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Review

5-Aminolevulinic acid-based fluorescence diagnostics of cervical preinvasive changes

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ABSTRACT

The purpose of this article is to review the diagnostic possibilities of 5-aminolevulinic acid (5-ALA)-based fluorescence diagnosis of preinvasive cervical changes.

Reviewed papers were selected from the PubMed database with keywords combining the terms individual cervical neoplasia and fluorescence diagnostics. The regular colposcopy procedure lacks specificity; therefore, new methods are continually sought for superior diagnosis of cervical pathology. 5-ALA-based fluorescence diagnostics is under investigation as an up-to-date diagnostic technique for cervical intraepithelial neoplasia (CIN). This method is grounded on the topical or systemic application of 5-ALA, which induces excess production of the endogenous photosensitizer protoporphyrin IX (PpIX) in tissues where carcinogenesis has begun. The conversion of PpIX to the heme is less efficient in tumors; therefore, higher amounts of PpIX tend to accumulate in premalignant and malignant tissues. Illumination with light of the appropriate wavelength initiates excitation of PpIX fluorescence, which in turn helps to localize PpIX-rich areas and identify potentially malignant tissues. A number of investigations suggest that because of its high selectivity for tumors and low toxicity to healthy tissues, 5-ALA-based diagnosis seems a promising tool for the noninvasive identification of cervical intraepithelial neoplasia.

1. Introduction

About 530,000 new cases of cervical cancer and 275,000 deaths from this disease are reported annually worldwide. The incidence of cervical cancer (CC) varies extensively between countries, with world age-standardized rates (WASR) ranging from <1 to >50 per 100,000 [1]. In the European Union, 34,000 new cases and 16,000 deaths due to cervical cancer are apprised every year. This maiming disease mostly affects younger women between the ages of 35 and 50 [2]. Data from the Lithuanian Cancer Registry proclaims that morbidity from

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cervical cancer reaches up to 19–23 cases per 100,000 (WASR), and mortality is 6–8 per 100,000 (WASR). In 2009, 457 new cases of CC were reported in Lithuania [3], and this is one of the highest rates of morbidity from cervical cancer rate among Baltic countries and also among all the Nordic countries [4].

Cervical intraepithelial neoplasia (CIN) is a precancerous condition of the cervix localized in the squamo-columnar junction. The main factor directly related to CIN development is chronic human papillomavirus (HPV) infection, mainly high-risk types 16 and 18 [5–7]. The classification of CIN ranges from CIN I (mild neoplasia) to CIN III (severe neoplasia). CIN is a condition that gradually leads to cancerous changes; however, those preinvasive changes might require several years [5,7,8]. If CIN is diagnosed at an appropriate time before cervical cancer manifestation, it may be cured and cervical cancer avoided [7–9].

With the aim of early CIN detection, screening programs have been organized worldwide that demonstrate their remarkable influence on cervical cancer morbidity and mortality [1,3,4]. Cervical cancer screening currently consists mostly of cervical cytology, diagnostic colposcopy and HPV testing, depending on national screening policies [2,3]. First and foremost, cytological testing is performed on all women of a particular age. If cytological alterations are found, colposcopy and biopsy of the cervical tissue are indicated. Colposcopy is based on recognizing specific markers in the margins, color, and vascular pattern of an epithelial lesion. This examination is focused on the differences between healthy, premalignant and malignant cervical epithelium and used to guide biopsies to an area or areas as needed [9,10]. There is an imperfect correlation between visual changes in the cervical epithelium and the severity of the intraepithelial neoplasia and cancer [10,11]. Several conditions can also interfere with the accuracy of colposcopic diagnosis (inflammation processes, atrophic changes, and anatomical features limiting the ability to examine a zone of transformation in the cervix) [10,12,13]. The main disadvantages of contemporary CIN detection methods are high false-negative rates with cytology and the low specificity of colposcopy. Conventional colposcopy demands long-term training and achieves no more than 48% specificity even in ''trained hands'' [8,11,12]. The low rates of positive predictive value seen with conventional colposcopy may result in unnecessary treatment, which causes unwarranted surgical procedures for patients and additional burden on cervical cancer screening programs. Various treatment methods that differ mainly in their complication rates and costs have proven satisfactory for CIN treatment [14–17]. The recent standard of care consists of excision, e.g., loop electrosurgical excision procedure (LEEP), cold knife excision of the transformation zone or local destruction by laser or cryotherapy. These procedures are commonly painful during treatment and may cause post-operative bleeding. The major drawback of these excision methods is the destruction of the cervical stroma, which may cause cervical insufficiency that may lead to premature delivery and low-birth-weight babies or, adversely, scar stricture with increased risk of infertility and cesarean section [15–17]. The potential complications of treatment procedures suggest that there is a need for new early diagnostic methods to differentiate CIN and avoid risky surgical procedures. The sensitivity of the method should be high enough to detect CIN in the early stages. However, the procedure should also be quite simple, painless and practical to perform during routine examination or screening programs.

