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Fluorescence detection of cervical intraepithelial neoplasia for photodynamic therapy with the topical agents 5-aminolevulinic acid and benzoporphyrin-derivative monoacid ring

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OBJECTIVE: The aim of this study was to determine whether 2 photosensitizers, benzoporphyrin-derivative monoacid ring and 5-aminolevulinic acid, are selectively absorbed by dysplastic cervical cells after topical administration.

STUDY DESIGN: This phase I clinical trial involved 18 women with biopsy-proven cervical intraepithelial neoplasia at the Beckman Laser Institute, Irvine, Calif. Colposcopically directed cervical biopsy specimens obtained after 1.5, 3, or 6 hours of exposure to a randomly assigned photosensitizer were evaluated for selective drug absorption with hematoxylin and eosin staining and fluorescence microscopy.

RESULTS: After exposure to 5-aminolevulinic acid, cervical tissue showed maximal fluorescence in dysplastic cells relative to normal cells, with negligible stromal fluorescence. According to our detection methods benzoporphyrin-derivative monoacid ring demonstrated nonselective, diffusion-driven uptake, with fluorescence appearing in the superficial cells, followed by nonselective drug absorption in the remaining cells and stroma of the epithelium.

CONCLUSION: Our data demonstrated selective absorption of 5-aminolevulinic acid by dysplastic cervical cells. This agent therefore represents a promising photosensitizing prodrug for the treatment of cervical intraepithelial neoplasia with photodynamic therapy. (Am J Obstet Gynecol 2001;184:1164-9.)

Key words: 5-Aminolevulinic acid, benzoporphyrin-derivative monoacid ring, cervical dysplasia, cervical intraepithelial neoplasia, cervix, photodynamic therapy, protoporphyrin IX

While routine cervical screening has led to an impressive decrease in the incidence of invasive cervical cancer, there has been a marked increase in the detection of preinvasive cervical disease.[1] Outpatient modalities currently used to treat premalignant lesions in the United States include cryotherapy, carbon dioxide laser ablation, and electrosurgical excision. Although the success rates of these techniques approach 90%,[2-8] they have been criticized for a number of reasons, including distortion of cervical anatomy, excessive destruction of cervical tissue, need for regional or general anesthesia, and possible side effects, such as cervical stenosis or incompetence, increased vaginal bleeding and discharge, and decreased production of endocervical mucus.

Photodynamic therapy consists of intravenous or topical administration of a photosensitizing drug that is selectively absorbed by malignant or premalignant cells. On activation by laser light at appropriate wavelengths, the intracellular drug generates highly reactive oxygen intermediates.[9] These intermediates irreversibly injure subcellular structures, including the plasma membrane and mitochondria, resulting in cell death and tissue necrosis.[10]

Photodynamic therapy is under investigation for a variety of clinical applications.[11] Potential advantages of photodynamic therapy for cervical disease relative to conventional treatments include the possibility that it could eliminate intraepithelial lesions without causing profuse bleeding, vaginal discharge, or a change in the location of the squamocolumnar junction. It is also possible that large or multifocal lesions or those lesions that extend into the endocervical canal could be targeted through selective drug uptake while sparing adjacent normal cervical tissue. A number of published studies have investi-

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gated the role of photodynamic therapy in the treatment of cervical dysplasia. Wierrani et al[12] observed cytologic improvement in the grading of Papanicolaou smears in 19 of 20 patients with biopsy-proven low-grade lesions (human papillomavirus and cervical intraepithelial neoplasia [CIN] I) and CIN II after photodynamic therapy with topically applied aminolevulinic acid (ALA).[12] Monk et al[13] reported that 68% of 24 patients with CIN (grades I to III) were disease free 12 months after photodynamic therapy after 24-hour exposure to topical Photofrin with subsequent illumination with an argon-pumped dye laser at 635 nm. Finally, Muroya et al[14] treated 56 patients with cervical dysplasia and carcinoma in situ with photodynamic therapy with a systemically administered photosensitizer followed by laser with an excimer dye laser. After 10 months of follow-up 96.4% of the patients had complete resolution of the cervical disease. Conversely, Hillemanns et al[15] found that 7 patients with high-grade squamous intraepithelial lesions had persistent disease after photodynamic therapy with 5-ALA and laser illumination with an argon-pumped dye laser. In their clinical trial the 7 patients were treated with 10 cycles of photodynamic therapy with an energy density of 100 J/cm^2 and a power density of 100 to 150 mW/cm^2. In addition, a cylindric applicator was used to treat the endocervical tissue, as well as the ectocervix. The contradictory results reported in the studies cited here probably reflect differences in treatment regimens, including different light doses, power densities, photosensitizing agents, drug application times, treatment schedules, and laser equipment, as well as the decision whether to include the endocervical tissue in addition to the ectocervical tissue when the cervix is illuminated. Once the ideal treatment parameters have been clarified, photosensitization of dysplastic cervical cells followed by photodynamic therapy could represent a new, nontoxic, nonsurgical ablative approach to the treatment of CIN.

