Therapeutic ROS Scavenging by Mitochondria-targeted Antioxidants SkQ1 and MitoTEMPO in the Mouse Model of CLP Sepsis

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1 Introduction

Sepsis and septic shock are not only costly to treat (Torio and Moore 2006), but also belong to the most frequent causes of death in critically ill patients. Despite the ongoing debate regarding the true incidence of sepsis syndromes (Gaieski et al. 2013), the recent data suggest that the incidence of sepsis is alarmingly high and grows (Fleischmann et al. 2016). Factors such as age, gender and comorbidities affect the survival rate (Levy et al. 2012, Gaieski et al. 2013); in uncomplicated sepsis mortality approximates 20% (Winters et al. 2010, Martin 2012), while in patients who develop multiple organ failure, mortality rates can reach 80% (Angus et al. 2001). Even if patients survive the initial phase of sepsis, they remain at high risk for an increased morbidity and mortality for up to five years after discharge (Hodgin and Moss 2008, Winters et al. 2010).

Several previous studies indicate that early diagnosis of sepsis followed by immediate standard antimicrobial and fluid treatment is necessary to improve outcomes (Otero et al. 2006, Freitas et al. 2008). A clinical trial on Early goal-directed therapy (EGDT) by Nguyen et al. showed a marked survival benefit (Nguyen et al. 2016), but a recent meta-analysis concluded that EGDT is not advantageous compared to classical care (PRISM Investigators et al. 2017). To date there is still no specific sepsis-treatment available (Ward and Bosmann 2012).

1.1 Definition of sepsis and septic shock

Sepsis is an immune response triggered by a bacterial, viral, fungal or parasitical infection. It also occurs as a secondary complication following noninfectious incidents, such as trauma. Sepsis was previously divided into four different stages: systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock. According to the latest sepsis definitions from early 2016 “The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)” the terms SIRS and severe sepsis were “unhelpful” and “superfluous” and as such they were deleted leaving only “sepsis” and “septic shock” in the definitions (Singer et al. 2016) (Fig. 1).

1.1.1 Sepsis

In Sepsis-3, sepsis has been defined as “life threatening organ dysfunction caused by a dysregulated host response to infection” (Singer et al. 2016). According to this document, a sepsis-related organ dysfunction can be identified by a Sequential/Sepsis-related Organ
Failure Assessment (SOFA) score. The score consists of the following parameters, segmented into six systems with a possible score from zero to four respectively:

- **Respiration:** \( \text{PaO}_2/\text{FiO}_2 \) (mmHg)
- **Coagulation:** Platelets \( \times 10^3/\text{mm}^3 \)
- **Liver:** Bilirubin
- **Cardiovascular:** Hypotension
- **Central Nervous System:** Glasgow Coma Score
- **Renal:** Creatinine or urine output

An acute change in total SOFA score of two or more points is considered to detect organ dysfunction (Singer et al. 2016). This scoring system is already in use in intensive care units (Vincent et al. 1996, 1998) where a high score is accompanied by an increased probability of mortality (Vincent et al. 1998).

To facilitate an early and rapid therapeutical action in patients outside of the ICU, Sepsis-3 has also introduced a new bedside index, the so-called quick SOFA (qSOFA), where patients with an increased risk of death can be identified as soon as two of the following three criteria are present:

- Respiratory rate \( \geq 22/\text{min} \)
- Altered mentation
- Systolic blood pressure \( \leq 100 \) mmHg

### 1.1.2 Septic shock

According to sepsis-3, septic shock can be identified in patients by the necessity for vasopressors to maintain a mean arterial pressure of at least 65 mmHg and a serum lactate level above 2 mmol/L in the absence of hypovolemia (Singer et al. 2016).

![Figure 1](current_sepsis_algorithm.png)  
**Figure 1** Current sepsis algorithm adapted from Singer et al. (Singer et al. 2016), illustrating the latest recommendations to identify patients with sepsis and septic shock. MAP, mean arterial pressure.
1.2 Sepsis pathophysiology

1.2.1 Immune responses in sepsis

Originating from the initial site of infection, most frequently lung and abdomen (Vincent et al. 2006, He et al. 2016), pathogens spread systemically. Binding of molecules either originating from penetrating microorganisms (pathogen-associated molecular patterns, PAMPs) or from necrotic cells (damage-associated molecular patterns, DAMPs) via pattern recognition receptors (PRRs) (Chousterman et al. 2017) subsequently leads to activation of the innate immune system followed by a systemic inflammatory response (Janeway and Medzhitov 2002, Akira et al. 2006). In vertebrates, four different PRRs have been described (i.e. Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs), and the C-type lectin receptors (CLRs)), from which the TLRs are the most thoroughly investigated. TLR4 binds to lipopolysaccharide (LPS, endotoxin) (Poltorak et al. 1998a, 1998b), which is embedded in the outer cell membrane of gram-negative microorganisms (Cohen 2002). The binding activates a signaling cascade which results in the activation of transcription factors, such as NFκB, and subsequently in the generation of pro-inflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α or IL-1β (Annane et al. 2005, Kawai and Akira 2011).

Gram-positive bacteria do not contain endotoxin, but peptidoglycan and lipoteichoic acid in their cell walls (Dziarski et al. 1998), to which another type of PRR can bind: The NOD-like receptors (NLRs), including NOD1 and NOD2. Following activation of the NLR pathway, pro-inflammatory cytokines are produced (Zamboni et al. 2006, Mariathasan and Monack 2007).

Cytokines promote the inflammatory process via auto-, para- and endocrine action leading to activation, proliferation and/or migration of immune cells (Dinarello 2007). Cytokines are small cell signaling molecules (<40 kDa), mainly produced by monocytes/macrophages, neutrophils, eosinophils, basophils and natural killer cells (NKs) (Chousterman et al. 2017). High levels of certain cytokines are also correlated with severity and outcome of sepsis and are therefore used as biomarkers, but they still lack specificity (Meisner 2014, Ricarte-Bratti et al. 2017).

The different categories of cytokines include interleukins, chemokines, interferons, tumor necrosis factor and growth factors, with interleukins playing the most important role during inflammation (Chousterman et al. 2017). They can be divided into pro- and anti-inflammatory interleukins: The former are responsible for activating cells, tissue damage and necrosis, including IL-1β, and IL-6.
Higher levels of IL-1β have been described in non-surviving septic patients, indicating a possible association of IL-1β levels and sepsis outcome (Mera et al. 2011).

IL-6 increases due to an activation cascade leading to an auto-amplifying production of pro-inflammatory cytokines ("cytokine storm") (Ferrara et al. 1993, Jones et al. 2011). As high levels of IL-6 can be seen in many inflammatory diseases they are not sepsis-specific, but similar to IL-1β they are associated with severity and worse outcome of sepsis (Kellum et al. 2007, Wu et al. 2009, Gouel-Chéron et al. 2012).

