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Authors

Chapman, Julia A
Tadir, Yona
Tromberg, Bruce J
[et al.](#)

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Effect of administration route and estrogen manipulation on endometrial uptake of Photofrin porfimer sodium

Julia A. Chapman, MD, Yona Tadir, MD, Bruce J. Tromberg, PhD, Calvin Yu, BS, Alberto Manetta, MD, Chung-Ho Sun, PhD, and Michael W. Berns, PhD

Irvine, California

OBJECTIVE: Our purpose was to evaluate the influence of the route of drug administration and target tissue vascularity on the distribution of a photosensitizer, Photofrin porfimer sodium, in the uterus.

STUDY DESIGN: The study was divided in two phases. In phase I 80 mature female rats were hormonally suppressed and then stimulated with estrogen. They were randomized to receive intravenous, intraperitoneal, or intrauterine Photofrin and killed 3, 6, 24, or 48 hours later. Drug distribution and levels were then determined. In phase II 40 female rats were randomized to receive hormonal stimulation, suppression, both, or neither. All received intrauterine Photofrin and were killed 24 hours later. Statistical analysis was performed with the unpaired *t* test and the two-way analysis of variance.

RESULTS: Intrauterine administration was determined to be the simplest and most effective method of delivery because it provided for optimal uptake and distribution ($p = 0.05$) within the uterus, at lower doses.

CONCLUSIONS: Selective localization of photosensitizer within the target tissue suggests that highly selective photodynamic destruction of endometrial tissue can be achieved. Furthermore, the combination of intrauterine administration of photosensitizer with estrogen adjuvant may minimize the most debilitating side effect of Photofrin, cutaneous phototoxicity. (AM J OBSTET GYNECOL 1993;168:685-92.)

Key words: Photofrin, estrogen, endometrium, photodynamic therapy

Photodynamic therapy is an experimental technique used in the treatment of certain cancers.¹ The process typically involves intravenous administration of a photosensitizing drug that is retained longer in malignant tumors. When light of sufficient energy and appropriate wavelength interacts with the sensitizer, highly reactive oxygen intermediates are generated.² These intermediates, primarily singlet molecular oxygen, irreversibly oxidize essential cellular components.³ The resulting photodestruction of crucial organelles in tumor cells and vasculature ultimately causes tumor necrosis.

In 1975 Dougherty et al.⁴ were the first to systematically describe, in laboratory animals, the therapeutic effect of the photosensitizer hematoporphyrin derivative. Since it was first used in cancer diagnosis,⁵ refinements in the preparation of hematoporphyrin derivative have led to its most recent and potent formulation,

Photofrin, a complex mixture of dihematoporphyrin esters and ethers. Photofrin is the most widely used photosensitizer, although it is not always the most efficacious. Phthalocyanins and chlorins, among others, have shown considerable therapeutic promise with few side effects (i.e., reduced cutaneous phototoxicity) in experimental animal tumors.² We selected Photofrin for the current work because it has been extensively characterized and it is being evaluated in multicenter human trials. In addition, the substantial effect Photofrin has on tumor microvasculature^{6, 7} suggests that it should be highly effective in the well-vascularized endometrium.

Currently, non-photodynamic therapy laser-induced tissue ablation is routinely performed on humans for a variety of conditions⁸⁻¹⁰ Drawbacks to these methods are that they require relatively high laser powers and have minimal tissue selectivity. In contrast, photodynamic therapy is a low-power, highly selective therapy. Several gynecologic applications have been described,¹¹ although to date it has been less extensively used on endometrial tissue. Promising results have been reported by Manyak et al.¹² who investigated photodynamic therapy of an endometriosis model, and Schneider et al.,¹³ who observed tissue binding enhancement when intravenously administered photosensitizer is supplied with estradiol to ovariectomized rats.

From the Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, and the Beckman Laser Institute and Medical Clinic, University of California, Irvine.

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Reprint requests: Yona Tadir, MD, Beckman Laser Institute and Medical Clinic, 1002 Health Sciences Road E., Irvine, CA 92715.

