Mitochondrial Function in Sepsis

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Introduction

Sepsis is defined as a systemic inflammatory response syndrome with evidence of infection characterized by abnormal variations in temperature, heart rate, respiratory rate and white blood cell count. Sepsis results from an exaggerated host inflammatory response to pathogens. In sepsis, the formation of a complex consisting of bacterial fragments and lipopolysaccharide-binding proteins activates neutrophils and macrophages to release cytokines which act as pro-inflammatory mediators. These mediators such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-12 and IL-6 promote the synthesis of prostaglandins and activation of complement and coagulation cascades. Physiologically, the action of pro-inflammatory cytokines is balanced by anti-inflammatory mediators. However, in sepsis, this balance is lost resulting in a systemic inflammatory response and oxidative stress commonly leading to multiple organ failure. Tissue hypoperfusion has been postulated to be the cause of multiple organ failure in sepsis. However,

despite adequate oxygenation, cells exhibit failure in oxygen utilization and this is proposed to be due to mitochondrial dysfunction\(^4\).

Physiologically, the mitochondria produce small amounts of reactive oxygen species (ROS) such as superoxide anions at complexes I and III during normal cellular respiration\(^5\). Superoxide anions are then dismutated by manganese superoxide dismutase to hydrogen peroxide which is then eliminated via several enzymatic pathways. In sepsis, production of superoxide anions is increased due to the inhibition of the respiratory chain at complex III by TNF-\(\alpha\)\(^6\). Simultaneously, excessive amounts of nitric oxide (NO) are produced by the inducible nitric oxide synthase\(^7\). NO is able to inactivate superoxide dismutase\(^8\) causing superoxide anions to accumulate in the mitochondria as they are unable to cross mitochondrial membrane. Superoxide reacts with NO to produce peroxynitrite which may mediate apoptosis, oxidize glutathione and induce DNA damage\(^9\).

In addition to that, superoxides are able to inactivate several metabolic enzymes, particularly the unligated-iron-atom-containing aconitase\(^10\). The oxidation of aconitase results in loss of an iron atom and formation of hydrogen peroxide (see Equation 1). This chelatable (free) iron atom can react with hydrogen peroxide to form the most reactive and damaging ROS that is the hydroxyl radical, via Fenton reaction (see Equation 2)\(^11\).

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\text{Equation 1 \[4\text{Fe-4S}]^{2+} + \text{O}_2^- + 2\text{H}^+ \rightarrow [3\text{Fe-4S}]^{+} + \text{Fe}^{2+} + \text{H}_2\text{O}_2 \]}
\]

\[
\text{Equation 2 \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- (\text{Fenton Reaction})}
\]

Hydroxyl radicals trigger a cascade of events which can result in inhibition of mitochondrial respiratory chain complexes, opening of mitochondrial permeability transition pores and induce irreversible structural changes\(^13\). These events subsequently cause dissipation in mitochondrial membrane potential and cessation

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\(^{5}\) Wendel, 'Mitochondrial function and dysfunction in sepsis', pp. 118-123.


\(^{7}\) Wendel, 'Mitochondrial function and dysfunction in sepsis', pp. 118-123.


\(^{9}\) Harrois, 'Alterations of mitochondrial function in sepsis and critical illness', pp. 143-149.


\(^{13}\) Wendel, 'Mitochondrial function and dysfunction in sepsis', pp. 118-123.
of electron transfer\textsuperscript{14}. As a result, cells are unable to utilize molecular oxygen for Adenosine-5'-triphosphate (ATP) production leading to cytopathic hypoxia and death\textsuperscript{15}.

