Differential cell photosensitivity in photodynamic therapy of the rat endometrium

Mathias K. Fehr,[1,2] Lars O. Svaasand,[1,3] Bruce J. Tromberg,[1] Phat Ngo,[1] Michael W. Berns,[1] Yona Tadir,[1,4]

1 Beckman Laser Institute and Medical Clinic, University of California, Irvine, CA 92 715
2 Department of Obstetrics and Gynecology, University Hospital, 8091 Zurich, Switzerland
3 Department of Physics, University of Trondheim, Norway
4 Department of Obstetrics and Gynecology, University of California, Irvine, CA 92 715

ABSTRACT

The purpose of this study was to determine the optical dose needed for both lasting endometrial destruction and prevention of implantation by photodynamic therapy (PDT) using 5-aminolevulinic acid (ALA) as photosensitizer.

3 hours after topical drug administration 74 female Sprague-Dawley received varying optical doses of 630 nm light delivered by an intrauterine cylindrical light diffusing fiber. Histologic evaluation of the endometrium 1 and 21 days after PDT as well as the number of implantation sacs after mating were assessed.

Irreversible endometrial destruction was determined measuring the thickness of the endometrial layer 3 weeks after treatment. An in situ dose of 64 J/cm^2 was required to eradicate endometrial glands and prevent regeneration. In contrast, a 43 J/cm^2 in situ dose visibly damaged the endometrial stroma and myometrium but the endometrial glands survived and the endometrium regenerated to its full thickness within 21 days. However, implantation potential was significantly reduced at these low light levels.

Due to differential cell photosensitivity, the optical threshold for lasting endometrial destruction is higher than for functional damage. For lasting endometrial destruction the endometrial glands must be destroyed, whereas for reproductive impairment, damage to the endometrial stroma seems to be sufficient.

Key Words: Photodynamic therapy, 5-aminolevulinic acid, endometrium, rat model, differential cell photosensitivity

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1. INTRODUCTION

Photodynamic endometrial ablation is currently being evaluated as a minimally-invasive procedure for treatment of dysfunctional uterine bleeding[1]. Photodynamic therapy (PDT) is based on light-induced oxidation reactions which lead to tissue necrosis[2]. Several animal studies[3-10] have shown that selective endometrial destruction using PDT is feasible and may provide a simple, cost effective and safe alternative to routinely performed surgical treatment modalities requiring anesthesia.

The photodynamic threshold for irreversible destruction of a given tissue is a complex function of the optical dose, intrinsic sensitizer characteristics, drug location and concentration, and tissue type[1]. For endometrial ablation, both systemic and topical application of various photosensitizers have been studied[3,5,7,8]. Intrauterine administration concentrates photosensitizer in the endometrium and minimizes systemic risks such as skin photosensitivity. Although the feasibility of endometrial PDT using topical (intrauterine) 5-aminolevulinic acid (ALA) has been described, many factors relevant to the photodynamic threshold have not been thoroughly examined.

ALA is endogenously converted to the fluorescent photosensitizer Protoporphyrin IX (PpIX)[6,7,8,11]. Pharmacokinetic studies in rats and rabbits showed peak endometrial fluorescence three hours after topical ALA administration[8,12]. Fluorescence of the endometrial glands significantly exceeded the fluorescence of the stroma or myometrium regardless of drug concentration. With 200 mg/ml topical ALA, saturation of endogenous Pp IX fluorescence was reached, suggesting that higher drug concentrations would not lead to additional tissue destruction[8]. However, following PDT, uncontrolled regional variations in endometrial regeneration indicated that maintaining the optical dose above the photodynamic threshold was critical to irreversible destruction[8].

In this study we examine the impact of light dose on endometrial PDT. Structural or functional damage was evaluated in a rat model by studying endometrial ablation and prevention of pregnancy, respectively. Results show that the photodynamic thresholds for structural and functional damage are not the same. Consequently, we suggest that the sensitivity of different uterine tissue types to photodynamic therapy can vary substantially.

2. MATERIAL AND METHODS

Seventy-four mature female Sprague-Dawley rats weighing 261-325 gm were placed in a controlled environment with free access to food and water and a 12-hour light /12-hour dark cycle. Guidelines for the care and use of animals approved by the Animal Review Committee of the University of California, Irvine were followed.