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Table I. Randomization characteristics for Sprague-Dawley rats

	<i>Suppression: Leuprolide acetate (0.05 mg/day subcutaneously for 10 days)</i>	<i>Stimulation: Estradiol valerate (500 µg/day intramuscularly for a single dose)</i>	<i>Photosensitization: Photofrin dose (mg/kg)</i>
Phase 1 (n = 80)			
Intravenous (n = 26)	Positive	Positive	7
Intraperitoneal (n = 12)	Positive	Positive	7
Intrauterine (n = 25)	Positive	Positive	0.7
Control (n = 17)	Positive	Positive	—
Phase 2 (n = 40)			
Group A (n = 10)	Negative	Negative	+ Intrauterine*
Group B (n = 10)	Positive	Positive	+ Intrauterine*
Group C (n = 10)	Positive	Negative	+ Intrauterine*
Group D (n = 10)	Negative	Positive	+ Intrauterine*

*Two rats served as internal controls and were not given Photofrin.

To better understand the determinants of tissue selectivity, we have systemically investigated the influence of estradiol and drug administration route on the distribution of Photofrin in rat uterine layers. Phase 1 of our studies evaluates the relative merits of intravenous, intraperitoneal, and intrauterine administration. In phase 2, we focus on the effect of estrogen on uptake and retention of the photosensitizer within uterine layers. Our results indicate that excellent drug localization can be achieved at extremely low Photofrin doses, thus suggesting that photodynamic treatment of selected endometrial conditions, such as menorrhagia and dysfunctional uterine bleeding, may be performed with minimal cutaneous phototoxicity.

Material and methods

Phase 1: Effect of route of administration on distribution. Eighty mature female Sprague-Dawley rats, weighing 263 to 330 gm, were placed in a control setting of 12 hours darkness followed by 12 hours light for 1 week. In this phase of the study all 80 rats were suppressed with 0.05 mg/day of subcutaneous leuprolide acetate for 10 days (TAP, North Chicago) followed by intramuscular administration of 500 µg estradiol valerate (Squibb, Princeton, N.J.) (Table I). Twenty-four hours later, 26 rats received 7 mg/kg Photofrin porfimer sodium (QLT, Vancouver) in normal saline solution intravenously and 12 rats received the same dose intraperitoneally. Twenty-five rats received 0.7 mg/kg intrauterine Photofrin. Intrauterine injection was performed through an open laparotomy, and Photofrin was placed in the right uterine horn. The skin was closed with staples, and all rats were housed individually in a dark room. Animals were killed 3, 6, 24, or 48 hours after Photofrin delivery. Seventeen rats that did not receive Photofrin served as controls.

Phase 2: Effect of estrogen on uptake and distribution of Photofrin. Forty mature female Sprague-Dawley rats were divided into four groups (Table 1). One group was used as a control and was neither hormonally

stimulated nor suppressed (group A). The second group (group B) was hormonally suppressed for 10 days with 0.05 mg/day of subcutaneous leuprolide acetate, followed by 500 µg of intramuscular estradiol valerate and 24 hours later by Photofrin. The third group received only leuprolide acetate for 10 days before receiving Photofrin (group C). In the final group (group D) animals did not receive leuprolide and were injected with estradiol valerate only, 24 hours before Photofrin administration. All rats received intrauterine Photofrin at a dose of 0.7 mg/kg, and all were killed by carbon dioxide inhalation 48 hours after Photofrin delivery. Within each group two rats were not given Photofrin and were used as baseline controls for each group. Intracardiac puncture and withdrawal of 3 to 5 ml of blood for estradiol levels were performed, followed immediately by surgical removal of the right uterine horn.

Vaginal smears (phase 1). Monitoring of estrogen suppression was achieved by means of vaginal smears. Rats were not injected with estradiol until after the smears were consistent with a hypoestrogenic state. Suppression was first noted after 5 days but was continued for 10 days to induce prolonged suppression. The 10-day interval was also used in phase 2 of the experiment.

Serum estradiol levels (phase 2). Blood samples obtained by an intracardiac puncture were immediately placed in serum separation tubes and then frozen at -20°C . After all samples were obtained, serum levels of estradiol were determined by direct radioimmunoassay (Pantex, Santa Monica, Calif.). The minimum detectable estradiol concentration was 10 pg/ml. The intraassay and interassay variability was $6\% \pm 1\%$ and $10\% \pm 3\%$, respectively.

