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Rat reproductive performance following photodynamic therapy with topically administered Photofrin

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A rat animal model was used for comparing the photodynamic efficacy of two formulations of topically administered Photofrin in the uterus: 0.7 mg/kg Photofrin and 0.7 mg/kg Photofrin + 4% Azone, a penetration-enhancing agent. Uterine structure and reproductive performance were evaluated following illumination with 80 J/cm² of 630 nm light. Fluorescence microscopy was employed to determine drug localization in frozen uterine sections at various times after drug administration. Functionality studies demonstrated a significant reduction in the number of implantations per treated uterine horn compared to controls. The mean number of implantations decreased systematically on increasing the interval between Photofrin administration and light application. At 72 h, 0.88 ± 0.52 gestational sacs per rat were recorded with Photofrin therapy, compared with 8.1 ± 1.12 (P = 0.01) on the untreated side, indicating nearly complete loss of reproductive capability. Similar results were achieved after only 3 h treatment with Photofrin+Azone (0.38 ± 0.26 sacs per rat versus 7.5 ± 1.07 on the untreated side; P = 0.01). This indicates that the effect of Photofrin can be enhanced either by extending the drug incubation period from 3 to 72 h or by adding the penetration-enhancing drug Azone. Fluorescence pharmacokinetic studies suggest that both forms of topically administered Photofrin are diffusely distributed throughout the endometrium at virtually the same rate. However, Azone may enhance the selectivity of photodynamic therapy by facilitating drug targeting to critical endometrial structures.

Key words: Azone/photodynamic therapy/Photofrin/reproductive performance

Introduction

Photodynamic therapy (PDT) is an investigational technique which has been applied to human tumours since the mid-1970s (Dougherty *et al.*, 1975; Kelly and Snell, 1976). PDT applications have expanded over the years to include a variety of tumours and hyperplastic diseases. Although the precise mechanism of photodynamic destruction has not been determined *in vivo*, several important features have been identified which have contributed to the rapid growth of PDT.

In general, the process involves i.v. administration of a photosensitizing drug that is retained in well-vascularized tissues. Tissue damage occurs when light of sufficient energy and appropriate wavelength interacts with the sensitizer, leading to the generation of highly reactive oxygen intermediates (Weishaupt *et al.*, 1976; Kimel *et al.*, 1989). These intermediates, primarily singlet molecular oxygen, irreversibly oxidize essential cellular components. Ultimately, selective tissue necrosis is observed in photosensitizer-containing structures (Kreimer-Birnbaum, 1989).

The human endometrium, which has unique proliferation and neovascularization characteristics, may be ideally suited to PDT. For example, PDT can provide a novel, selective means for studying endometrial physiology, particularly regeneration mechanisms. Endometrial tissue, depending on hormonal status, can vary between 2 and 9 mm in thickness (Gonen *et al.*, 1989). Thus, uterine light penetration can be modulated by manipulating the hormonal state. In addition, the substantial effect PDT has on tissue microvasculature (Star *et al.*, 1986; Chaudhuri *et al.*, 1987; Nelson *et al.*, 1988) suggests that it could be highly effective in the well-vascularized endometrium. Finally, from the structural standpoint, the thick myometrium surrounding the thin endometrial layer can serve as a 'barrier' to protect vital pelvic organs during illumination.

Several groups have studied the distribution of photosensitizers in rat (Schneider *et al.*, 1988; Chapman *et al.*, 1993; Yang *et al.*, 1993) and rabbit endometrium (Judd *et al.*, 1992; Bhatta *et al.*, 1992). Promising results have also been reported for the use of PDT to diagnose and treat surgically induced endometriosis in rabbits (Manyak *et al.*, 1989; Petrucco *et al.*, 1990; Vancaillie *et al.*, 1989). We anticipate that the primary clinical application of uterine PDT will be photodynamic treatment of dysfunctional uterine bleeding. In contrast to conventional tumour PDT, endometrial destruction has less stringent requirements for optimizing drug concentrations in target compared with normal tissue. Provided there is sufficient photosensitizer in critical cellular locations, depth of destruction is limited by our ability to deliver an effective photodynamic light dose throughout the endometrium. In general, higher tissue drug concentrations reduce light dose requirements. As a result, it is beneficial to increase endometrial drug concentration to the highest possible value. This can be most effectively accomplished by employing topical, rather than systemic, drug delivery schemes. An

additional benefit of topical application is that, unlike conventional i.v. administration, intra-uterine drug delivery does not result in cutaneous sensitivity. This is a particularly important consideration in managing patients with non-life-threatening conditions.