Forty-four animals were used for histologic evaluation of the endometrium after PDT and 30 rats for assessment of reproductive performance. The estrus cycle was monitored by obtaining frequent vaginal smears in order to synchronize the treatment to the day of diestrus[13]. Glandular proliferation occurs only during diestrus, stromal proliferation during diestrus and proestrus, and myometrial proliferation during proestrus[14]. All rats were anesthetized with 0.75 mL/kg i.m. ketamine/xylazine (2:1) and isoflurane/oxygen was provided during the surgical intervention. A lower abdominal midline incision was made and both uterine horns of the didelphic uterus were identified.

Crystallized 5-aminolevulinic acid hydrochloride (DUSA Pharmaceuticals, Inc., Denville, NJ) was diluted to 200 mg/mL in Hyskon^R (Pharmacia Inc., Piscataway, N.J.) prior to administration. The solution was titrated to pH 6 with 10 N sodium hydroxide. Hyskon^R is a viscous, hydrophilic, branched polysaccharide used to minimize spillage of photosensitizer through the cervix. 0.15 mL of the ALA solution was injected with a 25-gauge needle into the left uterine horn at the bifurcation of the didelphic uterus. Abdominal walls were closed in two layers with 4-0 absorbable suture.

Three hours after drug injection, PDT was performed by intrauterine illumination of the left uterine horn. Light from an argon-pumped dye laser (Spectra Physics, Mountain View, CA) with DCM dye (Exciton, Inc., Dayton, Ohio) operating at 630 nm was delivered to the uterine cavity via a quartz optical fiber terminated with a 1.2 mm-diameter, 3 cm-long cylindrical diffusing tip (model 4420-A02, PDT Systems, Inc., Santa Barbara, CA). The fiber was placed into the uterine horn through the same perforation site used for drug injection. Intrauterine fluid was evacuated by gentle pressure to make sure that the endometrium was in contact with the fiber surface. Under these conditions, the endometrial surface has no folds and is slightly distended. Hence, the entire endometrial surface is in contact with the surface of the light diffuser allowing prediction of the incident optical dose. The source power was adjusted to 100 mW/cm cylindrical diffusing fiber (300 mW total power coupled into the fiber). Exposure times were 30 s, 100 s, 150 s, 200 s and 600 s for equal groups of animals, resulting in an incident optical dose at the surface of the light applicator of 8, 27, 40, 53 and 160 J/cm^2, respectively. During PDT, the abdominal incision was reapproximated in order to cover the uterine horns with tissue and allow backscattering of light. Intrauterine temperature was monitored continuously during PDT in 4 animals using a 0.13 mm diameter copper/constantan thermocouple (Omega Engineering, Inc., Stamford, CT) placed along the light diffusing fiber inside the uterine cavity.

Each animal served as its own internal control by injecting the same amount of ALA solution into the right uterine horn immediately after light administration to the left horn. Additional control groups were established by instilling pure Hyskon^R into the left uterine horn before irradiation for 600 s. Temperature, pulse and respiration were monitored until the animals were ambulatory.

In order to evaluate the acute morphologic effect of PDT, four rats per exposure time (30, 100, 200, and 600 s) were sacrificed 24 hours after treatment. In order to assess endometrial regeneration, four rats per exposure time (30, 100, 150, 200, and 600 s) were sacrificed 21-24 days after PDT on the day of estrous. Endometrial cells are most differentiated on the day of estrus, thus morphologic differences between treated and untreated horns should be most prominent at this time. Uterine specimens were retrieved via laparotomy and placed in buffered 10% formalin immediately following euthanasia by asphyxiation with CO_2 gas .

Two samples of the middle portion of the uterine horns were paraffin embedded and sectioned transversely. After hematoxylin eosin staining, the thickness of the entire uterine wall as well as the distances from endometrial surface to myometrial-endometrial junction and myometrial-endometrial junction to serosal surface were measured using light a microscope with a calibrated ocular scale. Distances were measured along the largest uterine cross-sectional diameter (excluding the lumen), perpendicular to the mesometrium. The median of four measurements obtained from the four most ideal cross-sections of the left uterine horn was divided by the median obtained in the same manner from the right horn of the same animal.

