Cutaneous Mitochondrial Po_2, but Not Tissue Oxygen Saturation, Is an Early Indicator of the Physiologic Limit of Hemodilution in the Pig

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ABSTRACT

Background: Hemodilution is a consequence of fluid replacement during blood loss and is limited by the individual ability to compensate for decreasing hemoglobin level. We tested the ability of a novel noninvasive method for measuring cutaneous mitochondrial Po_2 (mitoPo_2) to detect this threshold early.

Methods: Anesthetized and ventilated pigs were hemodynamically monitored and randomized into a hemodilution (n = 12) or a time control (TC) group (n = 14). MitoPo_2 measurements were done by oxygen-dependent delayed fluorescence of protoporphyrin IX after preparation of the skin with 20% 5-aminolevulinic acid cream. Tissue oxygen saturation (StO_2) was measured with near infrared spectroscopy on the thoracic wall. After baseline measurements, progressive normovolemic hemodilution was performed in the hemodilution group in equal steps (500 ml blood replaced by 500 ml Voluven®; Fresenius Kabi AG, Germany). Consecutive measurements were performed after 20-min stabilization periods and repeated 8 times or until the animal died.

Results: The TC animals remained stable with regard to hemodynamics and mitoPo_2. In the hemodilution group, mitoPo_2 became hemoglobin-dependent after reaching a threshold of 2.6 ± 0.2 g/dl. During hemodilution, hemoglobin and mitoPo_2 decreased (7.9 ± 0.2 to 2.1 ± 0.2 g/dl; 23.6 ± 2 to 9.9 ± 0.8 mmHg), but StO_2 did not. Notably, mitoPo_2 dropped quite abruptly (about 39%) at the individual threshold. We observed that this decrease in mitoPo_2 occurred at least one hemodilution step before changes in other conventional parameters.

Conclusions: Cutaneous mitoPo_2 decreased typically one hemodilution step before occurrence of significant alterations in systemic oxygen consumption and lactate levels. This makes mitoPo_2 a potential early indicator of the physiologic limit of hemodilution and possibly a physiologic trigger for blood transfusion. **(ANESTHESIOLOGY 2016; 125:124-32)**

What We Already Know about This Topic

- Normovolemic hemodilution is common during major surgery, and the potential resultant reductions in oxygen supply can be associated with significant morbidity
- The ability to directly measure tissue oxygenation preferably at the intracellular and mitochondrial level would be a major advance for perioperative medicine

What This Article Tells Us That Is New

- The authors have developed a sophisticated technology to measure cutaneous mitochondrial oxygen tension, and hereby investigate the influence of hemodilution on the measurements in a porcine model
- The authors show that the measurement of cutaneous mitochondrial oxygen tension is feasible and that it may be a promising physiologic trigger to guide transfusion therapy and patient management

NORMOVOLEMIC hemodilution is a consequence of fluid administration to compensate blood loss and occurs commonly during major surgery, cardiopulmonary bypass interventions, and emergency medicine. One of the risks of hemodilution is tissue hypoxia due to a critical decrease in oxygen supply when hemoglobin levels drop below the individual-dependent threshold. Previous studies have shown serious consequences of insufficient oxygen supply, like stroke,[1] declined cognitive function,[2] kidney injury,[3,4] and cardiac complications.[5,6] Oxygen supply is commonly improved by the transfusion of erythrocytes. However, transfusion should be restricted to a minimum due to the risks involved in it, like bacterial[7,8] and viral infection,[9] transfusion-related acute lung injury,[10] transfusion-associated circulatory overload,[11] and febrile nonhemolytic and allergic reactions.[12,13] Despite the incidence of the previously mentioned complications having decreased significantly over the last decades, caution must be exercised due to the potential severity of complications.

The above-mentioned risks clearly indicate the need for a reliable and practical assessment of tissue oxygenation during hemodilution to support the decision for blood transfusion. Hemoglobin level determines the oxygen transport capacity of blood, and therefore is used as a trigger for

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blood transfusion. However, the level of hemoglobin below which oxygen supply becomes insufficient for the tissue, also known as "critical hemoglobin" or "critical hematocrit," is not unambiguous.[14] The balance of oxygen supply and consumption depends not only on the amount of erythrocytes, but also on cardiac output, gas exchange, and metabolic demand. As a consequence, critical hemoglobin differs interindividually and even per organ.[15,16] Compensatory mechanisms differ between individuals and so does the tolerance to anemia.[17]

For this reason, direct measurement of tissue oxygenation, and most preferably intracellularly at the mitochondrial level, could be of advantage next to the measurement of an indirect parameter like hemoglobin. Mitochondrial oxygen tension (mitoP_{O_2}) is a very important parameter for cellular function. With the protoporphyrin IX (PpIX)-triplet state lifetime technique, it is possible to measure mitoP_{O_2} *in vivo*. This technique has been used successfully *in vivo* and *in vitro* in several studies.[18–20]

In particular, monitoring of mitoP_{O_2} in the skin seems to be of special interest since the skin can be regarded as the canary of the body,[21] like the gastrointestinal tract.[22] Studies demonstrated that detection of subcutaneous oxygen tension could provide essentially the same information as invasively measured parameters of oxygen metabolism in the gut.[23,24] In addition, the skin can be easily noninvasively monitored with an optical technique in the clinic.

The aim of this study was to investigate the influence of hemodilution on cutaneous mitoP_{O_2}, compared to other common clinical parameters (mean arterial blood pressure [MAP], cardiac output, systemic oxygen consumption, and signs of anaerobic metabolism like lactate). To this end, mitoP_{O_2} measurements were related to hemoglobin level, mixed venous oxygen saturation, and systemic biochemical and physical signs of hypoxia. In addition to mitoP_{O_2}, we also measured tissue oxygen saturation (StO_2) on the thoracic wall as an already clinically available alternative. Our hypothesis was that ongoing normovolemic hemodilution would cause a decrease in cutaneous mitoP_{O_2} when the individual limits of compensatory mechanisms would be reached. The limits of these compensatory mechanisms are embodied by the parameters described in this paragraph.