Tissue extraction. Extraction of Photofrin from uterine tissue was conducted according to a modified porphyrin fecal extraction technique.¹⁴ After the right uterine horn was dissected, the specimen for frozen section was removed and the remaining portion was placed in a closed opaque container and frozen at -70°C .

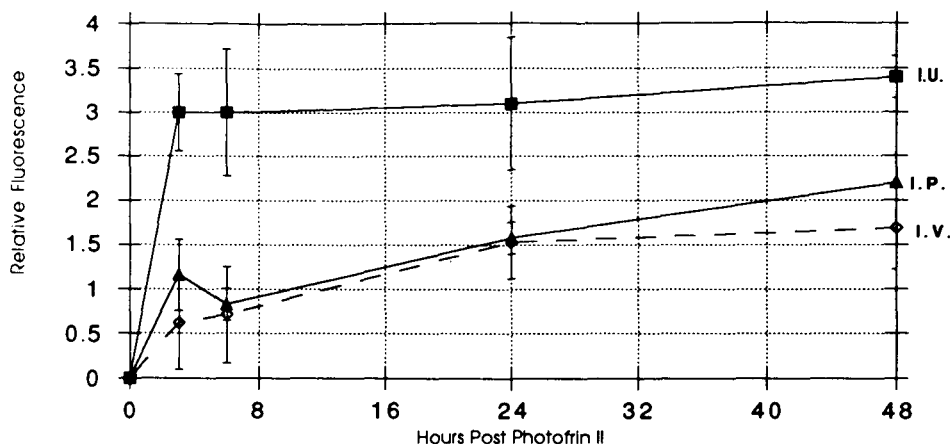


Fig. 1. Phase I. Surface endometrial fluorescence after intrauterine (■), intravenous (○), and intraperitoneal (▲) Photofrin 7 mg/kg.

Samples were lyophilized for 7 days, manually crushed, and weighed.

The samples were then placed in a glass vortex tube, to which 1 ml of 12 N hydrochloric acid was added, and mixed for 5 minutes. To ensure maximal dissolution, the tube was left standing for 45 to 50 minutes, with intermittent mixing. Ethyl ether, 3 ml, was added and the sample was remixed. After this, 3 ml of double-distilled water was added, mixed, and centrifuged for 10 minutes at 10,000 revolutions/min. The resultant mixture consisted of a top organic layer separated from an acid-water layer by a thin lipid-tissue layer. The lower Photofrin-containing layer was removed and analyzed for Photofrin content by means of either absorption or fluorescence techniques. Absorbance measurements were recorded at the absorption maxima (typically 405 to 407 nm). The emission wavelength peak was measured at 610 nm after excitation at 400 nm. Photofrin content was derived from calibration data and expressed as micrograms per gram dry weight of tissue. Drug recovery levels were determined from spiked tissue samples to range from 83% to 90%.

Frozen sections. Tissue samples used for fluorescence analysis were removed from the midportion of the original specimen and immediately placed in molds containing embedding medium for frozen specimens (OCT, Miles, Elkhart, Ind.). The blocks were rapidly frozen on dry ice and stored at -70°C . All specimens were handled in the dark. Tissues were sectioned in low diffuse light (Cryostat microtome, AO Reichert, Buffalo, N.Y.), with slices 6 μm thick taken from three locations, approximately 3 mm apart.

Frozen sections were analyzed for both histologic type and fluorescence. The cross section of the rat uterus was divided into different layers for comparative analysis. The first layer was the surface glandular endometrial cells, and the second layer was the underlying

endometrial stromal cells. The third layer was the myometrium, and the final layer was the serosa.

An epifluorescence microscope (Karl Zeiss, model RA, Oberkochen, Germany) was used for all tissue fluorescence studies. Fluorescence excitation was provided by a 100 W mercury arc lamp, and emission images were recorded by means of a low-light-level video camera (Karl Zeiss, model TV2M intensified newvicon). Video images were stored on one-half-inch VHS tape and analyzed for drug distribution and intensity. To evaluate relative fluorescence intensity in "real time," images were scored on a scale of 0 to 4 where 0 equaled no fluorescence. These levels were assigned by systematically attenuating the excitation intensity with a series of neutral-density filters. Autofluorescence values obtained from drug-free control animals were subtracted from experimental values to obtain a final fluorescence intensity score. This permitted a semiquantitative comparison of both tissue distribution and amount of Photofrin.

