Quantitative intracellular oxygen availability before and after 5-aminolevulinic acid skin photodynamic therapy

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A B S T R A C T

Background: During photodynamic therapy (PDT) oxygen is transformed into reactive oxygen species (ROS) to induce cellular apoptosis in (pre)malignant cells. Real time oxygen availability measurement is clinically available with the Cellular Oxygen Metabolism (COMET) monitor.

Methods: Primary objective is to show that mitochondrial oxygen availability (mitoPO2) measurement is possible during clinical ALA-PDT. The secondary aim was to determine the pain sensation, because it is the most commonly reported side effect of PDT. Before and after the two fraction PDT treatment, with a 2-hour dark period, mitoPO2 was measured and reported pain was documented with a visual analog scale (VAS) 0–100.

Results: Nine patients were included. Before the first PDT session the median signal quality was [IQR] 55.0% [34.2–68.0], which decreased after session one to 0% [0.0–10.0]. MitoPO2 was 40.0 [17.7–53.8] mmHg and increased afterwards to 61.8 [38.2–64.8] mmHg. This likely the result of the delay time between the illumination stop and the mitoPO2 measurements in a vasodilated, visibly red lesion. Before session two signal quality was 10.4% [0–20.15], 40% lower than at the start. In 5 patients the signal quality after session 2 was too low because of photobleaching and insufficient regeneration of PpIX, median 0% [0–10]. Subjects reported low median VAS scores, all below 3, directly after the mitoPO2 measurements.

Conclusion: With COMET we were able to reliably measure mitochondrial oxygen concentrations during photodynamic therapy. Signal quality drastically decreases after a PDT session because of PpIX deterioration during the illumination phase.

1. Introduction

Photodynamic therapy (PDT) is successfully used for treatment of superficial cutaneous cancers. The therapy requires three interdependent factors, light, photosensitizer, and oxygen. The photosensitizer absorbs light and reacts with oxygen, resulting in the creation of reactive oxygen species (ROS). The PDT results in cellular and vascular damage and an immunological response that leads to apoptosis and necrosis of (pre)malignant tissue [1]. The availability of oxygen is crucial for the amount of apoptosis induced during PDT [2].

PDT has a high complete clinical response rate of 90% in human superficial basal cell carcinoma [3]. Unfortunately the recurrence rate is around 21.1% and no clinical clearance is seen in 14.7% [4]. The cytotoxicity variation after PDT in vivo is multifactorial and depends on the photosensitizing molecule penetration, its localization, light dose and time, type of irradiation and time, and type of tumor and its level of oxygenation [5–7]. In order to improve the clinical response a two fraction treatment scheme, with a dark period in between two illumination sessions, can be used [8]. Such two-fraction treatment scheme is common in the Netherlands. However, although it has been proven to improve the clinical response the exact mechanism is not fully understood.

Because oxygen availability is one of the key factors for the induction of ROS and cellular apoptosis, it has been suggested to measure oxygen to be capable of adjusting the light dosimetry in real time for an optimal usage of the photosensitizer [9–11].

One of the most used photosensitizers in PDT is 5-aminolevulinic acid (ALA)-induced Protoporphyrin IX (PpIX). PpIX occurs naturally in cells and is endogenously produced in the mitochondrial heme-cycle. Under normal circumstances the accumulation of PpIX is avoided by a

Key message: Oxygen as key component in photodynamic therapy can be measured with mitochondrial oxygen tension.

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negative feedback mechanism of heme [12]. External administration of ALA bypasses this negative inhibition, which in combination with the rate-limiting incorporation of iron into PpIX for the formation of heme, results in PpIX accumulation in the mitochondria [13].

