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Differential cell photosensitivity in photodynamic therapy of the rat endometrium

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Structural and functional effects of endometrial photodynamic therapy in a rat model

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OBJECTIVE: Our purpose was to determine the optical dose required for irreversible endometrial destruction and prevention of implantation by photodynamic therapy with topical 5-aminolevulinic acid.
STUDY DESIGN: Three hours after drug application 74 female Sprague-Dawley rats received varying doses of 630 nm of light delivered by an intrauterine cylindric diffusing fiber.
RESULTS: A 64 J/cm^2 in situ optical dose resulted in long-term irreversible endometrial destruction; 43 J/cm^2 damaged endometrial stroma and myometrium but not glandular epithelium 1 day after photodynamic therapy. At this lower light dose endometrium regenerated to full thickness within 3 weeks; however, implantation sacs were significantly reduced.
CONCLUSIONS: Photodynamic destruction of glandular epithelium accompanies irreversible endometrial ablation, whereas isolated stromal damage leads to reproductive impairment only. The optical dose required for endometrial ablation is approximately 1.5-fold higher than for reproductive impairment (functional damage) because of differential cell photosensitivity. (Am J Obstet Gynecol 1996;175:115-21.)

Key words: Photodynamic threshold, optical dose, 5-aminolevulinic acid, protoporphyrin IX, rat model

Photodynamic therapy is currently being evaluated as a minimally invasive procedure for selective endometrial ablation.[1] This therapy is based on light-induced oxidation reactions, which lead to tissue necrosis.[2] Several animal studies[3-10] have shown that selective endometrial destruction with photodynamic therapy is feasible and may provide a viable alternative to routinely performed surgical treatment modalities requiring anesthesia.

The photodynamic threshold for irreversible destruction of a given tissue is a complex function of optical dose, intrinsic sensitizer characteristics, drug location and concentration, and tissue type.[1] For endometrial ablation both systemic and topical application of various photosensitizers has 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 photodynamic therapy with topical (intrauterine) 5-aminolevulinic acid has been described, many factors relevant to the photodynamic threshold have not been thoroughly examined.

5-Aminolevulinic acid is endogenously converted to the fluorescent photosensitizer protoporphyrin IX.[6-8, 11] Pharmacokinetic studies in rats and rabbits showed peak endometrial fluorescence 3 hours after topical 5-aminolevulinic acid 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 protoporphyrin IX fluorescence was reached, suggesting that higher drug concentrations would not lead to additional tissue destruction.[8] However, after photodynamic therapy 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 photodynamic therapy. To the best of our knowledge, this is the first report that correlates optical dose with structural or functional damage. These parameters were evaluated in a rat model by studying endometrial ablation and prevention of pregnancy, respectively.

Material and methods

Seventy-four mature female Sprague-Dawley rats weighing 261 to 325 gm were placed in a controlled

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environment with free access to food and water and a 12-hour light-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 photodynamic therapy and 30 rats for assessment of reproductive performance. The estrus cycle was monitored by obtaining frequent vaginal smears 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 intramuscularly 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, Denville, N.J.) was diluted to 200 mg/ml in Hyskon dextran 70 in dextrose (Pharmacia, Piscataway, N.J.) before administration. The solution was titrated to pH 6 with 10N sodium hydroxide. Hyskon is a viscous, hydrophilic, branched polysaccharide used to minimize spillage of photosensitizer through the cervix. A total of 0.15 ml of the 5-aminolevulinic acid 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 photodynamic therapy was performed by intrauterine illumination of the left uterine horn. Light from an argon-pumped dye laser (Spectra Physics, Mountain View, Calif.) with DCM dye (Exciton, Dayton, Ohio) operating at 630 nm was delivered to the uterine cavity by a quartz optical fiber terminated with a 1.2 mm diameter, 3 cm cylindric diffusing tip (model 4420-A02, PDT Systems, Santa Barbara, Calif.). The fiber was placed in 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 cylindric diffusing fiber (300 mW total power coupled into the fiber). Exposure times were 30, 100, 150, 200, and 600 seconds 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 photodynamic therapy the abdominal incision was reapproximated to cover the uterine horns with tissue and to allow backscattering of light. Intrauterine temperature was monitored continuously during photodynamic therapy in four animals with a 0.13 mm diameter copper-constantan thermocouple (Omega Engineering, Stamford, Conn.) placed along the light-diffusing fiber inside the uterine cavity.

