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Selective photosensitizer localization in the human endometrium after intrauterine application of 5-aminolevulinic acid

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OBJECTIVE: Our purpose was twofold: to determine the distribution of the endogenous photosensitizer protoporphyrin IX in the uterus and to ascertain the time interval leading to maximal endometrial fluorescence after intrauterine instillation of 5-aminolevulinic acid.

STUDY DESIGN: One milliliter of a 400 mg/ml 5-aminolevulinic acid–Hyskon solution was instilled into the uterine cavity of 27 women before hysterectomy. On frozen sections of uterine samples 5-aminolevulinic acid–induced fluorescence was measured with fluorescence microscopy.

RESULTS: 5-Aminolevulinic acid–induced fluorescence could first be detected in the superficial endometrial glands 75 minutes after drug injection. In the endometrial gland stumps fluorescence intensity peaked 4 to 8 hours after 5-aminolevulinic acid instillation and was >48 times higher than in the underlying myometrium.

CONCLUSIONS: Fluorescence in the endometrial glands suggests that selective photodynamic destruction of the endometrium may be possible 4 to 8 hours after intrauterine 5-aminolevulinic acid instillation. (Am J Obstet Gynecol 1996;175:1253-9.)

Key words: 5-Aminolevulinic acid, endometrium, photodynamic therapy, protoporphyrin IX, uterus

Photodynamic therapy is currently being evaluated as a minimally invasive procedure for endometrial ablation; it may not require anesthesia and therefore might be performed in an office setting.[1] Photodynamic therapy is based on light-induced oxidation reactions that 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. Both systemic and topical application of various photosensitizers have been studied.[3, 5, 7, 8] Intrauterine administration concentrates the photosensitizer in the endometrium and minimizes systemic risks such as skin photosensitivity. Exogenous administered 5-aminolevulinic acid is endogenously converted to the fluorescent photosensitizer protoporphyrin IX, leading to tissue photosensitivity.[6-8, 11] Fluorescence microscopy studies in rats and rabbits by our group[8] and others[7, 11] have shown higher 5-aminolevulinic acid–induced fluorescence of the endometrial glands, compared with stroma or muscle cells.

In this study we examined the conversion of 5-aminolevulinic acid and protoporphyrin IX in human uterine tissue after intrauterine instillation of an 5-aminolevulinic acid solution at different time intervals. To the best of our knowledge, this is the first report describing selectively induced fluorescence of the human endometrial glands and the time interval needed for peak fluorescence.

Material and methods

Twenty-four premenopausal (aged 40 ± 7 [SD] years) and 11 postmenopausal (aged 60 ± 5 [SD] years) patients scheduled for hysterectomy were included into the study after written informed consent was obtained. Three premenopausal and 3 postmenopausal women of the included patients served as a control group, and no intrauterine drug instillation was performed to determine autofluorescence of uterine tissue on frozen sections. The study was approved by the Human Subject Review Committee of the University of California, Irvine, and the

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University of Zurich. Indications for hysterectomy were uterine prolapse ($n = 3$), urinary incontinence procedure ($n = 3$), fibroids ($n = 10$), history of endocervical intraepithelial neoplasia grade 3 ($n = 7$), early cervical cancer ($n = 3$), early endometrial cancer ($n = 6$), and dysfunctional uterine bleeding ($n = 3$).