1.2.2 Immunosuppression in sepsis
In response to increasing IL-6 levels, the production of IL-10, the main anti-inflammatory cytokine (McGeachy et al. 2007) restrains the pro-inflammatory immune response. It is produced by immune or epithelial cells and attenuates the activity of Th1 cells, NK cells, and macrophages (Couper et al. 2008) by inhibiting the production of pro-inflammatory cytokines (Opal and DePalo 2000). IL-10 levels are directly related to severity of inflammation and associated with a phase of immunosuppression occurring during sepsis (Hotchkiss et al. 2013). Both pro- and anti-inflammatory cytokines occur simultaneously in the early phase of sepsis, where the proinflammatory response is still prevailing (Hotchkiss et al. 2013), (Hotchkiss et al. 2009). After the first days of sepsis progression, the anti-inflammatory response becomes predominant, leading to “immunoparalysis” and facilitating secondary infections (Volk et al. 2000). Besides the release of IL-10, there are various intertwining mechanisms of immunosuppression: Lymphopenia (Le Tulzo et al. 2002), T-cell exhaustion (Zajac et al. 1998), deactivation of monocytes (Volk et al. 1996), and loss of dendritic (Hotchkiss et al. 2002) and epithelial cells (Chawla et al. 2016) all contribute to the immunosuppressive state. In addition, Programmed Cell Death-1 (PD-1)/Programmed Death-ligand 1 (PD-L1) Pathway appear to play a role in sepsis-induced immunosuppression (Liu and Li 2017).

1.2.3 Organ dysfunction in sepsis
While the pathophysiology of sepsis and its various complications is not fully understood, the terminal stage of sepsis is often the multiple organ dysfunction syndrome (MODS). It can affect every organ in the body ranging from acute kidney injury (AKI) (Bagshaw et al. 2009) and acute lung injury (ALI) (Perl et al. 2011) over hepatic (Dhainaut et al. 2001) and brain injury (Wilson and Young 2003) to pathological cardiovascular changes (Levy et al. 2005) either via direct damage or secondary due to poor perfusion (Iskander et al. 2013).


1.2.4 **ROS production in sepsis**

ROS are a group of molecules, so-called free radicals, with an unpaired electron in their outer atomic electron shell, such as superoxide ($\text{O}_2^-$), hydroxyl (‘OH) and peroxynitrite (ONOO$^-$). They have the potential to damage a wide array of molecules by oxidation, such as amino acids, cell membranes and DNA. Following the release of pro-inflammatory cytokines during the first 30-90 minutes (e. g. after LPS recognition), reactive oxygen species increase (Cohen 2002).

There are several exogenous and endogenous sources of ROS. For example, during cellular energy production, the greatest amount of intracellular ROS is emerging from mitochondria as a byproduct of oxidative phosphorylation in the electron transport chain (ETC). The ETC is composed of five complexes (CI-CV), located in the inner mitochondrial membrane. Along this series of complexes, electrons are passed from a donor (e. g. Nicotinamide adenine dinucleotide, NADH) to a terminal electron acceptor ($\text{O}_2$), generating energy. Complexes I, III and IV use this energy to move protons from the mitochondrial matrix into the intermembrane space, creating a proton gradient. This electrochemical gradient enables CV, the enzyme adenosine triphosphate (ATP) synthase, to ultimately generate ATP via oxidative phosphorylation.

On their way through the ETC, electrons can leak from the electron carriers, forming $\text{O}_2^-$ (a single electron leaking) or $\text{H}_2\text{O}_2$ (a pair of electrons leaking) (Brand 2016), which can easily be converted into hydroxyl radicals. Complexes I, II and III are the main sites of ROS generation (Goncalves et al. 2015). Under physiological conditions, endogenous antioxidant enzymes tightly regulate the amount of emerging ROS to keep steady-state levels, facilitating cellular signaling pathways and regulation (Nordberg and Arnér 2001, Turrens 2003, Valko et al. 2007, Dan Dunn et al. 2015).

In contrast, severe inflammatory conditions like sepsis are associated with excessive ROS production, overwhelming the host’s antioxidant defenses and pushing the organism into oxidative stress, a destructive chain reaction, resulting in damaged cells and tissues (Phaniendra et al. 2015). Hence, mitochondrial oxidative stress is thought to play a major role in the development of sepsis-related organ failure (Turrens 2003, Murphy 2009). Such understanding of that phenomenon has defined mitochondria as a promising target for therapeutic interventions.
1.3 Mitochondria-targeted antioxidants as a therapeutical approach in sepsis

Numerous promising preclinical studies testing specific medications to treat sepsis failed to be successfully translated to clinics (Opal et al. 2014). In the last few years, preventing/neutralizing oxidative stress in sepsis has become a research focus and various antioxidants have been investigated in preclinical sepsis models, including selenium (Angstwurm et al. 1999, Sakaguchi et al. 2000), Vitamin C and E (Kim and Lee 2004, 2006) and N-acetylcysteine (NAC) (Hsu et al. 2006). In septic patients, only few antioxidants (i. e. levosimendan (Torraco et al. 2014) and melatonin (Galley et al. 2014) were investigated in clinical trials. Generally, antioxidants are recommended as beneficial co-therapies in sepsis (Prauchner 2017), but failed to significantly improve outcomes.

To scavenge ROS directly at its source of synthesis (i. e. inside the mitochondria), antioxidants have been enriched by conjugation to lipophilic cations like triphenylphosphonium (TPP), which have the ability to pass through biological membranes. Thanks to its positive charge, the conjugated antioxidant follows the potential gradient and initially accumulates inside the cytoplasm. Subsequently the cation passes the inner mitochondrial membrane (IMM) and accumulates inside the mitochondria drawn by the (in relation to the intermembrane space) negatively charged mitochondrial matrix (Murphy and Smith 2007) (Fig. 2).

It has been shown in vitro that targeted (i. e. regarding the precise destination of their effect/action), TPP-bound antioxidants provide better protection against damage caused by oxidative stress than untargeted agents (Kelso et al. 2001, Bedogni et al. 2003, Dhanasekaran et al. 2004). Mitochondria-targeted antioxidants (mtAOX) have also been successfully tested in different in vivo models (Smith et al. 2003, Galley 2011), including cardiovascular diseases (Adlam et al. 2005, Graham et al. 2009) and CLP-induced acute kidney injury (Patil et al. 2014).

Unfortunately, mtAOX failed to show significant survival benefit in clinical trials. To date, mtAOX as a treatment against inflammatory diseases have been tested in patients with Parkinson’s disease, showing no difference between the antioxidant treatment and placebo (Snow et al. 2010). In hepatitis C trial, oral treatment with mtAOX mitoquinone demonstrated only hints of decreasing necro-inflammation in the liver (Gane et al. 2010).

