An In Vitro Study on the Interactions of Pycnogenol® with Cisplatin in Human Cervical Cancer Cells

Öz

Amaç: Kanser tedavisinde antikanser etki artırmak ve sitotoksisiteyi azaltmak amacıyla kemoterapötik ilaçlar ile birlikte çeşitli bitkisel kökenli fenolik bileşiklerin kullanımı hedeflenmektedir. Pycnogenol® (PYC), bir fenolik bileşik olan Piknogenol® (PYC), birçok çalışmanın konusu olmaktadır. PYC'nin sisplatin ile etkileşimi tam olarak aydınlatılamadığı için insan serviks kanser hücrelerinde (HeLa) PYC'nin etkilerini belirlemeyi ve PYC'nin genotoksimetrik etkilerini değerlendirilmesini hedefledik.

Gereç ve Yöntemler: HeLa hücrelerinde, 24 ve 48 saatlik maruziyetlerde, PYC varlığında ve yokluğunda sisplatinin sitotoksisitesi 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolium bromür (MTT) yöntemi ile ölçüldü. Oksidatif DNA hasarına karşı PYC'nin etkisi Comet yöntemi ile değerlendirildi.

Bulgular: Sisplatinin IC50 degeri 24 saat ve 48 saat için sırasıyla 22,4 µM ve 12,3 µM idi. PYC'nin IC50 degerleri 24 saat ve 48 saat için sırasıyla 261 µM ve 213 µM idi. Yirmi dört saatlik maruziyet için, PYC'nin, seçilen konsantrasyonlarında (15,6-500 µM) sisplatinin IC50 degerini önemli ölçüde azalttı. Kırk sekiz saat maruziyet için, PYC sisplatinin sitotoksisitesini 15,6-125 µM arasındaki konsantrasyonlarda değişirmede, ancak 250 µM ve 500 µM konsantrasyonlarında önemli ölçüde azalttı. PYC tek başına 10 µM ve 25 µM konsantrasyonlarında DNA hasarını neden olmadı, ancak daha yüksek konsantrasyonlarında (50-100 µM) DNA hasarını önemli ölçüde indüksiyonu yapmadı. Ayrıca, çalışılan tüm konsantrasyonlarında (10-100 µM) 50 µM H2O2 tarafından indüksiyonu DNA hasarını önemli ölçüde azalttı.
INTRODUCTION
Oxidative stress is one of the hypotheses involved in the etiology of a number of diseases, including cancer. Considerable attention has been focused on antioxidant agents such as phenolic compounds in recent years, because it is stated that the development of oxidative stress-related diseases may be prevented or delayed by using them. It is commonly consumed as a dietary food supplement owing to its strong antioxidant activity. As shown in many studies, PYC has potential therapeutic and protective effects against cancer. However, there are not sufficient studies on the interactions between antineoplastic drugs and PYC. Antineoplastic drugs are clinically used in therapy of cancers, aiming to reduce tumor cell growth. Cisplatin (CIS) is a powerful antineoplastic drug to treat many types of cancer including esophageal, lung, breast, ovarian, bladder, cervical, and prostate cancers. Nowadays, combinatorial therapies have been investigated with the aim of increasing anticancer activity and minimizing drug resistance. Recent studies yielded positive findings using various phenolic compounds combined with an antineoplastic drug. Nevertheless, further investigations are needed to clarify the effects of phenolic compounds on cancer and the effects of combining them with antineoplastic drugs in different doses. The aim of the present study was to determine the effects of PYC on the cytotoxic profile of CIS in human cervical carcinoma (HeLa) cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The genotoxic/antigenotoxic effects of PYC against oxidative DNA damage were evaluated using alkaline single cell gel electrophoresis (Comet assay).

