Protective Effects of N-Acetyl Cysteine against Paclitaxel-Induced Cardiotoxicity Through Modulation of Transient Receptor Potential Melastatin 2 Channels

Geçici Reseptör Potansiyel Melastatin 2 Kanallarının Modülasyonu ile N-Asetil Sisteinin Paklitaksel Nedenli Kardiyotoksisiteye Karşı Koruyucu Etkileri

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ABSTRACT

Aim: In our study, we investigated the paclitaxel induced cardiotoxicity and alterations in Ca²⁺ influx, oxidative stress and apoptosis through transient receptor potential melastatin 2 (TRPM2) channels and modulator role of N-acetyl cysteine (NAC) in cardiomyocytes.

Material and Methods: All cells were cultured at 37°C. The cells were divided into seven main groups. Cells in the paclitaxel group were incubated with 2.5 μM Paclitaxel for 12 hours and cells in the NAC+Paclitaxel group were incubated with 2.5 μM Paclitaxel for 12 hours and then incubated with 10 μM NAC for 24 hours. Intracellular free calcium concentration, reactive oxygen species (ROS) production measurements and cell viability analyses were done according to the study protocol.

Results: Cytosolic calcium levels, apoptosis levels, intracellular ROS production levels were lower in paclitaxel+NAC group than in the paclitaxel group of cardiomyocytes. Also values were markedly lower in the paclitaxel+NAC+antranilic acid group when compared to the paclitaxel+NAC group.

Conclusion: We found that TRPM2 channels are overactivated during paclitaxel induced cardiotoxicity and NAC could show a cardioprotective effect through TRPM2 channel modulation.

Key Words: Apoptosis, paclitaxel, cardiomyocyte, transient receptor potential melastatin 2, N-acetyl cysteine

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ÖZET

Amaç: Biz çalışmamızda paklitaksel kaynaklı kardiotoksisiteyi ve kardiyomiyositlerde transient reseptör potansiyel melastatin 2 (TRPM2) kanalları üzerinden Ca²⁺ akışı, oksidatif stres ve apoptoz değerlerindeki değişimleri ve N-asetil sistein (NAC)’ın modulator rolünü araştırdık.

Materyal ve Method: Bütün hücreler 37 ° C’de kültürlendi. Hücreler yedi ana gruba ayrıldı. Paklitaksel grubundaki hücreler, 12 saat boyunca 2.5 μM Paklitaksel ile inkübe edildi ve NAC+Paklitaksel grubundaki hücreler, 12 saat boyunca 2.5 μM Paklitaksel ile inkübe edildi ve daha sonra 24 saat boyunca 10 μM NAC ile inkübe edildi. Çalışma protokolüne göre intraselüler serbest kalsiyum konsantrasyonu, reaktif oksijen türleri (ROS) üretim ölçümü ve hücre canlılığı analizleri yapıldı.

Bulgular: Kardiyomiyositlerdeki sitosolik kalsiyum seviyeleri, apoptoz seviyeleri, hücre içi ROS üretim seviyeleri; paklitaksel+NAC grubunda, paklitaksel grubuna göre daha düşüktu. Ayrıca paklitaksel+NAC grubu ile karşılaştırıldığında paklitaksel+NAC+antranilik asit grubunda daha belirgin olarak daha düşük değerler elde edildi.

Sonuç: TRPM2 kanallarının paklitaksel kaynaklı kardiyotoksisite sırasında aşırı aktif olduğunu ve NAC’in TRPM2 kanal modülasyonu ile kardiyoprotektif etki gösterdiklerine ulaştık.

Anahtar Sözcükler: Apoptoz, paklitaksel, kardiyomiyosit, transient reseptör potansiyel melastatin 2, N-asetil sistein

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INTRODUCTION

Cardiotoxicity is a potential complication of chemotherapy, and it can limit the clinical use of chemotherapeutic agents. These agents induce apoptosis and necrosis and lead to the suppression of angiogenesis or deterioration of repair mechanisms not only in cancer cells but also in cardiomycocytes. The generation of oxidative stress is a widely accepted pathophysiological mechanism underlying chemotherapy-induced cardiomyopathy (1, 2). Paclitaxel (PAC), an inhibitor of microtubule polymerization, is widely used as a treatment for multiple malignancies (3). The incidence of left ventricular dysfunction associated with PAC ranges from 5 to 15% (4). The underlying pathophysiological mechanism associated with PAC-induced cardiotoxicity is not well understood. According to some research, PAC can induce cardiac muscle damage by affecting subcellular organelles (5). Other research reported that PAC altered cytosolic calcium (Ca$^{2+}$) signaling by affecting mitochondrial permeability (6).