Materials and Methods

Animals

The protocol of the current study was approved by the Animal Research Committee of the Erasmus Medical Center, University Medical Centre Rotterdam, Rotterdam, The Netherlands (EMC protocol no. 129-13-05). Animal care and handling were performed in accordance with the national guidelines for the care of laboratory animals. For the experiments, 26 female Yorkshire pigs with mean body weights of 31.3 ± 0.3 kg (mean ± SD) were used.

Experimental Preparation

The animals were sedated with an intramuscular injection of tilatamine/zolazepam (2.5/2.5 mg/kg; Virbac Laboratories, France) and xylazine (2.25 mg/kg; AST Farma B.V., The Netherlands). After a 15-min induction period, an intravenous access was obtained in the left-ear vein with a 20G Venflon (Becton, Dickinson and Company, USA), and tracheal intubation was performed with a size 6.5 Portex® endotracheal tube (Smiths Medical International Ltd., United Kingdom). For maintenance of anesthesia, the animals received continuous infusion of ketamine (5 mg kg^{-1} h^{-1}; Alfasan Nederland B.V., The Netherlands), midazolam (1.5 mg kg^{-1} h^{-1}; Atavis Group PCT, Iceland), sufentanil (4 µg kg^{-1} h^{-1}; Janssen-Cilag B.V., The Netherlands), and rocuroniumbromide (4 mg kg^{-1} h^{-1}; Fresenius Kabi Austria GmbH, Austria). All animals received continuous infusion of crystalloid (Sterofundin® ISO 10 ml kg^{-1} h^{-1}; B. Braun, Germany). Each pig received a bolus of magnesium sulphate (500 mg; Pharmachemie, The Netherlands), as arrhythmia prophylaxis, added to the first bag of crystalloid solution. Animals' temperature, electrocardiogram, and Sp_{O_2} were continuously monitored.

To allow the measurement of mitoP_{O_2}, we first shaved 2 × 3 cm of the anterior chest wall bilaterally to remove hair. Second, we applied freshly prepared 5-aminolevulinic acid (ALA) cream (20%, 1 g on each side; 5-aminolevulinic acid hydrochloride, Fargon GmbH & Co. KG, Germany, in Lanette cream, Teva Nederland B.V., The Netherlands) to the shaved skin. The cream was covered by an IV3000 plaster (Smith & Nephew, United Kingdom) and by a layer of aluminum foil.

Pressure-controlled mechanical ventilation (Servo 300; Siemens-Elema, Sweden) was used with an F_{IO_2} of 0.4. Adjustments were made to maintain normocapnia (ET_{CO_2}: 35 to 45 mmHg). Temperature was maintained between 38° and 39°C, as measured rectally, with heating pads underneath the animal.

For continuous measurement of the arterial blood pressure, a 20G catheter (Arterial Leadercath, Vygon®, France) was placed in the left femoral artery by using the Seldinger technique. For blood withdrawal during hemodilution, a 9Fr introducer sheath (Arrow International Inc., USA) was placed in the right femoral artery. For monitoring of the cardiac output, another sheath in the right jugular vein was used as an introducer for placement of a Swan-Ganz catheter (Edwards Lifesciences, USA) in the pulmonary artery. The side port of this sheath was used for fluid administration. A lower midline laparotomy was performed in order to insert a catheter in the urinary bladder.

On the left lateral part of the chest wall, a near-infrared spectroscopy sensor was attached (InSpectra StO_2 Monitor, Hutchinson Technology, USA) for continuous measurement of tissue oxygenation (StO_2) at three different depths (2.5, 15, and 25 mm). In figure 1, an overview of the preparation is given.

Hemodynamic and Blood(-Gas) Measurements

Throughout the entire experiment, MAP, heart rate, continuous cardiac output (CCO), Spo_2, StO_2, and end-tidal carbon dioxide were continuously monitored. At baseline and at each step of the experimental protocol, an arterial and mixed-venous blood sample was obtained for measurement of Po_2, PCO_2, SO_2, lactate, pH, hemoglobin, and hematocrit. These measurements were performed with an ABL 800Flex (Radiometer, Denmark). From CCO, Pao_2, arterial oxygen saturation, mixed venous oxygen tension, and mixed venous oxygen saturation, we calculated the systemic oxygen consumption ($\dot{V}\mathrm{o}_2$) by the Fick principle ($\dot{V}\mathrm{o}_2 = \mathrm{CCO} \times (\mathrm{Cao}_2 - \mathrm{Cvo}_2)$). Oxygen delivery ($\mathrm{DO}_2$) was calculated using the CCO and arterial oxygen content ($\mathrm{DO}_2 = \mathrm{CCO} \times \mathrm{Cao}_2$).

Cutaneous MitoPo_2 Measurements

For the measurement of mitoPo_2, we used an optical technique based on the measurement of the delayed fluorescence lifetime of PpIX. A detailed description of the technique can be found elsewhere.[20,25] In short, we applied ALA cream to induce enhanced levels of PpIX in the cutaneous mitochondria (priming of the skin). By using a pulsed laser, we illuminated the primed skin at a wavelength of 510 nm (green) for photoexcitation of PpIX. With a gated photomultiplier tube, we collected the emitted red delayed fluorescence light. Instead of relaxation to the ground state by emission of a photon, excited PpIX can transfer its energy to oxygen (a process known as quenching), making the delayed fluorescence lifetime oxygen dependent. Due to these properties, we could calculate the mitoPo_2 from the lifetime of delayed fluorescence using the Stern–Volmer equation.[26]

Experimental Protocol

A total of 26 animals were divided into two groups, a hemodilution group (n = 12) and a TC group (n = 14), through randomization. Randomization was performed using "random numbers" in Microsoft Excel (Microsoft, USA). Randomization was not blinded because it is not feasible in the experimental design. Due to animal- or technology-related problems, we had to exclude three animals in the TC and two animals in the hemodilution group. Three hours after surgery and ALA cream application, the aluminum foil, the plaster, and the excess cream were removed. Afterward, we covered the prepared skin with heated (±38°C) infusion bags to prevent it from cooling. After 20 more min, which was necessary for the remaining cream to diffuse into the skin, we performed the first (baseline) set of measurements. One measurement consisted of a set of 10 mitoPo_2 measurements and an arterial and mixed-venous blood sample. In all animals of the hemodilution group, we withdrew 500 ml of blood *via* the sheath in the right femoral artery after the baseline measurement. Simultaneously, we infused 500 ml of heated (±38°C) colloid solution (Voluven®; Fresenius Kabi AG, Germany) using a pressure bag (200 mmHg) *via* the right jugular vein. Each hemodilution step was followed by a 20-min stabilization period. At the end of a stabilization period, a new measurement was performed, followed by a new hemodilution step. A total of eight hemodilution steps and measurements were performed. The animals of the TC group were not subjected to hemodilution. In those animals, a baseline and eight time-related measurements were performed (hemodilution 1, hemodilution 2, *etc.*). At the end of the experiment, the animals were euthanized with an overdose of potassium chloride (40 mM, Fresenius Kabi AG, Germany). A visual overview of the experimental protocol is given in figure 1.

