

# Selenium enhances the TRPM2 mediated effect of paclitaxel on human breast cancer cells

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## Abstract

**Aim:** Paclitaxel is widely used in adjuvant treatment of early breast cancer and second-line treatment of metastatic breast cancer. It has been reported that transient receptor potential melastatin-2 (TRPM2) channels are expressed intensively in breast cancer and has significant effects on oxidative stress. Selenium is an essential element and has effects on reproduction, toxicity, antiaging and DNA reproduction. In this study, we aimed to reveal the role of selenium and TRPM2 channels on apoptotic effects of paclitaxel in breast cancer cells.

**Material and Methods:** Breast cancer cells (MCF-7) were cultured and cells were divided into seven main groups. Cells were incubated with paclitaxel and selenium separately and together administrated on breast cancer cell cultures. Cell cultures incubated with TRPM2 channel antagonist anthranilic acid and stimulator cumene-hydroperoxyde. The effects of paclitaxel and selenium were investigated on molecular pathways of apoptosis.

**Results:** It was found that the levels of apoptosis in paclitaxel group were significantly increased in cancer cells compared to control group ( $p<0.001$ ). TRPM2 channel stimulator cumene-hydroperoxyde administration resulted in significantly increased apoptosis levels compared to the control group ( $p<0.001$ ) and it was found that in paclitaxel + selenium group the apoptosis level significantly increased compared to paclitaxel-only group ( $p<0.001$ ).

**Conclusion:** As a result of our study, it has been shown that paclitaxel significantly increases apoptosis in breast cancer cells, and this effect directly related the TRPM2 channels. It was found that the application of selenium in cell culture medium in non-toxic doses increased the TRPM2 mediated apoptotic activity of paclitaxel.

**Keywords:** Apoptosis; breast cancer; paclitaxel; selenium; TRPM2

## INTRODUCTION

Breast cancer is the most common cancer among women worldwide, with approximately 1.7 million new cases reported annually. Although significant progress has been achieved in recent studies on breast cancer treatment, it continues to be a major health problem in the world. Studies have improved mortality rates; however, survival in metastatic disease is rather low (approximately 24 months). Furthermore, it is expected that the incidence and mortality of breast cancer will increase in the next 5-10 years (1). Many different chemotherapy regimens have been used in the treatment of breast cancer preoperatively and postoperatively and one of these chemotherapeutic agents is taxanes (docetaxel, paclitaxel). Taxanes are chemotherapeutics used in early stage and metastatic breast cancer, increasing tubulin polymerization and maintaining microtubule stabilization.

Thus, they inhibit intracellular transport and replication and result in apoptotic cell death (2). It has been reported that paclitaxel, which is known to be effective in breast cancers, has high efficacy when used in combination. In addition, there are studies reporting that the effectiveness is enhanced when it is combined with some antioxidant agents (3,4).

At least 28 different types of transient receptor potential (TRP) channels have been reported in mammals, and these TRP channels are divided into 6 subfamilies based primarily on the amino acid structure they contain; TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPA (ançyrin) (5). Although there are some exceptions, the TRP channels have 6 distinct transmembrane domains and they contain cytoplasmic protein structures, long N and shorter C terminals (5). TRP channels act as gateways for ions

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and small molecules, beside they respond to hot, cold, mechanical force, physical and chemical stimuli as well. One of the ions in which TRP channels play an important role in intracellular spinning by stimuli such as receptor stimulation is calcium ion which is known to play an important role in oxidative stress and apoptosis (6). Increased expression of TRP channels in many cancers is well known. It has been reported that transient receptor potential melastatin-2 (TRPM2) channels are expressed intensively in breast cancer, head and neck, bladder, liver and lung adenocarcinomas (7). It has been shown that TRPM2 protein has significant effects on migration and cell death in tumor and immune system cells and leads to cell death by increasing cytokine and bacterial peptide activation through increasing direct cell migration and oxidative stress (8).