In previous work, we evaluated the uptake and distribution of Photofrin (Quadra Logic Technologies, Vancouver, B.C., Canada) in uterine tissue following i.v., i.p. and intra-uterine application in rats (Chapman *et al.*, 1993). Our conclusions were that topical intra-uterine application offers several advantages in terms of simplicity, preferential localization and minimal cutaneous toxicity (Dougherty, 1986). However, intra-uterine Photofrin administration results in disproportionately high drug concentrations in the superficial columnar epithelium and relatively slow drug diffusion in deeper endometrial layers.

Because of this observation, the current study was designed to evaluate the therapeutic potential of Photofrin-based PDT in the rat uterus. Particular attention was paid to enhancing the distribution and photodynamic efficacy of topically administered Photofrin by adding Azone, a penetration-enhancing agent (Chow *et al.*, 1984). Fluorescence microscopy of frozen sections was used to determine Photofrin localization in uterine tissue at various times after drug application. Photodynamic efficacy was determined by evaluating uterine structure and reproductive performance following intra-uterine irradiation with 80 J/cm^2 of 630 nm light.

Materials and methods

Animals

A batch of 124 mature female Sprague–Dawley rats weighing 270–325 g were placed in control day/night settings of 12 h each. The oestrous cycle was monitored by obtaining vaginal smears. On the day of di-oestrous, the drugs were applied topically by the intra-uterine route via laparotomy as described previously (Chapman *et al.*, 1993).

Pharmacokinetic study

Photofrin (Porfimer sodium; Quadra Logic Technologies, Vancouver, B.C., Canada) was stored as freeze-dried powder in the dark at 4°C until mixed with sterile water for injection. A volume of 0.15 ml at a dose of 0.7 mg/kg was administered. Azone (1-dodecylazacycloheptane-2-one; Whitby Research, Inc. Richmond, VA, USA) has been used in the past to increase haematoporphyrin derivative (HPD) penetrance in normal skin (Chow *et al.*, 1984). It is a clear colourless liquid that is mixed with Photofrin to make a 4% Azone solution. In all, 36 rats received intra-uterine Photofrin and 32 received an identical dose of Photofrin+Azone (0.7 mg/kg). Both uterine horns were treated.

Animals were killed and the uteri removed for fluorescence microscopy at different time intervals (0.5, 1.5, 3, 6 and 12 h) following drug administration. Data were obtained from three rats at each time point, and one group without any drug was assigned as a control to determine background autofluorescence levels. Three uterine specimens 3–4 mm thick were placed in moulds containing embedding medium for frozen sections (OCT media, Miles, Elkhart, IN, USA). The blocks were rapidly frozen on dry ice and stored at −70°C in the dark. Tissue sections, 6 μm thick, were prepared in low diffuse light (Cryostat microtome; AO Reichert, Buffalo, NY, USA).

Fluorescence microscopy

Low-light-level fluorescence imaging was performed with a slow scan, thermoelectrically cooled, charged coupled device camera system (Princeton Instruments, Trenton, NJ, USA) coupled to a Zeiss Axiovert 10 inverted fluorescence microscope (Carl Zeiss Inc., Germany). A ×10 objective (Zeiss Plan-neofluar, numerical 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) 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) was used to isolate the fluorescence emission.

