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ORIGINAL RESEARCH

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The cytotoxic, genotoxic and apoptotic effects of an adsorbent and anti-oxidative vaginal gel on human cervical cancer cell (HeLa) lines

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Abstract

To demonstrate the effect of silicon dioxide, citric acid and sodium selenite combination vaginal gel (DeflaGyn®) on critical parameters in carcinogenesis *in vitro* HeLa cell culture medium. Various parameters including cell viability, intracellular reactive oxygen species, genotoxicity, apoptosis, mitochondrial membrane potential and glutathione levels were evaluated by applying varying doses of vaginal gel to HeLa cell culture medium. The vaginal gel showed a dose-dependent cytotoxic effect on HeLa cells. The IC_{50} (half maximal inhibitory concentration) gel concentration for HeLa cells at 24 h was calculated on the concentration-response curve and was found to be 80.15% of DeflaGyn®. Increasing DeflaGyn® concentrations (12.5–100%) increased iROS (intracellular reactive oxygen species) levels 1.3-fold on average compared to the control (0.1% DMSO (dimethyl sulfoxide)). DNA damage and apoptosis levels increased significantly with concentration in a dose-dependent manner. Similarly, a decrease was observed in the mitochondrial membrane potential (MMP levels decreased statistically significantly up to 70.14%) and intracellular glutathione levels, and all changes were proportional to the dose. Although it is known that the combination of vaginal gel facilitates the regression of cervical dysplasia and clearance of high-risk HPV (Human Papilloma Virus) types, there is no detailed information on the mechanism by which it provides these effects. In the present study, we found that the specific vaginal gel had a dose-dependent effect on various parameters thought to be important in the process of cervical carcinogenesis. Further studies are needed to reveal the pathways through which the gel is effective and whether it has similar effects *in vivo*.

Keywords

HeLa; Adsorptive and antioxidative vaginal gel; Silicon dioxide; Sodium selenite; Cell viability; Apoptosis

1. Introduction

Cancer of the cervix is one of the most common cancers in women globally, approximately 300,000 women die from the disease every year. The fact that the disease is lethal creates a paradox as it is preventable. There is a strong association between high-risk HPV (*i.e.*, HPV types 16 and 18) infection and cervical cancer. Although 80–90% of the cases are transient and tend to clear approximately 24 months after the first detection, persistent infection progressing to cervical cancer is observed in 10–20% of cases. Although there is no consensus on the factors affecting persistent infection, there is strong evidence that an impaired immune response at the cellular level may play an important role [1, 2]. Preinvasive cervical lesions caused by HPV infection are mostly temporary. A different clinical course is observed depending on the degree of cervical intraepithelial neoplasia (CIN). For example, 57% regression, 32% persistence, and 12% progression were observed in CIN1 lesions, while 22% of CIN2 lesions reached the stage of carcinoma *in situ* and 5% of them progressed to invasive cancer. In CIN3 lesions, the cumulative incidence of invasive cancer is as high as 31% after 30 years [1].

CIN lesions are usually detected after abnormal cervical cytology results. Although low-grade cytological findings such as ASCUS (Atypical Squamous Cells of Udetermined Significance) and LSIL (Low-grade Squamous Intraepithelial Lesion), usually show spontaneous regression within 1–2 years, they may progress to more advanced lesions less frequently. ASC-H (Atypical Squamous Cells cannot rule out High grade lesion) or higher grade cytology may increase the risk of progression. Regardless of cytology, the progress

of the process hinges on supplementary factors such as age and persistent high-risk HPV infection. The follow-up of patients without any treatment for early lesions remains a valid practice in the current guidelines. However, such follow-up is a stressful period for both the physician and the patient [3, 4]. With an understanding of the role of persistent HPV infection in cervical pathologies, some therapeutic approaches that facilitate HPV clearance in infected individuals have been investigated. These include imiquimod, therapeutic HPV vaccines, 5-fluorouracil, curcumin, interferon, Vitamin D and lidocaine [5–11].