Statistics. The significance of differences was tested by means of the unpaired *t* test and two-way analysis of variance.

Results

As shown in Fig. 1, after Photofrin delivery the relative fluorescence of the endometrial glands gradually increased over time, regardless of route of administration. Endometrial fluorescence after intrauterine administration was significantly higher ($p < 0.05$) than with the other two routes of injection, and this was maintained over time (Fig. 2). As seen in Figs. 3 and 4, relative fluorescence of the endometrial stroma and myometrium also increased over time. There was no apparent difference between stromal fluorescence, regardless of route of Photofrin administration, over all time intervals (two-way analysis of variance, F 0.995, not significant). Myometrial fluorescence was significantly

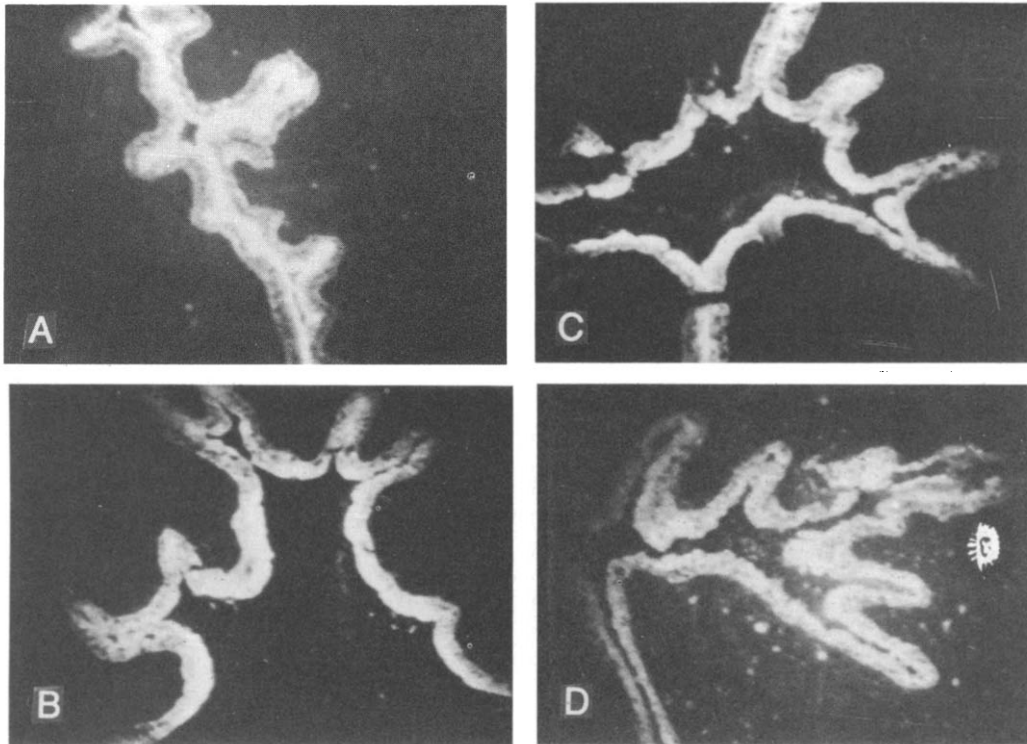


Fig. 2. Fluorescence micrographs of rat uteri after intrauterine administration of Photofrin. **A**, Collapsed surface endometrial layer 3 hours after injection. **B** and **C**, Fluorescence at 6 and 24 hours after injection. Uterine cavity distended because of estrogen stimulated secretions. **D**, Background fluorescence of deeper layers at 48 hours after injection.