Instrument control, image acquisition and processing were performed with a MacIntosh IIfx computer and IPlab software (Signal Analytics Corp., Vienna, VA, USA). Sample photodegradation was minimized in bright-field and fluorescence respectively by firstly locating the region of interest under low, orange/red light illumination, and secondly by limiting arc-lamp exposure to 1–2 s by electronically synchronizing camera and lamp shutters. In order to estimate 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 normalized by the following algorithm in order to correct for dark noise and non-uniform illumination:

$$\text{Normalized fluorescence image} = \frac{\text{Mean (background} - \text{dark noise)}}{\text{Image (background} - \text{dark noise)}} \times \text{Image}_{\text{(fluorescence} - \text{dark noise)}}$$

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

Cross-sectional images of the rat uterus were divided into two structurally distinct layers for comparative analysis: endometrium and circular muscle.

Reproductive performance

The second phase of this study was designed to evaluate firstly the effect of PDT on embryo implantation, and secondly whether there is a relationship between drug uptake (as demonstrated by the fluorescence study) and photochemical damage following light application. In all, 24 rats received Photofrin alone and eight rats received Photofrin+Azone. The drugs were administered to one (left) uterine horn in the same mode, volume and concentration as in the first phase. The 'drug–light' interval in the Photofrin-treated animals was 3, 24 and 72 h (eight animals each) and in the Photofrin+Azone group only 3 h. Prior to light treatment, the rats were re-anaesthetized and the left horns carefully exposed. A few millimeters distal to the uterine bifurcation, the uterine wall was punctured with a 20G needle to drain the cavity of traces of remaining fluid and allow passage of the diffusing fibre.

The light source was an argon-pumped dye laser tuned to emit 630 nm radiation (Spectra Physics, Mountain View, CA, USA). A clinical Hartridge Reversion spectroscope (Ealing Electro-

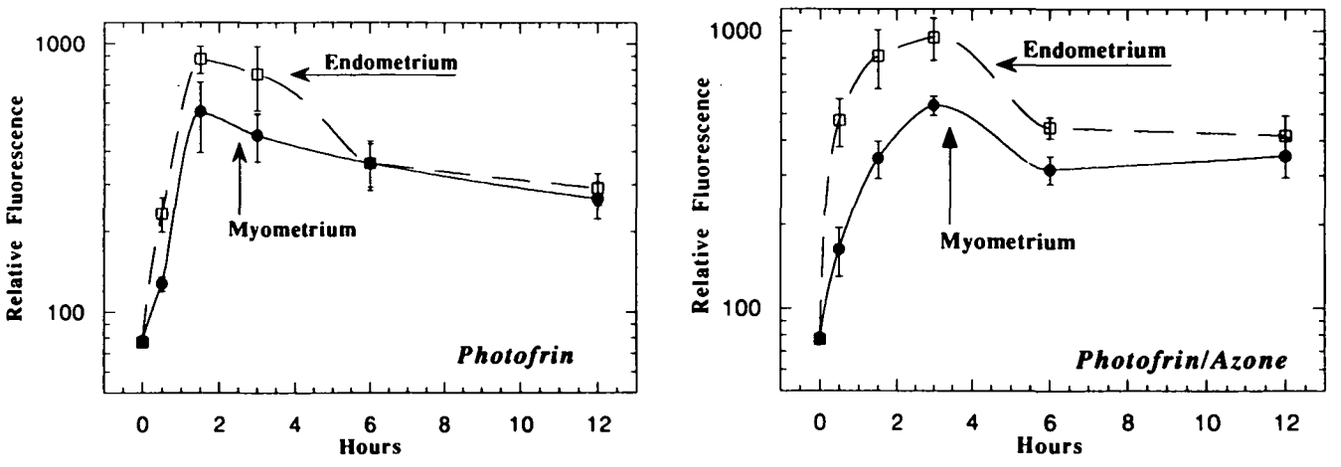

Fig. 1. Fluorescence microscopy curve for Photofrin (left) and Photofrin+Azone (right). Log fluorescence is reported in arbitrary units, as a function of time, for endometrium (excluding columnar epithelium) and myometrium.

