

Influence of Acetic Acid on a Pap smear of Dysplastic Lesion

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Abstract. *Background: Cervical cancer prevention is performed by taking Pap smears. The correct execution of the smear is crucial as an inadequate smear might result in underdiagnosis. The second means of cervical cancer prevention is visual inspection of the cervix uteri with acetic acid, while often both methods are combined. We investigated whether the application of acetic acid compromises the Pap smear. Patients and Methods: A total of 100 patients with dysplasia were prospectively included; Pap smears were obtained before and after the application of acetic acid. Results: We observed an alteration of the result of the Pap smear after acetic acid application in 41%. However, these alterations did not result in a dysplastic case being classed as a normal smear or vice versa. Conclusion: The application of acetic acid to the transformation zone of the cervix uteri may enhance changes of the Pap smear in dysplasia, however, the changes affect subgroups of dysplasia and thus do not change therapeutic management.*

Cervical cancer is responsible for approximately 275,000 deaths per year worldwide (1). As 85% of the approximately 530,000 annually new-diagnosed cases of cervical cancer occur in developing countries (2), emphasis has to be placed on cheap screening methods with high sensitivity (3).

After the introduction of special screening programs in a multitude of countries, the incidence of invasive cervical cancer decreased significantly, and mortality due to cervical cancer was reduced by 40 to 90% about one decade ago (3-6). In the U.S. in 2005, 98% of women reported ever having

a Pap smear, 90% reported having had a recent Pap smear (within three years), and 84% were adherent to U.S. Preventive Services Task Force (USPSTF) screening (7). In industrialized countries, screening is performed by an individual combination of visual inspection with acetic acid (VIA) cytology, histology, colposcopy and human papilloma virus (HPV) tests (8, 9). In particular, emphasis has to be placed on VIA for its high sensitivity in comparison to the Pap smear (10), cytology (11) and colposcopy (8, 12). The Papanicolaou test has low sensitivity but high specificity, whereas visual detection methods have a high sensitivity in addition to being cheaper (13-15). The established method for cytological diagnosis was first described in 1942 by George Papanicolaou (16) who soon introduced it for the diagnosis of uterine dysplasia and cancer (17) and thus pioneered cancer secondary prevention. In 1971, the so called Pap smear method for diagnosing cervical dysplasia was introduced as a procedure paid by health insurance companies once a year for women over 19 years of age in Germany. The average annual participation rate in Germany is about 50% and considering a 3-year interval, which constitutes the usual screening sequence in many other European countries, the coverage reaches about 80% (18). But despite standardized screening methods, preventive intervention, and short intervals between the examinations, Germany has one of the highest incidence rates of cervical cancer in Western Europe (19). Thus, a strong effort has to be made in order to optimize screening methods. Often a colposcope is used to assess the transformation zone, to take the smear directly and to differentiate a lesion from normal epithelium (12). Today, even experiments with automated image analysis of digital colposcopy for the detection of cervical neoplasia are used (15, 20). The narrow band imaging (NBI) system used in endoscopy can be used to obtain high-contrast vascular images (21). In developing countries, studies have shown that VIA without colposcopy or even without cytology has reasonable results (22). In our hospital, colposcopic findings are still interpreted by a medical doctor. The colposcopic examination usually

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includes an acetic acid test and sometimes additional Lugol's iodine. Immature metaplastic and dysplastic cervical squamous epithelia turn white after application of acetic acid during the exam. The whitening process occurs visually over several minutes and subjectively helps to discriminate between dysplastic and normal tissue. Acetic acid results in white epithelium if an increased ratio of nuclear material to cytoplasmic volume is present. Acetic acid has a dehydrating effect on cells and accentuates the nuclear/cytoplasmic ratio. Thus, areas with marginally elevated nuclear/cytoplasmic ratios will stand out more. Acetic acid is also mucolytic and assists in cleaning the cervix. The use of acetic acid has been discussed as possibly influencing the results of a Pap smear: Griffiths *et al.* put forward the idea that diagnostic epithelial cells can be removed or damaged by scraping with a sampling device, by vaginal digital examination, or as a result of application of acetic acid prior to a colposcopic examination (23). A recently published investigation showed that the sequence of acid application and taking a smear in non dysplastic cases did not change the ultimate result (24).

The assessment of the cervix may be limited when the cervix is contaminated *e.g.* with blood. This is often seen when performing the colposcopy after taking the Pap smear. However, screening procedures lack standard methodical guidelines concerning the sequence of acetic acid application and cytological sample taking. The present study investigated whether the dysplastic Pap smear (class III, IIID, IV and V) (Bethesda correlate Low-grade Squamous Intraepithelial Lesion, LGSIL, and High-grade Intraepithelial Lesion, HGSIL) is altered by acetic acid.