MATERIALS AND METHODS

Chemicals
The chemicals used and their suppliers were as follows: CIS from Koçak Farma (Turkey); dimethyl sulfoxide (DMSO), Dulbecco’s modified Eagle’s medium, ethanol, ethidium bromide (EtBr), ethylenediamine tetra acetic acid disodium salt dihydrate (EDTA-2Na), fetal bovine serum (FBS), hydrogen peroxide (35%) (H$_2$O$_2$), low melting point agarose (LMA), MTT, n-lauroyl sarcosinate, normal melting point agarose (NMA), penicillin-streptomycin, sodium chloride (NaCl), sodium hydroxide (NaOH), Tris, Triton X-100, trypsin-EDTA, RPMI-1640 medium, Dulbecco’s phosphate buffered saline (PBS) from Sigma (St. Louis, MO, USA); Millipore filters from Millipore (Billerica, MA, USA); all other plastic materials from Cornings (Corning Inc., NY, USA). PYC was purchased from Horphag Research Ltd. (Geneva, Switzerland). The quality of standardized PYC extract is specified in the United States Pharmacopeia (USP 28).

Cell culture
HeLa cells were obtained from the American Type Culture Collection (Rockville, MD, USA). HeLa cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl), and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO$_2$. The cells were subcultured in 75 cm$^2$ cell culture flasks. The medium was changed every 2-3 days. The passage numbers used in our study for both cell lines were between passage 8 and passage 10.

Determination of cytotoxicity
After growing for 2 weeks, the cells were plated at 1×10$^4$ cells/well by adding 200 µL of a 5×10$^4$ cells/mL suspension to each well of a 96 well tissue culture plate and allowed to grow for 24 h before treatment. The number of cells was calculated by trypan blue dye exclusion. The stock solution of PYC was freshly prepared in PBS and filtered with Millipore filters (0.20 µm). The cells were treated with PYC at a wide range of concentrations (1.95-2000 µM) or CIS (0.49-500 µM) in the related culture medium for 24 h and 48 h. Control experiments were carried out with the culture medium containing only PBS (1%). After the values of IC$_{50}$ were determined, the cytotoxic profiles of PYC on the IC$_{50}$ of CIS were evaluated at wide doses of PYC in HeLa cells for 24 h and 48 h.

The cytotoxicity of PYC and CIS was measured in HeLa cells using the MTT assay, which is a colorimetric assay that measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. At the end of the incubation (24 h and 48 h), 5 mg/mL MTT solution was added to each well, followed by incubation for another 4 h at 37°C. Then the medium was discarded. The formazan crystals were dissolved in 100 µL of DMSO and absorbance of each sample was measured at 570 nm using a microplate reader (SpectraMax M2, Molecular Devices Limited, Wokingham, UK).

The percentage of cell viability was calculated using the following formula:

\[
\text{Percentage of cell viability} = \left( \frac{\text{absorbance of sample}}{\text{absorbance of control}} \right) \times 100
\]

The cytotoxic concentration that killed cells by 50% (IC$_{50}$) was determined from the absorbance versus concentration curve.

Determination of genotoxicity
HeLa cells were incubated with PYC at noncytotoxic doses (0, 10, 25, 50, and 100 µM) for 2 h (preincubation) with/without...
genotoxic doses of H$_2$O$_2$ (50 µM) for 5 min. Thus, the possible protective effect of PYC against oxidative DNA damage induced by H$_2$O$_2$ was also evaluated. Moreover, 50 µM H$_2$O$_2$ was applied as a positive control. Medium containing 10% PBS was applied as a negative control. The comet assay was performed to assess DNA damage. The basic alkaline technique described by Singh et al.$^{15}$ was used for the detection of DNA damage in the cells. The concentrations of the cells were adjusted to 2×10$^5$ cells/mL, suspended in 5% LMA, and were then embedded on slides precoated with a layer of 1% NMA. The slides were allowed to solidify on ice for 5 min. The cover slips were then removed. All slides were immersed in cold lysing solution (pH 10) for a minimum of 1 h at 4°C. The slides containing the cells were removed from the lysing solution, drained, and then placed in a horizontal gel electrophoresis tank filled with freshly prepared alkaline electrophoresis solution (300 mmol/L NaOH, 1 mmol/EDTA-2Na, pH 13.0) for 20 min at 4°C to allow unwinding of the DNA and expression of DNA damage. Electrophoresis was then conducted at 4°C for 20 min at 24 V/300 mA. The slides were neutralized at room temperature by washing 3 times in neutralization buffer (0.4 mol/L Tris-HCl, pH 7.5) for 5 min. After neutralization, the slides were then incubated in 50%, 75%, and 98% of alcohol for 5 min successively. The dried microscope slides were stained with EtBr (20 µg/mL in distilled water, 60 µL/slide), covered with a cover glass prior to analysis with a fluorescence microscope (Leica DM1000, Wetzlar, Germany) equipped with an excitation filter of 515-560 nm. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, Version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize the DNA damage, the slides were examined at 400×. For each condition, 100 randomly selected comets from each of two replicate slides were scored (without knowledge of the group codes). DNA damage parameters were expressed as DNA tail intensity %.

