Title
In Vitro and In Vivo Testing of a TCM Treatment for Anal HSIL

Author
Jay, Naomi

Publication Date
2007-06-18

Peer reviewed|Thesis/dissertation

In Vitro and In Vivo Testing of A Traditional Chinese Medicine Treatment for Anal High Grade Squamous Intraepithelial Lesions

by

Naomi Jay (aka Jatovsky)

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nursing

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

I would like to acknowledge my dissertation committee members: Dr. Christine Miaskowski, chair of my dissertation committee and academic advisor at the University of California San Francisco, Dr. Marylin Dodd, chair of my qualification committee, Dr. Joel Palefsky, who has worked with me and mentored me throughout my research career, and Dr. Misha Cohen who introduced me to Chinese Medicine and served as the TCM expert on my committee. I would also like to acknowledge others who played an important role in the fruition of these projects including Dr. Karen Smith-McCune who mentored the laboratory research for these studies and Dr. Michael Berry who has worked alongside me in developing many of the insights for these projects. Mardi Marean RN, NP introduced me to colposcopy at a time when few nurse practitioners were doing this work during my preceptorship for my Master's degree. Finally, I am grateful and honored to have worked with the many patients and research participants who have inspired me to do this work and always made it worthwhile.

My friends and family are too numerous to name but my gratitude for your encouragement, and for all those times you put up with my crankiness, fed me, and took Elijah for play dates and sleepovers. A special thanks to Elijah, who has grown up watching his mother work on her dissertation and was always a good sport.

Finally, I wish to remember my mother who was there when I began this journey and would have loved to see the completion of this work

Abstract

Squamous intraepithelial lesions (SIL) of the anal canal are similar in etiology and pathology to SIL of the cervix. Cervical cytology screening and colposcopy examination were adapted and validated for anal canal lesions. It is hypothesized that treatment of anal high grade SIL (HSIL), considered the cancer precursor lesion in both the cervix and anus, will prevent their progression to cancer similar to the decreased incidence of cervical cancer that occurred with the advent of cervical screening programs. Anal cytology and high resolution anoscopy (i.e., colposcopy of the anal canal) are becoming the standard of care, particularly for populations considered at risk which include immunocompromised individuals and men who have sex with men (MSM). This approach has led to an increased diagnosis of HSIL, an increased demand for clinicians trained in procedures to identify and diagnosis HSIL, and an increased need for treatment. The papers in this dissertation evaluate methods to improve the detection of anal HSIL as well as in vitro and in vivo studies of a treatment of HSIL using Traditional Chinese Medicine (TCM).

The results indicate that lesion characteristics and Lugol's staining patterns associated with cervical HSIL are also associated with anal HSIL. The TCM ointment, used as part of a multi-modality treatment, was cytotoxic in three cervical cancer cell lines tested. Finally, the TCM treatment that consisted of weekly acupuncture and moxibustion, and daily self-applied TCM ointment and moxibustion was safe and feasible.

Table of Contents

Introduction

Fifteen years ago I was recruited by Dr. Joel Palefsky to work as a nurse practitioner in a natural history study of anal cancer and its precursors in HIV-seropositive and seronegative men who have sex with men (MSM). I was a women's health nurse practitioner with an interest, but no experience, in colposcopy and human papilloma virus (HPV). Male patients were not on my agenda. However, Dr. Palefsky enticed me with the words "the anus is just like the cervix". Since anal cytology smears and colposcopy were virtually unknown, prior experience was unnecessary and I was a perfect fit for the job. Funded by an NIH grant, Dr. Palefsky bought two 30-year-old Zeiss colposcopes and put me to work recruiting and examining a cohort of men from the San Francisco Gay Men's Health Study.

Several studies later and with the help of a few thousand patients, the natural history of anal squamous intraepithelial lesions (SIL) has been characterized and the tools for screening and identifying the range of anal disease associated with human papilloma virus (HPV) were developed and validated. Referral for High Resolution Anoscopy (HRA) is now considered standard practice in many clinics for patients with abnormal anal cytology. Histology remains the gold standard for diagnosis, but it relies on the clinician's evaluation of lesions to locate the highest grade of disease for submission to pathology. In addition, successful treatment of HSIL relies on the clinician to correctly identify lesions for treatment. The first manuscript in this dissertation is an analysis of characteristics used to describe anal lesions. The additional benefit of Lugol's staining is described and validated in this paper.

While the past decade of research has improved our understanding of the natural history of anal HPV and its associated diseases, treatment options lag. Ablation has been the mainstay of therapy although it is often an invasive procedure with potentially high rates of morbidity. It is contraindicated in patients with large and/or circumferential disease as well as in medically frail patients.

Soon after beginning my work with Dr. Palefsky, I was introduced to Dr. Misha Cohen, a well-known local acupuncturist who specialized in women's health and HIV care. As it were, we had many patients in common whom she treated for HIV-related problems. As more of these patients were diagnosed with anal HSIL through our studies, she began to develop Traditional Chinese Medicine (TCM) protocols for treatment of anal dysplasia based on her treatments for cervical dysplasia.

Treatment with TCM offers a non-ablative treatment option that was used in the community but had not been studied for safety, feasibility, or efficacy. The second manuscript in this dissertation describes the methodology developed to investigate the in vitro effects of the TCM ointment, developed by Dr. Cohen, on cervical cancer cell lines. The final dissertation paper describes a pilot study that determined the safety, feasibility, and efficacy of the TCM treatment protocol developed by Dr. Cohen for anal HSIL in fifteen patients with high grade squamous intraepithelial lesions (HSIL). This study was designed to investigate the treatment as it was used in the community, a multi-modality TCM intervention that consisted of weekly acupuncture and moxibustion as well as daily self-applied moxibustion and ointment.

The results of the treatment studies suggest that this TCM treatment protocol is viable. The ointment was cytotoxic against the three cancer cell lines tested. The

treatment was feasible and safe with an approximately 27% efficacy rate. However, a randomized clinical trial is warranted to demonstrate efficacy. The development of methods to evaluate TCM using rigorous research methodology will improve the collaborations between Western and complementary medicines.

Colposocopic Characteristics and Lugol's Staining Differentiate High and Low Grade Squamous Intraepithelial Lesions of the Anus

Naomi Jay[1], J Michael Berry[1], Chris Miaskowski[2], Marylin Dodd[2], Steve Paul[2], Misha Cohen[3], Elizabeth Holly[4], Teresa Darragh[5] and Joel Palefsky[1]

Department of Medicine UCSF[1], Department of Nursing UCSF[2], Department of Cancer Epidemiology UCSF[4], Department of Pathology UCSF[5], Chicken Soup Chinese Medicine, San Francisco Ca[3].

Corresponding author: Naomi Jay, Box 1699, Mount Zion Hospital UCSF, 1600 Divisadero, San Francisco, CA 94143; email Naomi.jay@ucsf.edu

Running Title: Colposcopic characteristics and Lugol's staining of anal lesions

Keywords: colposcopy, Lugol's solution, high resolution anoscopy

Abstract

Background: Colposcopic examination is the standard procedure used to identify cervical lesions following detection of abnormal cells on cervical cytology. The colposcope's magnification and lighting along with the application of acetic acid and/or Lugol's iodine result in recognizable epithelial and vascular changes associated with squamous intraepithelial lesions (SIL) which would otherwise be invisible. These characteristics create a colposcopic impression used to select lesions to biopsy for histologic evaluation which is considered the gold standard for the diagnosis of cervical dysplasia.

Based on similarities in the anatomy of the cervix and anal canal, and in the pathophysiology of human papilloma virus (HPV) disease, the cervix has been used as the hypothetical model for anal HPV associated lesions including LSIL, HSIL and cancers. Prior studies have validated the use of anal cytology for screening and high resolution anoscopy (HRA). HRA which is examination of the anus using the colposcope, is becoming the standard of care for diagnostic work-up following an abnormal cytology. This study evaluated the staining techniques used routinely in colposcopy for the detection of cervical lesions and determined the utility of Lugol's solution to improve the detection of anal SIL as well as its prognostic value for HSIL.

Methods: Lesions from participants enrolled in the UCSF natural history study of anal cancer and its precursors undergoing routine bi-annual HRA were included in this study. Lesions were described according to standard colposcopic criteria for their contour, surface patterns, vascular patterns and margins. Lesions were then stained with Lugol's solution and categorized as Lugol's negative, partial or positive. Only lesions submitted for histopathology were included in this analysis.

Results: The colposcopic characteristics of 835 biopsied anal lesions were described and correlated with histopathology. Significant differences ($p < .05$) were found for all lesion characteristics except margins. HSIL was associated with flat contour, smooth surface, punctation or mosaic vessels, and Lugol's negative (L-) staining. LSIL was associated with raised contour, granular, papillae or micropapillae and Lugol's Partial (LP) or Lugol's positive (L+) staining. The sensitivity and PPV for HSIL were highest in characteristics associated with cervical HSIL. L- staining increased the PPV in all characteristics but the increase was two to threefold in characteristics associated with cervical LSIL. A multiple logistic regression model indicated that flat, distinct lesions, mosaic and punctation vascularity, and L- staining were predictive for HSIL.

Conclusions: With the exception of margins, the colposcopic criteria used to distinguish cervical LSIL from HSIL are applicable to these lesions in the anal canal. Lugol's negative staining was independently associated with HSIL. Although nearly half of LSIL were L-, only 24% of LP and 9% of L+ were HSIL. This suggests that the addition of Lugol's staining to the HRA may help to differentiate HSIL lesions with more precision than reliance on the characteristics which appear with acetic acid alone. Since approximately 10% of lesions with LSIL appearance are histologically HSIL, L- stain may better differentiate those which are HSIL from LSIL.

Introduction

Colposcopic examination is the standard procedure used to locate and biopsy cervical lesions following detection of abnormal cells on cervical cytology. The colposcope's magnification and fiber-optic lighting along with the application of 3% to 5% acetic acid and/or Lugol's iodine results in recognizable epithelial and vascular changes or characteristics associated with squamous intraepithelial lesions (SIL) which would otherwise be invisible. These lesion characteristics are used to guide the selection of biopsy sites. Previous work has described the colposcopic-defined lesion characteristics that are associated with low (L) and high grade squamous intraepithelial lesions (HSIL) of the cervix [1, 2]. Although the sensitivity of cervical colposcopy in predicting the grade of disease varies widely, these lesion characteristics create a colposcopic impression used to select lesions to biopsy. Histology from colposcopic-directed biopsies is considered the gold standard for the diagnosis of cervical and genital dysplasia.

Based on similarities in the anatomy of the cervix and anal canal, and in the pathophysiology of human papilloma virus (HPV) disease, the cervix has been used as the hypothetical model for screening and diagnosis of HPV-induced abnormalities of the anal canal including LSIL, HSIL and cancers. The same range and types of HPV affect both the anus and cervix, and HSIL is considered the cancer precursor lesion in both sites. The anal canal like the cervix is composed of squamous epithelial cells adjacent to columnar epithelial cells in a transformation zone (TZ) that undergoes metaplasia (i.e., the normal process of change during which columnar epithelium is covered over and transformed to squamous epithelium). The cells are susceptible to abnormal

transformation during this dynamic process and the majority of squamous cell cancer precursor lesions and cancers originate in the TZ [1-3].

Prior studies have validated the use of anal cytology for screening of HPV-associated disease of the anus [4-6]. Examination of the anus with the colposcope, first described by O'Connor in 1977, is now called High Resolution Anoscopy (HRA). Gradually, HRA is becoming the standard of care for screening, diagnosis, and treatment of HPV-associated anal disease [7, 8]. Previous work suggested that the use of acetic acid and colposcopic-defined lesion characteristics for HSIL of the cervix predicted 49% to 61% of HSIL in the anus [9, 10]. However, 9% to 13% of biopsied lesions with LSIL characteristics were found on pathology to be HSIL [9,11]. In the present study, we sought to validate these findings as well as to determine if the application of Lugol's solution in addition to acetic acid improved the ability to detect anal SIL and specifically whether it had prognostic value for HSIL.

Lugol's staining is used in cervical colposcopy to differentiate cervical HSIL from LSIL and to determine the margins of disease for treatment in the cervix. Therefore, the central hypotheses of this study were: 1) that colposcopic-defined lesion characteristics associated with cervical HSIL and LSIL would be associated with anal HSIL and LSIL and 2) that Lugol's negative-stained anal lesions would correlate with HSIL, while Lugol's partial or positive-stained lesions would correlate with LSIL**.** While histology remains the gold standard for the diagnosis of disease, a histologic diagnosis relies on the clinician's ability to identify the lesions with the highest grade abnormality to biopsy. This study evaluated the staining techniques used routinely in colposcopy for the detection of cervical lesions submitted for histopathologic diagnosis.

Methods

The participants were 399 HIV-seropositive and 172 HIV-seronegative men who have sex with men (MSM) enrolled in a natural history study of anal cancer and its precursors who underwent routine bi-annual HRA examinations. Characteristics of this cohort have been described previously [5, 12]. Excluded from this analysis were 137 HIV-seropositive and 81 HIV-seronegative patients with a history of allergy to iodine, patients whose biopsies were scant and could not be interpreted, or patients who did not present with anal canal lesions. Forty lesions that were biopsied specifically to verify that the tissue was normal or atypical were excluded when the histologic diagnosis was confirmed. Perianal lesions were excluded because Lugol's iodine solution does not stain fully keratinized epithelium. The Committee on Human Research at the University of California San Francisco approved the collection of these specimens as part of the study protocol.

Procedures

Prior to the HRA, a moistened Dacron swab was inserted into the anal canal for cytology and HPV tests. Following a digital rectal examination using 2% lidocaine gel mixed with KY-jelly, an anoscope was inserted and a Q-tip wrapped in gauze and soaked in 3% acetic acid was placed in the anal canal. The anoscope was removed and the gauze was left in place for at least one minute to allow the acetic acid to soak into the mucosa of the entire anal canal. The gauze was then removed and the anoscope reinserted. Using the colposcope for lighting and magnification, the HRA was performed to identify potential LSIL, HSIL, or cancer. Discrete areas that turned white with the application of

3% acetic acid were considered acetowhite (AWE) lesions. Each lesion was evaluated and coded for the presence or absence of specific lesion characteristics (see below).

Full-strength Lugol's solution (i.e.,1 part iodine, 2 parts potassium iodide, and 300 parts water) was then applied to each lesion using a Q-tip and staining patterns were categorized (see below). In addition, Lugol's solution was applied to the entire transformation zone of subjects who had prior abnormal cytology and the absence of AWE lesions. In these subjects, the TZ was evaluated for the presence of Lugol's negative staining and these areas were biopsied. Finally, a series of 25 biopsies were taken in a subset of AWE lesions with different Lugol's staining patterns within a single lesion. The different areas were biopsied in order to determine if Lugol's negative staining defined the margins of HSIL for patients referred for treatment. Whenever possible photographs were taken before and after Lugol's staining using a video camera connected to the colposcope. Following the photographs, lesions were biopsied. All examinations and biopsies were done by one of two clinicians experienced in HRA.

A single pathologist, blinded to the lesion descriptions, clinician's impressions or the patients' history, provided the histopathologic diagnoses.

Lesion Categorization and Staining

Lesions were described using colposcopic criteria commonly used in gynecology and previously validated for anal lesions[9]. AWE was defined as an area in the internal anal canal demarcated by the application of acetic acid. Each AWE lesion was described based on specific characteristics in the following four categories: contour, surface patterns, vascular patterns and margins. Lesions were then stained with Lugol's solution and categorized as Lugol's Negative (L-) if no staining occurred, Lugol's Partial (LP) if

staining was not uniform in color, or Lugol's Positive (L+) if staining was uniformly dark mahogany (see Figure 1). Areas of L- staining without AWE were categorized as "non AWE L- lesions".

Characteristics hypothesized to be associated with HSIL were flat contour, smooth surface, punctation and/or mosaic vascular patterns, abnormal lesion margins, and L- staining. Characteristics hypothesized to be associated with LSIL were raised contour, surface patterns that were granular with papillae or micropapillae, warty vessels, indistinct margins, and LP or L+ staining. Using these characteristics and staining patterns, the lesions were assigned a colposcopic clinical impression as normal, atypia, LSIL, HSIL, or cancer. All biopsies were sent to pathology for a histologic diagnosis.

Statistical Analysis

Because of the multiple visits per patient that occur in a longitudinal study and the potential bias associated with repeated samples, only the first study visit in which a lesion was biopsied was included in this analysis. Multiple biopsies from the same patient were included if they occurred at the same visit and represented different lesions.

Data were analyzed using SPSS version 15.0 (SPSS, Chicago Ill.). For analysis purposes, characteristics were dichotomized into categories associated with either HSIL or < HSIL (i.e., LSIL, ATYPIA and Normal). Therefore, Lugol's staining was dichotomized as Lugol's negative versus Lugol's Partial or Positive. Margins were dichotomized as distinct versus indistinct, regular, or 'not scored' since raised lesions were not given a margin score. Lesions which presented with both characteristics of a dichotomized variable (for example flat and raised), were assigned to the category hypothesized to be associated with LSIL. The dichotomized distribution of histology

(HSIL versus < HSIL) and characteristics were tested for significance using the Chi square statistic. Sensitivity, specificity, positive predictive value (PPV), and negative predictive values (NPV) were calculated for each characteristic with HSIL as the dependent variable. To determine whether there was additional benefit from using Lugol's staining in the evaluation of lesions, these statistics were calculated with a derived variable "characteristic plus Lugol's negative" in which the positive result had both the characteristic and L- staining. Contingency tables were then calculated so that the True Positives were considered to be those characteristics with L- staining and HSIL histology while false positives were < HSIL. False negatives consisted of the remaining HSIL which did not have the characteristic or were not L-, and true negatives had neither the characteristic nor L- staining.

An analysis of lesion patterns was done by calculating the frequency of the common combinations of characteristics. All patterns with $\geq$ 10 lesions were included in this analysis. The PPV was estimated as the proportion of the pattern that had a histologic outcome of HSIL.

