



Bioimpedance for pain monitoring during cutaneous photodynamic therapy: Preliminary study

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Aminolevulinic acid (ALA);
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Summary

Background: Pain is a well-known problem associated with light exposure during topical photodynamic therapy (PDT). Different methods for dealing with the pain have been developed over the past years, ranging from cooling with air or water to nerve blocking. However, the mechanisms responsible for the pain induction have not yet been fully understood.

Aim: This study aims to evaluate bioimpedance *in situ* measurements of human skin as a method to shed light on pain-inducing real-time changes during light exposure during topical PDT.

Methods: Cream containing 20% aminolevulinic acid (ALA) was applied on forearms of ten healthy human volunteers. After 24 h incubation, the cream was removed and the spots were exposed to red laser light (636 nm, 300 mW/cm²). During light exposure bioimpedance measurements with a 4-electrode set-up were taken at two frequencies (10 Hz and 100 kHz).

Results: A significant drop in skin impedance at high and low frequencies coincided with onset of pain during light exposure of spots treated with ALA. A similar drop was not observed for controls.

Conclusions: Bioimpedance spectroscopy can provide valuable data for real-time observation of changes in skin, and may contribute to an increased understanding of the mechanisms responsible for induction of pain during topical PDT. Future studies are needed.

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Introduction

Topical photodynamic therapy (PDT) is a highly effective treatment modality for superficial basal cell carcinoma,

Bowen's disease and premalignant lesions actinic keratoses [1]. It is a selective, non-invasive treatment that offers high cure rates and good to excellent cosmetic outcome. The treatment consists of local application of cream containing a photosensitizer precursor (typically aminolevulinic acid (ALA) or its methyl ester (MAL)) followed by several hours of on-skin incubation. During the incubation hours the photosensitizer protoporphyrin IX (PpIX) is produced and accumulates inside rapidly dividing cells, i.e. neoplastic cells. Afterwards, the skin is exposed to red or blue/violet light which excites PpIX from the ground state into its triplet

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state. Excited triplet PpIX molecules transfer their energy onto biomolecules or to molecular oxygen present in its surroundings creating highly reactive oxygenized products and singlet oxygen, which in turn lead to cell death.

PDT kills cells both by apoptosis and necrosis [2]. During apoptosis cells “shrink” and disintegrate creating apoptotic bodies, which are then cleared by macrophages and dendritic cells [3]. In case of necrosis, cells swell and release their content due to cell membrane ruptures [4]. Each of these processes changes the ionic balance of the extracellular matrix, the total water and protein content of the extracellular matrix and other parameters influencing the electrical properties of skin. Real-time monitoring of such changes might help elucidating the mechanisms behind PDT-induced pain.

The concept of extracting information from tissue by monitoring its impedance has been recognized since the early 1900s [5]. Bioimpedance describes the passive electrical properties of biological materials and can often be measured in a relatively simple and non-invasive way. Since virtually any changes in tissue physiology or morphology will lead to changes in the impedance parameters, bioimpedance has a unique potential for characterizing or analyzing tissue when measured and interpreted correctly.

Electrical impedance spectroscopy has been used to characterize the electrical properties of a number of human and animal tissues, from a rat foot [6], through skin, testis, ovary, uterus, bladder, kidney, spleen, liver, lung and heart, to brain [7–11]. This has been useful for many clinical and prophylactic purposes. However, the studied samples were excised and/or measurements of bioimpedance were performed after the relevant treatments. In this article, for the first time, bioimpedance spectroscopy was used to observe *in situ*, real-time changes of the passive electrical properties inside human skin during topical PDT. This manuscript is a proof-of-concept work that aims to verify whether bioimpedance measurements of real-time changes in human skin during cutaneous photodynamic therapy session can provide helpful data in elucidating the mechanisms behind the pain.

Materials and methods

Chemicals

5-Aminolevulinic acid hydrochloride (ALA) was obtained from Sigma–Aldrich Norway AS (Oslo, Norway). The active compound was diluted in cream base (Unguentum®, Almirall Hermal GmbH, Reinbek, Germany) to reach 20% (w/w) of ALA concentration.

