ORIGINAL ARTICLE

GAMMA-GLUTAMYL TRANSPEPTIDASE ALTERATION AS A BIOMARKER OF OXIDATIVE STRESS IN PATIENTS WITH HUMAN PAPILLOMAVIRUS LESIONS FOLLOWING TOPICAL TREATMENT WITH SINECATECHINS

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Abstract

Human papillomavirus (HPV) penetrates the skin or mucous membranes and in most cases undergoes a natural clearance of virus, pertaining to various factors such as skin integrity and efficacy of the immune response. However, in persistent cases that escape the natural clearance mechanism, the infection leads to the development of skin and mucous lesions, including warts. Sinecatechins, an extract of green tea leaves from *Camellia sinensis,* is a newly approved therapeutic agent with recently proved efficacy in the treatment of warts. While the mechanism of action is not yet fully understood, it has been postulated that sinecatechins exhibit not only antiviral/antitumour activity, but also an antioxidant action contributing to its effectiveness. This study was conducted to evaluate the oxidative stress biomarkers before and after the treatment with 10% sinecatechins ointment in patients with cutaneous and genital warts. There were assessed the oxidative status (TOS), the antioxidative status (TAS), oxidative stress index (OSI) and gamma-glutamyl transpeptidase (GGT) activity as biomarkers of oxidative stress both before and after the topical treatment in patients with HPV lesions (n = 35) as well as in normal controls (n = 35). We have also assessed the correlations between GGT activity and the level of oxidative stress biomarkers. There were not registered significant changes in GGT level (U/L: 29.1 ± 15.9 *vs.* 20.8 ± 6.2, $p > 0.05$), TOS level (µmol H_2O_2 equivalent/L serum: 3.10 ± 0.29 *vs.* 3.08 ± 0.22, $p > 0.05$), and TAS level (µmol Trolox equivalent/L: 1.61 ± 0.31 *vs.* 1.68 ± 0.26, $p > 0.05$), accompanied by a statistically significant reduction in OSI level (1.93 ± 0.61 *vs.* 1.83 ± 0.46, $p < 0.05$) at the end of the treatment, compared to baseline values. A positive correlation between OSI and GGT in patients with HPV lesions prior to the initiation of treatment ($r = 0.312$, $p < 0.05$) was noticed, as well.

Rezumat

Virusul papilloma uman (HPV) penetrează tegumentele şi mucoasele şi, în cele mai multe cazuri, este eliminat în funcţie de diferiţi factori precum integritatea cutanată şi sistemul imun al gazdei; în cazurile de infecţie persistentă, care scapă mecanismelor naturale de eliminare, infecţia conduce la dezvoltarea unor leziuni cutanate şi mucoase, incluzând verucile. Un nou agent aprobat pentru tratamentul verucilor are la bază sinecatechinele dintr-un extract de ceai verde, provenit din frunzele de *Camellia sinensis*. Mecanismul de acţiune nu este pe deplin înţeles: se pare că sinecatechinele prezintă activitate nu doar antivirală şi antitumorală, ci şi o importantă activitate antioxidantă ce contribuie la eficacitatea tratamentului. Studiul a fost realizat pentru a evalua biomarkerii de stres oxidativ înainte şi după tratamentul topic cu unguent cu 10% sinecatechine la pacienţii cu veruci cutanate şi genitale. Am analizat statusul oxidativ (TOS), statusul antioxidativ (TAS), indicele de stres oxidativ (OSI) şi activitatea gamma-glutamil transpeptidazei (GGT) ca biomarkeri de stres oxidativ înainte şi după tratamentul topic. Am evaluat de asemenea corelaţia între activitatea GGT şi nivelul markerilor de stres oxidativ la pacienţi cu leziuni asociate cu HPV (n = 35) şi la grupul control (n = 35), înainte şi după tratament. Au fost observate modificări nesemnificative statistic ale nivelurilor GGT (U/L: 29,1 ± 15,9 *vs.* 20,8 ± 6,2, $p > 0,05$), TOS (µmol H_2O_2 Eq/L ser: 3,10 ± 0,29 *vs.* 3,08 ± 0,22, $p > 0,05$) şi TAS (µmol Trolox Eq/L: 1,61 ± 0,31 *vs.* 1.68 ± 0,26, $p > 0,05$), însoţite de o reducere semnificativă a nivelului OSI (1,93 ± 0,61 *vs.* 1,83 ± 0,46, $p < 0,05$) la finalul tratamentului, comparativ cu valorile iniţiale. S-a observat o corelaţie pozitivă, semnificativă statistic, între OSI şi GGT la pacienţii cu leziuni cauzate de HPV înainte de iniţierea tratamentului ($r = 0,312$, $p < 0,05$).