Real time oxygen availability measurement became possible with the introduction of the Triplet State Lifetime Technique (TSLT) [14]. As in ALA-PDT this technique also uses ALA-induced PpIX as an intra cellular endogenous oxygen probe. The PpIX exhibits prompt fluorescence, but also an oxygen-dependent delayed fluorescence in the same spectrum with a lifetime of several hundred microseconds [15]. Spontaneous regression via a bi-directional intersystem crossing from the excited triplet state, is accompanied by delayed fluorescence. Oxygen is a very effective quencher of the excited PpIX in the triplet state, it takes over the energy and PpIX will relax to its ground state without emission of a photon. The delayed fluorescent lifetime of the PpIX is therefore inversely proportional to the amount of intracellular oxygen [15]. The detailed description of the measurement technique has been published elsewhere [15–17]. Currently a device, called COMET, is available that has incorporated the TSLT and enables measurements of mitochondrial oxygen (mitoPO2) availability and oxygen disappearance rate (ODR) in the skin [17].

The primary aim of this pilot study is to demonstrate that the measurement of oxygen availability is possible with COMET in the current daily clinical ALA-PDT practice. The second aim was to determine the pain sensation caused by the oxygen measurement technique and of the PDT treatment [18].

2. Materials and methods

This single center study was ethical approved by ethical research board Rotterdam and registered at www.toetsingonline.nl [NL51187.078.14]. All adult patients (age > 18 years) who were scheduled for ALA-PDT in 2015 and 2016 in the Erasmus Medical Center Rotterdam were included. Patients came in around 9:00 am for 20% ALA gel application (Fagron, Oud Beijerland, The Netherlands) on the skin lesion. The area was subsequently covered with an occlusive dressing (Tegaderm, 3 M, Leiden, The Netherlands) for light protection. This coverage ensures a proper accumulation of PpIX, blocks the location for excessive sunlight, and prevents photo degradation PpIX.

Two illumination sessions were scheduled with the Omnilux light source (Waldmann Phototherapeutics, London, United Kingdom), emitting a wavelength of 633 nm with a bandwidth of 20 nm in a continuous mode. Light fractions were delivered at a fluence rate of 80 mW cm−2. Four hours after ALA application the first illumination session (20 J cm−2) started. Subsequently the second illumination session (80 J cm−2) was started after a two-hour dark interval.

Before and after each PDT illumination session the oxygen availability (as mitochondrial oxygen tension, mitoPO2) and oxygen disappearance rate (ODR) were measured with COMET (Photonics Healthcare BV, Utrecht, the Netherlands). One measurement is a sequence of 120 measurements (1 Hz). After approximately 15 s, pressure was applied on the probe to temporarily occlude the local microcirculation. In this period the mitochondria locally use the oxygen in the cells and therefore the oxygen disappearance rate can be determined, after 30 to 40 s pressure was released and the influx of oxygen into the cell can be seen. A detailed description of COMET, the measurement technique, and the signal quality calculation have been described elsewhere [17].

PDT is known to be painful in a selective group of patients. The sensation of the oxygen measurement with COMET specific was determined with a visible analogue scale (VAS score). The VAS goes from 0, no sensation, to 100, the worst pain ever felt. Before and after illumination a close-up digital photograph was taken. Patients needed to report their pain sensation with a pencil stroke between 0 and 100. Afterwards the length from 0 to the indication was measured with a digital caliper.

Oxygen availability was calculated as the average of six

<table>
<thead>
<tr>
<th>Table 1</th>
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<td>Diagnosis</td>
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AK: Actinic keratosis, BCC: Basal cell carcinoma, F: Female, M: Male, PDT: Photodynamic Therapy.,
measurements prior to the point where pressure was applied on the skin sensor to occlude the microcirculation. Oxygen disappearance rate was determined with a least square linear fit of the first part of the oxygen disappearance curve [19]. Because the availability of oxygen is unlimited for the consuming cells, the calculated coefficient dPO$_2$/dt approximates the cellular oxygen respiration. A detailed description of the analysis technique can be found in an article on cutaneous respirometry by Harms et al. 2016 [20].