Odds ratios (OR) were calculated for each of the characteristics individually using logistic regression. Then, multivariate logistic regressions were calculated using the characteristics with p values <.05 in the univariate analysis to model the cluster of characteristics associated with HSIL.

Results

Sample Characteristics

A total of 835 lesions were included in this analysis representing biopsies taken from 399 HIV-seropositive and 172 HIV-seronegative men. No differences were found in

any demographic characteristics between the men enrolled in the cohort study who did and did not undergo a biopsy or whose biopsy results were excluded. A larger percentage (8.5%) of men who were HIV-seronegative did not receive biopsies compared to HIV-seropositive men (2.2%).

Distribution of Anal Lesion Characteristics

The distributions of lesion characteristics and Lugol's staining patterns in relationship to histologic diagnoses are listed in Table 1. HSIL (46.6%) and LSIL (41.8%) were distributed evenly. The low number of lesions diagnosed as atypia (5.1%) and normal (6.5%) was congruent with the overall goal to biopsy HSIL and LSIL.

Significant differences ($p < .05$) in lesion characteristics and histology diagnoses were found for all of the characteristics except margins and granularity. A higher number of HSIL lesions were associated with the following characteristics: flat contour, smooth surface, punctation or mosaic vessels, and L- staining. In contrast, more lesions with diagnoses < HSIL had the following characteristics: raised contour, granular, papillae or micropapillae surfaces, warty vessels and LP or L+ staining. All margin characteristics were more frequently associated with HSIL, while lesions that were not scored for margins were mostly LSIL (See Table 1).

For all of the histologic diagnoses, the majority of the lesions were Lugol's' negative. However, within the L- lesions, differences in the distribution of histologic diagnoses were observed with 57% of the lesions diagnosed as HSIL compared to only 30% diagnosed as LSIL. In contrast, the distribution of the LP lesions was 24% HSIL compared to 68% LSIL while 83% of L+ lesions were LSIL compared to only 8.6% HSIL.

Sensitivity, Specificity and Predictive Value

For HSIL, the sensitivity, specificity, PPV, and NPV of the lesion characteristics, are summarized in Table 2. Characteristics with the highest sensitivities for HSIL were flat, smooth, punctation, and L- staining. The characteristics with the highest PPV for HSIL were flat, punctation, mosaic, and L- staining. The surface characteristic "smooth", while having the highest sensitivity also had the lowest specificity and a higher NPV than PPV indicating that it was not able to discriminate between HSIL and LSIL. The results for margins also indicated that this characteristic was a poor predictor of histology outcome. The characteristics with the highest sensitivities for LSIL were raised (59.0), smooth (92.3) and warty vessels (54.1). The sensitivity of a LP/L+ lesion for LSIL was 49.9%. The characteristics with the highest PPV for LSIL were raised (72.3), papillae (83.4) warty vessels (71.3) and Lugol's partial or positive stain (70.4). Specificity and NPV of LP/L+ for LSIL were 85% and 70.2% respectively.

The sensitivity, specificity, PPV and NPV of the lesion characteristics combined with L- staining are also summarized in Table 2. Sensitivity for detection of HSIL decreased in all categories while specificity and PPV increased in all categories with the use of Lugol's staining. In contrast, NPV decreased for those characteristics hypothesized to be associated with HSIL while it increased for those hypothesized to be associated with LSIL. Lugol's negative-stained characteristics with the highest sensitivities for HSIL were AWE, flat, smooth, punctation and non-distinct margin characteristics. Lugol's negative-stained characteristics with the highest PPV for HSIL were AWE, flat, smooth, punctation, mosaic and non-distinct margins (see Figure 2). The largest

increases in PPV for HSIL were seen in characteristics hypothesized to be associated with LSIL (e.g., raised, papillae, and warty vessels).

Logistic Regression Analyses

The results of the univariate logistic regression analysis indicating the relationship between anal lesion characteristics and HSIL are shown in Table 3. Flat contour, mosaic, and punctation vessels, smooth surface, and Lugol's negative staining all had OR > 1.0 and significant p values. Many of the characteristics had an OR signifying that they were "protective" (e.g., their presence meant HSIL was unlikely) as well as significant p values. Granular surface and margins were insignificant while smooth was borderline (p = .03). A multiple logistic regression analysis with all the variables is shown in Table 4a. The characteristics that remained significant were mosaic, punctation, flat, distinct, and L- stain. Distinct margins did not remain significant in a multiple logistic regression analysis of these variables. A final multiple logistic analysis with the remaining four variables is shown in Table 4b and it is considered the parsimonious model predictive for HSIL.

Patterns of Lesion Characteristics

Most anal lesions present with a combination of characteristics. The clinical impression of HSIL compared to LSIL is often based on evaluation of these combinations which are used to guide the choice of biopsy sites. Patterns of lesion characteristics were determined using a matrix analysis of all possible combinations of characteristics. Of the 835 lesions, 720 presented in 10 patterns of characteristics in which there were at least 10 lesions per pattern (see Table 5). The highest PPVs (67.4% to 68.2%) for HSIL were in the patterns that included a flat contour, mosaic vessels, and smooth surface with or

without punctation. Lesions that had a raised contour, with a smooth surface, warty vessels, and papillae or micropapillae had two to threefold increases in the PPV when L- stain pattern was present. Even though only 13.9% of these lesions were HSIL, 29% of those with L- staining were HSIL. The predictive probability for all lesion patterns increased with L- staining. For patterns containing warty vessels, papillae or micropapillae, the predictive probability nearly doubled in lesions that were L- compared to L+ and LP.

Additional Lugol's Staining

Lugol's staining of the transformation zone was done in situations in which a prior cytology result indicated the presence of SIL but acetowhite lesions were not seen during the HRA. A series of 93 biopsies were obtained of these "non-lesion L- stained areas". While the majority of these biopsies were normal (40%) or atypia (17%), 17% were HSIL and 26% were LSIL. These lesions were only visible with Lugol's staining.

A final series of 25 lesions were biopsied to determine whether Lugol's staining defined the borders for lesions with indistinct margins. In these areas of L- staining, lesions were biopsied as well as the adjacent LP or L-stained areas. In this series, 76% of the L- stained areas were HSIL and 88% of the LP or L+ areas were <HSIL. This finding indicates that the margins of HSIL may be accurately determined by Lugol's staining for treatment purposes.

Discussion

This study demonstrates that the colposcopic criteria used to distinguish cervical LSIL from HSIL may be used to distinguish between these lesions in the anal canal. The anal TZ and AWE lesions were discernible after application of 3% acetic acid to the anal

canal. The lesion characteristics commonly used to describe cervical lesions were visible in anal lesions and most were associated with the hypothesized grade of dysplasia. This study is the first to examine the use of Lugol's staining in anal mucosa. Lugol's solution distinguished anal HSIL from LSIL and better defined the lesion borders, similar to its utility in detection of cervical and esophageal disease.

Colposcopic detection of anal lesions has been reported by several groups[11,13,14]. O'Connor first described the use of the colposcope for detection of anal disease but did not use colposcopic methods such as application of acetic acid or Lugol's solution [13]. Others have used the colposcope with acetic acid [14]or modified colposcopic terminology [11, 15]. The colposcopic appearance of a lesion was not predictive of histology, but lesions were categorized only by contour [15]. Scholefield et al [11] correlated colposcopic features with histology in 213 women at risk for anal SIL and reported correlations between colposcopic predictors of normal or severe dysplasia but not mild to moderate dysplasia. It is difficult to compare these results with our own since the classification system was different than current standards. Friedlander et al [4] used HRA in 32 patients and reported an 81% correlation between HRA impression and histology but did not report the criterion used for colposcopic determination. A study of 385 biopsied anal lesions from 152 men was the largest series to be published to date [9]. In that study, the colposcopic appearance of anal lesions was shown to be similar to cervical lesions and correlated with expected grade of disease. The current study represents the largest series of patients and lesions described, biopsied, and correlated with histology diagnoses.

As hypothesized, four of the categories evaluated (i.e., contour, surface, vascular patterns, and Lugol's staining) were found to broadly distinguish between LSIL and

HSIL However, margins did not. Characteristics associated with cervical HSIL: flat contour, smooth surface, punctation or mosaic vessels, and L- staining were all associated with HSIL in the anus both as individual characteristics and together as a lesion pattern. However, 'smooth' did not remain significant in a multivariate logistic regression. Characteristics associated with cervical LSIL were found more often in anal lesions that were LSIL; namely raised contour, granular, papillae and micropapillae surfaces, warty vessels and LP or L+ staining. These are typical findings for cervical condyloma acuminate or flat warts.

Of note, the PPV and OR of these characteristics for HSIL increased with the addition of Lugol's staining, specifically with a L- stain. Importantly, the largest increases in PPV and OR were found in L- staining of lesions with otherwise "typical LSIL appearances". Prior studies have noted that almost 10% of lesions with a LSIL appearance were HSIL [9, 11]. In this series, 13.5% of lesions with a LSIL appearance were found to be HSIL on histology. However, 24.5% of these lesions which stained L- were HSIL compared to only 9.9% of those with L+ or LP staining. The PPV for HSIL was approximately twofold higher when individual characteristics (i.e., raised contour, papillae, micropapillae and warty vessels) were examined and found to be L- stained. Findings from this study suggest that the addition of Lugol's staining to the HRA may help to differentiate HSIL lesions with more precision than reliance on the lesion's appearance alone.

Only the margin characteristics were not associated with their hypothesized grade of disease. "Indistinct" margins, which are associated with cervical LSIL, were found in the majority of all histology categories and no single margin characteristic was

significantly associated with a histology diagnosis. In the cervix, margins are readily appreciated with the application of acetic acid. On the cervix, indistinct margins are thought to be associated with LSIL while distinct margins are associated with HSIL. In the anal canal, margins were difficult to evaluate. Frequently the lesion border was obscured or not fully seen. Nearly 70% of margins were considered "indistinct". Data were missing for approximately 31% of the lesions in this study because the lesions did not fit the margin descriptions that were prespecified for coding. For example, clinicians were not asked to code margins for raised lesions which resulted in a falsely inflated association between all of the margin characteristics and HSIL since most of the raised lesions were LSIL.

In some cases, the addition of Lugol's staining allowed the clinician to see distinct margins when only indistinct margins had been seen with acetic acid. The small series of lesions with histology results for L- areas adjacent to L+ or LP areas, showed that Lugol's staining helped define the lesion margins. In these lesions, L- areas were significantly associated with HSIL while the adjacent L+ or LP areas were associated with < HSIL. Lugol's staining in situations where diffuse margins are present may help to determine the extent of disease. While the margin category does not appear to distinguish HSIL from LSIL, further research is needed to determine whether lesion margins in the anus can be better defined using Lugol's staining.

While Lugol's staining is easier to discriminate than acetowhite epithelial changes induced by acetic acid, Lugol's staining alone is not adequate for determining sites for biopsy. While 86% of HSIL were L-, 43% of the L- lesions were not HSIL indicating a high false positive rate (FPR: 58.9%). The high FPR is not unique to anal lesions and has

been seen with cervical and esophageal lesions [16-18]. Several situations can cause the anal mucosa to have a L- stain in the absence of HSIL. The reaction of the Lugol's solution with glycogen induces the dark mahogany positive stain, conversely tissue without glycogen does not stain and will be Lugol's negative. Glycogen is absent in scar tissue, normal columnar or rectal epithelium and squamous metaplasia. A keratinized skin surface prevents the absorption of the Lugol's solution and will have a L- appearance. The L- appearance of rectal columnar tissue and squamous metaplasia can be recognized if the TZ is observed while the Lugol's solution is applied. Familiarization with these features will decrease the FPR but even very skilled clinicians will obtain false positive biopsies. In this study, biopsies may have been taken of L- scar tissue in prior areas of treatment to evaluate for recurrences or inadequate treatment. In such cases, a lesion would have been defined as L- but was unlikely to yield a positive result thus inflating the FPR. Finally, verrucous warty lesions may appear to be L- if the solution is not applied carefully to the uneven surface which may have contributed to a higher than expected rate of L- staining in the absence of HSIL.

Lugol's staining alone revealed the presence of some lesions. These were areas in which AWE had not been seen using acetic acid. Biopsies of these L- stained areas yielded additional HSIL (17.5%) or LSIL (20%) in 93 lesions which would not have otherwise been biopsied. These findings suggest that L- staining may help identify lesions in the absence of any other defined characteristics during an HRA following an abnormal cytology.

Visual inspection of the cervix using Lugol's iodine staining has been used in low resource settings in the absence of colposcopy. The sensitivity for HSIL ranged from

59.7% to 87.2% while specificity ranged from 63% to 88.4% in these studies [19-21]. In this study, the sensitivity was also 86% although specificity was much lower. However, the high NPV of L- staining for HSIL, along with the high sensitivity of LP or L+ staining for LSIL was notable. Only 14% of HSIL lesions were LP or L+. Indicating that in the absence of other criterion to evaluate, a LP or L+ lesion is unlikely to be HSIL.

Assessment of all the characteristics present in a lesion will improve the likelihood of obtaining HSIL. In this study compared to prior published data [9], a higher proportion of the characteristics hypothesized to be associated with HSIL were biopsied including flat lesions (71.6% versus 52.7%) mosaic patterns (31.3% versus 20%), and punctation (60.4% versus 43.2). This approach introduced a bias towards biopsies of lesions with characteristics thought to be HSIL based on the results from the prior study as well as common gynecologic practice. However, a higher proportion of these characteristics were histologically HSIL compared to prior results. HSIL was found in 58.2% versus 39.4% of lesions with flat contour, 67.4% versus 46.6% of mosaic patterns, and 60.4% versus 43.2% of punctation. The higher prevalence of HSIL compared to the previously published series may indicate the increased prevalence of HSIL in this cohort or that the clinicians became more skilled at distinguishing HSIL from LSIL. However, the logistic regression analyses, a statistic less influenced by prevalence, indicated that the characteristics hypothesized to be associated with HSIL were significant indicators for anal HSIL.

Patients with abnormal cytology may present with a large volume of disease and with varying types of lesions. It is not possible to biopsy each and every lesion to determine a patient's histology. As such the clinician must choose the lesions most likely

to provide the highest grade lesion for histopathologic diagnosis. A better understanding of the appearance of these lesions and their correlation with different lesion characteristics will maximize the likelihood of attaining the highest grade lesion. Because clinicians typically assume a lesion with warty appearance is LSIL, these are frequently not biopsied and may not be treated in deference to what is assumed to be higher grade disease. These findings underscore the importance of biopsying lesions even when HSIL is not suspected. It has particular importance in cases where cytology indicates the presence of HSIL but the lesions all have a LSIL appearance. L- staining in these circumstances may provide the means to distinguish HSIL from LSIL.

These results may not be generalizable to less experienced clinicians, as our own data indicated higher sensitivities and PPV in the lesions evaluated by the more experienced clinician (data not shown). In addition. this cohort represents men considered to be at high risk for anal HPV disease with a higher prevalence of HSIL compared to the general population, and results should be repeated in other settings. The preponderance of L- lesions revealed a bias. Systematic biopsies of L+ or LP and L- areas could decrease sampling bias which is a weakness in this study.

Conclusions

Colposcopic examination is considered standard practice for identification of SIL or cancer following an abnormal Pap smear. Swabbing with acetic acid, evaluation of the lesion for different characteristics, and Lugol's staining are commonly used in gynecology to guide the clinician's choice of biopsy site. Lugol's staining is standard practice to determine the margins for excision and treatment of cancer precursor lesions in both the cervix and esophagus. Based on screening programs for the prevention of

cervical cancer, screening programs for anal cancer and its precursor lesions are now established in many cities. HRA is becoming the standard of care for evaluation of the anus following an abnormal cytology.

Our study represents the largest series of anal lesion descriptors and corresponding histology results as well as the first systematic examination of Lugol's staining in anal lesions reported in the literature. The results indicate that colposcopic criteria developed for the cervix are useful in distinguishing anal lesions. Evaluating lesions systematically for their contour, surface, vascular patterns and Lugol's staining can help guide the clinician in choosing lesions for biopsy to submit for histologic diagnosis and may help maximize the likelihood of finding HSIL.

References

1. Coppleson M, Pixley E, Reid B. *Colposcopy: A scientific and practical approach to the cervix, vagina, vulva in health and disease*. Fourth ed. Springfield Ill: Charles C Thomas; 1986.

2. Reid R, Campion MJ. HPV-associated lesions of the cervix: biology and colposcopic features. *Clin Obstet Gynecol.* 1989;32(1):157-179.

3. de Ruiter A, Carter P, Katz DR, et al. A comparison between cytology and histology to detect anal intraepithelial neoplasia. *Genitourin Med.* 1994;70(1):22-25.

4. Friedlander MA, Stier E, Lin O. Anorectal cytology as a screening tool for anal squamous lesions: cytologic, anoscopic, and histologic correlation. *Cancer.* Feb 25 2004;102(1):19-26.

5. Palefsky JM, Holly EA, Hogeboom CJ, Berry JM, Jay N, Darragh TM. Anal cytology as a screening tool for anal squamous intraepithelial lesions. *J Acquir Immune Defic Syndr Hum Retrovirol.* Apr 15 1997;14(5):415-422.

6. Palefsky JM, Holly EA, Hogebbom CJ, Berry JM, Jay N, Darragh TM. Anal cytology as a screening tool for anal squamous intraepithelial lesions. *J Acquired Immune Deficiency Syndromes and Human Retrovirology.* 1997;14:415-422.

7. Fox PA. Human papillomavirus and anal intraepithelial neoplasia. *Curr Opin Infect Dis.* Feb 2006;19(1):62-66.

8. Chin-Hong PV, Palefsky JM. Human papillomavirus anogenital disease in HIV-infected individuals. *Dermatol Ther.* Jan-Feb 2005;18(1):67-76.

9. Jay N, Berry JM, Hogeboom CJ, Holly EA, Darragh TM, Palefsky JM. Colposcopic appearance of anal squamous intraepithelial lesions: relationship to histopathology. *Dis Colon Rectum.* 1997;40:919-928.