Two novel mtAOX, SkQ1 (lipophilic; accumulates in the inner mitochondrial membrane (IMM)) and MitoTEMPO (hydrophilic, accumulates in the mitochondrial matrix; Fig. 2), have the
ability to scavenge intracellular ROS directly at its source. Both mtAOX showed promising results in an endotoxemia model (Weidinger et al. 2015), but have not been further investigated in the long-term context of polymicrobial sepsis. This study continues the investigation of the two agents in a polymicrobial abdominal sepsis model: the aim was to investigate the effects of mtAOX SkQ1 and MitoTEMPO on outcome, organ function and inflammatory response in the acute and chronic phase of sepsis.

Figure 2 Chemical structure and scheme of intramitochondrial accumulation of mtAOX SkQ1 and MitoTEMPO. Driven by membrane potential and positive charge of triphenylphosphonium (TPP) both mtAOX accumulate in the mitochondrion: SkQ1 (lipophlic) in the inner mitochondrial membrane (IMM), MitoTEMPO (hydrophilic) in the mitochondrial matrix. S (blue): SkQ1; M (green): MitoTEMPO; ROS: reactive oxygen species; CI – CV: complex I – complex V; OMM: outer mitochondrial membrane; IMS: intermembrane space; ETC: electron transport chain; e⁻: electron; H⁺: proton; NADH/NAD⁺: Nicotinamide adenine dinucleotide in oxidized/reduced form; ADP+P: Adenosine diphosphate + phosphate; ATP: Adenosine triphosphate.
2 Publication
Research Article

Mitochondria-Targeted Antioxidants SkQ1 and MitoTEMPO Failed to Exert a Long-Term Beneficial Effect in Murine Polymicrobial Sepsis

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Mitochondrial-derived reactive oxygen species have been deemed an important contributor in sepsis pathogenesis. We investigated whether two mitochondria-targeted antioxidants (mtAOX; SkQ1 and MitoTEMPO) improved long-term outcome, lessened inflammation, and improved organ homeostasis in polymicrobial murine sepsis. 3-month-old female CD-1 mice (n = 90) underwent cecal ligation and puncture (CLP) and received SkQ1 (5 nmol/kg), MitoTEMPO (50 nmol/kg), or vehicle 5 times post-CLP. Separately, 52 SkQ1-treated CLP mice were sacrificed at 24 h and 48 h for additional endpoints. Neither MitoTEMPO nor SkQ1 exerted any protracted survival benefit. Conversely, SkQ1 exacerbated 28-day mortality by 29%. CLP induced release of 10 circulating cytokines, increased urea, ALT, and LDH, and decreased glucose but irrespectively of treatment. Similar occurred for CLP-induced lymphopenia/neutrophilia and the NO blood release. At 48 h post-CLP, dying mice had approximately 100-fold more CFUs in the spleen than survivors, but this was not SkQ1 related. At 48 h, macrophage and granulocyte counts increased in the peritoneal lavage but irrespectively of SkQ1. Similarly, hepatic mitophagy was not altered by SkQ1 at 24 h. The absence of survival benefit of mtAOX may be due to the extended treatment and/or a relatively moderate-risk-of-death CLP cohort. Long-term effect of mtAOX in abdominal sepsis appears different to sepsis/inflammation models arising from other body compartments.

1. Introduction

Sepsis is a deleterious clinical condition caused by a deregulated host response to infection associated with organ damage [1]. In immunocompetent individuals, sepsis provokes a robust systemic inflammatory response (which can coexist with concurrently developing immunosuppression). Various microbial, fungal, or viral components in the invaded host lead to a rapid, simultaneous release of pro- and anti-inflammatory mediators [2] and general activation of the innate/adaptive immunity. The acute phase of humoral and cellular response is accompanied by a rapid production of reactive oxygen species (ROS) [3]. Under physiological conditions, ROS are produced by different intra-/extracellular
sources [4] with mitochondria as one of the main sites [5, 6]. Tightly regulated by cellular antioxidant mechanisms, at low-to-moderate concentrations, ROS have potent beneficial effects as an intrinsic part of various intracellular signaling pathways [5, 7, 8]. In contrast, increased ROS concentration (e.g., caused by depletion of regulatory antioxidants, inadequate antioxidant response [9], and/or altered ROS release) can elicit a chain reaction cascade that can damage the cells and tissues of the host [4]. This oxidative stress has been considered as one of the key contributors to the development of organ failure in sepsis and linked to a deregulation of mitochondrial function [10–12].

Numerous preclinical studies demonstrated clear benefits of an antioxidant-dependent inhibition of ROS production/release in different pathophysiological conditions [13, 14] including sepsis [15–19]. In contrast, clinical trials either failed to show any survival benefit in septic patients or were inconclusive [20–22]. In general, the incomparability between preclinical and clinical studies (and among the clinical studies themselves) is due to numerous reasons including nonmatching models, erroneous study design, and/or non-specific therapeutics. First, endotoxia/LPS models are now considered inappropriate in recapitulating human sepsis syndromes [23]. Furthermore, due to a lack of clear dose-limiting toxicities, the dosages used in clinical trials are typically selected based on an “appeared feasible” subjective approach rather than preverified dose response studies [22]. For example, failure of a nonselective nitric oxide synthase inhibitor (46C88; L-NMMA acronym) phase 3 trial (versus placebo) to apparently achieve the target effect at much lower concentrations (e.g., caused by depletion of regulatory antioxidants, inadequate antioxidant response [9], and/or altered ROS release) can elicit a chain reaction cascade that can damage the cells and tissues of the host [4]. This oxidative stress has been considered as one of the key contributors to the development of organ failure in sepsis and linked to a deregulation of mitochondrial function [10–12].

2. Materials and Methods

2.1. Animals. Female, 3-month-old, outbred CD-1 mice (Charles River Laboratories; n = 142) were used for all experiments (the exact n/group is specified in the legend to each figure). The genetic diversity of the CD-1 outbred strain (e.g., compared to BALB/c) is closer to patient diversity and therefore clinically more relevant. We chose sexually mature, healthy females to exclude confounding variables such as comorbidities and young/old age and to follow on the NIH recommendation to study females given their underrepresentation in preclinical intensive care research. Experiments 1 and 2 were performed in Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (LB1), Vienna, Austria, and experiment 3 was performed in Institute of Surgical Research (ISR), University of Szeged, Faculty of Medicine, Szeged, Hungary. All mice were allowed to acclimatize to their new environment for at least one week after arrival. Mice were housed on a 12h light-dark cycle with controlled temperature (21–23°C) and provided with standard rodent diet and water ad libitum.