Three weeks after PDT, 6 animals per exposure time (30 s, 100 s, 200 s, 600 s) and a control group of 6 animals were bred for 4 days with mature male Sprague-Dawley rats in order to assess reproductive performance. Female rats were sacrificed in the second trimester of pregnancy (14-18 days after mating) as confirmed by palpation. The location and number of implantation sacs in the treated uterine horn and the control side were noted.

The in situ optical dose at a depth of 1 mm in the tissue for 630 nm light was calculated using a mathematical model describing the decay in fluence rate with increasing source-detector separation using cylindrical light diffusers[15]. The effective scattering coefficient used in our calculations was 0.73 mm^{-1}, a value determined from measurements of premenopausal human uterine tissue[16]. The penetration depth, d, for 630 nm light was determined for rat uteri using the following method: Four fresh uterine horns were opened longitudinally at the mesometrial insertion and sectioned to form squares of 2x2 cm. To facilitate backscattering, 4 sheets of uterine wall were laid on top of each other, perforated in the center of the square with a needle, and a spherical-tip detector fiber was pulled through the perforation site until the tip was flush with the surface of the tissue. The 200 mm core-diameter detector fiber (0.8 mm-diameter isotropic collection tip, PDT Systems, Inc., Santa Barbara, CA) was connected to a photomultiplier tube (Hamamatsu R 928, Bridgewater, NJ) to measure steady-state light intensity (fluence rate). 630 nm light was launched onto the center of the tissue (1 cm-diameter spot) and the fluence rate was recorded as a function of tissue thickness by stacking uterine layers on top of each other. The calculation of the penetration depth, d, from measured fluence

rates has been described elsewhere[15].

For statistical analysis the number of gestational sacs in the treated and untreated uterine horns were compared for each optical dose with the paired t test. Differences in sac numbers at different exposures times were examined with analysis of variance. If a significant overall difference was present, multiple comparisons were performed with Tukey's studentized range test[17]. The same methods were used to test for differences in the ranks of the ratios of the tissue thickness (whole uterine wall, endometrium, myometrium). The ranks of the ratios were used because the variability of the data differed by exposure time. Statistical significance was taken as $p < 0.05$. Data are presented as mean $\pm$ SE.

3.RESULTS

Table I shows the temperature rise, the incident optical dose and the calculated in situ optical dose at a depth of 1 mm from the endometrial surface for all light exposure times (mean endometrial thickness = 0.87 $\pm$ 0.2 mm, n = 74).

exposure time (s)	incident optical dose (J/cm^2)	in situ optical dose (J/cm^2)
30	8	13
100	27	43
150	40	64
200	53	85
600	160	260

The incident (irradiant) optical dose describes the source energy density while the in situ (tissue) optical dose characterizes the energy density deposited at a defined depth inside the tissue. These values differ due to the contribution of backscattered light which enhances the fluence rate in situ. The in situ optical dose was calculated from our rat uterus penetration depth measurements, d = 4.31 mm, and diffusion theory[15].

Baseline temperatures (31-33 °C) increased rapidly with irradiation, leveled off within 280s, and remained constant at about 6 °C above baseline for 600s. Temperatures during PDT never exceeded 40°C, a level that is insufficient for heat induced necrosis or apoptosis[24].