Optics, South Natick, MA, USA) was used to verify the wavelength to ± 1 nm. The laser beam was transmitted into the uterine cavity via a fibre with a 2.0 cm long cylindrical diffusing tip (Model 4420-A02, 400 micron core; PDT Systems, Buellton, CA, USA). By using a fibre splitter (model 1220; Laser Therapeutics Inc., Buellton, CA) connected to the laser source by a 200 μm core fibre (model 52120-A05; PDT Systems), two to three animals could be treated simultaneously. All animals received a total illumination dose of 80 J/cm^2 at a power density of 100 mW/cm^2. Before, during and after treatment the uterine horn was moistened with saline to prevent drying. At the end of the laser treatment, the abdominal wall was closed with Dexon 4–0 and the skin secured with staples. Recovery was monitored until return of normal activity. The rats were housed individually with free access to food and water.

In order to assess reproductive performance, rats were bred with mature male Sprague–Dawley rats between 21 and 28 days after PDT. Female rats were killed in the second or third trimester of pregnancy and the entire genital tract was excised and fixed in 10% buffered formalin after photo-documentation. The location and number of nidations were noted for each uterine horn. Specimens of the treated uterine horns were sectioned and stained with haematoxylin and eosin for histological evaluation. Control groups consisted of animals that received photosensitizer without laser irradiation (Photofrin, eight rats; Photofrin+Azone, six rats) and six animals that were treated with laser light and no drug.

Skin photosensitivity

All animals in the study group received a 100 J/cm^2 light dose (200 mW/cm^2) on a 1 cm^2 shaved area of the upper right abdomen. Light was delivered, as above, from a microlens-terminated, 400 μm diameter, fused silica fibre coupled to an argon-pumped dye laser (model 316; Spectra-Physics). The treated skin was checked immediately after light application, during recovery, and daily until the end of the study.

Statistical analysis

The number of gestational sacs in the treated and untreated areas was analysed with a paired two-tailed *t*-test and paired Wilcoxon signed rank test. Implantations with different therapeutic modalities were analysed using the Kruskal–Wallis test and the Mann–Whitney *U*-test. Results were considered to be statistically significant at $P < 0.05$. Cited results refer to the Wilcoxon signed rank test and the Mann–Whitney *U*-test respectively.

Results

Pharmacokinetic study

Figure 1 summarizes the results of frozen-section fluorescence microscopy studies for Photofrin (Figure 1a) and Photofrin+Azone (Figure 1b). Log fluorescence is reported in arbitrary units, as a function of time, for endometrium (excluding columnar epithelium) and myometrium. Each time-point represents average values for three animals, with the following exceptions: two animals at time-points 0.5 and 3 h in the Photofrin and Photofrin+Azone groups respectively, and four animals at time-points 6 and 12 h in the Photofrin+Azone group. The results indicated there was a sharp increase in drug uptake for endometrial and myometrial layers within the first 90 min of topical application. Fluorescence levels were higher for endometrium than for myometrium at early time-points, particularly in the case of Photofrin+Azone (Figure 1b). However, these values eventually converged and fluorescence remained elevated for both drugs at 12 h.

These observations were supported by representative frozen-section images of tissue treated with Photofrin+Azone (1.5 h post-injection), as shown in Figure 2. Examination of bright-field (Figure 2a) and fluorescent (Figure 2b) micrographs clearly showed the previously observed intense columnar epithelial fluorescence (Chapman *et al.*, 1993). In contrast, endometrial and myometrial fluorescence levels were substantially lower. However, as indicated in Figure 1, endometrium appeared to be brighter than the surrounding myometrium. Although Photofrin tissue distribution is not shown, the results were generally similar to Figure 2.