Selenium reproduction is one of the trace elements that have critical roles such as toxicity, antioxidant, antiaging and stimulating DNA reproduction. In addition, it is known to play a role in numerous degenerative processes including neurological, cardiovascular and inflammation (9). Although there are controversial results in studies on breast cancer and nutrition, it has been shown in many studies that there is a relationship between selenium and breast cancer (9,10).

In our study, by applying paclitaxel on breast cancer cell culture (MCF-7), we aimed to reveal the role of selenium in calcium ions ( $\text{Ca}^{2+}$ ) -mediated apoptosis in TRPM2 channels that are found in breast cancer cells and that are known to be susceptible to oxidative stress.

## MATERIAL and METHODS

### Cell culture, reagents and dyes

Human Breast Cancer (MCF-7) cell line was purchased from Culture Collection of Animal Cells, Foot and Mouth Disease (SAP) Institute, Ankara, Turkey and MCF-7 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640). All mediums contained 10% fetal bovine serum (FBS) (Fisher Scientific, and 1% penicillin/streptomycin (Thermo-Fischer). Cells were evenly distributed as  $1 \times 10^6$  cells in each of 8-9 flasks (filter cap, sterile, 5 ml, 25 cm<sup>2</sup>). Cells were incubated at 37°C at 5% CO<sub>2</sub> in a humidified incubator. After cells have reached 80–85% confluence, cells were incubated with the chemical compounds described in groups section. Cells were examined daily for evidence of contamination. After treatments, the cells were detached with %0.25 Trypsin-EDTA for analysis and split into the sterile falcon tubes for analyses.

Pluronic® F-127 was obtained from Biovision (San Francisco, USA). Caspase-3 (AC-DEVD-AMC) and Caspase 9 (AC-LEHD-AMC) substrates were obtained from Enzo (Lausen, Switzerland). APOPercentage assay with releasing buffer were purchased from Biocolor (Belfast, Northern Ireland). RPMI 1640, Trypsin-EDTA, Fetal Bovine Serum and penicillin-streptomycin and

Dimethyl sulfoxide, Dihydrorhodamine-123 (DHR 123) were obtained from Sigma Aldrich (St. Louis, MO), Fura 2 (AM) calcium fluorescent dye was purchased from Calbiochem (Darmstadt, Germany). A mitochondrial stain 5,50, 6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and Probenecid were obtained from Santa Cruz (Dallas, Texas, USA).

### Study Groups

The study was planned as 7 main groups below,

**Group 1 (Control):** None of the study drugs were used and were kept in a flask containing the same cell culture condition.

**Group 2 (PTX):** Cells in the flasks were incubated with 50  $\mu\text{M}$  Paclitaxel for 24 hrs (11).

**Group 3 (PTX+AA):** Cells in the flasks were incubated with 50  $\mu\text{M}$  Paclitaxel for 24 hrs and then incubated with TRPM2 channel antagonist Anthranilic acid (AA, 0.1 mM, 30 min).

**Group 4 (PTX+Se):** Cells in the flasks were incubated with 50  $\mu\text{M}$  Paclitaxel and 5  $\mu\text{M}$  Selenium (Sodium Selenite) for 24 hrs.

**Group 5 (PTX+Se+AA):** Cells in the flasks were incubated with 50  $\mu\text{M}$  Paclitaxel and 5  $\mu\text{M}$  Selenium (Sodium Selenite) for 24 hours and then incubated with TRPM2 channel antagonist Anthranilic acid (AA, 0.1 mM, 30 min).

**Group 6 (Se):** Cells in the flasks were incubated with 5  $\mu\text{M}$  Selenium (Sodium Selenite) for 24 hours (12).

**Group 7 (Se+AA):** Cells in the flasks were incubated with 5  $\mu\text{M}$  Selenium (Sodium Selenite) for 24 hours and then incubated with TRPM2 channel antagonist Anthranilic acid (AA, 0.1 mM, 30 min).