Keywords: HPV infection, sinecatechins, oxidative stress, gamma-glutamyl transpeptidase

Introduction

The skin acts as one of the most important barriers of the body and possesses numerous functions such as protection against external stimuli (i.e. mechanical, chemical, biological, thermal and electrical), thermo-regulation, sensorial and metabolic functions. The skin is also involved in the metabolism of toxic foreign substances through several key mechanisms such as: redox reactions, hydrolysis and conjugation, and it provides the transfer of fluids and electrolytes while removing the end products of their degradation [10, 15, 16, 26, 27, 35].

Human papilloma virus (HPV) penetrates the skin or mucous membranes through various inoculation pathways; afterwards, the infected subjects usually show no sign of infection, as the virus can be cleared by the host immune system, in people with a good immunity. However, in some cases, the clearance cannot be accomplished, and the persistent HPV infection that arises increases the risk of developing benign (palmar or plantar warts, genital warts, etc.) and malign (cervical carcinoma) lesions. Skin integrity, sexual behaviour, immune system, inflammation and oxidative stress all play an important role in establishing the course of HPV infection [2-4, 6, 8, 9, 11, 16, 19, 25, 28-32, 36, 37].

Many therapeutic methods are currently available for treating HPV lesions (e.g. curettage, trichloroacetic acid, podophyllin, lactic acid). However, all have various side effects and a high rate of reoccurrence of the lesions. Recently, the FDA has approved a novel agent (10% sinecatechins ointment) that showed good efficacy accompanied by mild side effects [14]. The main side effects include local redness, itching and pain; ulceration was rarely reported. The product is an extract of green tea leaves from *Camellia sinensis* and acts on the viral replication. Its main effects consist of the stimulation of cytokine release, inhibition of cell proliferation and modulation of apoptosis. Therefore the drug exhibits antiviral and antitumor activity; however, it has been postulated that sinecatechins also display a significant antioxidant action, considerably contributing to its efficacy [14, 22].

Gamma-glutamyl transpeptidase (GGT) is an enzyme that bears a central role in the preservation of skin architecture [1, 5, 7, 18, 21, 23, 24, 33, 34, 39]. GGT is a heterodimeric glycoprotein; while the main part resides in the cytoplasmic membrane, the catalytic centre of the enzyme is situated in the outer part of the membrane. This enzyme is expressed in most eukaryotic cells, being encountered in the liver, gallbladder, pancreas, spleen, kidney, mammary gland, brain, heart, malign tissues, lymphocytes and inflammatory cells [5, 18, 23, 39]. Under physiological conditions GGT is the only enzyme which can initiate the catabolism of glutathione (GSH) and GSH-containing molecules (the gamma-glutamyl cycle).

The functional maturation of GGT is influenced by the protein glycosylation status, presence of toxic substances, redox reactivity of transition metals, as well as ischemia, inflammation, infection, apoptosis, excess production of free radicals and damage of cell membranes [1, 5, 7, 18, 21, 23, 24, 33, 34, 39].

The level and catalytic activity of GGT influence the stability of the biomembranes [28, 30]. Under conditions of equilibrium, GGT initiates the catabolism of GSH and GSH-containing molecules and provides the cellular availability of cysteine [21, 39]. Low activity of GGT lead to oxidative damage of bio-molecules due to a reduced supply of cysteine in cells [15]. Cysteine-glycine, the compound which reduces the Fe^{3+} to Fe^{2+}, generates reactive species with pro-oxidant activity [1, 7, 18, 21, 34].

