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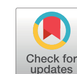
Non-invasive versus ex vivo measurement of mitochondrial function in an endotoxemia model in rat: Toward monitoring of mitochondrial therapy



Mark A. Wefers Bettink[a,*], Floor A. Harms[a], Nathalie Dollee[a], Patricia A.C. Specht[a], Nicolaas J.H. Raat[a], G.C. Schoonderwoerd[b], Egbert G. Mik[a]

[a] *Department of Anesthesiology, Laboratory of Experimental Anesthesiology, Erasmus University Medical Center Rotterdam, 's-Gravendijkwal 230, 3015 CE Rotterdam, the Netherlands*

[b] *Department of Clinical Genetics, Laboratory of Clinical Genetics, Erasmus University Medical Center Rotterdam, 's-Gravendijkwal 230, 3015 CE Rotterdam, the Netherlands*

A R T I C L E I N F O

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A B S T R A C T

Mitochondrial function has been predominantly measured *ex vivo*. Due to isolation and preservation procedures *ex vivo* measurements might misrepresent *in vivo* mitochondrial conditions. Direct measurement of *in vivo* mitochondrial oxygen tension (mitoPO_2) and oxygen disappearance rate (ODR) with the protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT) might increase our understanding of mitochondrial dysfunction in the pathophysiology of acute disease.

LPS administration decreased mitochondrial respiration (ODR) *in vivo* but did not alter mitochondrial function as assessed with *ex vivo* techniques (high resolution respirometry and specific complex determinations). PpIX-TSLT measures *in vivo* mitoPO_2 and ODR and can be applied non-invasively at the skin.

1. Introduction

Acute changes in mitochondrial function may play a role in the pathophysiology of sepsis (Fink, 2002). However, the options for monitoring mitochondrial function in patients are limited since only *ex vivo* techniques are available. The most common *ex vivo* technique measures oxygen consumption using oxygen electrodes (Estabrook, 1967), such as high-resolution respirometry (Gnaiger et al., 1995). Respirometry measures oxygen consumption in suspensions of isolated cells, isolated mitochondria or homogenates of small tissue biopsies and, therefore, might not adequately reflect the *in vivo* situation in acute changes of mitochondrial function (Jeger et al., 2013). A new non-invasive method to measure mitochondrial function could therefore improve the diagnosis of sepsis, and maybe even open the door to new therapies for which there is an urgent need (Jeger et al., 2013).

Techniques to study mitochondrial function *in vivo* are the Nuclear Magnetic Resonance (NMR)-technique and NADH fluorometry (Fosslien, 2001). NMR and NADH fluoroscopy both show determinants of metabolic state. The current inability of bedside monitoring and its costs make NMR less suitable for clinical use.

Changes of mitochondrial metabolic states measured by NADH fluoroscopy have been shown in a research setting. Despite these results, standard clinical monitoring of mitochondrial function by NADH fluoroscopy is not yet an option due to its sensitivity to artifacts and difficult interpretation.

An innovative method to monitor mitochondrial function *in vivo* has been developed and evaluated in our laboratory (Harms et al., 2013). The protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT) enables measurement of mitochondrial oxygen tension (mitoPO_2) in living cells and tissues (Mik et al., 2006, 2008). Further development of this technique allowed the detection of mitoPO_2 and mitochondrial oxygen disappearance rate (ODR) in the skin *in vivo* (Harms et al., 2011, 2012, 2013). Recently, the COMET monitor (Photonics Healthcare B.V., Utrecht, The Netherlands) has become commercially available. This device allows measurements of mitoPO_2 and ODR non-invasively in healthy volunteers and patients at the bedside based on PpIX-TSLT (Baumbach et al., 2018; Ubbink et al., 2016).

In two previous studies with the PpIX-TSLT we have measured a

Abbreviations: ALA, 5-Aminolevulinic acid; LPS, Lipopolysaccharide; LPS-FR, Lipopolysaccharide with fluid resuscitation; LPS-LR, Lipopolysaccharide with late resuscitation; LPS-NR, Lipopolysaccharide without resuscitation; mitoPO2, Mitochondrial oxygen tension; ODR, Oxygen disappearance rate; PpIX, Protoporphyrin IX; PpIX-TSLT, Protoporphyrin IX-triplet state lifetime technique; RDM, Rectangular distribution method

* Corresponding author at: Department of Anesthesiology, Laboratory of Experimental Anesthesiology, Erasmus University Medical Center Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

E-mail address: m.wefersbettink@erasmusmc.nl (M.A. Wefers Bettink).

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decreased ODR in the skin of LPS-induced rats (Harms et al., 2015a, 2015b). The effect of LPS administration causes macrohemodynamic and microcirculatory changes and inhibits mitochondrial complex I (Choumar et al., 2011). Until now, we did not directly compare an *ex vivo* muscle biopsy with our *in vivo* technique. This study therefore aims to perform this direct comparison of *in vivo* and *ex vivo* data in a clinically relevant model of sepsis.

Monitoring changes in ODR with the PpIX-TSLT during endotoxemia, as a model for sepsis, is promising. Showing changes in *in vivo* mitochondrial function due to an intervention would strengthen the PpIX-TSLT as a monitor. In classic respirometry the substrate succinate is used to study mitochondrial oxygen consumption linked to electron flow through complex II (Silva and Oliveira, 2011). In isolated mitochondria from endotoxemic rats the addition of succinate resulted in a normalization of mitochondrial oxygen consumption (Protti et al., 2007). We therefore measured with PpIX-TSLT whether succinate had similar effects on the mitochondrial respiratory chain *in vivo* in a rat model of endotoxemia with succinate pre-treatment.

Until now, two important questions remained unanswered. The first question is how our PpIX-TSLT measurements in skin relate to *in vivo* and *ex vivo* measurements in muscle, as the standard tissue for mitochondrial biopsies and measurements (experiment A). The second question is whether measurements performed with PpIX-TSLT besides detection of gross changes in mitochondrial respiration, are also sensitive enough to monitor therapeutic effects (experiment B). Successful *in vivo* measurement of mitochondrial parameters could help unravel the pathophysiology involved in critical illness. Additionally, it would provide a new technique for guiding therapies aimed at improving mitochondrial function.

