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## Modifications of protoporphyrin IX fluorescence during ALA-based photodynamic therapy of endometriosis

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### Abstract

Modifications of the porphyrin fluorescence during photodynamic treatment of ovarian cells, endometrial adenocarcinoma cells and rat uterus after administration of the porphyrin precursor  $\delta$ -aminolevulinic acid and exposure with 630 nm laser radiation have been studied. The intracellular and intratissue fluorescence was excited with 407 nm radiation of a krypton ion laser and detected by spectral microscopic imaging using a modified microscope equipped with a slow-scan cooled camera and a liquid crystal filter. In particular, singlet oxygen induced effects due to photobleaching as well as formation of chlorin-type fluorescent photoproducts were detected at around 500, 630 and 670 nm.

The photodynamically induced fluorescence modifications were found to vary between cell types. The major fluorescence peak of PP IX was photobleached 63% by a phototoxic fluence of about 20–40 J/cm<sup>2</sup> in cells and within rat endometrium. Higher fluences were required to bleach the fluorescence at around 670 nm due to the formation of fluorescent photoproducts. No significant photoinduced modifications of autofluorescence occurred around 500 nm.

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**Keywords:** 5-Aminolevulinic acid hydrochloride; Endometriosis; Fluorescence; Photobleaching; Porphyrin; Photoproduct; Photodynamic therapy

### Introduction

Photodynamic therapy (PDT) is a treatment modality where a light-activated photosensitizer interacts with

molecular oxygen via type I or type II photooxidation processes which results in the production of toxic reactive oxygen species (ROS) such as oxygen radicals and singlet oxygen [1]. Major applications of PDT are the treatment of cancer, of a variety of skin diseases and of age-related macula degeneration as well as the inactivation of pathogenic bacteria [2,3].

Typically, exogenous photosensitizers such as Verteporfin (Visudyne<sup>®</sup>, Novartis, Basel, Switzerland) or the porphyrin mixture Photofrin<sup>®</sup> (QLT Inc., Vancouver,

*Abbreviations:* ALA,  $\delta$ -aminolevulinic acid; ATCC, American Type Culture Collection; CHO, Chinese hamster ovary cells; FBS, fetal bovine serum; HEC-1-A, human endometrial adenocarcinoma cells; PDT, photodynamic therapy; PP IX, protoporphyrin IX