10. Mathews WC, Sitapati A, Caperna JC, Barber RE, Tugend A, Go U. Measurement characteristics of anal cytology, histopathology, and high-resolution anoscopic visual impression in an anal dysplasia screening program. *J Acquir Immune Defic Syndr.* Dec 15 2004;37(5):1610-1615.

11. Scholefield JH, Ogunbiyi OA, Smith JHF, Rogers K, Sharp F. Anal colposcopy and the diagnosis of anal intraepithelial neoplasia in high-risk gynecologic patients. *International Journal Gyencologic Cancer.* 1994;4:119-126.

12. Palefsky JM, Holly EA, Efirdc JT, et al. Anal intraepithelial neoplasia in the highly active antiretroviral therapy era among HIV-positive men who have sex with men. *Aids.* Sep 2 2005;19(13):1407-1414.

13. O'Connor JJ. The study of anorectal disease by colposcopy. *Dis Colon Rectum.* Oct 1977;20(7):570-572.

14. Scholefield JH, Sonnex C, Talbot IC, et al. Anal and cervical intraepithelial neoplasia: possible parallel [see comments]. *Lancet.* 1989;2(8666):765-769.

15. Surawicz CM, Kirby P, Critchlow C, Sayer J, Dunphy C, Kiviat N. Anal dysplasia in homosexual men: role of anoscopy and biopsy. *Gastroenterology.* 1993;105(3):658-666.

16. Connor MJ, Sharma P. Chromoendoscopy and magnification endoscopy for diagnosing esophageal cancer and dysplasia. *Thorac Surg Clin.* Feb 2004;14(1):87-94.

17. Chanen W, Pagano R. Diethylstilboestrol (DES) exposure in utero. *Med J Aust.* Oct 13 1984;141(8):491-493.
18. Sankaranarayanan R, Wesley R, Thara S, et al. Test characteristics of visual inspection with 4% acetic acid (VIA) and Lugol's iodine (VILI) in cervical cancer screening in Kerala, India. *Int J Cancer.* Sep 1 2003;106(3):404-408.
19. Sarian LO, Derchain SF, Naud P, et al. Evaluation of visual inspection with acetic acid (VIA), Lugol's iodine (VILI), cervical cytology and HPV testing as cervical screening tools in Latin America. This report refers to partial results from the LAMS (Latin AMerican Screening) study. *J Med Screen.* 2005;12(3):142-149.
20. Sankaranarayanan R, Nene BM, Dinshaw KA, et al. A cluster randomized controlled trial of visual, cytology and human papillomavirus screening for cancer of the cervix in rural India. *Int J Cancer.* Sep 10 2005;116(4):617-623.
21. Shastri SS, Dinshaw K, Amin G, et al. Concurrent evaluation of visual, cytological and HPV testing as screening methods for the early detection of cervical neoplasia in Mumbai, India. *Bull World Health Organ.* Mar 2005;83(3):186-194.

Table 1

Distribution of Anal Lesion Characteristics in Relationship to Histologic Diagnosis

AWE Lesion	HSIL N (%)	LSIL N (%)	Atypia N (%)	Normal N (%)	Total N
	389 (46.6)	349 (41.8)	43 (5.1)	54 (6.5)	835

a. Contour

Characteristic	HSIL N (%)	LSIL N (%)	Atypia N (%)	Normal N (%)	Total N
Flat	327 (59.5)	143 (26.0)	35 (6.4)	45 (8.2)	550
Raised*	62 (21.8)	206 (72.3)	8 (2.8)	9 (3.1)	285

***includes 48 lesions that were both Flat and Raised**

b. Surface

Characteristic	HSIL N (%)	LSIL N (%)	Atypia N (%)	Normal N (%)	Total N
Smooth	374 (47.5)	322 (40.9)	42 (5.3)	49 (6.3)	787
Not Smooth	15 (31.3)	27 (56.2)	1 (2.1)	5 (10.4)	48
Granular	27 (37.5)	38 (52.8)	1 (1.4)	6 (8.3)	72
Not Granular	362 (47.4)	311 (40.8)	42 (5.5)	48 (6.3)	763
Papillae	23 (13.6)	141 (83.4)	4 (2.4)	1 (.6)	169
No Papillae	366 (55.0)	208 (31.2)	39 (5.9)	54 (8.1)	666
Micropapillae	40 (35.1)	64 (56.1)	4 (3.5)	6 (5.3)	114
No Micropapillae	349 (48.4)	285 (39.5)	39 (5.4)	48 (6.7)	721

Table 1 (Continued)

c.Vascular Patterns

Characteristic	HSIL N (%)	LSIL N (%)	Atypia N (%)	Normal N (%)	Total N
Punctation	236 (60.3)	89 (22.8)	25 (6.4)	41 (10.5)	391
No Punctation	153 (34.5)	260 (58.6)	18 (4.1)	13(2.9)	444
Mosaic	176 (67.4)	50 (19.2)	18 (6.9)	17 (6.5)	261
No Mosaic	213 (37.1)	299 (52.1)	25 (4.4)	37 (6.4)	574
Warty	61 (23.0)	189 (71.3)	8 (3.0)	7 (2.6)	265
No Warty	328 (57.5)	160 (28.1)	35 (6.1)	47 (8.2)	570

d. Margin Patterns*

Characteristic	HSIL N (%)	LSIL N (%)	Atypia N (%)	Normal N (%)	Total N
Distinct	21 (43.8)	12 (25.0)	9 (18.7)	6 (12.5)	48
Regular	96 (75.0)	47 (36.7)	3 (2.3)	6 (4.7)	152
Indistinct	232 (58.0)	105 (26.3)	28 (7.0)	35 (8.7)	400
Not Scored*	40 (17.0)	185 (78.7)	3 (1.3)	7 (3.0)	235

***Raised lesions were not scored for margins**

e. Lugol's Staining*

Characteristic	HSIL N (%)	LSIL N (%)	Atypia N (%)	Normal N (%)	Total N
Negative	335 (56.9)	175 (29.8)	40 (6.8)	38 (6.5)	588
Partial	51 (24.1)	145 (68.4)	2 (.9)	14 (6.6)	212
Positive	3 (8.6)	29 (82.9)	1 (2.8)	2 (5.7)	35
Total	389	349	43	54	835

Table 2
Sensitivity, Specificity, Positive and Negative Predictive Values of Lesion Characteristics for anal HSIL Comparing No Stain to Lugol's Negative Staining

Characteristic	***Sensitivity***		***Specificity***		***PPV***		***NPV***	
	No Stain	Lug Neg	No Stain	Lug Neg	No Stain	Lug Neg	No Stain	Lug Neg
Contour								
Flat	84.1	74.8	50.3	60.3	59.5	62.2	78.2	73.3
Raised	15.9	11.3	50.0	83.0	21.8	36.7	40.5	51.7
Surface								
Smooth	96.1	82.8	7.4	47.8	47.5	58.0	68.8	76.2
Granular	6.9	5.9	89.9	94.6	37.5	48.9	52.6	53.6
Papillae	5.9	3.1	67.3	92.6	13.6	26.7	45.0	52.3
Micropapillae	10.3	8.5	83.4	93.3	35.1	52.4	51.6	53.9
Vessels								
Punctation	60.7	55.8	65.2	70.4	60.4	62.2	65.5	64.6
Mosaic	45.2	41.6	80.9	83.2	67.4	68.4	62.9	62.0
Warty	15.7	10.5	54.3	86.5	23.0	40.6	42.5	52.6
Margins								
Distinct	5.4	5.4	93.9	93.9	43.8	43.8	53.2	53.2
Lugols								
Negative	86.1		43.3		57.0		78.1	

Table 3

Univariate Logistic Regression Analysis of Anal Lesion Characteristic's Ability to Predict HSIL

Characteristic	*Odds Ratio*	*95 % Confidence Limit*		*P Value*
		Low	*High*	
Contour				
Flat	5.274	3.796	7.328	<.0001
Vessels				
Mosaic	3.509	2.576	4.782	<.0001
Punctation	2.896	2.185	3.838	<.0001
Warty Vessels	.221	.158	.307	<.0001
Surface				
Smooth	1.992	1.065	3.726	.0310
Granular	.665	.404	1.093	.1078
Papillae	.129	.081	.206	<.0001
Micropapillae	.576	.382	.869	.0086
Margins				
Distinct	.886	.492	1.593	.685
Lugol's Neg	4.732	3.358	6.669	<.0001

Table 4a
Multiple Logistic Regression Analysis of Anal Lesion Characteristics Ability to Predict HSIL

Characteristic	*Odds Ratio*	*95 % Confidence Limit*		*P Value*
		Low	*High*	
Contour				
Flat	2.236	1.318	3.792	.003
Vessels				
Mosaic	2.029	1.427	2.884	<.0001
Punctation	1.530	1.074	2.058	.019
Warty Vessels	1.073	.560	2.058	.831
Surface				
Smooth	1.770	.590	5.310	.308
Granular	1.905	.743	4.883	.180
Papillae	.546	.246	1.213	.137
Micropapillae	.937	.505	1.738	.836
Margins				
Distinct	.428	.223	.819	.010
Lugol's Neg	2.269	1.516	3.398	<.0001

Table 4b
Logistic Regression Model for Anal Characteristics Predictive of HSIL

Characteristic	*Odds Ratio*	*95 % Confidence Limit*		*P Value*
		Low	*High*	
Flat	2.745	1.886	3.995	<.0001
Mosaic	2.005	1.443	2.805	<.0001
Punctation	1.469	1.062	2.033	.0200
Lugol's Neg	2.318	1.570	3.422	<.0001

Table 5

Common Lesion Patterns: Positive Predictive Value and Logistic Regression Predictive Probablity for HSIL

Pattern	Lugol's Negative		Lugol's Partial/Pos		Total PPV For HSIL	Log Regression Predictive Probability	
	N	(n HSIL)	N	(n HSIL)		Lug P/+	Lug -
Flat, Mosaic, Smooth	75	(53)	13	(7)	68.2%	.474	.671
Flat, Mosaic, Punctation, Smooth	137	(92)	7	(5)	67.4%	.579	.757
Flat, Punctation, Smooth	157	(93)	27	(12)	57.1%	.404	.606
Flat, Smooth	58	(31)	11	(4)	50.7%	.307	.502
Flat, Warty Vessels, Smooth	6	(3)	5	(1)	36.4%	.322	.519
Flat, Warty Vessels, Smooth, Micropapillae	15	(6)	17	(5)	34.4%	.308	.503
Raised, Warty vessels, Smooth, Micropapillae	10	(5)	8	(1)	33.3%	.166	.311
Raised, Warty vessels, Smooth, Papillae	32	(7)	106	(11)	13.0%	.104	.209
Raised, Granular	10	(1)	10	(1)	10.0%	.176	.326
Raised, Warty vessels, smooth, Papillae, Micropapillae	1	(0)	15	(0)	0%	.098	.134
Total Lesions with <5 lesions per pattern	76	(35)	40	(10)	38.8%	NA	NA

Figure 1

Lugol's Staining Patterns in Anal Mucosa

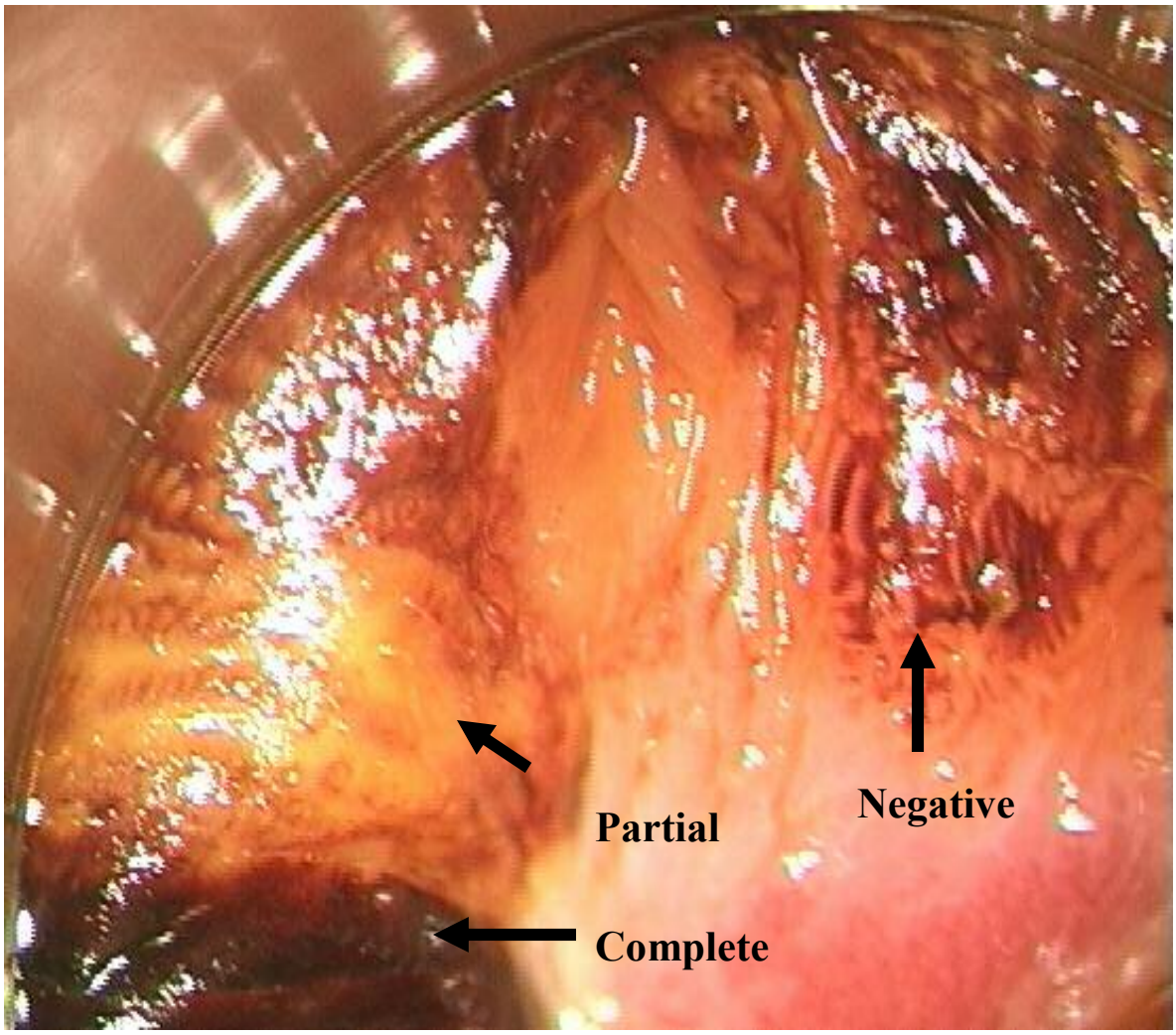


Figure 2

The Positive Predictive Values of Lesion Characteristics for HSIL before and after Lugol's Staining

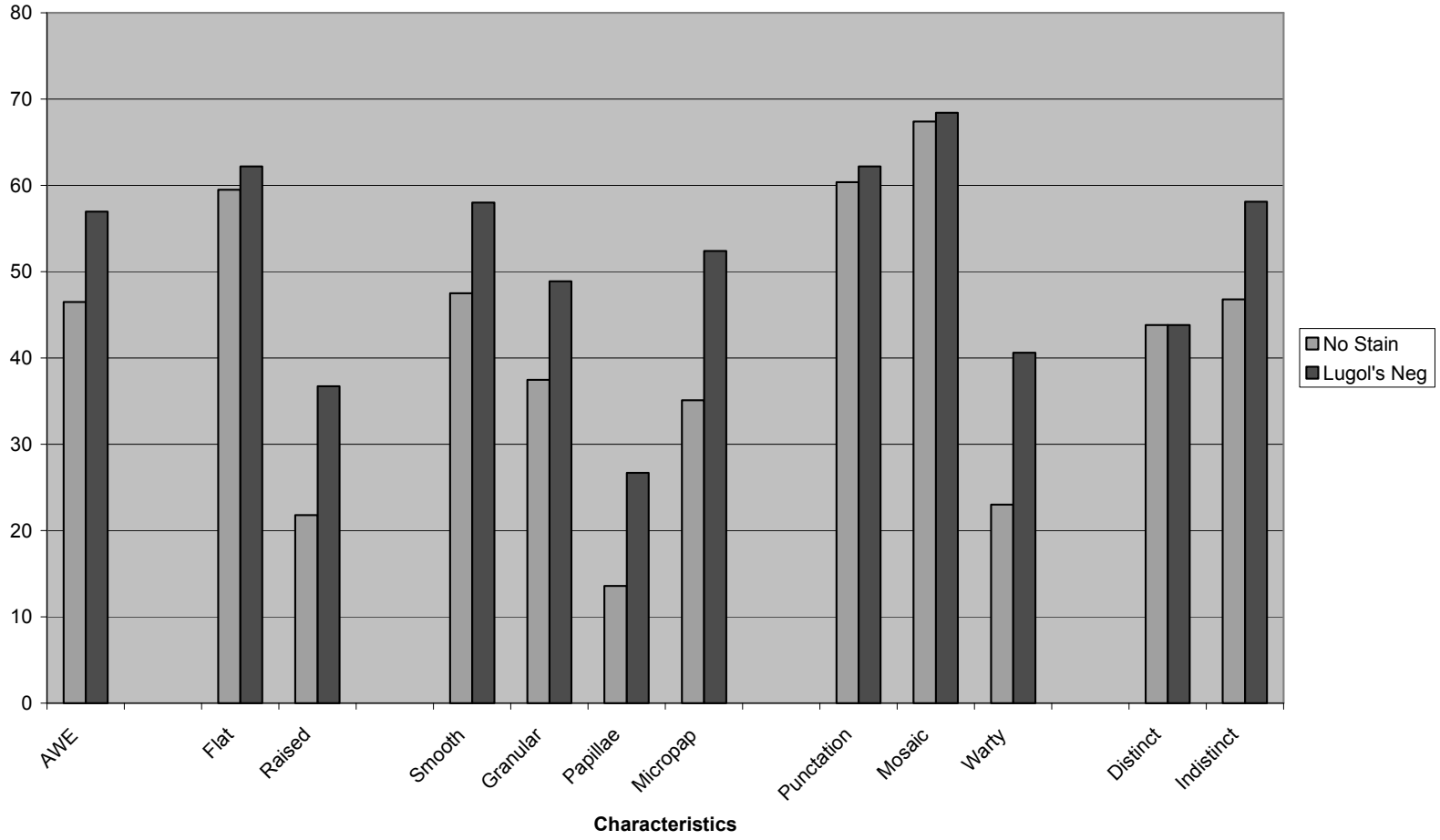
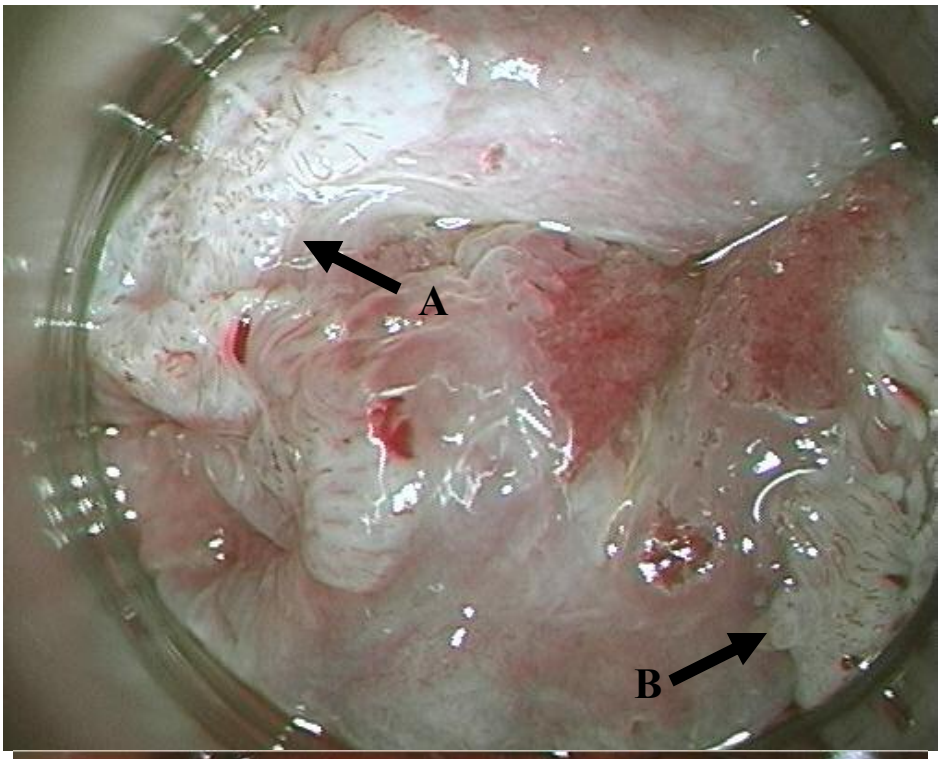