For Mitochondrial depolarization, Caspase 3 and Caspase 9, apoptosis, and intracellular reactive oxygen species analyses, the cells were further treated with TRPM2 channel agonist (CPx, 0.1 mM, 10 min) for activation of TRPM2 channel before related analysis. During calcium signaling analysis (Fura-2/AM), cells were stimulated on 20<sup>th</sup> cycles with 0.1 mM CPx in the existence of 1.2 mM Calcium and calcium free buffering extracellular environment.

### Measurement of intracellular free calcium concentration ( $[\text{Ca}^{2+}]_i$ )

Changes in  $[\text{Ca}^{2+}]_i$  were measured using the  $\text{Ca}^{2+}$ -sensitive UV light excitable Fura 2 acetoxyethyl ester dye as an intracellular free  $\text{Ca}^{2+}$  indicator. After cell culture treatments, MCF7 cells was incubated with HEPES-buffered saline [HBS; 5 mM KCl, 145 mM NaCl, 10 mM D-glucose, 1 mM MgCl<sub>2</sub>, 1,2 mM CaCl<sub>2</sub>, 10 mM HEPES and 0.1% (w/v) BSA; pH 7.4] containing 5  $\mu\text{M}$  fura-2 AM and 0.05% (w/v) Pluronic F-127 for 1 h at 37°C in the dark. The loaded cancer cells were washed twice with HBS and covered with 1000  $\mu\text{L}$  of HBS supplemented with 2.5 mM probenecid for at least 20 min at 37°C in the dark to allow for Fura-2 AM de-esterification. MCF-7 cells were

seeded in clear flat-bottom black 96-well culture trays (Grainer Cell Star, Life Sciences USA) at a density of  $4 \times 10^4$  cells/per well. Fluorescence emission intensity at 510 nm was determined in individual wells using a plate reader equipped with an automated injection system (Synergy TM H1, Biotek, USA) at alternating excitation wavelengths of 340 and 380 nm every 3 s for 50 acquisition cycles (cycle: 3 s; gain: 120) in response to agonists (CPx, 0.1 mM) added with the automated injector.  $[Ca^{2+}]_i$  in cells was expressed as the average emission at 510 nm in individual wells in response to excitation at 340 nm ( $Ca^{2+}$  bound) / 380 nm ( $Ca^{2+}$ -free Fura 2 AM) normalized to initial fluorescence emission obtained during the first 20 cycles. Measurement of  $[Ca^{2+}]_i$  including staining process modification was performed according to method of Martinez et al (13).

#### **Programmed Cell Death, Intracellular ROS production and Mitochondrial membrane potential (JC-1) measurement**

The APOPercentage™ which is used as a assay for the detection and quantification of apoptosis (Biocolor Ltd., Belfast, Northern Ireland) and the apoptosis analysis procedure was performed according to the manufacturer instruction and Ozdemir et al (14). APOPercentage dye is actively bound to phosphatidyl serine lipids and transferred into the cells and apoptotic cells are stained red. The cells were analyzed for apoptotic cells detection by spectrophotometry (multiplate reader) at 550 nanometer (SynergyTM H1, Biotek, USA).

The MCF-7 cells ( $10^6$  cells/ml for per group) were incubated with 20  $\mu$ M DHR 123 as fluorescent oxidant dye at 37°C for 25 min (15). The Rh123 fluorescence intensities were determined by using an automatic microplate reader (Synergy™ H1, Biotek, USA). Excitation and emission wavelengths of the analyses were 488 nm and 543 nm, respectively. We presented the data as fold increase over the level before treatment.

The quantification of mitochondrial membrane depolarization was carried out by measuring the fluorescence intensity of the JC-1 a cationic dye which was measured by a single excitation wavelength of 485 nm (green) and the emission wavelength of 535 nm, the red signal at the 540 nm (excitation) and 590 nm (emission) the wavelengths (Synergy™ H1, Biotek, USA) (16). Data are presented as emission ratios (590/535). Mitochondrial membrane potential changes were quantified as the integral of the decrease in JC-1 fluorescence ratio of experimental/control.