2.2. Ethics Statement. All animal procedures were approved by the Viennese (Austria) Legislative Committee (Animal Use Proposal Permission number: 006596/2011/11) and the Hungarian Scientific Ethical Committee on Animal Experimentation (Animal Use Proposal Permission number: V./148/2013) All experiments were conducted according to the National Institutes of Health guidelines. All mice were monitored by trained professionals at least three times per day and more whenever an animal’s condition deteriorated [38, 39]. In order to avoid unnecessary suffering of CLP mice facing imminent death, we use in our laboratory a custom-developed scoring approach that combines the mouse clinical assessment scoring system (M-CASS) and sequential body temperature (BT) measurements. In brief, well-being and general condition of mice (i.e., fur, posture, mobility, alertness, weight, and startle reflex) were assessed every 12 hours (starting 12h post-CLP), by assigning mice to one out of maximum three severity grades (i.e., 0, 1, or 2 points). Additionally, we measured inner BT. Mice were euthanized once the score indicated imminent death, that is, a score > 8 and/or inability to trigger the startle reflex and/or BT < 28°C (recorded in at least two sequential measurements; the score sheet in Supplementary Figure 1 available online at https://doi.org/10.1155/2017/6412682). This approach is generally in line with the recent mouse M-CASS philosophy proposed by Lilley et al. [40]. The BT-based prediction of outcome we have developed in our laboratory is very precise (AUC = 0.94; [41]). Combination of the BT-based prediction with our custom-developed
(M-CASS-like) scoring system prevents overliberal euthanasia decision-making, that is, allocation of potential survivors to the P-DIE group in survival studies.

2.3. Sepsis Model. Mice were subjected to cecal ligation and puncture (CLP) surgery according to the protocol by Wichertman et al. [42] with modifications described elsewhere [43]. We performed a medium severe CLP (18G needle) to reach an approximately 40% mortality (i.e., by day 28 post-CLP) that corresponds to mortality of human patients suffering from abdominal sepsis [44, 45]. Briefly, after opening the abdominal cavity via midline laparotomy, the cecum was exposed, ligated, and punctured twice (small amount of feces was extruded to ensure patency of the punctures). After repositioning of the cecum, the abdomen was closed with two single button sutures and Histoacryl® skin adhesive. Starting 2 hours post-CLP, all mice received subcutaneous broad-spectrum antibiotic therapy (25 mg/kg imipenem/cilastatin, Zienam®) and fluid resuscitation (1 ml Ringer’s solution) with analgesia (0.05 mg/kg buprenorphine, Bupaq®) twice daily (in approx. 12 h intervals) for five consecutive days (experiment 1) or until the time point of euthanasia (in experiments 2 and 3). Five-day course of antibiotic coverage is routinely employed in CLP studies [46].

2.4. Study Design

2.4.1. CLP Experiments. The entire study was performed in three separate experimental blocks: experiment 1 as the main survival part (SkQ1 and MitoTEMPO; Figures 1, 2, 3, 4, and 5) and follow-up experiment 2 (SkQ1 only; Figures 6 and 7; both in LBI) and experiment 3 (SkQ1 only; Figure 8; in ISR). To maximize reliability and minimize random effects in experiment 1, we conducted CLP in small groups of 15 mice per repetition (6 repetitions in total). Moreover, irrespective of location, all CLP surgeries were performed by the same operator (P.R.).

The follow-up experiments 2 and 3 had different sacrifice time points: at 48 h in experiment 2 and at 24 h post-CLP in experiment 3 (Figure 9 scheme). The time points were chosen based on the investigated endpoints: 48 h time point for bacterial load at the time of the maximal post-CLP mortality (experiment 2) and 24 h time point for potential mitophagy; the period closely preceding the most robust post-CLP deaths (experiment 3).

To ensure similar CLP severity between LBI (experiments 1 and 2) and ISR (experiment 3), we used BT profiling, that is, three (separate) pilot CLP runs were performed in ISR and the BT profiles of CLP mice (Supplementary Figure 1) were compared to the main CLP survival study (experiment 1). The third CLP run (i.e., experiment 3) showed nearly an identical BT profile to experiment 1 and was extended to reach the total \( n = 28 \).

2.4.2. Treatment. All animals (total of \( n = 90 \) in experiment 1; \( n = 24 \) in experiment 2; and \( n = 28 \) in experiment 3; detailed \( n \)/group distribution in legends to figures) were randomly assigned to receive (in a blinded manner) either SkQ1 (5 nmol/kg), MitoTEMPO (50 nmol/kg), or placebo treatment (saline) via intraperitoneal injection at maximally five subsequent time points (i.e., 1, 12, 24, 36, and 48 h) post-CLP (see Figure 9 scheme for detailed description of experimental groups). For SkQ1, this amounts to 0.0062 mg/kg/d for the first two days and 0.0031 mg/kg/d for the third day. For MitoTEMPO, this amounts to 0.051 mg/kg/d for the first two days and 0.0255 mg/kg/d for the third day.

For all experiments, SkQ1/MitoTEMPO was dissolved in ethanol (96%) and diluted in saline in further steps. Freshly prepared dilutions were divided into aliquots (one for each treatment time point separately) and stored frozen at \( -80^\circ \text{C} \) protected from light until use. The presence of SkQ1/MitoTEMPO molecules was verified on the first and the last days of experiments. The concentration of SkQ1 was tested spectrophotometrically as described in Antonenko et al. 2008 [30]. MitoTEMPO was measured with electron spin resonance spectroscopy as previously described [47].

We elected to administer the first antioxidant dose 1 h post-CLP to approach clinical relevance. We treated mice over the period of 48 h post-CLP given that it directly precedes/overlaps with the period of the most robust mortality in the acute CLP phase [48, 49]. Our treatment scheme is consistent with the one employed by Plotnikov et al. [13]. The selected antioxidant concentrations were adopted from Weidinger et al. [50]. Given that the effects of SkQ1 were predominantly examined in rats, we have compared the effects of increasing SkQ1 concentrations on the respiratory activity of mitochondria in liver homogenates from rats and mice. This was done to ensure that we remain within the nontoxic range of SkQ1 concentrations (Supplementary Figure 2).

In the follow-up experiments 2 and 3, SkQ1 (or placebo) was tested given that only SkQ1 treatment significantly exacerbated mortality in experiment 1. In experiment 2, mice received four injections (the last at 36 h post-CLP) and were sacrificed for peritoneal lavage and spleen collection. In experiment 3, mice received three injections (the last at...
24 h post-CLP) and were sacrificed to collect the liver for mitophagy assessment (Figure 9 scheme). Irrespective of sacrifice, the identical protocol for treatment and sampling procedures was used in experiment 1 and both follow-up experiments 2 and 3.

Of note, in experiment 2, to generate Figure 6(a), septic mice were compared using the above-described M-CASS-like criteria, that is, using our custom-developed scoring system and BT monitoring; we retrospectively assigned mice into either predicted-to-die (P-DIE; inner BT ≤ 28°C) or predicted-to-survive (P-SUR; inner BT ≥ 35°C) group. For maximally conservative approach, all mice featured in Figure 6(a) met the objective [41] BT cut-off criteria (i.e., mice meeting the euthanasia threshold based solely on the clinical assessment ≥ 8 points were not included).

2.5. Blood Sampling. Repeated low-volume blood sampling was used in all groups reducing the total number of mice
Blood was collected immediately before CLP (baseline) and at 6, 24, 48, and 72 h thereafter in experiment 1 and for shorter periods (depending on the sacrifice time point) in experiments 2 and 3 (Figure 9 scheme).