Volunteers

The influence of ALA–PDT on bioimpedance of human skin was investigated on 10 healthy volunteers (3 women and 7 men) with an average age of 30 (Fitzpatrick skin types II–III). All volunteers were informed about the procedure of the study, possible risks, protection of sensitive data and the right to withdraw any time they wanted. The project did not involve any health risk for the volunteers.

The study was approved by the local ethical committee, Regional komite for medisinsk forskningsetikk Sør-Norge (Ref. no. S-07434b).

Cream application

Four spots (a–d, 1 cm² each) were selected and marked on the forearm of each volunteer using a hydrocolloid dressing (DuoDERM®, ConvaTec, Deeside, UK). Approximately 0.1 g of cream containing 20% (w/w) ALA was applied on spots a and c. Spot b was covered with cream without the active compound and spot d was covered with occlusive dressing only. After 24 h, all creams were removed. Spots were washed with soap and warm water to remove possible occlusive dressing residue. PpIX accumulation in healthy tissue is slower than in neoplastic tissue, therefore a 24 h incubation regime was used to ensure high enough PpIX accumulation in all volunteers that guaranteed occurrence of pain.

Fluorescence measurements

The fluorescence of porphyrins produced in the skin of all volunteers was measured in all spots after cream removal using a fiber-optic probe coupled to a spectrofluorimeter (LS50B, PerkinElmer, Norwalk, CT). Excitation light was set on 407 nm, while emission was recorded at 636 nm. With this set-up the major part of the recorded fluorescence is related to PpIX molecules accumulated in the skin [12].

Light exposure

All spots were exposed to red light. For light exposure a red laser (632 nm, Cereles-Laborlaser, CeramOptec, Germany) with a power output set on 300 mW/cm² was used. This was the highest available fluence rate and it was used in order to reach the pain threshold as fast as possible. Time was accurately measured (in seconds) from the beginning of the light exposure to the onset of pain. Light exposure was interrupted when the volunteers judged the pain to be too strong for continuation of the exposure. Total fluence delivered during exposure of control spots (spots b and d) was 27 J/cm², while the total fluence delivered during exposure of test spots (spots a and c) varied depending on the time of the exposure since the exposure was interrupted on the onset of pain.

Bioimpedance measurements

Bioimpedance measurements at 10 Hz and 100 kHz were carried out using a 4-electrode system and a Solartron 1260 + 1294 impedance analyzer. Four Kendall Soft-E H59P (3M Health Care, Neuss, Germany) electrodes with conductive adhesive hydrogel were used. In order to reduce the effective electrode area (EEA) of the pick-up electrodes (PU), these were cut as narrow as possible. PU electrodes were placed next to each spot, with the unmodified current carrying electrodes (CC) on the outside of the pick-up ones, and perpendicular to the arm length of each volunteer (Fig. 1). The electrode modification was primarily done to

