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A RANDOMISED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL OF PHOTODYNAMIC THERAPY USING 5-AMINOLAEVULINIC ACID FOR THE TREATMENT OF CERVICAL INTRAEPITHELIAL NEOPLASIA

Adrian A. BARNETT[1], J. Christoph HALLER[2], Fiona CAIRNDUFF[3], Geoffrey LANE[4], Stanley B. BROWN[5] and David J.H. ROBERTS[1]*

[1]*School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom*
[2]*Department of Radiotherapy and Radiological Oncology, Heinrich-Heine-University, Duesseldorf, Germany*
[3]*Cookridge Hospital, Leeds, United Kingdom*
[4]*Department of Obstetrics and Gynaecology, St. James's University Hospital, Leeds, United Kingdom*
[5]*School of Biochemistry and Molecular Biology, University of Leeds, Leeds, United Kingdom*

Photodynamic therapy (PDT) using topical 5-aminolaevulinic acid (ALA) has been used to treat histologically confirmed cervical intraepithelial neoplasia (CIN-I and -I/II) in a randomised, double-blind, placebo-controlled protocol. Fluorescence microscopy revealed that topical application of 3% ALA in Intrasite Gel® to the cervix for 3 hr resulted in the accumulation of protoporphyrin IX in the cervical epithelium. Treatment of CIN with ALA-PDT was well tolerated, with only 3/12 patients in the PDT arm (0/13 in the placebo arm) reporting any discomfort during illumination. Histologic examination of the treated tissue following loop excision 3 months post-PDT indicated that 33% of patients had no evidence of CIN, 42% had no change in the grade of their disease, whilst 25% exhibited an apparent progression of disease. In the control group, the respective figures were 31%, 38% and 31%. There was no significant difference in response between the groups receiving ALA-PDT and those receiving placebo treatment.

Key words: *photodynamic; CIN; ALA*

Cervical intraepithelial neoplasia (CIN) is a condition involving partial or total replacement of the cervical squamous epithelium by cells showing various degrees of atypia. The treatment of cervical dysplasia has hitherto been either by a local destructive method (*e.g.,* laser, cold coagulator) or by excision (*i.e.,* cone biopsy or large loop excision of the transformation zone). Our study is designed to investigate photodynamic therapy (PDT) using 5-aminolaevulinic acid (ALA) as a new modality of treatment for CIN.

PDT is based on the light-induced activation of an otherwise inactive agent. It is thought to operate via the generation of highly reactive oxygen species that induce tumour necrosis by direct cytotoxicity and/or by indirect effects mediated via collapse of the tumour vascular supply.[1,2] Applying ALA cream to human skin cancers has been shown to induce the accumulation of the photosensitiser protoporphyrin IX (PpIX) in the tumour tissue, which can then be treated by illuminating with light of the appropriate wavelength.[3] Similarly, systemic administration of ALA induces PpIX accumulation in the metaplastic and dysplastic epithelium of Barrett's oesophagus.[4,5] PDT using ALA has been successfully used to treat Bowen's disease of the skin (squamous cell carcinoma *in situ*)[6] and dysplasia in Barrett's oesophagus.[4,7]

Several previous studies have examined the possibility of using ALA-based PDT (ALA-PDT) for treating CIN. The findings have proved equivocal, with Hillemanns *et al.*[8] reporting no improvement in patients with CIN-II ($n = 4$) or -III ($n = 3$) at 8–12 weeks post-ALA-PDT, whilst Wierrani *et al.*[9] found that 19/20 CIN-I ($n = 9$) or -II ($n = 10$) patients had normal cytology and colposcopic appearance 9 months after ALA-PDT. Unfortunately, neither of these studies was conducted in a double-blind controlled manner. This is particularly problematical with CIN, which exhibits a significant spontaneous regression rate. For example, Melnikow *et al.*,[10] in a review of 15 studies of low-grade cervical lesions, found that 47% of cases underwent spontaneous regression. At present it is not possible to identify which patients with CIN have malignant potential, so a blanket approach to treatment is generally taken.