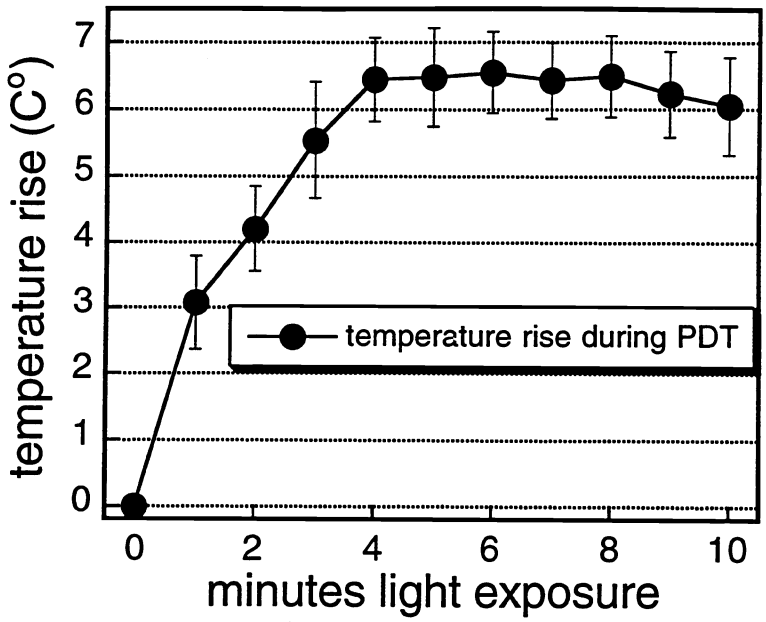


Table II summarizes morphological changes observed in treated uterine horns compared to untreated control horns 24 hours after PDT (numbers represent number of animals).

Exposure time	endometrial glands		edema of	edema/necrosis of	myometrium
	absent	reduced >50%	endometrial stroma	circular myometrium	focally absent
30 s (n=4)	0	0	0	0	0
100 s (n=4)	0	0	4	2	1
200 s (n=4)	2	4	4	4	3
600 s (n=4)	2	4	4	4	4

Endometrial edema was observed in all animals receiving a 100s light exposure. Although the size and number of stromal cells seemed diminished, most endometrial glands appeared intact. The luminal wall lining was devoid of surface epithelium and showed an inflammatory infiltrate. The lumen contained debris and white blood cells. Two of four animals showed myometrial damage and, in one animal, necrosis was observed focally throughout the myometrium. When light exposures equaled or exceeded 200s, all endometrial glands were grossly swollen or absent leaving debris in round stromal spaces. The cytoplasm of the inner muscular layer was consistently grossly swollen, though the overall cell configuration was preserved. Necrosis throughout the entire myometrium was

observed focally in most animals. Statistical analysis of uterine layer thickness measurements 24 hours post-PDT did not reveal significant differences with increasing exposure times (data not shown).

Table III summarizes histological changes observed in treated uterine horns compared to untreated control horns 3 weeks after PDT (numbers represent number of animals).

Exposure time	lumen obliterated	endometrial glands absent	endometrial glands reduced >50%	reduction in endometrial stroma cells / fibrosis	circular myometrium split by fibrotic tissue	myometrium focally absent
30 s (n=4)	0	0	0	0	0	0
100 s (n=4)	0	0	0	3	0	0
150 s (n=4)	1	0	4	4	1	0
200 s (n=4)	1	2	4	4	4	3
600 s (n=4)	3	3	4	4	4	4

The first significant morphological changes were endometrial fibrosis and a reduced number of stromal cells at 100s. By 150s, all animals showed a markedly reduced number of endometrial glands in addition to endometrial fibrosis and loss of cellularity. The inner myometrial layer was split by fibrous tissue or focally totally absent. In some cases the uterine lumen was replaced by scar tissue, although at 150s, this scar tissue embedded surviving or regenerated endometrial glands.

Figure 2 summarizes results of endometrial, myometrial, and uterine wall thickness measurements one day (left) and three weeks (right) after PDT. Analysis of variance showed no significant changes for all three variables 24 hours after PDT (left graph). 3 weeks after PDT a significant decrease for all 3 variables was noted with increasing exposure time. When exposure times increased from 100 to 150s, the decrease in wall thickness was attributed primarily to endometrial destruction since the myometrial thickness did not significantly change (Tukey's paired comparison procedure). No further differences in endometrial, myometrial, or wall thickness were seen between the 150, 200 and 600s groups (Tukey's paired comparison procedure). Endometrial thickness was significantly less than controls at 150, 200 and 600s light exposure times.

Similarly, increasing exposure times from 30 or 100 s to 200 or 600s produced significant reductions in endometrial thickness. In contrast, myometrial thickness was significantly less than controls at 600s light exposure times. Increasing exposure times from 100 to 600s also resulted in significant myometrial reduction.