One of the most promising techniques in early diagnostics is the so-called optical biopsy [18–22]. The term ''optical biopsy'' refers to any technique that uses the interaction of light and tissue to provide information about tissue morphology without the need for excision. Premalignant and malignant tissue differs from healthy tissue in its morphology and cell growth rate, which results in altered optical characteristics [13,20,23,24]. Most of the optical methods used in diagnostics are based on different types of spectroscopy such as fluorescence, near infrared, Raman, diffuse reflectance spectroscopy and similar techniques [25–28]; however, the most widely used techniques in the clinical practice are based on fluorescence phenomena [29,30]. The acceptance and suitability of these methods in the clinical arm is determined by the diagnostic effectiveness, simplicity and relatively low cost of the procedure. Moreover, they are noninvasive and can be repeated many times.

Another advantage is that the contrast between healthy and pathological tissue can be enhanced using exogenous fluorescence substances or their precursors [24,31–33]. Fluorescence diagnosis (FD) is also under investigation as an up-to-date diagnostic technique in various gynecological pathologies such as cervical, vulvar intraepithelial neoplasia, endometriosis, breast and ovarian cancer [34–36]. The effort is focused on creating a new noninvasive method to specify the grade of CIN. One of the most promising techniques for this purpose seems to be fluorescence diagnosis based on 5-ALA application [34,37–40]. 5-ALA is an endogenous agent that is metabolized in a chain of biochemical reactions to protoporphyrin IX (PpIX) and is also non-toxic at the levels that naturally occur in the body. PpIX is a precursor of heme in the cells and is also non-toxic in the absence of light of the appropriate wavelength [41–45]. Such a diagnostic method is grounded on the topical or systemic application of 5-ALA, which induces excess production of endogenous photosensitizer PpIX in tissues where carcinogenesis has begun [42,44–46]. This selectivity is partially explained by a lower ferrochelatase activity in tumor cells and a higher tumor level of porphobilinogen deaminase, which are the two key enzymes in regulating the heme pathway [42–44,46,47]. Illumination of the tissues with the appropriate wavelength of light initiates the excitation of PpIX fluorescence, which in turn helps localize PpIX-rich areas and identify potentially cancerous tissues [35,37,42,44].

High selectivity for the tumor and low toxicity to healthy tissues make 5-ALA-based diagnostics a promising tool for the noninvasive identification and staging of cervical intraepithelial neoplasia [48–50]. Moreover, the accumulated porphyrins could also be used for treatment purposes [47,51–53]. Using localized cytotoxic phenomena is the main idea behind photodynamic therapy (PDT), which can be applied as an alternative treatment for cervical intraepithelial neoplasia, avoiding the usual complications with excisional and destructive (laser or cryotherapy) procedures [14,47,54,55]. Various studies have demonstrated a 42%–95% response rate and a 31%–91% cure rate of 5-ALA-based PDT [53]. The therapeutic effect induced by laser light is well-confined to the illuminated area and, together with the short half-life of the generated

cytotoxic species, guarantees that the damage is mostly localized to the lesion while sparing the healthy surrounding tissue [42,47–49,55]. The fluorescence of the accumulated porphyrins could also be used during PDT and conventional treatment procedures for precise evaluation of tumor margins and serve as a guide for the surgeon to resect malignant tissues [35].