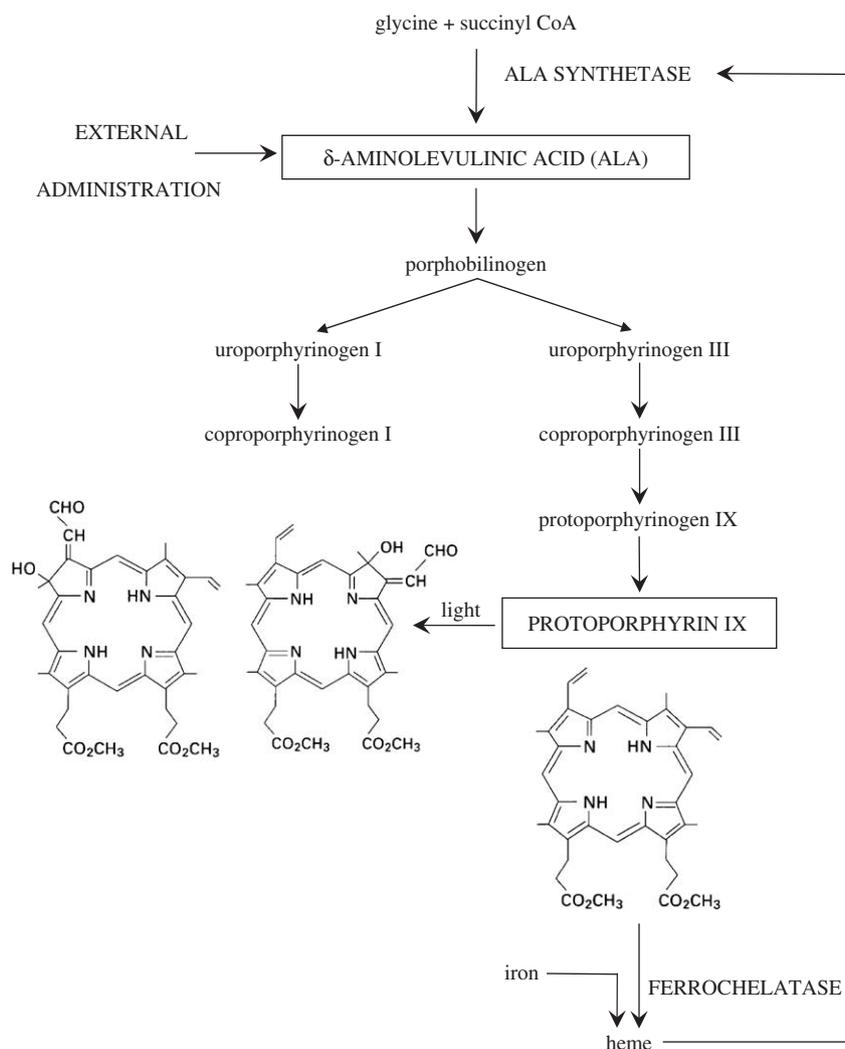
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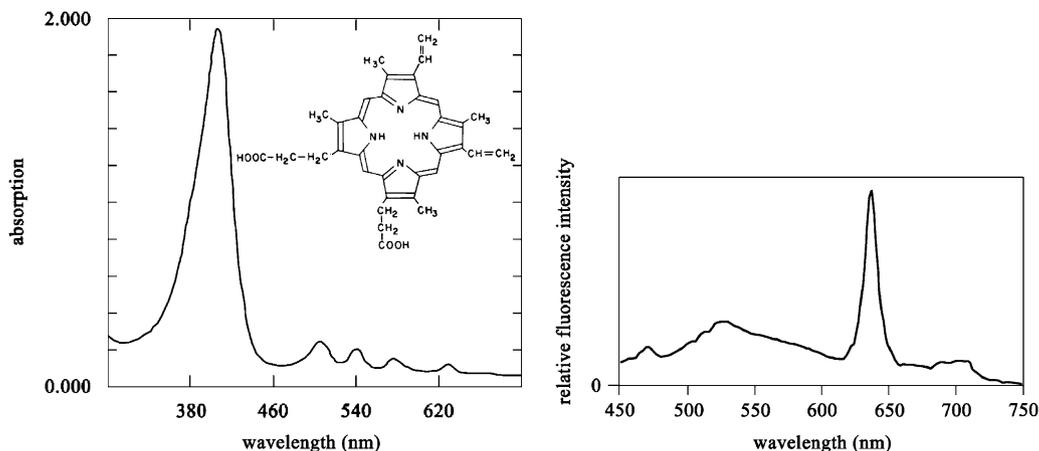
Canada) are applied. However, also endogenous photosensitizers such as protoporphyrin IX (PP IX) as the last intermediate in the biosynthetic pathway of heme and other tetrapyrrols can be used. In mammals, heme is synthesized in all nucleated cells. Typically, the concentration of intracellular PP IX is rather low (e.g. in blood about  $1 \mu\text{M}$ ). Only certain pathogenic bacteria [4] and certain cells in photosensitive patients with e.g. erythropoietic porphyria, a genetic disorder due to a deficiency of ferrochelatase (FECH) activity, possess high intracellular PP IX concentrations of  $10 \mu\text{M}$  and more. The mitochondrial enzyme FECH catalyzes the insertion of ferrous iron into PP IX to form heme.

In order to enhance the intracellular PP IX concentration in cells of interest for photodynamic treatment significantly, the porphyrin precursor  $\delta$ -aminolevulinic acid (ALA) can be applied, for example, as topically administered ointment in the case of skin cancer, as part of an inhalation procedure in the case of lung cancer and by instillation in the case of bladder tumor.

ALA is the first intermediate in the heme biosynthesis (Fig. 1). The exogenous ALA bypasses normal heme regulation and leads to enhanced PP IX concentration in mitochondria [5,6] which is used as photosensitizer in ALA-PDT. Due to the required high light penetration depth in photodynamic treatment, ALA-PDT is



**Fig. 1.** Schematic pathway of stimulated biosynthesis of PP IX and its photodegradation.  $\delta$ -aminolevulinic acid synthase (ALAS) catalyzes within the mitochondria the condensation reaction between glycine and succinyl CoA and forms  $\delta$ -aminolevulinic acid (ALA). After synthesis of porphobilinogen, uro- and coproporphyrinogen, the photosensitive and fluorescent porphyrin PP IX is formed. PP IX is converted into the photostable non-fluorescent heme when iron as ferrous ion is inserted into the protoporphyrin ring by FECH activity. The concentration of heme regulates the activity of ALAS. The external application of ALA bypasses this regulation and results in an increased concentration of intracellular PP IX. Light activation of PP IX leads to desired photodynamic reactions as well as in its photodestruction by photobleaching and the formation of chlorin-type porphyrin isomers (photoprotoporphyrin) by single oxygen involved vinyl group oxidation and hydrogen rearrangement.