The 2 second-generation photosensitizing agents that we chose to study in cervical tissue, 5-ALA and benzoporphyrin-derivative monoacid ring (BPD-MA), offer advantages relative to other agents, such as increased tissue penetration after topical administration, chemical purity, excitation at wavelengths >650 nm (BPD-MA), and rapid clearance from normal tissue. BPD-MA is synthetically derived from protoporphyrin IX dimethyl ester,[16-20] whereas 5-ALA is a prodrug that is converted intracellularly to protoporphyrin IX, which is the active, endogenous, photosynthetic compound in the biosynthesis of hemoglobin.[16]

The purpose of this study was to determine whether 5-ALA or BPD-MA would be selectively absorbed by dysplastic cervical cells relative to normal cervical tissue after topical administration to the cervix in women with biopsy-proven high-grade CIN. The selective uptake in preneoplastic cells would support further study of photodynamic therapy for the treatment of CIN through clinical trials.

Material and methods

After approval was obtained from the Food and Drug Administration and the institutional review board, 18 women with biopsy-proven CIN II or III were invited to participate in the study. Informed consent was obtained, and the patients were randomly assigned to receive either 1.5, 3, or 6 hours of exposure to either 5-ALA or BPD-MA (3 patients for each time interval with each drug). Because BPD-MA is metabolized in the liver, liver function tests were obtained before treatment for the patients who were randomly assigned to receive BPD-MA to avoid toxicity.

Each patient underwent colposcopy to confirm that the area of abnormality that had previously been documented by biopsy was still present. A cervical cap was then placed on the cervix with either 5-ALA (200 mg/mL) or BPD-MA (2 mg/mL) that had been reconstituted in Hyskon (Pharmacia, Inc, Peapack, NJ). Hyskon is a viscous, hydrophilic branched polysaccharide that is often used for uterine distention during hysteroscopy. In several previous studies we have demonstrated the feasibility of using this viscous fluid as a potential solvent for the topical application of BPD-MA and 5-ALA.[21-23] The doses were chosen after a careful review of the literature that evaluated different doses used in various animal and human models.

Although the volume placed on each cervix varied from 2 to 4 mL according to the cervical size and the corresponding cap required, the concentration of each agent, which determines drug uptake, was held constant. A small piece of gauze was placed in the bottom of each cervical cap to improve contact of the cervix with the drug.

After 1.5 hours all cervical caps were removed to avoid vacuum-related interference with blood supply to the area surrounding the transformation zone. The adherent solvent, Hyskon, in which the prodrug 5-ALA was dissolved, allowed long-term exposure of the cervix to the drug. Those patients randomly assigned to 1.5 hours of exposure underwent a cervical biopsy at the colposcopically defined area corresponding to the high-grade cervical intraepithelial lesion at this time. These patients were then treated conventionally with large loop excision of the transformation zone. Those patients randomly assigned to 3 or 6 hours of exposure similarly underwent cervical biopsy followed by large loop excision of the transformation zone but after the appropriate time had elapsed.