If successful, ALA-PDT may offer an alternative to large loop excision of the transformation zone (LLETZ, currently the standard treatment in many centres), possibly avoiding the intra- and postoperative bleeding and discomfort reported in up to 8% of patients undergoing such treatment.[11] Our current study has used a double-blind placebo-controlled protocol to determine the efficacy of ALA-PDT in the treatment of CIN-I and -II.

MATERIAL AND METHODS

Patients

Our study received local ethical committee approval, and all patients gave informed consent to inclusion.

Patients attending the St. James's University Hospital Colposcopy Clinic following a recent history of cytologic atypia were examined colposcopically and directed biopsies taken for histologic examination. Forty-one non-pregnant patients were recruited for inclusion. Three women subsequently opted for loop excision prior to PDT, and 2 failed to attend for PDT on 3 occasions and were subsequently referred back for standard treatment. One woman became pregnant following PDT, and the pregnancy continued without complication. Although her treatment was completed following her postnatal period, she was not included in the trial.

Of the 35 patients included, 32 (91%) had histologically confirmed CIN-I, and 3 (9%) had CIN-I and -II. None of the patients included had received any prior treatment for CIN.

Chemicals

ALA was synthesised in the Department of Colour Chemistry, University of Leeds. Final purification to clinical grade was undertaken by the Department of Pharmacy, St. James's University Hospital Leeds. For topical application, ALA was prepared as a 3% or 5% (w/w) solution in Intrasite Gel® (Smith & Nephew Healthcare Ltd., Hull, UK) immediately prior to use.

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*Correspondence to: School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, UK. Fax: +44-113-3434294.
E-mail: d.j.h.roberts@leeds.ac.uk

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Fluorescence microscopy

The first 10 patients recruited were entered into a pilot study to determine whether topically applied ALA could induce PpIX accumulation in the dysplastic cervical epithelium.

Ten grams of 3% (8 patients) or 5% (2 patients) ALA in Intrasite Gel® were applied to the cervix in a contraceptive cervical cap for a period of 4 hr prior to definitive excision by loop diathermy. Samples of the excised tissue were snap-frozen in melting *iso*-pentane and maintained at −85°C until required. Frozen sections (8 μm) were cut using a Leica cryostat and examined using a fluorescence microscope fitted with a slow-scan CCD camera (Astromed, Cambridge, UK). Fluorescence excitation was at 632 nm, and fluorescence was collected from 665–690 nm. All samples were examined by the same operator (DJHR) in a blinded manner.

Biopsies from lesions not exposed to exogenous ALA were used as negative controls (2 patients). Shade correction, correction for background and autofluorescence and determination of pixel intensities were performed using the Imager 2 software suite (Astromed).

Mean fluorescence intensity values were compared using unpaired 2-tailed Student's *t*-tests assuming unequal variances.

Photodynamic therapy

Twenty-five patients were randomised in a double-blind protocol to PDT using ALA or to a placebo group. Randomisation was conducted by the St. James's University Hospital Pharmacy Department using randomisation tables. The randomisation key was held by the Pharmacy Department until the end of the study.

Patients in the treatment arm (12 patients, mean age 30 years, range 21–41 years) received 10 g of 3% ALA in Intrasite Gel® applied to the cervix in a contraceptive cervical cap. Placebo group patients (13 patients, mean age 30 years, range 21–42 years) received 10 g of Intrasite Gel® containing no ALA. After 4 hr, the cervix was anaesthetised using a 4-quadrant injection of prilocaine hydrochoride (4%) and illuminated superficially with 100 Jcm^{-2} (100 $mWcm^{-2}$) 635 nm light from a diode laser (Ceralas PDT, Ceramoptec GmbH, Bonn, Germany) delivered through a microlens-tipped optical fibre.

All patients were asked to report any discomfort during treatment and were issued with a diary to record any pain, bleeding or discharge in the 2 weeks following PDT. Three months after treatment, patients were recalled to the clinic to undergo standard LLETZ treatment, at which time they were asked to complete a questionnaire regarding any symptoms they had experienced following PDT. A full histologic assessment of the excision specimen was made to determine whether there was any persisting dysplastic tissue. This assessment was conducted by the St James's University Hospital Histopathology Service within the system operating therein for the routine evaluation of LLETZ samples. The randomisation of patients to PDT or placebo arms was unblinded when all the LLETZ samples produced in the study had been assessed.