2. Material and methods

2.1. Subjects and preparation

The experimental protocols (A; DEC 129-14-03 and B; DEC 129-12-11) were approved by the Animal Research Committee of the Erasmus University Medical Center Rotterdam. Animal care and handling were performed in accordance with the guidelines for Institutional and Animal Care and Use Committees.

For this study, 54 male Wister rats (Charles River, the Netherlands; body weight 280-350 g) were used, 14 rats in protocol A (age 77–111 days) and 40 rats in protocol B (age 59–73 days). Anesthesia was induced by an intraperitoneal injection of a mixture of ketamine 90 mg kg^{-1} (Alfasan, Woerden, the Netherlands), medetomidine 0.5 mg kg^{-1} (Sedator, Eurovet Animal Health BV, Bladel, the Netherlands) and atropine 0.05 mg kg^{-1} (Centrofarm Services BV, Etten-Leur, the Netherlands). Tracheotomy was performed to enable mechanical ventilation. Ventilator settings were adjusted on end-tidal PCO_2, keeping the arterial CO_2 partial pressure between 35-45 mmHg; the inspired oxygen concentration was set at 40%. The right jugular vein was catheterized with a polyethylene 0.9 mm catheter for intravenous fluid administration. The left femoral artery was catheterized to monitor arterial blood pressure and heart rate, and for hourly blood gas analysis. Cardiac output was measured by a thermodilution method with a thermistor inserted in the right carotid artery. Anesthesia and fluid balance were maintained by continuous infusion of ketamine (50 mg $kg^{-1}h^{-1}$), a crystalloid (Ringer's, B. Braun Melsungen AG, Melsungen, Germany) and a synthetic colloid solution (2.5 ml $kg^{-1}h^{-1}$) (Voluven®, Fresenius Kabi, Bad Homburg, Germany). Body temperature was measured rectally and maintained at 38 ± 0.5°C by means of a heating pad.

2.2. Experimental procedures

Abdominal hair was removed by shaving followed by application of commercially available hair removal cream (Veet, Reckitt Benckiser Co., Slough, UK) for about 5 min. PpIX was induced by applying a freshly prepared 2.5% 5-aminolevulinic acid (ALA) (Sigma-Aldrich, St. Louis, MO, USA) in hydrophilic cremor lanette (Lanettecreme I FNA, Bipharma, Weesp, the Netherlands). The skin was covered with aluminum foil to protect PpIX from light exposure.

2.3. Experiment A; with muscle measurement

The skin above the m. quadriceps femoris was removed. The ALA applied on the skin and muscle were covered with an adhesive film to avoid oxygen diffusion from the surroundings. The ALA applied tissue was covered with aluminum foil to protect PpIX from light exposure.

The 14 rats were divided into 2 groups; a time-control group (M − TC, N = 6) and a LPS-induced endotoxemic group (M − LPS, N = 8). TC were matched on duration of the experiment, a lactate concentration above 2 mmol/l in the blood gas analysis was used as a marker for the second ODR measurement in the LPS group. Fluid resuscitation (Voluven®, 5 ml*kg^{-1}*h^{-1}) was given to prevent hemodynamic shock and a decline in mitoPO_2. In the LPS group endotoxemia was induced by intravenous LPS injection (4.5 mg/kg lipopolysaccharide from E.Coli 0127:B8, Sigma-Aldrich, St. Louis, MO, USA). After recording baseline values (T0), a solution of 1 mg/ml LPS was infused during 30 min. Fluid resuscitation was performed by doubling the maintenance colloid infusion directly after LPS application. The timeline of this experiment is shown in Fig. 1A.

2.4. Experiment B; without muscle measurement

The 40 rats were randomly divided into 5 groups (8 rats/group); two control groups consisting of a time control group (TC) and a control group receiving methyl-succinate (SC). Three LPS-induced endotoxemic groups consisting of a group in which only LPS was given (LPS --), a LPS group receiving fluid resuscitation (LPS + -), and a LPS group receiving fluid resuscitation and methyl-succinate (LPS + +). Fluid resuscitation (Voluven®, 5 ml*kg^{-1}*h^{-1}) was given to prevent hemodynamic shock and a decline in mitoPO_2. Succinate dimethyl ester (Brunschwig Chemie, Amsterdam, the Netherlands) was infused (concentration 0.67 M, rate 5 ml*kg^{-1}*h^{-1}) 2 h prior to the LPS infusion in the SC and LPS + + groups. For the remaining groups, the succinate solution was exchanged for saline at the same rate.

In all three LPS groups endotoxemia was induced by intravenous LPS injection (3 mg/kg lipopolysaccharide from E.Coli 0127:B8, Sigma-Aldrich, St. Louis, MO, USA). After recording baseline values (T0), a solution of 1 mg/ml LPS was infused during 15 min. Fluid resuscitation was performed by doubling the maintenance colloid infusion directly after LPS application and by an additional fluid bolus of 1 ml during 10 min prior to T1. The timeline of the experiment is shown in Fig. 1B.

2.4.1. Principle of MitoPO_2 and oxygen disappearance measurements

The background of the PpIX-TSLT is described in detail elsewhere (Mik et al., 2006, 2008). In short, PpIX is the final precursor of heme in the heme biosynthetic pathway. PpIX is synthesized in the mitochondria, ALA is the rate-limiting step in this pathway and therefore administration enhances the PpIX concentration substantially. PpIX possesses a triplet state that reacts strongly with oxygen, making its delayed fluorescence lifetime oxygen-dependent according to the Stern-Volmer equation (Mik et al., 2002).