The biopsy specimens were immediately snap-frozen in 2-methylbutane (Fisher Scientific Worldwide, Laboratory Projects Division, Springfield, NJ) over liquid nitrogen oriented in molds containing embedding medium for frozen sections (Tissue Tek OCT media; Miles Inc,

Elkhart, Ind) 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; Reichert Ophthalmic Instruments, A Division of Leica Microsystems Inc, Buffalo, NY) to obtain 6-μm-thick slices for fluorescence analysis. One slide from each patient was stained with hematoxylin and eosin to ensure that the proper CIN area was processed for low-light fluorescence microscopy.

Low-light fluorescence microscopy was performed with the slow-scan, thermoelectrically cooled, charge-coupled device camera system (Princeton Scientific Instruments, Inc, Monmouth Junction, NJ) coupled to a Zeiss Axiovert 10 (Carl Zeiss, Oberkochen, Germany) inverted fluorescence microscope. A ×10 objective (Zeiss Plan-neofluar, numeric aperture of 0.3) was used to visualize bright-field and fluorescence images of the frozen sections. A 100-W mercury arc lamp that was filtered through a 405-nm bandpass filter (20 nm bandwidth; Omega Engineering, Inc, 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 bandwidth, Omega Optical, Inc, 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 Power PC 8600/300 (Apple Computer, Inc, Cupertino, Calif) computer and software (Scanalytics, Inc, Fairfax, Va). Sample photodegradation was minimized by limiting sample exposure to excitation to 2 seconds by electronically synchronizing camera and lamp shutters (Uniblitz model T132; Vincent Associates, Rochester, NY). 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 and dark noise contribution was corrected for according to the following algorithm: Corrected fluorescence image = (Fluorescence image – Dark noise)/(Background image – Dark noise) × (Mean corrected background image), where mean corrected background image is the mean gray-scale value for the dark noise–corrected background image.

After fluorescence microscopy, the same frozen sections were stained with hematoxylin and eosin and reviewed by a pathologist for histologic diagnosis so that a direct comparison could be made between the grade of CIN and the degree of fluorescence. Fluorescence microscopy and hematoxylin and eosin staining were performed by 2 different evaluators who were blinded to the pathologic diagnosis and the photosensitizer used. All cervical specimens were evaluated by a pathologist to ensure that the lesion was included in the specimen.

Results

All patients tolerated the procedure well and did not have any side effects from either 5-ALA or BPD-MA. The cervical tissue exposed to 5-ALA consistently showed greater fluorescence in the dysplastic cells than in the normal cells and stroma. Fig 1 shows comparative fluorescence microscopic views and hematoxylin and eosin stains of cervical biopsy sections taken from 3 different patients after 1.5, 3, and 6 hours of exposure to 5-ALA. The CIN in the hematoxylin and eosin staining view *(arrows)* of each specimen (Fig 1, *b, d,* and *f*) corresponded exactly to the location of the brightly fluorescent dysplastic cells in the fluorescence microscopic view of the same section (Fig 1, *a, c,* and *e*). This observation persisted when the cervical biopsy was performed after 1.5, 3, or 6 hours of exposure to 5-ALA. There appeared to be preferential uptake of 5-ALA or conversion of 5-ALA to protoporphyrin IX by the dysplastic cells while sparing normal cervical cells throughout the epithelium at all time points examined.

In contrast, BPD-MA absorption and penetration by cervical intraepithelial neoplastic cells were not apparent in the fluorescence micrographs after 1.5 and 3 hours of exposure (Fig 2, *A* and *C, arrows*). However, there appeared to be some penetration and selective absorption after 6 hours of exposure (Fig 2, *E, arrows*). The cervical intraepithelial neoplasia seen in the hematoxylin and eosin staining images from these patients did not correspond with the brightly fluorescent cells present on the epithelial surface in the fluorescence microscopy of those specimens after 1.5 or 3 hours of exposure to BPD-MA (Fig 2, *B* and *D*). At 6 hours fluorescence was seen throughout the entire epithelium, with some increase in intensity in areas that corresponded to CIN; however, the intensity was not as marked, nor did it appear to correspond exactly with the CIN as was seen with 5-ALA (Fig 2, *E* and *F*). As the exposure time increased, the fluorescence appeared to spread from the superficial layer toward the epithelial basement membrane, exemplifying diffusion-driven drug delivery, where the depth of penetration is related to the contact time. It is possible that the delay in fluorescence could be related to intracellular proteins that inhibit fluorescence for some time.