GGT is also linked to various signal transduction mechanisms through activation of several intracellular pathways such as Ras, extracellular signal–regulated kinases (ERKs), P38 mitogen-activated protein kinases (p38MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (P13K); therefore, the enzyme is involved in the regulation of the intracellular redox status and the generation of signals for cell differentiation and proliferation [5, 15, 21, 24, 39].

GGT is involved in many metabolic processes, as well: the catabolism of GSH and its conjugates, the metabolism of mercapturic acid, the conversion of leukotrienes LTC4 to LTD4. It also catalyses the transfer of gamma glutamyl from GSH to other peptides; therefore it regulates the cell uptake of amino acids from the external environment through the generation of extracellular signals [1, 7, 24, 33, 34]. GGT induces oxidative stress [1, 15, 18, 21, 33, 39] and its catabolites are strong oxidants.

Because HPV infection induces malign or benign lesions only in certain cases, we investigated the mechanisms of adaptive response in HPV lesions and we focused on the detoxification process of harmful metabolites produced under conditions of oxidative stress and preservation of intracellular redox environment.

In order to assess the main catalytic actions of GGT in infected tissues, we aimed to perform a comparative study between patients with lesions caused by HPV and healthy subjects. The objective of the study was to assess the correlation between GGT activity and the levels of oxidative stress parameters in patients with lesions induced by HPV, before and after the initiation of the treatment with 10% sinecatechins ointment, in order to find out if the oxidative status (TOS), antioxidative status (TAS) or oxidative stress index (OSI) have an impact on this enzyme activity. We have also assessed the correlations between GGT activity and the levels of oxidative stress biomarkers in both groups of patients, with HPV

lesions as well as in normal controls, before and after treatment.

Materials and Methods

Study design

For the present study we have performed a rigorous selection of the study participants and distributed them in two groups with similar clinical/demographic characteristics. We have assessed GGT activity and oxidative stress biomarkers levels (TAS, TOS, OSI) in patients with lesions associated with HPV and in a control group, as follows: group 1 - n = 35 adults with HPV lesions (cutaneous and genital warts); group 2 - n = 35 healthy subjects (control group).

Selection criteria: otherwise healthy adults, adequate nutritional status, normoponderals, non-smokers.

Exclusion criteria (conditions widely known as being able to alter, and therefore interfere with stress parameters values): pregnancy, alcoholism, drug abuse, the use of a systemic therapy or nutritive supplements and the presence of a systemic disease.

All study participants gave their written informed consent for the use of their biological samples in research studies; the study protocol was approved by the Ethics Committee of the Institution.

The treatment was based on topical medication, 10% sinecatechins ointment, applied about an 0.5 cm strand to each wart, three times a day for a period of 8 weeks.

The processing of biological samples: blood samples were collected using a holder-vacutainer system under basal conditions, before any diagnostic or therapeutic procedures, as well as after 8 weeks of treatment with sinecatechins. Venous blood samples collected in tubes containing an anticoagulant (K_3EDTA) were used for haematological determinations; serum obtained from venous blood collected in vacutainer without an anticoagulant was used for biochemical, serological and immunological determinations. Samples were immediately processed or frozen at -60°C; haemolysed or lactescent samples were rejected.

Biochemical determinations

GGT activity was determined by a spectrophotometric method (Human reagents, HumaStar300 analyser); results were expressed in U/L serum. The GGT Activity Colorimetric Assay kit provides a simple and direct procedure for measuring GGT activity in a variety of samples. GGT activity is determined by a coupled enzyme assay, in which the GGT transfers the λ-glutamyl group from the substrate L-λ-Glutamyl-p-nitroanilide, liberating the chromogen p-nitroanilide (pNA, 418 nm) proportional to the GGT present in the samples. One unit of GGT is the amount of enzyme that will generate 1.0 µmole of pNA per minute at 37°C.