Pretreatment biopsies and LLETZ specimens from all patients entering the trial were evaluated histologically for signs of infection with the human papilloma virus (HPV).

The outcomes of patients in the ALA-PDT and placebo groups were compared by χ^2 analysis. A regression rate of 95% (19/20 patients) was observed in the uncontrolled study of Wierrani *et al.*[9] This compares with the spontaneous regression rate of 47% reported in the meta-analysis of Melnikow *et al.*[10] The sample sizes employed in the current study would detect such a difference in response rates at a significance level of 0.05 with a power of 0.8. Sample size calculations were carried out using the PS program (Vanderbilt University Medical Centre).

RESULTS

Fluorescence microscopy

Topical administration of 3% (w/w) ALA for 4 hr resulted in the accumulation of PpIX in squamous cervical epithelium (Table I). The mean fluorescence intensity in this tissue was 201 ± 41 counts/pixel. Much less PpIX accumulated in the underlying stroma (mean = 42 ± 13 counts/pixel, $p = 0.0058$).

In 2 patients treated with 3% ALA, biopsies were also removed from the upper region of the vagina. The mean fluorescence intensity in the squamous epithelium at this site was 50 ± 25 counts/pixel, whilst the mean level in the stroma was 4 ± 10 counts/pixel. Although the small sample size precludes reliable statistical analysis, the intensity of fluorescence in the vaginal epithelium appeared to be significantly less than that in the cervical epithelium ($p = 0.018$).

A further 2 patients were treated with 5% ALA. The mean fluorescence intensity in the cervical squamous epithelium was 164 ± 72 counts/pixel. Although there was a considerable difference in these levels between the 2 patients (Table I), there was no indication that increasing the dose of ALA had increased the levels of PpIX accumulating in the cervical epithelium compared to that observed in patients receiving 3% ALA ($p = 0.7$).

Photodynamic therapy

Of the 12 patients treated with ALA-PDT, 4 (33%) displayed no evidence of CIN 3 months following treatment, whilst 5 (42%) exhibited CIN of the same grade observed prior to PDT and 3 (25%) had evidence of higher grade CIN than before treatment (Table II). The results for patients in the placebo arm of our study did not differ significantly from those randomised to ALA-PDT ($p > 0.9$).

In 7 cases (4 placebo, 3 PDT), more advanced disease was identified in the LLETZ sample than in pretreatment biopsies. In 3

TABLE I – PpIX FLUORESCENCE IN THE CERVICAL AND VAGINAL TISSUES OF CIN PATIENTS TREATED WITH TOPICAL ALA IN INTRASITE GEL FOR 4 HR.[1]

Patient no.	ALA dose (%)	Tissue	Mean fluorescence intensity ± S.D. (counts/pixel)		Epithelium/stroma fluorescence ratio
			Epithelium	Stroma	
1	3	Cervix	143 ± 48	26 ± 29	6
2	3	Cervix	203 ± 72	44 ± 26	5
3	3	Cervix	143 ± 55	16 ± 26	9
4	3	Cervix	132 ± 43	27 ± 32	5
4	3	Vagina	25 ± 26	−6 ± 16	
5	3	Cervix	120 ± 106	19 ± 20	6
6	3	Cervix	173 ± 118	27 ± 46	6
7	3	Cervix	478 ± 141	126 ± 55	4
7	3	Vagina	75 ± 42	14 ± 19	5
8	3	Cervix	217 ± 69	47 ± 32	5
9	5	Cervix	236 ± 62	60 ± 26	4
10	5	Cervix	92 ± 42	1 ± 21	92

[1]All values are corrected for background and autofluorescence.