As the management of adverse cardiovascular events in patients treated with PAC is not well defined, it is important to understand the molecular mechanisms. Meshkini et al. reported that PAC-induced cytotoxicity was associated with the generation of reactive oxygen species (ROS) and glutathione depletion (7). At present, there is no reliable and effective preventive treatment for PAC-induced cytotoxicity.

We speculated that antioxidants could be used for cardioprotection. The present study focused on the potent antioxidant N-acetyl cysteine (NAC), which is a widely used drug in clinical practice. Previous studies demonstrated that NAC provided protection against drug-induced cardiac damage (8, 9). The mechanisms of NAC and its effects on cardiotoxicity are related to its antioxidant activity, effects on mitochondrial function, and regulation of cell survival and apoptosis (10).

Transient receptor potential (TRP) channels are unique ion channels, which influence cell apoptosis and survival. TRP melastatin 2 (TRPM2), a member of the melastatin TRP family, is widely expressed in many cell types, including cardiac cells. It is activated in response to oxidative stress, which can be initiated by pharmacological stimuli (11, 12). TRPM2 channels are permeable to Ca$^{2+}$ and have been investigated in several conditions associated with oxidative stress. The activation of these channels may play a major role in the pathogenesis of chemotherapy-induced cardiotoxicity.

Previous research demonstrated the role of TRPM2 channels and the protective effects of NAC against oxidative stress and Ca$^{2+}$ influx in other cell types (13). The aim of the present study was to investigate the effect of PAC therapy on Ca$^{2+}$signaling, apoptosis, and oxidative stress in cardiomycocytes and to evaluate the cardioprotective effect of NAC through TRPM2 channels at the cellular level in an in vitro model.

MATERIAL AND METHODS

Cell culture and reagents

A human cardiomycocyte cell line, (AC16), was purchased from ATCC. The cardiomycocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Fisher Scientific) and 1% penicillin/streptomycin from Fisher, MA, USA. The cells were evenly distributed (1 × 10$^6$ cells) in each of 8–10 sterile filter cap flasks (5 ml, 25 cm$^2$). The cells were then incubated at 37° C in a 5% carbon dioxide incubator in a humidified atmosphere. After the cells reached 75–85% confluence, they were distributed (1 × 10$^6$ cells) in each of 8–10 sterile filter cap flasks (5 ml, 25 cm$^2$). Cell culture and reagents

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Caspase 3 and caspase 9 activity assays

Caspase 3 and caspase 9 activity were measured using methods described previously (20, 21). Caspase 3 (AC-DEVD-AMC) and caspase 9 (AC-LEHD-AMC) substrates cleavages were determined using a SynergyH1 (Biotek) microplate reader at 360 nm and 460 nm wavelengths (excitation/emission). The values were evaluated as fluorescent units/mg protein and shown as the fold increase over the level before treatment (experimental/control).

Analysis of the mitochondrial membrane potential

The mitochondrial membrane potential was determined by using the fluorescent dye JC1. JC1 was purchased from Santa Cruz (TX, USA), and Fura 2 acetoxymethyl ester dye (Calbiochem) was as an intracellular free Ca$^{2+}$ indicator.

Measurement of the production of intracellular ROS

The measurement of intracellular ROS production was carried out in accordance with the experimental procedure of Espino et al. (18). The cells (10$^4$ cells/ml per group) were incubated with 20 μM of a noncharged, nonfluorescent dye (Rh 123) (excitation: 488 nm; emission: 543 nm) for 25 min (18). The DH123 dye easily passes through the cell membrane and is oxidized to cationic rhodamine 123 (Rh 123) inside the cardiomycocytes. Rh 123 localizes in the mitochondria and exhibits green fluorescence. A SynergyH1 (Biotek) automatic microplate reader device was used for determining the fluorescent intensities of Rh 123. The analyses were performed at 488 nm (excitation) and 543 nm (emission) wavelengths. The data are presented as the fold increase as compared with the level before the treatment.

