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International Journal of Gynecology and Obstetrics

journal homepage: www.elsevier.com/locate/ijgo



CLINICAL ARTICLE

Cryotherapy for HPV clearance in women with biopsy-confirmed cervical low-grade squamous intraepithelial lesions[☆]Bandit Chumworathayi^{a,*}, Jadsada Thinkhamrop^a, Paul D. Blumenthal^b, Bandit Thinkhamrop^c, Chamsai Pientong^a, Tipaya Ekalaksananan^a^a Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand^b School of Medicine, Stanford University, California, USA^c Faculty of Public Health, Khon Kaen University, Khon Kaen, Thailand

ARTICLE INFO

Article history:

Received 17 July 2009

Received in revised form 18 August 2009

Accepted 29 September 2009

Keywords:

Cryotherapy

HPV

Low-grade squamous intraepithelial lesions

LSIL

Uterine cervix

ABSTRACT

Objective: To compare the clearance rate of HPV infection among women aged older than 30 years with biopsy-confirmed cervical low-grade squamous intraepithelial lesions (LSIL) 1 year after cryotherapy with the spontaneous clearance rate (observation). **Method:** HPV DNA typing by polymerase chain reaction and reverse line blot hybridization were used to identify 14 high-risk types and 23 low-risk types. HPV DNA sequencing was also used for other types. **Result:** Between December 2007 and March 2009, 100 women were recruited to the study and 60 cases had positive results on HPV testing. Twenty-nine patients were randomly allocated to the cryotherapy group and 31 to the observation group. At 1 year, 89.7% (26/29; 95% CI, 78.6–100%) of the cryotherapy group and 90.3% (28/31; 95% CI, 79.9–100%) of the observation group had negative results on HPV testing (0.6% difference; 95% CI, –15.8 to 14.6%, $P=0.94$). **Conclusion:** Cryotherapy failed to increase the clearance of prevalent HPV infections among women with LSIL, although in both arms the clearance rates were above 80%. However, in coupling with visual inspection with acetic acid as a single visit approach, its effect on prevention of HSIL and cervical cancer is still promising. Therefore, cryotherapy should not be withdrawn from such programs. (ClinicalTrials.gov Identifier: NCT00566579).

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1. Introduction

Persistent human papillomavirus (HPV) infection has been shown to be the cause of cervical cancer. Nearly all (99.7%) cervical cancers are directly linked to previous infection with one or more types of HPV, one of the most prevalent sexually transmitted infections in the world [1]. Of the more than 50 types of HPV that infect the human genital tract, 15 to 20 types are linked to cervical cancer. Four of those types—16, 18, 31, and 45—are most often detected in cervical cancer cases, with type 16 accounting for half of the cases worldwide [2].

Often, HPV infections do not cause symptoms. The most common signs of infection are small pink or red warts that appear in the genital area and itching or burning. After a woman becomes infected with HPV, the infection may remain locally stable, may regress spontaneously, or, if the cervix is affected, may develop into low-grade squamous intraepithelial lesions (LSIL), which are also called mild cervical intraepithelial neoplasia (CIN 1) or early dysplasia. Unlike high-grade squamous intraepithelial lesions (HSIL or CIN 2–3), most low-grade

lesions disappear without treatment or do not progress, particularly those that occur in younger women [3]. These precancerous changes (LSIL and HSIL) are observed most frequently in women aged between 30 and 40 years [4,5].

Cervical cancer is still a major health problem in low-resource countries. It has been the most common female cancer in Thailand for many decades. Cervical high-risk HPV (HR-HPV) infections can lead to LSIL (10%), HSIL (0.8%), and finally, cervical cancers (0.16%) within 10–20 years [4].

When women have sexual intercourse with HPV-carrying sexual partners, significant percentages of them become infected with HPV. It has recently been shown that the prevalence of HPV infection in women in the United States is as high as 39% [6], while approximately 10% of Thai women have cervical high-risk HPV infections [7]. These data show that HPV is a common infection. About 50% of all new HPV infections may clear spontaneously within 12 months of initial infection. However, it is known that the older a woman is, the lower the rate of clearance [3,8,9]. Cryotherapy or the loop electrosurgical excision procedure (LEEP) can improve this HPV clearance rate to about 80% in LSIL using cryotherapy, and to about 95% in HSIL after LEEP [10].