Specifically, 30 μl of blood was drawn by puncturing the facial vein (vena submandibularis) with a 23 gauge needle. Samples were then collected with a pipette rinsed with ethylenediaminetetraacetic acid (K$_3$-EDTA) (diluted 1:50) and immediately diluted 1:10 in PBS. After centrifugation (1000 × g, 5 min, 22°C), 270 μl of plasma was removed and stored at −80°C until further analysis.

2.6. Abdominal Lavage. In experiment 2, after sacrifice (isoflurane anesthesia followed by cervical dislocation), we performed abdominal lavages with 5 ml cold PBS/3% fetal calf serum, using an adapted protocol from Ray and Dittel [52]. The collected cell suspension was deposited in tubes and kept on ice until further FACS analysis.

2.7. Complete Blood Count. After plasma was removed, we resuspended the remaining blood pellet with 180 μl Cell-Dyn buffer with EDTA and a complete blood count with differential (erythrocytes, hemoglobin, platelets, white blood cells, neutrophil granulocytes, and lymphocytes) was performed with a Cell-Dyn 3700 counter (Abbott Laboratories, Illinois, USA) as previously described [51].

2.8. Cytokine and Chemokine Measurement. Interleukin (IL)-1β, IL-5, IL-6, IL-10, and IL-12p70, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1α, chemokine ligand (KC; CXCL-1), and

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**Figure 3:** Comparison of circulating chemokines between SkQ1-, MitoTEMPO-, and placebo-treated control mice. (a–c) Plasma levels of CXCL1, MCP-1, and MIP-1α in treated mice (SkQ1 or MitoTEMPO) at 6, 24, 48, and 72 h post-CLP were compared to the placebo group (CLP + NaCl). For (a–c): at 6 h: control n = 19, SkQ1 n = 19, MitoTEMPO n = 19; at 24 h: control n = 19, SkQ1 n = 17, MitoTEMPO n = 15; at 48 h: control n = 13, SkQ1 n = 12, MitoTEMPO n = 10; at 72 h: control n = 13, SkQ1 n = 11, MitoTEMPO n = 9. Data points shown as mean ± SEM.
monocyte chemoattractant protein-1 (MCP-1) were analyzed from plasma samples according to the manufacturer’s protocol using FlowCytomix™ Multiplex Kits (eBioscience, USA).

2.9. Metabolic and Organ Function/Cell Injury. Plasma levels of urea, glucose, lactate dehydrogenase (LDH), and alanine transaminase (ALT) were analyzed with Cobas c111 analyzer (Roche, Switzerland). The lower detection limit for urea was 3 mg/dL, for glucose 1.98 mg/dL, for LDH 10 U/L, and for ALT 2 U/L. The inner body temperature was measured (at least twice per 24 h) using a Fluke 52 Series II thermometer (Fluke, USA) with a rectal probe.

2.10. Nitrate/Nitrite (NOx) Measurement. The Nitric Oxide Analyzer (NOA) (Nitric Oxide Analyzer Sievers 280i, GE Analytical Instruments, USA) consisted of a glassware system and an electronic detection unit. Plasma samples were injected through a septum into the glass vessel, where NO species were converted selectively to NO\(_2\) by a redox active reagent (VCl\(_3\)). Following the reduction step, NO molecules are carried to the reaction chamber of the detection unit by N\(_2\), an inert gas which was constantly purged through the mixture during measurements. Inside the reaction chamber, NO from the gas phase reacted rapidly with O\(_3\) to form NO\(_2\) in an excited state (NO + O\(_3\) \rightarrow NO\(_2\) + O\(_2\)). As the excited e\(^-\) returned to its ground state, a photon was emitted and detected as chemiluminescence (hv) (NO\(_2\) \rightarrow NO\(_2\) + hv). Emitted light was detected and amplified by a photo multiplier tube (PMT) to generate an electric signal. The detection limit for NO and related species in liquid samples was \(\sim\)1 pmol.
spleens were homogenized using pellet pestles (Dsoy-S-15, Biozym, Oldendorf). After centrifugation at 13000 rpm for 10 minutes, supernatant was removed and serially diluted with PBS for subsequent plating on lysogeny broth (LB) agar plates (Miller, USA) and incubation overnight at 37°C. Colony-forming units were counted after 24h.

2.13. Western Blotting. Mice were euthanized at 24h post-CLP, and liver samples were immediately snap-frozen in liquid nitrogen and stored at -80°C for further analysis. All analyses were performed in Jena University Hospital (Germany). Total protein content was isolated from the frozen tissue samples by a protein isolation buffer pH 7.5 (10 mM Tris, 250 mM saccharose, 1 mM EDTA, 1% phosphatase-inhibitor cocktail, and 1% protease inhibitor 2) using a dounce homogenizer (Kimble Chase, Mexico). Homogenates were centrifuged at 900g for 10 min at 4°C to obtain the post nucleus fraction, followed by 100,000g for 1 h at 4°C to separate the cytosolic and microsomal fractions. Protein content in the post nucleus supernatant was determined using the bicinchoninic acid (BCA) assay (Sigma Aldrich, Hamburg, Germany). Fractions were used as indicated. 20 µg protein was loaded on a sodium dodecyl sulfate (SDS) gel and transferred to a polyvinylidenfluorid (PVDF)- membrane (Roth, Karlsruhe, Germany). Membranes were incubated with 5% N,O-bis(trimethylsilyl)acetat (BSA) or skim milk (Sigma-Aldrich, Hamburg, Germany) in tris-buffered saline with Tween20 (TBST) for 1 h at room temperature. Primary antibodies in 5% BSA or skim milk were incubated on a shaker overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) were incubated for 1 h at room temperature. The following primary antibodies were used: anti-LC3B, TOM20, cytochrome C (Cell Signaling Technology®, Danvers, USA), and p62 or anti-β-actin (Abcam®, Cambridge, UK). Detection of the secondary antibody by chemiluminescence was performed using HRP substrate (Immobilon Western HRP Substrat, Merck Millipore, Darmstadt, Germany) by the LAS3000 (GE Healthcare, Frankfurt, Germany). Protein content was quantified using software ImageJ (National Institutes of Health, Bethesda, MD, USA). β-Actin or Coomassie-stained gel assembled as loading control to calculate the differences in the protein expression between the groups. To quantify the level of autophagy, the LC3B-II : LC3B-I ratio was calculated based on a densitometric analysis and normalized to β-actin.

2.14. Statistical Analysis. The Kaplan–Meier method was used to plot and compare the 28-day survival in experiment 1. All other data sets were tested for normality before further analysis. Abnormally distributed data sets were log-transformed to achieve normal distribution. Comparisons among the three groups were made using one-way ANOVA and Bonferroni’s Multiple Comparison Test for the parametric data and Kruskal-Wallis Test and Dunn’s Multiple Comparison Test for the nonparametric data. To compare the SkQ1 with the placebo group, we used the unpaired Student t-test for parameters with parametric distribution or the Mann–Whitney Test for parameters with nonparametric distribution. The level of significance
was set at $p < 0.05$. All statistical analyses and graphs were made with GraphPad (San Diego, USA).