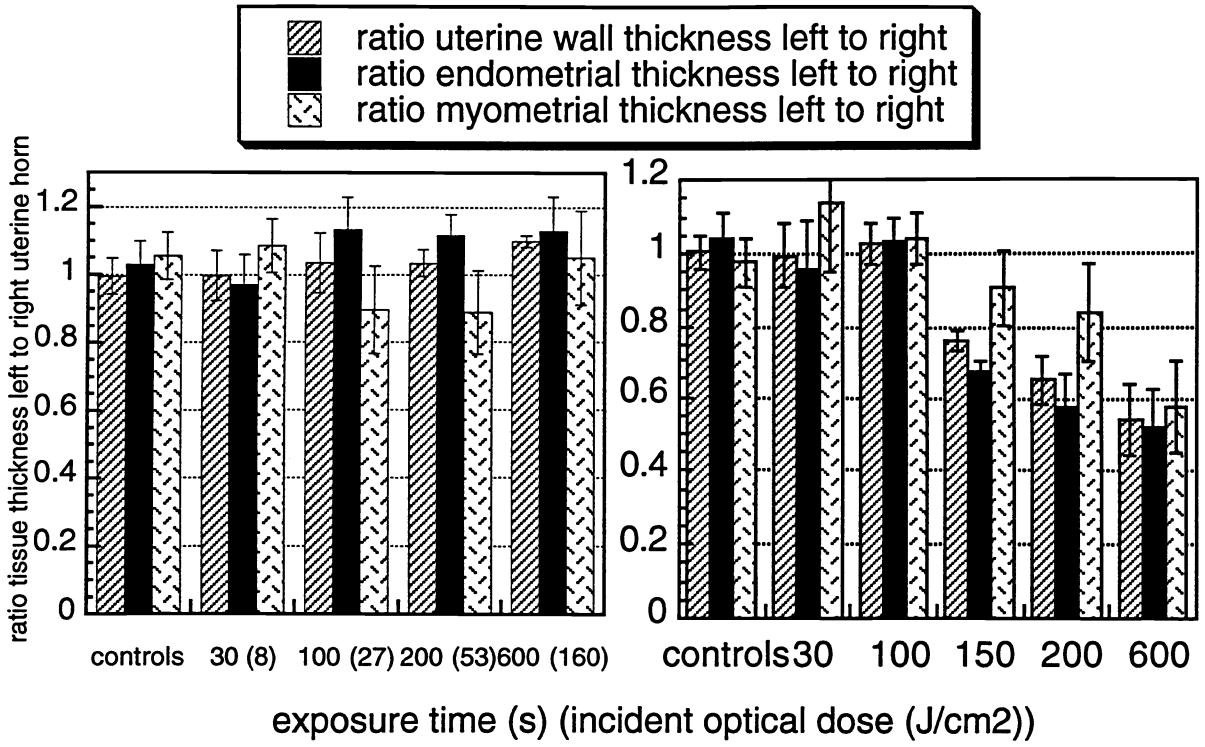
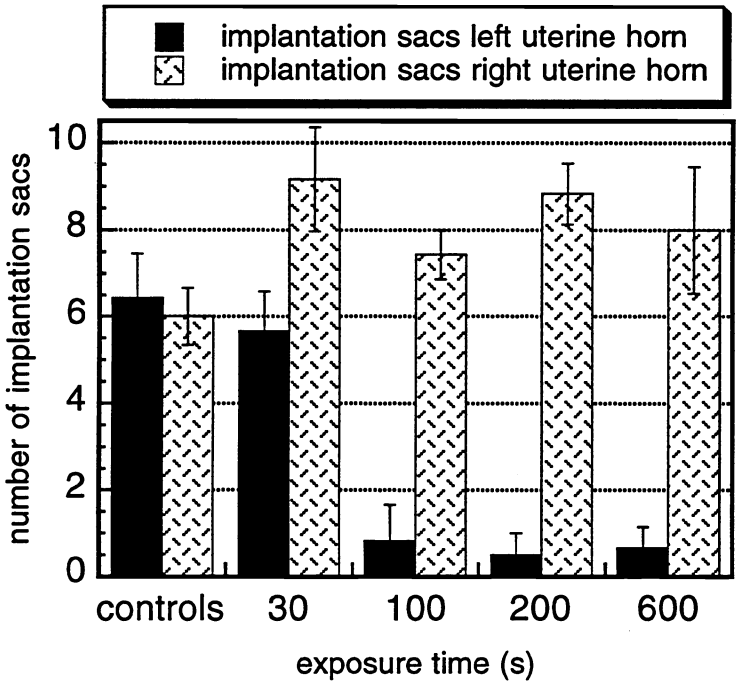


Figure 3 shows the number of implantation sacs counted in left (experimental) and right (control) uterine horns 14-18 days after mating (5-6 weeks post-PDT).