However, currently no 5-ALA product has been dedicated specifically for gynecological applications and to date no generally accepted protocol has been demonstrated to be the most appropriate for cervical neoplasia detection. Some commercially produced preparations of ALA (Levulan, Metvix) are used for FD and PDT in gynecology, but those products are mostly made up for use in the dermatology and urology fields [36,44].

The aim of this review was to analyze the existing experience of 5-ALA usage for cervical neoplasia detection with respect to diagnosing effectiveness, type of 5-ALA, administration ways and safety.

2. Material and methods

For this purpose the Pubmed database was searched using different combinations of the search phrases: ''cervical neoplasia diagnostics,'' ''cervical dysplasia optical diagnostics,'' ''cervical neoplasia fluorescence diagnostics,'' ''cervical neoplasia ALA diagnostics,'' ''gynecology fluorescence diagnostics,'' ''gynecology ALA diagnostics,'' ''cervical neoplasia photodynamic diagnostics,'' ''cervical neoplasia fluorescence imaging,'' etc. Altogether 15 articles were found and analyzed where 5-ALA or its derivatives were used for cervical neoplasia diagnosis in cases of abnormal cytology and pathological colposcopic findings. The main criteria for the search were local usage of 5-ALA and its derivatives for CIN diagnostics in vivo. We did not find clear recommendations for CIN fluorescence diagnostics, because different scientists use distinct preparations, doses and forms of application of 5-ALA, as well as different light sources and incubation times.

3. Results

The Pubmed database search identified only 4 articles corresponding to our predefined criteria for ALA usage.

Hillemanns et al. [37] compared 5-ALA-based FD with colposcopy in 68 randomly selected women for CIN diagnosis. A 0.5% or 1% aqueous solution of 5-ALA was applied to the cervix with a swab. Two areas, the portion of the cervix and the upper vagina, were evaluated by semi-quantitative fluorescence image analysis and spectrum analysis using a special endoscope combined with a color filter and a spectrometer. Biopsies were guided with a colposcope and taken from suspicious places. In cases where a 0.5% 5-ALA solution was applied, the fluorescence was insufficient for clinical use. With the 1% 5-ALA solution the optimal application time was estimated to be 60–90 min. Sensitivity and specificity were 95%/50%, respectively, for colposcopy alone and 94%/51% for fluorescence image analysis. For the same sensitivity, fluorescence spectrum analysis presented a specificity of 75%. Moreover, evaluation of peak fluorescence intensities revealed significantly higher values for CIN compared with normal tissue and for CIN II/III compared with CIN I.

Collinet et al. [36] observed 14 patients with histologically proven CIN, planned for electrosurgical conization. 5-ALA, as methylaminolevulinate (MAL) 160 mg/mL cream (Metvix) was used for FD. A thick layer of MAL cream was applied on the cervix for 35–150 min (the mean application time was 73 min). Examination of the cervix was performed using an endoscope system with a white light and blue light mode (wavelength 380–440 nm). Fluorescence imaging demonstrated red fluorescent foci in 71.4% of cases (10/14), and the neoplastic status of the fluorescent foci was confirmed histologically in 80% (2- CIN I (25%), 3- CIN II (37.5%) and 3- CIN III (37.5%)). However, the discrimination of different CIN grades by fluorescence measurements was not attempted. Patients were monitored for systemic and local toxicity with a postoperative examination at 6–8 h and at 4–6 weeks. No toxicity from MAL application (either local or systemic side effect) was observed.

Szafińska-Dolata et al. [38] compared 5-ALA-based FD with colposcopy for 73 patients (43 with CIN changes and 30 in the control group with no CIN). A 3% 5-ALA gel was applied topically to the cervix for 4 h. Fluorescence was excited using a light source with a 405 nm wavelength. The cytology, colposcopy and fluorescence imaging results were compared with the clear histological diagnoses from direct biopsies and endocervical curettage. Those results concluded that FD improved the detection of CIN, with higher sensitivity (91%) than colposcopy (79%) and higher specificity (93%) than cytological diagnosis (43%). FD allows the location of a CIN lesion to be identified accurately and its extension, borders and multifocal character to be evaluated; consequently, FD may help to localize sites for direct biopsy.