3. Results

3.1. Application of Mitochondrial ROS Scavengers Exacerbated Mortality. Survival was monitored for 28 days post-CLP. To be consistent with clinical severity of abdominal sepsis, mice were subjected to a medium-severe CLP with 38% mortality (average of six CLP repetitions). Neither MitoTEMPO nor SkQ1 exerted a protracted survival benefit. Conversely, SkQ1 treatment exacerbated mortality to 67% ($p = 0.03$) by day 28 (Figure 1). This negative effect was consistent in all six CLP repetitions (Supplementary Figure 3); it was apparent immediately in the acute phase (i.e., within 1–5 days post-CLP; in four CLP repetitions) and continued into the chronic phase until the end of the observation period. MitoTEMPO effect was inconsistent among individual repetitions (not shown) and did not reach statistical significance ($p = 0.24$; Figure 1).

3.2. Application of Mitochondrial ROS Scavengers Did Not Modulate the Inflammatory Response to Polymicrobial Sepsis. We examined a comprehensive set of circulating cytokines that are typically upregulated in both human and mouse sepsis [54, 55]. In general, CLP induced an increase of all cytokines measured; the strongest concentration was recorded for IL-6, MCP-1, CXCL-1, and MIP-1$\alpha$ at 24 h (Figures 2 and 3). None of those post-CLP increases was further significantly modulated by either SkQ1 or MitoTEMPO.

Following acute sepsis, a strong rearrangement of cell populations occurs. We found out that neither SkQ1 nor MitoTEMPO affected the CLP-induced lymphopenia (at 24 h: placebo 1.2 pg/ml versus SkQ1 1.1 pg/ml versus MitoTEMPO 0.9 pg/ml) and neutrophilia (at 24 h: placebo 0.4 pg/ml versus SkQ1 0.4 pg/ml versus MitoTEMPO 0.3 pg/ml) when compared to the placebo group (Supplementary Figure 5).

3.3. Application of Mitochondrial ROS Scavengers Did Not Change Organ Function/Injury and Metabolic Parameters. Functional tissue damages are hallmarks of sepsis
We followed temporal fluctuations of glucose (GLU), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and urea in septic mice after CLP. In general, all groups displayed similar trajectories at all time points (Figure 4). Specifically, ALT, LDH, and urea peaked in all treatment groups at 24 h (ALT: placebo 111 U/l versus SkQ1 119 U/l versus MitoTEMPO 115 U/l; LDH: placebo 940 U/l versus SkQ1 1097 U/l versus MitoTEMPO 773 U/l; urea: placebo 89 mg/dl versus SkQ1 97 mg/dl versus MitoTEMPO 93 mg/dl), while blood glucose decreased by approx. 63% (BL: 128 mg/dl versus 24 h: 49 mg/dl) in the placebo group. Hypoglycemia persisted until 72 h post-CLP (placebo 68 mg/dl versus SkQ1 64 mg/dl versus MitoTEMPO 76 mg/dl), while the other parameters recovered.

3.4. Application of Mitochondrial ROS Scavengers Did Not Affect NOx Concentration in Plasma. Nitric oxide (NO) and its metabolized forms nitrite (NO$$\text{_2}^-$$) and nitrate (NO$$\text{_3}^-$$; both referred to as NOx) play a central role in inflammation and correlate with severity of sepsis. We
measured NOx concentration in plasma at BL, 6, 24, 48, and 72 h post-CLP (Figure 5); there were no significant inter-group NOx changes. Similar to the organ function parameters, NOx peaked at 24 h post-CLP (approx. 4-fold higher than baseline).

3.5. SkQ1 Treatment Did Not Impair the Bacterial Clearance in the Spleen. Because of the consistent survival deterioration after SkQ1 (but not MitoTEMPO), we only compared the SkQ1 and placebo groups in experiment 2. First, to verify the bacterial clearance, we determined colony-forming units (CFUs) in the spleen homogenates. Mice that were predicted to die post-CLP had approximately 100-fold more CFUs in the spleen than mice that were predicted to survive CLP (Figure 6(a); \( p = 0.03 \)). However, this effect was not related to antioxidant treatment (Figure 6(b)).

3.6. SkQ1 Treatment Did Not Change Macrophage or Granulocyte Counts from Peritoneal Lavages. In experiment 2, to identify potential treatment-related differences in the abdominal leukocyte populations, we performed peritoneal lavages at 48 h post-CLP and analyzed cells by FACS. In general, the cell counts were similar between both groups (Figures 7(a) and 7(b)): macrophages (CD11b+/F4/80+) reached 11% in placebo and 16% in SkQ1, while granulocytes (Ly6G+) reached 56% in placebo and 45% in SkQ1.

3.7. SkQ1 Treatment Did Not Modify the Mitochondrial Autophagy. Finally, we investigated mitophagy and integrity of mitochondria in the liver (as a relatively sensitive organ) to establish whether SkQ1 exerted a direct detrimental effect upon this organelle. Western blot analysis of key proteins involved in autophagy (i.e., LC3B, p62) [56], mitochondrial membrane integrity (i.e., TOM20; Figures 8(a), 8(b), and 8(c)), and apoptosis (i.e., cytochrome C; Supplementary Figure 3) was performed. Compared to CLP mice treated with placebo, SkQ1 failed to reveal any modulation in the expression of those markers in the liver at 24 h post-CLP. This implies that the SkQ1-induced exacerbation of CLP mortality was not caused by direct mitochondrial damage.

4. Discussion

Recently, Sepsis-3 guidelines redefined sepsis as a “life-threatening organ dysfunction caused by a dysregulated host response to infection” [1]. ROS production/release is thought to play a major part in the progression of organ failure and mitochondrial dysfunction [12, 57]. Therefore, we aimed to improve the outcome of mice with polymicrobial sepsis by selectively inhibiting ROS at one of its key formation sites, the mitochondrion. Surprisingly, none of the tested antioxidants demonstrated any benefits, while SkQ1 markedly increased CLP mortality.

Best to our knowledge, this is the only study demonstrating a negative long-term impact of targeted mitochondrial antioxidant scavenging (i.e., by SkQ1) upon abdominal sepsis survival. The majority of rodent studies showed benefits of SkQ1 and/or MitoTEMPO in acute inflammatory conditions including pyelonephritis [13], gastritis [58], cardiomyopathy [31], SIRS [59], and sepsis [35]. Similar was true in a rat model of pneumosepsis [60] and endotoxemia [14]. Antioxidant (not mitochondria targeted) treatment did not improve survival only in three mouse studies, acute pancreatitis [61], pancreatic cancer [62], and CLP [63] models. While surprising, our protracted study design (Supplementary Figure 3) allows postulating that the reduction of survival due to SkQ1 was not a coincidental observation.