The oxygen disappearance rate is measured directly after local occlusion of the oxygen supply. The reflection probe was mounted on a height-adjustable frame, above the ALA-treated skin, providing different settings of the probe distances to the skin. Local occlusion of the microcirculation in the skin was obtained by local pressure with the measurement probe. This simple procedure created reproducible stop-flow conditions and induced measurable oxygen disappearance rates, due to cessation of microvascular oxygen supply and ongoing cellular

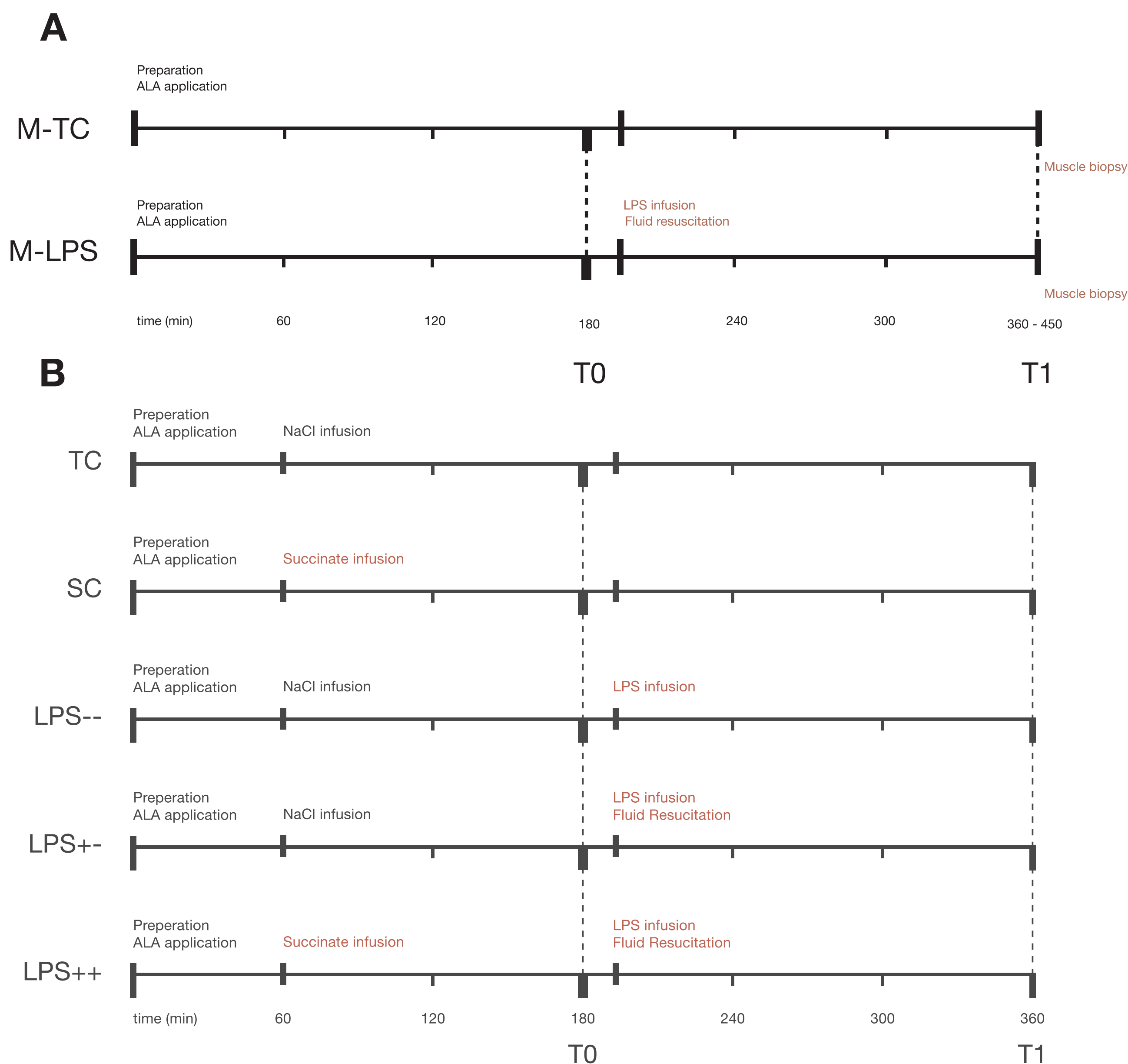

Fig. 1. Schematic timeline of the experimental protocol. Panel A: Experiment A. with muscle measurement. M − TC: time control, M − LPS: endotoxemia with fluid resuscitation. Panel B: Experiment B. without muscle measurement. TC; time control, SC; succinate control, LPS --; endotoxemia, LPS + -; endotoxemia with fluid resuscitation, LPS + +; endotoxemia with fluid resuscitation and succinate. ALA; 5- aminolevulinic acid, LPS; lipopolysaccharide, T0 and T1 are the time points of mitoPO2 and ODR measurement.

oxygen consumption. MitoPO_2 was measured before, during and after application of pressure at an interval of 1 Hz and using 1 laser pulse per measurement. We have described the fundamental principles behind the technology and have provided a working implementation of the technique for ODR measurements *in vivo* (Harms et al., 2013) and a method to calculate ODR from the mitoPO_2 kinetics. On each time point mitoPO_2 and ODR are presented as average of 4 replicate measurements. In the time control group at T1 a starting mitoPO_2 of around 50 mmHg was chosen, to match the LPS group, for fitting of the ODR. This was done to prevent potential negative effects of increased noise at higher mitoPO_2 readings on ODR.

2.5. Mitochondrial assay in muscle biopsies

At the end of the experiment a muscle biopsy was taken from the m. quadriceps femoris. The biopsy was transferred to mitochondrial respiration buffer (MRB, a hybrid buffer consisting of 110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 10 mM monobasic potassium phosphate, 3 mM magnesium chloride, 20 mM HEPES, 1 mM EGTA, and 0.1% (w/v) BSA at pH 7.1 at 37°C) on ice, homogenized using a Potter-Elvehjem PTFE pestle and glass tube, and injected in the respirometer. A small piece of muscle was snap-frozen in liquid nitrogen and stored at −80°C for later determination of complex concentration and activity.

2.5.1. High-resolution respirometer

Oxygen consumption was measured using a high-resolution respirometer (Oxygraph O2k, Oroboros, Innsbruck, Austria). Prior to homogenate loading the instrument was calibrated following the manufacturer instructions and loaded with 2.1 ml MRB. An oxygen solubility in water of 0.92 was used to calculate oxygen levels. 0.1 ml of muscle homogenate was added to the respirometer, and the chamber

was closed. All chemicals for the respirometer experiments were obtained from Sigma-Aldrich (Darmstadt, Germany). To determine mitochondrial complex activity, first pyruvate (5 mmol/l final concentration) and malate (2 mmol/l final concentration) were added. Subsequent addition of ADP (0.25 mmol/l final concentration) showed full activity of complex 1. After addition of rotenone (0.5 micromol/l final concentration) to the chamber complex 1 activity was stopped and succinate (5 mmol/l final concentration) was added to measure complex 2 activity. The ATPase was inhibited by adding oligomycin (2.5 micromol/l final concentration) and subsequently FCCP (1 mmol/l solution) titration was performed to determine maximal oxygen consumption. The oxygen consumption measured by the high-resolution respirometer was corrected for citrate synthase activity, an indicator of the number of mitochondria was measured according to Srere (1969) (Srere and Lowensteijn, 1969) of the homogenate to enable objective comparison between samples.