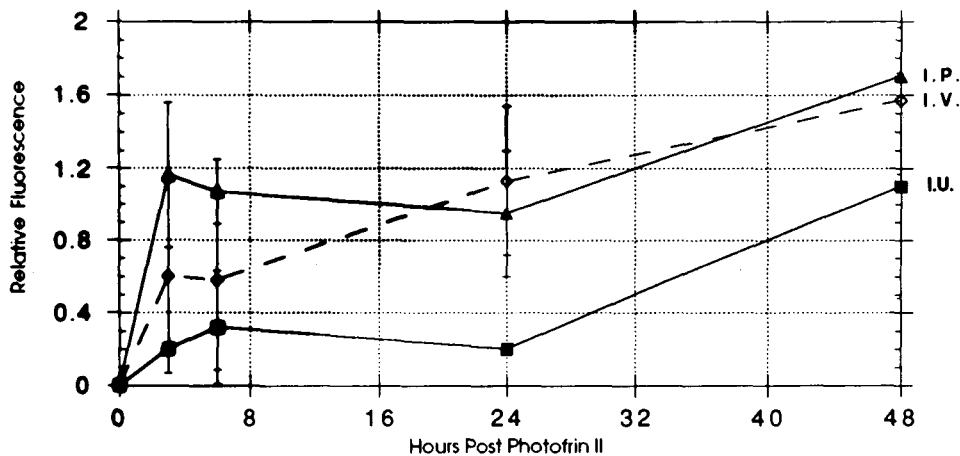


Fig. 3. Phase 1. Endometrial stromal fluorescence after intrauterine (I.U.) (■), intravenous (I.V.) (○), and intraperitoneal (I.P.) (▲) Photofrin 7 mg/kg.

higher after intraperitoneal injection of Photofrin ($p < 0.05$), at 3 hours and 6 hours, and after intravenous administration ($p < 0.05$) at 24 hours and 48 hours, as compared with the intrauterine route (Fig. 4).

As shown in Table II, total porphyrins extracted 3 and 48 hours after intrauterine drug administration (10.45 ± 16.1 , 30.9 ± 20.1) was not significantly different ($p > 0.2$) than that of either the intravenous

or intraperitoneal route (7.4 ± 9.61 , 17.9 ± 18.5 ; 18.57 ± 7.93 , 9.3 ± 8.11). The Photofrin dose used was tenfold higher in both the intraperitoneal and intravenous routes of delivery (7 vs 0.7 mg/kg intrauterine), such that intrauterine delivery resulted in the highest concentration of Photofrin absorbed per milligram of Photofrin.

After analyzing the data from phase one, the second

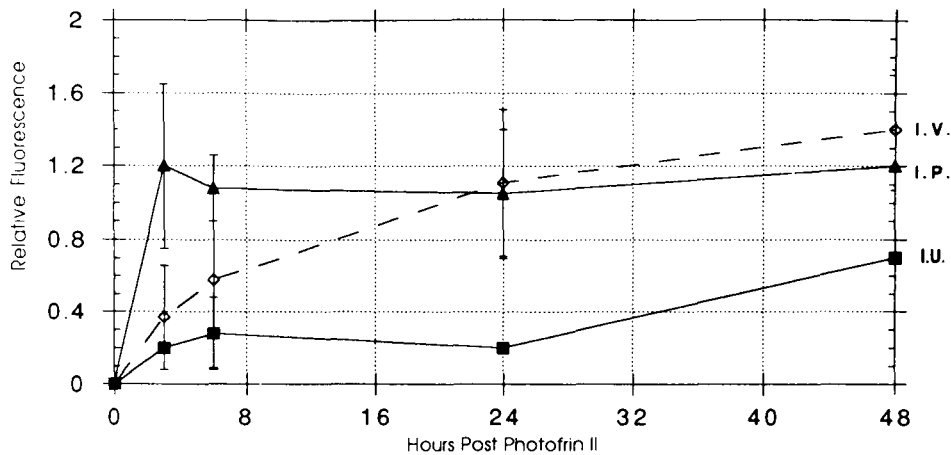


Fig. 4. Phase 1. Myometrial fluorescence after intrauterine (I.U.) (■), intravenous (I.V.) (○), and intraperitoneal (I.P.) (▲) Photofrin 7 mg/kg.

Table II. Extraction of total porphyrins at 3 and 48 hours after administration of Photofrin

Photofrin route	Dose (mg/kg)	Extracted total porphyrins				Significance	
		3 hr		48 hr		3 hr	48 hr
		Mean	SD	Mean	SD		
Phase 1							
Intravenous	7	7.4	9.6	17.9	18.5	p = 0.03*	p = 0.31*
Intraperitoneal	7	18.57	7.93	9.3	8.11		
Intrauterine	0.7	10.45	16.1	30.9	20.1	p = 0.11†	p = 0.18† p = 0.22‡

Values are levels over background, which were obtained from controls.