Patients and Methods

Between September 2009 and May 2010, 100 of the patients with dysplasia being referred to our special dysplastic consultation-hour were prospectively included in this investigation. The protocol of the study was approved by the local Review Board (09-141). Only patients who provided written informed consent participated in the study. For every patient, anamnesis was taken and consecutively the cervix was visualized using a bivalve speculum. A colposcopy was then performed. The transformation zone was identified and a Pap smear was taken with a cytobrush. A 5% acetic acid solution was then applied to the transformation zone. Again, a colposcopy was carried out and after 30 sec, a second Pap smear was taken. All of the smears and the colposcopic examinations were performed by the same person. The two smears obtained were fixed directly. After staining, they were examined by an experienced cytologist who analyses all the gynaecological cytology in our hospital. The smears were blinded and the cytologist was not aware of the order in which the respective smears were taken. The smears were classified according to the Münchner Nomenklatur II (25). In 92 of the patients, a tissue sample was taken out of the transformation zone when there was a suspicious area detected.

Statistics. Data (scores before and after application of acid) are presented as frequencies. According to the histological result, the

Table I. Change of smear grade after acetic acid application.

	Without acetic acid, n	After acetic acid, n
PAP III	24	13
PAP IIID	64	70
PAP IVa	11	16
PAP V	1	1

PAP: Papanicolaou.

respective ratios (true-positive, false-negative *etc.*) were computed as accuracy measures. All statistical analyses were performed with SAS (SAS Institute Inc., North Carolina, USA, Version 9.2 July 12, 2011).

Results

A total of 100 patients were examined, 200 smears were performed. The patients' median age was 33.6 years (range 19.8-74.5 years). In all patients, the previously detected dysplasia was confirmed in the smear. In 41 patients (41%), the Papanicolaou grade of the Pap smear changed after application of acetic acid (Table I). In five cases it changed from a worse to a better grade of dysplasia (from PAP IVa to PAP IIID), in nine patients from a better to a worse grade (from PAP IIID to PAP IVa) (Table II). In eight patients, the result of the Pap smear changed from a clear moderate dysplasia (PAP IIID) to an unclear grade (PAP III). In 18 patients it changed from the undefined grade PAP III to dysplastic PAP IIID; in one patient from PAP III to IVa. Class PAP III (3) in the Münchner Nomenklatur II means the cells are degenerated or altered by infection. A clear statement about dysplasia for this class is not possible and therefore it is usually recommended to repeat the smear within two weeks or to obtain tissue for a histological evaluation. We additionally present the tissue samples we obtained from 92 of the patients. The histological results with the corresponding smears are shown in Table III. In 29 patients, no dysplasia was found after biopsy. The results were infection, condylomata acuminata, or normal tissue without alterations. Table IV demonstrates the accuracy ratios of the smears and the histological results.

Discussion

Cervical cancer is the second most common female tumor worldwide, and its incidence is disproportionately high (>80%) in the developing world. In the United States, in which Pap tests have reduced the annual incidence to approximately 11,000 cervical cancers, >60% of cases are reported to occur in medically-underserved populations as part of a complex of diseases linked to poverty, race/ethnicity, and/or health disparities (26). Thus,

Table II. *Change of smear after acetic acid application (subgroups).*

Smear without acetic acid	Smear after acetic acid
PAP III=24 Patients	5 Patients (20.8%) no change 18 Patients (75.0%) PAP IIID 1 Patient (4.2%) PAP IVa 0 Patient (0.0%) PAP V
PAP IIID=64 Patients	8 Patients (12.5%) PAP III 47 Patients (73.4%) no change 9 Patients (14.1%) PAP IVa 0 Patient (0.0%) PAP V
PAP IVa=11 Patients	0 Patient (0.0%) PAP III 5 Patients (45.5%) PAP IIID 6 Patients (54.5%)no change 0 Patient (0.0%) PAP V
PAP V=1 Patient	1 patient, no change
PAP: Papanicolaou.	