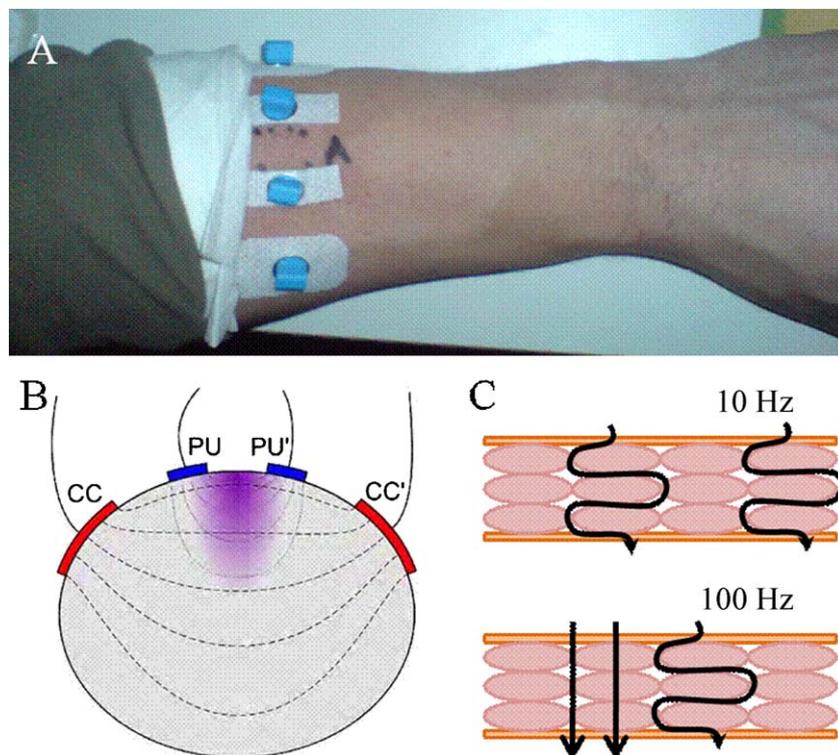


Fig. 1 (A) Placement of electrodes in 4-electrode setup. Note size of pick-up electrodes (PU and PU') (on each side of the marked skin spot) and bigger, current carrying electrodes (CC and CC') placed outside of the pick-up electrodes, perpendicular to the arm length. The other spots prepared are covered to prevent PpIX bleaching from the room lighting. (B) 4-Electrode system in idealized, homogenous volume conductor. Volume of highest positive sensitivity is emphasized with a darker color. Negative sensitivity (between PU and CC electrodes) is left out to simplify the illustration. (C) Schematic illustration of current paths through tissue during high and low frequency bioimpedance measurements.

increase the CC EEA relative to the PU EEA – which gives a better signal-to-noise ratio on the recorded signal.

For our investigations we chose to monitor the impedance at 10 Hz and 100 kHz, which fall within the range where the current conduction through tissue is mainly determined by tissue structure, i.e. the extra- and intra-cellular compartments and the insulating cell membranes [6]. Whereas the 10 Hz data will mainly reflect properties of the extracellular space, the 100 kHz data will also include the intracellular volume (Fig. 1C).

Measurements on control spots were taken for 180s before the beginning of light exposure, during 90s of light exposure and 90s after light exposure. On test spots, measurements were taken for 3min before light exposure, during light exposure and up to a total time of 25 min after the end of irradiation. The rationale behind this time differences are of purely practical reasons. The measurements lasted about 3–4h per volunteer and it was very tiring for their arms. Ever since we saw that the bioimpedance measurements on the control spots showed no changes with light exposure, we limited the monitoring time after light exposure to 90s.

Statistics

Data are presented as means \pm S.E. (standard error) from 10 volunteers. Raw data vectors were imported into Mat-

Lab, where two types of filtration were used. Due to the high-frequency noise present in recorded impedance data, a median filter was utilized first (Medfilt1, with a parameter setting of 30). To filter out artefact-related impedance changes, a filter for step removal was also applied for those vectors where artefacts were found (custom filter).

Results

Skin bioimpedance changes at high (100 kHz) and low (10 Hz) frequencies were monitored during and after light exposure on spots b and d (Fig. 2). For both spots statistically significant changes in bioimpedance were not found at high frequency, neither during nor after light exposure (Fig. 2). At the low frequency, a decrease in skin bioimpedance with time was found in spot b (Fig. 2). However, this decrease was statistically insignificant and did not seem to be associated with the light exposure. It is worth noting that cream incubation on the skin (spot b) led to larger interpersonal variations as compared to dry skin (spot d) (shaded areas in Fig. 2).

On skin areas pre-treated with ALA-cream for 24h prior to measurements an increase in the impedance occurred before the light exposure, which continued until the onset of pain at low frequencies (extracellular compartments), but stopped to rise with the beginning of light exposure at high frequencies (extra- and intracellular compartments). Subse-