Figure 3a and 3b

Raised warty lesions: Arrow A indicates area of lugol's negative stained area correlated with HSIL histology, Arrow B indicates area with Lugol's positive/partial staining which correlated with LSIL histology.



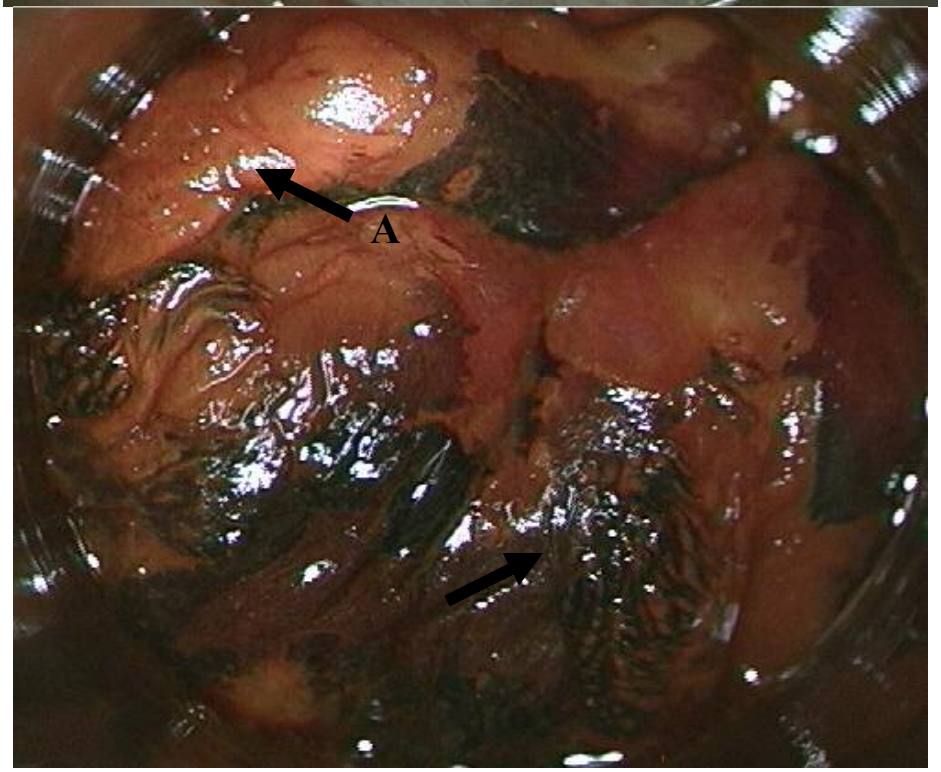
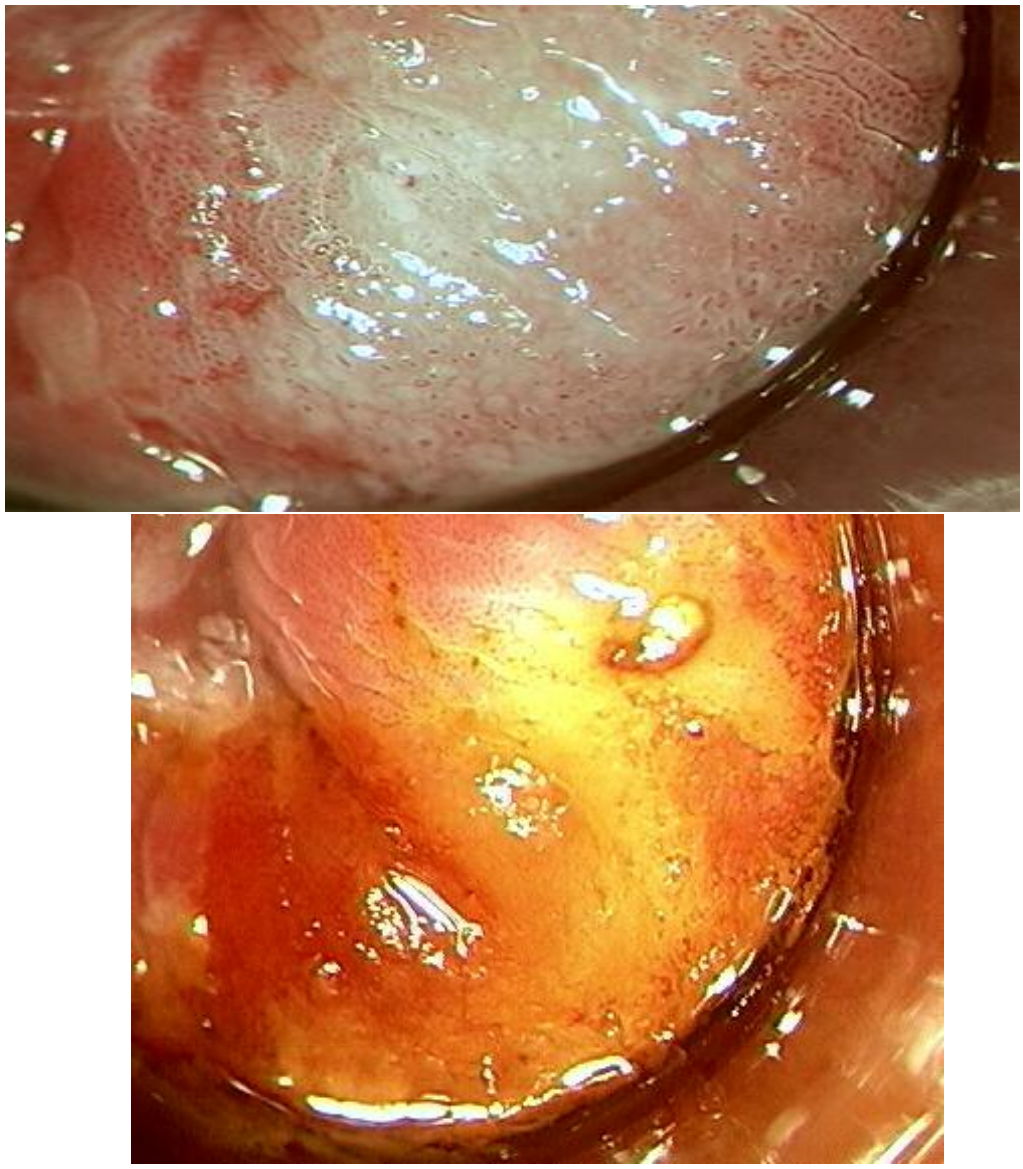


Figure 4

Typical HSIL with flat contour, smooth surface, mosaic and punctation vascular patterns, and lugol's negative staining.



Cytotoxic Activity of a Traditional Chinese Medicine Ointment in Cervical Cancer Cell Lines

Naomi Jay[1], Misha Cohen[4], Margaret Takedo[3], Joel Palefsky[1], Marylin Dodd[2], Christine Miaskowski[2] and Karen Smith McCune[3]

Departments of Medicine[1], Nursing[2], and Gynecology[3] University of California San Francisco, and Chicken Soup Chinese Medicine[4]

Running Title: Cytotoxic Activity of TCM Ointment

Keywords: Traditional Chinese Medicine, Cervical Cancer

Acknowledgements

This paper was supported in part by a doctoral fellowship grant from the American Cancer Society.

Abstract

Arnebia Indigo Jade and Pearl (AIJP) Ointment was developed by Misha Cohen, LAc, and Andrew Ellis of Springwind Herbs for treatment of anal and cervical high grade squamous intraepithelial lesions (HSIL). The ingredients were chosen based on their properties according to traditional Chinese Medicine therapeutics. The ointment consists of 10 ingredients including 7 herbs-infused in sesame oil, along with an additional 3 powdered ingredients added to the herb-infused oil to create the ointment. AIJP was manufactured in accordance with traditional methodology by Vitality Works (Albuquerque, NM) and distributed by Springwind Inc. (Berkeley, Ca.).

HSIL is considered to be the precursor lesion for both cervical and anal cancer. The decreased rate in cervical cancer over the past several decades is attributed to treatment of these precursor lesions, and it is hypothesized that anal cancer can be prevented by treatment of HSIL as well. The standard of care in Western medicine is removal of the lesion using ablative techniques such as laser therapy or more recently with experimental therapies such as aldara or efudex creams. The herbs in AIJP have been used in ointment form for a variety of dermatologic conditions including skin cancers. As a preliminary trial we sought to see if the ointment would have an effect on cervical cancer cell lines in vitro.

Methods: A series of four experiments were performed. 1) pilot studies in 96-well plates using the herb-infused oil, powdered ingredients, and AIJP; 2) control experiments to demonstrate false positive cell readings and determine mechanisms for delivering treatment to cells; 3) herb-infused oil treatments of the three cell lines in 96-well plates and 4) AIJP ointment in CASKi and HELA cells in 6 well-plates. The cells were prepared

according to standard recommendations The cell lines were treated for 24 – 72 hours and percentage of surviving live cells were then counted using an MTT assay.

Results: The herb-infused oil and ointment were effective in arresting cell growth in all 3 cancer cell lines. Increasing doses of both treatment oil and ointment correlated with a decreased percentage of surviving cells, indicating a dose response effect. The study also validated methodology for in vitro testing of TCM ointments in cell culture.

Introduction

Worldwide, cervical cancer is the third most common cancer in women with mortality rates of approximately 200,000 per year. In developing countries, it is the leading cause of cancer deaths. In North America, its incidence has decreased significantly with the advent of cytology screening. This reduction is attributed to targeted treatment of precancerous high grade squamous intraepithelial lesions (HSIL) which prevents their progression to cancer. The natural history of anal cancer is similar to cervical cancer. Like cervical disease, it is associated with the human papilloma virus (HPV), primarily types 16 and 18. In addition, HSIL is considered a precursor lesion as it is frequently found adjacent to or in association with anal cancer [1, 2]. Therefore, it is hypothesized that treatment of these anal lesions will prevent progression to cancer.

The mainstay of therapy for cervical HSIL has been ablation of the lesions using loop electrosurgical excision (LEEP), laser, or cryotherapy. Similar ablative therapies are used for anal canal HSIL. However, anal lesions are more difficult to treat effectively and are associated with increased morbidity. For these reasons, many patients prefer a non-invasive approach and may turn to complementary alternative therapies (CAM) for treatment.

A decade ago, it was estimated that 629 million people in the United States used some form CAM which exceeded total visits to primary care providers [3]. Traditional Chinese Medicine (TCM) is one CAM approach that includes the use of herbs or other substances. The selection of these herbs is derived from "historically-based" evidence that was collected over centuries of use. Despite this rich tradition, little empiric evidence exists to support the safety and efficacy of these herbal treatments.

Arnebia, Indigo, Jade, and Pearl (AIJP) Ointment was developed by a specialist in TCM dermatology and a licensed acupuncturist (LAc) for the treatment of cervical and anal HSIL. It is applied topically to the lesion as part of a multi-modal treatment that may include acupuncture, dietary therapy and/or moxibustion. AIJP ointment is based on traditional formulas found in the TCM literature and combines Jade Pearl Cream, *Huang Lian Gao,* and *Zi Gui Yu* ointments into a single ointment. These latter two formulas are referenced as early as the Chin Dynasty (circa 1830) and were used traditionally for dermatologic disorders such as inflammation, infections, and burns[4, 5]. Jade Pearl Cream has been used in Chinese and Tibetan medicines for skin rejuvenation and for treatment of precancerous or malignant skin lesions[6, 7].

TCM source books on dermatology cite the use of the components of AIJP individually and in combination for a wide range of indications including: seborheic dermatitis; contact dermatitis; herpes; rectal abscesses; keloids; skin and genital ulcerations; eczema; psoriasis; fungal infections; mastitis; burns; tonsillitis; hemorrhoids; and genital warts[5, 8-12]. Several hundred different prescriptions exist that include one or more of these ingredients as traditional remedies for both internal and external application to all mucous membranes, including all genital sites[5, 8, 11, 12]. A review of the literature confirms that the ingredients used in AIJP have known mechanisms of action that could be beneficial in the treatment of SIL due to their anti-viral, anti-tumor, or immune stimulation effect (see Table 1). However, the effect of these components in combination has not been tested.

In vitro testing of the effects of individual herbs in cell culture is well documented. Many of the individual AIJP herbs distilled in alcohol or water, or the

synthesized active chemical [13-15]. However, the effect of herbs on HPV-associated cancers or HSIL has not been well studied [16-20] . However, TCM commonly uses a combination of ingredients to potentiate the effects or ameliorate the side effects of an individual herb, or in TCM terms, to balance the Yin and Yang of the herbs. Therefore, prior to use in a clinical trial of anal HSIL, the entire formulation of AIJP ointment was tested to determine whether it had any effect on cell cultures. This study describes the methods that were developed to perform in vitro testing of AIJP and documents its cytotoxic activity in three cervical cancer cell lines (i.e. C33A, CASKI, and HELA). CASKI and HELA are, respectively, HPV 18 and 16 DNA-immortalized epithelial cells while C33A is an HPV-negative cervical cancer cell line.

Methods

The AIJP ointment consisted of 10 ingredients (i.e., 8 herbs and 2 minerals). It was manufactured according to traditional TCM methods by Vitality Works (Albuquerque, NM) and distributed by Springwind Inc. (Berkeley Ca). Following verification of the ingredients, seven herbs (i.e., arnebia, coptis, safflower, angelica, peach pit, rehmannia, licorice) were soaked (infused) in sesame oil for 6 weeks. The herbs were then pressed from the oil and removed. The herb-infused oil was heated to 160^0F. Once heated, the herb-infused oil along with the three remaining powdered ingredients (i.e., indigo, jade, pearl) were whipped into beeswax which had been heated to 190^0F. The ointment was poured into sterile 4-ounce glass jars and sealed. The ratio of herbs, minerals, oils, and beeswax was determined by a proprietary blend (Drug Master File held by Springwind Inc.). Springwind Inc. provided the researchers with samples of the infused oil, powders, and ointment for use in these experiments.

Cancer Cell Lines

Human cervical cancer cell lines C33A, CASKI, and HELA were obtained from American Type Culture Collection (ATCC). The cell lines were maintained in ATCC recommended medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. After incubation for 7 to 10 days at 37°C, cells were trypsinized in log phase growth, counted, and added to a 96-well tissue culture plate at a concentration of 2,000 cells per well or to a 6-well plate at a concentration of 10,000 cells per well. Cells were than incubated an additional 1 to 3 days at 37°C. Herbal treatments were added to the cells in varying dilutions and incubated for an additional 24 to 72 hours. Medium was aspirated and replenished every 48 hours.

Experimental Conditions

A series of four experiments were performed. 1) pilot studies in 96-well plates using the herb-infused oil, powdered ingredients (i.e., indigo, jade, pearl), herb-infused oil with powders to simulate the active ingredients in the ointment formula without inert beeswax, and the ointment; 2) control experiments to demonstrate false positive ELISA cell readings and determine the optimal approach to deliver treatment to cells adjusted to determine the point at which no change was noted in the number of living cells post-treatment; 3) herb-infused oil treatments of the three cell lines in 96-well plates using dilutions of 1:10 to 1:1280; and 4) ointment treatment of CASKI and HELA cells using filters in 6 well-plates in doses of .25µ to .40µg.