**Fig. 2.** Absorption spectrum of PP IX (left) and fluorescence spectrum of CHO cells 4 hours after incubation with ALA (right).

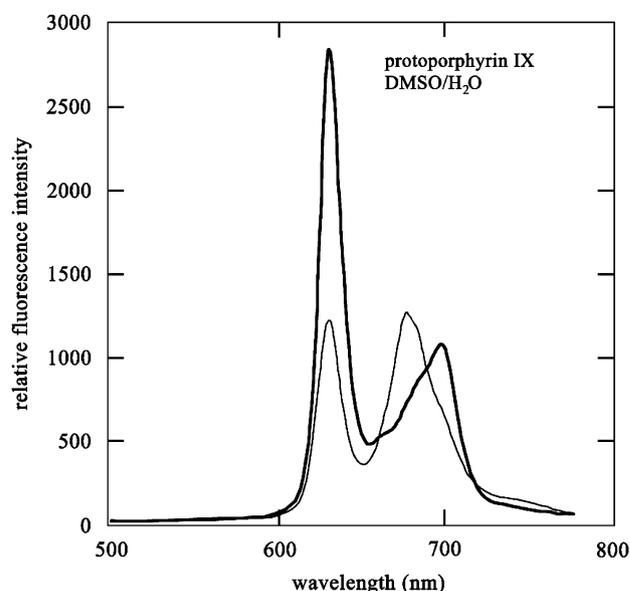
performed with red light at around 630 nm with respect to the long-wavelength absorption band (Fig. 2).

PP IX is a fluorescent photosensitizer with major emission bands at 635 and 710 nm and an optimum fluorescence excitation wavelength around 405 nm. The detection of the fluorescence can be used to study the biosynthesis of ALA into PP IX and to monitor the intracellular and intratissue accumulation of the fluorescent photosensitizing porphyrin. Interestingly, the occurrence of fluorescent photoproducts (photoporphyrin, Fig. 1) [7] as well as fading effects [8,9] during light exposure of PP IX have been reported. The photoconversion rate of PPIX in its photoproducts (Fig. 3) can be increased if H<sub>2</sub>O is exchanged with D<sub>2</sub>O where the lifetime of photoinduced singlet oxygen is enhanced. The formation of photoproducts can be avoided if the singlet oxygen quencher NaN<sub>3</sub> is added to the solvent.

Finley et al. [10] observed a fluorescence emission peak at 620 nm due to uroporphyrin/coproporphyrin response to ALA-PDT-induced mitochondrial damage.

Moan et al. [11] reported that 70–95% of PP IX was degraded in a particular cancer cell line after 40–200 J/cm<sup>2</sup> exposure at 630 nm. Van der Veen et al. [12] used green light to expose mammary tumors in rats and concluded in this paper that there is no correlation between fluorescence intensity and the PDT-induced vascular damage. In contrast, Orenstein et al. reported in 1997 that fluorescence monitoring is very appropriate for the definition of an optimal ALA-PDT clinical protocol. They studied PP IX fluorescence after topical ALA administration of basal and squamous cell carcinoma in 60 patients. They observed a re-appearance of fluorescence after PDT. Such phenomenon was also described by the Dutch group of Star in the same year [13].

Using fluorescence analysis, Roy et al. [14] investigated the concentration of PP IX in the rat uterus after different ways of ALA incubation and concluded that



**Fig. 3.** The light exposure of a PP IX solution (solvent: DMSO and water) results in significant changes of the fluorescence spectrum due to the formation of fluorescent photoproducts (photoporphyrin) with an emission maximum at around 670 nm.

the highest PP IX level can be achieved by administration of 25 mg ALA as bolus injection. Fluorescence spectroscopy with the aim to diagnose cervical intraepithelial neoplasia was performed on 68 women 60–90 min after topical application of 1% ALA [15].