Apopotosis assay

APOPercenage (Biocolor Ltd.) dye was used for the detection and quantification of apoptosis. The dye actively bound to phosphatidyl serine lipids and was transferred into the cells. The apoptotic cells were stained red. An apoptosis assay was performed according to the manufacturer's instructions and the method of Martinez et al. (16).

Measurement of the intracellular Ca$^{2+}$ concentration

The intracellular Ca$^{2+}$ concentration was determined using a microplate spectrophotometer at 550 nm (SynergyH1; Biotek).

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The optical density was estimated using a Synergy™ H1 (Biotek) automatic microplate reader at a test wavelength of 490 nm and a reference wavelength of 650 nm to nullify the effect of cell debris. The obtained data are shown as the fold increase over the level before the treatment (experimental/control).

**Statistical analysis**

All data are presented as mean ± standard deviation (SD). To compare the different treatments, statistical significance was calculated by a one-way analysis of variance and the Mann–Whitney U test. All the data were analyzed using the SPSS statistical program, version 9.05 software (SPSS Inc., IL, USA). A value of $p < 0.05$ was considered statistically significant.

## RESULTS

**Effects of PAC and NAC administrations on cytosolic calcium levels through TRPM2 channel activation in cardiomyocytes**

The effect of PAC and NAC administrations on cytosolic calcium levels in cardiomyocyte cells are shown in figure 1A-B. The TRPM2 channel antagonist antranilic acid (ACA) was used to evaluate the receptors related to involvement of Ca$^{2+}$ increase through TRPM2 channels. As shown in figure 1A-B, the Ca$^{2+}$ concentration in cardiomyocytes was ($p<0.001$) higher in the PAC group than in the control. The Ca$^{2+}$ concentration was lower in the NAC+ACA group compared to the control ($p<0.001$). Also cytosolic Ca$^{2+}$ concentration was lower in the PAC+NAC group than in the NAC+ACA groups than in the PAC group ($p<0.001$).

In addition, cytosolic Ca$^{2+}$ concentration in the cardiomyocytes was markedly lower in the PAC+NAC+ACA group compared to the PAC+NAC group ($p<0.001$).

**Figure 1A-B.** The effect of paclitaxel (PAC) (2.5 micromolar, 12 hrs) and N-acetyl cysteine (NAC) (10 µM, 24 hrs) on the [Ca$^{2+}$]i concentration (A) and cytosolic calcium release (B) in cardiomyocyte cells. Cells are stimulated by cumene hydroperoxide (CMPx 0.1 mM and on 20th cycle) and cells in the antranilic acid groups (PAC+ACA, PAC+NAC+ACA, NAC+ACA) were inhibited with antranilic acid (ACA 0.04 mM for 30 min) (mean ± SD and n=10). $^a$p<0.001 vs control, $^b$p<0.001 vs PAC, $^c$p<0.001 vs PAC+NAC and $^d$p<0.001 vs NAC.

PAC: Paclitaxel group, PAC+ACA: Paclitaxel+antranilic acid group, PAC+NAC: Paclitaxel+N-acetyl cysteine group, PAC+NAC+ACA: Paclitaxel+N-acetyl cysteine+ antranilic acid group, NAC: N-acetyl cysteine group, NAC+ACA: N-acetyl cysteine+antranilic acid group

**Effects of paclitaxel and NAC administrations on apoptosis levels in cardiomyocytes**

Effects of paclitaxel and NAC administrations on apoptosis levels are shown in figure 2. The apoptosis values were significantly higher in the paclitaxel group than control. The apoptosis values were significantly lower in the NAC and the PAC+NAC group than in the PAC group of cardiomyocytes ($p<0.001$). Also the values were lower in the PAC+NAC+ACA group when compared with the PAC+NAC group of cardiomyocytes ($p< 0.001$).
Figure 2. The effect of paclitaxel (PAC) (2.5 micromolar, 12 hrs) and N-acetyl cysteine (NAC) (10 µM, 24 hrs) on apoptosis levels in the cardiomyocyte cells. Cells are stimulated by cumene hydroperoxide (CMPx 0.1 mM for 10 min) and cells in the antranilic acid groups (PAC+ACA, PAC+NAC+ACA, NAC+ACA) were inhibited with antranilic acid (ACA 0.04 mM for 30 min) (mean±SD and n=10). *p<0.001 vs control, †p<0.001 vs PAC, ‡p<0.001 vs PAC+NAC and §p<0.001 vs NAC.