Statistical Analysis

The number of animals used was based on previous experience in pilot studies. Data are presented as mean ± SD, unless otherwise indicated. When we were interested in group differences and therefore in minimizing the overlap of error bars between the groups, we used mean ± SEM[27] to improve interpretation in our figures. Normal distribution was assessed both visually and using Kolmogorov–Smirnov testing. For comparison of animal characteristics and changes between both groups, we used the unpaired Student's *t* test. To examine the effect of hemodilution

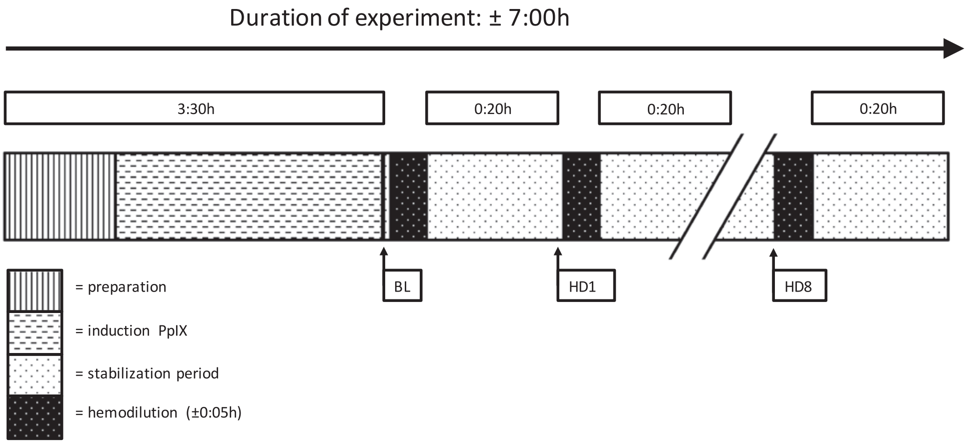

Fig. 1. Time schedule of the experiment. BL = baseline; HD# = hemodilution step number; PpIX = protoporphyrin IX.

over time on mitoPo$_2$ and other relevant parameters, linear mixed-effect models were used. The models included subject as random effect and hemodilution, time and hemodilution * time as fixed within-subject effects. Linear mixed-effect models provide unbiased and efficient estimates (assuming data are missing at random) and can accommodate intermittently missing values. For *post hoc* analysis of the timing of occurrence of significance, we used paired Student's *t* test. Correction for repeated measurements was performed with Bonferroni correction. Statistical analysis was performed with SPSS Statistics (Version 23, SPSS, USA). Graphics were produced with GraphPad Prism 6 (GraphPad Software Inc., USA).

Results

Survival

All data of the 11 TC and 10 hemodilution animals are presented in our data table (table 1). In the hemodilution group, four animals did not survive all the eight hemodilution steps. We did not exclude these animals because it is likely that the sensitivity toward hemodilution differs per animal. In the TC group, the number of animals is constant (n = 11). Due to the fact that four animals in the hemodilution group did not survive all eight hemodilution steps, the number of animals in this group is lower at hemodilution step 7 (n = 8) and step 8 (n = 6). In the last column, we presented the results at the terminal point for some of the parameters. The terminal point represents the value of the concerning parameter at the point the animal is pre terminal. Because not all of the animals died at the same time point, these values represent the condition of the animal at its most critical hemoglobin level.

Hemoglobin Levels and Systemic Response

In table 1, the course of the hemoglobin level is shown for both the TC and hemodilution groups. As expected, we see

Table 1. Values Represent Mean ± SD

	BL	HD1	HD2	HD3	HD4	HD5	HD6	HD7	HD8	Terminal
MAP (mmHg)										
Control	83±14	82±16	80±15	81±14	80±14	79±14	79±13	78±12	79±13	
Experimental	79±5	81±7	88±34	75±8	79±19	79±35	68±31	68±26	63±22	30±10*
MitoPo$_2$ (mmHg)										
Control	21.6±4.1	17.0±3.9	17.4±3.9	17.9±3.7	17.9±3.6	18.2±3.4	17.9±3.5	18.2±3.6	18.3±3.2	
Experimental	23.6±5.3	20.3±5.3	22.9±10.3	18.7±5.4	14.8±4.3*	12.9±2.8*†	12.1±3.9*†	11.8±2.5*†	9.9±2.0*†	9.3±3.6*
Hemoglobin (g/dl)										
Control	8.1±0.6	7.9±0.5	7.9±0.5	7.9±0.5	7.9±0.5	7.9±0.5	7.9±0.5	7.7±0.5	7.9±0.5	
Experimental	7.9±0.6	5.6±0.5*†	4.5±0.5*†	3.7±0.3*†	3.1±0.2*†	2.6±0.2*†	2.3±0.3*†	2.3±0.5*†	2.1±0.6*†	2.7±0.8*
Hematocrit (%)										
Control	24±2	24±2	24±2	24±1	23±2	23±1	23±2	23±1	23±2	
Experimental	23±3	15±3*†	13±2*†	11±1*†	9±1*†	8±1*†	7±1*†	7±1*†	7±1*†	7±1*
HR (beats/min)										
Control	77±16	74±14	73±12	72±14	73±13	71±11	70±8	71±10	71±9	
Experimental	77±22	75±16	79±15	83±15	87±13†	90±12†	96±16*†	112±18*†	112±11*†	108±25*
CCO (l/min)										
Control	3.5±1.1	3.4±0.6	3.4±0.6	3.4±0.7	3.4±0.5	3.4±0.6	3.4±0.6	3.3±0.6	3.5±0.7	
Experimental	3.8±1.0†	4.7±0.9†	5.3±0.8†	5.8±0.6†	6.0±0.8†	6.0±1.2†	5.9±2.5†	6.3±2.3†	5.5±2.9†	2.6±1.7
Lactate (mM)										
Control	1.3±0.3	1.2±0.3	1.1±0.3	1.1±0.3	1.0±0.3	0.9±0.3*	0.9±0.3*	0.8±0.3*	0.7±0.3*	
Experimental	1.1±0.4	0.9±0.4	0.8±0.4	0.8±0.4	0.8±0.4	1.2±1.0	1.5±1.4	2.1±2.6	2.0±1.5†	4.5±1.7*
StO$_2$ (%)										
Control	59±11	58±16	59±15	58±12	57±13	60±9	56±15	55±20	58±14	
Experimental	57±15	58±16	57±19	60±17	60±16	56±16	54±21	57±19	54±22	40±15
$\dot{V}\text{o}_2$ (ml/min)										
Control	123±76	114±56	126±59	121±51	132±57	128±52	133±46	130±51	137±59	
Experimental	125±29	117±22	109±23	113±28	113±25	111±20	98±36	99±43	81±28	73±57*
DO$_2$ (ml/min)										
Control	401±142	380±70	381±76	371±71	380±63	370±69	279±82	367±85	383±97	
Experimental	417±85	377±66	346±57	312±38*†	277±35*†	241±50*†	208±87*†	213±73*†	165±78*†	107±76*
N=										
Control	11	11	11	11	11	11	11	11	11	
Experimental	10	10	10	10	10	10	10	8	6	6