2.5.2. Complex determination

To obtain an indication of the function of the oxidative phosphorylation the activity of citrate synthase (CS) and the activity of complex 1 and 2 were analyzed. Activities of the complexes were based on the amount of CS to make objective comparison possible.

Tissue homogenates were prepared from frozen muscle in 0.25 M sucrose, 10 mM N-[2-hydroxyethyl] piperazine-N′-[2 ethylsulfonic acid](HEPES) and 1 mM Ethylene Diamine Tetraacetic Acid (EDTA), pH 7.4. Enzyme activities were measured in the homogenates. Activities of the complexes of the mitochondrial respiratory chain were determined in muscle homogenates by spectrophotometric methods as described before (Scholte et al., 1995) Complex I or NADH-Coenzyme Q reductase was determined kinetically by following the rotenon sensitive decrease in the amount of NADH. Complex II or Succinate Coenzyme Q reductase was measured by following the TTFA dependent reduction of DCPIP after addition of succinate. Citrate synthase was used as an indicator for the number of mitochondria to enable an objective comparison.

2.5.3. Statistical analysis

In experiment A data are expressed as median with interquartile range. For intragroup analysis the Wilcoxon matched pair signed rank test was used, for intergroup analysis the Mann-Whitney test was used. For experiment B data are expressed as means with standard deviation (SD), unless stated otherwise. A paired t-test was used to detect intragroup differences. Two-way ANOVA with repeated measurements, using post hoc multiple comparison with Bonferoni correction, was used to detect intergroup differences. For the hemodynamic data only intergroup differences were tested compared to time control. Normality was tested by Q-Q analysis and the Shapiro-Wilk test. A p-value < 0.05 was considered statistically significant. Statistical calculations were performed using Graphpad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Experiment A

3.1.1. Hemodynamic parameters

Table 1 presents data on hemodynamic parameters of experiment A. At baseline (T0) all rats were hemodynamically stable and no significant difference was observed between the groups. Three hours after LPS infusion heart rate ($p = 0.0293$) and lactate ($p = 0.0007$) increased compared to the time-control group.

3.1.2. MitoPO_2 measurements

In accordance with previous experiments (Harms et al., 2015a), the application of ALA increased the amount of PpIX in the mitochondria to detectable levels in all animals. MitoPO_2could be measured in the abdominal rat skin for the duration of the experiment (3 h after application of ALA). MitoPO_2 in the abdominal skin didn't change for 3 h (T0 → T1) in both the time control (M − TC) and endotoxemia (M − LPS) group (Fig. 2A). However, muscle mitoPO_2 at T1 in the M − TC group was markedly higher, 82 [42] mmHg vs 46 [27] mmHg, compared to the M − LPS group (Fig. 2B).

Table 1
Hemodynamic parameters experiment A.

	T0	T1
MAP (MmHg)		
M − TC	95 [14]	80 [6]
M − LPS	93 [6]	89 [26]
Cardiac output (ml/min)		
M − TC	118 [39]	142 [67]
M − LPS	148 [60]	167 [60]
Heart rate (bpm)		
M − TC	286 [41]	271 [42]
M − LPS	284 [37]	314 [56][a]
Lactate (mmol/L)		
M − TC	1.1 [0.1]	0.9 [0.4]
M − LPS	0.9 [0.1]	2.4 [0.6][b]

Values are median [IQR], M − TC, time control; M − LPS, endotoxemia + fluid resuscitation.
[a] = $P < 0.05$ compared to TC.
[b] = $P < 0.001$ compared to TC.

3.1.3. ODR measurements

ODR was derived from the mitoPO_2 slope during blockage of microcirculatory blood flow. At T0 the initial mitoPO_2 was approximately 60 mmHg. Blocking the microcirculation by local pressure with the measurement probe caused a drop in mito$PO_2$from 55 mmHg to approximately 10 mmHg in 10 s, this resulted in a ODR of 5.8 mmHg*s^{-1} (Fig. 3). In experiment A, at baseline no difference was found in skin and muscle between M − TC and M − LPS group (Fig. 2A and B). In contrast, at T1, a significant lower value was measured in skin (LPS; −4.2 [3.0] mmHg*s^{-1} vs TC; −7.1 [1.5] mmHg*s^{-1}) and in muscle (LPS −6.7 [2.4] mmHg*s^{-1} vs TC −9.7 [0.5] mmHg*s^{-1}) (Fig. 2B).

4. Mitochondrial assay in muscle biopsies

Muscle biopsies were performed at the end of the experiment. We found no difference in complex 1 and 2 activity in the high-resolution respirometer of the homogenized muscle in both groups, as shown in Fig. 2C. We found no difference in complex activity corrected for citrate synthase activity measured in the snap frozen biopsies.

4.1. Experiment B

4.1.1. Hemodynamic parameters

At baseline (T0) all rats were hemodynamically stable and no significant difference was observed between the groups, as shown in table 2. Three hours after LPS infusion (T1), significant hemodynamic changes were observed in the LPS groups, reflected by an elevation in serum lactate levels in all three endotoxemia groups. A decrease in mean arterial blood pressure (MAP) was seen in the LPS – and the LPS + + group compared to TC, but not in the LPS + - group. Cardiac output decreased in the LPS – ($p = 0.049$) and LPS + + ($p = 0.039$) groups (102 ± 59 and 100 ± 39 bpm vs 140 ± 56 bpm TC) compared to time control. The succinate control group showed a significant decrease in heart rate ($p = 0.0245$), 267 ± 11 vs 290 ± 13 bpm, compared to time control.