Statistical analysis
All experiments were carried out in quadruplicate. The results were given as the mean ± standard deviation. The statistical analysis was performed with SPSS 10.5 (SPSS, Chicago, IL, USA). The distribution of the data was checked for normality using the Kolmogorov-Smirnov test. The means of data were compared by One-way variance analysis test and post hoc analysis of group differences was performed by least significant difference test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Pycnogenol cytotoxicity
The results of PYC cytotoxicity are given in Table 1 and Figure 1. PYC did not cause significant cytotoxic effects at the concentration range of 1.95-125 µM when compared to the negative control for 24 h and 48 h incubation; however, the cell viabilities were significantly decreased above 250 µM concentrations of PYC (p<0.05) (Table 1). The IC$_{50}$ values of PYC were 261 µM and 213 µM for 24 h and 48 h, respectively (Figure 1).

Cisplatin cytotoxicity
The results of CIS cytotoxicity are given in Table 2 and Figure 2. CIS did not cause significant cytotoxic effects at the concentration range of 0.49-7.81 µM or at the concentration range of 0.49-3.91 µM when compared to the negative control for 24 h and 48 h, respectively; however, the cell viabilities were significantly decreased above 15.2 µM and 7.81 µM of CIS for 24 h and 48 h incubation, respectively (p<0.05) (Table 2). The IC$_{50}$ values of CIS were 22.4 µM and 12.3 µM for 24 h and 48 h, respectively (Figure 2).

Effects of Pycnogenol on cisplatin cytotoxicity
The effects of PYC at the concentration range of 15.6-500 µM on CIS cytotoxicity in HeLa cells are shown in Figure 3 for 24 h and 48 h incubation. As shown in Figure 3a, at all studied concentrations (15.6-500 µM) PYC significantly decreased the

![Figure 1. Effects of pycnogenol on the cell viability of HeLa cells for 24 h and 48 h*](image)

* Values are given as the mean ± standard deviation (n=4), p<0.05, compared to negative control (Pharmaceutical Benefits Scheme). PVC: Pycnogenol

![Table 1. Effects of pycnogenol on the cell viability of HeLa cells for 24 h and 48 h*](table)

| Group       | 24 h (%) | 48 h (%) |
|-------------|----------|----------|
| (-) Control | 100.0±0  | 100.0±0  |
| 1.95 µM PYC | 91.9±12.9| 94.4±6.2 |
| 3.91 µM PYC | 84.1±96  | 87.0±3.3 |
| 7.81 µM PYC | 84.0±6.9 | 86.6±11.9|
| 15.63 µM PYC| 92.2±7.6 | 89.9±8.2 |
| 31.25 µM PYC| 91.8±4.7 | 92.3±7.9 |
| 62.5 µM PYC | 89.6±9.7 | 86.2±12.9|
| 125 µM PYC  | 86.2±10  | 84.3±10.4|
| 250 µM PYC  | 52.2±10.8| 36.1±5.5 |
| 500 µM PYC  | 7.0±1.6  | 3.8±0.3  |
| 1000 µM PYC | 6.6±0.7  | 3.7±0.4  |
| 2000 µM PYC | 5.7±0.8  | 2.8±0.7  |

