

Ellagic acid and *Annona muricata* in the chemoprevention of HPV-related pre-neoplastic lesions of the cervix

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Abstract. Ellagic acid is a phenolic compound naturally present in nuts and berries. Several studies have demonstrated that this bioactive compound has antioxidant, chemopreventive and antiviral activity. *Annona muricata* is a type of fruit tree with a long history of traditional use. A number of properties have been attributed to different parts of the plant, including anticancer and antioxidant activities. In the current study, a complex based on ellagic acid, *Annona Muricata* and antioxidant factors (an ellagic acid complex) was administered to a group of human papilloma virus (HPV) infected women with and without cervical lesions, for 12 months. Its effect on HPV clearance and cervical cytological outcomes was assessed and a group of women with the same clinical features who did not receive the ellagic acid complex served as a control. A positive correlation was observed between intake of ellagic acid complex and negative Pap test following 6 and 12 months of treatment (χ^2 test: 0.041 and 0.014, respectively). Women treated with the ellagic acid complex were less likely to be diagnosed with an abnormal Pap smear at 6 months [Odds ratio (OR): 0.39; 95% confidence interval (CI) 0.14-1.06] and 12 months (OR: 0.35; 95% CI 0.13-0.89), compared with the control group. After adjusting for confounding factors including age and smoking habit, this association remained significant. No effect was observed on HPV clearance or viral integration. The data from the current study suggest a protective effect of the ellagic acid complex on cervical cells, possibly through apoptosis, cell cycle arrest and repair mechanisms.

Introduction

Cervical cancer is the second most common cancer among women in the world (1). The major etiological factor in cervical carcinogenesis is unanimously recognized to be the human papillomavirus (HPV) (2,3). Previous studies have demonstrated that persistent HPV infection, particularly with HPV 16 and 18, may predict cervical intraepithelial neoplasia (4). However, HPV infection alone is not sufficient to induce cervical cancer.

A number of factors influence the progression of HPV infection and/or low-grade cervical pre-invasive lesions. Among these factors, age, immune system status, HIV infection, smoking, nutritional factors, concomitant sexually transmitted diseases, estroprogestinic use, viral genotype (low or high-risk types) and viral integration into the host cell genome appear to be the most relevant (5-10).

Various reactive oxygen species are involved in mutagenesis and carcinogenesis (11) and a number of previous studies have demonstrated that a high daily intake of natural antioxidants may significantly decrease the risk of such cellular degenerative processes occurring, particularly in the female and male urogenital tract (12-17). It has been observed that synergic positive action by ellagic acid, *Annona Muricata*, *Epilobium Parviflorum*, green tea, lycopene, vitamin E, vitamin A, pyridoxine, selenium and β -carotene, lead to the efficient maintenance of the body's natural defense mechanisms, with consequent organ-cytoprotection (17-25). In addition, it has been observed that ellagic acid induces G1 cell cycle arrest and apoptosis in human cervical carcinoma Caski cells (22). Therefore, it has been demonstrated that antioxidants can protect the organism from biological insults deriving from endogenous and exogenous free radicals and may be protective against proliferative diseases.

The aim of the current study was to evaluate the effect of ellagic acid and *Annona Muricata* (ellagic acid complex), on viral clearance and/or cytological lesions in a group of women with HPV cervical infection with or without cytological cervical alterations [atypical cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL) or high-grade squamous intraepithelial lesions (HSIL)].

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As a control, HPV infection and Pap test were also monitored in a group of women with the same clinical features who did not receive the ellagic acid complex.

Patients and methods

Clinical study design. Participants were selected from the daily list of outpatients of the Colposcopy and Cervix-Vaginal Pathology Surgery of the Policlinico Tor Vergata, Rome, Italy, from January 2010 to April 2014. A total of 106 women were enrolled, with a mean age of 33.8 years (median, 32 years; range 20-55 years).