Reproductive performance following PDT

Figure 3 shows that, in all study groups, there was a significant difference between the number of implantations per rat in the

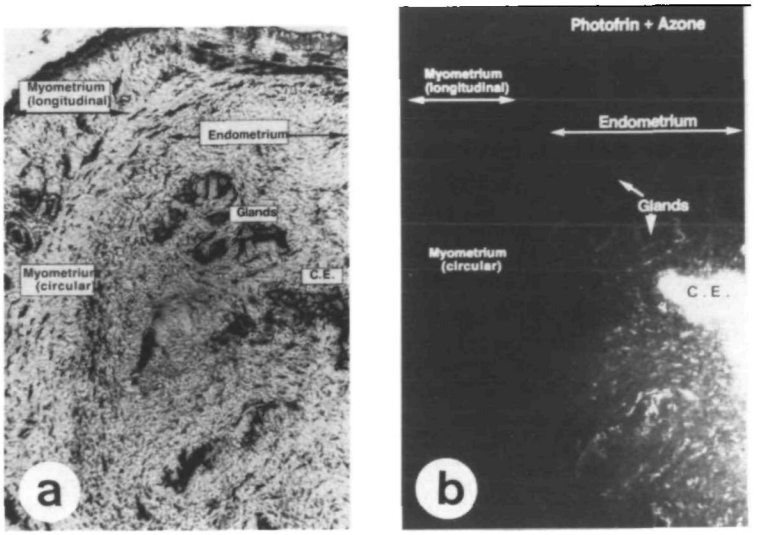

Fig. 2. Bright-field image (**a**) and fluorescence micrograph (**b**) of the rat uterus at 1.5 h following topical application of Photofrin with 4% Azone. CE = columnar epithelial fluorescence.

treated left uterine horn compared with the corresponding, untreated control right side ($P < 0.05$). A more effective outcome with Photofrin as a single agent was obtained by extending the time interval between the drug administration and the light application to 72 h. In the case of Photofrin, the mean number of gestational sacs per uterine horn was 2.38. The mean ± SE was 2.38 ± 0.92 in the treated side and 5.12 ± 0.74 in the control side ($P = 0.04$) at 3 h. The 24 h interval resulted in 1.5 ± 0.68 implantation sites for the left side and 5.9 ± 0.88 for the control ($P = 0.02$); at 72 h the corresponding values were 0.88 ± 0.52 compared with 8.1 ± 1.12 ($P = 0.01$). The difference between 3 and 72 h was significant ($P < 0.02$).

In the case of Photofrin+Azone, the mean ± SE number of gestational sacs per treated area was 0.38 ± 0.26 in the treated side and 7.5 ± 1.07 in the untreated side ($P = 0.01$). The Photofrin+Azone combination was more effective than Photofrin alone in the short (3 h) interval ($P = 0.02$). No significant difference was observed between the number of implantations per treated horn for 3 h Photofrin+Azone and 72 h Photofrin. Figure 4 clearly shows the impact of PDT on reproductive performance. Multiple gestational sacs were visible in the control horn and one sac appeared in the distal end of the photodynamically treated left horn. The single pregnancy in the treated side (Photofrin+Azone, 3 h) was located in an area that was exposed to a reduced light intensity. This artefact of fibre geometry and placement was presumed to result in the delivery of a sub-threshold light dose for irreversible photodynamic damage.

In the three control groups (two drug combinations with no light and light without drug), there was a tendency for fewer implantations in the manipulated horn, though the difference was not statistically significant. PDT was tolerated well by all rats. No signs of any adverse effects were noticed during treatment, recovery, or the subsequent pregnancy.

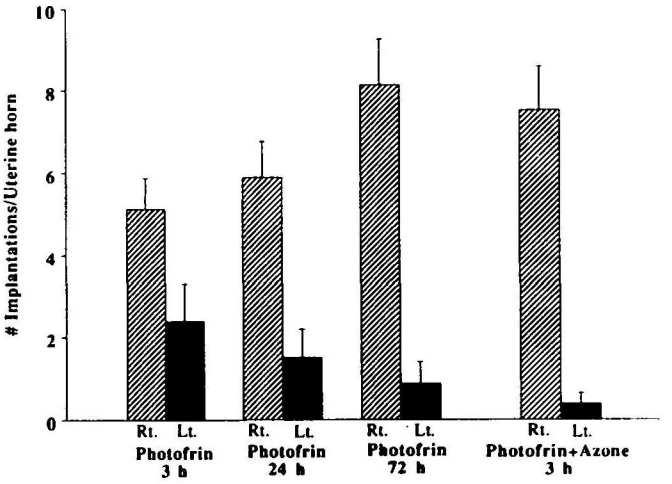

Fig. 3. Number of gestational sacs implanted in the treated area of the left uterine horns (solid bars) and the corresponding, untreated control right sides (hatched bars).