Each animal served as its own internal control; the same amount of 5-aminolevulinic acid solution was injected into the right uterine horn immediately after light administration to the left horn. Additional control groups were established by instilling pure Hyskon solution into the left uterine horn before irradiation for 600 seconds. Temperature, pulse, and respiration were monitored until the animals were ambulatory.

To evaluate the acute morphologic effect of photodynamic therapy, four rats per exposure time (30, 100, 200, and 600 seconds) were killed 24 hours after treatment. To assess endometrial regeneration, four rats per exposure time (30, 100, 150, 200, and 600 seconds) were killed 21 to 24 days after photodynamic therapy 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 by laparotomy and placed in buffered 10% formalin immediately after the animal was killed by asphyxiation with carbon dioxide 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 and the distances from endometrial surface to myometrial-endometrial junction and myometrial-endometrial junction to serosal surface were measured with a light 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 photodynamic therapy six animals per exposure time (30, 100, 200, and 600 seconds) and a control group of six animals were bred for 4 days with mature male Sprague-Dawley rats to assess reproductive performance. Female rats were killed in the second trimester of pregnancy (14 to 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.

To know the optical dose given to the endometrial gland stumps, the in situ optical dose at a depth of 1 mm in the tissue for 630 nm of light had to be calculated. We used a mathematic model describing the decay in fluence rate with increasing source-detector separation with a cylindric light diffuser as light source.[15] As an effective scattering coefficient we used in our calculations 0.73

Table I. Incident optical dose at surface of endometrium, calculated in situ optical dose at depth of 1 mm, and temperature rise at surface of endometrium

Exposure time (sec)	*Incident optical dose (J/cm^2)*	*In situ optical dose (J/cm^2)*	*Temperature rise (°C ± SE)*
30	8	13	2.8 ± 0.5
100	27	43	3.6 ± 0.3
150	40	64	4.9 ± 0.2
200	53	85	5.1 ± 0.3
600	160	260	5.8 ± 0.5

Table II. Morphologic changes observed 24 hours after photodynamic therapy (numbers represent number of animals)

	Endometrial glands				
Exposure time	*Absent*	*Reduced >50%*	*Edema of endometrial stroma*	*Edema/necrosis of circular myometrium*	*Myometrium focally absent*
30 sec ($n = 4$)	0	0	0	0	0
100 sec ($n = 4$)	0	0	4	2	1
200 sec ($n = 4$)	2	4	4	4	3
600 sec ($n = 4$)	2	4	4	4	4

mm^{-1}, a value determined from measurements of premenopausal human uterine tissue.[16]

The penetration depth, δ,[1] for 630 nm of light was determined for rat uteri by the following method. Four fresh uterine horns were opened longitudinally at the mesometrial insertion and sectioned to form squares of 2 × 2 cm. To facilitate backscattering, four 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 μm core diameter detector fiber (0.8 mm diameter isotropic collection tip, PDT Systems) was connected to a photomultiplier tube (Hamamatsu R 928, Bridgewater, N.J.) to measure steady-state light intensity (fluence rate). After 630 nm of light was launched onto the center of the tissue (1 cm diameter spot), 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, δ, 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 by 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 ± SE.

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 ± 0.2 mm, $n = 74$). The incident (irradiant) optical dose describes the source energy density, whereas the in situ (tissue) optical dose characterizes the energy density deposited at a defined depth inside the tissue. These values differ because of 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, $\beta = 4.31$ mm, and diffusion theory.[15] Baseline temperatures (31° to 33° C) increased rapidly with irradiation, leveled off within 280 seconds, and remained constant at about 6° C above baseline for 600 seconds. Temperatures during photodynamic therapy never exceeded 40° C, a level that is insufficient for heat-induced necrosis or apoptosis.[18]