Data were analyzed using R version 3.4.2 [21]. Boxplot shows the median in the middle with hinges first and third quartiles (25 and 75% percentiles). Whiskers are min max value within 1.5*IQR. Group comparison for mitoPO$_2$ levels was done with Wilcoxon-Mann-Whitney test. Significance was determined by p-values < 0.05. All values reported are expressed as median with the first and third inter quartile range. Due to the exploratory nature of this study, no corrections for multiple comparisons were applied.

3. Results

Nine patients were measured. Seventy eight percent (78%) of

Fig 2. An example of mitoPO$_2$ measurement (black) and signal quality (blue), before photodynamic therapy session 1. MitoPO$_2$ is 51.2 mmHg calculated as average from the 6 measurement points before pressure is applied to the sensor resulting in a drop in the mitoPO$_2$.

Fig 3. A) Oxygen availability in the lesion (MitoPO$_2$). B) Oxygen disappearance rate. C) Signal quality.
patients were treated with ALA-PDT for basal cell carcinoma (BCC) and 22% for actinic keratosis (AK). The overview of demographic data can be found in Table 1. In one occasion COMET failed to measure in the 22% for actinic keratosis (AK). The overview of demographic data can be found in Table 1. In one occasion COMET failed to measure in the morning because of high humidity in the summer period, after acclimatization in the air-conditioned area it started working, therefore only before and after the second illumination phase the mitoPO\textsubscript{2} was measured. In another case the pain in session one was non-bearable and the PDT session was stopped and no measurements could be done afterwards. The data and patient flow scheme can be found in Fig 1. An example of an oxygen availability measurement with an oxygen disappearance rate measurement is shown in Fig 2.

3.1. Illumination session 1

Before the first PDT session the signal quality was adequate (defined as $>20\%$ [17]) in all patients, median [IQR] 55.0% [34.2–68.0] and decreased after the PDT session to 0% [0.0–10.0]. Oxygen availability before the start of PDT was 40.0 [17.7–53.8] mmHg \( n = 8 \) in the lesion. After the first PDT session 3 patients did have insufficient signal quality and no mitoPO\textsubscript{2} could be gathered. The oxygen availability increased after session one to 61.8 [38.2–64.8] mmHg \( n = 5 \). Oxygen disappearance rate (ODR) before was 5.6 [3.0–8.4] mmHg/s and increased to 6.4 [1.8–6.6] mmHg/s. In one patient the oxygen consumption measurement could not be performed after the PDT session because of insufficient signal quality in subsequent measurements to calculate the ODR.

3.2. Illumination session 2

After a 2-hour interval in the dark, to re-accumulate PpIX, one patient regained a signal, the mitoPO\textsubscript{2} increased further to 86.3 [41.8–164.1] mmHg \( n = 6 \) before the second PDT illumination. It decreased to 60.0 [30.0–82.7] mmHg, \( n = 3 \). The signal quality started with a median 10.4% [0–20.15] what is 40% lower compared to the start illumination 1. In 5 patients the signal quality after session 2 was too low to measure mitoPO\textsubscript{2} and resulted in a median 0% [0–10]. The ODR after the 2 h dark period was 6.6 [5.4–8.1] mmHg/s, and decrease to 1.4 [0.7–5.7] mmHg/s. An overview is given in Fig 3.

3.3. Measurement sensation

Subjects were asked to report the sensation after the measurement with COMET specific. Time points before the illumination session tend to be close to zero VAS median with IQR 1.5 [0.6–3.5], VAS-scores afterwards have the tendency to be higher 2.3 [0.9–9.7]. Before session 2 it was lower compared to session 1 VAS 1.6 [0.6–1.6] and after the PDT session VAS 1.9 [1.2–2.6]. It was hard for the subjects to distinguish between the pain felt after the PDT session and during the COMET measurement itself, an overview can be found in Fig 4. Photographs of the lesions before the first and after the second PDT session are seen in Fig 5.