4.1.2. MitoPO_2 measurements

A mitoPO2 value around 60 mmHg was observed for all experimental groups at T0. In the LPS – group a significant decline in mito$PO_2$from 62 ± 9 mmHg (T0) to 41 ± 12 mmHg (T1) was

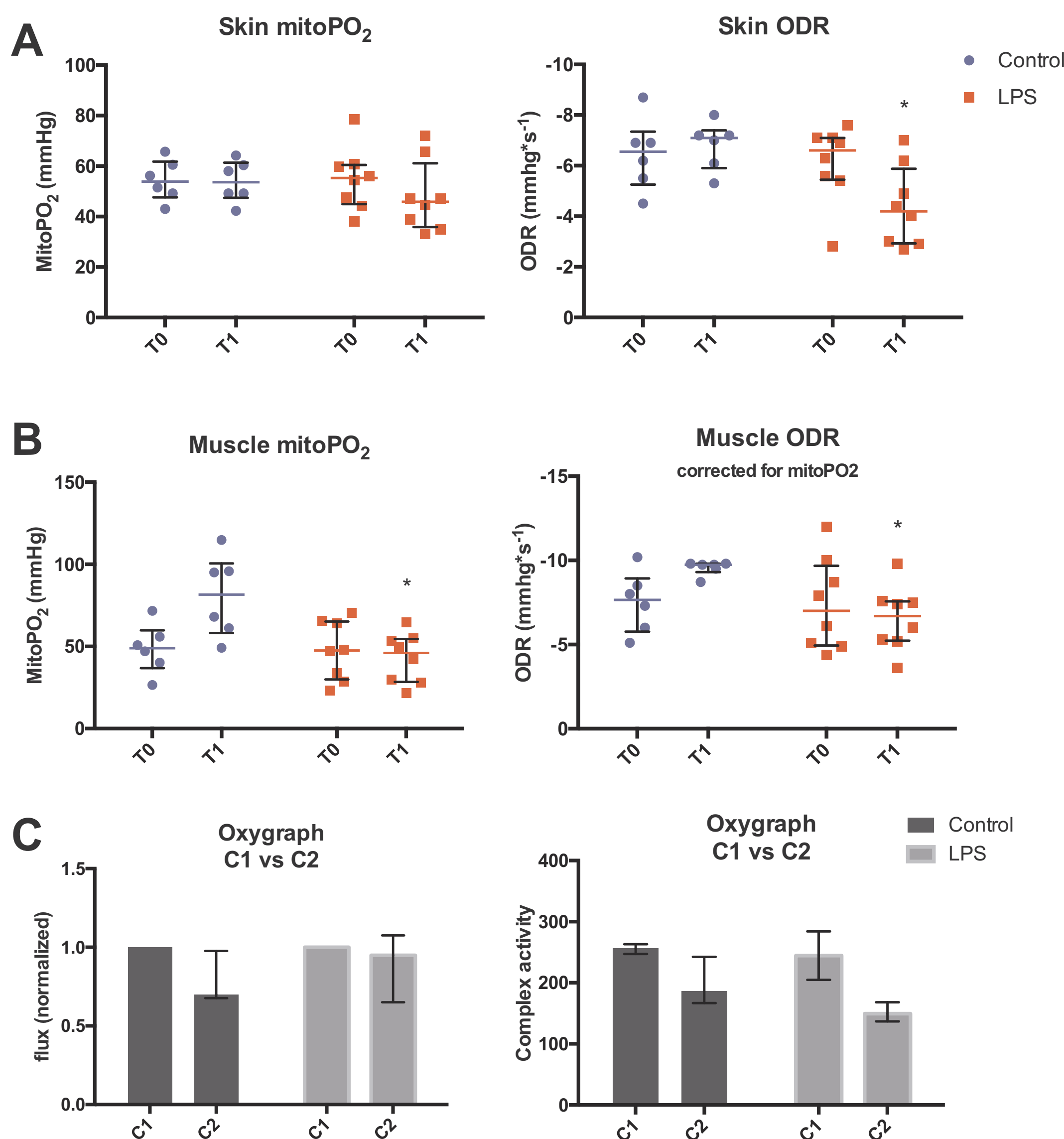

Fig. 2. Panel A: MitoPO$_2$ and ODR measurements in skin at T0 and T1 in the different experimental groups, ODR M − LPS (T1) vs M − TC (T1) ($p = 0.0093$) Panel B: MitoPO$_2$ and ODR in muscle at T0 and T1 in the different experimental groups, mitoPO$_2$ M − LPS T1 versus M − TC T1 ($p = 0.127$) and ODR decreased M − LPS T1 vs M − TC T1 ($p = 0.0127$). Panel C: comparison of flux (normalized) between complex 1 and complex 2 corrected for protein concentration, and comparison of complex activity in the snap frozen muscle biopsy corrected for citrate synthase. TC; time control, LPS; endotoxemia with fluid resuscitation, LPS; lipopolysaccharide, ODR; mitochondrial oxygen disappearance rate. T0 and T1 are the time points of ODR measurement. Data are presented as median ± IQR, * significant difference compared to time control measurement ($p < 0.05$); (TC; 6 rats, LPS; 8 rats).

measured. At T1 a significant lower value of mitoPO$_2$ was measured in the LPS – and LPS + - ($p = 0.0006$) groups compared to time control, of 41 ± 12 mmHg (LPS –) and 54 ± 21 mmHg (LPS + -) compared to 74 ± 17 mmHg (TC) respectively (Fig. 4A). The individual changes in mitoPO$_2$are presented in Fig. 4B.

4.1.3. ODR measurements

The ODR measurements at baseline (T0) did not differ significantly between the groups. In three groups ODR at T1 decreased compared to the baseline measurement (T0); the succinate control decreased from 4.6 ± 1.2 mmHg*s^{-1} to 3.6 ± 0.5 mmHg*s^{-1}, the LPS – group decreased from 4.5 ± 1.0 mmHg*s^{-1} to 2.4 ± 1.6 mmHg*s^{-1}, and the LPS + - group decreased from 4.2 ± 1.3 mmHg*s^{-1} to 3.1 ± 1.0 mmHg*s^{-}. However, infusion of methyl-succinate maintained ODR in the endotoxemia group (LPS + +) at baseline values (Fig. 5).

5. Discussion

The major findings of this study are that *in vivo* respirometry and *ex vivo* respirometry provide different results in our endotoxemic rat model and that PpIX-TSLT provides a sensitive means to measure aspects of mitochondrial function *in vivo*. LPS decreased *in vivo* muscle ODR at the measured timepoints compared to a time control group, but no effect of LPS was found in *ex vivo* mitochondrial function tests. No change in mitochondrial function was found using the high-resolution respirometer nor using direct complex activity measurements. In an LPS-induced rat endotoxemia model we showed a decrease in *in vivo* mitochondrial function (ODR) using the PpIX-TSL technique, that could be prevented by pre-treatment with methyl-succinate. This result confirms the previously measured beneficial effects of methyl-succinate in *ex vivo* biopsies from endotoxemic rats (Protti et al.). In addition, it shows the feasibility of the PpIX-TSLT as an *in vivo* monitoring tool to determine the therapeutic effect of mitochondrial targeted drugs.