*Values are given as the mean ± standard deviation (n=4), p<0.05, compared to negative control (Pharmaceutical Benefits Scheme). PVC: Pycnogenol
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IC_{50} value of CIS (20 µM, approximately) in a dose-dependent manner (1.53 fold, 1.84 fold, 1.87 fold, 2.28 fold, and 2.86 fold for 15.6 µM, 31.3 µM, 62.5 µM, 125 µM, and 500 µM, respectively, vs. the positive control) when compared to the negative control for 24 h incubation (p<0.05). As shown in Figure 3b, when compared to the negative control, PYC did not change the IC_{50} value of CIS (10 µM, approximately) at the concentration range of 15.6-125 µM for 48 h incubation; however, the IC_{50} value of CIS was significantly reduced at concentrations of 250 µM and 500 µM of PYC (1.11 fold and 1.57 fold for 250 µM and 500 µM, respectively, vs. the positive control) (p<0.05).

Effect of pycnogenol on DNA damage

The results of genotoxicity and antigenotoxicity of PYC at noncytotoxic doses (10 µM, 25 µM, 50 µM, and 100 µM) in HeLa cells using the comet assay were evaluated. DNA damage, expressed as DNA tail intensity in the HeLa cells, is shown in Figure 4. We observed that PYC did not significantly increase DNA damage at all studied concentrations when compared to the negative control (p>0.05). In addition, PYC significantly decreased the DNA damage induced by H_{2}O_{2} (50 µM) in a dose-dependent manner at all studied concentrations (10 µM=36.6%; 25 µM=36.7%; 50 µM=40.1%; 75 µM=50.8%; 100 µM=58.6%) when compared to the positive control (p<0.05).