TABLE II – PRE- AND POSTTREATMENT DETAILS OF PATIENTS RANDOMIZED TO PLACEBO OR ALA-PDT GROUPS[1]

Group	Pretreatment diagnosis		Diagnosis 3 months after PDT (based on examination of LLETZ sample)							Outcome		
	I	I/II	None	I	I/II	II	II/III	III	I/II/III	Normal	No change	Apparent progression
Placebo	12	1	4 (31%)	4 (31%)	3 (23%)	1 (8%)	1 (8%)	0	0	4 (31%)	5 (38%)	4 (31%)
PDT	10	2	4 (33%)	5 (42%)	0	0	2 (17%)	0	1 (8%)	4 (33%)	5 (42%)	3 (25%)

[1]The outcomes of patients in the 2-arms were not significantly different.

patients diagnosed with CIN-I, the tissue removed by LLETZ included CIN-I/II (2 patients) or CIN-II (1 patient). In another patient initially diagnosed with CIN-I, the LLETZ sample was reported as CIN-II/-III, whilst another contained CIN-I, -II and -III. Two of the 3 patients presenting initially with CIN-I/II exhibited CIN-II/III in the LLETZ sample.

Histopathologic changes consistent with HPV infection were detected in the pretreatment biopsies of 10 patients who subsequently received PDT. Such changes were detected in the LLETZ samples of 4 of these patients (60% eradication rate). In the placebo arm, 9 patients exhibited histologic evidence of pretreatment HPV infection, with 3 LLETZ samples displaying such evidence (66% eradication rate). There was no significant difference between the eradication rates in PDT and placebo groups ($p > 0.75$).

All patients tolerated treatment well. During illumination, 3 patients in the PDT group described slight vaginal discomfort, but none requested that the treatment be halted or that analgesia be administered. None of the patients in the placebo group reported any discomfort during illumination.

At follow-up 14 days after illumination, 3 patients reported having a watery vaginal discharge lasting from 3–10 days. One of these patients also reported vaginal/pelvic pain lasting 4 days after treatment, but no analgesia was required. On decoding the randomisation at the end of our study, it was found that all 3 of these patients had received PDT. Patients who had been in the placebo group reported no discharge or pain. Patients in both groups reported no abnormal vaginal bleeding or effect on menstruation.

DISCUSSION

Semiquantitative fluorescence microscopy indicated that topical application of 3% ALA in Intracite Gel® for a period of 3 hr resulted in PpIX accumulation in the cervical epithelium of 8 patients presenting with early dysplasia (Table I). Increasing the dose of ALA to 5% made no apparent difference to the accumulation of PpIX, although this was only examined in 2 further patients. Based on these observations, it was decided to use 3% ALA for the subsequent clinical study.

In all cases, PpIX fluorescence in the epithelium was greater than in the underlying lamina propria, suggesting differential sensitisation of the abnormal epithelium and its supporting connective tissue. Samples taken from the vagina adjacent to the cervix in 2 patients revealed greater fluorescence in the cervical epithelium than that of the vagina, possibly due to retention of ALA gel in the contraceptive cap during application. It was not possible to determine whether there was any differential PpIX accumulation between dysplastic and adjacent normal cervical epithelium. However, Pahernik *et al.*,[12] examining loop excision samples, observed fluorescence ratios of 1.3 for CIN-1:normal and 1.21 for CIN-2:normal. These observations were qualitatively similar to those we have made in the epithelium of Barrett's oesophagus following oral administration of 30 $mgkg^{-1}$ ALA.[5] In this case, no consistent difference in PpIX fluorescence intensity was identified between the normal and dysplastic oesophageal epithelium, both of which exhibited significantly greater levels of fluorescence than the underlying lamina propria.

Histopathologic analysis of LLETZ samples 3 months following PDT indicated no significant difference in outcome between patients who had received PDT and those in the placebo arm of the trial. These findings are in contrast to the successful results we have obtained treating both nonmelanoma skin cancers[6] and Barrett's oesophagus.[4] They are, however, very similar to those of Hillemanns *et al.*,[8] who examined CIN patients colposcopically, cytologically and histologically 8–12 weeks following ALA-PDT in an uncontrolled Phase-I/II trial. None of the 7 patients treated by Hillemanns *et al.*[8] showed regression of CIN (initially grade II or III in all cases). In contrast, Wierrani *et al.*[9] found that ALA-PDT normalized the transformation zones of 19/20 patients with CIN-I or -II examined colposcopically and cytologically up to 9 months following treatment.