Nowakowski et al. [56] examined 68 patients with different grade CIN changes. The cytology, colposcopy and fluorescence diagnosis results were compared and verified by histological findings. A 15% 5-ALA cream was spread on the cervix for 2 h before the FD procedure. Fluorescence was excited using a 400–420 nm wavelength light. In 41 patients with low grade changes and cancer the results from cytology, colposcopy, FD and histology were correlated. Among 27 patients with high grade changes the conformity of histological results with colposcopic examination was 55.6% and the positive fluorescent effect during FD was 63%. The authors also concluded that the efficacy, specificity and sensitivity of FD are analogous to colposcopy in diagnosing severe neoplasia.

Many more studies have been performed using 5-ALA and its derivatives for CIN diagnostic purposes ex vivo. This methodology is used to improve diagnostics in the pathology field [40,50,57–60].

Pahernik et al. [59] topically applied 10 ml of 3% 5-ALA with a cervical cap prior to conization of the cervix due to CIN. The incubation time was 1–6 h. The fluorescence intensity was then measured on histological slides. Several specific fluorescence bands (634 and 704 nm, characteristic of PpIX) were detected in the dysplastic epithelium of the ectocervix. The fluorescence was limited to the epithelium and was not seen in the underlying stroma. A significant selectivity was found between CIN III lesions and normal tissue. The peak fluorescence for high-grade lesions was determined after

180 min. Either longer or shorter application intervals showed reduced selectivity. In some cases where CIN II/III changes were grown in to cervical glands, topically applied 5-ALA accumulation was detected in these glands.

Keefe et al. [40] examined cervical biopsies for CIN received after 1.5, 3, or 6 h of topical application of benzoporphyrin-derivative monoacid ring (BPD-MA) or 5-ALA acid. The samples were evaluated for selective drug accumulation with hematoxylin and eosin staining and fluorescence microscopy. It was found that after 1.5 h of 5-ALA accumulation the cervical tissue showed maximal fluorescence intensity in dysplastic cells compared to normal cells, with moderate stromal fluorescence. In contrast, BPD-MA showed selective accumulation only after 6 h. This data demonstrated that 5-ALA is more convenient for clinical applications because of its short incubation times.

In a study by Bogaards et al. [50], a double ratio (DR) fluorescence imaging technique was employed for noninvasive staging of CIN. In this analysis two different excitation wavelengths were used for fluorescence spectroscopy to separate normal and dysplastic tissue. The intensity ratios calculated for the two excitation wavelengths helped to localize areas where the CIN was noticed colposcopically. The conclusion was made that the value of DR determined at the site of biopsy correlated in a statistically significant way with the histopathologically determined stage of the disease.

During another study by Hillemanns et al. [58], hexaminolevulinate dissolved in thermolabile pluronic F 127 gel was applied locally to 24 women with different levels of CIN. The gel was applied at concentrations of 4 mmol/L or 10 mmol/L for 5–720 min before cervical conization. Fluorescence intensity was then evaluated ex vivo in histological samples at a wavelength of 635 nm. CIN tissue had a more pronounced fluorescence peak at 635 nm compared with normal tissues. The intensity of fluorescence was found to increase over time, reaching a peak after 180–540 min. Additionally, the fluorescence intensity in CIN tissues was significantly higher after 10 mmol/L hexaminolevulinate gel application than after application of 4 mmol/L ($P < 0.05$).

Andrejevic-Blant et al. [60] explored PpIX accumulation in neoplastic cervical epithelium at different times after local 0.5% HAL cream application. The accumulation of PpIX in the tissues was explored ex vivo using fluorescence microscopy. High epithelial fluorescence and a significant selectivity between the epithelium and underlying lamina propria were observed for nearly 100 (±10) min. However, no significant spectral difference between normal and neoplastic tissues or between low and high-grade epithelial neoplasia was observed.