The treatment options for LSIL are either observation or ablative surgery [11,12]. However, in the authors' institution, cryotherapy, which is a form of ablative surgery, is more frequently used to treat

[☆] Presented at the Thailand Research Expo 2009, August 26–30 2009, Bangkok, Thailand.

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patients. It is not only effective, but safe, with only minimal adverse effects—watery leukorrhea for 2–4 weeks and local cervical infection occurring in not more than 1% of patients [13]. Although cryotherapy can be performed for any lesion on the cervix, it is less effective when lesions are very large or extend deep into the cervical canal [14]. Therefore, contraindications to this procedure include active cervical infection, lesions that extend more than 2 mm beyond what the probe would cover, lesions inside the cervical os, and suspected cervical cancer [4,15].

Additionally, in low-resource countries such as Thailand, this treatment has been shown to be safe, acceptable, feasible, and effective. As a result, cryotherapy has become an integral component of programs utilizing a “single visit approach” to cervical cancer prevention, most commonly involving visual inspection with acetic acid (VIA) and the offer of cryotherapy to women who test positive and are eligible for the procedure. Particularly in rural areas where physicians, and especially obstetricians-gynecologists, are less accessible, patients with a positive test can also have this treatment provided by nurses who have undergone a competency-based training course [13,15].

Because of their high recurrence rate, it has previously been felt that HPV infections, not LSIL, have no truly effective management except for spontaneous clearance [4]. This is more likely to occur only in cases of new infections or in younger women [3,8], but less likely to occur in cases of chronic persistent infection or in older women [9]. One recent report showed that cryotherapy is not only able to clear LSIL, but is also able to clear the HPV infections—its necessary cause [10]. However, no randomized controlled trials have compared the clearing ability of cryotherapy compared with untreated (observed) controls.

A randomized controlled trial is important to demonstrate a potential new property of cryotherapy: to eradicate HPV infection. Findings from such a trial would contribute enormously to the care plans for older women who are already infected with HPV. Aside from preventing cervical cancer in treated women, cryotherapy could also give such patients relief from worrying about having a persistent infection with a potentially cancer-causing HPV in the cervix. Therefore, the aim of the present study was to compare the clearance rate of HPV infections among women older than 30 years diagnosed with biopsy-confirmed uterine cervical LSIL 1 year after cryotherapy (intervention group) with the spontaneous clearance rate (observation group).

2. Materials and methods

Following research protocol approval by the Institutional Review Boards at Khon Kaen University (IRB00001189, FWA00003418), Khon Kaen Hospital, and Roi Et Hospital, the patients were recruited into the study if they did not have contraindications to cryotherapy. Each patient received verbal and written information about the purpose, procedures, and potential risk and benefit of the study, and signed a consent form before recruitment into the study.

Patients meeting the following eligibility criteria were recruited consecutively: women aged older than 30 years who had histopathological confirmation of LSIL from a uterine cervical biopsy following an abnormal cervical cancer screening test (either Pap smear or VIA), with a positive result at HPV testing. Those with no contraindications to cryotherapy were randomly allocated by blocks of 2, 4, and 6 using computerized randomization to treatment with either cryotherapy or observation only. Those with negative HPV test results were excluded.

After admission to the study, all patients were assessed for HPV infection approximately 1 month before undergoing cryotherapy. A cervical brush sample was collected from the endo- and ectocervices. The brush was put in a plastic tube with 1 mL of phosphate-buffered saline solution containing a 5-mmol/L ethylenediaminetetra-acetic acid buffer, then immediately refrigerated at 0–4 °C, and later

transferred to a –70 °C storage tank within the same day for future analysis within the next 2 weeks [10].

Cryotherapy was performed using carbon dioxide as the refrigerant in the treatment group only. The cryotip was applied to the cervix and a standard “double-freeze” procedure of two 3-minute freezes with 5-minutes thawing in between was carried out [4]. Follow-up visits were scheduled at 6 and 12 months after allocation to the treatment or observation group.

At the follow-up visits, the women were examined by the same investigator who had examined them at baseline. Cervical brush sampling was conducted using the same method as the baseline visit, before a Pap smear, colposcopy, and, if necessary (i.e., in case of colposcopic or cytologic signs of CIN) a punch biopsy was also performed. At each follow-up visit, all women were asked about any new sexual partners since the previous consultation.