All prospective participants underwent a cervical cytological exam (known as a Pap test), HPV DNA and mRNA E6/E7 tests, colposcopy and, if necessary, cervical biopsy. Inclusion criteria were: Positive HPV DNA and/or mRNA E6/E7 tests with a normal or abnormal Pap smear (ASCUS, LSIL or HSIL). Exclusion criteria were: The presence of *in situ* or invasive cervical carcinoma, previous surgical treatments for high-grade cervical lesions and patients being in an immunosuppressive state.

Women were randomly divided into two groups, with 50 treated and 53 untreated patients respectively. One group was administered 16 mg ellagic acid, 100 mg *Annona muricata* and antioxidant factors as a single tablet (Oasit-K, Bio-STILOGIT Pharmaceuticals, Florence, Italy) daily for 12 months, while the second group received no medication. The tablet was taken once a day during the main course. The therapy was not prescribed to patients with a known allergy or intolerance to ≥ 1 of its components. Smoking habits and estrogenic therapy were taken into consideration. The groups were monitored for HPV infection by Pap smear, HPV DNA, mRNA E6/E7 of HPV 16, 18, 31, 33 and 45 and colposcopy. Molecular tests were performed at baseline and 12 months; Pap tests and colposcopy were conducted at baseline and at 6 and 12 months. All patients gave informed consent and ethical approval (RS178/15) was granted by the local Ethical Committee of the Polyclinic Tor Vergata, Rome, Italy.

Cervical cytology. All patients enrolled in the study were admitted to the Colposcopy and Cervical-Vaginal Pathology unit of the Policlinico Tor Vergata (Rome, Italy). During each visit, ecto- and endo-cervical cells were taken using an Ayre's spatula and Cytobrush, respectively. The cells were streaked out on two distinct sections of a glass slide, spray fixed and sent to the Pathology Department of the same hospital. Smears were classified according to the 2001 Bethesda system. [ASCUS, atypical squamous cells (ASCH), LSIL, HSIL] (26).

HPV DNA testing and typing. HPV screening and genotyping were performed at the Laboratory of Molecular Virology, Policlinico Tor Vergata. Cervical cells were collected from the transition zone of the cervical canal, suspended in 20 ml of PreservCyt solution (Hologic, Marlborough, MA, USA) and stored according to the manufacturer's instructions until analysis. One aliquot of 5 ml was transferred into a collection tube and centrifuged at 839 x g for 3 min. The supernatant was then discarded and the cellular pellet stored at -80°C prior to analysis. Total DNA/RNA was extracted using a NucliSENS® easyMag® automatic extractor (BioMerieux, Marcy l'Etoile,

France). HPV-DNA was detected with the HPV Sign kit (Diatech LabLine SRL, Jesi, Italy) according to the manufacturer's instructions. HPV type was assigned by pyrosequencing using the PyroMark Q96 ID system (Qiagen GmbH, Hilden, Germany). The assay targets a hypervariable region of 30 nucleotides within the L1 gene. The obtained product is aligned with an HPV library and the viral type assigned using the software IdentiFire SW 1.0 (Qiagen GmbH).

Detection of the E6/E7 transcripts of HPV 16, 18, 31, 33 and 45. The presence of the oncogenic transcripts of HPV 16, 18, 31, 33 and 45 was detected by the NucliSense EasyQ® HPV kit (BioMerieux) following the manufacturer's instructions. Six different molecular beacons were used to identify and amplify the corresponding five HPV types and the *UIA* control gene. Two different fluorophores; 6-carboxyfluorescein (6-FAM) for HPV 16, 31 and 33 and 6-carboxy-X-rhodamine (6-ROX) for *UIA*, HPV 18 and 45, allow simultaneous duplex amplifications. Dedicated software (NucliSENtral™ HPV software V1.1) was used to reveal the presence or absence of the viral target.