Recently, a solution of silicon dioxide, citric acid and sodium selenite vaginal gel (DeflaGyn®) was developed. Silicon dioxide is pharmacologically inert, but acts as an adsorbent. The addition of citric acid and sodium selenite results in antioxidative gel properties. This preparation has been successfully used in the treatment of herpes simplex-related lesions and irritations caused by conditions such as insect bites or sunburn. Oxidative stress leads an essential role in carcinogenesis. HPV plays a key part in HPV DNA assimilation, which is a crucial step in malignant conversion. Oxidative damage to DNA is important in the progression of CIN1 lesions to CIN3. Therefore, early treatment of CIN1 and CIN2 lesions with topical application of this propriety antioxidative and anti absorbent gel may be an excellent option to prevent progression [1, 12]. Theoretically, it can be assumed that an agent that slows down carcinogenesis will have a similar effect at any stage of the process [13]. From this point of view, live human cervical cancer (HeLa) cell cultures can be considered an ideal environment to reveal the possible effects of any agent in the preinvasive process.

In our study, we aimed to reveal the effect of vaginal gel on various mechanisms that play a role in carcinogenesis using HeLa cell culture.

2. Materials and methods

2.1 Test material and chemicals

DeflaGyn® vaginal gel (Exeltis Germany GmbH, Ismaning) is an acidic aqueous solution with a pH of 3.0. The main components were silicon dioxide SiO_2 (2 mg/mL) and the anti-oxidant sodium 2-selenite pentahydrate (0.166 mg/mL). Hydroxyethylcellulose, potassium sorbate and sodium benzoate are additional constituents that can serve as preservative/gelling agents. Human cervical adenocarcinoma cell cultures (HeLa, ATCC-CCL-2™) were bought from the ATCC Cell Bank (Germany). Cell culture medium and complements were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). 2,7-dichlorodihydrofluorescein-diacetate, JC-1, penicillin-streptomycin (Istanbul, Turkey), ethidium bromide, and acridine orange were obtained from Sigma-Aldrich (Seelze, Germany). The CellTiter-Glo™ Luminescent Cell Viability Assay Kit, and Glutathione Assay were purchased from Promega Corp. (Madison, WI, USA). (DeflaGyn®) gel was purchased from Hiperfarma (Istanbul, Turkey). DMSO was used as the positive control and NAC (N-acetylcysteine) as the negative control in all experiments.

2.2 Cell viability assay

The cytotoxic activity of melatonin on cells was examined by measuring ATP (Adenosine triphosphate) levels using a luminescence test (Cell-Titer-Glo Luminescent Cell Viability Assay, Promega). HeLa cells were maintained in Eagle's Minimum Essential Medium supplemented with 10% FBS (fetal bovine serum), 1% penicillin, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured in an atmosphere of 5% CO_2 at 37 °C. The cells were grown in 75 cm^2 culture bottles. HeLa cells were implanted in 96-well white plates (1×10^4 cells/well) for cell viability assays. After 24 h, the cells were incubated with different gel concentrations. After 24 h of incubation, luciferin derivative converted a light signal proportional to the current amount of ATP. Luminescence was measured using a Multiplate Reader (Varioscan Flash Multimode Reader, Thermo, Waltham, MA, USA) and was normalized to that of the control (0.1% DMSO). The IC_{50} gel concentration for HeLa cells at 24 h was calculated on the concentration-response curve and was found to be 80.15% of DeflaGyn® respectively.

2.3 Intracellular reactive oxygen species measurement

Intracellular reactive oxygen species (iROS) production levels were measured by the fluorometric method using a probe, 2′7′-dichlorofluorescein diacetate HeLa cells (1×10^4 cells/well) were seeded in each well and incubated. They were then treated with vaginal gel at different concentrations and incubated again. The cells were then washed with phosphate-buffered saline incubated with 1× dPBS (Phosphate buffered saline) containing 5 μM H_2DCF-DA for 30 minutes at 37 °C in the dark. After 30 min of incubation, the absorbance was measured for iROS content using a fluorimeter (Varioskan Flash Multimode Reader, Thermo Scientific) at Ex./Em. = 488/525 nm. After normalizing the concentration-dependent iROS levels to the control 100× iROS/control (0.1% DMSO), the results were normalized to cytotoxicity (iROS/ATP).