*P < 0.0055 (paired Student's *t* test with Bonferroni correction) *versus* baseline. †P < 0.05 (unpaired Student's *t* test) *versus* control.

BL = baseline; CCO = continuous cardiac output; DO$_2$ = oxygen delivery; HD# = hemodilution step number; HR = heart rate; MAP = mean arterial blood pressure; MitoPo$_2$ = cutaneous mitochondrial oxygen tension; StO$_2$ = tissue oxygen tension; $\dot{V}\text{o}_2$ = systemic oxygen consumption.

a stable hemoglobin level in the TC group. In contrast, in the hemodilution group, we observed a significant decrease in hemoglobin level during hemodilution, with hemoglobin showing an exponential decline in the first hemodilution steps and leveling off around a value of 2 g/l in the final hemodilution steps. Systemic compensation for the loss in oxygen-carrying capacity of the blood is apparent from hemodilution 4 as both heart rate and CCO increase. Nevertheless, DO_2 decreases gradually from the start of hemodilution and becomes significantly lower compared to the baseline at hemodilution 3 (417 ± 85 *vs.* 312 ± 38; $P = 0.0033$).

Cutaneous MitoPo$_2$

As shown in figure 2, based on linear mixed-effect models, there is no significant time effect in mitoPo$_2$ in the control group. In the hemodilution group, we found a decreasing trend in mitoPo$_2$ after a number of hemodilution steps, which reaches significance level starting at hemodilution 4 (23.6 ± 5.3 *vs.* 14.8 ± 4.3; $P = 0.0033$). Because four hemodilution animals died before they reached the eighth hemodilution step, the eighth point in this figure does not offer an adequate view on the mitoPo$_2$ at the point the animal dies. To create this view, we took all the terminal values of the animals that died during the hemodilution and plotted these values as "terminal."

MitoPo$_2$ as Early Indicator of Hemodilution Limit

In figure 3, the mitoPo$_2$ can be compared to the hemoglobin level and other parameters that are known or expected to be an indicator for reaching the physiologic threshold of hemodilution. The dotted line is placed at the point where mitoPo$_2$ started to become significantly lower than the baseline. It is evident that this event preceded the trends in increase in lactate, drop in MAP, and drop in $\dot{V}o_2$ by two hemodilution steps. Importantly, none of the other parameters changed significantly unless the animals were pre terminal (table 1). While lactate, MAP, and $\dot{V}o_2$ at least showed a declining trend at the final hemodilution steps, StO_2 did not show a response.

Oxygen Delivery and Consumption

After calculating the $\dot{V}o_2$ (fig. 4A), we found only a significant difference at the terminal point. However, the DO_2 (fig. 4B) shows a significant decrease with ongoing hemodilution from the fourth hemodilution step onward. When we made the comparison between $\dot{V}o_2$ and DO_2 (fig. 4C), we found that the $\dot{V}o_2$ tends to remain stable during decrease in DO_2 in the first hemodilution steps. At the sixth hemodilution step, the $\dot{V}o_2$ tends to decrease during progression of subsequent hemodilution.

Individualized MitoPo$_2$

The previous data presentation clearly shows the dependency of mitoPo$_2$ on hemoglobin after hemoglobin dropped below a certain threshold. While mitoPo$_2$ appears to decline gradually with ongoing hemodilution, on the individual level, mitoPo$_2$ typically dropped much more acute. Figure 5A shows an example in an animal in which mitoPo$_2$ already showed a steep decline from 24 mmHg to 8 mmHg in one hemodilution step, relatively early in the protocol (between hemodilution 3 and 4). This acute drop preceded hemodynamic instability in the next hemodilution steps, and the animal did not survive after hemodilution 6. Therefore, to create a somewhat better visual presentation of the real course of mitoPo$_2$ during hemodilution, we aligned the individual curves of the experimental animals on their deflection point. In figure 5, we presented the mean ± SEM of these realigned curves.