*Intravenous versus intraperitoneal.

†Intraperitoneal versus intrauterine.

‡Intrauterine versus intravenous.

phase of the experiment, involving estradiol's effect on Photofrin uptake and localization, was performed. As shown in Table III, the average serum estradiol in group A (control, n = 10) was < 10, which was equivalent to that in group C (n = 10). The average serum estradiol in groups B and D (n = 10, each group) was significantly higher (p < 0.05) at 237 ± 95 and 361 ± 108 pg/ml, respectively. There was a significant difference in serum estradiol levels between the two estrogen-stimulated groups (p = 0.01). The average serum estradiol level in rats ranges from < 10 pg/ml immediately after estrus to 20 to 50 pg/ml during estrus.¹⁵ The estrus cycle in the rat is from 4 to 5 days. Endometrial thickness was measured to document end-organ effect. Also shown in Table III, the endometrial depth of the two estrogen-stimulated groups (groups B and D) were 585 ± 132 and 735 ± 155, respectively, which were significantly higher (p < 0.05) than those of the two nonstimulated groups (490 ± 114, 372 ± 60).

Table III. Serum estradiol levels and endometrial thickness at time of death, 48 hours after intrauterine administration of Photofrin

Group	Serum estradiol (pg/ml)	Endometrial depth (μm)
Phase 2		
A (-L, -E)	< 10 (n = 10)	490 ± 114 (n = 7)
B (+L, +E)	237 ± 95* (n = 10)	585 ± 132† (n = 9)
C (+L, -E)	< 10 (n = 10)	372 ± 60 (n = 7)
D (-L, +E)	361 ± 108* (n = 10)	735 ± 155* (n = 7)

Estradiol valerate (E), 500 μg, was administered intramuscularly 24 hours before Photofrin in groups B and D. Leuprolide acetate (L), 0.05 mg/day, was administered subcutaneously for 10 days in groups B and C.

*p < 0.05, compared with groups A and C.

†p < 0.05, compared with group C.

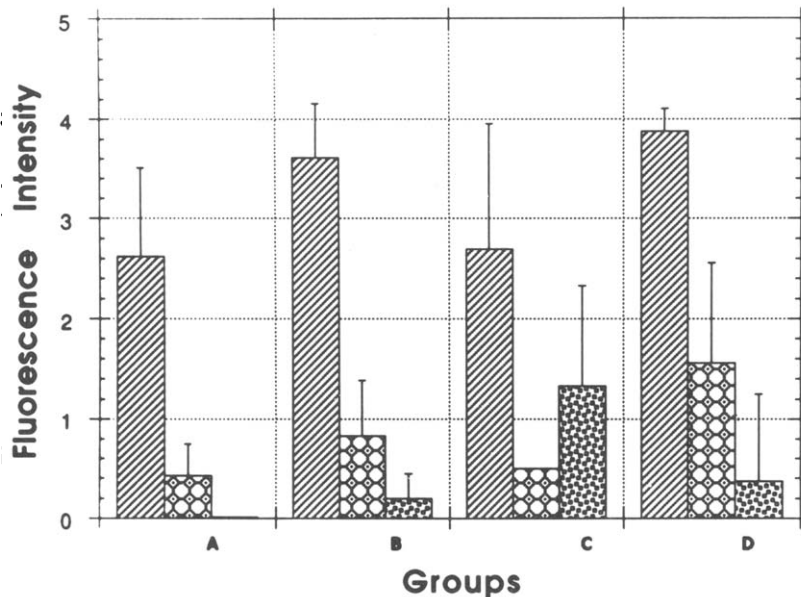


Fig. 5. Phase II. Uterine fluorescence after intrauterine administration of Photofrin (0.7 mg/kg) within the columnar epithelium (▨), endometrial stroma (▩), and myometrium (▧). Group A, Leuprolide or estradiol; group B, leuprolide and estradiol; group C, leuprolide only; group D, estradiol only.