2.4 Genotoxicity assay

An alkaline single-cell gel electrophoresis assay (Comet Assay) was carried out to assess the genotoxic effects of the vaginal gel on HeLa cells. The cells were placed on 6-well cell culture plates and incubated at 37 °C in 5% CO_2. Concentrations below the IC_{50} (50% inhibitory) value of the gel samples were then added to the wells and incubated again. Afterwards, the cells were collected using 0.25% trypsin/EDTA (ethylenediamine tetraacetic acid) and bathed with 1× dPBS. Eighty microliters of 0.6% low melting point agarose and 12 μL of cell suspension were mixed and placed on 1% normal melting point agarose pre-coated slides. Cold lysis buffer (1% Triton X-100, 2.5 M NaCl, 10 mmol L^{-1} Tris, 0.1 M EDTA, Sigma-Aldrich) was applied for 1 hour on ice. The slides were again incubated in an alkaline solution (0.3 M NaOH, 1 mM EDTA, Sigma-Aldrich, MA, USA) with accompanying cooling blocks to unravel DNA. After incubation, electrophoresis was performed at 0.70 V/cm (26 V, 300 mA) for 20 minutes. Thereupon the slides were neutralized in Tris buffer (0.4 M Tris, pH: 7.5)

and dehydrated with ethanol. The slides were subsequently dyed with Ethidium Bromide (2 μg/mL in distilled H_2O, 70 μL/slide), The scoring was performed utilizing a fluorescence microscope (Leica DM 1000, Solms, Germany).

2.5 Apoptosis analysis: acridine orange/ethidium bromide (AO/EB), double staining method

The apoptotic effect of the vaginal gel samples was studied by the double-staining acridine orange/ethidium bromide (AO/EB) technique. This technique is a basic and cost effective method to differentiate apoptotic cells from living cells [14]. According to the cytotoxicity experiments, concentrations below the IC_{50} values were applied to cells for 24 h. The cells were then removed from the plates and stained with the AO/EB dye (Sigma Aldrich, USA). Stained cells were examined using a fluorescence microscope, and the apoptotic cell ratios were determined by evaluating a minimum of 100 cells for each sample.

2.6 Mitochondrial membrane potential and glutathione analysis

Mitochondrial membrane potential (MMP) is an important indicator of mitochondrial function. A decrease in MMP implies endangered integrity of the mitochondria, and thus is an indicator of pro-apoptotic signaling [15]. Dihexyloxacarbocyanine iodide DiOC6(3), is a membrane-permeable, fluorescent agent. Incubation with different concentrations (under IC_{50}) of the gel, followed by another round of incubation was performed. Washing was performed three times utilizing 1$\times$ dPBS. The fluorescent density was calculated with an Ex: 484 nm/Em: 501 nm fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, USA). The results were compared with the control group in terms of ATP (MMP/ATP). All doses were replicated four times.

To analyze intracellular glutathione levels in this study, a luminometric glutathione kit was used. The commercially obtained GSH-Glo™ Glutathione Assay luminescence kit converts the luciferin-NT substrate into luciferin. ATP is an end product of this process. Luciferin transforms into oxyluciferin with the aid of a recombinant luciferase enzyme and emits luminescence. Different concentrations (under IC_{50}) of the vaginal gel were applied, and a repeat incubation for 24 h was carried out. Afterwards, glutathione solution was applied, and luminescence was measured using a Multiplate Reader (Varioscan Flash Multimode Reader, Thermo, Waltham, MA, USA). Results were analyzed in terms of ATP (GSH/ATP) in comparison to the 0.1% DMSO-containing control group. All doses were replicated four times.

2.7 Statistical analysis

The findings are expressed as the mean values (mean $\pm$ standard deviation). Statistical analysis was conducted using one-way analysis of variance (ANOVA), and Tukey's test was used for *post-hoc* test analysis. Statistically significant variations with a probability value of $p < 0.05$ were considered meaningful. Statistical Package for Social Sciences (SPSS) version 25 (IBM Corp, Armonk, NY, USA) was used for analysis.

3. Results

3.1 Cell viability assessment

HeLa cells were treated with different concentrations (12.5–100% of DeflaGyn®) of the gel. The effects on cell viability were measured after 24 h using an ATP cell viability assay. As shown in Fig. 1a, increasing the gel dose was more effective in decreasing cell viability. Treatment with high doses of vaginal gel resulted in a higher cytotoxic effect (45.56 $\pm$ 8.31) on HeLa cells. The IC_{50} gel concentration for HeLa cells at 24 h was calculated on the concentration-response curve and was found to be 80.15% of DeflaGyn® respectively.