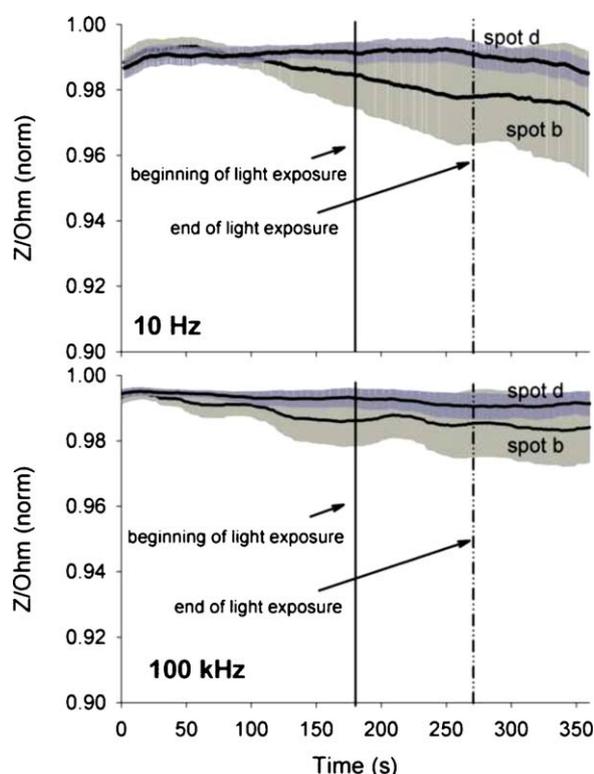


Fig. 2 Baseline measurements at low frequency (10 Hz) and high frequency (100 kHz). Shaded areas represent standard error values for given points. Spot d is the spot covered only with occlusive dressing during the incubation time. Spot b is the spot covered with cream without active compound (ALA) during the incubation time.

quently, a decrease in bioimpedance was observed at both frequencies (Fig. 3). After the end of the light exposure, bioimpedance kept decreasing until it reached a “stable” level and did not rise during the rest of the measurements (25 min). Although the abovementioned changes are true for both low- and high-frequency measurements, they are much more pronounced for the low frequency, in spite of large interpersonal variations (note error bars in Fig. 3). One change, however, occurred only at the low frequency. Between 470th and 566th second of measurements (8th and 9th minute), the bioimpedance levelled off before it started to decrease again. This plateau was surprisingly consistent in all volunteers.

Protoporphyrin IX fluorescence was measured at 636 nm and compared with the time needed for pain to occur (Fig. 4). A correlation between the time for pain to occur and PpIX fluorescence was found. There was, also, a positive correlation between the time for pain to occur and the total drop in bioimpedance (Fig. 5). This correlation was stronger for the high frequency than for the low one, but was present in both.

Discussion

Light exposure during topical PDT often causes pain, which is a pressing problem. This pain can be characterized as a burning and stinging sensation arising within seconds after

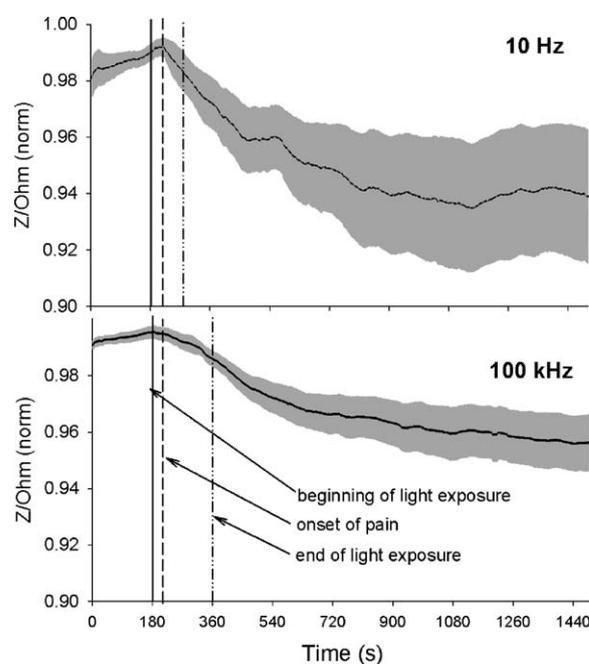


Fig. 3 *In situ* skin bioimpedance measurements during light exposure at 10 Hz (low frequency) and 100 kHz (high frequency). Shaded areas represent standard error values for each given point. Light exposure commenced at 180th second, the marked “onset of pain” is a moment when volunteers defined the sensation as pain. The “end of exposure” point marks out a moment when the pain sensation was too strong to continue the exposure.