Table IV. Extraction of total uterine porphyrins 48 hours after intrauterine Photofrin (0.7 mg/kg) administration

Group	Phase 2: Total uterine porphyrins ($\mu\text{g/gm dry weight}$)
A	36.5 ± 9.8
B	31 ± 12.9
C	44.4 ± 12.9
D	198.6 ± 118

There was also a significant difference ($p = 0.01$) between the control group and the leuprolide-only (suppressed) group. There was no significant difference ($p = 0.97$) in the endometrial depth of the two estrogen-stimulated groups.

As seen in Fig. 5, the fluorescent intensity of the columnar epithelium after estrogen stimulation (group B 3.61 ± 0.55 , group D 3.88 ± 0.23) is significantly higher ($p > 0.05$) than in the absence of estrogen (group A $2.62 \pm .89$, group C 2.7 ± 1.26). Prolonged leuprolide suppression did not inhibit uptake in the presence of estrogen. After estradiol stimulation there was no significant effect on uptake of Photofrin in the endometrial stroma layer ($p > 0.05$). In spite of this lack of statistical significance, the estrogen-only group had a more diffuse and homogeneous pattern of stromal uptake as compared with the sparse pattern of fluorescence in the other three groups. Comparing the two estrogen-stimulated groups, group D had a significantly higher ($p = 0.04$) uptake of Photofrin. All four

groups consistently showed minimal fluorescence in the myometrium (Fig. 5). Estrogen had no effect on increasing or preventing uptake by the myometrium when there was no prolonged suppression (group A 0, group D 37 ± 0.88 ; $p > 0.05$). Estrogen stimulation after prolonged leuprolide suppression showed a significantly lower level of myometrial uptake, as compared with that of leuprolide suppression alone (group B 0.2 ± 0.25 vs group C 1.33 ± 1.29 ; $p > 0.05$), while increasing uptake within the endometrium ($p = 0.04$).

Extraction of total porphyrins from the rat uteri is seen in Table IV. Higher extracted levels of porphyrin corresponded to a relatively higher fluorescence. The only exception is in the group receiving both leuprolide acetate and estrogen stimulation.

Comment

Our purpose was to evaluate the effects of route of administration and estrogen manipulation on the uptake and distribution of a photosensitizer (Photofrin) in an estrogen-dependent tissue, to develop diagnostic and treatment modalities for endometrial pathologic conditions. Several delivery routes were compared to determine whether site-specific delivery would minimize the systemic side effects of Photofrin while maximizing endometrial uptake. Systemic application of Photofrin inherently involves a higher level of possible adverse reactions, primarily skin photosensitivity. In addition, because the drug distribution predominantly limited to the endometrium (glands and stromal tissue) is desired, an effective delivery system was needed.

After determination of the most effective route, estrogen manipulation was evaluated to determine if the hormone could be used to selectively increase Photofrin uptake or retention within uterine layers. Of concern here is whether states of high proliferative activity are more likely to retain the photosensitizer. Potential recipients of this therapeutic approach would be women with menorrhagia, dysfunctional uterine bleeding, or other endometrial disorders. Because of the high tissue destruction specificity characterized by photodynamic therapy, an endometrial application might replace other surgical approaches.

In phase 1 of the experiment, all animals received the gonadotropin-releasing hormone analog leuprolide acetate, followed by estradiol. This was done to suppress endogenous estrogen secretion and to synchronize the hormonal status of the experimental subjects, followed by endometrial proliferation and neovascularization at the time of photosensitization. Systemically administered (intravenous) Photofrin proved to be the least effective on a per-weight Photofrin-absorbed basis (Table II). Furthermore, intravenously injected Photofrin showed diffuse fluorescence within the uterus, regardless of time killed (Figs. 1 to 4). In spite of the fact that fluorescence increased over time, intravenous injection of the photosensitizer did not appear to promote endometrial selectivity. Myometrial fluorescence uptake (Fig. 4, intravenous injection) was not significantly different ($p > 0.05$) from endometrial uptake, and this lack of significance persisted over time. Higher myometrial fluorescence was observed when all other routes were compared with intrauterine delivery ($p > 0.05$). Intraperitoneally administered Photofrin resulted in a definite pattern of uptake and redistribution within the uterus, as well as a higher overall Photofrin uptake than with intravenous Photofrin (Figs. 1, 3, and 4; Table II). This trend suggested that initially there was a high concentration of the drug in the serosa (data not shown); however, as time elapsed, the fluorescence shifted toward the endometrium. Again, myometrial uptake and retention persisted up to 48 hours, although it was not significantly higher ($p > 0.05$) than intrauterine delivery. It is not clear whether this redistribution was caused by diffusion or absorption into the vascular system and subsequent redistribution. Intrauterine delivery of the photosensitizer appeared to allow for more selective retention within the surface endometrial cells over all time intervals (Fig. 1, $p < 0.05$) and minimized myometrial uptake (Fig. 4, $p < 0.05$). On the basis of fluorescent intensity, the drug remained within the surface endometrial glands with limited diffusion into the deeper stromal layers. Uptake by the endometrial stroma was not significantly different at 48 hours as compared with intravenous administration. However, the relative distribution favors