Methylthiazolatetetrazolium (MTT) Assay

At the conclusion of each time point, the medium was aspirated, then 200 µl of fresh medium and 50 µl of a 5mg/ml MTT solution was added to each well and the plate was incubated at 37°C for 4 hours in the dark. The resultant MTT-formazan crystals were dissolved in 200 µl/well of a solution of 22.4µl of DMSO and 2.24 ml of glycine buffer (0.1M glycine, 0.1M NaCl, pH 10.5). The absorbance at 560 nm was read on a spectrophotometer ELISA plate reader which quantifies the color in colormetric assays. The percent of cell survival was calculated by dividing the average absorbance of the treated wells by the average of the non-treated wells. All experimental dilutions of treated cells were performed in 6 to 12 wells each, and compared with untreated or control wells treated with plain sesame oil. All experiments were repeated in triplicate or quadruplicate.

Results

Pilot Studies

Pilot studies were conducted to determine the experiment's feasibility. Beginning with C33A, a series of experiments were completed with the herb-infused oil, jade, pearl and indigo powders, and the AIJP ointment. We began with the herb-infused oil (containing 7 of 10 active ingredients) which was easier to pipette into the plate wells and treatment doses could be measured more accurately to evaluate a dose-response.
In these experiments, dilutions of the herb-infused oil ranged from 1:10 to 1:1280 and C33A cells were plated for 24 to 72 hours. Plates examined microscopically before and after incubation with the MTT solution indicated that a large percentage of cells treated with the herb-infused oil were dead compared to controls, and that a dose response that correlated to the dilutions was evident. However, the response as quantified by the ELISA was inconsistent (see Figure 1a). Additional experiments with the herb-infused oil and powders used as single agents produced inconsistent ELISA readings despite the effect seen microscopically (Figure 1B). The AIJP ointment could be placed in the wells with a pipette tip, but its viscosity made it difficult to measure or quantify treatment dilutions. In addition, it did not disperse into the medium but adhered to the well walls or floated. A clear effect on cell viability was seen microscopically even without dispersion of the ointment. However, the ELISA readings for these experiments did not correlate with the microscopic examination.

Control Experiments

We hypothesized two possible explanations for these results. Gross examination of the plates demonstrated that the oil was adhering to the walls which may have caused

inconsistent dosing because the oil was not dispersed evenly into the wells. Alternatively, we hypothesized that the red-coloring of the herb-infused oil and ointment that adhered to the plates, was read by the ELISA as "live cells" since the MTT assay is colormetric and specifically for red/purple colors. Therefore, experiments were initiated: 1) to determine a vehicle that had minimal effect on cell viability and could be used to dilute the herb-infused oil to attain even dispersion in the wells with less adherence to the plate walls and 2) to determine whether the herb-infused oil and ointment were being read as false positive live cells by the ELISA reader.

New experiments were first replicated in C33A cells with medium compared to dimethyl sulfoxide (DMSO) and plain sesame oil (see Figure 2a). The DSMO had a moderate cytotoxic effect (32% decrease in live cells) while sesame oil showed only a 9% decrease in live cells compared to the control. Experiments using different dilutions of sesame oil verified that it had a minimal effect on cell viability (see Figure 2B). Less than a $< 10\%$ difference in cell survival was found in the lowest compared to highest dilutions of sesame oil. Because the sesame oil and herb-infused oil had similar viscosities, using the sesame oil for dilutions of the treatment oil provided consistent quantities and more even dispersion of treatments into the plates.

Another series of experiments were done that mimicked the methods used for plating and treating cell-lines but without any cells to determine whether the ELISA plate reader was reading the color of the herb-infusted oil or ointment as false positive live cells. Medium was pipetted into 96-well plates followed by treatment with herb-infused oil using plain sesame oil in dilutions that ranged from 1:20 to 1:640 and compared with

AIJP Ointment, plain sesame oil, and medium. These plates were incubated for 24 to 48 hours followed by treatment with the MTT solution as described above.

As shown previously, gross examination of the plates demonstrated adherence of the herb-infused oil and ointment to the plate walls. The ELISA reading indicated the presence of "live cells" in a dose-response that correlated with decreasing dilutions of the herb-infused oil and with the AIJP ointment having the highest false positive reading of "live cells". While it was not possible to quantify the false positive effect, nutating the herb-infused oil with the sesame oil provided a more consistent dilution and cell walls appeared to be evenly coated with oil. The lower range of dilutions had more red-coating of the walls compared to the higher range of dilutions which was consistent with the dose as well as the results (see Figure 3).

The powders could not be added in any consistent dose to the 96-well plates although a range of experiments were tried including the herb-infused oil with each powder individually or all three in combination, as well as the powders alone and in combination without herb-infused oil. These results indicated that Indigo was the most cytotoxic, Pearl had a minimal effect, and Jade was protective with an increase in live cells over control (see Figure 1B).

Herb-Infused Oil and Ointment Experiments

Based on the control experiments, sesame oil was added in increasing amounts to the herb-infused oil and nutated. Dilutions of herb-infused oil (i.e., 1:10 to 1:1280) were used to treat the cell plates. In the C33A cell line, no differences in cytotoxic effect were found between the 1:10 and 1:20 dilutions. The effect leveled or dropped off at a dilution of 1:640. Subsequent experiments were done with dilutions between 1:20 and 1:640.

With the CASKI and HELA cell lines, the dose response leveled off at the 1:80 dilution (see Figure 4). The dilutions which resulted in a 50% reduction in live cells were determined based on the dilution where the MTT assay reading was 50% of the reading for the untreated cells. The dilution that resulted in a 50% reduction of live cells at 24 and 48 hours are shown in Table 2. Dilutions as low as 1:10 resulted in 50% reduction of live cells compared to controls.

For testing of the complete formula that consisted of the 10 active ingredients experiments were initiated with the AIJP ointment. The pilot studies indicated that the AIJP ointment had a cytotoxic effect evidenced microscopically and that the flat response found by the ELISA reader was due to false positive readings caused by the ointment adhering to the plate walls. To eliminate these false positive readings, larger 6-well plates were used and the ointment was placed in small plastic filters that floated on top of the medium, thereby avoiding any contact of the ointment with the cell walls. These results were compared to controls in which the filters were placed without ointment. The plates were treated with two doses for 24 and 48 hours. When the filters and ointment were removed at the conclusion of the treatment times, no visible staining of the ointment was evident on the plates. The 48 hour treatments resulted in a 25% to 30% reduction in live cells compared to controls in both cell lines tested. The dose response was not as consistent in the 24 hour treatments (Figure 5)

Discussion

Combinations of herbs and other substances are frequently prescribed in TCM because the resulting blend, balances the yin and yang of the herbs and provides the best treatment. In Western science, herbs are tested individually to determine cytotoxicity or mechanisms of action. The herbs considered to be effective are then analyzed to determine the "active" ingredient which leads to new treatments for many diseases. However, because the blended formulas are used in TCM, it is important to determine the effects of the combination of substances in the manner in which they were developed and used, as was done in these experiments. Specifically, this study determined the methods for in vitro testing of an herb-infused oil that consisted of 7 herbs and substances, as well as a beeswax-based ointment that consisted of the herb-infused oil with three additional herbs and substances. Once the experimental methods were refined, the results were reproducible and demonstrated that both blends produced a dose-response effect on all three cancer cell lines tested.

The herb-infused oil contains the active ingredients for Springwind Ointment, produced by Springwind Inc., Berkeley Ca. and is prescribed for a variety of skin conditions including precancerous skin lesions. AIJP contains this oil with the added ingredients of jade, pearl and indigo and has been used specifically for cervical and anal HSIL. These experiments showed that both Springwind and AIJP ointments had an in vitro cytotoxic effect. However, direct comparison between the two products is not possible because they were not tested under comparable conditions (e.g., both as ointments using the methods developed in this study). Future experiments can test the two formulas in similar conditions to determine whether one product produces more

cytotoxic effects. In addition, a larger range of ointment doses would better determine a dose response curve. Finally, it is not known whether different batches of the ointment would produce consistent results.

Conclusion

The effectiveness of TCM ointments for primary treatment of anal or cervical HSIL has not been investigated in a clinical trial. However, these ointments and other TCM treatments are used by large numbers of subjects. This project was undertaken in an attempt to provide empiric evidence that these ointments can be tested and that they have effects in vitro. In vivo tests of these formulas will determine their safety and efficacy.

References

1. Berry JM, Palefsky JM, Welton ML. Anal cancer and its precursors in HIV-positive patients: perspectives and management. *Surg Oncol Clin N Am.* Apr 2004;13(2):355-373.

2. Palefsky JM, Holly EA, Gonzales J, Berline J, Ahn DK, Greenspan JS. Detection of human papillomavirus DNA in anal intraepithelial neoplasia and anal cancer. *Cancer Res.* 1991;51(3):1014-1019.

3. Eisenberg DM, Davis RB, Ettner SL, et al. Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. *Jama.* Nov 11 1998;280(18):1569-1575.

4. Qian W. *The Golden Mirror of Medicine Tradition (Yi Zong Jin Jian)*; 1742.

5. Shen D, Wu XF, Nissi W. *Manual of Dermatology in Chinese Medicine*. Seattle: Eastland Press; 1995.

6. Bensky D, Gamble A. *Chinese Herbal Medicine Materia Medica*. Revised ed. Seattle: Eastland Press; 1993.

7. Hsu H. *Oriental materia medica: a concise guide (Chien ming yao ts'ai hsueh)*. Long Beach: Oriental Healing Arts Institute; 1986.

8. Zhang ZJ. *Synopsis of Prescriptions of the Golden Chamber: a classic of traditional Chinese medicine (circa 25-220)*. Beijing: New World Press; 1987.

9. *Complete External Therapies of Chinese Drugs*. Beijing: Foreign Languages Press; 1998.

10. Boik J. *Cancer and natural medicine: a textbook of basic science and clinical research*. Princeton: Oregon Medical Press; 1996.

11. Chang ST. *The Great Tao*. San Francisco: Tao Publishing; 1985.

12. *The Divine Farmer's Material Medica: a translation of the shen nong ben cao jing*. Boulder: Blue Poppy Press; 1998.

13. Gaddipati JP, Mani H, Shefali, et al. Inhibition of growth and regulation of IGFs and VEGF in human prostate cancer cell lines by shikonin analogue 93/637 (SA). *Anticancer Res.* 2000;20(4):2547-2552.

14. Chung JG, Chen GW, Hung CF, et al. Effects of berberine on arylamine N-acetyltransferase activity and 2-aminofluorene-DNA adduct formation in human leukemia cells. *Am J Chin Med.* 2000;28(2):227-238.

15. Shibata T, Morimoto T, Suzuki A, Saito H, Yanaihara T. [The effect of Shakuyaku-kanzo-to on prostaglandin production in human uterine myometrium]. *Nippon Sanka Fujinka Gakkai Zasshi.* 1996;48(5):321-327.

16. Zheng J, Deng YP, Lin C, Fu M, Xiao PG, Wu M. Arsenic trioxide induces apoptosis of HPV16 DNA-immortalized human cervical epithelial cells and selectively inhibits viral gene expression. *Int J Cancer.* Jul 19 1999;82(2):286-292.

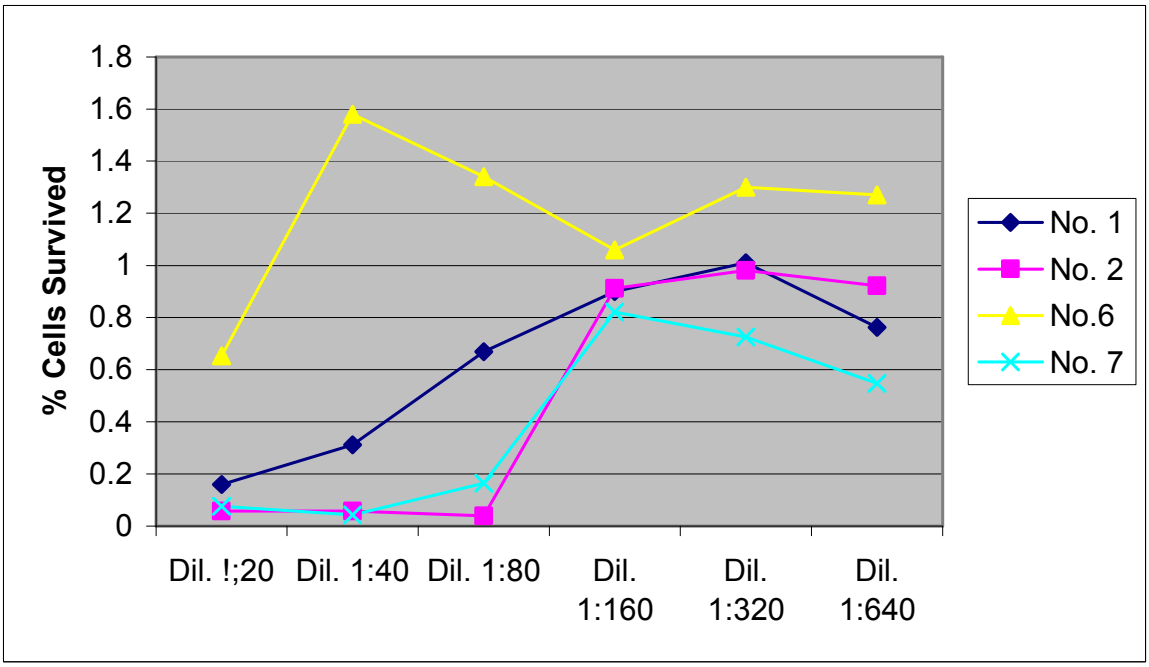
17. Deng WP, Chao MW, Lai WF, et al. Correction of malignant behavior of tumor cells by traditional Chinese herb medicine through a restoration of p53. *Cancer Lett.* Feb 28 2006;233(2):315-327.
18. Sun Y. [An experimental study of the promoting effects of extracts from Chinese medicinal herbs and tung oil (HHPA)]. *Zhonghua Bing Li Xue Za Zhi.* Mar 1986;15(1):9-11.
19. Liu CJ. [Long term results of preventive and curative treatment of cervical carcinoma with Chinese medicinal herbs--cui-tuo-ding]. *Zhonghua Zhong Liu Za Zhi.* Nov 1984;6(6):450-452.
20. Yang XZ. [Pharmaco-conization with Chinese traditional drugs for the treatment of early carcinoma of cervix uteri--observation of late results]. *Zhong Xi Yi Jie He Za Zhi.* May 1983;3(3):156-158.

Table 1. Mechanisms of Action of Arnebia Indigo Jade and Pearl Cream Ingredients

Herbs/ Minerals	Anti-tumor	Immune Stimulat-ing	Antiviral	Anti-Bacterial	Anal-gesic	Wound Healing	Anti-inflam-matory
Arnebia	X		X	X	X	X	X
Chinese Angelica	X	X		X	X		X
Coptis	X	X		X			
Indigo	X	X	X	X			X
Jade	X		X			X	
Licorice	X	X	X				X
Pearl			X				X
Peach			X				X
Rehman-nia	X			X			X
Safflower	X				X		X

Figures 1A and 1B: Pilot experiments exhibited inconsistent results.

Figure 1A. Results of four experiments with Herb-Infused Oil.



1B. Results of experiments of herb-infused oil and powders

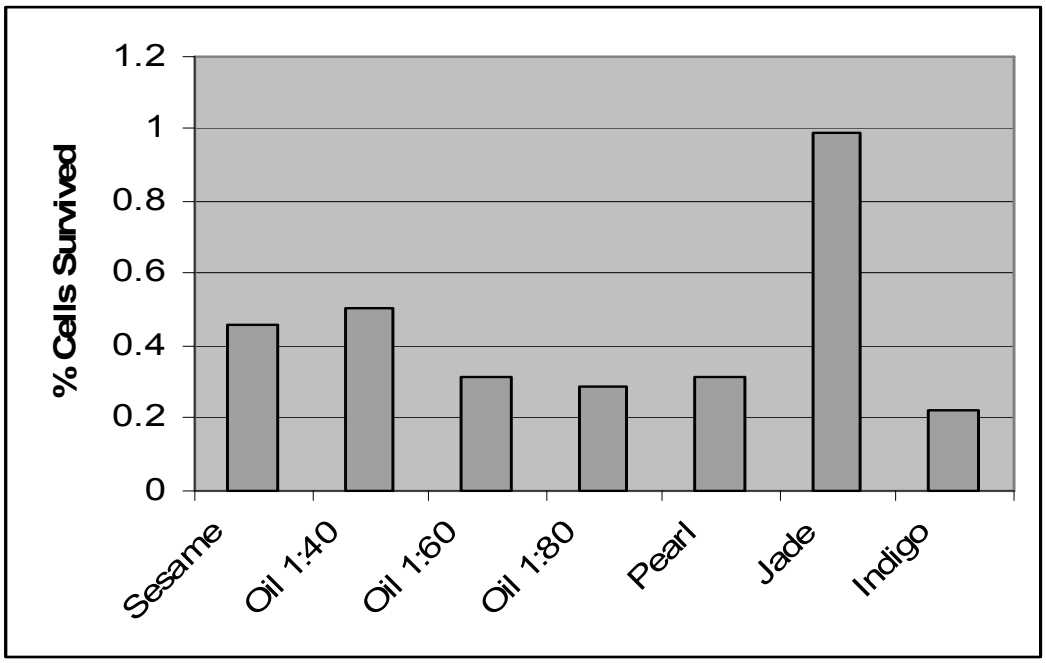


Figure 2A: The effects of control solution, sesame oil and DMSO on cell survival at 24 hours of incubation.