Immediately before instillation 1 gm of crystallized 5-aminolevulinic acid hydrochloride (Dusa Pharmaceuticals, Denville, N.J.) was dissolved in 0.9 ml of Hyskon (Pharmacia, Piscataway, N.J.) and buffered to pH 6 with 0.8 ml of sterile 8.4% Sodium Bicarbonate Injection U.S.P. (Abbott Laboratories, North Chicago, Ill.) and 620 mg of Sodium Bicarbonate Powder U.S.P. (Spectrum Chemical, Gardena, Calif.) under sterile conditions. The resulting 2.5 ml of 400 mg/ml 5-aminolevulinic acid solution was pressed through a sterile 0.22 μm filter unit (Millex-GS, Millipore, Bedford, Mass.) into a sterile syringe. A solution of 400 mg/ml was found to be the highest soluble 5-aminolevulinic acid concentration in Hyskon possible. Topical application of the 5-aminolevulinic acid solution was performed with the patient in the lithotomy position 45 minutes to 24 hours before the scheduled hysterectomy. 5-Aminolevulinic acid instillation was performed immediately after induction of anesthesia for surgery in 14 patients, and in 13 subjects instillation was performed without anesthesia on the ward. A standard bivalve speculum was placed. The cervix was cleansed with povidone-iodine solution. A Cook hysterosalpingography catheter (Cook, Billerica, Mass.) with an outer diameter of 2 mm was inserted into the cervical canal. To avoid spillage, the balloon was filled with 1 ml of air and the catheter was slightly retracted to block the os internum. A total of 1 ml of 400 mg/ml 5-aminolevulinic acid–Hyskon solution was injected slowly into the uterine cavity over 30 seconds at a uniform flow rate by slow manual push. No dilation of the cervix was attempted.

Immediately after hysterectomy the specimen was brought to the clinical pathology laboratory where two sections of the uterine wall from the corpus and fundus were obtained. Specimens were immediately placed in molds containing embedding medium for frozen sections (Tissue Tek, ornithine carbamyltransferase media, Miles Laboratory, Elkhart, Ind.), snap frozen on dry ice, and stored at −70° C in a light-impermeable container. All specimens were handled in the dark. Tissues were sectioned in low diffuse light (Cryostat microtome, AO Reichert, Buffalo, N.Y.) to obtain slices 6 μm thick for fluorescence analysis.

Low-light-level fluorescence microscopy was performed with a slow-scan, thermoelectrically cooled, charge-coupled device camera system (Princeton Instruments, Trenton, N.J.) coupled to a Zeiss Axiovert 10 inverted fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany). A 10× objective (Zeiss Plan-neofluar, numeric aperture 0.3) was used to visualize bright-field and fluorescence images of frozen sections. A 100 W mercury arc lamp filtered through a 405 nm bandpass filter (20 nm band width, Omega Engineering, Stamford, Conn.) provided excitation light. A dichroic filter (Zeiss, FT 420) was used to separate excitation from emission signals and a 635 nm broad bandpass filter (55 nm band width, Omega Optical, Brattleboro, Vt.) was used to isolate the fluorescence emission. These wavelengths were chosen because protoporphyrin IX has a strong absorption peak at 405 nm and a fluorescence peak at 635 nm. Instrument control, image acquisition, and processing were performed with a Macintosh IIfx computer and IPLab software (Signal Analytics, Vienna, Va.). Sample photo degradation was minimized by limiting arc-lamp exposure to 2 seconds by electronically synchronizing camera and lamp shutters (Uniblitz, model T132, Vincent Associates, Rochester, N.Y.). To correct for light distribution, background images were acquired from blank slides under conditions identical to those used for sample measurements. Dark noise levels were determined by acquiring images without source illumination. All fluorescence images were corrected for both nonuniform illumination and dark noise contribution by the following algorithm:

$$\text{Corrected fluorescence image} = \frac{\text{Fluorescence image} - \text{Dark noise}}{\text{Background image} - \text{Dark noise}} \times \text{Mean (Background image} - \text{Dark noise)}$$

where mean (background-dark noise) is the mean gray-scale value for the dark noise–corrected background image.