#### **Assay for caspase 3 and caspase 9 activities**

The determination methods of Caspase 3 and Caspase 9 activity were based on previously reported (17,18). Caspase 3 (ACDEVD-AMC) and Caspase 9 (AC-LEHD-AMC) substrates cleavages were calculated with Synergy™ H1 microplate reader (Biotek, USA) with 360 nm and 460 nm wavelengths (excitation/emission). The values were evaluated as fluorescent units/mg protein

and shown as fold increase over the level before treatment (experimental/control).

#### **Statistical analyses**

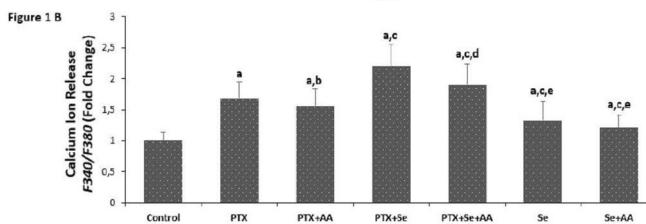
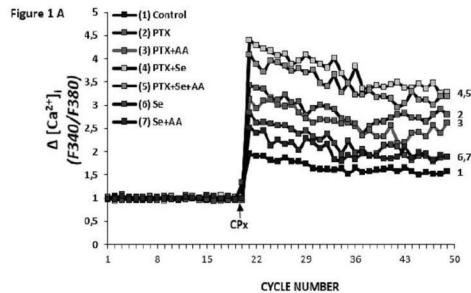
All data were expressed as means  $\pm$  standard deviation (SD). Significant values in the groups were assessed with one-way ANOVA. Statistical analyses were calculated using GraphPad Prism version 7.04 for windows (GraphPad Software, San Diego California, the USA).  $p < 0.05$  was considered significant.

#### **RESULTS**

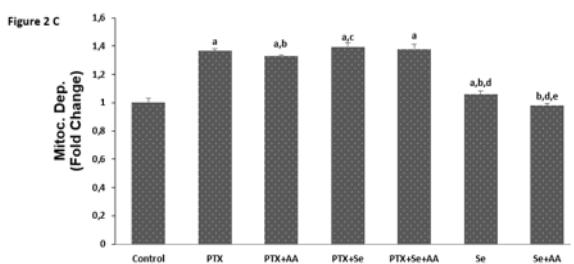
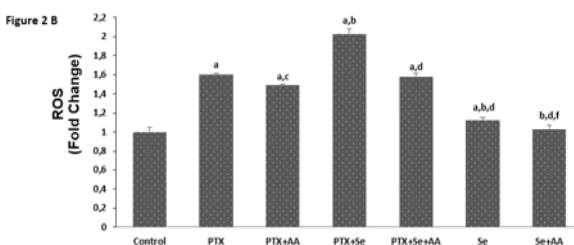
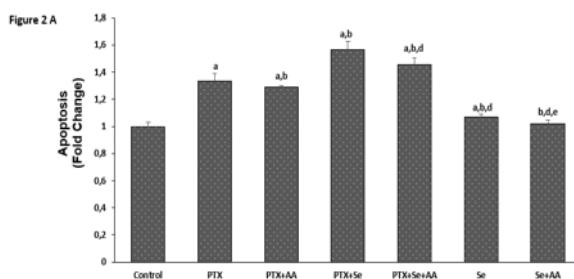
It was found that the application of the chemotherapeutic agent paclitaxel on MCF-7 cells increased intracellular  $Ca^{2+}$  levels significantly compared to the control group ( $p < 0.001$ ). In the cancer cell, intracellular  $Ca^{2+}$  levels were elevated significantly with the use of TRPM2 channel stimulator CPx compared to paclitaxel group ( $p < 0.05$ ). The use of TRPM2 channel inhibitor AA showed a significant decrease in intracellular  $Ca^{2+}$  levels ( $p < 0.05$ ). As a result of the application of selenium along with paclitaxel to MCF-7 cells, intracellular  $Ca^{2+}$  levels increased significantly compared to paclitaxel alone group ( $p < 0.001$ ) and channel inhibitor AA administration significantly decreased intracellular  $Ca^{2+}$  levels compared to paclitaxel+selenium group. ( $p < 0.05$ ) (Figure 1A, B).