Comparison of left and right horns indicated that differences in the number of nidations were only significant for 100 s (p=0.002), 200 s (p=0.0003) and 600 s (p=0.007) exposure time groups (paired t test). Tukey's studentized range test showed that the number of implantation sacs in the left horn were significantly lower than controls or the 30-second group at exposure times equal to or exceeding 100s.

4. DISCUSSION

Endometrial PDT can produce dramatic structural changes, such as tissue ablation, or more subtle functional alterations, such as implantation prevention. The minimum optical dose required for each effect differs substantially. A relatively high in situ optical dose of 64 J/cm^2 caused glandular necrosis and, by 3 weeks, incomplete regeneration. Endometrial glands survived lower optical doses and the endometrium regenerated to its full thickness in spite of obvious damage to the stroma and myometrium one day after PDT. In human endometrial regeneration, the surface epithelium is derived from simultaneous proliferation of the exposed ends of basal glands, where stem cells are believed to exist, and from the persistent surface lining of cornual and isthmic regions[19]. Our observation that regeneration can only be prevented using an optical dose sufficient for glandular necrosis agrees with the concept that glandular crypts of the basal endometrial layer must be destroyed to prevent regeneration.

At an in situ optical dose of 43 J/cm^2, we observed edema of the endometrial stroma acutely, followed by fibrosis, lack of cellularity and necrosis of the circular myometrial layer 3 weeks after PDT. Because endometrial glands remain morphologically intact at this optical dose, our results indicate that uterine cells do not have uniform photosensitivity. This is not unreasonable since differential photosensitivity has been reported in both normal and malignant cell studies using ALA[20] as well as other porphyrin photosensitizers[21,22]. It is well known that endothelial cells are more sensitive to photodynamic treatment than smooth muscle cells[21] or adenocarcinoma cells[23]. However, our observations are somewhat unexpected since fluorescence microscopy studies by our group[8] as well as others[7,12] show that the highest photosensitizer fluorescence is in the endometrial glands (versus stroma or muscle cells). While fluorescence data suggest that there should be a lower optical dose threshold for glandular damage, in vivo PDT results clearly indicate that there is no strict correlation between Pp IX content and phototoxicity. This incongruity may be due to superior oxidative stress repair mechanisms or differences in Pp IX sub-cellular localization which, in comparison to stromal cells, provide glandular cells with enhanced photo damage protection.

Functional damage to the endometrium as determined by reproductive performance occurred at a

lower optical dose than needed for structural damage. Following PDT with an in situ dose of 43 J/cm^2, the number of implantation sacs in the treated uterine horn was significantly reduced, but no lasting thinning of the endometrial layer or reduction of endometrial glands could be noted at the time of mating. The only morphological change noticed was fibrosis of the stroma with reduction of cellularity. Obviously, intact endometrial glands are not the only requirements necessary for successful implantation. Occlusion of the lumen was not responsible for failure of implantation since uterine horns irradiated for 100 s did not cause lumen obliteration. Furthermore, 2 of 6 animals irradiated with the highest optical dose showed implantation sacs in the proximal portions of the left uterine horns which were not reached by the light diffusing fiber. We do not believe that PDT-induced deterioration in epithelial cell function compromised implantation since the estrus cycle of the rat is around 4 days and the endometrial glands must have been replaced at least once before mating. More likely, lack of stromal cells and/or endometrial microvasculature are responsible for the impairment in reproductive performance.

Measurements of uterine layer thickness may not be ideal for determining tissue damage. Viable cells are replaced by fibrous tissue and measured thickness values can underestimate the degree of destruction. However we believe that this is the only quantitative technique for assessing endometrial regeneration.

In conclusion, the photodynamic threshold for lasting endometrial destruction is higher than for functional damage.Endometrial glands appear to be more resistant to PDT than stroma due to differential cell photosensitivity. Irreversible endometrial ablation requires glandular destruction, whereas damage to the endometrial stroma is sufficient for reproductive impairment.

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