To subtract autofluorescence of uterine tissue and debris, we acquired a corrected fluorescence image with a 590 nm bandpass filter of each examined frozen section. The same exposure time was used and the image was acquired immediately after the 635 nm bandpass filter fluorescence image. Then the corrected fluorescence image with the 590 nm bandpass filter was subtracted from the corrected fluorescence image with the 635 nm bandpass filter correcting for different illumination intensities, which are due to the use of different filters:

$$\text{5-Aminolevulinic acid–induced fluorescence image} = \text{Corrected image (635 nm)} - \frac{\text{Illumination (635 nm)}}{\text{Illumination (590 nm)}} \times \text{Corrected image (590 nm)}$$

On the 5-aminolevulinic acid–induced fluorescence images, fluorescence intensity measurements of different anatomic layers were made for comparative analysis. Mean fluorescence intensities of the superficial endometrial gland (immediately adjacent to the lumen), the deep endometrial glands (immediately adjacent to the endometrial-myometrial junction), and the myometrium underlying the endometrium were measured. Because

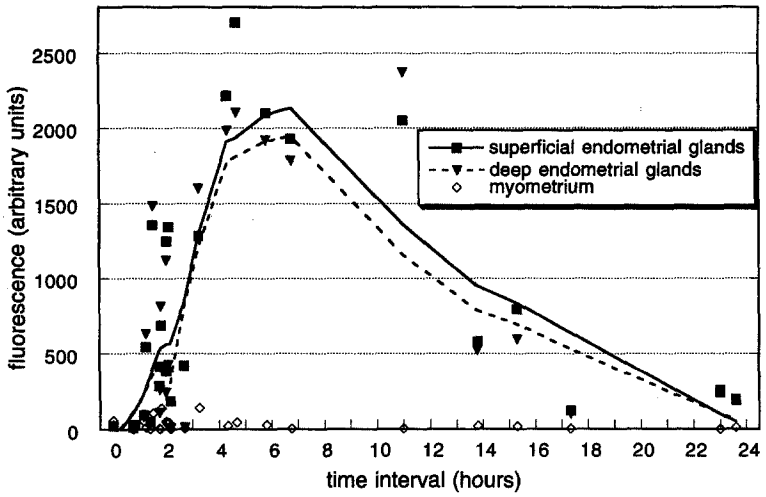

Fig. 1. Mean fluorescence values of superficial endometrial glands, deep endometrial glands, and myometrium at endometrial-myometrial junction of 26 patients operated on at different time intervals after intrauterine instillation of a 400 mg/ml 5-aminolevulinic acid–Hyskon solution. Curves represent Lowess weighted curve smoothing by use of local linear regression with span size of 50% ($f = 0.5$) and default of 0.75.[13]

the endometrial stroma is a loosely built tissue and often showed artificial holes on the frozen sections, the endometrial stroma was not included in analysis. After fluorescence microscopy the frozen sections were stained with hematoxylin-eosin, reviewed for histologic diagnosis by one of the authors (F.L.), and endometrial thickness was measured.

After instillation of the 5-aminolevulinic acid solution in the uterine cavity, urine was collected in 17 consecutive patients for the following 24 hours and on the second postoperative day for analysis of urinary 5-aminolevulinic acid excretion. This was measured by spectrophotometry after condensation of 5-aminolevulinic acid with acetylacetone to form a pyrrole, which then reacts with Ehrlich's reagent and is quantitated colorimetrically.[12]

For statistical analysis of differences in fluorescence intensities at different time points we used analysis of variance. Statistical significance was taken as $p < 0.05$. If a significant overall difference was present, multiple comparisons were performed with Sheffé's multiple comparison procedure. For comparison of the fluorescence intensities of premenopausal and postmenopausal endometrium, matched pairs at the same time intervals were formed and the paired two-tailed t test was used. Data are presented as mean ± 1 SD.

Results

In 2 of 29 patients intended to have drug application, intrauterine instillation could not be performed because of cervical stenosis. In the 27 remaining patients frozen sections of endometrium could be evaluated by fluorescence microscopy and was confirmed as nonneoplastic by histopathologic review. None of the patients complained of pelvic pain either during instillation or during the time span before surgery. One patient had knee pain and numbness in the thigh after drug instillation. Surgery was postponed and immediate physical examination did not reveal any neurologic signs or other pathologic disorders. The patient underwent hysterectomy 21 days after drug instillation.

