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ARAŞTIRMA / RESEARCH

Neuroprotective effects of phloretin and phloridzin on paclitaxel-induced neuronal damage in primary neuron cells

Floretin ve floridzin'in primer nöron hücrelerinde paklitaksel ile indüklenen nöron hasarındaki koruyucu etkileri

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Abstract

Purpose: Paclitaxel, is one of the most commonly used chemotherapeutic, causes neuron damage with some serious side effects such as neutropenia and peripheral neuropathy. In current study, we used phloretin and phloridzin to investigate their neuroprotective effects on paclitaxel-induced neuronal damage.

Materials and Methods: The neuroprotective effects of phloretin and phloridzin has been analyzed on cell culture of primary neuron cells and evaluated by testing cell viability, total oxidant and total antioxidant capacities and expression of caspase-3, caspase-9 and TNF-α. Paclitaxel administration caused cell death and significant increase of total oxidant levels and activation of apoptotic genes such as caspase-3, caspase-9 and TNF-α.

Results: Phloretin and phloridzin treatments at micromolar concentrations reduced paclitaxel-induced cell death by increasing total antioxidant levels. Also these two flavonoids protect neuron cells from apoptosis by decreasing caspase-3, caspase-9 and TNF-α gene expression. For this reason, these molecules may recover the oxidative damage, and restore normal cellular conditions.

Conclusion: This study shows the promising neuroprotective ability of the phloretin and phloridzin able to protect neuron cells from injury induced by paclitaxel, actively increasing antioxidant capacity, normalizing oxidant levels and consequently avoiding cell death.

Keywords: Paclitaxel, phloretin, phloridzin, primary neuron cell

Öz

Amaç: Kemoterapide sıklıkla kullanılan ilaçlardan biri olan paklitaksel, nötropeni ve periferik nöropati gibi bazı ciddi yan etkilerle nöron hasarına neden olur. Biz bu çalışmada, floretin ve floridzin' in paklitaksel kaynaklı nöronal hasar üzerindeki nöroprotektif etkilerini araştırdık.

Gereç ve Yöntem: Floretin ve floridzin' in nöroprotektif etkileri primer nöron hücreleri üzerindeki etkileri, hücre canlılığı, total oksidan ve antioksidan kapasiteleri ve kaspaz-3, kaspaz-9 ve TNF-α ekspresyonu test edilerek değerlendirilmiştir.

Bulgular: Paklitaksel uygulaması hücre ölümüne, toplam oksidan seviyelerinin önemli ölçüde artmasına ve kaspaz-3, kaspaz-9 ve TNF-α gibi apoptotik genlerin aktivasyonuna neden oldu. Mikromolar konsantrasyonlardaki floretin ve floridzin tedavileri, toplam antioksidan seviyelerini artırarak paklitaksel kaynaklı hücre ölümünü azalttı. Ayrıca bu iki flavonoid, kaspaz-3, kaspaz-9 ve TNF-α gen ekspresyonlarını azaltarak nöron hücrelerini apoptozdan korudu. Bu nedenle, bu moleküller oksidatif hasardan geri kazanımı indükleyebilir ve normal hücresel koşulları düzenleyebilir.

Sonuç: Bu çalışma, floretin ve floridzin' in nöron hücrelerini paklitaksel kaynaklı hücre hasarından koruyabilen, antioksidan kapasitesini aktif olarak arttıran, oksidan seviyelerini normalleştiren ve sonuç olarak hücre ölümünü önlemede umut verici nöroprotektif potansiyelini göstermektedir.

Anahtar kelimeler: Floretin, floridzin, paklitaksel, primer nöron hücresi

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INTRODUCTION

Antineoplastic drugs are known for their cytotoxic effects. These drugs provide chemotherapy for many types of cancer but they adversely affect life during treatment because of their serious side effects[1]. Paclitaxel is an important chemotherapeutic that is used alone or in combination with other chemotherapeutics in ovarian[2], breast[3], lung[4] and many other cancer patients[5]. Besides this chemotherapeutic effect, paclitaxel causes neuron damage with some serious side effects such as neutropenia and peripheral neuropathy[6,7]. However, chemotherapy-induced neuronal damage could not be prevented yet. Therefore, cyto protective and antioxidant agents should be used in combination with anticancer drugs to prevent their side effects[8]. Todays, new strategies are being developed, especially strong antioxidant substances are being discovered and activities are being tried to prevent the toxic effects of chemotherapeutics[9,10].