The formation of hydroxyl radicals have been found to be prevented by the use of iron chelators in several studies\textsuperscript{16}. These studies demonstrate how iron chelators can protect cells against oxidative stress by preventing the Fenton reaction. Here in this study we sought to determine the potential of two mitochondria-specific compounds, N.N'-Di(2-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid-HCl-H\textsubscript{2}O (HBED) and 5-[[4-(2-Hydroxyethyl)-1-piperazinyl[methyl]-8-quinolinol (VK-28) in chelating free iron in a cell model of sepsis. We used Lipopolysaccharide/Peptidoglycan (LPS/PepG), a polymicrobial septic insult, to simulate sepsis in human umbilical vein endothelial cells (HUVEC). Iron release and cell viability assays were then conducted following treatment of HUVEC with HBED and VK-28. The optimum concentration and time of incubation with HBED and VK-28 were determined by preliminary experiments involving the use of Menadione, a ROS-producing compound. We predicted that both VK-28 and HBED are able to chelate mitochondrial free iron in HUVEC following a septic insult.

**Methods**

1. **Preparation of HUVEC Growth Media**

A one-litre autoclaved bottle was placed in the tissue culture hood after being sprayed with 70% ethanol. 500ml of DMEM high glucose media and 500ml Ham's F12 solution were poured into the bottle. 1ml of Gentamicin and two 5ml aliquots of Amphotericin were added to the media. 20ml of the solution was aliquoted into a 50ml tube. 0.1g heparin, 1.2g arginine and 0.65g glucose were added to the 20ml aliquot. This mixture was then vortexed until all the compounds had dissolved. The

\textsuperscript{14} Harrois, 'Alterations of mitochondrial function in sepsis and critical illness', pp. 143-149.
\textsuperscript{15} ibid.; Fink, 'Bench-to-bedside review: Cytopathic hypoxia', pp. 491-499.
mixture was then syringed into the rest of the media through a microscopic filter. 100ml of foetal-calf serum was poured into the media. The bottle was inverted several times and refrigerated at 4°C.

2. Preparation of HUVEC in 96-Well Plates

Two large flasks of cultured HUVEC were taken out of the incubator and placed in a sterile hood. Growth media from both flasks was disposed. Cells in the flasks were washed with 25ml of Phosphate Buffered Saline/ Ethylenediaminetetraacetic acid (PBS EDTA). After the first wash, cells were incubated with PBS EDTA for five minutes to uncouple cells adjoined by gap junctions via chelation of calcium and magnesium. After five minutes, the PBS EDTA was disposed and substituted with 9ml of PBS EDTA and 1ml of trypsin 10x. After four minutes of incubation, the flasks were ‘slapped’ to dislodge cells from the electrostatic surface of the flasks. 20ml of culture media was added to each flask, pipetted and released several times to disperse cells with shear force. The contents of each flask were poured into two 50ml tubes respectively and centrifuged for 10 minutes at 500xg to pellet the cells. The supernatant was disposed from both tubes. Both tubes were tapped to disperse the cell pellets. 30ml of media was added into each tube and inverted several times. 10ml of the contents from each tube was pipetted into two 50ml tubes respectively. 40ml of media was then added to all six tubes. Cells from one tube were poured into a petri dish. 100ul of the mixture was pipetted into every well of a 96-well plate with a multichannel pipette. This last step was continued until all the mixture has been used. The 96-well plates with containing HUVEC were incubated at 37°C for 24 hours.

3. Treatment with 50uM Menadione of HUVEC incubated with HBED and VK-28 to test for cell viability and iron release

a. Preparation of working stocks of Menadione, HBED and VK-28

The mass of Menadione, HBED and VK-28 powders needed to produce the desired concentration for working stocks were calculated. Masterstocks were made by dissolving the desired amount of powders in their respective solvents. The volume of masterstock of Menadione needed to produce a 15uM solution in growth media was calculated.

b. Preparation of dilutions for HBED and VK-28
It was determined that the dilutions of chelators with which to treat HUVEC consist of the following concentrations (in uM): 0, 5, 10, 10, 30, 40, 50, 60, 70, 80, 90, and 100. The volume of the working stock needed to be added to growth media was calculated.

c. Pre-treatment of HUVEC with chelators

Four 96-well plates of HUVEC prepared previously were removed from the incubator and placed in the tissue culture hood. Growth media from each plate were disposed. Each dilution of the chelators was used to treat two 96-well plates. Once all 96-well plates of HUVEC have been treated, they were placed in the incubator for an hour at 37°C.

d. Treatment with 50uM Menadione

96-well plates of HUVEC treated with the chelators were taken out of the incubator and placed in a tissue culture hood. Growth media from each plate were disposed. 50uM of Menadione in growth media was pipetted into each well with a multichannel pipette. The 96-well plates were then incubated at 37°C for 24 hours.