It was found that paclitaxel administration on MCF-7 cells significantly increased apoptosis, ROS and mitochondrial depolarization levels compared to the control group ( $p < 0.001$ ). Using TRPM2 channel stimulator CPx has led to significant increase in apoptosis, ROS and mitochondrial depolarization levels compared to the paclitaxel group, and these levels were significantly reduced with the use of TRPM2 channel inhibitor AA ( $p < 0.001$ ). Application of selenium in combination with paclitaxel to MCF-7 cells resulted in a statistically significant increase in apoptosis, ROS and mitochondrial depolarization levels compared to paclitaxel alone group ( $p < 0.001$  for apoptosis and ROS;  $p < 0.05$  for mitochondrial depolarization). Channel inhibitor AA, on the other hand, significantly reduced these levels compared to the paclitaxel +selenium group. ( $p < 0.001$ ) (Figure 2A, B, C).

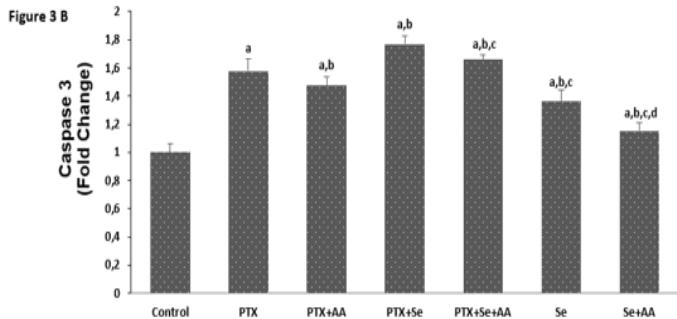
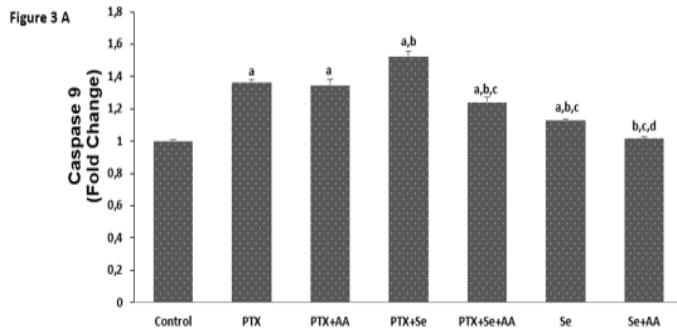
It was found that paclitaxel administration on MCF-7 cells significantly increased caspase 9 and caspase 3 levels compared to the control group ( $p < 0.001$ ). Using TRPM2 channel stimulator CPx has led to significant increase in caspase 9 and caspase 3 levels compared to the paclitaxel group, and these levels were significantly reduced with the use of TRPM2 channel inhibitor AA ( $p < 0.001$ ). Application of selenium in combination with paclitaxel to MCF-7 cells resulted in a statistically significant increase caspase 9 and caspase 3 levels compared to paclitaxel alone group ( $p < 0.001$ ). Channel inhibitor AA, on the other hand, significantly reduced these levels compared to the paclitaxel + selenium group. ( $p < 0.001$ ) (Figure 3A, B).