prevention strategies require low costs, practicability and effectiveness at the same time. Cytology presents an economic method for secondary prevention. False-negative results in Pap smears for early detection of the disease therefore have to be as few as possible. Both cervical intraepithelial neoplasia grade I (CIN I) (mild dysplasia) and human papillomavirus (HPV) lesions are grouped together as low-grade squamous intraepithelial lesions (LGSIL), while moderate and severe dysplasia (CIN II and III) belong to the high-grade squamous intraepithelial lesion (HGSIL) category. Atypical squamous cells of undetermined significance (ASCUS) and atypical glandular cells of undetermined significance (AGCUS) need further qualification as to whether they favor a reactive or neoplastic process (27). In most cases, cytological results according to the Münchner Nomenklatur II correspond to analogous histopathological findings. PAP II plus HPV infection corresponds to LGSIL, PAP IIw corresponds to ASCUS, respectively AGCUS when there is an HPV infection, PAP IIID and PAP IV correspond to HGSIL. There is no counterpart for PAP III, which means the cells are degenerated or altered by infection. A statement about dysplasia for this class is not possible and therefore it is usually recommended to repeat the smear within two weeks or to obtain tissue for histological evaluation (28). According to the individual Pap smear and the patient's history, guidelines based on the Bethesda system suggest certain procedures, *e.g.* either wait and see with examinations at certain intervals, or histopathological diagnostic assessment by cervical conization (27, 29). Thus, misinterpretation of Pap smear due to technical aberration,

Table III. *Histological results and corresponding smear result.*

Histological result, n	Smear without acetic acid, n	Smear after acetic acid test, n
29, Non-dysplastic histologies (infection, condylomata acuminata, normal tissue)	6, PAP III 21, PAP IIID	2, PAP III 3, PAP IIID 1, PAP IVa 3, PAP III 15, PAP IIID 3, PAP IVa 2, PAP IVa
7, CIN 1	2, PAP IVa 2, PAP III 5, PAP IIID	2, PAP IVa 2, PAP IIID 4, PAP IIID 1, PAP IVa
22, CIN 2	7, PAP III 14, PAP IIID	2, PAP III 5, PAP IIID 2, PAP III 9, PAP IIID 3, PAP IVa
31, CIN 3	1, PAP IVa 6, PAP III 19, PAP IIID 6, PAP IVa	1, PAP IIID 1, PAP III 5, PAP IIID 3, PAP III 15, PAP IIID 1, PAP IVa 3, PAP IIID 3, PAP IVa
3, Squamous cell carcinoma	1, PAP IIID 1, PAP IVa 1, PAP V	1, PAP IVa 1, PAP IVa 1, PAP V

CIN: Cervical intraepithelial neoplasia, PAP: Papanicolaou.

might lead to redundant operations or inadequate therapy. False-negative Pap smear results can be attributed to several factors. Among the variables are sampling errors, *e.g.* cell sample collection, and mistakes in transferring cells to slides, laboratory errors, *e.g.* errors in performing the hematoxylin staining, and interpretation errors, *e.g.* errors in the individual interpretation by the cytologist (30). Considerable numbers of false-negative Pap smears may occur with the traditional Pap technique, mostly due to sampling errors. More recently, the use of liquid-based technologies such as ThinPrep and AutoCyte Prep have gained popularity, in part because of evidence suggesting a reduction in the incidence of inadequate smears (31-32). Acetic acid has a dehydrating effect on cells and accentuates the nuclear/cytoplasmic ratio. Thus, areas with marginally elevated nuclear/cytoplasmic ratios will stand out more in the colposcopic examination. The principle of VIA consists in these changes in the macroscopic pattern. Thus we hypothesized there might be a change in the microscopic pattern, *e.g.* in the Pap smear as well, due to changes of the cellular architecture and problems with interpreting the smear because of cell loss or cell damage. Griffiths *et al.*

Table IV. *Histological results and accuracy ratios.*

Histological result	Smear without acetic acid				Smear with acetic acid			
	True-positive	False-negative	True-negative	False-positive	True-positive	False-negative	True-negative	False-positive
Non dysplastic	-	-	-	29/29*	-	-	-	29/29*
CIN 1	7/7	-	-	-	6/7	-	-	1/7
CIN 2	21/22	-	-	1/22	18/22	-	-	3/22
CIN 3	6/31	23/31	-	-	4/31	25/31	-	-
SCC	3/3	-	-	-	3/3	-	-	-

*All smears of the collective were dysplastic because of the selection criteria. Therefore all of the dysplastic smears with corresponding non-dysplastic histological results in tissue samples were false positives. CIN: n; SCC: n.