A theoretical approach to describe photobleaching of ALA-induced PPIX was undertaken by Jongen and Sterenberg [16]. They calculated a photobleaching dose constant of 33 J/cm<sup>2</sup> and concluded that monitoring of photobleaching during PDT is valuable for dosimetry of PDT.

Goal of this study was the investigation of in vivo fluorescence modifications, in particular photobleaching, during PDT of ALA-sensitized endometrial tissue

and their comparison with effects on a single cell level. The ALA-PDT and ALA-fluorescence diagnostics of the human endometrium may become a promising novel photomedical procedure in gynecology [17–23].

## Materials and methods

**Animals:** Six female Sprague–Dawley rats at a weight of around 300 g were treated with 58 mg/kg ALA which was kindly provided by Deparnayl USA Inc., Parsippany, NJ. ALA was topically administered into each uterine horn (0.15 ml) through a midline incision. The animals were anesthetized with ketamine/xylazine (2:1) 0.75 ml/kg intramuscular. PDT was performed 3 h after ALA administration.

**Cell lines:** Chinese hamster ovary (CHO) cells (*Cricetulus griseus*; ATCC CCL 61) were routinely cultured in Minimum Essential Medium with Earle's salts and non-essential amino acid (Gibco 410-1500EL) supplemented with 10% fetal bovine serum (FBS). Well-differentiated human endometrial adenocarcinoma cells (HEC-1-A, ATCC HTB 112) were bought from American Type Culture Collection (ATCC). The cells were grown in complete culture medium 199 with Earles salts (Irvine Scientific, Irvine, CA) supplemented with 10 mM L-glutamine (Gibco), 100 µg/ml streptomycin, 50 µg/ml gentamycin (Gibco) and 10% FBS. Cells were incubated with 5-aminolevulinic acid hydrochloride (Sigma chemical Co., St. Louis, MO, USA) in the exponential growth stage at a concentration of 0.025 mg/ml (CHO) and 0.5 mg/ml (HEC-1A) for 6 h. The higher concentration in the case of HEC-1A cells was required in order to obtain similar fluorescence levels. At the end of the incubation time, the medium was removed, the cells were washed with PBS twice, detached from the culture dishes and treated with laser light. Then fluorescence intensities of the detached cells were measured and the bleaching fluence was determined.

**Bleaching parameter:** We defined the value where the fluorescence intensity dropped down to  $1/e = 0.37$  of the initial value as bleaching fluence.

**Equipment:** Large-scale cell fluorescence measurements were performed on the Epics V flow cytometer from Coulter Electronics Inc., equipped with a Coherent Innova 90 argon ion laser emitting at 488 nm. Fluorescence was detected in the red spectral range (LP 610). Fluorescence intensities were measured on the log integrated red fluorescence (LIRF) channel for 10,000 cells/sample and converted to a linear scale according to the 3.00 decade channel conversion table provided by Coulter Electronics. The “background” natural autofluorescence obtained from cells without ALA incubation was subtracted from the values of ALA-treated samples. In order to obtain a fluorescence spectrum of

ALA-incubated cells, the 407 nm excited fluorescence of a cell suspension was recorded by means of an optical multichannel analyzer equipped with a polychromator.

Single cell studies and animal studies have been performed by fluorescence microscopy at different spectral ranges. In particular, fluorescence images were taken with a slow-scan cooled CCD camera (model ST-180, Princeton Instr., Trenton, NJ) equipped with a birefringent tuneable liquid crystal filter (VariSec, Cambridge Research and Instr.) to acquire spectrally resolved fluorescence microscopy images. A Zeiss Axiovert 10 microscope was used. Liquid filter properties include an out-of-band-transmission below  $10^{-4}$ , a typical bandwidth of 20 nm, a transmission of 20% and a minimum tuning speed of 50 ms.

The 407 nm radiation (25 mW) of a krypton ion laser as fluorescence excitation light was transmitted via multimode fiber to the target on the microscope stage. The light intensity was  $5 \text{ mW/cm}^2$  and the exposure time for fluorescence excitation 1 s.