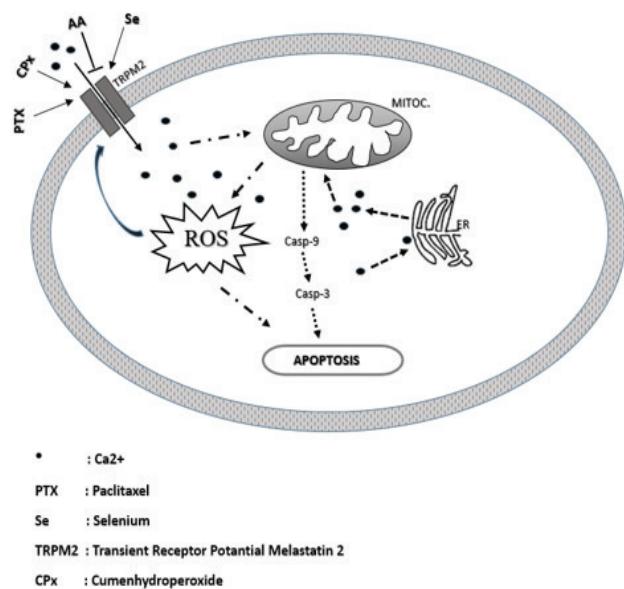
**Figure 1 A-B.** The effect of Paclitaxel (50  $\mu$ M, 24 hrs) and Se (5  $\mu$ M, 24 hrs) on cytosolic calcium levels in MCF-7 cells. Cells are stimulated by Cumene hydroperoxide (CPx 0.1 mM and on 20th cycle) but they were inhibited by Antranilic Acid (AA 0.1 mM for 30 min) (mean  $\pm$  SD and n=3). <sup>a</sup>p<0.001 vs control, <sup>b</sup>p<0.05 and <sup>c</sup>p<0.001 vs PTX, <sup>d</sup>p<0.05 and <sup>e</sup>p<0.001 vs PTX+Se and <sup>f</sup>p<0.05 vs Se group



**Figure 2 A-B- C.** The effect of Paclitaxel (50  $\mu$ M, 24 hrs) and Se (5  $\mu$ M, 24 hrs) on apoptosis (A), ROS (B), mitochondrial depolarisation (C) levels in the MCF-7 Cells. Cells are stimulated by Cumene hydroperoxide (CPx, 0.1 mM for 10 min) but they were inhibited by Antranilic Acid (AA 0.1 mM for 30 min) (mean  $\pm$  SD and n=10). <sup>a</sup>p<0.001 vs control, <sup>b</sup>p<0.001 and <sup>c</sup>p<0.05 vs PTX group, <sup>d</sup>p<0.001 vs PTX+Se group and <sup>e</sup>p<0.001 and <sup>f</sup>p<0.05 vs Se group



**Figure 3 A-B.** The effect of Paclitaxel (50  $\mu$ M, 24 hrs) and Se (5  $\mu$ M, 24 hrs) on caspase 9 (A) and caspase 3 (B) levels in the MCF-7 Cells. Cells are stimulated by Cumene hydroperoxide (CPx, 0.1 mM for 10 min) but they were inhibited by Antranilic Acid (AA 0.1 mM for 30 min) (mean  $\pm$  SD and n=10). <sup>a</sup>p<0.001 vs control, <sup>b</sup>p<0.001 vs PTX group, <sup>c</sup>p<0.001 vs PTX+Se group and <sup>d</sup>p<0.001 vs Se group



Graphical Abstract: The effect of paclitaxel and selenium (sodium selenite) on MCF-7 cells through TRPM2 channels. The paclitaxel and selenium causes the activation of TRPM2 channels indirectly and reactive oxygen species overproduction in mitochondria because of increasing the cytosolic calcium in MCF-7 cells. Apoptosis occurs in the cell after activation of the caspases. The TRPM2 channels blocker (Antranilic acid) reduce the amount of calcium ions entering the cell.