Fig. 1 shows the measured mean fluorescence of the superficial endometrial glands, the deep endometrial glands, and the myometrium immediately underlying the endometrium as a function of time interval between 5-aminolevulinic acid instillation and uterine removal. Fluorescence above the values of autofluorescence was first detected in the superficial endometrial glands 75 minutes after drug application, whereas fluorescence of the deep endometrial glands increased after 2 hours and peaked at the same time as the superficial endometrial glands. By grouping the patients for statistical analysis, fluorescence of the deep and superficial endometrial glands was highest 4 to 8 hours after 5-aminolevulinic acid instillation (Table I) and showed high selectivity to endometrial tissue (Fig. 2). Mean fluorescence of superficial and deep endometrial glands was 155 ± 75 (61 to 386) times and 140 ± 73 (48 to 357) times, respectively, higher than mean myometrial fluorescence (Table I). In the myometrium no significant changes in fluorescence could be detected with increasing time interval. Eleven hours and more after drug instillation glandular fluorescence became inhomogeneous (Fig. 3) with highly fluorescing glands lying next to poorly fluorescing glands. In the patient who had hysterectomy postponed by 21 days, no 5-aminolevulinic acid–induced fluorescence could be detected in the uterine tissue.

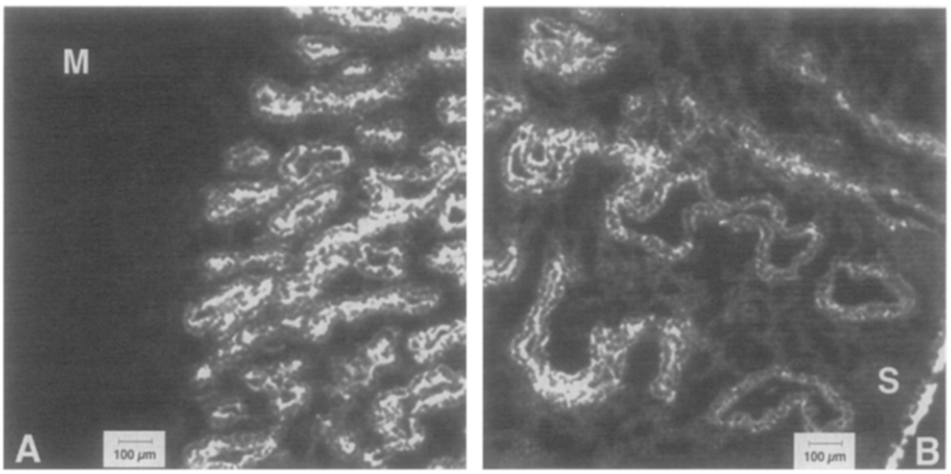

Fig. 2. Fluorescence microscopy image of endometrial frozen section sampled 4 hours after intrauterine 5-aminolevulinic acid instillation. **A,** Area at endometrial-myometrial junction. *M,* Myometrium. Endometrial gland stumps are highly fluorescent. **B,** Area near endometrial surface *(S).* Lumen of uterine cavity is at *right edge.*

Table I. Mean fluorescence signal of superficial endometrial glands, deep endometrial glands, and myometrium after grouping for time intervals

