Combination of Anti α-Gal-PpIX Conjugate and Photodynamic Therapy for the Diagnosis and Elimination of HeLa and CaSki Cells

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Abstract: The photodynamic therapy (PDT) is a specific and alternative treatment for premalignant and malignant diseases. The α-Gal epitope is expressed in red blood cells and nucleated cells of placental mammals not primates, but absent in old world monkeys, apes and humans. α-Gal epitope has been found in cervical biopsies HPV positive, in biopsies at different stages of cervical intraepithelial neoplasia and in cell lines such as HeLa and CaSki. The aim of this work was to obtain IgG anti α-Gal monoclonal antibody (mAb) and evaluate its usefulness in the photodynamic therapy in order to diagnosis and eliminate infected cells by HPV, types 16 and 18. From the 4C1F6D5G7B8 hybridoma line was obtained a producer hybridoma of IgG3, the mAb was conjugated with PpIX (IgG-PpIX). The IgG-PpIX conjugated was capable of identify the α-Gal epitope present at the membrane of HeLa and CaSki cell lines by immunocitochemistry. The cell lines were exposed to IgG-PpIX and irradiated with a LED system at 635 nm at an energy dose of 64.3 J/cm^2. The PDT using IgG anti α-Gal monoclonal antibody has effect on the viability of cervical carcinoma cells; the mortality results obtained were, in descendent order, HeLa (66.9 %) and CaSki (50.8 %). It was possible to conclude that PDT using the immunoconjugate IgG anti α-Gal-PpIX is effective to diagnosis and eliminate cervical carcinoma cells.

Keywords: Monoclonal antibody, photodynamic therapy, Human papilloma virus, IgG, LED system.

INTRODUCTION

The infection with human papillomavirus (HPV) is the most common risk factor of cervical carcinoma [1]. In Mexico, 3840 deaths were reported in 2013 because of this disease [2]. In 1996 was described the presence of α-Gal epitopes in cervical biopsies HPV infection, in biopsies at different stages of cervical intraepithelial neoplasia and in cell lines such as HeLa and CaSki [3]. This epitope is expressed in red blood cells and nucleated cells of marsupials and placental mammals not primates, but absent in non-mammalian vertebrates, in old world monkeys, apes and humans [4, 5]. Photodynamic therapy (PDT) is considered as a specific treatment and is a new alternative in the treatment of premalignant and malignant disease. PDT requires a photosensitizer (PS), light, energy and oxygen [6, 7] together; they initiate a photochemical reaction producing singlet oxygen ($^1O_2$). This can cause toxicity leading to cell death *via* apoptosis or necrosis [8]. In recent years have been synthesized and characterized immunoconjugates with PS to increase the specific accumulation of PS in host cells, and reduce unwanted PDT side effects [9]. Photoinmunoconjugates (PIC) have been synthesized against EGFR, [10-12], HER2 [13-15], carcinoembryonic antigen [16], VEGF [17] and CA-125 (mucin 16) [18]. In 2007, our working group generated a monoclonal antibody (mAb) class IgM anti α-Gal which was conjugated with PS protoporphyrin IX, and the effectiveness of photodiagnosis in cell lines and biopsies infected with HPV 16 and 18 was evaluated by Juárez-Palafox[1]. The aims of this study were to obtain an IgG mAb from a clone obtained by our working group in 2007 and evaluate its usefulness in the diagnosis of HPV 16 and 18 by inmunofluorescence, as well as its use in treatment with photodynamic therapy using a light light-emitting diodes (LED) system.

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MATERIALS AND METHODS

Production and Selection of Hybridomas

In 2007 Balb/c mice were used for hybridoma production as described elsewhere [19]. Briefly, Balb/c mice (8-12 weeks of age) were immunized with four intraperitoneal injections of α-Gal in Freund´s complete adjuvant (Sigma, USA) (first injection) or incomplete adjuvant (Sigma, USA) (other injections) (50 µg, every week). Three days after the last injection, spleen cells were fused with X63-Ag8.653 myeloma cells (ATCC), using polyethylenglycol (PEG 1500) (Sigma, USA). Hybridomas were grown in RPMI 1640 culture medium (Sigma, USA) containing 10 % Fetal Bovine Serum (FBS) (Hyclone), penicillin (100 IU/mL) and streptomycin (100 µg/mL) and supplemented with hypoxantine (1×10^{-4} M), aminopterin (4×10^{-7} M) and thymidine (1.6×10^{-5} M) (HAT) (Sigma, USA). Ten to 14 days after fusion, secreting hybrids were identified by analysis of culture supernatants by the ELISA technique described below. The producer clones of anti alpha gal antibody were selected by limiting dilution assay [20]. Clones secreting antibody of desired reactivity were expanded in 75 cm^2 flasks (Nunc, Denmark), harvested and cryopreserved in 90 % FBS and 10 % dimethylsulfoxide (DMSO) (Sigma, USA).