First, we examined two main elements of the systemic CLP response, inflammation, and organ function, to discern the deleterious mechanism(s). We repeatedly measured over twenty relevant parameters in mice enrolled in the survival study. This ensured maximal synchrony between the end-outcome effect and detection of a potential mechanism-of-action. CLP-induced dysregulation of the inflammatory and organ function system was consistent with data reported by us [64, 65] and others [66]. Surprisingly, we failed to observe any additional consistent changes caused by the SkQ1 and/or MitoTEMPO. This is suggestive of two things. First, the increased mortality in the SkQ1 mice did not appear to be caused by an evidently exacerbated early systemic inflammatory response, especially given that the four-day cytokine trajectories preceded/overlaid with the period of the highest acute mortality (days 1–3 post-CLP). Second, hepatocyte damage and/or impaired kidney function as well as dysregulation of carbohydrate metabolism was not a culprit either. The latter, however, does not exclude potential late derangements occurring in those and other organs (e.g., heart, lungs, and intestines) and/or systems (e.g., microcirculation, coagulation) that we did not examine. The protracted readout of the CLP-induced NOx fluctuations in the blood was consistent with the above changes. Similar to other works [67], the post-CLP peak of circulating nitrate/nitrite occurred in our study at 24 h time point but it was not further modified by antioxidants.

Next, we tested whether SkQ1 modulated the abdominal cellular characteristics and/or impeded bacterial clearance. We performed two-tier retrospective comparison: (1) treatment versus control as well as (2) predicted-to-die versus predicted-to-live using the body temperature-based prediction of outcome [41]. Such an approach allows more precise verification of the potential treatment effect upon the lethal phenotype. Our data show that compared to (predicted-to-be) survivors, predicted-to-die mice had much more pronounced bacterial load in the spleen. Yet again, the placebo treatment comparison revealed that SkQ1 neither altered the abdominal recruitment of phagocytes nor interfered with the bacterial clearance.

Impaired mitochondrial function was not always detected in CLP models, particularly in rodents [68]. Yet, the absence of a visible mitochondrial dysfunction in such cases was explained by activation of the effective autophagy process and removal of nonfunctional mitochondria. Thus, the autophagy appeared to us as a very sensitive sensor of the potential impairment of mitochondrial function. The final experiment 3 showed, however, that SkQ1 treatment was not by itself harmful to mitochondria (in the liver) given that neither autophagy (i.e., mitophagy) nor mitochondrial membrane integrity was altered by SkQ1 at the administered dose. This is in line with the nonotoxicity
assessment of SkQ1 in liver homogenates of healthy mice and rats (Supplementary Figure 2).

This leaves us with a negative effect of SkQ1 upon sepsis survival and insufficient understanding what this effect can be attributed to. There are several ways to explain the absence of the expected antioxidant benefits as well as the uncertain mechanism-of-action behind the worsened mortality. First, a proper dose fine tuning of the antioxidants appears important. For example, at micromolar concentrations, cationic quinone derivatives have a strong pro- not antioxidant effect [29]. Thus, it is unlikely that a higher dose would have lessened (reversed) the observed adverse SkQ1 effect. We rather consider inapt treatment timing and/or relatively low-risk-of-death sepsis CLP cohort as culprits. Specifically, we used a repeated treatment to cover the first two post-CLP days, the phase with the most frequent acute sepsis mortality. It seems now that such a blanket coverage might have been too aggressive and/or wide ranging, especially in a nonoverly lethal sepsis environment of our study. A minimal threshold of mitochondrial ROS (mtROS) is key for maintaining intra-cellular defense signaling pathways [69, 70] to enable, for example, a proper response to bacterial invasion [71]. Although we did not see a post-SkQ1 exacerbation of microbial load in the spleen, a complete blockage of mtROS (not detrimental to mitochondria themselves) could have impeded bacterial clearance at other sites. Likewise, blanket mtROS scavenging could have deregulated transcription of protective hypoxia inducible factors [72, 73] that we did not examine. Of note, there is an emerging role of cell-free hemoglobin in sepsis-induced organ failure [74]. For example, the recent clinical trial showed that blocking ferryl hemoglobin with acetaminophen improved sepsis outcome [75]. Thus, in addition to mtROS, rich amounts of ROS are generated by different intra/extracellular sources such as NADPH-oxidase, myeloperoxidase, and ferryl hemoglobin. Future studies need to explain precise site- and cell-specific roles of oxidative stress in sepsis. Of note, recent antioxidant treatment using N-acetylcysteine and butylated hydroxyanisole (unspecific ROS scavengers) improved survival of mice with specific deletion of the iron sequestering protein ferritin heavy chain (FTH); restoration of hepatic glucose production was identified as the key mechanism of this benefit [76]. Given that our CLP mice did not enter a prolonged (potentially deadly) hypoglycemia, this beneficial effect would not have applied.

The two recent sepsis studies offer some relevant clues regarding the antioxidant treatment timing/dosage and model severity [13, 63]. At first, CLP mice were given a single-bolus coenzyme Q10 (strong mitochondria-targeted antioxidant) at 5h and 20h post-CLP. Only the latter attenuated organ injury and (insignificantly) improved survival [58]. The authors also noted that when sham (also control) mice were treated with coenzyme Q10, their injury score doubled (versus untreated sham/control) and a (statistically insignificant) decline in survival occurred in sham + coenzyme Q10 mice. This indicates a predilection for detrimental effects of coenzyme Q10 in a nonlethal milieu. The second study was performed in rats with acute pyelonephritis (APN) who benefited from SkQR1 (we reproduced their treatment protocol) [13]. There are four major differences between that study and ours: cumulative dosage (500 versus 25 nmol/kg), species (rat versus mouse), origin of sepsis (urosepsis versus abdominal), and the infection severity. We consider the latter two elements as essential. First, the rat APN model displayed approx. 70% while our model reached a total 40% mortality by day 28. We believe that treatment benefits are much more likely to occur in a lethal versus moderate severity. For example, inhibition of murine leukotriene B4 synthesis improved CLP survival in severe sepsis [77], while the same agent worsened survival by 50% in a sublethal CLP [78]. We recently showed that only CLP mice predicted-to-die benefited from dexamethasone [49], whereas PAI-1 inhibition exacerbated CLP mortality in mice that were predicted to survive [79]. A similar response scenario cannot be ruled out in our study given that on average surviving mice outweighed those with the lethal CLP phenotype as opposed to the (beneficial) coenzyme Q10 treatment (65% mortality) [63], Mito-TEMPO mouse experiment (60% mortality), and SkQR1 APN rat study [13]. Likewise, in the recent SIRS study, 48 h pretreatment with SkQ1 improved mouse survival in a lethal but not in 50% mortality setup [59].