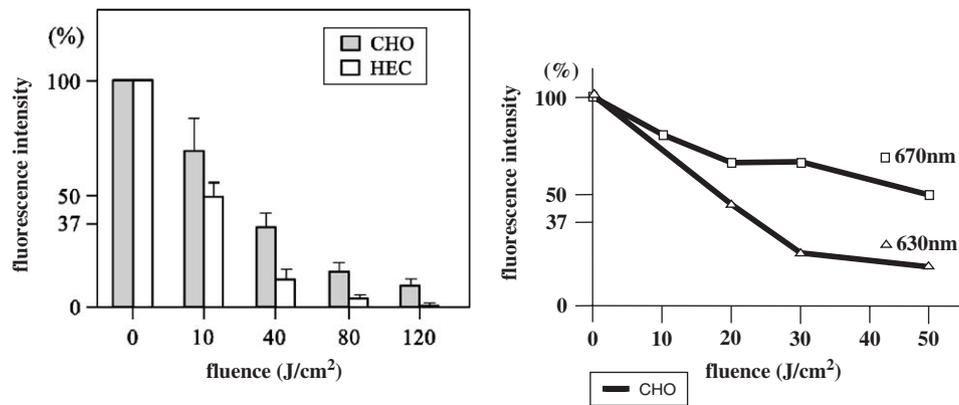
Photodynamic treatment was conducted with fiber-transmitted laser radiation of a Coherent Dye Laser 599 at 630 nm which corresponds to the long-wavelength absorption maximum of PP IX. In cell studies, photodynamic treatment was performed with light doses between 10 and  $120 \text{ J/cm}^2$  at an intensity of  $100 \text{ mW/cm}^2$  at different durations. In animal studies, PDT was performed with a light intensity of  $60 \text{ mW/cm}^2$  and an exposure area of  $1.8 \text{ cm}^2$ . The light fibre was positioned to the microscope stage. In the case of in vivo animal studies, the fibre was introduced into the uterine cavity.

## Results

### Photobleaching during photodynamic treatment of cells

The biosynthesis of PP IX could be clearly detected in ALA-incubated cells by fluorescence spectroscopy (Fig. 2, right). The red fluorescence intensity reached a maximum at ALA concentrations between 0.01 and 0.1 mg/ml medium for CHO and between 0.1 and 1 mg/ml medium for HEC-1-A. CHO exhibited twofold higher fluorescence compared to HEC-1-A.

Flow cell cytometry based on the 488 nm excited PP IX fluorescence was performed on 630 nm laser exposed cells ( $n > 10,000$ ). The laser-induced modifications of ALA-induced intracellular PP IX are demonstrated in Fig. 4 left. Interestingly, significant differences in the bleaching behavior occurred. In both cases, the fluorescence decreased nearly monoexponentially with increasing fluence. However, the bleaching fluence of about  $40 \text{ J/cm}^2$  in CHO was found as double as high as in the case of HEC-1-A (around  $20 \text{ J/cm}^2$ ).



**Fig. 4.** Fluorescence modifications of ALA-incubated CHO and HEC cells due to 630 nm laser exposure. Left: results of flow cell cytometry showing the decay of the total fluorescence intensity in the red spectral range. Right: Typical spectrally resolved fluorescence modification in a single CHO cell demonstrating a different bleaching behavior at 630 nm (protoporphyrin) and 670 nm (protoporphyrin and photoporphyrin). In order to obtain corrected fluorescence values, the mean value of the fluorescence signal of control cells (no ALA) was subtracted from fluorescence values of ALA-sensitized cells.

In order to study the variations of fluorescence modifications on a single cell level and in dependence on the emission wavelength, spectral microscopic imaging was performed. As revealed by the liquid crystal filter technique, red fluorescence arose from the cytoplasm, in particular from regions of high concentration of mitochondria.

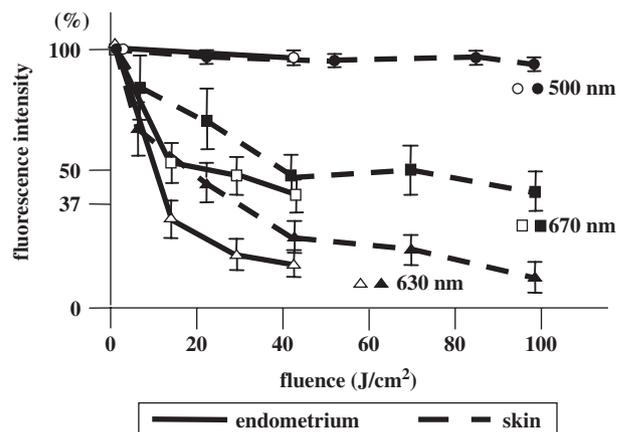
Typically, fluorescence was recorded in the range of 620–640, 660–680 and 490–510 nm. In order to obtain fluorescence images, the exposure with red laser light was interrupted for 1–2 s. As seen from Fig. 4 (right), the fluorescence around 630 nm and around 670 nm decreased differently. The bleaching fluence at around 630 nm of about 20 J/cm<sup>2</sup> was found to be lower than at 670 nm with values above 50 J/cm<sup>2</sup>. This indicates the existence of a multicomponent system.