Statistical analysis. Comparisons were made between patients with a cytological diagnosis of HSIL, LSIL, ASCUS or negative and a positive HPV-DNA and/or mRNA tests who took the prescribed therapy, and those with the same clinical characteristics who did not receive the therapy.

Patients were also matched for smoking habits, age and the use of estrogenic therapy. The relationship between high-risk genotypes, viral integration for HPV 16, 18, 31, 33 and 45, and clinical outcome was evaluated by estimating the odds ratio (OR) and 95% confidence intervals (CIs). In order to adjust for potential confounding factors multivariate logistic regression was used to calculate adjusted odds ratios (AORs).

The χ^2 test or Fisher's exact test were used to assess the statistical association between therapy administration and regression of cytological lesions and viral clearance. $P < 0.05$ was considered to indicate a statistically significant difference. All analyses were performed using STATA software ver. 11 (StataCorp LP, College Station, TX, USA).

Results

Cytological outcomes in patients treated with ellagic acid. At baseline, of the 50 enrolled cases, 29 had an abnormal Pap smear (1 HSIL, 20 LSIL, and 8 ASCUS), while 21 exhibited normal cytology. At 6 months' follow-up, the single HSIL case was treated by loop electrosurgical excision procedure (LEEP), while out of the 20 LSIL cases, 11 reverted to normal cytology, 2 regressed to ASCUS and 3 still exhibited a cellular morphology compatible with a diagnosis of LSIL. There was no data at 6 months for 4 of the LSIL cases: 3 did not repeat the Pap test and there was 1 drop out due to diarrhea, a side effect related to the consumption of ellagic acid complex. Out of the 8 ASCUS cases, repeat cytology indicated a regression to normal cytology in 6 cases, while 2 patients developed a LSIL. Of the 21 cases with normal cytology at baseline, repeat cytology performed at 6 months confirmed normal cytology in 18 patients, while 1 case had evolved to LSIL. The Pap test was not repeated in 2 patients in this group.

At 12 months' follow-up, the initial HSIL case treated with LEEP at 6 months tested negative following repeat cytology. Among the group of LSIL cases, the number of cases exhibiting negative cytology increased from 11 to 17. The remaining 2 patients were diagnosed with ASCUS and LSIL. In the ASCUS group, 5 patients still exhibited negative cytology, 1 did not undergo the Pap test and 2 were treated by LEEP due to a diagnosis of cervical squamous intraepithelial neoplasia (CIN) 2.

In the negative Pap smear group, the patient that progressed to LSIL after 6 months reverted to normal at 12 months. The 2 patients that did not undergo the Pap test at 6 months exhibited negative results when it was performed at 12 months. Of the 18 cases with normal cytology at 6 months, 16 still presented a normal Pap smear at 12 months follow-up while 2 were classified as ASCUS and LSIL. One patient did not perform the Pap test. Cytological follow-up is summarized in Table I.

After 6 and 12 months of treatment, a statistically significant association was observed between intake of ellagic acid complex and negative cytology. Patients treated with ellagic acid complex were less likely to be diagnosed with an abnormal Pap smear at 6 months (OR: 0.39; 95% CI, 0.14-1.06; P=0.041) and at 12 months (OR, 0.35; 95% CI, 0.13-0.89; P=0.014). After adjusting for age and smoking status the AORs and their 95% CI were 0.38 (0.14-0.97) and 0.32 (0.13-0.77) at 6 and 12 months, respectively. No statistically significant association was observed between ellagic acid complex intake and detection of HPV DNA, oncogenic transcripts or colposcopy outcome.

Cytological outcomes in untreated patients. In the untreated group, at baseline, a cytological diagnosis of LSIL was made in 24 cases, ASCUS in 11 and negative cytology in 18 cases. At 6 months follow-up, of the 24 patients with initial LSIL lesions, 8 still presented a LSIL lesion, 3 progressed to HSIL, 2 regressed to ASCUS and 11 became cytologically negative. The 3 cases that progressed to HSIL were treated by LEEP following colposcopy and biopsy was subsequently performed, which confirmed the presence of a CIN2 lesion, thus these cases did not undergo further testing.