Duska et al. [57] analyzed the possibility of administering 5-ALA orally for CIN fluorescence diagnosis. During the study ALA was administered to 14 patients with abnormal Pap smears. Fluorescence in the cervix was detected at a 10 mg/kg dose and there were no noticeable side effects such as nausea or photosensitivity. Optimal fluorescence intensity was assessed at the 3-h time period. However, fluorescence correlated with neoplasia on biopsy only in some cases and the effectiveness of this method has not been evaluated. Nevertheless, the study demonstrated that the preparation is well tolerated and could be used for CIN fluorescence diagnosis and also for photodynamic therapy.

4. Discussion

A number of studies have been performed on the use of 5-ALA and its derivatives for cervical pathology diagnosis. Most of these were carried out ex vivo and showed a reliable diagnostic value for CIN detection in tissue specimens; however, only a handful of groups in various countries have tried to perform investigations of ALA-based FD in vivo. Different scientists used individually prepared study protocols. The main parameters that influenced value of FD were the incubation time and the particular ALA compound, including its pharmaceutical form and mode of application. Three main types of ALA compounds have been used in cervical pathology diagnosis: 5-ALA, methylaminolevulinate, and hexaminolevulinate. ALA is hydrophilic and does not easily penetrate through intact cell membranes; thus, the efficiency of PpIX production is low. Meanwhile, ALA esters are more lipophilic; therefore, they are more specific than conventional 5-ALA [44,45,61,62].

Due to anatomical localization of the cervix, administering the ALA poses a clinical problem. Typically 3 pharmaceutical forms are used: liquid, gel and cream. Some of them are branded, some produced on order. However, the preparations have limited validity and therefore must be used as soon as possible after manufacture. The liquid form is the most convenient to prepare, but its application is complicated, while the cream is convenient to apply but more difficult to manufacture. Moreover, the cream has the longest permeation time. The introduction of thermogels to the market has made the application of ALA simpler. Andikyan et al. [63] demonstrated that using a thermogel Lutrol F-127 (thermolabile bioadhesive, 19% poloxamer 407 gel), that was liquid at 4 °C and sticky at temperatures >31 °C, the adhesion of 5-ALA solution to cervix tissues could be improved. Such 5-ALA preparation is easy to prepare and apply, and it also has good permeability properties. In terms of clinical usage, the comfort gel seems to be the most promising pharmaceutical form.

The final parameter that directly affects the efficiency of ALA-based diagnostics is the incubation time. The incubation time, depending upon the mode of application, pharmaceutical form and the ALA compound itself, has varied in different studies from 0.5 to 12 h. Different authors have relied on different incubation times regardless of the application method and ALA compound; therefore, to date there is no strictly recommended incubation time [37–39,42,59,60].

Irrespective of the methodology used, all the cited authors have shown the potential of FD to provide a noninvasive means to diagnose cervical pathology [34,36–40,50,57]. Cervical regions of neoplasia tend to have higher fluorescence, with CIN III possessing the most intense fluorescence. The method demonstrates improved accuracy in detecting precancerous cervical lesions compared with either standard cytology or colposcopy. By using appropriate light sources, FD could also improve the specificity of conventional colposcopy, meanwhile having no side effects. As the aforementioned investigations suggest, due to its high tumor selectivity and low toxicity, fluorescence diagnosis seems a promising tool for the recognition of cervical intraepithelial neoplasia and can be used in noninvasive staging of CIN.

5. Concluding remarks

The most effective diagnostics could be performed when combining FD with conventional diagnostic methods. This allows a more precise diagnosis to be obtained and, if needed, guide the treatment (loop, electrosurgical excision, laser conization or PDT). It also allows the accuracy of the procedure to be evaluated during treatment. Using optical methods for diagnostics and evaluation, the ''see and treat'' principal could be fulfilled. However, the use of this technique as a routine diagnostic tool for CIN is limited today by the lack of appropriate equipment and the time needed for ALA to accumulate. Moreover, additional unified studies are required to determine appropriate FD procedural parameters and shift this promising method to regular clinical practice. It also should be noted that, prior to introducing FD into clinical practice, it should be compared with other alternative diagnostic methods amending conventional colposcopy, including other types of spectroscopy or impedancometry. Such a study would compare the properties of the new techniques and discuss the advantages and disadvantages of different methods, allowing the most appropriate method for a particular case to be chosen.

Conflict of interest

The authors state no conflict of interest.

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