### In vivo animal studies

Before photodynamic treatment, a first fluorescence image at 630 nm was taken. The PP IX fluorescence was found to be more intense in glands than in stroma and myometrium (data not shown).

Rats were exposed to 630 nm laser light at an intensity of 60 mW/cm<sup>2</sup>. In time intervals of 4.1 min (endometrium) and 5.5 min (skin), which corresponds to 15 and 20 J/cm<sup>2</sup> fluence, respectively, the PDT treatment was stopped for 1 s by blocking the laser beam in order to perform fluorescence imaging.

As seen from Fig. 5, the mean fluorescence intensity at 630 nm of PDT-treated endometrium, which was calculated from the fluorescence images, dropped significantly within the first interval of 15 J/cm<sup>2</sup> to a value of less than 1/e of the initial value (bleaching fluence). Also the fluorescence at 670 nm dropped, however, the



**Fig. 5.** Modification of fluorescence in endometrium and skin of PDT-treated living rats ( $n = 6$ ). No significant photoinduced changes of the natural occurring autofluorescence at around 500 nm occurred.

bleaching fluence was not achieved at an energy density as high as 40 J/cm<sup>2</sup>.

Studies on skin revealed a slower fluorescence decay. The naturally occurring autofluorescence at around 500 nm remained nearly constant during 630 nm laser exposure.

### Studies on tissue cryosections

Interestingly, measurements on 630 nm exposed cryosections of ALA-sensitized endometrium and skin biopsies under the same conditions revealed an increase of fluorescence intensity at 670 nm with a maximum at 40 J/cm<sup>2</sup> red light exposure. At 630 nm, the fluorescence decreased nearly mono-exponentially with a bleaching fluence of about 40 J/cm<sup>2</sup> (Fig. 6).

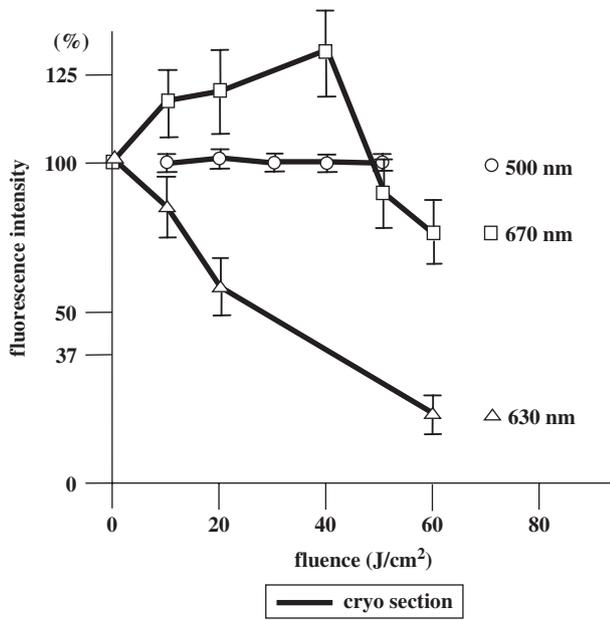


Fig. 6. Spectrally resolved fluorescence kinetics of 630 nm exposed cryosections ( $n = 6$ ).

## Conclusion

Photosensitizers do not remain unaffected by the formation of cytotoxic species which they induce after their light excitation and interaction with molecular oxygen. Exposure of ALA-incubated cells and tissues with laser light at around 630 nm results in photodegradation of the ALA-induced photosensitizer PP IX. This can be detected by fluorescence. Interestingly, the red laser-induced modification of fluorescence varies with cell type and between in vivo and ex vivo conditions. However, photobleaching of the 635 nm fluorescence band is the dominant process as shown by microscopic spectral imaging. Around 670 nm, the bleaching process is less pronounced likely due to the formation of fluorescent photoproducts. As shown in former studies, the photoproduct is less photosensitive than PP IX [3,4].