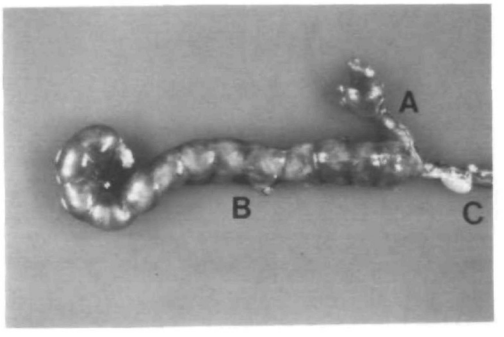

Fig. 4. Rat uterus with multiple gestational sacs in the control horn and a single sac in the distal end of the left uterine horn, which received photodynamic therapy (PDT). A = PDT horn, B = control horn, C = cervix.

Morphological changes after PDT

Immediately following light application there was a blanching effect of the treated area. By 6–8 weeks later the treated left uterine horn of most animals showed signs of atrophy, with various degrees of decreased diameter. Two animals in the Photofrin+Azone group with marked atrophy had pseudo-cystic

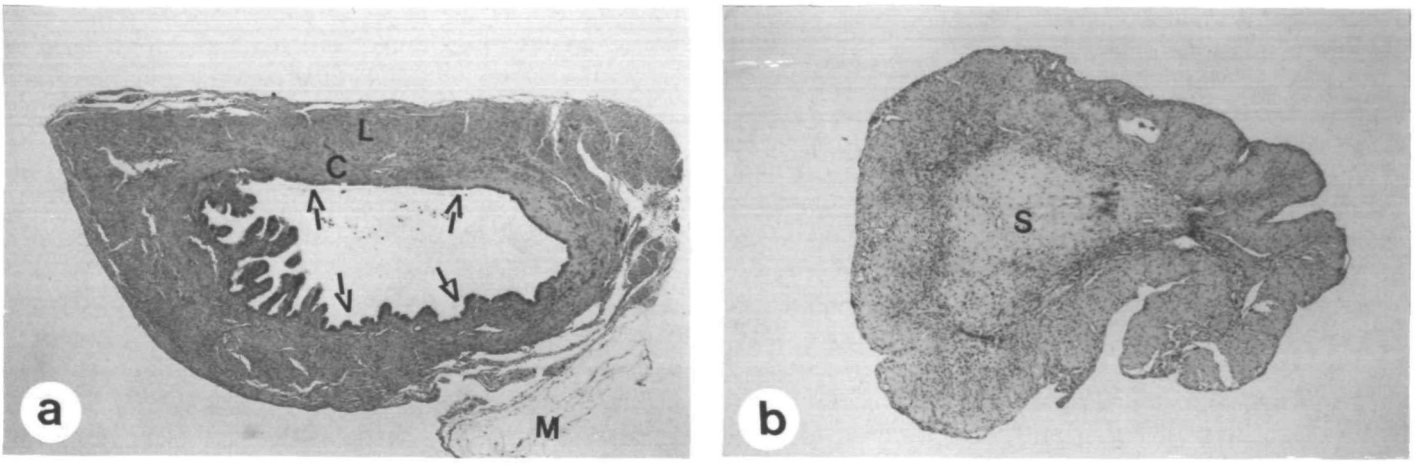

Fig. 5. Cross-sections of rat uterus (haematoxylin and eosin stained, ×10). (**a**) 72 h interval between Photofrin and light, the rat having been killed 5.5 weeks post-photodynamic therapy (PDT). Endometrial stroma is destroyed on the right side of the specimen. On the left side, the endometrium is well-stimulated, indicating insufficient damage (arrows). (**b**) 3 h interval between the administration of Photofrin+Azone and illumination, the rat having been killed 7 weeks post-PDT. This treatment resulted in destruction of the entire endometrium, it being replaced by scar tissue. S = scar tissue, L = longitudinal muscle, C = circular muscle, M = mesometrium.