4C1F6D5G7B8 Hybridoma Culture

The h**y**bridoma produced in 2007[1] was thawed and cultured in RPMI-1640 culture medium (Gibco, USA) and 10 % Fetal Bovine Serum (FBS) (GIBCO Invitrogen, USA) at 37 °C, in 5 % of CO_2.

Detection of IgG by Indirect ELISA

Briefly, a microtiter polystyrene plate (Maxi-sorp, Nunc) was coated with 10 µg/mL of α-Gal in carbonates buffer. The plate was blocked with 0.5 % bovine serum albumin (BSA) in phosphate buffered saline (PBS) pH 7.2. Then 100 µL of culture supernatant were added and incubated at 37°C for 2 h. Later 100 µL of HRP-conjugated rabbit anti-mouse IgG antibody at a dilution of 1:5000 were added to each well and incubated at 37 °C for 1.5 h. The reaction was revealed with O-phenylenediamine dihydrochloride (OPD) (Sigma, USA) substrate. Finally, the reaction was stopped with H_2SO_4 2 N and the Optical Density (OD) measured by an ELISA reader at 490 nm. The producing cultures of the antibody IgG selected were cloned by limiting dilution assay [20]. The clones secreting antibody of desired reactivity were expanded in 75 cm^2 flasks (Nunc, Denmark).

Subclass Determination of IgG by Direct ELISA

Each well of a microtiter polystyrene plate (Maxi-sorp, Nunc) was coated with 100 µL of culture supernatant and it was incubated at 37 °C for 2 h. Afterwards were blocked with 200 µL of 0.5 % BSA in PBS pH 7.2. Then 100 µL of HRP-conjugated goat antimouse IgG1, IgG2a, IgG2b and IgG3 at 1:3000 dilution were added to the wells and incubated at 37 °C for 1.5 h. The reaction was revealed with OPD (Sigma, USA) substrate. Finally, the reaction was stopped with H_2SO_4 2 N and the Optical Density (OD) measured by an ELISA reader at 490 nm.

Antibody Concentration and Purification

The culture supernatant obtained from the hybridoma was placed in a dialysis membrane of molecular weight cut of 6-8 KDa. Subsequently, the supernatant was concentrated by osmotic dehydration whereby the membrane was placed in contact with sucrose; this created a gradient of chemical potential between water in the supernatant and water in the sucrose, causing the flow of water from inside the membrane. The volume of the supernatant was reduced to 5 mL. Anti α-Gal mAb was purified from culture supernatant by affinity chromatography using Montage® Antibody Purification Columns with PROSEP G Media kit. Briefly, culture supernatant was filtered through 0.45 µm filter. The eluted antibody was dialyzed against PBS at pH 7.2.

Conjugation of IgG with Protoporphyrin IX

2.66 mg of protoporphyrin IX was dissolved in 125 µL of PBS pH 7.2 plus 80 µL of N, N-dimethylformamide and mixed at low speed for 1 h at room temperature and protected from light. Subsequently 2.66 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl were added dissolved in 80 µL of PBS pH 7.2 and stirred for 30 min at room temperature. Then 1.5 mg of purified IgG was added. This reaction was carried out for 5 h at 8 °C and stopped by adding 6.6 µL of diethanolamine. The IgG anti α-Gal coupled PpIX was dialyzed using a dialysis membrane of molecular weight cut of 6-8 KDa. Subsequently, the membrane was placed in contact with PBS pH 7.2 and incubated at 8 °C during four days. Finally the dialyzed conjugated IgG-PpIX was collected in fractions and stored at -20 °C protected from light.