At 12 months follow-up, of the 8 cases with LSIL lesions, 3 became negative, 4 were still LSIL and 1 skipped the control. The 2 ASCUS cases remained so at 12 months follow-up, while the 11 cytologically negative smears at 6 months evolved as following: 8 remained negative, 2 became LSIL and 1 ASCUS.

A total of 11 ASCUS cases were enrolled at baseline. At 6 months follow-up, 7 became negative while 4 evolved towards a LSIL lesion. At 12 months follow-up, of the 7 patients with negative Pap smear only 6 repeated the Pap test and the results were: 1 HSIL and 5 negative. Of the 4 LSIL cases, 3 were still classified as LSIL at 12 months follow-up, while 1 was treated by LEEP as the lesion had progressed to CIN3 (HSIL).

Of the 18 cases with normal cytology at baseline, 15 had still a normal Pap smear at 6 months follow-up and 3 had evolved towards LSIL. At 12 months' follow-up, of the 15 patients with a normal Pap smear, 14 were still negative while 1 had progressed to LSIL. Of the 3 LSIL cases, 1 was treated by LEEP due to the insurgence of a CIN3 (HSIL) lesion and 2 remained LSIL. Table I summarizes the results of the Pap test follow-up.

Table I. Cytological outcomes in treated and untreated patients.

Time	Negative cytology, no. (%)		P-value	ASCUS, no. (%)		LSIL, no. (%)		HSIL, no. (%)		No Pap test no. (%)		S Stop EA complex, no.		Total no.	
	Treated	Control		Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Baseline	21 (42)	18 (34)		8 (16)	11 (21)	20 (40)	24 (46)	1 (2)	0 (0)	0 (0)	0 (0)	0	50	53	
6 months	36 (74)	33 (63)	0.041 ^a	2 (4)	2 (4)	6 (12)	15 (28)	0 (0)	3 (6)	5 (10)	0 (0)	1	49	53	
12 months	40 (82)	30 (60)	0.014 ^a	2 (4)	3 (6)	2 (4)	12 (24)	3 (6)	3 (6)	2 (4)	2 (4)	0	49	50	

^aP<0.05; ASCUS, atypical cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; EA, ellagic acid.

Table II. HPV detection in treated and untreated patients at baseline and 12 months follow-up.

Treated group, n=50	HPV types, total no.	P-value	mRNA E6/E7	P-value
Time, baseline	14 high-risk (51) 6 low-risk (13)		19 positive 30 negative 1 NA	
Time, 12 months	8 high-risk (18) 4 low-risk (5) 22 Negative 7 NA	0.600	6 positive 32 negative 12 NA	0.977
Control group, n=53				
Time, baseline	14 high-risk (68) 3 low-risk (3)		10 positive 31 negative 12 NA	
Time, 12 months	11 high-risk (24) 7 low-risk (9) 18 negative 7 NA		7 positive 21 negative 25 NA	

NA, data not available; HPV, human papilloma virus.

Detection of HPV DNA and mRNA E6/E7. HPV DNA was assessed at baseline and at 12 months follow-up. In the control group, at baseline, HPV DNA was detected in 52 out of 53 cases (35 single infections, 17 multiple infections). The only case that tested negative for HPV DNA was positive for mRNA E6/E7 of HPV 16. At 12 months follow-up, 7 patients did not undergo the molecular test. Of the remaining 46, HPV DNA was still detected in 28/46 (61%) cases while 18/46 (39%) had cleared the virus spontaneously.