uptake within the endometrium with limited uptake by the myometrium. It appears the elevated mitotic activity and increased protein production within the surface endometrial cells (glandular) and deeper stromal cells increased the concentration and retention of the drug by cells in these two layers. Finally, in spite of a tenfold reduction in dose, intrauterine injection yielded a significant increase in extracted Photofrin, lending support to the hypothesis that site-specific delivery of the photosensitizer can achieve selective retention of the drug at a much reduced dose (Table II). The distribution of fluorescence after intravenous injection is in partial agreement with previous work by Schneider et al.,¹³ who followed intravenously administered iodine 125-labeled dihematoporphyrin ether in both estrogen-primed and non-estrogen-primed ovariectomized rats. Pharmacokinetic differences may be caused, in part, by our use of Photofrin rather than I¹²⁵-labeled dihematoporphyrin ether.

In phase 2 of the experiment, on the basis of the above findings, all rats received intrauterine photosensitizer. Fluorescent activity within the surface endometrial glands is most prominent in the estradiol-stimulated rats (Fig. 5). There is some fluorescence in the deeper stromal cells, with all groups showing some pockets of bright fluorescence ($p < 0.05$ for group D only). However, except for a more homogeneous distribution and slightly more intense fluorescence in the estrogen-only group, there is no significant difference. Photofrin, however, tended to be excluded from the myometrial layer especially after estrogen stimulation, and this appears to be due to the presence of an active, thicker endometrial layer. In the leuprolide-only group (group C) the endometrial depth is significantly reduced (Table III, $p < 0.05$), which may account for the higher myometrial uptake in this group. There are possible explanations for the prolonged retention of Photofrin within the epithelium. As a relatively hydrophobic compound, once it is inside the epithelial cellular lining, it binds to the metabolically active components within the cytosol (recently stimulated production by estradiol). It is unclear whether estradiol affects the initial uptake. Schneider et al.¹³ noted the distribution of photosensitizer to be similar regardless of estrogen status but that the intensity of the fluorescence was greater in the estrogen-stimulated group. This could account for the higher Photofrin levels after estrogen stimulation. It appears the estradiol effect is indirect and secondary to the end-organ effect. It may also be a diffusion defect, because Photofrin appears not to traverse the tight gap junctions between the epithelial lining. The uptake within the endometrium itself would be greater if this were the case. Most likely, with time the Photofrin will diffuse out of the surface endometrial cells and enter the deeper stromal layer. If the endo-

metrium has been recently stimulated by estradiol, stromal cells and endometrial glands will exhibit prolonged binding of the Photofrin. The distribution pattern of fluorescence within the endometrial stroma may reflect products produced from the breakdown of Photofrin within the surface endometrial cells that diffuse into the stroma, while still retaining their fluorescent photodynamic properties.

Although estradiol stimulation yielded equivocal increases in fluorescence in the endometrial layer (columnar epithelium and stroma), it did increase the overall amount of porphyrins retained within the uterus, indicative of greater photosensitizer uptake. This model system can be used to further the study of a site-delivered system for photodynamic therapy. First, estradiol appears to promote the uptake and retention within the surface endometrial glands. When the photosensitizer enters the deeper endometrium, it appears to be retained longer. Second, this model illustrated that site delivery was successful. This study, in addition to encouraging further preclinical studies with intra-uterine site delivery of light, may lead to the clinical application of photodynamic therapy for endometrial ablation.

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