Phloretin (PH) and its glucoside form phloridzin (PZ) are members of the chemical class of dihydrochalcones, which are phenylpropanoids[11]. The apple tree (*Malus sp.*) contains high amounts of PH and PZ and it is thought that PH and PZ are responsible for the antidiabetic effect of apples[12]. Chalcone derivatives have shown lots of protective effects[13]. Anti-inflammatory[14], anti-oxidant[15] and neuroprotective[16] effects of PH has been announced in previous studies[17,18]. PZ has also been investigated to provide neuroprotective and cytoprotective effects[16]. Besides the cytoprotective and anti-oxidant effects, PH and PZ showed anti-cancer effect[19,20]. It has also been shown that PH and other chalcone derivatives protect liver cell from toxicant-induced liver injury[13]. PH and PZ protect cell from the toxicity because of their anti-inflammatory and anti-oxidant effects[17]. In the literature there a lot of studies about the protective effects of PH and PZ, however, there is no information about the effect of PH and PZ on neurotoxicity due to cancer chemotherapy. Because of their neuroprotective and anti-cancer effects, we aimed to search, PH and PZ treatment in combination with chemotherapeutics to treat chemotherapy-induced neuronal damage.

MATERIALS AND METHODS

The Institutional Animal Care and Use Ethics Committee of Kafkas University approved the study on 25.10.2018, which was conducted in accordance with protocol number 2018/080.

Animals and neuron cell culture

Animals were obtained from Ataturk University Experimental Animal Laboratory. Totally 10 newborn male Sprague Dawley rats were decapitated and brain cortexes were removed[21]. Cortexes were divided into pieces and trypsin was added 1:1 (v/v) upon the tissue fragments. To isolate cortex neurons, the liquid mixture incubated 30 min. at incubator under the 5% CO_2, 98% humidity and 37°C condition. Then, incubated mixture was centrifuged three times and supernatant discarded at all step. Cortex neuron cells were seeded to 96-well plates as $2x10^4$ cells per well in 200 µl neuronal medium (1% Penicillin-Streptomycin, 2% B27 supplement and 10% Fetal Bovine Serum) (Fig.1). Half of medium changed every 3 days during two weeks. Two weeks later drugs were treated to related wells into the 96-wells plate[10].

Groups and drugs

1. Control	**9**. PH10+PAC8
2. Methanol	**10**. PH20+PAC8
3. PH10 (10 µM doses of Phloretin)	**11**. PZ10+PAC8
4. PH20 (20 µM doses of Phloretin)	**12**. PZ20+PAC8
5. PZ10 (10 µM doses of Phloridzin)	**13**. PH10+PAC7
6. PZ20 (20 µM doses of Phloridzin)	**14**. PH20+PAC7
7. PAC8 (10^{-8} M doses of Paclitaxel)	**15**. PZ10+PAC7
8. PAC7 (10^{-7} M doses of Paclitaxel)	**16**. PZ20+PAC7

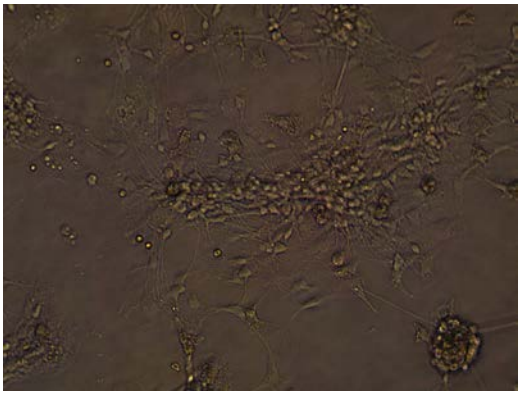

Figure 1. Light microscopy imaging of primary neuron cells.

Phloretin and Phloridzin (dissolved with methanol) were administered to primary neuron cells at the doses of 10 and 20 μM[16]. One hour after the drug administration toxicity was induced with paclitaxel at high (10^{-7} M) and low (10^{-8} M) doses as described previously[9].