Of the 28 HPV positive patients, 6 belonged to the ASCUS group, 11 to the LSIL group and 11 to the group with negative cytology. mRNA E6/E7 was detected in 7 patients: 3 HPV 33, 2 HPV 16, 1 HPV 18 and 1 HPV16/18. In the 3 cases positive for mRNA E6/E7 HPV 33, the HPV types detected at the DNA level were HPV 58, 81 and 18. In the remaining cases, the same genotype was detected at both the DNA and RNA levels.

In the group treated with the ellagic acid complex, at baseline, all cases tested positive for HPV DNA (38 single infections and 12 multiple infections) and in 19 of them, the oncogenic transcripts E6/E7 were detected. At 12 months follow-up, 7 patients did not undergo the HPV DNA test: 1 dropped out due to diarrhea induced by treatment with the ellagic acid complex, 6 were lost to follow-up for unknown reasons. Of the 43 tested patients, 22/43 (51%) cleared the virus, whereas 21/43 (49%) remained HPV positive. Of the 21 HPV positive patients, 14 belonged to the group with negative cytology, 1 to the ASCUS group and 6 to the LSIL group. The patient in the HSIL group did not undergo further testing at the molecular level following LEEP performed at 6 months. Among the HPV positive patients, it was observed that the virus was integrated in 6 patients: 1 HPV 31, 2 HPV 45 and 3 HPV16. The same viral type was detected at DNA level. HPV and mRNA E6/E7 detection are summarized in Table II.

The administration of ellagic acid complex did not affect viral clearance as well as virus integration compared to the control group, $P=0.600$ for HPV DNA (OR, 0.80; 95% CI, 0.32-2.00) and 0.977 for mRNA E6/E7 (OR, 0.98; 95% CI, 0.31-3.26).

Discussion

A number of previous studies investigating the role of diet and nutritional status in cervical HPV have suggested that certain natural compounds provide a protective effect against cervical dysplasia and HPV persistence (8,14,15,20,23). Among the compounds studied, the antioxidant and chemopreventive activity of ellagic acid and *Annona muricata* have been demonstrated (12-17,25). It has been demonstrated by *in vitro* models that both compounds induce cell cycle arrest in the G1 phase, DNA repair and apoptosis (22,25).

The current study assessed the chemopreventive and antiviral activity of the ellagic acid complex in a group of HPV infected women with and without abnormal Pap smears. As a control, Pap smears and HPV infection were monitored in a group of women with the same clinical features who did not receive the ellagic acid complex. A statistically significant association was observed between intake of ellagic acid complex and negative Pap test following 6 and 12 months of treatment compared with the control group ($P=0.041$ and 0.014, respectively), suggesting that treated women were less likely to be diagnosed with abnormal Pap results. However, some molecular data were missing and this may have impacted on the statistical significance of the association, given the small sample size. No effect was observed on viral clearance and/or virus integration when the two groups were compared.

It has been reported that oxidative stress may cooperate with oncogenic HPV in cervical carcinogenesis, possibly

through two different cooperative mechanisms: i) Genotoxic activity related to oxidative stress and genomic instability induced by HPV occurs independently of the generation of molecular damage that causes the emergence of neoplastic clones; ii) oxidative stress cooperates with the molecular stages of neoplastic initiation and/or progression induced by HPV (27). The progression from mild dysplasia to severe dysplasia, and onwards to invasive cancer, is promoted by increasing levels of oxidative stress (27,28). Proteomics analysis of HPV-16 infected tissues have indicated that dysplasia is characterized by an increased oxidative environment compared to normal or neoplastic tissue, resulting in the oxidative modification of DNA and proteins involved in cell morphogenesis and terminal differentiation, thus providing ideal conditions for disease progression and cancer (28). The use of antioxidants including ellagic acid and *Annona muricata* may counterbalance the damaging effects of oxidative stress by arresting the cell cycle, promoting DNA repair and inducing apoptosis, thus hampering disease progression. Further studies on larger sample size are required to validate the results of the current study. The results of the present study suggest that ellagic acid and *Annona muricata* may have chemopreventive action.

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