the beginning of the light exposure [13,14]. Due to this pain, many patients cannot complete the PDT treatment, since the discomfort becomes unbearable [15]. Several different approaches to achieve pain relief have been studied. So far, only two proved effective: cooling of the areas with air or water [16,17] and nerve blocking by intradermal or cranial injections [18]. Pain relief with the use of cooling has to be well balanced since a decrease in skin temperature can negatively influence the PpIX accumulation in the skin [19]. In fact, preheating of the area prior to the treatment has been shown to enhance the therapy outcome [20]. Nerve

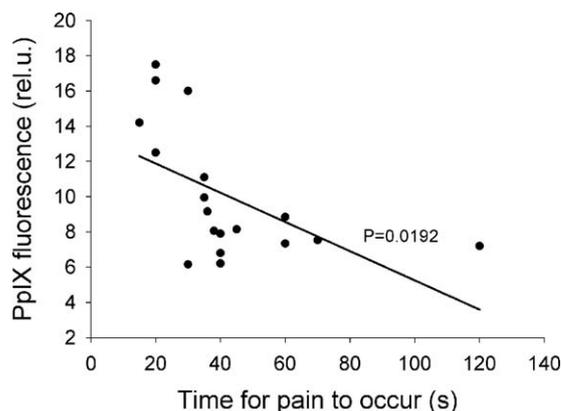


Fig. 4 A positive correlation was found between the PpIX fluorescence intensity at the beginning of the light exposure and the time for pain to occur.

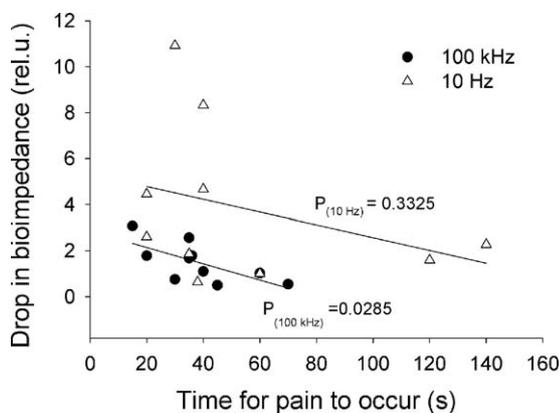


Fig. 5 A positive correlation was found between the drop in bioimpedance at high frequency and the time needed for pain to occur.

blocking, although more effective than cold air analgesia [21], is an invasive technique that have potential adverse events including haematoma through vessel trauma or paresis by direct nerve injury [18]. Detailed information about the mechanism behind this pain would provide a more specific management of this pain.

Over the past decades many studies have attempted to elucidate the mechanisms behind this pain. Tissue damage and/or nerve stimulation [22], the acidity of the cream, i.e. change in local pH [23], a combination of inflammation and heat [24] and ALA transport into nerve endings by GABA transporters [23] have been proposed. Furthermore, total light dose [25], total protoporphyrin IX fluorescence intensity level [26], localization, type and size of the lesion [27] and, lately, the skin type [13] have been considered predictors of the severity of the pain. However, the exact mechanisms leading to the pain remain unknown.

Electrical impedance spectroscopy can provide valuable information about biological systems, ranging from cell suspensions to total body composition measurements [5].

In this work a 4-electrode system was used for studies of skin impedance. This set-up largely eliminates the influence from electrode polarization impedance in the measured data [28]. It also ensures that the sensitivity field is mainly focused in the viable epidermis and upper dermis, and the measurements are not significantly influenced by the *stratum corneum* [29].

In order to validate our set-up, a comparison of the impedance of skin pre-treated with cream without active compound with skin pre-treated with ALA-cream was made. The impedance of skin pre-treated with cream falls slightly during measurements at low frequencies (Fig. 2). However, it does not fall during the measurements at high frequencies (Fig. 2). This is in contrast to the declines in bioimpedance observed for both frequencies in skin during ALA-PDT (Fig. 3), and indicates that these reductions in tissue impedance reflect actual changes on the cellular level, thus validating our set-up.

During the time course of the treatment, the volunteers were seated comfortably in a dark room (to avoid PpIX photobleaching) during the measurements and were asked not to move to avoid artefacts related to change in the geometry of measured volume. The fact that, just before commencing

measurement, the volunteers were seated quietly in a chair with their arm elevated on the table probably caused a decrease in the blood volume in the arm. This would lead to increased impedance, since blood is a good conductor, and may explain the observed increase in bioimpedance before the start of the light exposures (Figs. 2 and 3).