3.2 Intracellular reactive oxygen species (iROS) generation assessment

The intracellular ROS generated by different gel concentrations were analyzed by the fluorometric method using H_2DCF-DA dye. The results showed that the gel increased intracellular ROS production in HeLa cells dose-dependently (Fig. 1b). Intracellular ROS levels were found to increase 1.3-fold (1.31 $\pm$ 0.1) on average compared to the control (0.1% DMSO).

3.3 Genotoxic assessment

DNA damage after gel treatment was measured using the Comet Assay method. HeLa cells were treated with different gel concentrations for 24 h and then analyzed. Fragmented DNA forms a comet head and tail, whereas undamaged DNA remains round. The data obtained showed that increased gel concentration significantly increased the tail intensity (%), as shown in Fig. 1. Increasing the gel concentration significantly increased the extent of DNA damage (Fig. 1c). These findings indicate that the DNA-damaging effects are related to the anticancer effects of the gel. All concentrations have been found to be DNA damage. Even at 25% gel concentration, there is a statistically significant increase in DNA damage (% DNA tail: 12.24 $\pm$ 2.57).

3.4 Apoptosis assessments

Apoptosis, an indirect marker of tumor development and treatment resistance, is one of the most critical types of programmed cell death. In the current study, we performed AO/EB double staining experiments to detect apoptosis in cancer cells treated with vaginal gel. Cells were stained with the AO/EB dye mix and examined under a fluorescence microscope to observe the morphological appearance of apoptosis (Fig. 2). Our results indicated that the number of viable cells decreased, and the number of apoptotic and necrotic cells increased dose-dependent (Fig. 2a). The apoptosis rate increased from 1% (Control, 0.1% DMSO) to 34% (100% of DeflaGyn®) at the highest concentration.

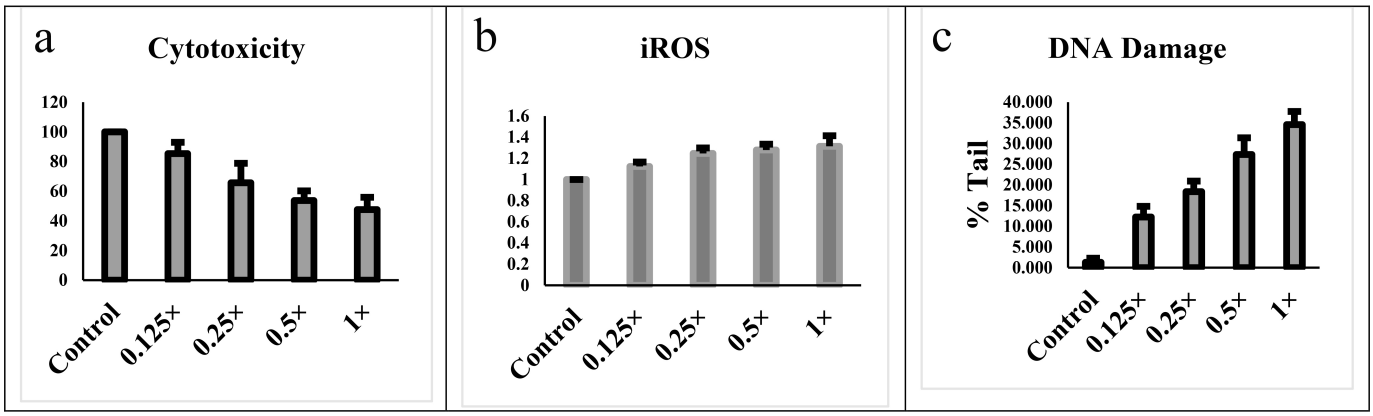

FIGURE 1. Effects of the gel on cervical cancer cell viability (a), intracellular ROS production (b) and DNA damage (c). All values are expressed as mean ± SD. iROS: intracellular reactive oxygen species.