DISCUSSION

As is well known, CIS is clinically used in the therapy of many types of cancers (including esophageal, lung, breast, ovarian,
bladder, cervical, prostate, etc.), aiming to reduce tumor cell viability. However, it has important side effects, mainly nephrotoxicity.7 Side effects and drug resistance are two of the major problems in antineoplastic therapy; hence recent studies have focused on new approaches, like combinational therapies with phenolic compounds, in order to prevent drug resistance, minimize side effects, and increase anticancer activity.8-12 PYC, a phenolic compound, is commonly consumed as a dietary food supplement because of its strong antioxidant activity. It has potential therapeutic and protective effects against cancer, as shown in many studies.5,6 However, there are not sufficient studies on the interactions between antineoplastic drugs and some natural phenolic compounds, including PYC. In the present study, after the determination of the cytotoxicities of PYC and CIS alone, the effects of PYC in combination with CIS were evaluated. The cytotoxicity of PYC and CIS increased approximately 1.22 fold and 1.82 fold, respectively, after 48 h incubation, when compared to 24 h incubation. The cytotoxicity profiles of PYC and CIS alone were different. It seems that the cytotoxicities of PYC and CIS are dose and time dependent. In our study, PYC (15.6-500 µM) significantly decreased the cytotoxicity of CIS in a dose-dependent manner with 24 h incubation. However, for 48 h incubation, PYC did not increase the cytotoxicity in the cells treated with CIS (10 µM, approximately) at the concentration range of 15.6-125 µM when compared to the negative control; however, the cell viability was reduced significantly at concentrations of 250 µM and 500 µM of PYC in the CIS-treated cells (p<0.05). According to our results, PYC seems to have the desired effect on the cytotoxic profile of CIS in HeLa cells for anticancer activity in a time- and dose-dependent manner. The possible mechanism underlying the cytotoxic effect of PYC has been associated with apoptosis.16,17 In the study investigating the apoptotic effects of PYC, PYC induced apoptosis in human fibrosarcoma cells (HT1080), using flow cytometric analysis and RNA microarray.18 In another study, it was reported that PYC significantly decreased cell viability and also induced caspase-independent apoptosis. Furthermore, PYC induced the translocation of apoptosis-inducing factor into the nucleus and regulated apoptosis.19 In a study investigating the antitumor effect of PYC, the IC50 values of PYC in human leukemia cells (HL-60, U937, and K562) were reported to be 150 µg/mL (~516.8 µM), 40 µg/mL (~137.8 µM), and 100 µg/mL (~344.5 µM), respectively, for 24 h incubation, by propidium iodide exclusion.18 In another study, in which the apoptotic effect of PYC in human oral squamous carcinoma (HSC-3) cells was investigated by the MTS assay, the IC50 value of PYC was reported as 20 µg/mL (~68.9 µM) for 24 h incubation.19 However, the IC50 value of PYC was determined to be 285 µg/mL (~982 µM) for 24 h incubation in Chinese hamster ovary cells by Neutral Red Uptake test.20 The genotoxicity and antigenotoxicity potential of PYC was evaluated with the commonly used alkaline comet assays at noncytotoxic doses in the HeLa cells. In the present study, we observed that PYC alone did not induce DNA damage at concentrations below 50 µM. However, it significantly reduced H2O2-induced DNA damage at all studied concentrations (10-100 µM). Our study using the comet assay showed that PYC might have a protective effect against H2O2-induced DNA damage in cells. The results were in good correlation with those of studies conducted previously. The antigenotoxic studies using the comet assay show that PYC may have a protective effect against oxidative DNA damage. For instance, Taner et al.21 reported that PYC caused no genotoxic effects alone at low concentrations (5-50 µg/mL) as compared with the controls, and it might reduce H2O2-induced chromosome breakage and loss and DNA damage in cultured human lymphocytes in the comet assay. It seems that PYC may have potential for the treatment of diseases related to oxidative DNA damage. The IC50 value of CIS in the selected human cancer cells was reported to be 54.07 µM and 96.38 µM in cervical cancer cells (HeLa and Caco-2, respectively), 97.20 µM and 85.66 µM in pancreatic cancer cells (MIA PaCa-2 and BxPC-3, respectively), and 14.87 µM and 77.89 µM in hepatocellular carcinoma cells (Hep-G2 and SK-HEP-1, respectively), for 24 h incubation, using the MTT method.22 Although there are some in vitro studies on the protective effect of PYC on CIS cytotoxicity, there are limited in vivo studies on the chemotherapeutic activity of PYC.23 It has been reported that in CIS cytotoxicity CIS-induced prooxidant enzymes (myeloperoxidase, xanthine oxidase), malondialdehyde, and nitric oxide levels were corrected by PYC and chromosomal defects were reduced. These findings suggest that PYC may be a protective agent against CIS-induced oxidative, inflammatory, and genotoxic damage.24 It has also been suggested that increased oxidative damage through radiotherapy can be prevented by strong antioxidant activity of PYC.25 It was shown that grape seed extract (GSE), a polyphenolic compound like PYC, exerted synergistic anticancer effects with doxorubicin in human breast carcinoma (MCF-7 and MDA-MB468) cells.26 In that study, GSE and doxorubicin alone and in combination strongly inhibited cell growth but there was no increase in apoptotic cell death caused by doxorubicin. These results suggest a strong possibility of synergistic anticancer effects of GSE and doxorubicin in combination for breast cancer treatment and also promising effects of combination of PYC and CIS for cancer. In recent studies, it has been aimed to decrease cytotoxicity and to increase anticancer activity using various phenolic compounds with antineoplastic drugs.8,10,19 Many researchers have reported that CIS has positive effects in combination with antioxidants to increase its efficacy in cancer chemotherapy, reduce resistance development, and reduce toxicity. Nevertheless, more investigations are necessary to clarify the effects of phenolic compounds on cancer and the effects of combining with antineoplastic drugs in different doses.11,24

CONCLUSION

At the end of the study, it was considered that the use of PYC in the treatment of CIS revealed positive effects on HeLa cells. These findings suggest that PYC might contribute to the anticancer effect of CIS in cervical carcinoma. Therefore, combinational therapy may be therapeutically used in order to increase anticancer activity and minimize drug resistance
and side effects. It will be a new point of view in anticancer treatment, and further in vivo studies with other cancer cell lines as well as in vitro studies are suggested.

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