3.4. Typical finding

One of the measurements had a typical finding showing that the oxygen availability was 0 mmHg after the usage of an anesthetic lidocaine with adrenaline. In session 1 so much pain was experienced that the patient asked for an anesthetic. Before and after the PDT session with an adequate signal quality, 0 mmHg mitoPO\textsubscript{2} was measured, seen in Fig 6.

3.5. Discussion and conclusion

This study was designed to explore the usability of COMET during ALA-PDT. For the mitochondrial oxygen availability measurement, a sufficient conversion of ALA in PpIX is necessary to get an adequate delayed fluorescence signal. The signal quality before ALA-PDT was sufficient to determine the mitoPO\textsubscript{2} and ODR adequately.

Before session one, eight patients were measured with COMET. The use of COMET in a non-air-conditioned room during summer with high humidity led to occasional laser miss fire of the excitation light source in the COMET device. Before the illumination session the signal quality was sufficient for every patient to measure reliable mitoPO\textsubscript{2}. After session one an increase of mitoPO\textsubscript{2} was seen. In the majority of cases the signal quality decreased drastically after the PDT illumination and resulted in non-reliable measurements. It was expected that the signal quality would decrease after PDT sessions because of PpIX photo-bleaching and photodegradation of PpIX [22].

One of the main mechanisms of PDT is the utility of oxygen conversion into reactive oxygen species that leads to apoptosis and necrosis. Therefore, we expected that the oxygen would be depleted after an illumination phase. Surprisingly, the median oxygen availability increased after the first PDT session. This is likely the result of the delay between the illumination stop and the mitoPO\textsubscript{2} measurements. The inflow of oxygen into the microcirculation is in order of seconds and could occur right after the stop and is reflected in the increased mitoPO\textsubscript{2} after session 1. To substantiate this theory, the skin was macroscopically erythematous after the session, which is a consequence of vasodilatation and increased blood flow.

Extensive pain is one of most severe and most often reported side effects during PDT treatment. All patients experience some discomfort or even unbearable pain during the PDT. VAS scores found for PpIX PDT were $\geq 70$ in 58% of the cases [23] and in 85% of the cases reported by Piffaretti et al. [24], in comparison the COMET did not induce any significant pain sensation with a median VAS of 1.6 and 1.6 seen in Fig 4. Bias is introduced in the VAS-score after a PDT session because it was...
hard to distinguish between the pain felt from the ALA-PDT and the oxygen measurement. Therefore the VAS scores after the treatment were higher compared to pre-PDT sessions, but still negligible compared to the pain felt during PDT.

One patient that started with a low mitoPO$_2$ stood out, due to extreme pain during the photodynamic session. No measurements were done after the first session and the second session was not started. These low mitoPO$_2$ (<10 mmHg) values were not seen in any other measured subject, maybe the initial low oxygen and the fast usage of all the oxygen have led to nerve signaling with a pain sensation. A second interesting case was the use of lidocaine with adrenaline shown in Fig 6. After using the anesthetic with an adequate signal quality 0 mmHg oxygen availability was measured. It is likely that due to vasoconstriction of the adrenaline a small amount of oxygen enters the lesion and is consumed right away. Therefore results in 0 mmHg, since PDT efficacy is oxygen dependent, this may negatively influence the PDT treatment effectiveness.

The application of the 20% ALA-gel 4 h before the PDT treatment was done proportional to the lesion size. Most lesions measured a few millimeters in diameter. Therefore, the measurement location was also a few mm$^2$ in size. The skin sensor head of COMET is not designed for such small lesions. A high signal quality indicated the head was positioned properly. It was hard to find location with good signal quality with on these small lesions, and when found, to measure and hold the Skin Sensor steady in position.

A smaller skin sensor, steady secured, over the lesion would increase the reproducibility of the measurements. It is known that the mitoPO$_2$ is heterogenic distributed in the skin surface so not the exact same volume of cells is measured before and after the PDT treatment [16,25].