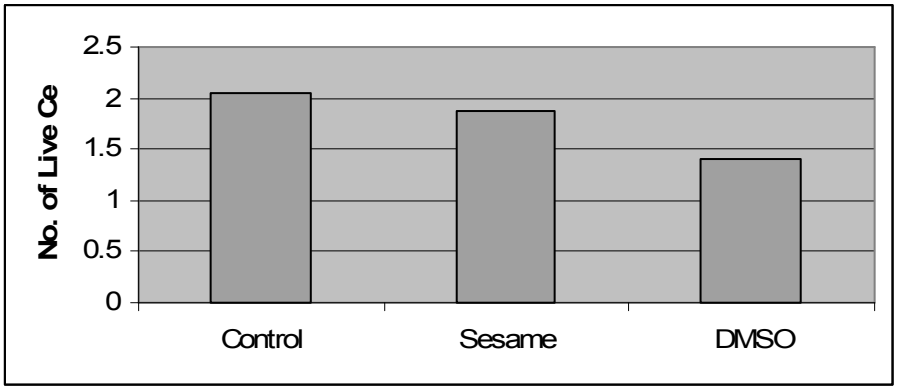


Figure 2B. The effects of plain sesame oil tested in various dilutions on cell viability at 48 hours.

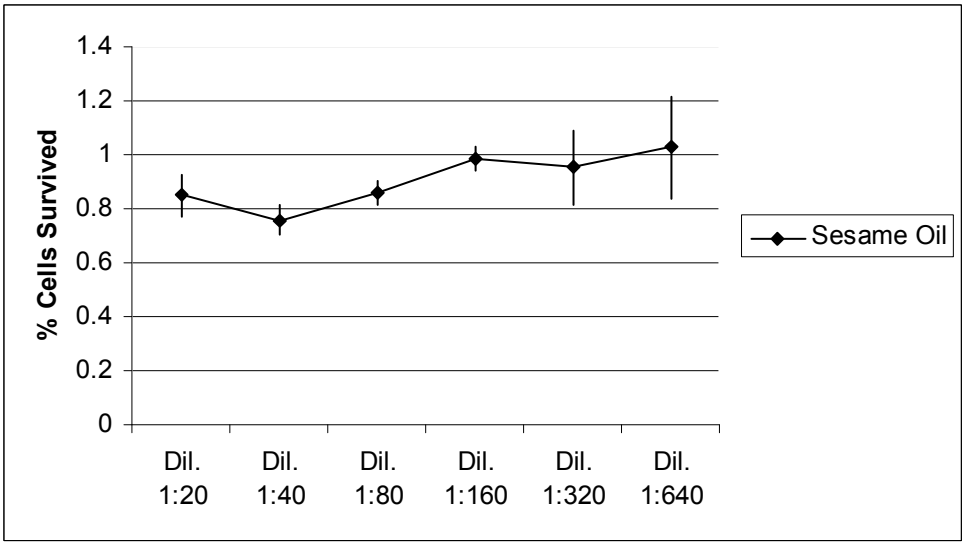


Figure 3: Percentage of cells read as “alive” by ELISA in 96-well plates when no cells had been plated using medium and plain sesame oil for controls compared to a range of herb-infused oil dilutions and AIJP ointment.

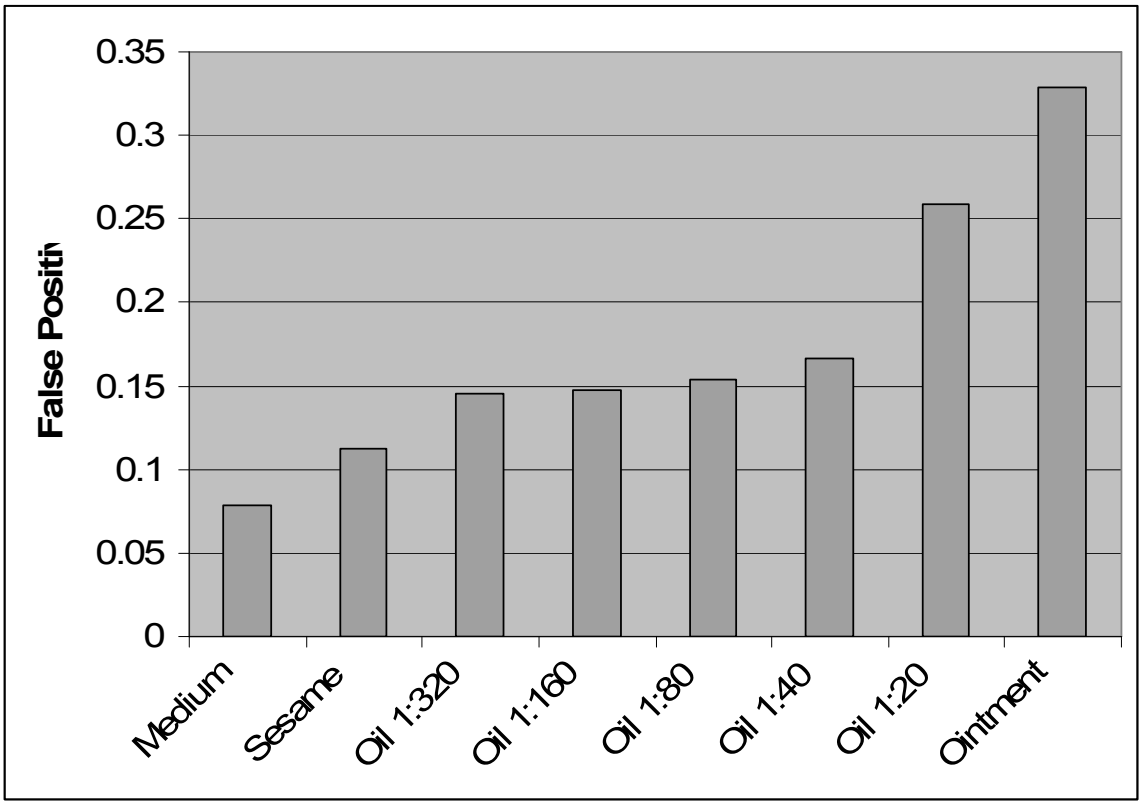
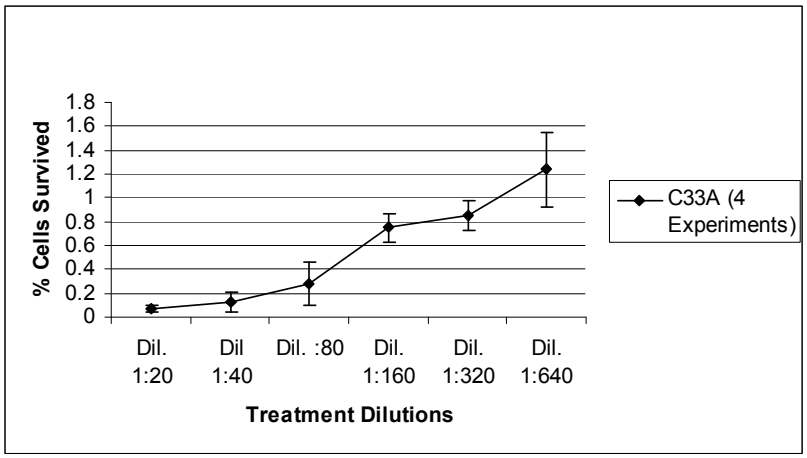
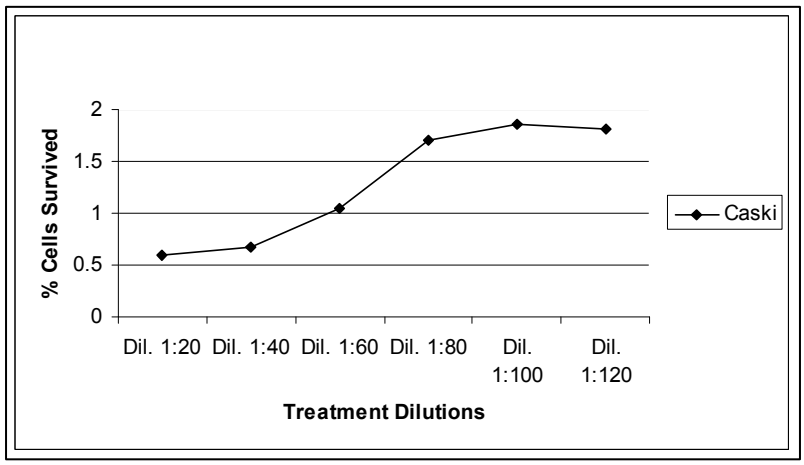


Figure 4. Dose response curves in herb-infused oil treatments of 3 cervical cancer cell lines..





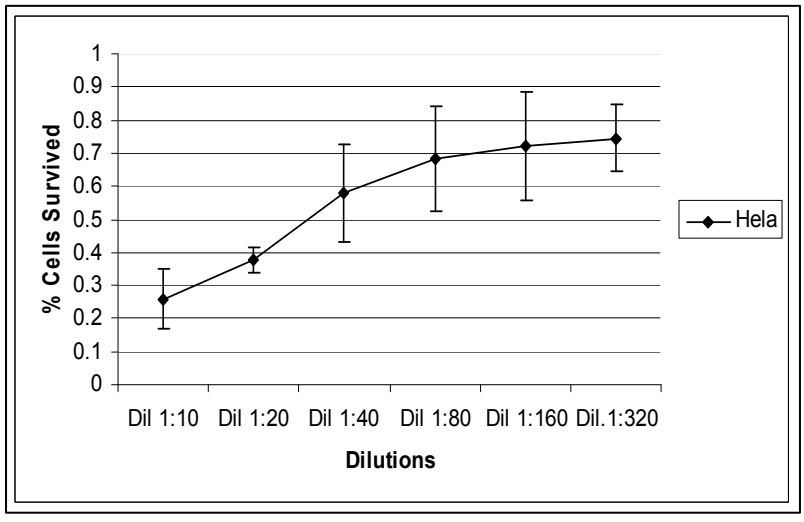
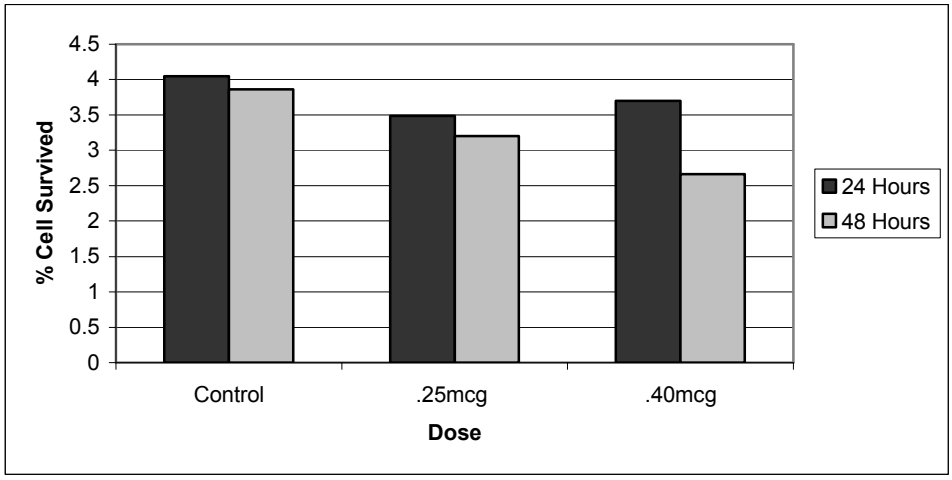


Figure 5
A. Ointment Treatments of HELA Cells



b. Ointment Treatment of CASKI Cells

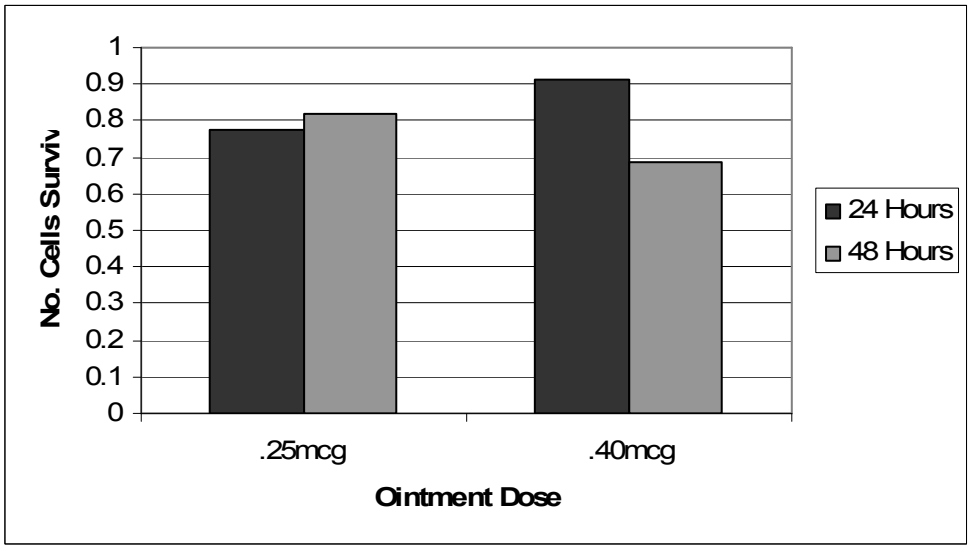


Figure 6
Dilutions for <50% Survival

TIME	C33A	CASKI	HELA
24 Hours	1:40 or less	1:10	1:20 or less
48 Hours	1:40 or less	1:160 or less	1:20 or less

The Safety, Feasibility and Efficacy of a Traditional Chinese Medicine for Treatment of Anal High Grade Squamous Intraepithelial Lesions (HSIL)

Naomi Jay[1], J. Michael Berry[1], Teresa Darragh[3], Maria Da Costa[1], Joel Palefsky[1], Marylin Dodd[2], Christine Miaskowski[2] and Misha Cohen[4]

Departments of Medicine[1], Nursing[2], and Pathology[3] University of California San Francisco, and Chicken Soup Chinese Medicine[4]

Corresponding author: Naomi Jay, Box 1699, Mount Zion Hospital UCSF, 1600

Divisadero, San Francisco, CA 94143; email Naomi.Jay@ucsf.edu

Running Title: TCM Treatment of Anal HSIL

Keywords: Traditional Chinese Medicine, Treatment, Anal, HSIL

Acknowledgements

This study was funded in part by a Nursing Doctoral Research Fellowship from the American Cancer Society and a grant from the National Cancer Institute of the National Institute of Health.

Abstract

Background The incidence of anal cancer is increased in human immune-deficiency viral-positive men and women. High grade squamous intraepithelial lesions (HSIL) are considered to be the precursor lesion for both cervical and anal cancer. It is hypothesized that treatment of anal HSIL will prevent anal cancer, similar to the decrease in cervical cancer attributed to treatment of cervical HSIL. The standard of care is ablative therapy, which is invasive, painful, and associated with frequent recurrences. Many patients seek TCM as a non-invasive holistic approach. The primary goals of this pilot study were to evaluate the safety and feasibility of a 9-month treatment regimen consisting of weekly acupuncture, Moxibustion, and self-applied herbal ointment. The main secondary aim was an evaluation of treatment efficacy.

Methods Fifteen patients with histologically proven anal HSIL were enrolled in a 9-month treatment study that consisted of weekly acupuncture, Moxibustion, and an herbal cream self-applied twice daily to the HSIL. Patients were followed every 3 months with cytology, histology, and monitored for adherence and safety.

Results Thirteen men and 2 women (14 HIV-positive) enrolled in the study. Adherence was good in all but one who discontinued treatment during a 2-month episode of unrelated illness. No adverse events occurred. Complete regression to normal or partial regression to low grade SIL was noted in 4 of 15 patients. Two additional patients with regression developed subsequent metachronous HSIL and a third patient had a recurrence in the area of a prior regressed HSIL.

Conclusions The TCM treatment was safe and feasible. These preliminary results suggest that the TCM therapy may be an effective treatment for anal HSIL. However, additional research is needed to determine if regression will be sustained. In addition, larger,

randomized trials are required to determine the immediate and long-term efficacy of TCM as treatment for anal HSIL

Introduction

Invasive squamous cell anal cancer is a relatively rare disease with an estimated incidence rate of < 1/100,000 persons (Wexner, Milsom et al. 1987). However, its incidence is elevated in certain high-risk populations including immune-comprised patients and men who have sex with men (MSM). Prior to the onset of the HIV epidemic, the incidence of anal cancer was estimated at 35/100,000 among MSM (Daling, Weiss et al. 1987), which is similar to the rates of cervical cancer in women prior to the introduction of cytology screening programs, and several fold higher than current rates of cervical cancer in the United States (Franco 1996). Subsequent data suggest that the incidence of anal cancer among HIV-positive MSM may be twofold higher than that of HIV-seronegative MSM making the rates of anal cancer in HIV-seropositive men 70 times to 90 times higher than in the general population (Goedert, Cote et al. 1998; Biggar 2001). Iatrogenically immune-compromised women have higher incidence rates for all anogenital squamous cancers (Ogunbiyi, Scholefield et al. 1994). More recently the relative risk of anal cancer among HIV-seropositive women was estimated to be 6.8 (Frisch, Biggar et al. 2000).

HSIL is hypothesized to be the precursor lesion to anal cancer and treatment of anal HSIL may prevent the development of anal cancer. This approach is analogous to the decreased incidence in cervical squamous cell cancer (SCC) attributed to the implementation of routine screening and subsequent treatment of cervical HSIL, considered the precursor lesion for cervical SCC. While no studies have demonstrated the direct progression of anal HSIL to invasive anal cancer, the natural history of cervical HSIL and its relationship to invasive cervical cancer is well established (Schiffman, Bauer et al. 1993; Walboomers, Jacobs et al. 1999; Ylitalo, Sorensen et al. 2000).