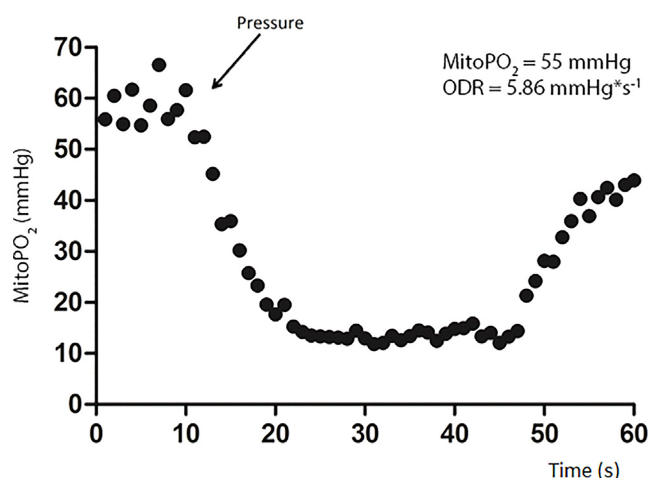

Fig. 3. Typical example of *in vivo* mitochondrial respirometry measured at T0 on the abdominal skin of a rat. The ODR was determined from the linear part of the oxygen disappearance curve by fitting equation 2. $MitoPO_2$ was the mean $mitoPO_2$ before the start of tissue compression.

Table 2
Hemodynamic parameters experiment B.

	T0	T1
MAP (MmHg)		
TC	107 ± 8	90 ± 11
SC	97 ± 15	80 ± 7
LPS --	100 ± 12	73 ± 15[a]
LPS +-	102 ± 11	81 ± 13
LPS + +	103 ± 10	71 ± 4[a]
Cardiac output (ml/min)		
TC	153 ± 58	140 ± 56
SC	145 ± 62	135 ± 55
LPS --	158 ± 26	102 ± 59[a]
LPS +-	165 ± 34	148 ± 46
LPS + +	126 ± 35	100 ± 39[a]
Heart rate (bpm)		
TC	263 ± 19	290 ± 13
SC	258 ± 16	267 ± 11[a]
LPS --	267 ± 20	294 ± 30
LPS +-	276 ± 16	306 ± 16
LPS + +	259 ± 15	305 ± 15
Lactate (mmol/L)		
TC	1.3 [0.3]	1.1 [0.3]
SC	0.9 [0.5]	1.0 [0.3]
LPS –	1.1 [0.5]	2.6 [1.5][a]
LPS+-	1.3 [0.4]	1.8 [1.3][a]
LPS + +	1.2 [0.1]	2.5 [1.8][a]

Values are means ± SD (MAP, heart rate and cardiac output), and for lactate; median [IQR].
TC, time control; SC, succinate control; LPS –, endotoxemia; LPS +-, endotoxemia + fluid resuscitation; LPS + +, endotoxemia + fluid resuscitation + succinate.
[a] = P < 0.05 compared to TC; Lactate tested with Kurskal-Wallis test with Dunn's test for multiple comparison.

The administration of LPS leads to inhibition of complex I of the mitochondrial electron transport chain (Choumar et al., 2011), measured as a decrease of mitochondrial oxygen disappearance rate (Harms et al., 2015a). We did not replicate the detrimental effect of endotoxemia on *ex vivo* mitochondrial respiration experiments as shown by Protti et al. However, Protti et al used a different model (cegal ligation and puncture) to initiate an endotoxemia with no fluid resuscitation. Furthermore, their experiment lasted longer and they only used clinical severity grade as a marker for sepsis at 48 h. This discrepancy with our results might imply that changes in ODR precede the changes measured with *ex vivo* mitochondrial function tests.

In the measurement of $mitoPO_2$ directly on the muscle a marked increase of $mitoPO_2$ on T1 was found compared to T0 in the time control group but not in the LPS treated group. In 6 h a wound starts with it first phases of the healing process, it is unclear if a higher or lower oxygen concentration is expected since both are mentioned

(Gottrup et al., 1984; Niinikoski et al., 1972). Endotoxemia has a marked diminutive effect on the pathophysiology of wound healing, this may explain the relatively low $mitoPO_2$ found in the LPS group compared to the time control group (Kawaguchi et al., 1995).

In our *in vivo* respirometry experiments, the administration of LPS resulted in decreased ODR. Fluid resuscitation in the LPS+- group prevented macro-hemodynamic deterioration, although a significant lower value of $mitoPO_2$ was found compared to time control, this may be in part explained by a relatively high $mitoPO_2$ found in the time control group at T1. A decrease in MAP and cardiac output often occurs after rapid and/or long-term LPS infusion (Harms et al., 2015a). In all our endotoxemia groups, lactate was significantly higher compared to the control groups. Lactate is only a crude marker of disease, depicting the balance between aerobic and aerobic metabolism in the tissues at the one site and on the other site an indication of slowing of liver metabolism. An increase of $mitoPO_2$ in the time control group, although not significant, may overestimate the changes found compared to the other experimental groups. The $mitoPO_2$ of 74 ± 17 mmHg at T1 in the time control group may be explained by a relative liberal fluid regime (succinate infusion was replaced with extra saline infusion in the other groups) used in this experiment. A constant $mitoPO_2$ in the succinate control group indicates unaltered and adequate tissue oxygenation. Despite maintained $mitoPO_2$values at T1, the ODR of the succinate control group, the LPS – group and the LPS with fluid resuscitation group (LPS + -) showed a significant decrease compared to baseline measurements. Unclear is why succinate decreased ODR slightly compared to baseline, of interest is the decline of standard deviation compared to time control. Moreover, no intergroup differences were found on T1 in the ODR measurements. However, the greatest difference was observed between the LPS +- and the succinate and fluid resuscitated LPS-group (LPS+ +), in which ODR values did not decline from baseline values. Importantly, previous PpIX-TSLT measurements showed that mitochondrial respiration is independent of $mitoPO_2$ levels (Harms et al., 2013). Therefore, a distinction between problems related to oxygen supply or oxygen consumption on the cellular level in critical illness could be made using the PpIX-TSLT technique.