dilatation of the distal part of the uterine horn. No morphological changes were detected in the controls. Taking into consideration the time-consuming manipulations of the left uterine horn during treatment, we found surprisingly few adhesions of any clinical significance. Even the peritoneal serosa covering the treated region of the uterus was smooth and unchanged. However, at the site of fibre insertion into the uterus, there were some forms of mild filmy adhesions in 86% of the PDT animals and the laser controls. No uterine perforation was observed.

Skin photosensitivity

Skin photosensitivity was not observed in any of the treated animals at any time during follow-up.

Structural damage

In order to determine the extent of structural damage, we examined rat uteri from each group several weeks after PDT. Figure 5 shows haematoxylin/eosin-stained sections (× 10) for 72 h Photofrin (Figure 5a) and 3 h Photofrin+Azone (Figure 5b) at 5.5 and 7 weeks post-PDT respectively. The endometrium was destroyed on the right side of the specimen in Figure 5a, as indicated by the arrows. Only columnar epithelium was left, along with the circular and longitudinal muscle layer and peritoneal serosa. On the left side the endometrium was well stimulated, indicating insufficient damage. Uneven damage was occasionally observed, regardless of time-point, and may have been the result of inhomogeneous light delivery to the tissue. In Figure 5b, Photofrin+Azone treatment resulted in destruction of the entire endometrial stroma. There was substantial scar tissue formation which effectively replaced the lumen.

Discussion

The limited information available on the use of PDT for selective endometrial ablation is based mainly on systemic Photofrin studies (Schneider *ét al.*, 1988; Chapman *et al.*, 1993). In our estimation, the most likely candidates for uterine PDT are women with benign endometrial disorders leading to dysfunctional uterine bleeding. In order to treat these cases successfully, drug concentrations should be as high as possible throughout the endometrium. For a given optical dose (provided it is above a threshold value), high drug concentrations permit damage to greater depths, an important requirement for complete destruction of the endometrial zone. In order to achieve comparable effective drug concentrations using i.v. administration, patients would be subjected to the unacceptable risk of skin photosensitivity, which is the main side-effect for PDT and a limiting factor for its clinical application. As a result, we have investigated topical administration strategies for photodynamic destruction of endometrial tissue.

In previous work (Chapman *et al.*, 1993), we demonstrated that intra-uterine administration of Photofrin is feasible; however, it resulted in relatively high uptake and retention by the superficial columnar epithelium, and slow penetration to deeper uterine layers. In this study, we attempted to expand our earlier findings by improving Photofrin tissue distribution and photodynamic efficacy with Azone, a penetration-enhancing agent. Azone has been used with both hydrophobic and hydrophilic molecules. Optimum concentration can vary, but 1−5% Azone solutions appear to be appropriate for most formulations (Chow *et al.*, 1984).

Pharmacokinetic studies of the two drug formulations (Figure 1) showed similar fluorescence patterns. There was a rapid rise in fluorescence, followed by a plateau at about 1.5−3 h, during which maximum contrast between endometrial stroma and circular myometrium was observed. Drug levels persisted for the entire 12 h observation period. Examination of Figure 1b indicates that the Photofrin+Azone combination may have had slightly greater endometrial selectivity. Overall, the pharmacokinetics and structural distribution were quite similar for both formulations. These findings are clearly illustrated in the frozen-section images (Figure 2). In addition, this figure

shows that the columnar epithelium does not appear to inhibit Photofrin+Azone diffusion substantially.