Immunocytochemistry with Antibody IgG-PpIX in Cell Lines

The cell lines HeLa (18-HPV), CaSki (16-HPV) and HaCaT (normal whitout HPV) were used. In 24 well

plates where placed coverslips in the bottom of each well. Then 6 x 10^3 cells in each well were seeded and cultured in DMEM culture medium (Gibco, USA) with 10 % FBS (GIBCO Invitrogen, USA) at 37 °C, in atmosphere of 5 % CO_2 for 24 h. The cells were fixed with 100 µL of 4 % paraformaldehyde at room temperature for 20 min and the cells were blocked with 100 µL of 3 % BSA in PBS at 37 °C for 1 h. Later, 10 µL/well of IgG-PpIX diluted 1:10 in PBS were added in order to recognize the α-Gal epitope on cell membranes. The plates were incubated at 4 °C overnight in a humid chamber protected from light. 20 µL of 50 % glycerol in PBS were placed on slides and the coverslips with fixed cells were placed. The edges of the slides were sealed. The fluorescence was read at 600 nm.

Cellular Death Using PDT with IgG-PpIX

In all conditions described above, HaCaT, HeLa and CaSki cells were exposed in 96 well plates and irradiated for 10.72 min with a LED system at 50 mW. Each circular well has an area of 0.5 cm^2. The total light dose was 64.3 J/cm^2 at 635 nm; this energy density was calculated with equations 1 and 2.

$$\text{Power density} = \frac{\text{LED power (mW)}}{\text{Irradiation area (cm}^2\text{)}} \quad (1)$$

$$Energy\,(Jcm^{-2}) = Power\,(Wcm^{-2}) \times t(s) \quad (2)$$

The used groups were: viability control (cells without IgG-PpIX and irradiation), irradiation control (irradiated cells only), treated cells group (cells with IgG-PpIX and irradiated). The last condition was analyzed with cells exposed to IgG-PpIX for 24 h before the PDT.

Cell Viability Determination: Method of Reducing the Tetrazolium Salt (MTT)

After exposing cells to the different conditions mentioned above, cell viability was measured. Cellular viability was estimated by means of the method of reducing the spectrophotometric tetrazolium assay [21]. The medium containing IgG-PpIX was removed from the wells and replaced with 100 µL of fresh medium per well containing 100 µg/mL MTT. The plates were then returned to the incubator for 4 h. The medium was subsequently discarded avoiding removing the formed crystals, and the cultures were washed with 100 µL of isopropanol pH 4.0. The plate was shaken and left to stand at room temperature for 10 min. The absorbance of the solubilized salt was subsequently read at 630 nm. Control wells were prepared in parallel, and these cells were exposed to MTT, but not to IgG-PpIX or irradiation. The percentage of viable cells in the cell population at each condition was calculated by means of the equation:

$$\%viability = \frac{Mean\ absorbance\ treated\ cells}{Mean\ absorbance\ of\ control\ cells} \times 100 \quad (3)$$

Statistical Analysis

To analyze the results of the determination of cell viability a comparative analysis for more than two groups with two-factor test way analysis of variance with *Post hoc* Tukey test was performed. The level of significance was set at $p < 0.05$. The tests were performed with Sigma Stat v3.5 for Windows (Jandel Scientific).

RESULTS

Determination of Specific IgG Anti α-Gal in Cell Clones and Subclones from Hybridoma B8 Anti α-Gal

Seven days after cell cloning 12 wells were selected. Supernatants from the selected clones were evaluated by ELISA assay to determine specific IgG antibodies anti α-Gal in the 12 clones obtained. It was observed that only 11 clones produced IgG class antibodies anti α-Gal. B8-D11, E7-B8 and B8-F8 clones were selected for submission to cell cloning by limiting dilution technique. Finally, 21 subclones B8 α-Gal were obtained and evaluated by ELISA to detect what subclone produced the biggest title of antibodies. Thus, two subclones were selected, B8-D11C11 and B8-B8-F8F10.

Determination of the IgG Subclass of Anti α-Gal Antibodies Produced by Subclones B8 α-Gal

To determine the subclass of IgG antibodies the direct ELISA was used. It was determined that the two subclones B8-D11C11 and B8-F8F10 D11C11 produce IgG3 subclass

Purification of IgG Anti α-Gal by Affinity Chromatography with Protein G

The collected fractions from the purification of IgG by affinity chromatography were read at 280 nm in order to identify the best subclone producing IgG. Based on the recorded readings (data not shown), B8-F8F10 subclone was selected. A total volume of 17.5

mL was obtained. It was determined, by Bradford method, that the purified antibody concentration was 1.5 mg IgG contained in a volume of 17.5 mL. The volume was reduced approximately 5-fold in a dialysis membrane of molecular weight cut-off of 12-14 KDa.

