to protect mammalian cells from hydrogen peroxide-induced apoptosis [87, 88]. In another study, the effects of MitoQ were tested at first in vitro in an endothelial cell model of sepsis and afterwards in vivo in a rat model of sepsis. In vitro, MitoQ decreased oxidative stress and protected mitochondria from damage as indicated by a lower rate of ROS formation and by maintenance of the mitochondrial membrane potential. In vivo, MitoQ treatment resulted in lower levels of biochemical markers of acute liver and renal dysfunction [89]. The hypothesis that the administration of MitoQ would prevent endotoxin-induced reductions in cardiac mitochondrial and contractile function was tested in adult rodents. Endotoxin induced reductions in mitochondrial state 3 respiration rates, the respiratory control ratio, and ATP generation. These effects were ameliorated in the MitoQ-treated animals [90]. There are other substances conjucated to triphenylphosphonium cation as well, like vitamin E (MitoVitE), or ebselen, a selenium-containing compound with peroxidase activity (MitoPeroxidase) [91, 92]. Despite their promising properties, data on human studies are lacking.

Another option is the use of SOD mimetics. SOD mimetic M40401 improved vascular reactivity to vasopressors, reduced cytokine production, and improved mortality in a rat model of septic shock [93]. The ability of another SOD mimetic, the MnIIITE-2-PyP5+, to enter the mitochondria in vivo at levels sufficient to exert its antioxidant action was established by another study in rats [94]. These results encourage the development of SOD mimetics as therapeutic agents for sepsis.

TEMPOL was also used in animal studies with promising results [95, 96], but human studies are lacking. Antiapoptotic properties and ROS scavenging may explain its beneficial action.

5.5. NOS Inhibitors. The crucial role of NO in sepsis development and organ dysfunction led to the implementation of therapeutic strategies capable of reducing NO levels. NOS inhibition can be nonselective or selective for iNOS, which is predominantly synthesized during inflammation. In animal studies, nonselective NOS inhibition improved haemodynamics but increased mortality [97, 98]. The use of nonselective NOS inhibitors in patients with septic shock was terminated early because of increased mortality [99]. The inhibition of eNOS may explain the negative results of the study. The finding that the overexpression of eNOS is beneficial in septic animals [100, 101] led to the hypothesis that it is the excessive NO production by iNOS that is harmful and stimulated a research for selective iNOS inhibitors. Treatment with the selective iNOS inhibitor aminoguanidine inhibited the LPS-induced bacterial translocation by ameliorating intestinal hyperpermeability [102]. The rate of oxygen consumption was significantly restored in endotoxemic rats treated with aminoguanidine as compared with vehicle-treated endotoxemic rats [103]. Furthermore, in a porcine model of bacteremia where selective iNOS blockade was used, sepsis-induced plasma nitrate/nitrite concentrations were inhibited, hypotension was prevented without affecting cardiac output, and progressive deterioration in ileal mucosal microcirculation was blunted without mucosal acidosis [104]. An interesting alternative is ketanserin, a serotonin receptor antagonist. Several studies suggest that the administration of ketanserin is beneficial in septic animals. Mechanisms involved are the restoration of baroflex function [105] and the inhibition of iNOS expression via the MEK/ERK pathway [106]. The administration of ketanserin in septic patients resulted in improved microcirculatory perfusion assessed by direct visualization of the microcirculation with sidestream dark-field imaging [107]. The promising results deserve further evaluation in randomized trials.

5.6. Melatonin. Melatonin is the major hormone secreted by pineal gland predominantly at night. Melatonin has significant anti-inflammatory and antiapoptotic effects, but it can also act as an antioxidant scavenger for radical oxygen and nitrogen species [108, 109]. There are several animal studies depicting these beneficial antioxidant properties of melatonin in LPS or cecal ligation and puncture- (CLP-) induced septic shock [110–112]. Another important finding is the protection of mitochondrial dysfunction. Melatonin

administration decreased mitochondrial NOS activity and inhibition of complexes I and IV in LPS-treated rats [113]. Furthermore, the results from another study suggest that melatonin can also prevent mitochondrial damage from the inducible isoform of mitochondrial NOS in septic mice [114]. Finally, it can restore mitochondrial production of ATP [115]. When healthy volunteers received melatonin before the administration of LPS, several markers of inflammation and oxidative stress were reduced [116]. In another study, melatonin treatment in septic newborns resulted in lower concentrations of lipid peroxidation products and other favorable outcomes [117]. In conclusion, melatonin has beneficial effects in sepsis that encourage the development of human studies since relevant data are lacking.

6. Conclusion

Oxidative stress mechanisms in sepsis are highly complicated. ROS and RNS play a pivotal role in sepsis evolution, but their specific role and importance remain obscure. Nevertheless, hyperpermeability, hypotension induced by reduced vascular tone, and mitochondrial impairment of respiration are key elements for multiorgan failure and thus mortality in septic patients. Several therapies were tested in clinical trials. Results are not sufficient for the implementation of these therapies in a clinical setting. An explanation may be that animal models do not completely resemble human sepsis. Further research is needed to answer questions about the underline mechanisms. Nevertheless, the increasing insight may alter our perception in sepsis development and management.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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