Table II summarizes morphologic changes observed in treated uterine horns (Fig. 1, *A*) compared with untreated control horns (Fig. 1, *B*) 24 hours after PDT. Endometrial edema was observed in all animals receiving a 100-second 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 200 seconds, all endometrial glands were grossly swollen or absent, leaving debris in round stromal spaces, as shown in Fig. 1, *A*. The cytoplasm of the

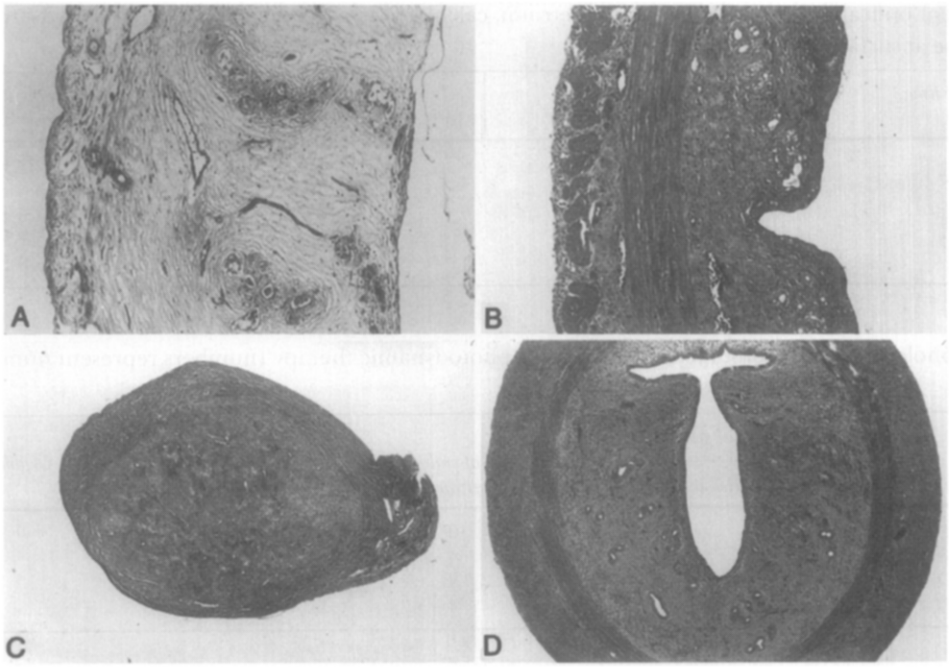

Fig. 1. Hematoxylin-eosin stained sections of photosensitizer and light treated, *left* (**A, C**) and control, *right* (**B, D;** photosensitizer only) rat uterine horns 1 (**A, B**) and 21 days (**C, D**) after photodynamic therapy, 200 second light exposure. Luminal surfaces of **A** and **B** (original magnification ×38) are on *right*. In **A** edema of entire uterine wall, loss of cellularity, and disintegration of glands are seen. In **C** lumen is obliterated, endometrial glands are absent, stroma is replaced by fibrous tissue, and myometrium is partially absent.

Table III. Morphologic changes observed 3 weeks after photodynamic therapy (numbers represent number of animals)

Exposure time	*Lumen obliterated*	*Endometrial glands*		*Reduction in endometrial stroma cells/fibrosis*	*Circular myometrium split by fibrotic tissue*	*Myometrium focally absent*
		Absent	*Reduced >50%*			
30 sec ($n = 4$)	0	0	0	0	0	0
100 sec ($n = 4$)	0	0	0	3	0	0
150 sec ($n = 4$)	1	0	4	4	1	0
200 sec ($n = 4$)	1	2	4	4	4	3
600 sec ($n = 4$)	3	3	4	4	4	4

inner muscular layer was consistently grossly swollen, but 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 after photodynamic therapy did not reveal significant differences with increasing exposure times (data not shown).

Table III summarizes histologic changes observed in treated uterine horns (Fig. 1, *C*) compared with untreated control horns (Fig. 1, *D*) 3 weeks after photodynamic therapy. The first significant morphologic change was endometrial fibrosis with a reduced number of stromal cells and capillary vessels at 100 seconds. By 150 seconds 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 was totally absent. In some cases the uterine lumen was replaced by scar tissue (Fig. 1, *C*), although at 150 seconds this scar tissue embedded surviving or regenerated endometrial glands.