	Time interval						
	0 hr (Controls, n = 6)	*0.75-1.5 hr (n = 5)*	*1.5-2 hr (n = 5)*	*2-4 hr (n = 6)*	*4-8 hr (n = 4)*	*11-24 hr (n = 6)*	*Overall difference*
Endometrial thickness (single layer)	2 ± 1	1.5 ± 1.1	1.5 ± 0.9	3.1 ± 2.4	2.8 ± 0.9	2 ± 0.9	NS
Fluorescence superficial glands	22 ± 33	145 ± 224	627 ± 434	765 ± 587	2235 ± 330	664 ± 726	$p < 0.0001$
Compared with controls		NS	NS	NS	$p < 0.0001$	NS	
Compared with 0.75 to 1.5 hr			NS	NS	$p < 0.0001$	NS	
Compared with 1.5 to 2 hr				NS	$p = 0.0001$	NS	
Compared with 2 to 4 hr					$p = 0.01$	NS	
Compared with 4 to 8 hr						$p = 0.001$	
Fluorescence deep glands	24 ± 34	161 ± 264	583 ± 571	553 ± 551	1948 ± 133	674 ± 852	$p = 0.0003$
Compared with controls		NS	NS	NS	$p = 0.0008$	NS	
Compared with 0.75 to 1.5 hr			NS	NS	$p = 0.003$	NS	
Compared with 1.5 to 2 hr				NS	$p = 0.036$	NS	
Compared with 2 to 4 hr					$p = 0.047$	NS	
Compared with 4 to 8 hr						$p = 0.045$	
Fluorescence myometrium	58 ± 51	14 ± 24	61 ± 61	40 ± 54	25 ± 16	11 ± 8	NS

Fluorescence values in arbitrary units (mean ± 1 SD) and endometrial thickness in millimeters. *NS,* Not significant.

Premenopausal endometrium seemed to convert 5-aminolevulinic acid faster into fluorescing protoporphyrin IX than did postmenopausal endometrium (Fig. 4). By forming matched pairs of premenopausal and postmenopausal mean fluorescence of superficial endometrial glands at the same time points (±5 minutes), a significant higher fluorescence was found in premenopausal endometrial glands ($p = 0.01$).

In 6 of 17 24-hour urine samples (35%) collected after drug instillation 5-aminolevulinic acid excretion was above the reference value of 7 mg per 24 hours. All values of 24-hour urine samples collected on the second postoperative day were below the reference value. Side effects that are not related to the study included wound cellulitis treated successfully with intravenous antibiotics and a case of uterine fibroids that required blood transfusion because of intraoperative hemorrhage. No phototoxic skin reactions were observed, especially not on the skin exposed to intense illumination during operation.

Comment

5-Aminolevulinic acid is a precursor of protoporphyrin IX in the biosynthetic pathway for heme. Normally, the synthesis of heme regulates the synthesis of 5-aminolevu-