Comment

Photodynamic therapy for cervical dysplasia has been evaluated in the past with different photosensitizers and different lasers. Dihematoporphyrin ether administered intravenously in earlier studies resulted in significant cutaneous photosensitivity, whereas topical administration required longer application times than the photosensitizers evaluated in this study.[12-15] The advantages of using newer, second-generation agents such as 5-ALA and BPD-MA include rapid plasma and tissue clearance, increased selectivity in dysplastic cells, and improved light absorp-

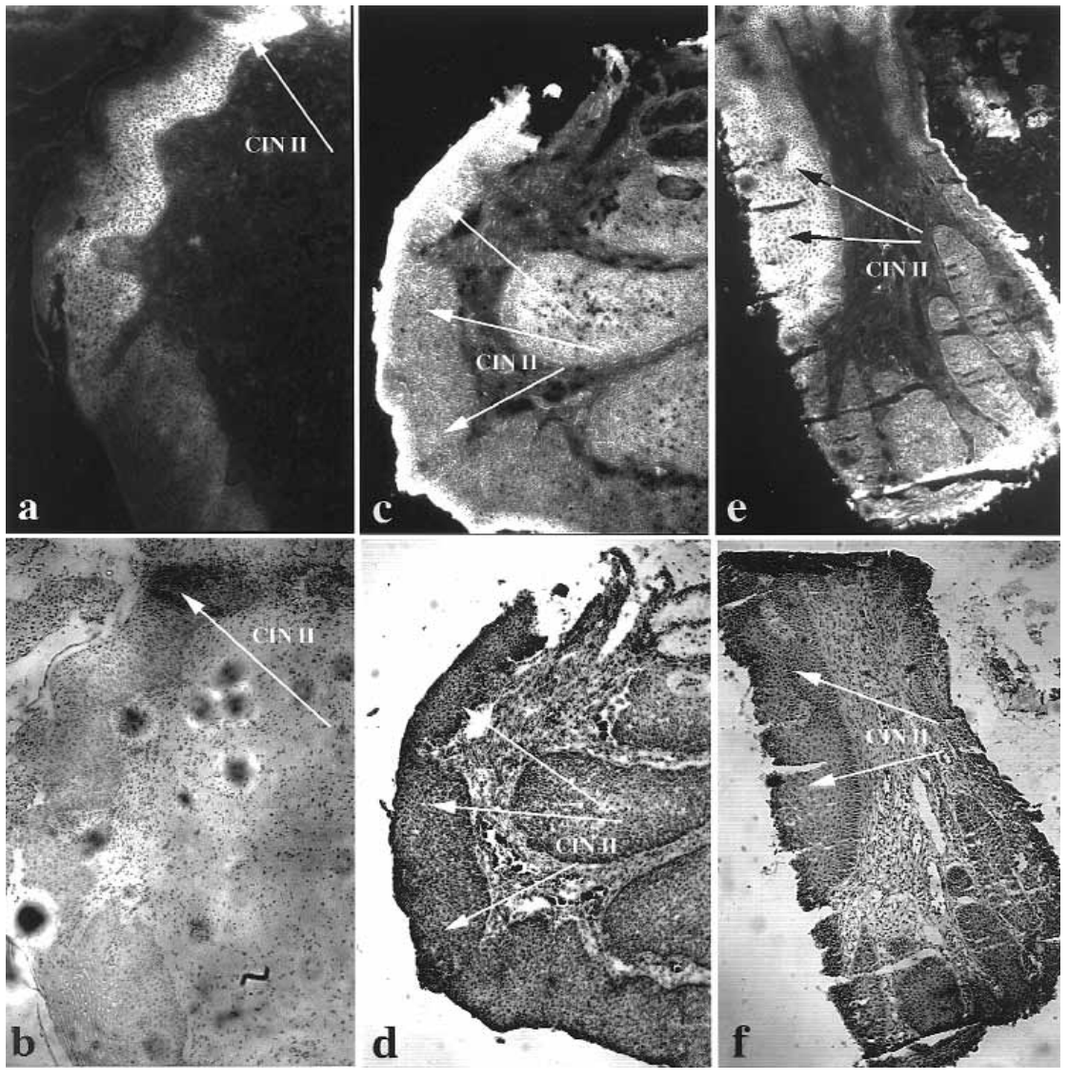

Fig 1. Fluorescence microscopy **(a, c,** and **e)** and hematoxylin and eosin staining images **(b, d,** and **f)** of cervical biopsy sections obtained from 3 different patients after exposure to 5-ALA for 1.5 hours (patient 1, **a** and **b**), 3 hours (patient 2, **c** and **d**), and 6 hours (patient 3, **e** and **f**). *Arrows* point to corresponding areas of CIN in fluorescence microscopy and hematoxylin and eosin staining images.

tion in the visible red and near-infrared region of the electromagnetic spectrum. BPD-MA is activated at 690 nm, and 5-ALA (after its conversion to protoporphyrin IX) results in effective photosensitization at excitation wavelengths of 630 to 635 nm.

Our data suggest that the prodrug 5-ALA undergoes a more rapid conversion to protoporphyrin IX in abnormal cells (as seen with the higher fluorescence of dysplastic cells) than in normal cells after 1.5, 3, or 6 hours of exposure to 5-ALA. The selective absorption of 5-ALA or rapid conversion of 5-ALA to protoporphyrin IX at the shortest duration, 1.5 hours, has clinical relevance because it would allow photodynamic therapy to be performed quickly after drug application, thus reducing total treatment time. In addition, treatment after 1.5 hours of exposure could minimize the destruction of the adjacent normal cells, because fewer would have absorbed 5-ALA and converted it to protoporphyrin IX. Pahernik et al[24] confirmed increased uptake in CIN lesions relative to normal tissue after 5-ALA was topically applied to the cervix in a cervical cap for 1 to 6 hours.

In contrast, BPD-MA did not appear to be selectively absorbed by CIN cells after 1.5 or 3 hours, although there may have been some selective absorption after 6 hours. In addition, even at 6 hours the fluorescence did not correspond exactly with the neoplastic cells in the hematoxylin and eosin staining image, and there was nonspecific stromal uptake as well. BPD-MA appeared to diffuse from the