In the last decade, an emerging body of evidence has shown that a parallel clinical phenomenon exists in the anal canal. The analogy to cervical disease is based on several biologic similarities between the cervix and anus. There is a common embryonic origin between the two sites and the presence of a transformation zone where the squamous epithelium meets the columnar epithelium. The etiology and histology of anal and cervical SIL are quite similar. Likewise, invasive anal cancer is histologically similar to invasive cervical cancer. As with invasive cervical cancer, many cases of invasive anal cancer have concurrent HSIL, suggesting that HSIL is the lesion from which the cancer arose (Palefsky, Holly et al. 1991). The relationship between HPV and anal disease has been documented and is similar to the relationship between HPV and cervical disease. The range of approximately 30 HPV subtypes found in the anal canal is similar to those found in the cervix (Williams, Darragh et al. 1994). These HPV subtypes have been categorized as "low risk" or "high risk" types according to their potential oncogenic risk. In both the anal canal and cervix, the "low risk" types such as 6 or 11 are associated with LSIL and are rarely found in association with HSIL or cancer. The "high risk" types are most commonly found in cervical and anal HSIL and cancer. HPV 16 is the predominant "high risk" HPV type found in anal and cervical cancers (Zaki, Judd et al. 1992; Frisch, Glimelius et al. 1997). Finally, like cervical cancer, anal cancer has been epidemiologically linked to sexual activity (Daling, Weiss et al. 1987; Holly, Whittemore et al. 1989).

Based on the analogy to cervical disease, it seems likely that treatment of anal HSIL would prevent its malignant transformation. While no treatment algorithms or standards of practice exist for anal HSIL, treatment of cervical HSIL is considered standard care. The mainstay of treatment for cervical HSIL is ablative therapy. Removal of the precancerous

lesion(s) by loop electrosurgical excision procedure (LEEP), laser, cryotherapy, or chemotherapeutic methods is thought to prevent its further progression to cancer. The mainstay of treatment for anal HSIL has also been ablation most commonly utilizing electrocautery to fulgarate the affected area. Several studies have reported using laser and photodynamic therapy and infrared coagulation (IRC) (Brittain, Carlson et al. 1994; Webber and Fromm 2004; Goldstone, Kawalek et al. 2005; Goldstone, Hundert et al. 2007). Surgical-based treatments have several limitations including the usual risks and costs associated with a day-surgery setting and high rates of postoperative morbidity. The IRC offers an office-based ablative therapy, and although it has less associated morbidity than surgical fulgaration, it is still an invasive procedure.

However, ablative therapy may not be appropriate for all patients including those who present with large lesions or circumferential disease patterns. These lesions are difficult to treat with ablative therapy because of the risk of inducing strictures or because of the inability to adequately treat all areas of disease. In addition, patients with concomitant neutropenia or thrombocytopenia are also candidates for ablative therapy. The diagnosis of anal HSIL in the absence of treatment options is a source of frustration for both physicians and patients and has led to increased interest in TCM as a non-invasive alternative therapy. TCM may provide effective treatment without the risks of ablative therapies. However, they have not been adequately evaluated for feasibility, safety, and efficacy in the treatment of anal disease.

Traditional Chinese Medicine

The primary goal of TCM therapies, originating from the Taoist philosophy of Yin-Yang theory, is to restore and create harmony and balance within the patient. Disease is a manifestation of imbalance. Treatment often includes a variety of modalities

that are chosen for their ability to achieve balance. The TCM treatment in this study has been used for treatment of cervical and anal HSIL in the community based on principles of TCM theory. It includes the following three treatment modalities: acupuncture moxibustion, and Arnebia Indigo Jade and Pearl (AIJP) ointment. Acupuncture is the art of inserting fine sterile metal filiform needles into certain points in order to control the flow of energy in the meridians. Acupuncture is best known for its ability to relieve pain, but it also assists the body to heal itself of organic syndromes and symptoms by changing its energy patterns. The energetic effects and physiologic effects of acupuncture are not distinguished separately in TCM. Moxibustion is the burning of the common herb mugwort, artemisia vulgaris, over particular points or channels to stimulate or warm these areas. Topical herbal medicines are part of the rich tradition of Chinese herbal medicine and are used regularly in TCM for diseases that manifest externally or in orifices.

Arnebia, Indigo, Jade, and Pearl (AIJP) Ointment was developed by Andrew Ellis, a specialist in TCM dermatology, and Misha Cohen, a licensed acupuncturist (L.Ac) for the treatment of cervical and anal HSIL. AIJP ointment is based on traditional formulas found in the TCM literature and combines Jade Pearl Cream, *Huang Lian Gao,* and *Zi Gui Yu* ointments into a single ointment. These latter two formulas are referenced as early as the Chin Dynasty (circa 3rd century common era) and were used traditionally for dermatologic disorders such as inflammation, infections, and burns(Qian 1742; Shen, Wu et al. 1995). Jade Pearl Cream has been used in Chinese and Tibetan medicines for skin rejuvenation and for treatment of precancerous or malignant skin lesions(Hsu, Chen et al. 1986; Bensky and Gamble 1993).

Individualized treatments using multiple modalities are the norm in TCM. As an exploratory study, we sought to evaluate a TCM therapy for anal HSIL as it is used in the community. Research on TCM treatments have been challenging for several reasons. Recruitment of patients to randomized trials can be difficult, particularly for cancer related therapies, because the treatment is available in the community without the concern of potential randomization to the placebo arm. Treatment is frequently long-term and it is unknown whether patients will adhere to long-term therapies if a short-term option is available. Evaluation of TCM therapies is challenging from a Western scientific perspective, as the therapies may be unique per provider, or per patient, or per treatment application.

The primary goal of this study was to evaluate the safety and feasibility of a nine month TCM treatment protocol. The primary aim was completion of the protocol without significant adverse events. Secondary endpoints included clinical response evidenced as lesion regression and effect on anal HPV DNA. A final endpoint was the validation of TCM diagnoses associated with HSIL.

Methods

This study was a phase I non-randomized open-label trial of a nine-month TCM therapy for anal HSIL. The treatment consisted of weekly acupuncture and moxibustion treatments, and self-applied daily herbal ointment and Moxibustion. Men and women, aged 18 or older were enrolled in this study if they had a histologically-confirmed anal or peri-anal HSIL within 12 weeks of enrollment. Patients had to refuse or be ineligible for ablative therapy. HIV-seropositive patients were required to be on a stable treatment regimen for at least 8 weeks. Patients were excluded if they had a history of cervical, vaginal, vulvar, or anal cancer or had a granulocyte count of $<1500mm^3$, or a platelet count $<70000mm^3$.

A convenience sample was recruited from the University of California San Francisco (UCSF) dysplasia clinic and from referring clinicians in the community. Safety studies, anal examinations, and specimens were obtained at the UCSF General Clinical Research Center (GCRC) and TCM evaluation and acupuncture treatments were done at Chicken Soup Chinese Medicine, San Francisco. Approval for this study was obtained from the UCSF Committee on Human Research (CHR). A total of 24 patients were evaluated for the study and fifteen met the inclusion criterion.

Safety Studies

Preliminary safety studies were performed with the first 6 patients who were evaluated for grade 3 or 4 adverse events during the first four weeks of therapy. The remaining patients were enrolled when no adverse events occurred in these six participants. All HIV-seropositive individuals received HIV RNA viral loads twice before treatment was initiated, and at two and four weeks as well as during the 3-monthly

intervals. Safety was monitored by laboratory studies including complete blood counts (CBC) and liver function tests (LFT) every three months. Women were instructed to avoid pregnancy during the study.

TCM Procedures

Patients received a TCM evaluation at baseline and at their final study treatment. The evaluation consisted of an extensive interview, as well as a tongue and pulse analysis according to traditional TCM diagnostics. The following diagnoses were hypothesized to be associated with anal HSIL based on the TCM literature for cervical HSIL: Toxic Heat, Kidney or Liver Yin Deficiency, and Heat Dampness in the Lower Triple Burner (Yu, Zhang et al. 1991; 1998). The TCM treatment plan consisted of individual acupuncture points chosen according to the TCM diagnoses as well as a standardized acupuncture treatment protocol based on TCM Channel Theory. In Channel Theory, acupuncture points are chosen according to the Qi (or energy) flow in the affected channels. For anal HSIL, this included the following acupuncture points: Du1, Du20, UB57, UB31-34, UB60, and Ki3. The practitioner chose two of the Baliao (UB31-34) at each visit and needled these bilaterally. UB60 and Ki3 were also needled bilaterally, resulting in a total of 11 needles used for anal dysplasia at a given visit. Additional acupuncture points, determined by the TCM diagnosis and unrelated to the anal HSIL, were also used in some patients. At the weekly visits, patients also received moxibustion over the sacrum. Patients received the AIJP ointment and moxibustion sticks for self-applied therapy. They were instructed to apply ¼ teaspoon of ointment twice daily to the affected area (internal or peri-anal or both) and to self-apply the moxibustion heat near the sacrum twice daily. Patients were given treatment logs to monitor adherence and to record side effects.

Adherence and adverse events were also monitored at three-month intervals by interview at the time of HRA evaluations.

A single batch of AIJP ointment was manufactured by a facility that specialized in the processing of TCM ointments according to traditional methods. Seven herbs (i.e., arnebia, coptis, safflower, angelica, peach pit, rehmannia, licorice) were soaked (infused) in sesame oil for 6 weeks. The herbs were then pressed from the oil and removed. The herb-infused oil was heated to 160^0F. Once heated, the herb-infused oil along with the three remaining powdered ingredients (i.e., indigo, jade, pearl) were whipped into beeswax which had been heated to 190^0F. The ointment was poured into sterile 4-ounce glass jars and sealed. The ratio of herbs, minerals, oils, and beeswax was determined by a proprietary blend (Drug Master File held by Springwind Inc.). An investigational new drug (IND) license was approved by the FDA for treatment of anal or cervical HSIL (IND #68903). The proportion of ingredients in AIJP is considered a proprietary blend. A Drug Master File (DMF) was submitted to the FDA and is held by Andrew Ellis of Springwind Inc., Berkeley California.

Efficacy Evaluation

Patients received a High Resolution Anoscopy (HRA) examination, cytology, and biopsies at baseline and every three months as described previously (Jay, Berry et al. 1997). Patients with histologically-proven HSIL were enrolled in the study. Histology was evaluated by a single pathologist who was blind to the patient's clinical status or prior history. Patients whose lesions resolved received had biopsies in the same location if no lesion was seen. Efficacy was evaluated by histology, and changes in the number of lesions or percentage of disease. The examining clinician coded the number and location

of all lesions. Percentage of disease was coded as <25%, 25% to 50%, 50% to 75%, and > 75% or circumferential. Comparison of examinations was evaluated using digital photographs.

HPV DNA Analysis

HPV was analyzed from aliquots taken from the cytology medium and evaluated by PCR for high risk and low risk HPV types. A crude DNA preparation was made from the Cytyc Thinprep specimen. First the specimens were gently mixed to suspend cells and 1.5 ml were removed to a separate tube and spun for 15 minutes at 14K. The tube was inverted and the sample allowed to dry overnight. The sample was then suspended in 100 µl STM containing 0.2 mg/ml proteinase K (Invitrogen Life Technologies) and incubated at 56°C for one hour. The proteinase K was heat inactivated at 95°C for 10 minutes. Five µl of this was used in the HPV consensus PCR.

PCR was performed using MY09/MY11 consensus HPV L1 primers as well as primers for amplification of the human beta-globin as an indicator of specimen adequacy as described previously (Palefsky, Holly et al 98). After 40 amplification cycles, specimens were probed with a biotin-labeled HPV L1 consensus probe mixture. A separate membrane was probed with a biotin-labeled probe to the human beta-globin gene. Specimens were also typed by hybridizing to 29 different HPV types, 6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, 82 variant, 83, 84, as well as the following 10 types together in a probe mixture: HPV 2, 13, 34, 42, 57, 62, 64, 67, 72, and 82. Specimens negative for beta-globin gene amplification were excluded from analysis. The results of PCR were recorded on a scale from 0 to 5 rather than as positive or negative. The strength of the PCR signal was recorded on a

scale from 0 (negative) to 5 based on the intensity of the signal on the dot-blots, as described previously (Morrison EA, 1992).

Statistical Analysis

Adherence with the TCM regimen was determined by calculating the percentage of adherence with each modality (i.e., self-applied herbal ointment, self-applied Moxibustion, and weekly acupuncture treatments). Good adherence was considered >80%, moderate adherence was considered to be between 80% and 50%, and fair adherence was considered < 50%. Comparisons of pre- and post-treatment measures of the patients' hematologic, renal and hepatic function, and in HIV seropositive patients their CD4+. CD8+ lymphocytes counts, HIV viral load were done to evaluate safety. The effectiveness of the intervention was evaluated by assessing the number of patients with a complete or partial response. Complete clinical response was defined as regression from HSIL to normal anal cytology and histology and the absence of visible lesions at HRA. Partial response was defined as regression from HSIL to histology showing LSIL or atypical squamous cells of undetermined significance (ASCUS). Disease recurrence was defined as a recurrence of HSIL after complete or partial regression. Comparisons pre- and post-treatment of the percent of disease and number of lesions was also used to assess improvement. Responses were compared with disease burden, number of lesions, HPV strength and quantity, and TCM diagnoses.

Results

Feasibility and Safety

Fourteen HIV-seropositive and one-HIV seronegative patients enrolled in the study. Twelve patients were male and three were female, the mean age was 39 years (range of 32 to 59 years). All patients completed the study. Thirteen patients completed all acupuncture and office moxibustion treatments. Two patients completed 36 or 37 of the scheduled treatments. One patient required an additional two months to complete the study as she had ceased treatment during a two-month period of illness unrelated to the study. Thus, all patients but two completed the target goal of 40 weeks of therapy.

All patients successfully self-applied the AIJP ointment. Adherence was considered good in 10/15 patients who applied it twice daily at least 90% of the time. Adherence was considered moderate in an additional 4 patients who used the ointment at least 50% of the time. Some of these patients used it consistently but only once daily. The remaining patient used the ointment only for the first 3 months and infrequently. However the amount of ointment applied appeared to vary widely. Although patients were instructed to use and were given a ¼ teaspoon measure to use per dose, the number of jars used varied from 15 jars in one patient to one jar in two patients (see Table 1).

Adherence with self-applied moxibustion was less successful. Only 3/15 patients used it twice daily >80% of the time. A fifth of the patients reported using it ≤ 25% of the time. The remaining 6 patients reported using it approximately 50% of the time, either once daily, or twice daily but intermittently. Patients reported that it was technically difficult to apply or that they did not like the residual smell.

Adverse events were minimal. One patient received a mild burn during an office moxabustion treatment which did not require intervention other than a topical cream and was considered a grade 2 adverse event. An additional patient complained of an intermittent peri-anal rash that resolved with an additional topical TCM cream and was considered a grade 2 adverse event. No serious adverse events occurred. In HIV-seropositive patients, no changes were found from the baseline HIV RNA viral load that could be attributed to the medication. The only significant change in viral load was found in a patient in his second measurement that occurred before the initiation of treatment and was therefore unrelated to the treatment.

Disease Burden and HPV

The number of lesions ranged from one to circumferential disease in which lesion number could not be determined, although most patients (11 of 15) had two to three lesions (see Table 2). All but one patient had internal anal canal disease (i.e., one patient had both internal and external disease). The burden, or percentage, of disease, was estimated by the examiner as < 25%, 25% to 50%, 75% or circumferential. In 9 of 12 patients, the percentage of disease was $\leq$ 25% while in 5 patients it was $\geq$ 50% including one patient with circumferential internal disease and another with circumferential external disease. The remaining patient had a disease burden estimated at between 25% to 50%. Comparison of the disease burden pre- and post-treatment showed that the percentage of disease decreased in 5 patients, was stable in 9 patients, and worsened in 1 patient.

HPV status at baseline was positive in 13 patients, negative in two patients, and not determined in one patient who was betaglobin negative indicating that the sample was

inadequate. Of the HPV positive patients, all but one had a signal strength of +4 which is a semi-quantified indication of a high HPV viral load. Only four patients had HPV 16 or 18, which are the two strains most commonly associated with anal cancer and HSIL. Other common stains were HPV 53 in 4 patients and HPV 6, 33, and 70 which were each found in 3 patients. The number of HPV strains varied from one to seven. All patients with >50% disease had at least 4 HPV viral types. The exception was the one patient whose HPV test was inadequate.

Histologic Response

Table 3 shows the histology or cytology results for each time point. Cytology was used as the definitive diagnosis if it was more severe than the histology result. Two patients had a complete regression of HSIL to normal and two patients had a partial regression of HSIL to LSIL or atypia. Three patients had recurrence of HSIL following regression. Of these three patients, two recurrences were in metachronous lesions indicating a new lesion developed unrelated to the treated lesion, the third case was considered a true recurrence. No progressions to cancer occurred and the remaining 8 patients had persistent HSIL. Therefore, the efficacy rate based on the 4 patients with partial or complete regression was 26.6%. Most of the responders were patients with a lower disease burden (<25%) and those with a greater numbers of lesions did not respond histologically although the percentage of disease decreased in most of these patients.

TCM Diagnoses

Of the four diagnoses that were hypothesized to be associated with anal HPV, Toxic Heat was diagnosed in 11 patients, Kidney Yin Deficiency in 3 patients, and Liver Yin Deficiency or Dampness in the Lower Triple Burner in 1 patient each. Conversely,

several TCM diagnoses that had not been specifically hypothesized for patients with anal HSIL were found including, Spleen Qi Deficiency and Liver Qi Stagnation in 11 patients each, and Kidney Qi deficiency in 5 patients.

Discussion

This study is the first to evaluate a TCM treatment for anal HSIL. The nine-month TCM treatment using acupuncture, Moxibustion, and AIJP ointment was safe and feasible. However, adherence with the various modalities was variable. The acupuncture treatments were completed by nearly all of the patients. Daily application of the ointment also showed good adherence in most patients while self-applied moxibustion had very poor adherence in most patients.

The dose of AIJP varied widely based on the number of jars used per patient. It is interesting to note that responses were obtained in a patient who used one jar as well as in a patient who used 15 jars of ointment. However, the latter patient had a metachronous lesion develop. Several patients complained that the ointment leaked, which may indicate over-treatment in that the amount applied was not absorbed by the anal mucosa. Subsequent studies will need to re-evaluate the dose and standardize the method of application.