Our results show that the impedance at low frequency (10 Hz) started to fall from the onset of pain rather than from the beginning of the light exposure (Fig. 3). Knowing that the primary sites of PpIX localization in the cell are mitochondria and membranes, one can expect that within the first few seconds after commencing the light exposure, cell membranes will be compromised, which will lead to decreased bioimpedance. This observation is in agreement with histological data published by Molocovsky and Wilson [8] on liver tissue subjected to Photofrin-PDT. They have found that immediately after PDT the tissue samples contained widely spaced normal-sized cells and nuclei, some compromised cell membranes, an overabundance of vacuoles and swollen endoplasmic reticulum [8]. Photofrin has a component of similar lipophilicity as PpIX and even contains some PpIX. Another possible explanation of the observed bioimpedance decrease is the reactive oxygen-induced degranulation of mast cells and release of inflammation mediators, like histamine or prostaglandins. Histamine has been identified as a mediator involved in the immediate pain response during PDT [30].

The observed decrease in bioimpedance at the high frequency (Fig. 3) may be associated with a reallocation of the intracellular fluid volume, or with changes in the state of intracellular membranes. As mentioned above, the intracellular localization of PpIX promotes damage to membranes of the endoplasmic reticulum and the mitochondria. ALA-PDT-treated rat foot tumours (DS-sarcoma), investigated by the use of a 2-electrode bioimpedance measurements set-up in the 37 Hz to 3.7 MHz frequency range, showed that the impedance decreased immediately upon commencement of light exposure, which indicated the development of a pronounced intracellular oedema [6].

A decrease in skin impedance was found both with high- and low-frequency measurements (Fig. 3). Since the high frequency results are influenced by processes taking place in the intra- and in the extracellular space, and since the observed drop in bioimpedance was larger at low frequency, it is likely the critical changes in the initial phase of light exposure mainly take place in the extracellular space.

It is also interesting to note that there seems to be a delay, possibly related to a threshold of induced damage, before the pain was felt and the bioimpedance decrease was observed (Fig. 3). This delay may indicate that a small increase in temperature during light exposure may be needed to activate TRPM8 channels (menthol receptors) that lead to nerve responses. Another possible explanation may be that there is a certain time delay needed between H_2O_2 -induced mast cells degranulation and histamine release which leads to C-fibers activation.

The sudden plateau in the bioimpedance time series (Fig. 3) may be explained by a transition from vasoconstriction to vasodilatation, which, accompanied by platelet aggregation, takes place in tissue immediately after PDT [31].

A correlation between fluorescence intensity at the start of light exposure and time to pain was observed, in agreement with previous studies (Fig. 4) [26]. Moreover, we found a correlation between the skin bioimpedance and the time for pain to occur (Fig. 5). This correlation was significant only for high frequency measurements. Since the low frequency measurements reflect extracellular processes only, whereas the high frequency measurements reflect both intracellular and extracellular properties, our results suggest that the pain inducing changes occur mostly (but not only) in the extracellular space.

Bioimpedance spectroscopy proved to be a very accurate method for establishing a time line of crucial changes in the skin, reflecting occurrence of pain. Despite the fact that there is a well-known interpersonal variation in response to PDT as well as the heterogeneity of the measured tissue, the bioimpedance succeeded to show consistent data for all volunteers.

In conclusion, the pain-inducing changes in the healthy human skin, arising during light exposure in topical photodynamic treatment, may originate mainly (but not only) in the extracellular compartment of the tissue and perhaps require a time-delay, possibly a threshold, for occurrence.

In the future, measurements of skin impedance during cutaneous PDT on skin cancer patients would be beneficial for comparison with already obtained data from healthy human skin (like those presented in the present study). It would also be interesting to compare bioimpedance data for pain-inducing and non pain-inducing conditions, i.e. where the PpIX fluorescence level is similar, but light fluences differ. It would be interesting to see the amount of photo-bleached PpIX fluorescence correlating with pain intensity, which may also correspond to PDT effect. Furthermore, it would be interesting to attempt to correlate pain intensity through bioimpedance spectroscopy and predict clinical PDT outcome in skin.

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