Several mitochondrial respiration studies have shown that mitochondrial oxygen consumption recovers after administration of succinate (Protti et al., 2007; Silva and Oliveira, 2011). Protection of ODR by methyl-succinate administration can be explained by several effects. Methyl-succinate could increase mitochondrial membrane permeability, increase substrates in the citric acid cycle, or increase the activity of complex II.

In our *in vivo* experiments, the infusion of methyl-succinate in the LPS-treated rats (LPS+ +) prevented a decline in ODR from baseline values. This demonstrates that *in vivo* ODR measurements are able to show changes in mitochondrial respiration which are subtler than changes in lactate. We reproduced the beneficial effect of methyl-succinate on mitochondrial oxygen consumption as previously found in isolated mitochondria (Protti et al.). We therefore think that our novel measurement method enables efficient monitoring of *in vivo* changes in mitochondrial respiration.

In our study, methyl-succinate was already administered before the LPS mediated endotoxemia was induced, which is not comparable to the clinical situation. Therefore, our data mainly demonstrates the feasibility of our experimental technique to detect subtle changes in mitochondrial respiration. Further research is needed to determine whether the protective effect of methyl-succinate is still present when administered after endotoxemia has already developed.

The clinical applicability of PpIX-TSLT (Harms et al., 2016; Mik, 2013) allows non-invasive real time monitoring of mitochondrial

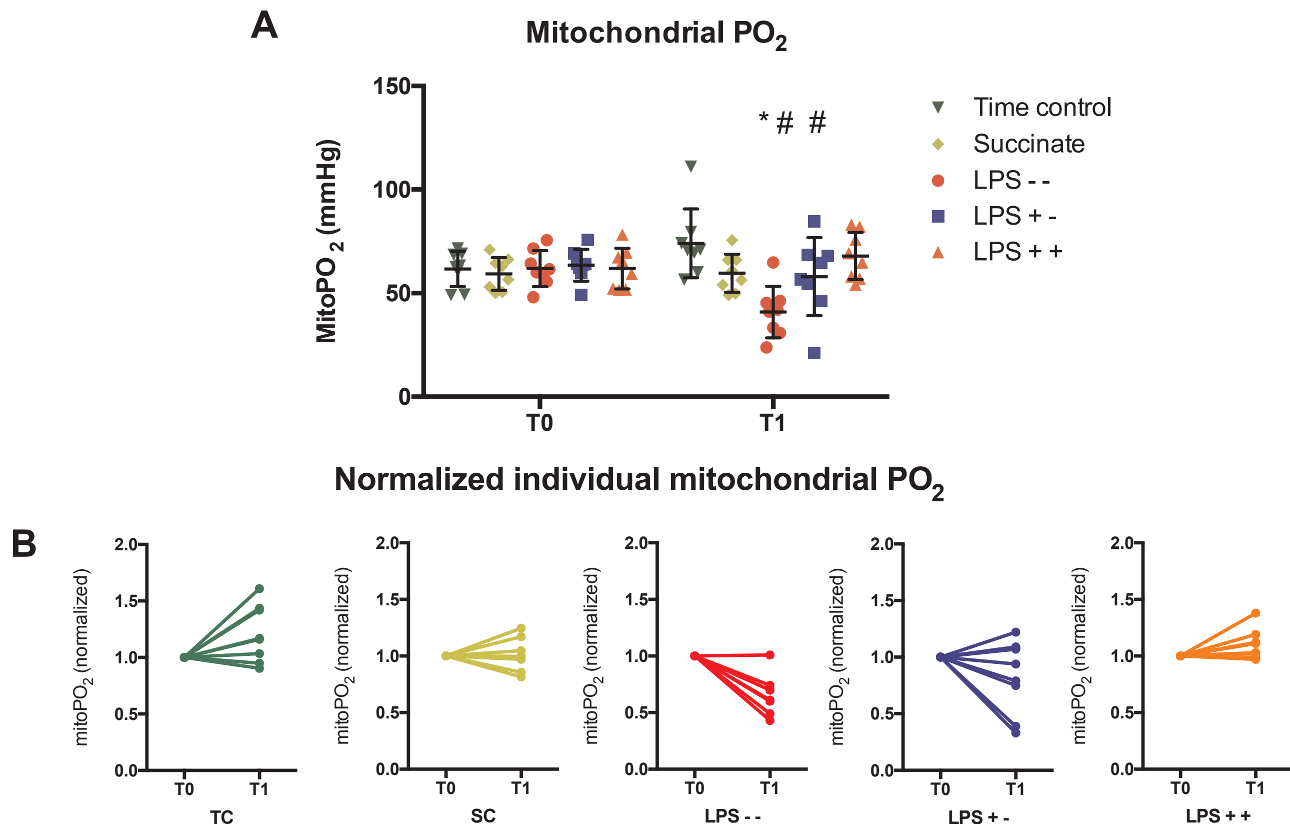

Fig. 4. Panel A: MitoPO$_2$ at T0 and T1 in the different experimental groups. Panel B: Percentage change in individual mitoPO$_2$. TC; time control, SC; methyl-succinate control, LPS –; endotoxemia, LPS + -; endotoxemia with fluid resuscitation, LPS + +; endotoxemia with fluid resuscitation and methyl-succinate administration, LPS; lipopolysaccharide, mitoPO$_2$; mitochondrial oxygen tension. T0 and T1 are the time points of the mitoPO$_2$ measurement. Data are presented as mean ± SD, * significant difference compared to baseline measurement, # significant difference compared to time control measurement ($p < 0.05$); (8 rats/group). LPS – T1 vs LPS – T0 ($p = 0.0015$), LPS – T1 vs TC T1 ($p < 0.0001$) and LPS +- T1 vs TC T1 ($p = 0.0006$).

function. The absence of tissue damage and functional loss may overcome some of the current disadvantages experienced with biopsies (Jeger et al., 2013) and enables bedside monitoring. A limitation of the technique is the need to build up the PpIX signal following ALA application for at least 3 h (Harms et al., 2015b). The recent release of a monitor based on the PpIX-TSLT technique (Ubbink et al., 2016) provides a bedside clinical tool to monitor the deterioration and recovery of mitochondrial function in critically ill patients. Other potential clinical uses of PpIX-TSLT are related to resuscitation and blood transfusion management (Römers et al., 2016).