There are a number of possible reasons why Wierrani *et al.*[9] observed better clinical responses than in either our present study or that of Hillemanns *et al.*[8] Spontaneous regression of CIN lesions has been well documented previously[10] and is illustrated further here by the reversion to normal of 31% of patients receiving placebo treatment. The meta-analysis of Melnikow *et al.*[10] indicated that 47% of patients (95% confidence interval 36–59%) with low-grade cervical dysplasia undergo spontaneous regression. However, it seems unlikely that this factor alone could account for all of the regression observed by Wierrani *et al.*[9] It is possible that the treatment regime employed by Wierrani *et al.*[9] was superior to that of Hillemanns *et al.*[8] or that used here. For example, Wierrani *et al.*[9] employed 12% ALA in saline applied for 8 hr whilst 3% ALA in a gel applied for 4 hr was used here. Although our observations of PpIX distribution in the cervical tissue were qualitatively similar to those we made of Barrett's epithelium,[5] they were quantitatively very different, with the cervical epithelium typically exhibiting fluorescence 10–40 times less intense than the Barrett's epithelium of patients given 30 $mgkg^{-1}$ ALA orally 4 hr before endoscopic biopsy. This may suggest that the concentration of PpIX accumulating in the cervical epithelium was not sufficient to support effective PDT.

It may also be significant that Wierrani *et al.*[9] did not, in contrast to our present study, rely solely on superficial illumination of the cervix but illuminated the cervical canal separately via an inserted optical fibre tipped with a cylindrical diffuser. Although Hillemanns *et al.*[8] also illuminated the endocervical canal by inserting a cylindrical applicator, they employed a much lower fluence rate than Wierrani *et al.*[9] (150 $mWcm^{-2}$ *vs.* 300 $mWcm^{-2}$). Marijnissen *et al.*[13] observed a temperature rise of 11.5°C at a depth of 3.5 mm from cylindrical diffusers delivering light at 400 $mWcm^{-2}$ into rat tumours, and it is generally recommended that fluence rate should be kept below 200 $mWcm^{-2}$ if hyperthermia in the surrounding tissue is to be avoided.[14] The possibility that the lesions treated by Wierrani *et al.*[9] might have been influenced by hyperthermic effects cannot be discounted.

One further factor that should be considered when comparing the current findings to those of Hillemanns *et al.*[8] and Wierrani *et al.*[9] is the method used to assess clinical outcome. In both our current study and that of Hillemanns *et al.*,[8] evaluation was by histologic analysis. In the study of Wierrani *et al.*,[9] follow-up was restricted to colposcopy and cytology. The one patient who Wierrani *et al.*[9] did assess histologically was found to have persistence of CIN.

Wieranni *et al.*[9] observed an 80% HPV eradication rate in their uncontrolled trial. In our present double-blind randomised placebo-controlled environment, however, we observed an eradication rate

of 60–66% that was not significantly different between PDT-treated and placebo groups.

In the study of Hillemanns *et al.,*[8] 3 lesions initially diagnosed as CIN-II were graded as CIN-III following examination of the conation specimen. In our current study, 3 (25%) of the patients receiving PDT exhibited apparent progression of disease, with 4 (31%) in the control arm doing so. It is not clear whether these changes in grade represent genuine progression or sampling error when the initial diagnostic biopsies were collected. The observations of Holowaty *et al.*[15] suggest that progression is a relatively slow process, with the risk of progression from CIN-I to CIN-III or cervical cancer being only 1% per year and the risk of progression from CIN-II to CIN-III being 16% within 2 years. The possibility of biopsy sampling error is supported by the observation of Chia *et al.*[16] of a large disparity in histologic findings between colposcopically directed punch biopsies and LLETZ samples.

In conclusion, although preliminary fluorescence microscopy revealed PpIX accumulation in the cervical epithelium following topical application of ALA, there was no significant difference in the outcome of patients randomised to receive ALA-PDT and those receiving placebo treatment. These results are in close agreement with those of an earlier noncontrolled trial. Alternative ALA delivery protocols, designed to enhance the accumulation of PpIX in the cervical epithelium, may yield more encouraging clinical outcomes.

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