Discussion

Our data support our main hypothesis that a decrease in mitoPo$_2$ is a direct and early indicator for the development of tissue hypoxia due to failing compensation mechanisms during ongoing hemodilution. The fact that cutaneous mitoPo$_2$ decreased abruptly at a certain stage of hemodilution instead

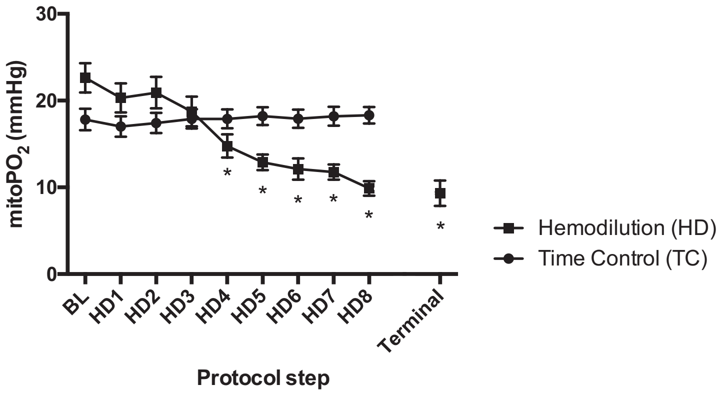

Fig. 2. MitoPo$_2$ (cutaneous mitochondrial oxygen tension) during the ongoing hemodilution (mean ± SEM). Mixed model analysis: group effect: estimate, 5.15; CI, 2.1 to 8.2, $P = 0.002$; Time effect: estimate, 0.11; CI, −0.09 to 0.32, $P = 0.283$; Group × time interaction: estimate, −1.73; CI, −2.03 to −1.44, $P < 0.001$. MitoPo$_2$ values in HD group at different hemodilution steps were compared to baseline levels using a paired Student's *t* test with Bonferroni correction ($^*P < 0.0055$). BL = baseline; HD# = hemodilution step number; TC = time control; Terminal = mean value at the point the animals died.

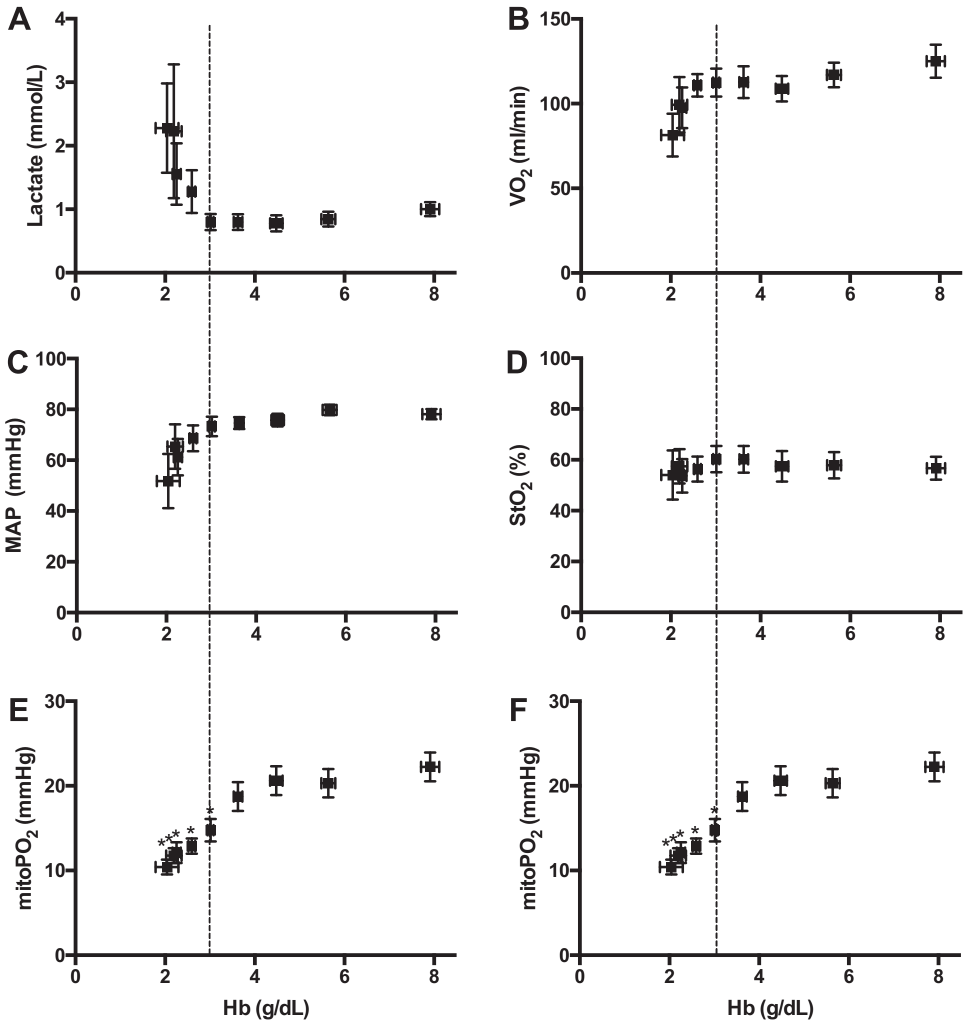

Fig. 3. Experimental animals. (*A*) Lactate *versus* hemoglobin (Hb); (*B*) $\dot{V}o_2$ (systemic oxygen consumption) *versus* hemoglobin; (*C*) mean arterial blood pressure (MAP) *versus* Hb; (*D*) StO$_2$ (tissue oxygen tension) *versus* Hb; E/F mitoPo$_2$ (cutaneous mitochondrial oxygen tension) *versus* Hb (mean ± SEM). *Dotted line* is placed at a significant decrease of mitoPo$_2$. Levels in hemodilution group at different hemodilution steps were compared to baseline levels using a paired Student's *t* test with Bonferroni correction (*$P < 0.0055$).

of showing a more gradual decline was a surprising finding. This could prove a very valuable behavior of mitoPo$_2$ when aiming to use it as physiologic transfusion trigger. The unexpected nonresponse of StO$_2$ shows that measuring quantitative Po$_2$ at the intracellular level provides different and complementary data.

The measurement of mitoPo$_2$ during hemodilution has never been performed before. The significant decrease in mitoPo$_2$ with ongoing hemodilution is in line with previous microvascular Po$_2$ (μPo$_2$) measurements by van Bommel *et al.*[16] Measuring μPo$_2$ has the disadvantage that it is not suitable for clinical use because the oxygen sensor dye Pd-porphyrin is exogenous and potentially nephrotoxic. Furthermore, after realignment of the mitoPo$_2$ data of the individual hemodilution animals, we found an even more convincing abrupt decrease in mitoPo$_2$ with decreasing hemoglobin levels. This suggests a strong relationship between the hemoglobin level and cutaneous mitoPo$_2$ in the pig.