Despite the length and intensity of the treatment, most patients adhered with the treatment regimen with the exception of self-application of the moxibustion therapy. Only a few studies have described adherence with TCM regimens. Treatment with moxibustion as a single-modality therapy is described for reversal of breech fetal presentation in several studies(Cardini and Weixin 1998; Kanakura, Kometani et al. 2001). Although, moxibustion is described as a simple self-applied, inexpensive method to reverse breech presentation, none of these studies involved self-application of the moxibustion. No standard measurements of adherence were found to compare these results with, either for single or multi-modality treatment in Western or TCM. Although,

the accuracy of self-reported adherence may be compromised by bias to avoid being perceived as uncooperative, these data showed that patients willingly reported varied levels of adherence for different modalities.

Several findings suggest that the treatment was effective. The overall efficacy rate based on histology or cytology was 26.6% including two patients with complete regression to normal and two with partial regression to LSIL. An additional 20% of the patients had an initial response followed by a recurrence although two of these recurrences were clearly metachronous lesions. Two of these occurred three months post-treatment and only one recurrence of a metachronous lesion occurred during treatment. Post-treatment recurrences may indicate successful regression, but that the response was not sustained without continued treatment. Post-treatment persistence or recurrences rates from 53% to 79% in HIV sero-positive individuals treated with ablative therapies for anal HSIL have been documented in other studies as well (Chang, Berry et al. 2002; Goldstone, Hundert et al. 2007) A response rate of 77% was reported in a study using imiqumod suppositories for treatment of LSIL or HSIL (Wieland, Brockmeyer et al. 2006).

Other findings suggest that the treatment had a clinical effect even in the absence of histologic improvement. In 5 patients, the percentage of disease decreased, including most of the patients with the highest burden of disease at their baseline visit. Other clinical improvements included decreases in the amount of Lugol's negative staining, and decreases in lesion size and numbers. The decrease in disease burden, in some cases, meant that ablative therapy was viable following the TCM treatment. Two patients with extensive disease pre-treatment, who did not respond histologically had significant

improvement in their disease burden and were able to be treated with ablative therapy at the conclusion of the study. Measurements of lesion size and disease burden are subjective findings. It is difficult to accurately measure anal lesions, due to normal fluctuations in the mucosa (e.g. hemorrhoidal swelling). Although other studies have correlated the number of lesions with outcome (Goldstone, Kawalek et al. 2005), this is the first study to report on the measurement of disease burden and outcome.

High risk HPV viral types were found in all patients, but HPV 16 and 18 were found in only four patients. The treatment did not appear to affect the HPV viral load and it remained high (+4) in most patients. Several patients showed a decrease in the number of HPV types including one patient who had a partial response and two patients whose disease burden improved. Only one of the four responders became HPV negative, and one who was HPV negative (at baseline and 3 months) became HPV positive despite lesion regression. Changes in HPV viral load, or the number of HPV types in relation to efficacy of treatment for anal disease has been reported in a few studies. In these studies, using ablation and topical imiqumod, HPV viral load decreased in responders compared to non-responders (Palefsky, Berry et al. 2006; Wieland, Brockmeyer et al. 2006). In our study with only 15 patients and 4 responders, it is difficult to draw any conclusions from the lack of change in HPV status. Long term follow up will determine whether patients who responded but remained HPV positive have recurrences.

Only one of the four TCM diagnoses hypothesized to be associated with anal HPV was found in most patients, Toxic Heat which is a common finding associated with chronic viruses or pestilences according to TCM theory (Cohen and Doner 1996). The other most common diagnoses were Spleen Qi Deficiency which is a common finding in

HIV-seropositive individuals and Liver Qi Stagnation, commonly associated with high stress lifestyles (Cohen 1998). The TCM diagnoses did not correlate with response. It was also unknown whether improvement was noted by the TCM practitioners for the parameters assessed by them such as the tongue and pulse qualities because they were not asked to rate improvement. This omission underscores the need to improve data collection tools for TCM indicators in future studies.

Conclusions

Anal HSIL is being diagnosed with increased frequency for several reasons. Populations who are at risk for anal cancer have been identified and screening programs are being implemented. However, few treatment options exist and none are non-invasive. Findings from this pilot study suggest that this TCM intervention is feasible, safe, and has some efficacy. However, it requires additional testing in a larger placebo-controlled trial. In this study, a comprehensive TCM intervention was developed based on the directive of the NIH Working Group on Quantitative Methods in Alternative Medicine, which suggested that entire complementary systems of medicine be conceptualized as integrated wholes for the purpose of evaluation within a research trial (Levin, Glass et al. 1997). Studies of the single component therapies of a TCM intervention may be easier to evaluate statistically but create a reductionist bias for a holistic treatment, and as such may not provide a meaningful test of the therapy or intervention as it is used in the community. For these reasons, our study was designed so that patients received a comprehensive TCM intervention for 9 months.

Our protocol integrated a TCM treatment into a Western scientific study. Although TCM often advocates individualized treatments with acupuncture and herbal therapies, standardized treatment approaches are also used in TCM. This study used a standardized treatment approach with individualization for acupuncture, consistent with the diagnostic and treatment principles found in traditional Chinese texts, such as the *Nei Jing*. While recruitment and retention were completely successful in this study, it is unknown whether a placebo-controlled study would also be feasible. The effect size of 26.6% will help determine power for future studies. Based on the patients' moderate to

poor adherence with self-applied moxibustion in this study, it may need to be removed from future protocols.

Although it is important to study TCM as a holistic system, it is also important to understand whether one component or another has an effect in and of itself. One of the responders for example, used acupuncture consistently but none of the other modalities. It was also difficult to measure adherence with a multi-modality treatment. The AIJP ointment can most easily be studied as a single modality in a double-blind placebo study. However, this study would not be a study of a holistic comprehensive TCM treatment.

TCM will continue to be sought by patients eager to avoid allopathic medical treatments perceived to be toxic (e.g., chemotherapy) or invasive. The integration of research using TCM or other complementary medicines will help determine their safety and efficacy.

.

References

(1998). The Divine Farmer's Material Medica: a translation of the shen nong ben cao jing. Boulder, Blue Poppy Press.

Bensky, D. and A. Gamble (1993). Chinese Herbal Medicine Materia Medica. Seattle, Eastland Press.

Biggar, R. J. (2001). "AIDS-related cancers in the era of highly active antiretroviral therapy." Oncology (Huntingt) **15**(4): 439-48; discussion 448-9.

Brittain, P., J. Carlson, et al. (1994). "Laser ablation of squamous cell carcinoma in situ of the anal canal. A case report." J of Reproductive Medicine **39**(11): 913-914.

Cardini, F. and H. Weixin (1998). "Moxibustion for correction of breech presentation: a randomized controlled trial." Jama **280**(18): 1580-4.

Chang, G. J., J. M. Berry, et al. (2002). "Surgical treatment of high-grade anal squamous intraepithelial lesions: a prospective study." Dis Colon Rectum **45**(4): 453-8.

Cohen, M. (1998). The HIV Wellness Sourcebook: An East West Guide To Living Well with HIV/AIDS. New York, Henry Holt.

Cohen, M. and K. Doner (1996). The Chinese Way To Healing: Many Paths to Wholeness. New York, Berkley Publishing Group.

Daling, J. R., N. S. Weiss, et al. (1987). "Sexual practices, sexually transmitted diseases and the incidence of anal cancer." New Engl J Med **317**: 973-977.

Franco, E. L. F. (1996). "Epidemiology of anogenital warts and cancer." Obstetrics and Gynec Clinics of North America **23**(3): 597-623.

Frisch, M., R. J. Biggar, et al. (2000). "Human Papillomavirus-Associated Cancers in Patients With Human Immunodeficiency Virus Infection and Acquired Immunodeficiency Syndrome." J Natl Cancer Inst **92**(18): 1500-1510.

Frisch, M., B. Glimelius, et al. (1997). "Sexually transmitted infection as a cause of anal cancer." NEJM **337**: 1350-1358.

Goedert, J., T. Cote, et al. (1998). "Spectrum of AIDS-associated malignant disorders." Lancet **351**: 1833-1839.

Goldstone, S. E., J. S. Hundert, et al. (2007). "Infrared Coagulator Ablation of High-Grade Anal Squamous Intraepithelial Lesions in HIV-Negative Males Who Have Sex with Males." Dis Colon Rectum.

Goldstone, S. E., A. Z. Kawalek, et al. (2005). "Infrared coagulator: a useful tool for treating anal squamous intraepithelial lesions." Dis Colon Rectum **48**(5): 1042-54.

Holly, E. A., A. S. Whittemore, et al. (1989). "Anal cancer incidence: genital warts, anal fissure or fistula, hemorrhoids, and smoking." J Natl Cancer Inst **81**(22): 1726-31.

Hsu, H. Y., Y. P. Chen, et al. (1986). Oriental Materia Medica: a concise guide, Oriental Healing Arts Institute.

Jay, N., J. M. Berry, et al. (1997). "Colposcopic appearance of anal squamous intraepithelial lesions: relationship to histopathology." Dis Colon Rectum **40**: 919-928.

Kanakura, Y., K. Kometani, et al. (2001). "Moxibustion treatment of breech presentation." Am J Chin Med **29**(1): 37-45.

Levin, J. S., T. A. Glass, et al. (1997). "Quantitative methods in research on complementary and alternative medicine: a methodological manifesto. NIH office of Alternative Medicine." Medical Care **35**(11): 1079-1094.

Morrison EA, Goldberg GL, Kadish AS, Burk RD (1992). "Polymerase chain reaction detection of human papillomavirus: quantitation may improve clinical utility." Journal of Clinical Microbiology **30**:2539-43.

Ogunbiyi, O. A., J. H. Scholefield, et al. (1994). "Prevalence of anal human papillomavirus infection and intraepithelial neoplasia in renal allograft recipients." Br J Surg **81**(3): 365-7.

Palefsky, J. M., J. M. Berry, et al. (2006). "A trial of SGN-00101 (HspE7) to treat high-grade anal intraepithelial neoplasia in HIV-positive individuals." Aids **20**(8): 1151-5.

Palefsky, J.M., Holly EA, et al. (1998). "Prevalence and risk factors for human papillomavirus infection of the anal canal in human immunodeficiency virus (HIV)-positive and HIV-negative homosexual men.". J Infect Dis **177** (2):361-7/

Palefsky, J. M., E. A. Holly, et al. (1991). "Detection of human papillomavirus DNA in anal intraepithelial neoplasia and anal cancer." Cancer Res **51**(3): 1014-9.

Qian, W. (1742). The Golden Mirror of Medicine Tradition (Yi Zong Jin Jian).

Schiffman, M. H., H. M. Bauer, et al. (1993). "Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia." Journal of the National Cancer Institute **85**(12): 958-964.

Shen, D., X. F. Wu, et al. (1995). Manual of Dermatology in Chinese Medicine. Seattle, Eastland Press.

Walboomers, J. M., M. V. Jacobs, et al. (1999). "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide." J Pathol **189**(1): 12-9.

Webber, J. and D. Fromm (2004). "Photodynamic therapy for carcinoma in situ of the anus." Arch Surg **139**(3): 259-61.

Wexner, S. D., J. W. Milsom, et al. (1987). "The demographics of anal cancers are changing: identification of a high risk population." Dis Colon Rectum **30**: 942-946.

Wieland, U., N. H. Brockmeyer, et al. (2006). "Imiquimod treatment of anal intraepithelial neoplasia in HIV-positive men." Arch Dermatol **142**(11): 1438-44.

Williams, A. B., T. M. Darragh, et al. (1994). "Anal and cervical human papillomavirus infection and risk of anal and cervical epithelial abnormalities in human immunodeficiency virus-infected women." Obstet Gynecol **83**(2): 205-11.

Ylitalo, N., P. Sorensen, et al. (2000). "Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study." Lancet **355**(9222): 2194-8.

Yu, S. Y., L. A. Zhang, et al. (1991). "Dialectic classification of syndrome diagnosis in traditional Chinese medicine used as new criterion for evaluating prognosis of patients with cervical cancer." J Tongji Med Univ **11**(2): 123-5.

Zaki, S. R., R. Judd, et al. (1992). "Human papillomavirus infection and anal carcinoma. Retrospective analysis by in situ hybridization and the polymerase chain reaction." Am J Pathol **140**(6): 1345-55.

Table 1. Adherence Rates of different TCM Treatment Modalities

Subject	AIJP	No. Jars dispensed	Moxibustion	Acupuncture	Logs Kept
1004	>90%	5	>80%	100%	100%
1005	>90%	3	50%	100%	100%
1007	>50%	3	>80%	100%	100%
1009	100%	15	0%	100%	0%
1010	>90%	3	0%	100%	100%
1011	10%	1	0%	100%	0%
1012	>90%	6	33%	100%	50%
1013	100%	3	>50%	100%	50%
1016	>90%	3	>50%	100%	100%
1018	>90%	2	50%	100%	0%
1019	50-75%	1	<50%	100%	0%
1021	100%	3	100%	95%	100%
1022	>90%	2	50%	100%	50%
1023	>70%	2	25%	95%	0%
1024	>70%	7	25%	95%	0%
MEAN	79.7%	3.9	39.5%	99%	50%
MODE	>90%	3	50%	100%	100%, 0%

Table 2. Baseline Disease Status

Subject	Location of HSIL Internal (I) External (E)	Number of Lesions (L or HSIL)	% Disease	HPV Strength (+ = Betaglobin present	HPV No. Types
1004	I	2	50-75%	NA	NA
1005	I	2	50%	+4	33
1007	I	3	<25%	+0	0
1009	E	3	25%	+4	16,33,53
1010	I	1	<25%	+3	53,58
1011	I	2	<25%	+4	52,53,58,70,2
1012	I	2	25%	+0	NA
1013	I	3	25%	+4	16,4
1016	I	2	<25%	+4	53,70
1018	I	7	>75%	+4	6,16,52,68
1019	I	2	<25%	+4	Mix*
1021	I	5	50-75%	+4	6,11,18,31,39,45,59
1022	I,E	Int 4 Ext >10	I 25-50%, Ext >75%	+4	32,33,39,59,68,70,Mix*
1023	I	2	25-50%	+4	16
1024	I	2	25%	+4	6

NA for Strength = betaglobin negative, unable to determine presence/absence of HPV
* Mix contains HPV types 2,13,35,42,57,62,64,72,82; specimens are not evaluated for specific type in the mix

Table 3. Efficacy of TCM Treatment for anal HSIL

Participant	HIV Status	Baseline	3 mos.	6 mos.	9 mos.	12 mos.	Final Response
04	Pos	H	H	H	H	H	Stable
05	Pos	H	H	H	L	H	Recurrence
07	Pos	H	H	H	H	L	Partial
09	Neg	H	N	N	H (pap)	H	Recurrence
10	Pos	H	A	L (pap)	L (pap)	N	Complete
11	Pos	H	H	H	H	A	Partial
12	Pos	H	H	H	H	H	Stable
13	Pos	H	H	H	H	H	Stable
16	Pos	H	L	L	H	H	Recurrence
18	Pos	H	H	H	H	H	Stable
19	Pos	H	N	L	N	N	Complete
21	Pos	H	H	H	H	NA	Stable
22	Pos	H	H	H	H	H	Stable
23	Pos	H	H	H	H	H	Stable
24	Pos	H	H	H	H	H	Stable

Table 4. Pre- and Post-Treatment Clinical Disease Characteristics in Responders To TCM Treatment

Patient Number	Disease Burden		Number Lesions	HPV Signal Strength		# HPV Types		Compliance		
	Pre	Post		Pre	Post	Pre	Posts	AIJP	Mox	Acup
COMPLETE										
1010	<25%	<25%	1	+3	+4	2	1	Good	Poor	Good
1019	<25%	<25%	2	+4	0	Mix	0	Mod.	Mod.	Good
PARTIAL										
1007	<25%	<25%	3	+0	+4	0	3	Mod	Good	Good
1011	<25%	<25%	2	+4	+4	5	1	Poor	Poor	Good
RECURRENCE										
1005	50%	<25%	2	+4	+4	1	1	Good	Mod	Good
1009	25%	<25%	3	+4	+4	3	2	Good	Poor	Good
1016	<25%	<25%	2	+4	+4	2	2	Good	Mod	Good

Conclusions and Implications for Nursing Research and Practice

Screening for anal cancer and its precursor lesions is becoming the standard of care for populations considered at risk including MSM and immunocompromised men or women. In these populations, the incidence of HSIL, considered the precursor lesion for anal cancer, is greatly increased as is anal cancer. As more people are screened, there is a greater need for trained clinicians to provide HRA. Nurse practitioners provide colposcopy in many different clinic settings. HRA is a natural extension of advanced practice nursing. The heightened interest shown by mid level practitioners in this field is reflected in the large volume of requests for training, and filled-to-capacity courses.

Screening for anal disease has also exposed the need for more viable treatment options. Patients seeking alternatives to invasive procedures commonly seek TCM. Yet little is known regarding the efficacy and safety of these treatments. The studies presented in this manuscript demonstrate the safety of the treatment but randomized clinical trials are needed to ascertain the efficacy.

The purpose of these studies was to explore tools for identification of anal disease, and apply them in a pilot treatment study using TCM.

Understanding the appearance of anal lesions will improve the clinician's ability to identify HSIL. The findings in these studies showed that anal lesions can be identified by lesion patterns. Further studies will be necessary to validate these findings and determine whether the characterizations can be systematized to clinically distinguish HSIL, LSIL and normal histology. The sensitivity and specificity of HRA is unknown yet treatment studies rely on HRA to determine

the presence or absence of disease following treatment. Measurement tools for HRA were piloted in these studies and further research as to their utility should be investigated.

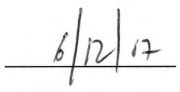
These studies also exposed the need for improved methods for measuring adherence in TCM studies which may be complicated by multiple modalities. While study designs of single modality treatments may be easier, it behooves us to meet the challenge of determining good research methods for studies of TCM or other complementary treatments as they are used in the community.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses and dissertations. Copies of all UCSF theses and dissertations will be routed to the library via the Graduate Division. The library will make all theses and dissertations accessible to the public and will preserve these to the best of their abilities, in perpetuity.

I hereby grant permission to the graduate Division of the University of California, San Francisco to release copies of my thesis or dissertation to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

Author Signature



Date