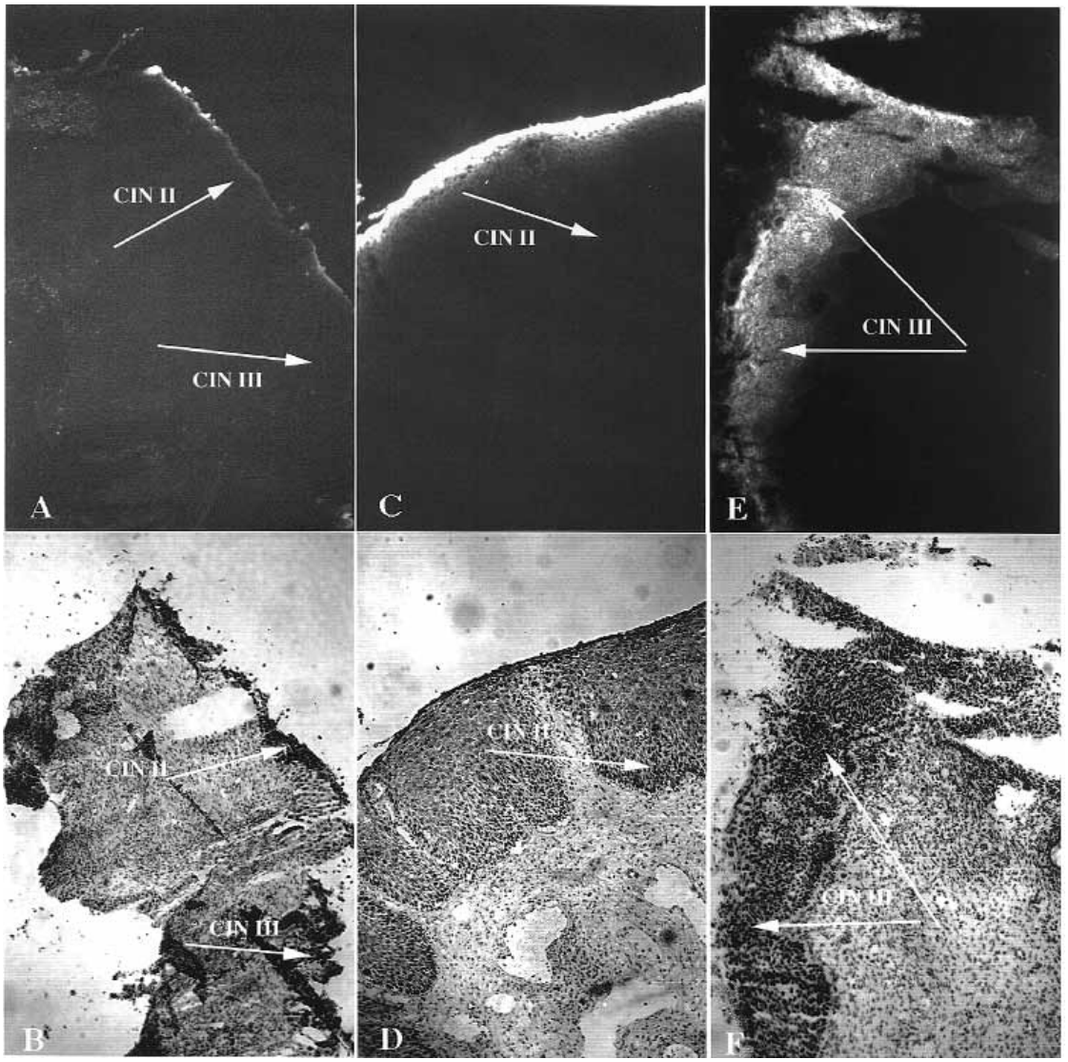

Fig 2. Fluorescence microscopy **(A, C,** and **E)** and hematoxylin and eosin staining images **(B, D,** and **F)** of cervical biopsy sections obtained from 3 different patients after exposure to BPD-MA for 1.5 hours (patient 4, **A** and **B**), 3 hours (patient 5, **C** and **D**), and 6 hours (patient 6, **E** and **F**). *Arrows* point to corresponding areas of CIN in fluorescence microscopy and hematoxylin and eosin staining images.

epithelial surface toward the basement membrane in a diffusion-dependent fashion. One explanation for the lack of fluorescence could be that BPD-MA is selectively absorbed by CIN cells but our detection methods are not able to detect the fluorescence. Conversely, this formulation of BPD-MA has not been tested in cervical tissue previously, so the cervical cells may not be absorbing the BPD-MA at the concentration used in this study, which was 100 times lower than the concentration of 5-ALA used. In addition, the solubility characteristics of BPD-MA in Hyskon may differ from those of 5-ALA, which could lead to decreased absorption into CIN cells. We previously demonstrated similar solubility characteristics for 5-ALA and BPD-MA in the endometrium.[25] However, these data are most likely not applicable to the cervix because of the difference in tissue characteristics between the endometrium and the cervix. Until selective absorption into the cervix and fluorescence of BPD-MA can be demonstrated, the utility of this drug in the diagnosis and treatment of CIN is limited.

Only the dysplastic cells in the cervical epithelium exposed to 5-ALA fluoresced, which demonstrates the selective nature of 5-ALA after its application to the cervix with a cervical cap. The selective fluorescence of dysplastic cells after selective absorption or rapid conversion of 5-ALA to protoporphyrin IX makes 5-ALA a highly promising drug for the selective treatment of CIN.

In this phase I study the prodrug 5-ALA was effective in selectively targeting dysplastic cells after 1.5, 3, or 6 hours of topical application. The rapid penetration of 5-ALA into the cervical tissue 1.5 hours after topical application in a cervical cap and the subsequent selective conversion to protoporphyrin IX further suggests that photodynamic therapy with 5-ALA might be a practical approach to the treatment of CIN. It may also be a useful diagnostic technique.[24] A phase I/II study is currently underway at our institution to evaluate the efficacy of this photosensitizer in the treatment of cervical dysplasia. The use of photodynamic therapy to selectively ablate abnormal cervical cells offers benefits to both the patient and physician and potentially represents an important addition to current treatment options for cervical dysplasia.

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