After the specimen was sent to the laboratory, all cervical samples from the baseline and follow-up visits were thawed and the tubes were vortexed to dispense mucus and cells from the brush. The brush was then removed from the tube in a sterile manner while the remaining mucus and cells on the brush were pressed toward the edge of the tube. After vortexing, 350 µL of the sample was transferred to a new tube, frozen, and analyzed for HPV DNA. The analyzing laboratory was blinded to the identity of the samples, but an analysis order list ensuring that samples from the same women would be analyzed side-by-side was provided.

Because of the difference between the two treatments, only the assessors measuring the HPV testing could be blinded to the treatments. The specimens were sent to the assessors without the patient's name or hospital number that could be linked to the treatment received. They were provided only with the study ID number for which only the principal investigator could link it to the treatment received. The main outcome, HPV clearance, was measured by standardized HPV testing [16]. This was performed only at baseline and 12 months later.

Testing for HPV was performed using an HPV general primer GP5 +/6+-mediated polymerase chain reaction (PCR)–enzyme immunoassay method, which is also similar to the method used by Elfgrén et al. [10]. The HPV DNA typing by PCR and reverse line blot hybridization were used to identify 14 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and 23 low-risk types (6, 11, 26, 34, 40, 42, 43, 44, 53, 54, 55, 57, 61, 70, 71 [CP8061], 72, 73, 81 [CP8304], 82/MM4, 82/IS39, 83 [MM7], 84 [MM8], and CP6108) [16]. HPV DNA sequencing was also used for other HPV types. Three times the mean optical density value of the PCR-negative controls was used as a cutoff point.

Testing for beta-globin to ensure that adequate samples containing intact human DNA was also undertaken. The method used is described in detail elsewhere [17]. Women were defined as having cleared their HPV infection if the type of HPV infection at the baseline visit could not be detected at the 12-month follow-up visit. If they did not attend for the 12-month follow-up visit, their 6-month follow-up specimen was used instead. For women positive for multiple HPV types at baseline, clearance of all HPV types was required [10].

Based on the reported spontaneous clearance rate of 53.6% at 1 year in women older than 34 years [9] and the reported clearance rate of 83.9% at 1 year after cryotherapy [10], in the sample size calculation we varied the clearance rate in the observed group from 50%–60% and in treated group from 70%–90%. The estimated sample size of 25–103 patients in each arm (about 100–300 patients recruited for testing) was estimated to have a power of 80% to detect a 20%–40% difference in the rates of HPV clearance after 1 year of treatment between the two groups, with a significance level of 0.05 and a 2-sided test. Recruitment of 300 patients was selected at first to have the best precision. However, recruitment to the trial was stopped by the steering group (without knowledge of the study outcomes after 60 HPV-positive women were randomized) because of slow recruitment

of patients and because the limited financial resources available for the trial were exhausted. Therefore, the analysis was undertaken when only 100 patients (33%) were recruited for screening and the 60 were followed-up. The Z-test using STATA software version 10.0 (StataCorp LP, College Station, TX, USA) was used to calculate the difference between the clearance rates and its 95% confidence interval (CI).

3. Results

During December 2007 to March 2009, 103 patients who had been diagnosed with LSIL from cervical biopsy histopathology were recruited and 3 of them declined to participate. Sixty of these 100 patients had positive results on HPV testing. Of these, 29 (48.3%) were randomly allocated to receive cryotherapy and 31 (51.7%) were allocated to observation (Fig. 1). Demographic data for the 2 groups were all comparable (Table 1).

Median follow-up time was 12 months (range, 10–14 months). At 12 months after treatment, one patient from each group did not attend. The patient in the cryotherapy group (HPV type 52 and HIV-positive) died from pneumonia at 9 months after treatment. For the patient in the observed group (HPV type 16/18), only the 6-month specimens were available and these were HPV negative. This meant that their HPV infections were cleared, according to our definition. In 2 HIV-positive patients in the cryotherapy group, all of their HPV infections were cleared; however, in 3 HIV-positive patients in the observed group, only 1 patient was clear of their HPV infection.