TOS levels were determined by a spectrophotometric method (Randox reagents, HumaStar 300 analyser); results were expressed in µmol H_2O_2 equivalent/L serum [13]. The method is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidative species in acidic medium and the measurement of the ferric ion by xylenol orange.

TAS levels were determined by a spectrophotometric method (Randox reagents, HumaStar 300 analyser); results were expressed in µmol Trolox equivalent/L serum [12].

OSI values were calculated using the following formula:

$$\text{OSI (arbitrary units)} = \text{TOS (µmol } H_2O_2\text{Eq/L)}/\text{TAS (µmol Trolox Eq/L)}.$$

Statistical analysis

The comparison of the obtained experimental data between groups was carried out using Student t-test or ANOVA. The correlations between variables were determined by linear regression. The relationship between pairs of two parameters was assessed by Pearson's correlation coefficient (r). We choose a significance level (p) of 0.05 (5%) and a confidence interval of 95% for the hypothesis testing.

Results and Discussion

In this step of our study, we assessed: the baseline GGT activity and oxidative stress biomarkers levels in patients with lesions associated with HPV before the administration of 10% sinecatechins ointment and in the control group; data on the levels of GGT activity and oxidative stress biomarkers levels in patients with HPV lesions after 8 weeks of treatment with 10% sinecatechins ointment; correlations between GGT activity and the level of oxidative stress biomarkers in patients with HPV lesions before and after treatment and in the control group.

GGT activity (U/L) in the group of patients with lesions caused by HPV did not vary significantly compared to the values recorded in controls (26.0 ± 7.1 *vs.* 19.8 ± 5.5, $p > 0.05$) (Table I). These results suggest that GGT is not involved in the pathogenesis of HPV lesions and it cannot be used as a biomarker for diagnosis and monitoring of these patients.

The levels of TOS (µmol H_2O_2 Eq/L) in the group of patients with lesions caused by HPV did not vary significantly compared to the values recorded in controls (3.11 ± 0.33 *vs.* 3.09 ± 0.30, $p > 0.05$) (Table I). A decrease in TAS levels (µmolTrolox Eq/L) in patients with HPV *versus* controls was observed, however, we found no statistically significant differences between the analysed groups (1.61 ± 0.33 *vs.* 1.78 ± 0.18, $p > 0.05$) (Table I). The OSI levels (arbitrary units) presented a special interest given that there were significantly different values between the patients with HPV and controls (1.96 ± 0.61 *vs.* 1.84 ± 0.36, $p < 0.05$) (Table I). These results

indicate that oxidative stress may contribute to the development of lesions associated with HPV.

Table I

GGT activity and oxidative stress biomarkers in patients with HPV lesions *versus* controls

Parameter	Patients with HPV lesions	Controls	p value
GGT (U/L)	26.0 ± 7.1	19.8 ± 5.5	0.421
TOS (µmoli H_2O_2 Eq/L)	3.11 ± 0.33	3.09 ± 0.30	0.392
TAS (µmoli Trolox Eq/L)	1.61 ± 0.33	1.78 ± 0.18	0.062
OSI (arbitrary units)	1.96 ± 0.61	1.84 ± 0.36	**0.039**

The values of the studied parameters were analysed according to age and sex. To avoid variations depending on these parameters the study participants were divided by age. GGT activity increased with age both in patients with lesions caused by HPV ($p > 0.05$) and control group ($p > 0.05$) without a statistically significant difference (Table II). TOS values increased in both groups according to age, but the variations were not statistically significant ($p > 0.05$). A special mention must be made for variations of TAS in the two groups. A significant reduction in TAS depending on age was noticed both in patients with HPV ($p < 0.05$) and the control group ($p < 0.05$). OSI variation depending on age in the studied groups was statistically significant ($p < 0.05$) (Table II).