Effects of paclitaxel and NAC administrations on intracellular ROS production
in cardiomyocytes

We show intracellular ROS production of groups in figure 3. Intracellular ROS levels were significantly higher in the paclitaxel group than control. The values were lower in the PAC+ACA (p<0.001), the PAC+NAC (p<0.001) and the PAC+NAC+ACA (p<0.001) than in the PAC group. Also the ROS production was markedly lower in the PAC+NAC+ACA group when compared to the PAC+NAC (p<0.001).

Effects of paclitaxel and NAC administrations on caspase 3 and 9 activities, mitochondrial depolarization levels and cell viability (MTT) values in cardiomyocytes

Mitochondrial membrane depolarization levels, caspase 3 and caspase 9 activities and cell viability (MTT) values of groups are shown in figure 4 (A-B-C-D) respectively. It has been shown that caspase 3 and 9 activities have an important role in the mitochondrial apoptotic pathways. Also they are associated with mitochondrial cytochrome c releasing during the apoptotic cascade. MTT values were significantly lower in the PAC group than control (p<0.001). The values were significantly higher in the PAC+ACA (p<0.001) and PAC+NAC+ACA (p<0.001) groups than in the PAC group.
Figure 4A-D. The effect of paclitaxel (PAC) (2.5 micromolar, 12 hrs) and N-acetyl cysteine (NAC) (10 µM, 24 hrs) on mitochondrial membrane depolarization (A), Caspase 3 (B), Caspase 9 (C) and MTT-Cell Viability (D) levels in the cardiomyocyte cells. Cells are stimulated by cumene hydroperoxide (CMPx 0.1 Mm, for 10 min) and cells in the antranilic acid groups (PAC+ACA, PAC+NAC+ACA, NAC+ACA) were inhibited with antranilic acid (ACA 0.04 mM for 30 min) (mean±SD and n=10). *p<0.001 vs control, **p<0.001 vs PAC, ***p<0.001 vs PAC+NAC and ****p<0.001 vs NAC.

PAC: Paclitaxel group, PAC+ACA: Paclitaxel+antranilic acid group, PAC+NAC: Paclitaxel+N-acetyl cysteine group, PAC+NAC+ACA: Paclitaxel+N-acetyl cysteine+antranilic acid group, NAC: N-acetyl cysteine group, NAC+ACA: N-acetyl cysteine+antranilic acid group.
Paclitaxel, an anitmitotic drug, is used for the treatment of breast, ovarian, and non small cell lung cancers (25). Previous research reported that taxanes, such as PAC, can cause early left ventricular dysfunction and heart failure within 2 d of onset of therapy (26). Reported adverse effects of this chemotherapeutic agent on the heart included cardiomyocyte death, which led to cardiomyopathy or cardiac arrhythmias. The clinical cardiotoxicity of PAC was reported to range from 5–30% (25), and co-administration of PAC and doxorubicin increased the incidence of congestive heart failure to 20% (27).

Despite the aforementioned data on PAC, the molecular mechanism of PAC-induced cardiotoxicity remains unclear. Some studies suggested that both ROS and oxidative stress seemed to play a crucial role in chemotherapy-induced cardiotoxicity and subsequent cardiac dysfunction (28, 29). Studies also reported that widely used anticancer drugs, including PAC, induced mitochondrial dysfunction in vivo and in vitro (30, 31).

Mitochondria play an important role in cell survival and apoptosis. Santulli et al. reported that Ca
to overload was associated with mitochondrial dysfunction and impaired cardiac function after a myocardial infarction (32). Research showed that Ca
to homeostasis was essential for the cellular physiology and pathophysiology in mitochondria, with mitochondrial Ca
to uptake controlling Ca
to signals and having a major impact on cell death (33, 34). High levels of Ca
to stimulated respiratory chain activity and were associated with increased levels of ROS (35). In a study by Kidd et al., PAC affected cytosolic Ca
signals by opening mitochondrial permeability transition pores (36). They concluded that the side effects of PAC could be associated with mitochondrial dysfunction and Ca
to signal cascades (36). Pan et al. demonstrated that PAC-induced changes in Ca
to significantly promoted apoptosis in breast cancer cells (37, 38).