Clearly in the data is seen that the signal quality is dramatically decreased after a PDT session. This is expected because the red-light illumination during PDT will use all the available PpIX and will also bleach and destroy the PpIX ring integrity.

Over the years several mechanisms have been developed to improve PDT and implement a form of individual dosimetry. Singlet oxygen measurement, oxygen phosphorescence with palladium porphyrin, and photobleaching are a few examples of this [2,24,26].

One of the potential clinical improvements for PDT with COMET is dosimetry with oxygen availability feedback mechanism. The PDT treatment depends mainly on the creation reactive oxygen. The COMET could provide feedback to ensure enough oxygen inflow in the lesion. This may improve the individual PDT dosimetry and therefore outcomes [10,27-29].

Recently a pilot has been published by Scholtz et al. (2020) [11] that uses a delayed fluorescence imaging system during ALA-PDT to detect mitochondrial oxygen tension in tumors in mice. It showed that oxygen is tumors is heterogenous, and that the redistribution of oxygen in the lesion after a light dosage of 5 J/cm$^2$ can take up to 10–60 min. Furthermore the study showed that the oxygen depletion rate was dependent on the PpIX concentration, which in itself was heterogenous distributed in the lesions [11].

Photobleaching-based PDT dosimetry is a technique to provide dosimetry feedback. It can be used when sufficient or a high concentration of O$_2$ is available. Especially with low oxygen concentrations, this approach becomes unreliable and is therefore not practical in clinical PDT dosimetry [30].

Fig 5. Photograph before photo dynamic therapy (PDT) and after illumination.
Another technique to evaluate PDT dosimetry is photon count from \( ^1 \text{O}_2 \) singled oxygen luminescence (SOL). It is stated that the amount SOL is related to the cell toxicity [2]. Unfortunately, this technique is costly, complex and measures a very weak signal, therefore it is unlikely to be clinically available in the short term. This technique showed that if the illumination is increased and the oxygen availability depleted no extra apoptosis will be induced. Rather a time in the dark to reintroduce oxygen in the lesion, is beneficial for lesion apoptosis [2].

An existing well-known technique to measure intravascular oxygen tension, is the phosphorescence technique with palladium porphyrin, introduced in PDT over 2 decades ago [26]. The phosphorescence lifetime is detectable besides the fluorescence of the PpIX and can be used simultaneously with the PDT illumination. A drawback of this technique is the extra infusion of potential toxic metalophorphyrin in the bloodstream. Second compared to mitoPO\(_2\) the vascular compartment is measured, instead of the intra cellular compartment where its used.

In conclusion, the COMET measurement system can be used in ALA photodynamic therapy to measure in vivo mitochondrial oxygen concentration. Signal quality was adequate in all patients before the PDT session started. Afterwards the signal quality was drastically decreased. It was safely applicable; in small lesions it was hard to perform steady measurements, because of a large skin sensor in comparison. Since only a small number of patients was included for this pilot, no conclusions could be drawn about the relationship between mitoPO\(_2\) and clinical outcome.

It is recommended to further look into the oxygen availability and the treatment effect of PDT. A study should be designed to compare the illuminated area with a non-illuminated area. Small intermittent stops could be introduced in the PDT illumination to evaluate the oxygen concentration. This may act as a feedback loop to further recupare oxygen into the lesion in the dark intermittent phase. This may individualize treatment per lesion or individual. After this exploration this may open a new monitoring tool for PDT to deliver personal and lesion-specific light dosimetry guided by oxygen delivery in the lesion.

**Declaration of Competing Interest**

R. Ubbink: The author is shareholder of Photonics Healthcare, a company aimed at making the delayed fluorescence lifetime technology available to a broad public. 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 Erasmus Medical Center in Rotterdam, The Netherlands.

E. G. Mik: The author is a one of the founders and shareholder of Photonics Healthcare, a company aimed at making the delayed fluorescence lifetime technology available to a broad public. 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 Erasmus Medical Center in Rotterdam, The Netherlands.

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