Table II

GGT activity and oxidative stress biomarkers in the studied groups divided by age

Age group (years)	GGT (U/L)		TOS (µmoli H_2O_2 Eq/L)		TAS (µmoli Trolox Eq/L)		OSI (arbitrary units)	
	Patients with HPV lesions	Controls	Patients with HPV lesions	Controls	Patients with HPV lesions	Controls	Patients with HPV lesions	Controls
20 - 29	16.4 ± 3.8	16.3 ± 2.2	2.77 ± 0.08	2.78 ± 0.07	1.91 ± 0.03)	1.94 ± 0.01	1.45 ± 0.05	1.44 ± 0.04
30 - 39	16.9 ± 2.7	17.4 ± 1.9	2.94 ± 0.12	2.98 ± 0.16	1.96 ± 0.01	1.82 ± 0.03	1.50 ± 0.07	1.64 ± 0.06
40 - 49	36.1 ± 2.2	16.3 ± 1.7	3.22 ± 0.21	3.08 ± 0.15	1.46 ± 0.06	1.86 ± 0.05	2.21 ± 0.12	1.66 ± 0.1
50 - 59	25.2 ± 9.1	21.2 ± 5.3	3.07 ± 0.25	2.99 ± 0.05	1.49 ± 0.05	1.73 ± 0.06	2.06 ± 0.10	1.73 ± 0.09
60 - 69	29.3 ± 6.7	20.3 ± 2.8	3.24 ± 0.21	3.31 ± 0.04	1.57 ± 0.07	1.76 ± 0.06	2.07 ± 0.08	1.88 ± 0.06
70 - 79	32.3 ± 4.3	27.4 ± 1.1	3.44 ± 0.08	3.38 ± 0.01	1.29 ± 0.01	1.57 ± 0.01	2.67 ± 0.03	2.16 ± 0.02

Of the 35 patients with HPV lesions enrolled in the study, 4 abandoned; consequently, 31 patients were evaluated in the therapeutic and post-therapeutic period (Table III).

Table III

GGT activity and oxidative stress biomarkers in patients with HPV lesions, before and after treatment

Parameter	Before treatment (n = 31 cases)	After treatment (n = 31 cases)	p value
GGT (U/L)	29.1 ± 15.9	20.8 ± 6.2	0.086
TOS (µmol H_2O_2 Eq/L)	3.10 ± 0.29	3.08 ± 0.22	0.721
TAS (µmol Trolox Eq/L)	1.61 ± 0.31	1.68 ± 0.26	0.097
OSI (arbitrary units)	1.93 ± 0.61	1.83 ± 0.46	**0.049**