In particular, the fluorescence behavior of ALA-incubated endometrium was studied with respect to a novel potential treatment modality. As documented in cell and animal studies, most (63%) of the photosensitizer PP IX is already bleached within 40 J/cm<sup>2</sup>. Typically, PDT is performed with a fluence of 100 J/cm<sup>2</sup> or more. At that fluence, the photosensitizer molecules should be nearly completely degraded. The ongoing biosynthesis of PP IX including the phenomenon of re-appearance and recovery of PP fluorescence [24], different oxygen levels as well as diffusion mechanisms within living 630 nm exposed rats are likely the reason for the differences to fluorescence modifications during studies on cryosections.

## Zusammenfassung

### Modifikationen der Protoporphyrin-IX-Fluoreszenz bei 5 – ALA induzierter Photodynamischer Therapie von Endometriose

Es wurden Modifikationen der Porphyrin-Fluoreszenz während der photodynamischen Behandlung von Ovarzellen, endometrischen Adenokarzinom-Zellen und des Rattenuterus nach Applikation des Porphyrin-Precursors  $\delta$ -Aminolävulinsäure und 630-nm-Laserbestrahlung untersucht. Die intrazelluläre und die Intragewebs-Fluoreszenz wurde mit der 407 nm-Strahlung eines Krypton-Ionenlasers angeregt und mittels spektraler mikroskopischer Bildgebung auf der Basis eines modifizierten Mikroskops mit gekühlter slow-scan Kamera und einem Flüssigkeitskristallfilter nachgewiesen. Insbesondere wurden Singlettsauerstoff induzierte Effekte wie Photobleaching und die Bildung fluoreszierender Photoprodukte vom Chlorintyp bei 500 nm, 630 und 670 nm detektiert.

Die photodynamisch induzierten Fluoreszenzänderungen hingen vom Zelltyp ab. Der Hauptfluoreszenzpeak unterlag einem Photobleaching um 63% in Zellen und innerhalb des Rattenendometriums, wenn Strahlungsdosen von 20–40 J/cm<sup>2</sup> appliziert wurden. Höhere Dosen wurden aufgrund der Bildung von fluoreszierenden Photoprodukten erforderlich, um die 670-nm-Fluoreszenz zu bleichen. Es wurden keine signifikanten photoinduzierten Änderungen der Autofluoreszenz um 550 nm detektiert.

**Schlüsselwörter:** 5-Aminolävulinsäurehydrochlorid; Endometriose; Fluoreszenz; Photobleaching; Porphyrine; Photoprodukte; Photodynamische Therapie

## Resúmen

### Modificación en la fluorescencia de la protoporfirina IX durante el tratamiento de la endometriosis mediante Terapia Fotodinámica con ALA

Se han estudiado las modificaciones en la fluorescencia de la porfirina durante el tratamiento fotodinámico de células de ovario, células de adenocarcinoma endometrial y útero de rata, luego de la administración de su precursor (ácido  $\delta$ -aminolevulínico) y la exposición a una radiación láser de 630 nm. La fluorescencia intracelular e intratejido fue excitada a 407 nm con un láser de cripton y detectado por microscopia espectral utilizando un microscopio modificado equipado con una cámara "slow-scan" refrigerada y un filtro de cristal líquido. En particular se detectaron los efectos inducidos por singletes de oxígeno como la fotodegradación, así como a la formación de fotoproducidos tipo clorina a una longitud de onda de alrededor de 500 nm, 630 nm y 670 nm.

Se determinó que las modificaciones inducidas foto-dinámicamente en la fluorescencia varían con el tipo celular. El pico máximo de fluorescencia de PP IX fue fotodegradado en un 63% por una fluencia fototóxica de aproximadamente 20–40 J/cm<sup>2</sup> en las células y en el endometrio de rata. Para degradar la fluorescencia de alrededor de 670 nm, fueron necesarias fluencias mayores debido a la formación de productos fotofluorescentes. No hubo modificaciones significativas de la autofluorescencia alrededor de 500 nm.

**Palabras clave:** Ácido 5-aminolevulinico hidroclicorídico; Endometriosis; Fotodegradación; Fluorescencia; Porfirina; Fotoproductos; Terapia fotodinámica

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