Conjugation of IgG with PpIX

In order to verify the conjugation between the IgG and the PpIX was conducted the absorption spectra of anti α-Gal IgG purified, PpIX dissolved in 1.5 N HCl, and the IgG-PpIX-conjugated. The three spectra were obtained and are shown in Figure **1a**, **b**.

Spectra showed that both the IgG and the PpIX moved towards increased absorption wavelength when they are conjugated. The IgG shows two absorption peaks 275 and 555 nm; while the PpIX exhibits more peaks: 390, 410, 550 and 610 nm. The conjugated IgG-PpIX showed two major peaks at 305 and 606 nm.

Immunocytochemical with IgG-PpIX in HeLa, CaSki and HaCaT Cells

Immunocytochemistry for all cell lines were performed with a 1:10 dilution of the antibody IgG-PpIX. Fluorescence images are shown in Figure **2**.

It can be observed that the antibody has a high affinity for HeLa (18-HPV positive) (Figure **2A**, **B**) and CaSki (16-HPV positive) cells (Figure **2C**, **D**). In HaCaT cell line (HPV negative) no fluorescence is observed (Figure **2E**, **F**).

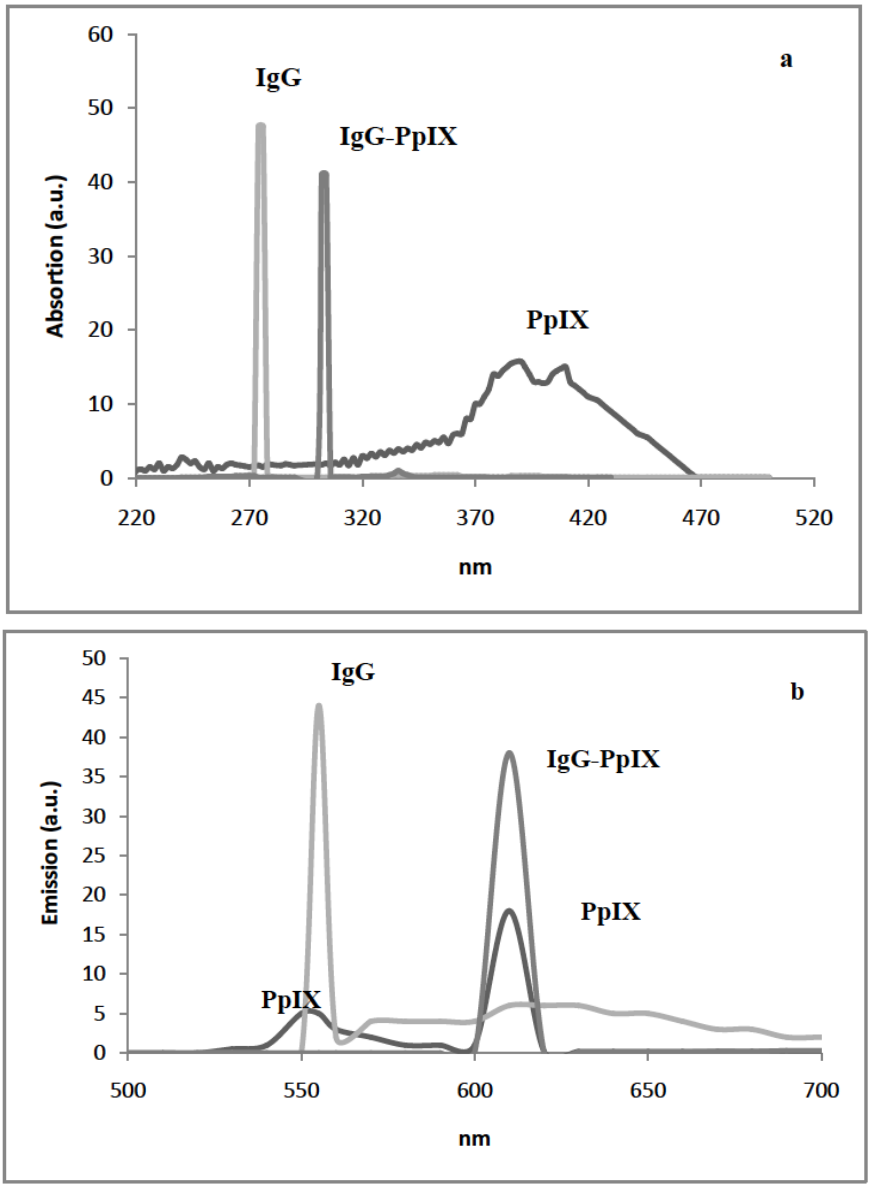

Figure 1: Absorption (a) and emission (b) spectra of IgG anti α-Gal, PpIX and IgG-PpIX.

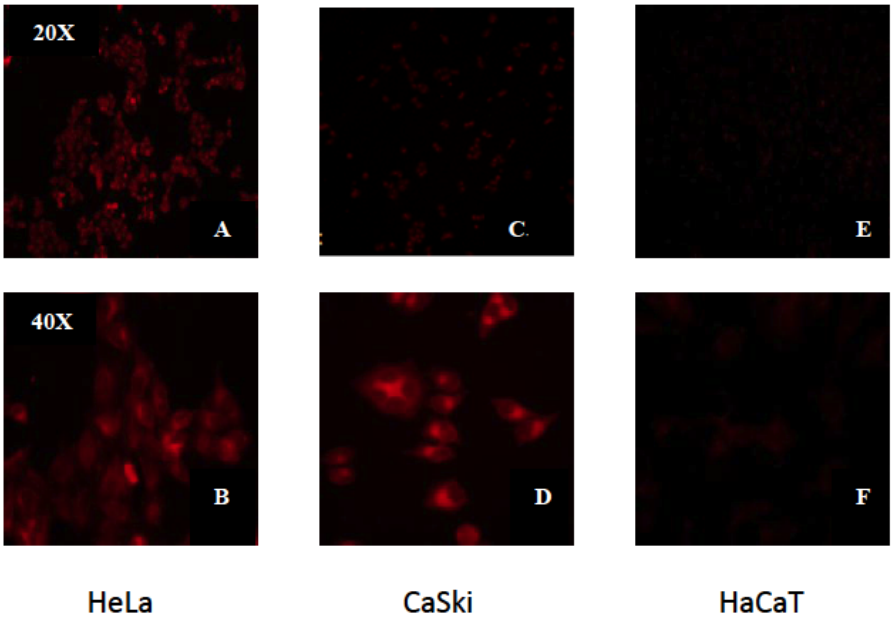

Figure 2: Immunocytochemistry of positive and negative HPV cells with anti α-Gal-PpIX conjugated. Images of red fluorescence obtained by exciting at 560 nm and detecting emission at 600 nm. A and B: HPV-18 positive HeLa; C and D: HPV-16 positive CaSki and E and F: HPV negative HaCaT.

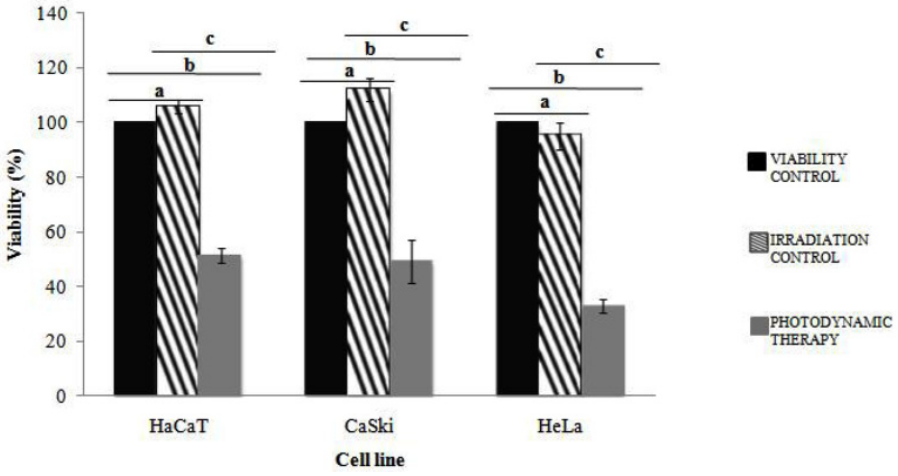

Figure 3: Effect of PDT on cell lines at a wavelength of 635 nm. VC: viability control, IC: irradiation control, PDT: photodynamic therapy. There is a significant difference ($p < 0.05$) between pairs: a (VC and IC), b (VC and PDT) and c (IC and PDT).