Cell viability

Live/dead cells were analyzed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation kit (CAYMAN-10009365). To analyze cell viability, $1x10^4$ cells (in 150 μl medium) were added to all proper group wells of the 96 well plates[22]. 24 hours after paclitaxel administration neuron cells were analyzed in accordance with directives of the kit. Then, the absorbance of each sample was measured at 570 nm using a 96 well plate reader. We determined best toxicity and treatment groups according to the cell viability analyses and further analyses carried out these groups (Fig. 2).

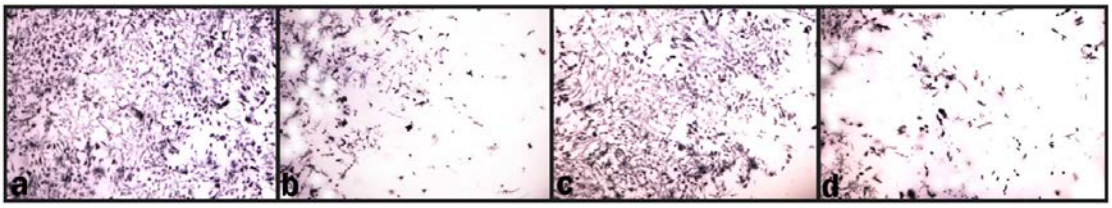

Figure 2. Light microscopy imaging after MTT analyses.

Total oxidant and anti-oxidant capacity

The total oxidant capacity (RELASSAY-Mega02) and anti-oxidant capacity (RELASSAY-Mega01) was analyzed according to directives of the commercial kits. Primary neuron cells ($1x10^4$ cells in 150 μl medium) were added to wells of the 96 well plates. At first drugs were administered to the wells and 1 hour after, toxicity was induced with paclitaxel. 24 hours after paclitaxel administration total oxidant and anti-oxidant levels were analyzed with a 96 well plate reader.

Real Time PCR gene expression analysis

Neuron cells ($1x10^5$ cells/2 ml medium/well) were added to the 6 well plates. Drugs were administered to the wells and toxicity was induced with paclitaxel. 6 hours after paclitaxel administration, cells were scraped and RNA isolation (Qiagen, RNaeasy mini kit-74104) and cDNA synthesis was performed (Qiagen RT-HT First Strand cDNA synthesis kit-330404) by the kits.

Caspase-3, Caspase-9 and Tumor necrosis factor alpha (TNF-α) expression analysis were performed with StepOne Plus Real Time PCR System technology (Applied Biosystem) using cDNA synthesized from neuron cell RNAs. PCR amplification was analyzed with TaqMan Gene Expression Assays JN174232 for rat Caspase-3, JN174233 for rat Caspase-9, JN157530 for rat TNF-α and JN174235 for rat β-actin (Primer Design). β-actin was used as endogenous control gene. For each cell group, triplicate determinations were performed in a 96-well optical plate for all targets (Caspase-3, Caspase-9, TNF-α and β-actin) using 2,5 μl of cDNA (200 ng), 1 μl of TaqMan Gene Expression Assay, 10 μl of TaqMan PCR Master Mix (Applied Biosystems), and 6.5 μl of RNase free water in each 20 μl reaction[23]. All data were expressed as fold-change in expression compared to the expression in other groups, using the $2^{-\Delta\Delta Ct}$ method[24].

Statistical analysis

Independent samples are groups and dependent samples are MTT, TAS, TOS, TNF-α, Cas-3 and Cas-9 parameters. Firstly, statistical analyses between the groups were performed according to one-way analysis of variance (ANOVA) in all parameters. After evaluation statistically significance of the parameters between the groups, if ANOVA test (p) <0.05, differences among the groups were obtained using post hoc Duncan multiple comparison test in all parameters and they were considered statistically significant at ($p<0.05$). All data were expressed as mean ± standard deviations (SD).