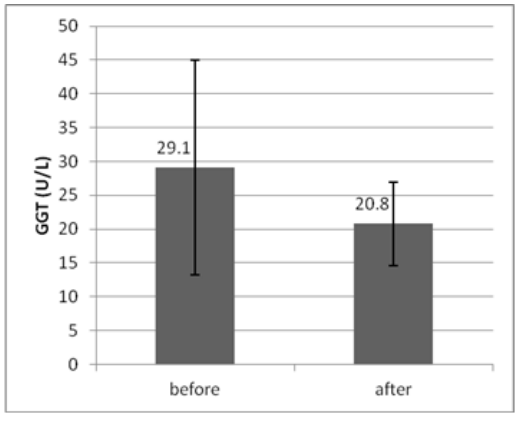

Figure 1.
GGT activity before and after treatment in patients with HPV lesions

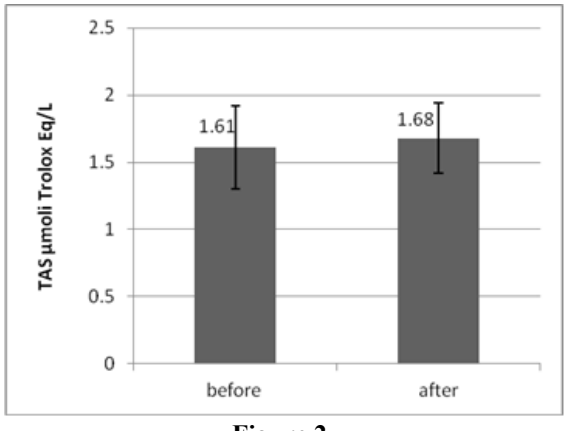

Figure 2.
TAS levels before and after treatment in patients with HPV lesions

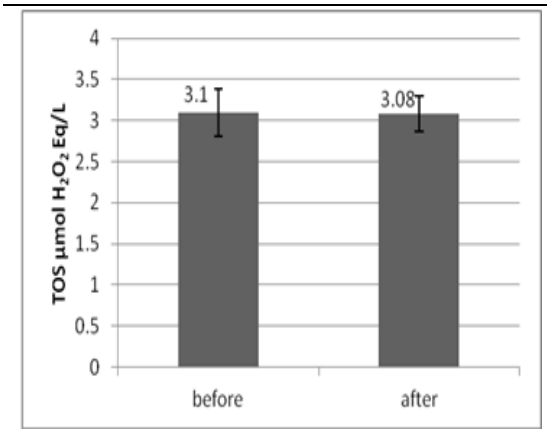

Figure 3.
TOS levels before and after treatment in patients with HPV lesions

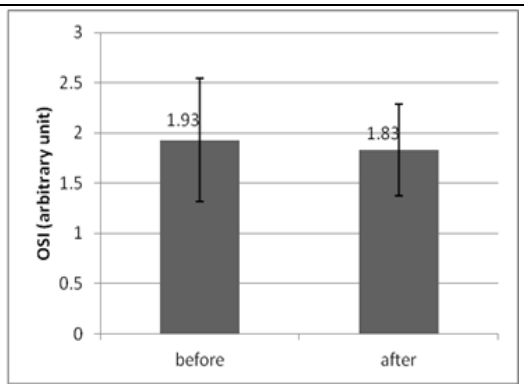

Figure 4.
OSI levels before and after treatment in patients with HPV lesions

In this study, at the end of the treatment compared to the values measured before the initiation of the treatment we have observed a decrease in GGT activity (29.1 ± 15.9 *vs.* 20.8 ± 6.2, $p > 0.05$) (Figure 1), no differences in TOS levels (3.10 ± 0.29 *vs.* 3.08 ± 0.22, $p > 0.05$) (Figure 2), no significant reduction in TAS levels (1.61 ± 0.31 *vs.* 1.68 ± 0.26, $p > 0.05$) (Figure 3), and a statistically significant reduction in OSI levels (1.93 ± 0.61 *vs.* 1.83 ± 0.46, $p = 0.049$) (Figure 4).

Persistent infection with HPV induces a functional adaptation of gamma-glutamyl transpeptidase, an observation supported by the statistically significant positive correlation noticed between OSI and GGT in patients with lesions caused by HPV, prior to the initiation of the specific treatment ($r = 0.312$, $p < 0.05$) (Table IV). The lack of correlation between GGT and oxidative stress parameters at the end of therapy is consistent with the behaviour of these biomarkers. The normalization of the balance between pro- and antioxidants in the monitored patients was observed.

Table IV
Statistical correlations between GGT and the biomarkers of oxidative stress in patients with HPV lesions before and after treatment and in the control group

Variables	Patients with HPV lesions before treatment	Patients with HPV lesions after treatment	Controls
TOS/GGT	r = -0.009 p = 0.837	r = 0.016 p = 0.698	r = 0.003 p = 1.0
TAS/GGT	r = 0.127 p= 0.166	r = 0.111 p = 0.250	r = 0.017 p = 0.987
OSI/GGT	**r = 0.312** **p= 0.038**	r = 0.104 p= 0.206	r = 0.019 p = 0.527

The data obtained in this work support the idea that the main role of GGT in HPV infected cells is the modulation of immune and inflammatory response. Thus, GGT is synthesized in the affected tissue as a response to HPV infection, inflammation or oxidative stress, reaching higher serum levels compared to controls. GGT promotes the synthesis of GSH in the cytosol, being involved in the preservation of intracellular redox potential. The moderate increase in GGT level may be considered a mechanism of adaptive response in HPV lesions, involved in the removal of harmful metabolites produced under conditions of oxidative stress.