4. Cell viability assay

Two 96-well plates of HUVEC treated with 15uM Menadione and pre-treated with HBED and VK-28 respectively were removed from the incubator and placed on a lab bench. The media from both plates were disposed and the cells were washed twice with PBS. 100ul of p-nitrophenylphosphate in acid-base buffer (0.1M Sodium Acetate and 0.1% Triton-x-100) was pipetted into each well. Following an hour of incubation 25ul of sodium hydroxide (NaOH) was pipetted into every well. Both plates were read on a spectrophotometer at 405nm to measure absorbance.

5. Iron release assay

Two 96-well plates of HUVEC treated with 15uM Menadione and pre-treated with HBED and VK-28 respectively were removed from the incubator and placed on a lab bench. The media from both plates were disposed and the cells were washed twice with PBS. 100ul of Rhodamine B-[(1,10-phenanthrolin-5-yl)-aminocarbonyl]benzylester (RPA) in PBS was pipetted into each well. The 96-well plates were then incubated at 37°C. After 15 minutes both plates were removed from
the incubator and tipped to remove the RPA solution. Wells were washed with PBS twice and added with 200ul of PBS before 10 minutes of incubation. The PBS from both plates was disposed. 100ul of PBS was later added to each well before the plates were placed onto a fluoroscan, excited at 544nm and read at 620 nm.

6. Treatment of HUVEC with Menadione 0-100 microM for iron release assay

Fourteen 96-well plates of HUVEC treated with the following concentration (uM) of Menadione in growth media were prepared: 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100. Every day for seven days a cell viability and iron release essay were done on two plates respectively to determine the optimum concentration and period of incubation with Menadione. The results of this test were used in an experiment where cells were pre-treated with HBED and VK-28.

7. Treatment of HUVEC with 30uM Menadione following pre-treatment with HBED and VK-28

HUVEC were pre-treated with HBED and VK-28 as per experiment 3. The cells were then incubated for 48 hours after being treated with 30uM Menadione.

8. Treatment of HUVEC with LPS/ PepG for days 1-7

Two 96-well plates of HUVEC in high glucose media were prepared. Every day for seven days one column of cells beginning with column 1 was treated with 2ug/ml LPS and 20ug/ul PepG. After seven days, a cell viability essay and an iron release assay were conducted on the plates respectively to determine the optimum concentration and period of incubation with LPS/ PepG.

9. Treatment of HUVEC with LPS/PepG, HBED and VK-28

Eight sets of dilutions consisting of 2.5ug/ml LPS and 25ug/ul PepG in growth media were prepared. HBED was added to one half of the sets and VK-28 added to another half to achieve the following concentration of chelators (uM): 0, 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100. 100ul of each dilution was pipetted into the corresponding well on a twelve 96-well plates of HUVEC. Cell viability and iron release assays were conducted on two plates for days 1, 4 and 7 of incubation at 37°C.
10. Treatment of HUVEC with diethylene triamine pentaacetic acid (DTPA), LPS/ PepG, VK-28 and HBED followed by mitochondrial volume assays

Experiment 9 was repeated with the addition of 200uM of DTPA in the treatment schedule. Two plates of HUVEC treated with HBED and VK-28 respectively were taken out of the incubator and disposed of their growth media. The cells were washed twice with 200ul PBS before being incubated with 10nM Mitotracker Green in the dark at room temperature. After 30 minutes, cells in both plates were washed twice with 200ul PBS. 100ul of PBS was added to each well before both plates were placed on the fluoroscan. The Mitotracker Green was first excited at 485nm and read at 520nm followed by excitation at 485nm and reading at 590nm.