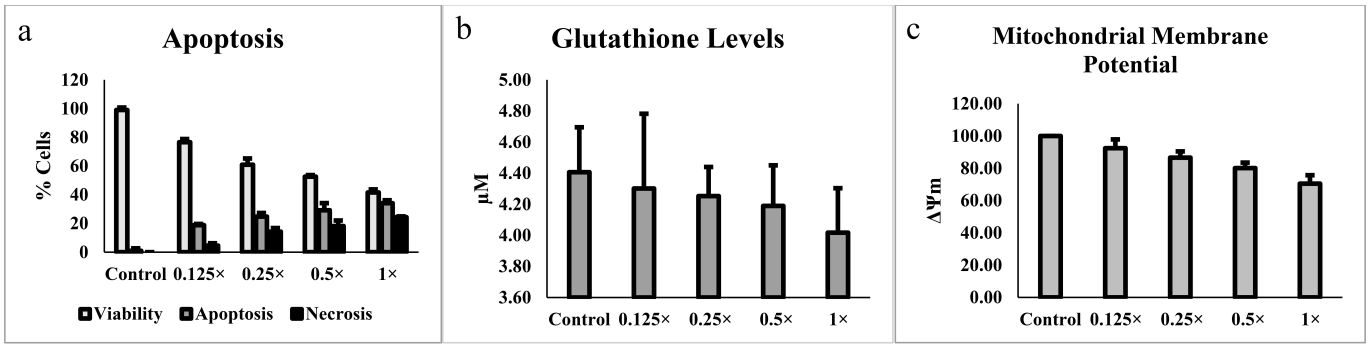

FIGURE 2. Effect of the gel on apoptosis (a), intracellular glutathione levels (b) and mitochondrial membrane potential (c) of cervical cancer cells. All values are expressed as mean ± SD.

3.5 Mitochondrial membrane potential and glutathione analysis

We found that the GSH levels in HeLa cells decreased with increasing gel concentrations (Fig. 2b). GSH levels at maximum Deflagyn concentration (100%) 4.01 ± 0.28 μM. In addition, the mitochondrial membrane potential of HeLa cells decreased with increasing gel concentrations (Fig. 2c). MMP levels decreased statistically significantly up to 70.14% (100% of DeflaGyn®).

4. Discussion

It has been suggested that mucus production and function are important along with the length of the cervix, and therefore surgical treatment of cervical precancerous lesions leads to premature birth, dyspareunia, infertility and adversely affects the psychological state of the patients [16]. Thus, treating precancerous lesions at an early stage with a non-destructive method has come to the fore [17]. Various treatment approaches have been investigated to prevent the progression of low-grade lesions in cervical cancer and to facilitate regression. These include many options, such as 5-fluorouracil, curcumin, imiquimod and interferons. In recent years, a vaginal gel based on a combination of silicon dioxide, citric acid and sodium selenite has been added to armamentarium.

In a study by Huber *et al.* [18] of 186 PAP (Papanicolaou) III and 119 PAP IIID cases, remission rates were 6% and 11%, respectively, in untreated patients, while these rates were 77% and 71%, respectively, in patients treated with a vaginal gel containing a combination of citric acid, silicon dioxide and sodium selenite pentahydrate. Regarding the topical mechanism of action of the gel applied in this study in patients with cervical dysplasia; it has been suggested that the silicon dioxide microparticles bind potentially pathogenic particles, thus preventing the cells with dysplasia from spreading on the vaginal mucosa and cervix surfaces. In addition, it has been emphasized that citric acid and sodium selenite neutralize and eliminate pro-oxidative agents. In our study, it was found that when this gel was applied to cervical cancer cells, it showed a dose-dependent increased ROS production and cytotoxic effect on the cells. It was also found to cause statistically significant high DNA damage in cancer cells, leading to apoptosis.

In the Munich classification, PAP III includes atypical squamous cells (ASC) or atypical glandular cells of undetermined significance (AGUS), whereas PAP IIID corresponds to CIN1 or CIN2 [19, 20]. Another study demonstrated that the 3-month use of a vaginal gel containing SiO_2 sodium selenite and citric acid in 100 women with various epithelial cell abnormalities such as ASCUS, LSIL, ASC-H or HSIL (High-grade squamous intraepithelial lesion) improved cytological findings in 80% of the patients and 53% of the patients showed high-risk HPV clearance [21]. Vaginal gel was applied to 75 of 172 patients diagnosed histologically with CIN2 or p16 positive CIN1 lesions, and 97 patients were followed up without any treatment. At the end of three months, the cytological regres-