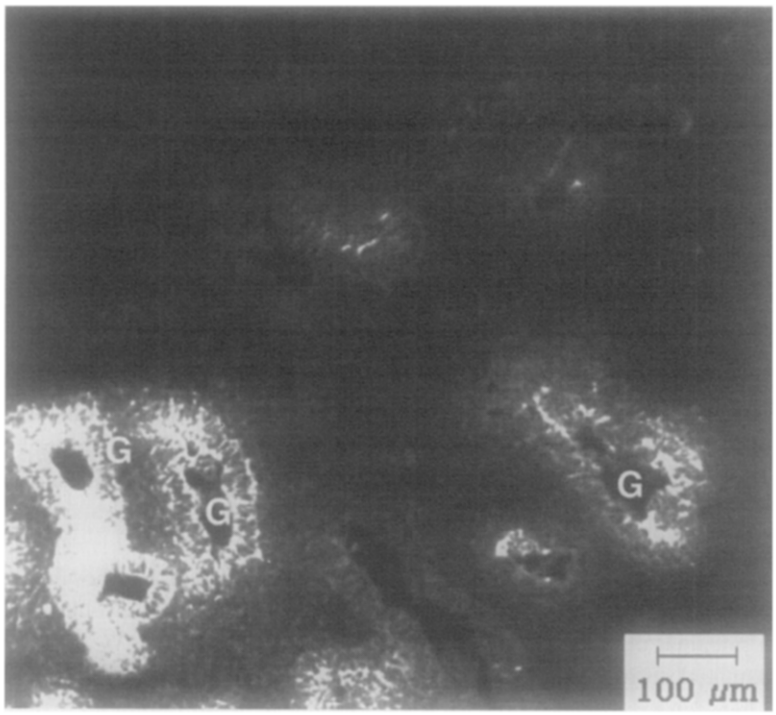

Fig. 3. Fluorescence microscopy image of endometrial frozen section sampled 11 hours after intrauterine 5-aminolevulinic acid instillation. Fluorescence signal of endometrial glands *(G)* is inhomogeneous with highly fluorescent glands lying next to poorly fluorescent endometrial glands.

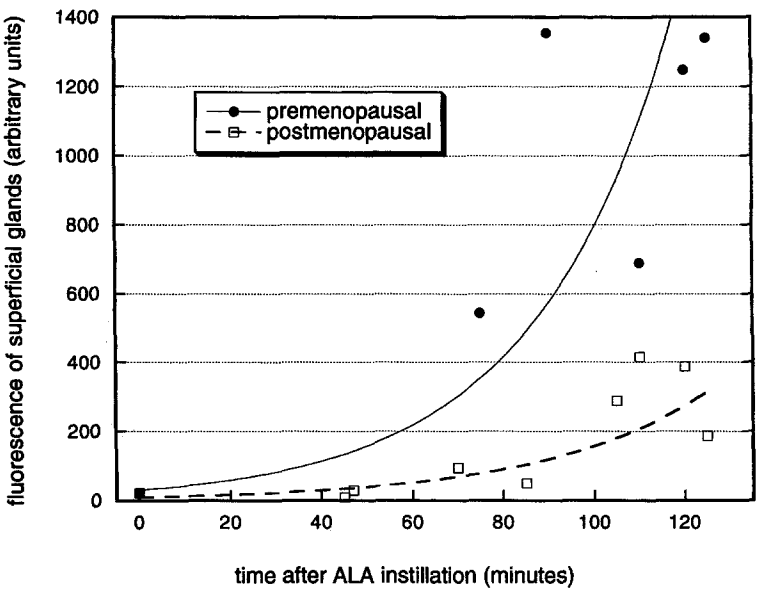

Fig. 4. Mean fluorescence values of superficial endometrial glands of premenopausal and postmenopausal patients undergoing hysterectomy at different time intervals after intrauterine instillation of a 400 mg/ml 5-aminolevulinic acid *(ALA)*–Hyskon solution. Marks at *time point zero* represent 3 premenopausal and 3 postmenopausal patients of control group.

linic acid by feedback control. The step of converting protoporphyrin IX into heme is a relatively slow process. Therefore the administration of exogenous 5-aminolevulinic acid bypasses the feedback effect of heme and induces the accumulation of protoporphyrin IX, causing tissue photosensitivity.[14] Lasting endometrial ablation and prevention of implantation by photodynamic therapy with 5-aminolevulinic acid–induced protoporphyrin IX as photosensitizer have been successfully achieved in animal models of rats and rabbits by our group and others.[6-8] In this study the time interval leading to maximal photosensitizer accumulation in the human endometrial glands and tissue selectivity of photosensitizer localization was determined by monitoring 5-amino-

levulinic acid–induced fluorescence after intrauterine application.