Nevertheless, due to the extremely high columnar epithelium fluorescence, it was difficult to determine the precise impact of Azone on Photofrin penetrance. Fluorescence microscopy-based pharmacokinetic studies provide visual evidence of drug localization but are not necessarily predictive of structural damage during PDT. This is particularly true for Photofrin, since it is a mixture of compounds which vary in photodynamic efficacy, subcellular localization and fluorescence quantum yield. In addition, Photofrin's most photoactive components are probably least fluorescent. Ultimately, photo-damage is a consequence of drug association with oxidizable molecules and critical structures in cells and organelles. This information is difficult to infer from frozen-section fluorescence micrographs.

The reproductive performance study, illustrated in Figures 3 and 4, is a better indicator of functional photodynamic damage. These results show that the number of implantations per treated area was significantly reduced in all treated uterine horns. Substantially impaired reproductive performance was demonstrated for Photofrin PDT at all time-points (drug−light intervals: 3, 24 and 72 h), as well as for 3 h Photofrin+Azone. However, implantations decreased significantly when the Photofrin drug−light interval was increased from 3 to 72 h, indicating that treatment efficacy increased with incubation time. Interestingly, 72 h Photofrin results were comparable to those obtained for animals in the 3 h Photofrin+Azone group. These observations suggest that the primary consequence of combining Photofrin with Azone may be to enhance the localization of 'active' Photofrin components in essential, oxidizable cellular structures. Without Azone, the systemic contribution of residual recirculating Photofrin at 72 h may play a role in enhancing efficacy. In addition, Photofrin fluorescence at 24 and 72 h may be derived from more photodynamically active components (compared to 3 h), since the spatial distribution of fluorescence appears to be the same for both formulations regardless of time.

Evaluation of long-term structural effects shows similar damage patterns for 3 h Photofrin+Azone and 72 h Photofrin. Endometrial stroma was clearly destroyed using both formulations. Only columnar epithelium, muscle and peritoneal serosa remained post-irradiation. Both formulations resulted in substantial scar tissue formation which, in the case of Photofrin+Azone, replaced the lumen. However, our occasional observation of uneven damage, regardless of time-point, underscores the importance of uniform light delivery. Endometrium can easily regenerate if the photodynamic damage threshold of the tissue is not exceeded. Regenerating tissue can originate in a single location and eventually proliferate throughout the entire uterine cavity. This observation is further confirmed by pregnancy study results using 2 cm long diffusing fibre tips. Since the uteri were somewhat longer than 2 cm, regions proximal and/or distal to the tip were exposed to light at a reduced intensity (Figure 4). These areas of sub-threshold irradiation exhibited more pregnancies as compared with the fully irradiated uterine regions.

We conclude that the penetration-enhancing drug Azone can be used to modulate the efficacy of topically applied Photofrin during uterine PDT. The mechanism of action is not clear solely from the results of fluorescence-imaging, pharmacokinetic studies. However, when these observations are considered in conjunction with tissue histology and post-irradiation pregnancy tests, we surmise that Azone facilitates the delivery of photoactive Photofrin components to critical cellular structures. Combining Photofrin with Azone may be of practical use since it seems to permit earlier treatment following topical administration of the drug. The use of effective, topical sensitizers is desirable due to the fact that they involve relatively low doses and do not cause skin photosensitivity. However, selectivity and tissue damage must be carefully evaluated using topical applications, since drug localization mechanisms and photodynamic targets are probably different from those associated with i.v. administration strategies.

In order to optimize topical endometrial PDT it is therefore essential to evaluate several factors. In the case of human applications, where large, irregularly shaped uterine cavities are encountered, particular attention should be paid to drug and light distribution in the tissue. Uterine tissue optical properties should be determined in order to design delivery systems which can uniformly illuminate the entire cavity. Adequate illumination should result in light levels that are above the threshold photodynamic dose for a given drug. Finally, animal model studies designed to measure a functional endpoint, such as the contraceptive effect of PDT, should be used to compare different photosensitizers. These issues must be considered in order to ensure a long-term effect in human tissue.

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