When mitoPo$_2$ is compared with other parameters proposed to be the indicators of reaching the physiologic limit of hemodilution, it is found that mitoPo$_2$ values are decreasing in an earlier phase of the hemodilution. When MAP, lactate level, $\dot{V}o_2$, and DO$_2$ start to decrease, there is already anaerobic metabolism as indicated by increased lactate levels. Directly measuring the detrimental effects of hemodilution on cutaneous mitochondrial oxygenation provides a means to detect the individual limit without systemic signs of decompensation. Before the experiments were performed, we assumed that the StO$_2$ measured by near-infrared spectroscopy could be an early indicator as well. However, during the experiments, the StO$_2$ remained unaltered until the animals became hemodynamically unstable. So the decrease

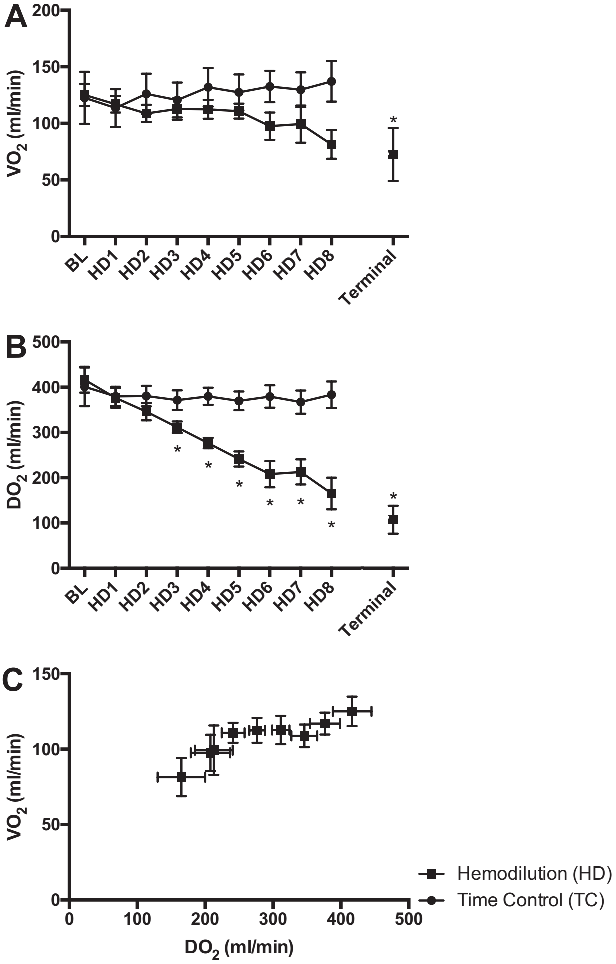

Fig. 4. (*A*) $\dot{V}o_2$ (systemic oxygen tension) *versus* protocol step; (*B*) DO_2 (oxygen delivery) *versus* protocol step; (*C*) $\dot{V}o_2$ *versus* DO_2 (mean ± SEM). Levels in hemodilution group at different hemodilution steps were compared to baseline levels using a paired Student's *t* test with Bonferroni correction (* = $P < 0.0055$). BL = baseline; HD# = hemodilution step number; Terminal = mean value at the point the animals died.

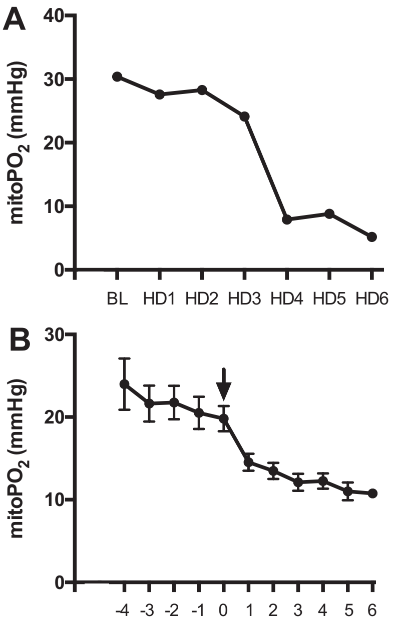

Fig. 5. (*A*) Example of an abrupt decrease in mitoPo_2 (cutaneous mitochondrial oxygen tension) early in the hemodilution protocol in an animal that survived only until hemodilution step 6. (*B*) Realigned mitoPo_2 (mean ± SEM) of all HD animals. Data arranged in relation to deflection point. On the x-axis, negative numbers are protocol steps before deflection point, and positive after deflection point. BL = baseline; HD# = hemodilution step number.

in StO_2 appears more related to the overall hemodynamic collapse than the reduced hemoglobin level *per se*.

Looking at the $\dot{V}o_2$ and DO_2 data, we found results comparable with the results published by van Bommel *et al.*[28] Pearce *et al.*[21] presented an illustrative picture of the relationship between DO_2 and $\dot{V}o_2$ in which the $\dot{V}o_2$ remains stable during hemodilution while the DO_2 decreases. When DO_2 decreases further, it will eventually compromise $\dot{V}o_2$. In our experiments, we have found a similar, but less obvious, trend in $\dot{V}o_2$ and DO_2, with $\dot{V}o_2$ only becoming significantly reduced in the nonsurviving animals. In a continuous and relatively fast hemodilution protocol in pigs, a critical hemoglobin (Hb_{crit}) value of 2.7 g/l has been reported by Lauscher *et al.*[15] Although it is difficult to directly compare our results to this study, from table 1, it appears that the trend in total body $\dot{V}o_2$ starts to decline somewhere between hemodilution 5 and 6. At this point, the trend in lactate also starts to increase. This would correspond with a Hb_{crit} around 2.4 to 2.5 g/l, which is not far from the previously reported value of 2.7 g/l. The difference might be attributed to the contrasting hemodilution protocols (stepwise *vs.* continuous) and, probably even more important, the different Fio_2 values (0.4 and 0.21, respectively). The fact that we used a stepwise form of hemodilution could be a limitation of our study design. A continuous form of hemodilution, as for example, used by Lauscher *et al.*,[15] is more comparable with clinical practice. In this study, we chose a stepwise form to be able to perform measurements in-between the successive hemodilution steps.