We showed that the induced fluorescence of the endometrial glands was highly selective compared with the adjacent myometrium in spite of instillation of a highly concentrated 5-aminolevulinic acid solution. Although the endometrial stroma also showed fluorescence, quantification was not attempted because this loosely built tissue showed artificial disruption by frozen sectioning, as shown in Fig. 2. Peak fluorescence of the entire endometrial glands, including the gland stumps, was reached 4 to 8 hours after drug instillation. The suggestion that sufficient light illumination at this time interval may lead to lasting endometrial ablation is based on two assumptions. First, endometrial regeneration primarily originates by proliferation of stem cells supposed to be located in the gland stumps[15]; therefore photodynamic destruction of the entire endometrial glands results in lasting endometrial ablation. The second assumption is that peak fluorescence correlates with maximal phototoxic effect. Although studies in malignant and normal cell lines did not show a strict correlation between protoporphyrin IX cellular content and 5-aminolevulinic acid–induced phototoxicity, there appeared to be a "threshold" effect of cellular protoporphyrin IX content above which a consistent phototoxic response was noticed.[16] This "threshold" protoporphyrin IX cellular content may be reached when maximal protoporphyrin IX fluorescence is present. In animal models for photodynamic endometrial ablation maximal tissue destruction was induced at sites of maximal fluorescence.[7, 8]

The high interpatient variability of mean fluorescence may be due to differences in menopausal status and day of menstrual cycle at treatment resulting in differences in endometrial thickness and metabolic activity of the endometrial cells (Table I). Because fluorescence is measured in arbitrary units per given area and the threshold value needed for initiation of the phototoxic effect is not known, the critical question is at which time interval photosensitizer accumulation in the gland stumps is maximal and present in all patients. Diffusion of 5-aminolevulinic acid into endometrial cells and its conversion into the fluorescent photosensitizer protoporphyrin IX seems to require around 75 minutes in vivo (Fig. 4). Because inactive atrophic endometrium of postmenopausal women showed lower fluorescence at the same time points than premenopausal endometrium, conversion of 5-aminolevulinic acid into protoporphyrin IX seems to depend on cellular metabolic activity (Fig. 4). This is in agreement with the enhancement of protoporphyrin IX accumulation by mitogenic stimulation in cell lines.[17]

To induce protoporphyrin IX accumulation in the endometrial gland stumps, 5-aminolevulinic acid has to diffuse through the lumen of endometrial glands before cellular uptake for conversion. Interestingly, no significant differences between fluorescence intensities of superficial and deep endometrial glands could be observed (Table I), which can only be explained by rapid endoluminal 5-aminolevulinic acid diffusion. Endoluminal diffusion seems to be facilitated by the low molecular weight and hydrophilic nature of 5-aminolevulinic acid because diffusion velocity is dependent on molecular weight. As a result of the low volume of instilled drug solution and slow injection rate, we do not think that intracavitary pressure influenced endoluminal 5-aminolevulinic acid propagation. Accordingly, in pathologic endometrial disorders such as endometrial cancer or polyps where the communication of endometrial glands to the lumen is disrupted, homogeneous photosensitization of the endometrial tissue by topical 5-aminolevulinic acid application may be inhibited.

At time intervals >11 hours measurement of glandular fluorescence became problematic because of the patchy pattern of the signal. We speculate that the 5-aminolevulinic acid solution penetrated certain glands better resulting in an intraluminal drug pool that sustained cellular fluorescence for a longer period than in other glands where no such intraluminal drug pool was present. Photodynamic therapy at these time points may lead to inhomogeneous endometrial destruction followed by regeneration.

Weak autofluorescence of blood vessel walls and extracellular debris was demonstrated in the corrected fluorescence images by use of the 635 nm bandpass filter in preliminary studies (unpublished data). To eliminate this autofluorescence, a corrected fluorescence image was used as described earlier. With this method the measured signals of the control tissues not exposed to exogenous 5-aminolevulinic acid were near zero (Table I). Hence the measured values in uterine tissues exposed to exogenous 5-aminolevulinic acid primarily represent 5-aminolevulinic acid–induced protoporphyrin IX fluorescence.

We conclude that selective photosensitizer accumulation in the human endometrial glands is feasible after intrauterine administration of 5-aminolevulinic acid and that maximal fluorescence of the endometrial gland stumps is observed 4 to 8 hours after instillation. At this point lasting endometrial photodynamic ablation may be feasible if a sufficient light dose can be delivered to the entire endometrium with an appropriate intrauterine light delivery device.[18]

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