Results

1. Treatment with 50uM Menadione of HUVEC incubated with HBED and VK-28 to test for cell viability and iron release

HUVEC demonstrated no significant changes in cell viability in relation to increasing concentration of chelators. The fluorescence of RPA—which is inversely proportional to the amount of iron—progressively declined with response to increasing chelator concentration.

2. Treatment of HUVEC with Menadione 0-100uM

Cell viability of HUVEC was 100% up to a Menadione concentration of 40uM for days 1, 2 and 3 of incubation. The amount of iron released increased with respect to duration of incubation and concentration of Menadione.

3. Treatment of HUVEC with 30uM Menadione fo 48 hours following pre-treatment with HBED and VK-28

Viability was 100% for HUVEC treated with HBED and VK-28 respectively. Surprisingly, the amount of iron released declined according to increasing chelator concentration.

4. Treatment of HUVEC with LPS/ PepG for days 1-7
Cell viability for HUVEC remained at 100% for days 1 to 7 of incubation with LPS/PepG. The amount of iron released showed a decline from days 1 to 4 but increased thereafter.

5. Treatment of HUVEC with LPS/PepG, HBED and VK-28 for Days 1, 4 and 7

The viability of HUVEC was 100% for the first day of incubation for cells treated with HBED and VK-28 respectively. However, cell viability reduced on days 4 and 7 of incubation for high concentrations of both chelators. Iron release for HBED for days 1, 4 and 7 showed an initial decline followed by a gradual increase across an increasing concentration of HBED. Iron release for VK-28 for days 1, 4 and 7 however, increased with response to increasing chelator concentration.

6. Treatment of HUVEC with diethylene triamine pentaacetic acid (DTPA), LPS/PepG, VK-28 and HBED followed by mitochondrial volume assays

HUVEC viability was 100% for day 1 of incubation. Iron release for cells incubated with HBED and VK-28 respectively increased with higher amounts of chelators. Mitochondrial volume for cells treated with HBED increased with respect to increasing chelator concentration. When corrected for mitochondrial volume, iron release for cells treated with HBED continued to increase with higher chelator concentration. Mitochondrial volume for HUVEC treated with VK-28 did not display significant changes across increasing VK-28 concentration. Iron release corrected for mitochondrial volume for VK-28 treated HUVEC showed an erratic pattern with response to an increase in chelator concentration.

Discussion

We sought to find the effects of two mitochondria-specific compounds in chelating free iron following a septic insult. Mitochondrial iron concentration is inversely proportional to the fluorescence of RPA, a mitochondria-specific dye quenched by iron. Oxidative stress was induced by the use of Menadione in initial experiments to determine the optimum time of incubation and concentration of both chelators. In later experiments, LPS/PepG was used.

We expected the availability of free iron would reduce with increasing chelator concentration. However, our first experiment with Menadione showed the opposite. We postulated that the conditions of incubation of Menadione may not be optimal.
later experiment involved incubation of HUVEC with increasing concentrations of Menadione over several days. The optimal concentration and time of incubation for Menadione were determined by the concentration and time at which cell viability and iron release were highest. This was found to be 30uM at two days of incubation.

This new data was used in another experiment when introducing oxidative stress to HUVEC pre-treated with HBED and VK-28. Despite changes made to the design, iron release continued to show an increase despite increasing chelator concentration. The same result was observed when cells were incubated with LPS/PepG. It was hypothesized that the results obtained were due to either a change in mitochondrial volume or chelation of iron in the growth media itself. A change in experiment design was made whereby free iron in growth media was chelated by the use of DTPA and iron release was corrected for mitochondrial volume. To our surprise, the results obtained from this new set of experiment showed a similar trend to previous experiments despite correction for mitochondrial volume.

The results shown so far may be due to the fact HBED and VK-28 are simply not chelating iron released from aconitase inactivation. Another explanation for our observations is that both chelators may be inducing oxidative stress themselves. Further experiments may be required in the future to prove that both compounds are able to chelate mitochondrial iron and are not toxic to cells.