With respect to Hb_{crit} and the critical hematocrit (cHct), it is important to realize that reported values in animal studies differ somewhat per species, with values for cHct ranging from 10 to 15%.[14,29–31] Pigs are clearly on the lower end of the spectrum, with recent studies reporting cHct and Hb_{crit} around 10%[14] and 2.7 g/dl,[15] respectively. Likewise, in our study, mitoPo_2 dropped significantly between hemodilution steps 3 and 4, the point where hematocrit decreased below 11%. Thereafter, the animals became hemodynamically unstable during ongoing hemodilution. Due to interspecies differences, care should be taken when translating results of animal experiments to humans. Pigs are relatively anemic (normal hemoglobin in the range 8 to9 g/dl), and in humans, the reference range for hemoglobin is twice as high.

Hb_{crit} in humans is also different. For example, in healthy volunteers, a slight reversible cognitive dysfunction was seen during acute anemia at hemoglobin concentrations as high as 5 to 6 g/dl.[32]

Following the concept of previous experimental studies (*e.g.*, Lauscher *et al.*[15]; Meier *et al.*[29]) in our study design, a decrease in total body oxygen consumption was interpreted as the absolute limit of hemodilution. MitoPo_2 reacts abruptly well before reaching this point, and therefore, it might be a valuable parameter for indicating an individual's transfusion need. This tempting idea needs thorough further evaluation, especially considering the fact that vital organs show different dependencies on hemoglobin level.[15,16,28] Future studies should, therefore, next to incorporation of transfusion groups, include the evaluation of a set of clinically relevant endpoints regarding organ function and damage.

The different sensitivities of organs to anemia pose a challenge when considering a local mitoPo_2 measurement as a potential transfusion trigger. One has to look at an organ that is one of the first to get into trouble and one of the last to normalize. Based on previous findings, the skin certainly qualifies as such an organ. Using phosphorescence-quenching technology, simultaneous microvascular Po_2 (μPo_2) measurements on different organs have been performed in the past,[16] showing intestinal μPo_2 to decrease earlier during hemodilution compared to cardiac μPo_2 in rats. In pigs, it was demonstrated that during hemodilution intestinal serosal μPo_2 became impaired at an earlier stage than cerebral μPo_2.[28] Since oxygenation measurements in the skin can provide similar information to intestinal measurements,[23,24] the skin is hypothesized to be a valuable early indicator ("canary").

However, whether the above assumption of mitoPo_2 in the skin being a valuable canary holds for all organs, especially the kidney, remains to be seen. In previous studies in rats, μPo_2 dropped in very early stages of hemodilution in which hematocrit level did not even decrease clinically significantly.[16,33] In pigs, Konrad *et al.*[3] showed that normovolemic hemodilution to a hematocrit of 15% with a crystalloid significantly impaired renal function. Importantly, in the latter study, renal function loss seemed a result of tissue edema formation and not of anemia *per se* since hemodilution with a colloid preserved renal function. Also, biases due to other factors influencing skin perfusion and oxygenation (like temperature and centralization of the circulation) might be potential limitations for the use of cutaneous mitoPo_2 as a transfusion trigger in clinical practice.

In conclusion, looking at individual experiments, there is an abrupt decrease in mitoPo_2 with ongoing hemodilution. Furthermore, mitoPo_2 precedes changes in other parameters, which could make mitoPo_2 a clinically useful physiologic trigger for the need for erythrocyte transfusion. The concept of using cutaneous mitoPo_2 as a potential individual blood transfusion trigger is promising but obviously needs further research.

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Competing Interests

Dr. Mik is founder and shareholder of Photonics Healthcare B.V. (Utrecht, The Netherlands), a company aimed at developing a clinical monitoring device based on the delayed fluorescence lifetime technology for measuring mitochondrial oxygen. Photonics Healthcare B.V. holds the exclusive licenses to several patents related to this technology, filed and owned by the Academic Medical Center in Amsterdam and the Erasmus Medical Center in Rotterdam, The Netherlands. The other authors declare no competing interests.

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References

1. Tsai CF, Yip PK, Chen CC, Yeh SJ, Chung ST, Jeng JS: Cerebral infarction in acute anemia. J Neurol 2010; 257:2044–51
2. Mathew JP, Mackensen GB, Phillips-Bute B, Stafford-Smith M, Podgoreanu MV, Grocott HP, Hill SE, Smith PK, Blumenthal JA, Reves JG, Newman MF; Neurologic Outcome Research Group (NORG) of the Duke Heart Center: Effects of extreme hemodilution during cardiac surgery on cognitive function in the elderly. ANESTHESIOLOGY 2007; 107:577–84
3. Konrad FM, Mik EG, Bodmer SI, Ates NB, Willems HF, Klingel K, de Geus HR, Stolker RJ, Johannes T: Acute normovolemic hemodilution in the pig is associated with renal tissue edema, impaired renal microvascular oxygenation, and functional loss. ANESTHESIOLOGY 2013; 119:256–69
4. Ranucci M, Romitti F, Isgrò G, Cotza M, Brozzi S, Boncilli A, Ditta A: Oxygen delivery during cardiopulmonary bypass and acute renal failure after coronary operations. Ann Thorac Surg 2005; 80:2213–20
5. Habib RH, Zacharias A, Schwann TA, Riordan CJ, Durham SJ, Shah A: Adverse effects of low hematocrit during cardiopulmonary bypass in the adult: Should current practice be changed? J Thorac Cardiovasc Surg 2003; 125:1438–50
6. Ranucci M, Biagioli B, Scolletta S, Grillone G, Cazzaniga A, Cattabriga I, Isgrò G, Giomarelli P: Lowest hematocrit on cardiopulmonary bypass impairs the outcome in coronary

surgery: An Italian Multicenter Study from the National Cardioanesthesia Database. Tex Heart Inst J 2006; 33:300–5