RESULTS

As shown in Fig. 3A, 10^{-8} M paclitaxel inhibited cell viability of neuron cells at the end of the 24 hours ($p<0.05$). However 10^{-7} M paclitaxel administration showed higher toxic effects on neuron cells compared to the 10^{-8} M paclitaxel group ($p<0.05$). Pretreatment of PH and PZ alone groups for 24 hours resulted in activation of cell proliferation primary neuron cells in a dose dependent manner (Fig. 3B). High doses of PH and PZ administration significantly increased cell viability compared to 10^{-8} M paclitaxel treated group (Fig. 3C) ($p<0.05$). While both doses of PH significantly showed protective effects in a dose dependent manner after 10^{-7} M paclitaxel administration, PZ treatment showed protective effects only in high doses as shown in Fig. 3D ($p<0.05$). Therefore we determined 10^{-7} M paclitaxel for toxicity, 20 μM PH and 20 μM PZ for treatment groups during oxidative stress, inflammation and caspase protein examinations.

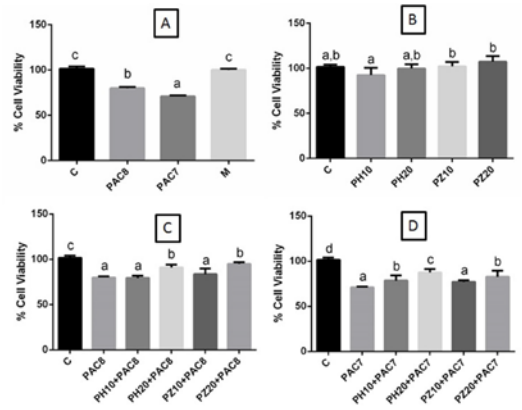

Figure 3. The effects of Phloretin, Phloridzin and Paclitaxel on neuron cell viability.

Paclitaxel significantly increased the total oxidant level (Fig. 4A) while decreased the total antioxidant level (Fig. 4B) compared to Control group ($p<0.05$). PH and PZ alone groups did not significantly change the total oxidant and total antioxidant levels compared to Control ($p<0.05$). After the paclitaxel toxicity PH and PZ treatments significantly decreased total oxidant level while increased antioxidant level compared to PAC7 group ($p<0.05$). In Fig. 4A, it was also shown that PZ decreased total oxidant level more than PH compared to PAC7 group ($p<0.05$).

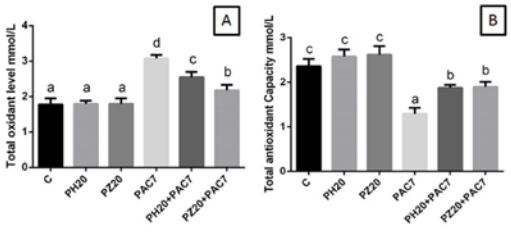

Figure 4. The effects of Phloretin, Phloridzin and Paclitaxel on oxidant and anti-oxidant levels in neuron cells.

Looking at the TNF-α gene expressions in Fig. 5A, it was shown that Paclitaxel significantly up regulated the TNF-α levels compared the Control group ($p<0.05$). PH and PZ administrations significantly decreased TNF-α levels compared to PAC7 group ($p<0.05$). There was no any significantly difference between PH and PZ groups ($p<0.05$). Looking at the Caspase 9 expressions in Fig. 5B, it was shown that PH and PZ alone treatments down regulated Caspase 9 levels while Paclitaxel up regulated when compared to Control group ($p<0.05$). When compared the PAC7 group, it was shown that PH and PZ significantly decreased Caspase 9 levels ($p<0.05$) but PZ significantly decreased more than PH administration ($p<0.05$). In Fig. 5C, Caspase 3 gene expressions were increased with Paclitaxel while were not significantly changed with PH and PZ alone treatments compared to Control group ($p<0.05$). It was shown that PZ administration significantly improved Caspase 3 levels while PH did not change compared to PAC7 group ($p<0.05$).

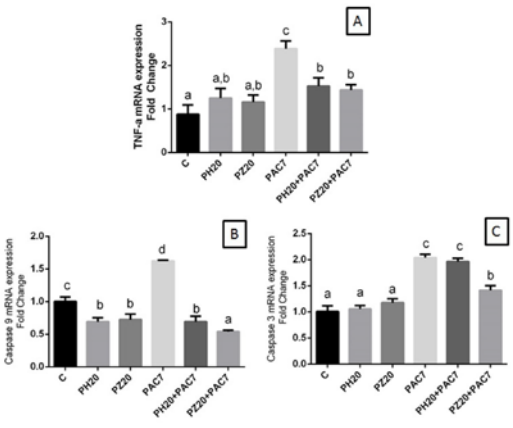

Figure 5. The effects of Phloretin, Phloridzin and Paclitaxel on gene expression levels in neuron cells.