Previous studies also demonstrated that Ca
to was a major intracellular messenger and that it played an essential role in cardiomyocyte homeostasis and survival. Elevated levels of cytosolic Ca
to stimulated ROS and led to the release of proapoptotic factors, which caused apoptosis (37, 38).

TRP channels belong to a family of plasma membrane transporters of Ca
ions. They are unique ion channels, which influence cell death rates and cell survival (39). Some channels are constitutively open, whereas others are activated by Ca
to overload (31, 32). TRPM2 is widely expressed in many cell types, including brain, heart, and endothelial cells (11, 40). Li et al. reported that TRPM2 channels were activated in response to oxidative stress, which could be initiated by pharmacological stimuli (12). Hoffman et al. showed that the modulation of Ca
to entry via TRPM2 channels was important for maintaining mitochondrial function and reducing ROS levels in cardiomyocytes (41). Other studies demonstrated that TRPM2 channels were involved in myocardial ischaemia-reperfusion injury (42, 43). In these studies, the authors suggested that TRPM2 channels were mainly associated with Ca
to overload, mitochondrial dysfunction, and the apoptosis signaling pathway (42, 43).

In the present study, TRPM2 channels were present in cardiomyocytes, and they were stimulated by cumene hydroperoxide and blocked by antranilic acid, respectively. Moreover, the results showed that Ca
to overload was associated with mitochondrial components and initiated apoptosis in cardiomyocytes. NAC reduced the effectiveness of oxidative stress-sensitive TRPM2 channels in cardiomyocytes due to its antioxidant property. Previous research demonstrated a close relationship between heart failure and oxidative stress. ROS-induced changes in heart failure included myocardial hypertrophy, fibrosis, and apoptosis (44). When ROS production exceeded the capacity of control mechanisms, including superoxide dismutase, glutathione, and catalase, it damaged mitochondrial components and initiated apoptosis in the heart (45). As the major role of ROS in this pathology is well known, free radical scavengers, such as NAC, may be able to reduce this damage. Neri et al. showed protective effects of NAC on postprandial oxidative stress and endothelial dysfunction in patients with untreated type 2 diabetes mellitus (46). Although the importance of oxidative stress and antioxidant therapy in chemotherapy-induced cardiotoxicity is well known, its role in the modulation of TRPM2 channels has not been evaluated in cardiomyocytes before. Understanding the molecular mechanisms of chemotherapy-induced cardiotoxicity is necessary to improve preventive strategies. To the best of our knowledge, no previous studies have examined the effect of a combination of PAC and NAC on oxidative stress, apoptosis, and Ca
to entry through TRPM2 channels in cardiomyocytes. The present study indicated that NAC modulated PAC-induced oxidative stress and apoptosis through regulation of TRPM2 channels. Furthermore, NAC suppressed mitochondrial depolarization levels and provided protection against the rate of programmed cell death, as determined by caspase 3 and caspase 9 measurements.

Oxidative stress is associated with oxidative damage, which leads to Ca
to influx into cells through TRPM2 channels. ROS, such as hydrogen peroxide, can affect the functions of several proteins and TRPM2 channels by oxidation of cysteine residues (47). These damaging effects of ROS on TRPM2 channels could be controlled by endogenous antioxidants, such as reduced glutathione (GSH). NAC is a sulphydryl donor, with a free thiol group, which enhances glutathione antioxidant capacity. NAC is hydrolyzed to cysteine in cells, thereby stimulating GSH synthesis. By this mechanism, NAC can prevent GSH depletion in cells. (48). Previous studies reported that an increase in glutathione peroxidase activity was cardioprotective against left ventricular remodeling and failure after a myocardial infarction (49, 50). Özgil et al. showed an activator role of GSH depletion on Ca
to entry through TRPM2 channels in dorsal root ganglia and demonstrated protective properties of NAC on Ca
to entry through regulation of these channels (13).

In conclusion, the results showed that PAC can induce apoptosis and oxidative stress and result in increased Ca
to levels in cardiomyocytes. In addition, NAC showed protective effects against PAC-induced cardiotoxicity through modulation of TRPM2 channels. Given the absence of an effective treatment for chemotherapy-induced cardiotoxicity, understanding of the underlying pathophysiological mechanism of cardiotoxicity and potential therapeutic targets are important in patients treated with chemotherapeutic agents.

Conflict of interest
No conflict of interest was declared by the authors.

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