sion rates in the treatment and follow-up groups were 76% and 25%, respectively. p16/Ki-67 positivity changed to negative in 77% and 21% of cases, respectively, while high-risk HPV positivity decreased from 87% to 44%, it increased from 78% to 84% in the follow-up group; It was observed that cytological regression findings and p16/Ki-67 negativity continued at 6 months [3]. Major *et al.* [12] compared the results obtained in women in the control group who were followed up without any treatment with the group treated with a combination of adsorptive silicon dioxide, sodium selenite, and citric acid in histologically diagnosed CIN1 and CIN2 cases. Regression was observed in CIN lesions in 78 (72%) of 108 patients in the treatment group and in 27 (25%) of 108 patients in the control group. Although the prevalence of high-risk HPV decreased from 87% to 40% in the treatment group, there was a slight increase (from 79% to 83%) in the prevalence in the control group [12]. In these studies, it was suggested that oxidative stress triggered by infection and inflammation is the main mechanism in cervical dysplasia and cancer, and that the applied gel blocks this mechanism with its antioxidative effect, leading to healing in dysplastic cells. However, in our study, we observed a healing effect on cancer cells through the activation of mechanisms that trigger cell death rather than an antioxidant effect.

Our study highlights the advantages of using *in vitro* models, especially for understanding the process of apoptosis induction in cancer cells. Cancer cell lines derived from human cervical cancer tissues provide a controlled environment for detailed study of cancer cell behavior and the effects of various treatments. In *in vitro* studies, cancer cell lines allow for precise manipulation of variables such as drug concentration and genetic expression, providing a clearer understanding of how these factors affect cancer cell behavior, especially in triggering apoptosis. At the same time, *in vitro* studies using these cell lines have demonstrated the effects of apoptosis in cancer cells. It enables the rapid identification of potential drugs that can induce [22]. This is what we aimed for with our *in vitro* experiment, but while these studies provide valuable information, they lack the full tumor microenvironment found *in vivo*. This limitation is crucial because the tumor environment significantly affects cancer progression and treatment response. Therefore, further studies are necessary to translate laboratory findings into effective clinical treatments.

Collectively the clinical implications of our present findings may provide evidence for the antitumor effects of DeflaGyn® in human cervical cancer HeLa cell lines through; apoptosis induction as potential mechanisms. Further studies will be required to investigate the effects of DeflaGyn® both *in vitro* and *in vivo*. Although it has been shown that a combination of silicon dioxide, citric acid and sodium selenite vaginal gel facilitates the regression of cervical dysplasia and clearance of high-risk HPV types [23, 24], there is no detailed information on the mechanism by which it provides these effects. In our study, it was observed that vaginal gel had a dose-dependent effect on parameters such as cell viability, intracellular ROS production, and genotoxicity, which are effective in the process of cervical carcinogenesis. Likewise, while programmed cell death was facilitated in cancer cells in a dose-dependent manner, the mitochondrial membrane potential and glutathione levels decreased in HeLa cells.

This is among the first studies to investigate the effects of a combination of silicon dioxide, citric acid and sodium selenite vaginal gel on carcinogenic processes at the cellular level. The most important limitation of the study is that due to its *in vitro* design, it does not mean that the results obtained will be replicated *in vivo*. However, the general advantages of *in vitro* studies (*e.g.*, tight control of chemical and physical conditions, and lower cost) also apply to our study.

5. Conclusions

Our work provides insight into the mechanisms by which vaginal gel based on silicon dioxide, citric acid and sodium selenite combination facilitates regression in cervical dysplasia. To understand the pathways through which the gel exerts its reversal effect on carcinogenesis, more comprehensive and detailed studies at the molecular level are needed.

ABBREVIATIONS

ROS, Reactive oxygen species; HPV, Human papillomavirus; CIN, Cervical intraepithelial neoplasia; ASCUS, Atypical squamous cells of undetermined significance; LSIL, Low-grade squamous intraepithelial lesion; ASC-H, Atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion; ATP, Adenosine triphosphate; PBS, Phosphate buffered saline; EDTA, Ethylenediaminetetraacetic acid; MMP, Mitochondrial membrane potential; ANOVA, Analysis of variance; SPSS, Statistical package for social sciences; GSH, Glutathione; AGUS, Atypical glandular cells of undetermined significance.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

GK—designed the research study. EMG, AK—performed the research. NUK, FBT—provided help and advice on methodology. EMG, AK—analyzed the data. GK and AFGK—wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Bezmialem Vakif University Research Committee, Project Number: 20210203. Owing to the nature of *in vitro*-cell culture study, ethical approval was not applicable.

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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