DISCUSSION

Paclitaxel, which has recently been used in cancer chemotherapy, can also be used in combination with many chemotherapeutic agents[2]. However, the increased risk of drug-induced toxicity after chemotherapy has led to an increase in alternative approaches to chemotherapeutic drugs[1]. The neurotoxicity is one of the side effects of Paclitaxel and other chemotherapeutic agents[1,7]. In the present study, we demonstrated the protective effects of Phloridzin and Phloretin which have strong anti-oxidant and anti-inflammatory activities, in paclitaxel-induced primary neuron damage as biochemical and molecular.

Previous studies show that paclitaxel using may cause neurotoxicity depending on dose and time[6,7]. After the paclitaxel treatment axons disintegrate by swelling[25] and neuron death occurs[26,27]. Previous studies have also shown that paclitaxel reduces axon length and directly causes axonal degeneration by stimulating environmental factors[26]. However, the mechanism of damage has not yet been fully elucidated.

Cell viability, is one of the most important indicators of cell toxicity studies. MTT is widely used for analyzing the cell viability. The succinate-dehydrogenase enzyme in the mitochondria in living cells breaks down the tetrazolium ring to form of insoluble formazone salts. As the cells multiply, the absorbance value increases due to the formation of formazone salt[28,29]. These absorbance levels can use in MTT to show cell viability.

Previous studies showed that paclitaxel reduces the viability of primary neurons due to its cytotoxic effect[30,31]. In our study, paclitaxel administration decreased the viability of primary neuron cells depending on the dose. PH and PZ, when administered alone, did not show any toxic effects on neuron cells at both doses. Even, they increased the cell proliferation. PH and PZ treatments maintained cell viability by preventing paclitaxel-induced neuron damage. Previous studies showed that PH and PZ prevent toxicity in many different models[13,16]. In these studies, PH and PZ demonstrated protective effects due to theirs anti-oxidant and anti-inflammatory effects.

In the present study, we performed biochemical and molecular analyzes in order to understand the toxic effect of paclitaxel on primary neuron and by which mechanisms PH ad PZ showed these protective effects. One of the most important effects of paclitaxel on neurotoxicity is the increasing of oxidative stress and free radicals[30,32]. Increased free radicals due to oxidative stress cause the membrane damage that leads to cell death. There are anti-oxidant defense systems in our body to prevent these harmful effects of free radicals. If the antioxidant defense system decreases, oxidative stress damage exacerbates. Therefore, substances with antioxidant activity are thought to prevent damage due to oxidative stress[33,34]. Many methods are used as indicators of oxidative stress and antioxidant system. Total Anti-oxidant Status (TAS) and Total Oxidant Status (TOS) measurements are widely used in the literature[35,36]. Previous studies showed that the level of TOS increases and the level of TAS decreases in paclitaxel-induced neurotoxicity[30]. In our study, it was shown that PH and PZ alone treatments did not affect the TAS and TOS while paclitaxel administration decreased the TAS and increased the TOS compared to the control group. As shown in the PH+PAC and PZ+PAC groups, both of PH and PZ prevented the effect of paclitaxel by decreasing oxidative stress, ameliorated the anti-oxidant capacity and prevented neuronal damage.

Besides the oxidative stress, inflammation is also an important indicator of neuron damage and we analyzed inflammatory cytokines on the second part of this study. As it is known, accompanied by oxidative stress, inflammation increases the severity of damage in drug-induced toxicities. In the inflammatory process many cytokines have roles, but TNF-α is the maestro. TNF-α, a cytokine placed in the membrane, is known for its pro-inflammatory effects and extrinsic apoptotic effect[37]. The most important effect of TNF-α is the induction of a non-specific inflammatory answer[38]. Initiating the inflammatory process increased TNF-α leads to an exacerbation of the damage with macrophages and monocytes[39]. It also induces apoptosis by binding to TNF-α receptor 1 or 2 on the cell surface[40]. Neurotoxicity studies show that paclitaxel increases the production of the proinflammatory cytokine TNF-α[41,42]. This suggests that paclitaxel can both cause inflammation and lead to apoptosis of neuron cells. In our study, paclitaxel administration significantly increased TNF-α expression compared to the control group while PH and PZ significantly ameliorated this effect. Preventing effect of PH and PZ on increased TNF-α expression shows that besides anti-oxidant effect they have significant anti-inflammatory effects. Indeed, anti-inflammatory