The negative effect of SkQ1 can be also attributed to the specificity of CLP; SkQ1 at the identical (pretreatment) dose was protective in the LPS rat model [50]. CLP only reproduces polymicrobial human sepsis of abdominal origin, and it cannot be reflexively extended to other sepsis types [2]. SkQ1 was never tested in CLP, and it is plausible that its negative effects may arise only in sepsis originating from this particular body compartment. Of note, our study monitored survival for 28 days. Although advised in preclinical sepsis [2], a protracted followup has never been tested before for targeted antioxidant treatment in sepsis. Had we used a 7-day observation, the SkQ1-induced effect would have been statistically insignificant.

There are several limitations in our study. First, only placebo versus verum CLP mice were compared and sham controls were not employed. Second, we did not assess the mitochondria-and/or cell-derived ROS. Third, mitophagy assessment was limited to a single (i.e., 24 h post-CLP) time point. Finally, CLP experiments were performed at two different locations (i.e., LBI and ISR) and the potential influence of macrobiome fluctuations was not verified.

It is also possible that the rodent CLP model is not an appropriate testing model for antioxidant therapies given the overall higher resistance of rodents to trauma/infection [2] and the qualitative difference in ROS release [80] under infectious and/or endotoxic stimuli (rodents versus humans). Thus, the sudden ROS release might not be as damaging in septic rodents as it is in patients. Long-term mouse studies using pneumosepsis, CASP, and candidiasis models as well as testing the mitochondria-specific antioxidants in various sepsis severity protocols will verify whether the negative effect observed here can be attributed to the CLP model itself, species used, the magnitude of the sepsis insult, or combination thereof. Finally, our study can be also considered as cautionary; it suggests that in certain severe inflammatory states, mtROS scavenging can be therapeutically inefficient and/or deleterious.
Disclosure

Pia Rademann’s current address is Center for Experimental Medicine, Medical Faculty, University of Cologne, Cologne, Germany.

Conflicts of Interest

Vladimir P. Skulachev is a board member of Mitotech LLC (Russia), a company developing SkQ1-based pharmaceuticals. The other authors declare that there is no conflict of interests regarding the publication of this paper.

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References


3 Summary

3.1 German summary

In Mitochondrien entstehende freie Sauerstoffradikale werden als ein wichtiger Faktor in der Sepsis-Pathogenese erachtet. In dieser Studie haben wir untersucht, ob zwei auf Mitochondrien ausgerichtete Antioxidantien (mtAOX) SkQ1 und MitoTEMPO, in einem Mausmodell für abdominal induzierte Sepsis, das langfristige Überleben und die Aufrechterhaltung der Organfunktionen verbessern, sowie die Entzündungsreaktion reduzieren. Neunzig drei Monate alte, weibliche CD-1 Mäuse wurden einer CLP (cecal ligation and puncture)-Operation unterzogen um eine Sepsis hervorzurufen. Daraufhin erhielten sie zu fünf verschiedenen Zeitpunkten eine intraperitoneale Injektion mit entweder SkQ1 (5 nmol/kg), MitoTEMPO (50 nmol/kg) oder Natriumchlorid (NaCl). Zusätzlich wurden weitere 52 Mäuse auf diese Weise behandelt und 24, bzw. 48 Stunden nach CLP euthanasiert um weitere Daten zu erheben. Weder SkQ1 noch MitoTEMPO hat das Überleben der Tiere in dieser Studie verbessert. Im Gegenteil, SkQ1 verschlechterte das Überleben an Tag 28 um 29 %. Unabhängig von der Behandlung induzierte die CLP die systemische Freisetzung von zehn verschiedenen Zytokinen, erhöhte Urea, Alanin-Aminotransferase (ALT), Lactatdehydrogenase (LDH) und reduzierte Glukose. Ebenso zeigte sich die durch CLP erzeugte Lymphopenie und Neutrophilie, sowie die intravasale Freisetzung von Stickstoffmonoxid (NO) unabhängig von der Behandlungsgruppe. Als sterbend eingeordnete Mäuse hatten 48 Stunden nach CLP ca. 100 mal mehr koloniebildende Einheiten (CFUs) in der Milz als die als überlebend eingestuften Individuen, jedoch war auch diese Tatsache nicht mit der mtAOX-Behandlung in Zusammenhang zu bringen. Die Anzahl der Makrophagen und Granulozyten aus Peritoneallavagen war 48 Stunden nach CLP ebenfalls erhöht, jedoch wieder ohne Einfluss der mtAOX. Auch die Mitophagie in der Leber wurde 24 Stunden nach CLP nicht durch die mtAOX verändert. Das Fehlen einer positiven Wirkung auf das Überleben könnte auf den relativ langen Behandlungszeitraum und/oder auf die relativ milde Mortalität (ca. 40 %) der durchgeführten CLP zurückzuführen sein. Die Langzeiteffekte von mtAOX in der abdominalen Sepsis scheinen sich von denen in anderen Sepsismodellen zu unterscheiden.

3.2 English summary

Mitochondrial-derived reactive oxygen species have been deemed an important contributor in sepsis pathogenesis. We investigated whether two mitochondria-targeted antioxidants (mtAOX; SkQ1 and MitoTEMPO) improved long-term outcome, lessened inflammation, and
improved organ homeostasis in polymicrobial murine sepsis. Three-month-old female CD-1 mice (n = 90) underwent cecal ligation and puncture (CLP) and received SkQ1 (5 nmol/kg), MitoTEMPO (50 nmol/kg), or vehicle five times post-CLP. Separately, 52 SkQ1-treated CLP mice were sacrificed at 24 h and 48 h for additional endpoints. Neither MitoTEMPO nor SkQ1 exerted any protracted survival benefit. Conversely, SkQ1 exacerbated 28-day mortality by 29%. CLP induced release of ten circulating cytokines and increased urea, ALT, LDH, and glucose but irrespectively of treatment. Similar occurred for CLP-induced lymphopenia/neutrophilia and the NO blood release. At 48 h post-CLP, dying mice had approximately 100-fold more CFUs in the spleen than survivors, but this was not SkQ1 related. At 48 h, macrophage and granulocyte counts increased in the peritoneal lavage but irrespectively of SkQ1. Similarly, hepatic mitophagy was not altered by SkQ1 at 24 h. The absence of survival benefit of mtAOX may be due to the extended treatment and/or a relatively moderate-risk-of death CLP cohort. Long-term effect of mtAOX in abdominal sepsis appears different to sepsis/inflammation models arising from other body compartments.
### 4 Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<td>ALT</td>
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<td>Analysis of Variance</td>
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<td>Acute Kidney Injury</td>
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<td>Antioxidants</td>
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<td>Phycoerythrin</td>
</tr>
<tr>
<td>PMT</td>
<td>Photo Multiplier Tube</td>
</tr>
<tr>
<td>P-SUR</td>
<td>Predicted to Survive</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidenfluorid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered Saline with Tween20</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of Outer Membrane</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
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5 References


Chousterman BG, Swirski FK, Weber GF. 2017. Cytokine storm and sepsis disease