In conclusion, we showed the feasibility to monitor changes in mitochondrial respiration in endotoxemic rats by cutaneous PpIX-TSLT measurements. A decrease in ODR in the presence of preserved or restored mitoPO$_2$ suggests that mitochondrial dysfunction may be at the basis of the metabolic failure in sepsis, even in the absence of hemodynamic shock. The ability to measure mitochondrial ODR and mitoPO$_2$ in the clinical setting using bedside monitoring PpIX-TSLT is expected to contribute to a better understanding of mitochondrial dysfunction.

In addition, it will allow to monitor existing therapeutic approaches and develop improved therapeutic approaches aimed at restoring aerobic metabolism and cellular function.

Author contribution

MAWB designed and performed experiments, analyzed data, and wrote the manuscript. FAH participated in the design of the study and contributed to the manuscript. ND performed experiments, analyzed data, and contributed to the manuscript. PACS performed experiments, analyzed data, and contributed to the manuscript. NJHR participated in calculating the ODRs, contributed to the manuscript and revised the manuscript critically. GCS superficed the high-resolution respirometer experiments and performed the mitochondrial complex determinations. EGM conceived and supervised the study, designed the model, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interest

MD, Mark A. Wefers Bettink; Conflict of interest: None
MD, PhD Floor A. Harms: Conflict of interest: None
MD, N. Dollee: Conflict of interest: None
P.A.C. Specht: Conflict of interest: None
PhD Nicolaas J. Raat: Conflict of interest: None
MD, PhD G.C. Schoonderwoerd: Conflict of interest: None
MD, PhD Egbert G. Mik:
Dr. E.G. Mik is founder and shareholder of Photonics Healthcare B.V., Utrecht, The Netherlands. Photonics Healthcare B.V. holds the exclusive licenses to several patents regarding this technology, filed and owned by the Academic Medical Center in Amsterdam and the Erasmus Medical Center Rotterdam, the Netherlands.

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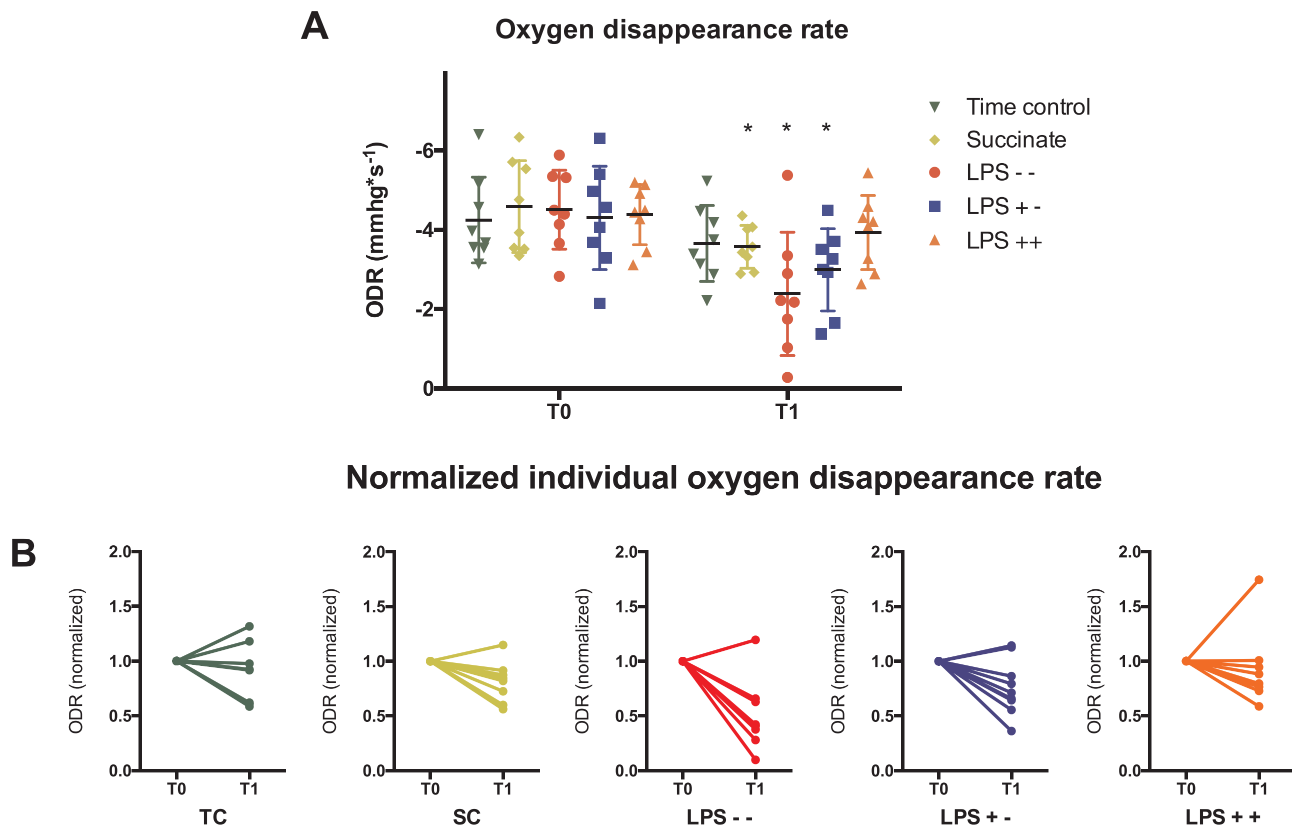

Fig. 5. Panel A: ODR at T0 and T1 in the different experimental groups. Panel B: Percentage change in ODR. TC; time control, SC; methyl-succinate control, LPS –; endotoxemia, LPS +-; endotoxemia with fluid resuscitation, LPS ++; endotoxemia with fluid resuscitation and methyl-succinate administration, LPS; lipopolysaccharide, ODR; mitochondrial oxygen disappearance rate. T0 and T1 are the time points of ODR measurement. Data are presented as mean ± SD, * significant difference compared to baseline measurement, # significant difference compared to time control measurement ($p < 0.05$); (8 rats/group).intergroup analysis T1: SC vs TC ($p = 0.034$), LPS – vs TC ($p = 0.0034$) and LPS+- vs TC ($p = 0.0223$).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2019.11.003.

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