Effect of the Application of PDT using a System of LED in the Cell Lines

No significant difference was found among the control irradiation (CI) within the same cell line or between the three cell lines ($p < 0.05$). In Figure **3** are described all the significant differences between the different treatments within the same cell line.

Mortality of cervical carcinoma cells was compared between cell lines after PDT was applied; the results obtained were, in descendent order, HeLa (66.9 %) CaSki (50.8 %) and HaCaT (48.6 %).

DISCUSSION

Photodynamic therapy is used in the treatment of localized cancers. However, normal cells can internalize non-targeted PS. To overcome the poor selectivity of PS, antibodies and ligands for receptors on tumor cells were tested to target PS into tumor cells [22].

In this study we assessed if the α-Gal IgG3 class antibody coupled with protoporphyrin IX, has higher affinity for cervix adenocarcinoma cells HeLa and CaSki HPV 18 and 16 positive respectively, compared

to HaCaT cells negative to HPV by an immunocyto-chemistry assay.

It was determined that PDT at a dose of energy of 64.3 J/cm^2 with IgG-PpIX removed approximately 25 to 74 % of cervical cancer cells (Lopez-Cardenas, 2005)[2]. The application of the red light (635 nm) in PDT has a greater effect on cell metabolism, by interacting the light with its basal fluorochromes [23]. The absorption spectrum of PpIX-IgG conjugate showed that the absorption peaks of the purified IgG shifted toward the red when it is conjugated with the PpIX. This indicates that the conjugated absorbs light of a longer wavelength (to red) when the antibody is not conjugated.

It was observed the highest percentage of viability after PDT in CaSki and HaCaT cell lines, with no significant difference between them; from this it can be concluded that the CaSki cells (derived from a metastatic site of squamous cell carcinoma) were more resistant to PDT in comparison to HeLa cells (cervical adenocarcinoma), we could infer that cervical cancer cells in advanced stage of disease are more resistant to this treatment modality. In the case of the HaCaT line (noncancerous control line) it was found that the red light itself stimulates a positive effect on their proliferation, this was observed in the control irradiation (viability 105.0 %) and was statistically significant; this result is different as compared with the HeLa line; the red light itself decreased cell viability. However, applying the PDT at 635 nm with IgG-PpIX PIC, decreased viability of HaCaT cells in almost 50 %, in other studies where cells were exposed to 5-aminolevulinic acid and PDT was applied, viability decreased by 87.2 % and 65 % [24, 25]. We believe that the cell line HaCaT can incorporate the PIC through phagocytosis however, further studies are necessary. On the other hand, HaCaT is not a suitable negative control in PDT assay because these cells are human keratinocytes from skin and HeLa and CaSki cells are from cervix, is necessary repeat this assays using a different control such as C33-a cell line and analyze the PDT effect. In PDT apparently IgG-PpIX PIC binds nonspecifically, this therapy is applied in living cells, so the IgG-PpIX PIC could penetrate into the cells and accumulates; also free chains mAb and free PpIX may penetrate cells.

In immunocytochemistry the IgG-PpIX PIC bound specifically to the HPV infected cells and does not bind to uninfected cells; however, in PDT apparently IgG-PpIX PIC binds nonspecifically, PDT is applied in living cells, so the IgG-PpIX PIC could penetrate into the cells and accumulates; also free chains mAb and free PpIX may penetrate cells. In immunocytochemistry, the IgG-PpIX PIC can not accumulate because the cells are fixed and also several washes are performed to prevent nonspecific binding.

In summary, the results generated from this study show that anti α-Gal IgG-PpIX is capable of identifying by fluorescence infected cells with HPV 16 and 18, and it is a useful tool that could be used in the protocols for screening of HPV-premalignant lesions or can be used with cytological and molecular assays to increase the sensitivity and specificity of early diagnosis of infection with HPV in the mass screening [26]. Also shows that when this is used in PDT induces citotoxicity. On the other hand, the results presented suggest that infected cells are more sensitive to PDT when is used the PIC IgG-PpIX, further testing are necessary to determine if it can be used in the treatment of HPV infections and malignant lesions associated with the presence of this virus.

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