efficacy of PH and PZ are also supported in previous studies[13,14]. That's why; it was thought that PH and PZ can reveal many beneficial effects on the chemotherapeutic agent-induced neurotoxicity.

In this study, we recently demonstrated the role of apoptosis in paclitaxel-induced neurotoxicity. As it is known that paclitaxel stabilizes microtubules and stops cell division and eventually the cell undergoes apoptosis[43]. This effect of paclitaxel also affects healthy cells because of it is not selective for cancer cells. Paclitaxel induces apoptosis by activating both intrinsic and extrinsic pathways of apoptosis[44]. Paclitaxel activates the extrinsic pathway, in particular by inducing TNF-α[45]. There are many different markers of apoptosis in cells. Caspase proteins are responsible for both intrinsic and extrinsic mechanisms[46]. It was shown that paclitaxel induced neuronal damage by inducing caspase proteins in neurons[47]. In parallel with the literature, our results showed that paclitaxel increased both caspase 3 and caspase 9 expression compared to the control group and thus caused cell death. In the literature, it was also demonstrated that some anti-oxidant molecules showed protective effects on chemotherapy-induced neurotoxicity[48]. Anti-oxidant molecules are known with their chemo protective effects[48,49]. We also showed the chemo protective effects of PH and PZ on primary neuron cells. In combine administration of paclitaxel with PZ treatment significantly decreased both caspase 3 and 9 expressions while PH decreased only caspase 9 expression. These results showed that PH and PZ can have anti-apoptotic effects on neuron cells. In one study in the literature, it was shown that PH and PZ protected neuroblastoma cells from the pesticide induced neurotoxicity[16]. In this study, researchers showed antioxidant effects of PH and PZ and also showed that PH and PZ decreased the caspase 3 expression. But they used SHSY-5Y cancer cells to show neuroprotective effects of these molecules[16]. Cancer cells have a lot of different mechanisms from the normal cells like caspases protein signaling. In cancer cells caspases already activated spontaneously[46]. So using cancer cells is not a useful way to show neuroprotection of drugs or molecules. In our study we used healthy and primary neuron cells and showed protective effects of PH and PZ via decreasing oxidative stress and inflammatory cytokine TNF-α on paclitaxel-induced neurotoxicity model.

Phloretin and phloridzin protects neuron cells from the paclitaxel toxicity. These affects may originate from their chemical structure. Previous studies showed the protective effects of natural compounds as flavonoids and dihydrochalcones. Pharmaceutical importance of natural products is north worthy especially at last decades. Phloretin and its glycoside form phloridzin may be useful if they are used with the chemotherapeutic drugs. It is well known that all chemotherapeutic drugs have serious side effects and patient need secondary treatment for these side effects. We showed a protection against the neuropathy with phloretin and phloridzin in primary neuron cells. However these treatments need more pre-clinical studies as in vivo and in vitro studies.

From all this information, the current findings indicated that paclitaxel appears to cause neurotoxicity with many different mechanisms. PH and PZ inhibited paclitaxel induced neurotoxicity by its strong antioxidant, anti-inflammatory and anti-apoptotic effect. This study has increased the physiological importance of phloretin and phloridzin in the field of health and it has been concluded that it will be supported by advanced clinical studies.

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Author Contributions: Concept/Design : MY, HÜ, DB; Data acquisition: MY, HÜ, DB; Data analysis and interpretation: MY; Drafting manuscript: MY, HÜ, DB; Critical revision of manuscript: MY, HÜ, DB; Final approval and accountability: MY, HÜ, DB; Technical or material support: MY, HÜ, DB; Supervision: MY; Securing funding (if available): n/a.
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