Fig. 2 summarizes results of endometrial, myometrial, and uterine wall thickness measurements three weeks after photodynamic therapy. Analysis of variance showed a significant decrease for all three variables with increasing exposure time. When exposure times increased from 100 to 150 seconds, the decrease in wall thickness was attributed primarily to endometrial destruction because 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 600-second groups (Tukey's paired comparison procedure). Endometrial

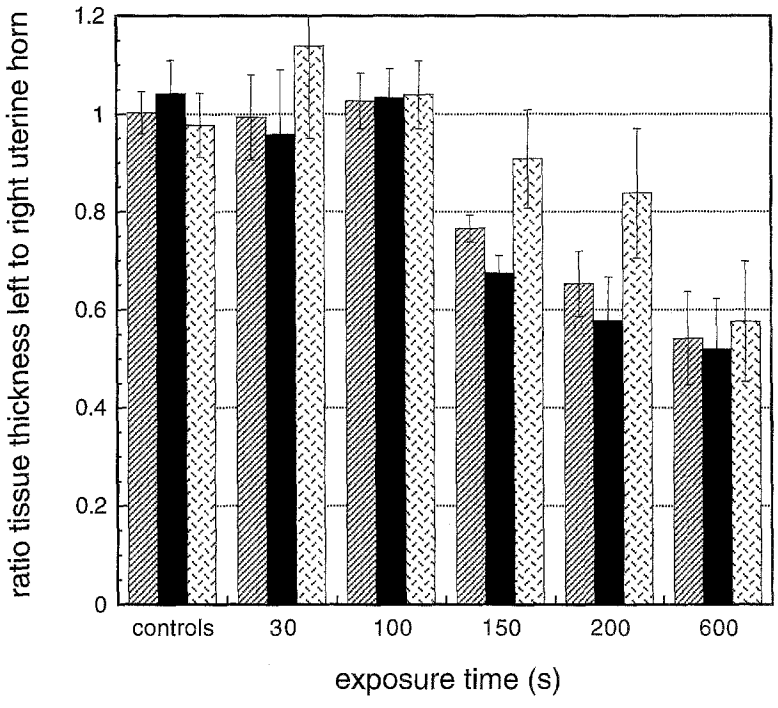

Fig. 2. Ratio of measured thickness of left uterine wall (□), endometrial (■), and myometrial (□) layer to corresponding values of right horn 3 weeks after photodynamic therapy. *Columns,* Mean ± SE.

thickness was significantly less than controls at 150, 200, and 600 seconds of light exposure. Similarly, increasing exposure times from 30 or 100 seconds to 200 or 600 seconds produced significant reductions in endometrial thickness. In contrast, myometrial thickness was significantly less than controls at 600 seconds of light exposure. Increasing exposure times from 100 to 600 seconds also resulted in significant myometrial reduction.

Fig. 3 shows the number of implantation sacs counted in left (experimental) and right (control) uterine horns 14 to 18 days after mating (5 to 6 weeks after photodynamic therapy). Comparison of left and right horns indicated that differences in the number of nidations were only significant for 100 seconds ($p = 0.002$), 200 seconds ($p = 0.0003$), and 600 seconds ($p = 0.007$) of exposure (paired t test). Tukey's studentized range test showed that the number of implantation sacs in the left horn were significantly lower than in controls or the 30-second group at exposure times ≥100 seconds.

Comment

Photodynamic endometrial ablation may provide a simple, cost-effective alternative to surgical techniques for treatment of dysfunctional uterine bleeding or for sterilization. However, development of minimally invasive endometrial photodynamic therapy for human applications requires a quantitative understanding of the relationship between drug dose, optical dose, and tissue response. In previous studies concentration-dependent kinetics of 5-aminolevulinic acid conversion in rat and rabbit animal models were studied.[8, 12] This article complements our earlier work by describing the precise light dose (photodynamic threshold) required for endometrial damage with topical 5-aminolevulinic acid.