pointed out the possible advantages of taking smear samples after colposcopy and acetic acid application: first, avoiding possible bleeding induced by scraping with a sampling device, secondly avoiding epithelial cell loss and thirdly taking cytological samplings exactly at the acetowhite suspicious lesions identified by colposcopy (23). The authors found an elevated false-negative rate in post-acetic acid smears of 53% in comparison to 16% in pre-acetic acid smears. For these reasons, it is important to know possible influences on the cytological result. Other agents used in the course of the colposcopic examination have been investigated because they are believed to interfere with Pap tests. There is level I evidence that modest lubrication of the external surface of the speculum does not impair cytological and infectious evaluation of the cervix (33). We demonstrated that prior acetic acid treatment does not influence non-dysplastic Pap smears (24). In the present study, 41% of all Pap smears were clearly altered after acetic acid application. Although the results were not significant, this striking ratio must be further analyzed. In the present study, conspicuous Pap smears were detected, irrespective of the application of acetic acid. Pap smears revealing LGSIL or HGSIL were not changed to normal Pap smears by acetic acid. Thus, the sensitivity of cytology was not altered by prior acetic acid application. Considering the results of the Pap smears and their particular diagnostic and therapeutic consequences, changes can be observed regarding PAP III, PAP IIID and PAP IVa of the Münchner Nomenklatur. A PAP IIID result should be clarified after an interval of three months, whereas a PAP IVa should directly be histologically examined, *e.g.* a biopsy or conization should be performed. In the present study, we considered the respective prognostically worse smear as the valid result. Thus, when PAP IIID became PAP IVa after application of acetic acid and *vice versa*, a histological sample was obtained. However, when PAP IIID and PAP IVa are transcribed into the Bethesda classification, both become HGSIL. Thus, there would be no change in diagnostic procedure or therapy if the Bethesda classification

were applied (28). On the other hand, we hypothesized that the application of acetic acid might render a smear difficult to analyze and difficult to assign to a certain class of the Münchner Nomenklatur for the pathologist. Thus, the proportion of PAP III, *e.g.* smears which are suspect, but not clearly analyzable due to disruptive factors such as infections or menstrual blood, was expected to be elevated after application of acetic acid in comparison to native smears. However, only eight smears became PAP III, all of them were native PAP IIID. Neither PAP II nor PAP IVa was changed to PAP III. Interestingly, in contrast, 20 of the PAP III native smears were changed to PAP IIID after the application of acetic acid, *e.g.* there might even be a better assessment after the application of acetic acid. However, the results of our study are statistically not significant and further study should be performed in order to confirm that there is a better assessment of Pap smears after acetic acid application in comparison to native smears. A total of 23 (25,8%) of the 89 histological samples were non-dysplastic tissue although the Pap smears indicated dysplasia (regardless of acetic acid use). This result is in line with the study by Szurkus *et al.*, who performed loop excisions without a prior biopsy (34). Nevertheless, we should critically evaluate whether the tissue samples were taken from the correct area of the cervical transformation zone in these patients. All these patients were asked to return for another colposcopic examination within three months. Regarding our results presented in Table IV, all smears of the collective were dysplastic according to selection criteria. Therefore all of the dysplastic smears with corresponding non-dysplastic histological results in tissue samples were false-positives. However, these data of false-positives must not be interpreted as quantitative evidence concerning the correlation of dysplasia in smears and biopsies, as the end point of the study aimed at different evidence. The apparent evidence is due to selection criteria and does not reflect any disqualification of the Pap smear as an established diagnostic technique.

Conclusion

Secondary prevention of cervical cancer in medical practice is performed by obtaining cytological smears from the outer cervical os, the Pap smear. In Germany, an examination is performed once a year when there is an inconspicuous result and the Pap smear is classified according to the Münchner Nomenklatur II. In cases of abnormal Pap smears, the patient should be referred for special dysplastic consultation where a colposcopy is performed and, if necessary, a tissue sample is obtained. As the result of the Pap smear provides the basis for further diagnostic proceedings or therapy, a high methodical sensitivity is required. The application of acetic acid constitutes a major step in the diagnostic approach when performing a colposcopy, the VIA. The aim of the present study was to investigate whether the application of acetic acid changes the result of the Pap smear, or not. We already demonstrated that acetic acid does not influence non dysplastic Pap smears (24). In the present study, we observed an alteration of the result of the Pap smear after acetic acid in 41%. However, these alterations did not change a dysplastic PAP smear into a normal PAP smear finding nor *vice versa*. The alterations concerned subclasses (of the Münchner Nomenklatur II) of HGSIL according to the Bethesda classification. After the application of acetic acid there was no impairment of assessment, there were even fewer smears classified as PAP III (unclear grade). The sequence of sample taking and VIA does not seem to influence the diagnostic and therapeutic proceedings based on the result of a Pap smear. In conclusion, the application of diluted acetic acid to the cervix neither enhances the systematic misinterpretation of the Pap smear nor influences the diagnostic accuracy of the smear in correlation to the corresponding histological findings. Further analysis should be performed in order to evaluate whether VIA should even be performed prior to smear taking in order to provide for adequate smear samples.

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