7. Kuehnert MJ, Roth VR, Haley NR, Gregory KR, Elder KV, Schreiber GB, Arduino MJ, Holt SC, Carson LA, Banerjee SN, Jarvis WR: Transfusion-transmitted bacterial infection in the United States, 1998 through 2000. Transfusion 2001; 41:1493–9
8. Perez P, Salmi LR, Folléa G, Schmit JL, de Barbeyrac B, Sudre P, Salamon R; BACTHEM Group; French Haemovigilance Network: Determinants of transfusion-associated bacterial contamination: Results of the French BACTHEM Case-Control Study. Transfusion 2001; 41:862–72
9. Kleinman SH, Lelie N, Busch MP: Infectivity of human immunodeficiency virus-1, hepatitis C virus, and hepatitis B virus and risk of transmission by transfusion. Transfusion 2009; 49:2454–89
10. Vlaar AP, Hofstra JJ, Determann RM, Veelo DP, Paulus F, Kulik W, Korevaar J, de Mol BA, Koopman MM, Porcelijn L, Binnekade JM, Vroom MB, Schultz MJ, Juffermans NP: The incidence, risk factors, and outcome of transfusion-related acute lung injury in a cohort of cardiac surgery patients: A prospective nested case-control study. Blood 2011; 117:4218–25
11. Clifford L, Jia Q, Yadav H, Subramanian A, Wilson GA, Murphy SP, Pathak J, Schroeder DR, Ereth MH, Kor DJ: Characterizing the epidemiology of perioperative transfusion-associated circulatory overload. Anesthesiology 2015; 122:21–8
12. Geiger TL, Howard SC: Acetaminophen and diphenhydramine premedication for allergic and febrile nonhemolytic transfusion reactions: Good prophylaxis or bad practice? Transfus Med Rev 2007; 21:1–12
13. Rohde JM, Dimcheff DE, Blumberg N, Saint S, Langa KM, Kuhn L, Hickner A, Rogers MA: Health care-associated infection after red blood cell transfusion: A systematic review and meta-analysis. JAMA 2014; 311:1317–26
14. Hiebl B, Mrowietz C, Ploetze K, Matschke K, Jung F: Critical hematocrit and oxygen partial pressure in the beating heart of pigs. Microvasc Res 2010; 80:389–93
15. Lauscher P, Kertscho H, Schmidt O, Zimmermann R, Rosenberger P, Zacharowski K, Meier J: Determination of organ-specific anemia tolerance. Crit Care Med 2013; 41:1037–45
16. van Bommel J, Siegemund M, Henny ChP, Ince C: Heart, kidney, and intestine have different tolerances for anemia. Transl Res 2008; 151:110–7
17. Madjdpour C, Spahn DR, Weiskopf RB: Anemia and perioperative red blood cell transfusion: A matter of tolerance. Crit Care Med 2006; 34(suppl 5):S102–8
18. Balestra GM, Mik EG, Eerbeek O, Specht PA, van der Laarse WJ, Zuurbier CJ: Increased *in vivo* mitochondrial oxygenation with right ventricular failure induced by pulmonary arterial hypertension: Mitochondrial inhibition as driver of cardiac failure? Respir Res 2015; 16:6
19. Harms FA, Bodmer SI, Raat NJ, Mik EG: Cutaneous mitochondrial respirometry: Non-invasive monitoring of mitochondrial function. J Clin Monit Comput 2015; 29:509–19
20. Harms FA, Bodmer SI, Raat NJ, Stolker RJ, Mik EG: Validation of the protoporphyrin IX-triplet state lifetime technique for mitochondrial oxygen measurements in the skin. Opt Lett 2012; 37:2625–7
21. Pearce FJ, Waasdorp C, Hufnagel H, Burris D, DeFeo J, Soballe P, Drucker WR: Subcutaneous PO2 as an index of the physiological limits for hemodilution in the rat. J Appl Physiol (1985) 2005; 99:814–21
22. Dantzker DR: The gastrointestinal tract. The canary of the body? JAMA 1993; 270:1247–8
23. Mellstrom A, Månsson P, Jonsson K, Hartmann M: Measurements of subcutaneous tissue PO2 reflect oxygen metabolism of the small intestinal mucosa during hemorrhage and resuscitation. An experimental study in pigs. Eur Surg Res 2009; 42:122–9
24. Venkatesh B, Morgan TJ, Lipman J: Subcutaneous oxygen tensions provide similar information to ileal luminal CO2 tensions in an animal model of haemorrhagic shock. Intensive Care Med 2000; 26:592–600
25. Harms FA, de Boon WM, Balestra GM, Bodmer SI, Johannes T, Stolker RJ, Mik EG: Oxygen-dependent delayed fluorescence measured in skin after topical application of 5-aminolevulinic acid. J Biophotonics 2011; 4:731–9
26. Mik EG, Johannes T, Zuurbier CJ, Heinen A, Houben-Weerts JH, Balestra GM, Stap J, Beek JF, Ince C: *In vivo* mitochondrial oxygen tension measured by a delayed fluorescence lifetime technique. Biophys J 2008; 95:3977–90
27. Nagele P: Misuse of standard error of the mean (SEM) when reporting variability of a sample. A critical evaluation of four anaesthesia journals. Br J Anaesth 2003; 90:514–6
28. van Bommel J, Trouwborst A, Schwarte L, Siegemund M, Ince C, Henny ChP: Intestinal and cerebral oxygenation during severe isovolemic hemodilution and subsequent hyperoxic ventilation in a pig model. Anesthesiology 2002; 97:660–70
29. Meier J, Kemming GI, Kisch-Wedel H, Wölkhammer S, Habler OP: Hyperoxic ventilation reduces 6-hour mortality at the critical hemoglobin concentration. Anesthesiology 2004; 100:70–6
30. Welte M: [Is there a “critical hematocrit”?]. Anaesthesist 2001; 50(suppl 1):S2–8
31. van Bommel J, Siegemund M, Henny CP, Trouwborst A, Ince C: Critical hematocrit in intestinal tissue oxygenation during severe normovolemic hemodilution. Anesthesiology 2001; 94:152–60
32. Weiskopf RB, Kramer JH, Viele M, Neumann M, Feiner JR, Watson JJ, Hopf HW, Toy P: Acute severe isovolemic anemia impairs cognitive function and memory in humans. Anesthesiology 2000; 92:1646–52
33. Johannes T, Mik EG, Nohé B, Unertl KE, Ince C: Acute decrease in renal microvascular PO2 during acute normovolemic hemodilution. Am J Physiol Renal Physiol 2007; 292:F796–803