At 12-month follow-up, 3 patients in the cryotherapy group (HPV type JEB2, HPV type 52, HPV type 16) and 4 patients in observed group (HPV type 16, HPV type 31, HPV type 16, HPV type 52) had positive HPV results. However, the last patient described was infected with HPV types 70/83 at baseline and, therefore, according to our definition, this previous infection had been cleared. There were 2 LSIL and 2 HSIL Pap smear results in both groups, while colposcopic findings and biopsy were normal in 4 women: 2 with LSIL and 1 with HSIL results from the cryotherapy group, and 1 LSIL result from the observed group.

We also investigated the histologic changes independent of HPV clearance; 1 patient from the cryotherapy group and 2 patients from the observed group were found to have HSIL on LEEP. The persistent LSIL patient was the one whose HPV infection could not be cleared (Table 2). In all 3 HSIL cases, high-risk HPV infections from types 59, 18, and 58, were cleared.

In summary, at 12 months after treatment, 89.7% (26/29; 95% CI, 78.6–100%) of the cryotherapy group and 90.3% (28/31; 95% CI, 79.9–100%) of the observed group had negative results on HPV testing for the previous infection types. The difference in the HPV clearance rate between the 2 groups was inconclusive because of the wide confidence

Table 1
Demographic characteristics of the cryotherapy and observation groups.^a

Characteristics	Cryotherapy (n = 29)	Observation (n = 31)
Age, y	42.17 ± 7.86	40.71 ± 7.51
Weight, kg	59.07 ± 11.09	54.74 ± 9.24
Parity 0	1	3
1	2	7
2	18	15
3	6	4
4 or more	2	2
Duration of sexual activity, y	20.69 ± 8.83	19.13 ± 8.77
Duration with patient's partner, y	16.66 ± 10.69	16.77 ± 10.31
Monthly income, Thai Baht	5757 ± 6715	5839 ± 6737
Education of college or more	14 (48.3)	14 (45.2)
Patient's smoking status	1 (3.4)	0 (0)
Partner's smoking status	19 (65.5)	16 (51.6)
Patient's reported HIV positive	2 (6.9)	3 (9.7)
Partner's reported MSP status	9 (31.0)	12 (38.7)
Patient's reported MSP status	12 (41.4)	8 (25.8)
High-risk HPV types	27 (93.1)	25 (80.6)

Abbreviation: MSP, multiple sexual partners.

^a Values are given as number (percentage) or mean ± SD.

interval (0.6% difference; 95% CI, -15.8 to 14.6%; $P = 0.94$). HSIL rates found by LEEP in the cryotherapy and observed groups were both low (3.4% vs 6.5%; $P = 0.58$) (Table 2). Even if the study had reached its sample size, the difference between the cryotherapy and the observed groups is not significant. This was another reason that the study was stopped permanently.

4. Discussion

In the original sample size, we postulated clearance rates in the observed group to be 50%–60% and in the cryotherapy group to be 70%–90%. The sample size of 25–103 in each arm (about 100–300 patients recruited for HPV testing) would have had a power of 80% to detect a 20%–40% difference in rate of HPV clearance after 1 year of treatment between the two groups. Recruitment of 300 patients was targeted at first to have the best precision, but because of slow recruitment of patients and lack of funding, the trial was stopped and this analysis was performed when only the first 100 patients recruited for testing and 60 who were HPV-positive had completed their 1-year follow-up. A similar obstacle was found by Crowther et al. [18]. Early divergence of treatment effects against the new treatment was also found [19] as clearance rates in both groups were very similar at approximately 90% (0.6% difference; 95% CI, -15.8 to 14.6%; $P = 0.94$), although inconclusive because of the wide confidence interval.

Since the time from diagnosis of LSIL to HPV testing ranged from 1–6 months (mean 3.5 months), 40 of 100 women would have cleared their HPV infection leading to a negative test. The clearance rate of 89.7% in the cryotherapy group was not surprising as rates of 70.1% and 83.9% were reported by Aerssens et al. [20] and Elfgrén et al. [10], respectively; however, the rate of 90.3% in observed group was unexpected. Clavel et al. [9] reported this finding in only 53.6% of women older than 34 years. Moscicki et al. [8] also reported that the

Table 2
Clearance, LSIL, and HSIL rates found at 12-month follow-up in each group.^a

	Cryotherapy (n = 29)	Observation (n = 31)	Rates difference	P value ^b
Clearance rate	89.7	90.3	-0.6 (-15.8 to 14.6)	0.94
LSIL rate	0	3.2	-3.2 (-9.4 to 3)	0.33
HSIL rate	3.4	6.5	-3.1 (-14 to 7.8)	0.58

Abbreviations: LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions.