Endometrial photodynamic therapy 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 1 day after photodynamic therapy. 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 with 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 photodynamic therapy. Because endometrial glands remain morphologically in-

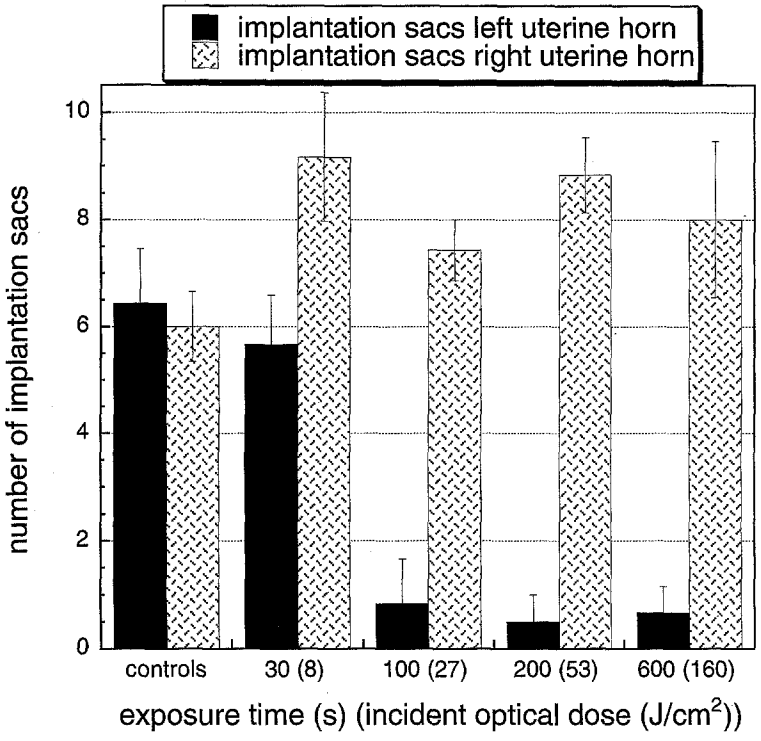

Fig. 3. Implantation sacs counted in treated left (■) and untreated right (□) uterine horn. Six animals for each exposure time. *Columns,* Mean ± SE.

tact at this optical dose, our results indicate that uterine cells do not have uniform photosensitivity. This is not unreasonable because differential photosensitivity has been reported in both normal and malignant cell studies with 5-aminolevulinic acid[20] and 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 because fluorescence microscopy studies by our group[8] and others[7, 12] show that the highest photosensitizer fluorescence is in the endometrial glands (vs stroma or muscle cells). Although fluorescence data suggest that there should be a lower optical dose threshold for glandular damage, in vivo photodynamic therapy results clearly indicate that there is no strict correlation between protoporphyrin IX content and phototoxicity. This incongruity may be due to superior oxidative stress repair mechanisms[21] or to differences in protoporphyrin IX subcellular localization[20] which, in comparison with stromal cells, provides glandular cells with enhanced photodamage protection.

Functional damage to the endometrium as determined by reproductive performance occurred at a lower optical dose than needed for structural damage. After photodynamic therapy with an in situ dose of 43 J/cm^2, the number of implantation sacs in the treated uterine horn was significantly reduced (Fig. 3), but no lasting thinning of the endometrial layer or reduction of endometrial glands could be noted at 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 because uterine horns irradiated for 100 seconds did not cause lumen obliteration. Furthermore, two of six 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 photodynamic therapy–induced deterioration in epithelial cell function compromised implantation because 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 or endometrial microvasculature is 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. Variations in the extent of tissue damage may be due to optical dose irregularities. Although we were careful to irradiate in a closed abdomen, air pockets trapped in the peritoneal cavity could reduce backscattering, and the actual optical dose would be four to five times less than calculated in situ values.[1] This would not occur in the human uterus be-

cause, in contrast to the rat, the relatively thick muscular layer surrounding the endometrium would provide unlimited backscattering.

In conclusion, endometrial glands appear to be more resistant to photodynamic therapy than stroma. Irreversible endometrial ablation requires glandular destruction, whereas damage to the endometrial stroma is sufficient for reproductive impairment. As a result, the photodynamic threshold for lasting endometrial ablation is higher than for functional damage.

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