Under conditions of equilibrium, GGT initiates the catabolism of GSH and GSH-containing molecules (GSSG, S-glutathione conjugates and GSH complexes) and provides the cellular availability of cysteine [21, 39]. Low levels of GGT lead to oxidative damage of biomolecules due to a reduced supply of cysteine in cells [30]. The massive cleavage of the extracellular GSH causes release of cysteine-glycine, the compound which reduces the Fe^{3+} to Fe^{2+}, generating reactive species with pro-oxidant activity [1, 7, 18, 34]. There are studies in literature supporting the role of GGT in the preservation of cell architecture, cell signalling, participation in certain metabolic pathways, tissue protection against oxidative stress [1, 5, 7, 12, 13, 18, 20, 21, 23, 24, 33, 34, 39].

Alterations of oxidative status in patients with HPV lesions were reported in several studies. Therefore there have been identified: low levels of superoxide dismutase and high levels of malonildialdehyde in plantar warts *versus* normal tissue [3]; increased levels of catalase, superoxide dismutase, glucose 6 phosphate dehydrogenase and malonildialdehyde in serum of individuals with cutaneous warts [29]; altered tissue expression of 8-nitroguanine, 8-oxo-7,8-dihydro-2'-deoxyguanosine and p16 protein in

condyloma acuminatum versus normal tissue [19]; faster clearance of the virus in patients with HPV lesions who had elevated levels of malonildialdehyde and anti-deoxyuridine [31]; polyphenols limit oxidative stress in HPV infected cells through their antioxidant effect, induction of apoptosis, prevention of cell growth, down-regulation of some redox-sensitive transcription factors [9, 28]; upregulation of oxidative DNA lesions [19, 20, 30, 36], the amount of -SH groups [8, 16] nitrites/nitrates ratio, NO, NO synthase, NFkB p65 [8, 32, 37], serpin B3 [8], protein carbonyls [8], Zn reduction [25], the induction of glutathione S-transferase and thioredoxin reductase in HPV infected [8] *versus* normal tissues.

Considering our data and the aforementioned studies, the evaluation of oxidative stress in HPV infected patients is useful in understanding the pathogenesis of the infection and establishing the most effective therapeutic methods. Our results regarding the GGT, TOS, TAS and OSI levels variation enhance the current data in the field.

As future perspectives, several technologies are being developed, based on encapsulation of bioactive molecules with antioxidant effect derived from plants. Thus, rosmarinic acid, an agent used in drug carrier systems encapsulated in liposomes or nano-spheres can provide a high bioavailability and prolonged antioxidant activity [38]. Phenolic compounds (gentisic acid, isoquercitrin, rutoside, quercetin, luteolin, apigenin, p-coumaric acid, ferulic acid, kaempferol) isolated from species of *Cytisus nigricans* and *Cytisus albus* are important sources of antioxidants for the pharmaceutical domain [17].

Conclusions

GGT could be regarded as a "molecular switcher", which is involved in the glutathione turnover and in the maintenance of intracellular redox status. GGT acts against HPV infection removing harmful metabolites formed in excess under conditions of oxidative stress.

Persistent infection with HPV induces a functional adaptation of GGT, materialized in the alteration of the enzyme capacity to reduce the toxic effects of free radicals or neutralize unstable species of oxygen and prevent certain molecules from oxidation in infected tissue. The direct correlation observed between GGT activity and OSI can be a biomarker of molecular imbalance, useful in the early detection of persistent HPV infection. The normalization of the redox status after treatment was correlated with a good clinical outcome and validated the hypothesis that restoring the balance between pro- and anti-oxidants could be an important element in the treatment of HPV lesions.

To the best of our knowledge, this is the first report in medical literature on GGT variation following the treatment of cutaneous and genital warts with topical sinecatechins; this work may contribute to establishing the theoretical basis for sinecatechins use in the treatment of HPV lesions.

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Conflict of interests

The authors declare no conflict of interests.

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