^a Values are given as percentages (95% CI).

^b Using the Z-test.

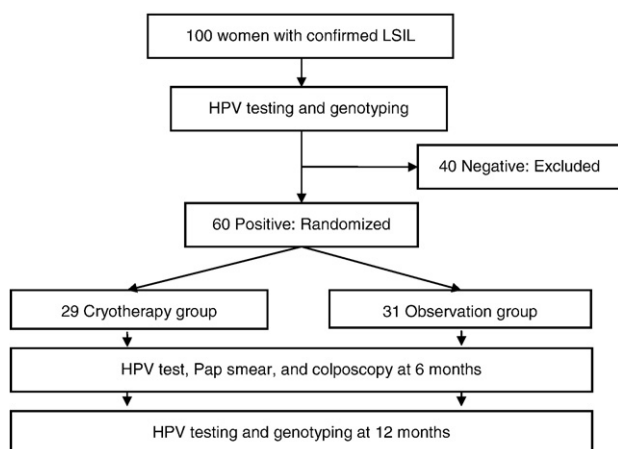


Fig. 1. Study flow diagram.

spontaneous HPV clearance rate in even younger women (less than 30 years old) at a longer period of 24 months was only 70%. Why was this so high in these patients? One explanation might be that frequent Pap smears trigger some immunologic processes that can overcome cervical HPV infections in these LSIL patients [21] and those effects were strong enough to be comparable with cryotherapy. It is also possible that there is a genetic predisposition to clearance of these viral subtypes among Thai women (or perhaps just in women from this part of Thailand) while this same genetic proclivity was not present in the study by Moscicki et al.

Currently, health personnel are using cryotherapy to treat dysplasias and not the infection. Using cryotherapy in the presence of infection without dysplasia would be difficult to promote given that HPV infection is highly prevalent and most will clear spontaneously, as found in the present study. HPV infection, which is detected by HPV DNA testing, is redundant until it has affected the cellular processes to initiate dysplasia and progress to cancer.

Although the study was stopped prematurely and the clearance rate in the observed group was surprisingly high [19], based on the observed difference in clearance of only 0.6% there was more than a 95% chance that the real difference would never be more than 20%. This finding suggests that the differences in the HPV clearance rates found in the previous studies [8–10,20] were overestimated. Indeed, a large sample size would be needed to demonstrate statistically significant differences between treatment and observation if authors continued to detect only a 10%–15% difference between the groups.

The difference in HSIL rate found at 12-month follow-up could be an indicator of treatment efficacy, although it could also be an indicator of the adverse effects of observation. There were only 1 and 2 patients in each group, and these numbers are too small to allow for conclusions about any real differences. However, comparing this was not an objective of the study. The patient found in the cryotherapy group could result from either under diagnosis before recruitment or treatment failure. The 2 patients found in the observed group could also result from either under diagnosis before recruitment or progression during observation. If the latter is correct, this could be considered as an adverse outcome attributable to observation. Fortunately, although most HPV infections were cleared, Pap smears could still detect these lesions and the patients with HSIL were treated before progression to cervical cancer. These findings also support use of the Pap smear in conjunction with HPV testing [22].

Despite these data we do not recommend discontinuing the use of cryotherapy, which is an integral component of VIA and cryotherapy-based cervical cancer prevention programs in several regions around world. Although cryotherapy did not seem to be better than observation for HPV clearance in patients with LSIL, coupled with VIA as a single visit approach and while LSIL and HSIL cannot be discriminated, its effect on prevention of HSIL and cervical cancer is still promising [5,23]. Furthermore, despite the future potential for HPV-based testing in low-resource countries, such testing and confirmatory diagnostic testing remain impractical and infeasible in many areas. Therefore, in low-resources settings where colposcopy, biopsy, or follow-up with Pap smears cannot be done effectively, cryotherapy, as part of a single visit approach is still needed.

Acknowledgement

The study was funded and monitored by the Royal Golden Jubilee-PhD Program of the Thailand Research Fund and the Clinical Epidemiology Unit of the Faculty of Medicine, Khon Kaen University.

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