## DISCUSSION

The presence of previously reported TRPM2 channels in numerous organs and tissues, including breast, brain, spleen, liver, heart, pancreas, lung and bone marrow, plays a crucial role in the intracellular and extracellular regulation of  $\text{Ca}^{2+}$ , which plays an important role in apoptosis. It has also been reported that there is a strong relationship between the N terminal of the TRPM2 channel and the calcium sensor calmodulin (19). Increased intracellular reactive oxygen species (ROS) due to increased intracellular  $\text{Ca}^{2+}$  triggers oxidative stress and apoptosis in the cell is induced as a result of the increase in mitochondrial membrane depolarization, leading to irreversible changes in components such as intracellular lipid, protein and nucleic acid (20). TRP channels are known to play important roles in cancer cells. TRPM2-mediated  $\text{Ca}^{2+}$  release in pancreatic cancers increases cytokine release, which is involved in migration and angiogenesis (21). It's also reported that TRPM2 channel activation plays an important role in ATP release and DNA damage in lung cancer (22) and TRPM2 channels play a crucial role in cellular death occurring in neuroblastomas due to oxidative stress (23).

There are studies investigating the relationship between breast cancer and TRP channels, which have been shown to have overexpression and roles in many cancer types. Overexpression of TRPC1, TRPM2, TRPM7, TRPV2, TRPV4 and TRPV6 channels compared to normal breast tissue has been reported in breast cancers (24). In a study, TRPM2 expression was reported to be higher in in-situ breast cancer compared to normal breast tissue. Furthermore, it has been reported that TRPM2 expression in invasive breast cancer was six times higher than in-situ cancer. It has also been reported that TRPM2 can also be used as a molecular biomarker for breast cancer invasion. In the same study, it was found that TRPM2 channels play a role in tumor proliferation in breast cancer; they can be used as an invasion marker and may play a role as a prognostic factor (8).

Paclitaxel is a highly effective cytotoxin and is used in the treatment of many cancers such as melanomas, lung, cervix, ovary, and breast cancer. Paclitaxel, which has an antitumor effect higher than cisplatin and fluorouracil, also has immunoregulatory activity. Paclitaxel is widely used in adjuvant treatment of early breast cancer and second-line treatment of metastatic breast cancer (3,25). Taxanes cause microtubule stabilization by increasing tubulin polymerization, and lead to apoptosis by inhibiting intracellular transport and cell replication. Taxanes also cause cell death by blocking the antiapoptotic effect of the bcl-2 gene family and TP53 activation (2). There are scarce number of studies in the literature regarding the association of paclitaxel with TRP channels, and in these studies, the relationship between peripheral neuropathy which is an important side effect of paclitaxel and TRPV1 and TRPA1 channels was investigated. It was reported that

TRP channel antagonists may be effective in peripheral neuropathy which occurs due to taxanes (26).

In our study, paclitaxel was applied to breast cancer cell culture (MCF-7) and its effects on cell death via TRPM2 channels were investigated. Specific stimulators (CMPx) and inhibitor (AA) were applied to TRPM2 channels, and the stages of apoptosis and the levels of apoptosis during the programmed development of apoptosis in cancer cells were compared with the control group. As a result of the analysis, paclitaxel use resulted in TRPM2 channel activation, and there was a significant increase in TRPM2 channel mediated intracellular  $\text{Ca}^{2+}$  levels, mitochondrial depolarization, intracellular ROS caspase 9 and caspase 3 levels of cancer cells and apoptosis levels due to oxidative stress compared to the control group. As a result of TRPM2 channel inhibition, a statistically significant decrease in these levels was observed ( $p<0.05$ ).

According to our investigations, there are only a limited number of studies in the English literature examining the effects of chemotherapeutic agents on TRP channels in breast cancer. In a study, it was found that TRPC5 channel activated by oxidative stress plays an important role in the efficiency of 5 fluorouracil on colon cancer cells (27). In another study examining the apoptotic activity of 5 fluorouracil on breast cancer cells, it was reported that TRPV1 channel activation increased intracellular  $\text{Ca}^{2+}$  levels and showed similar effect in all stages of apoptosis molecular pathway (28). In another study, doxorubicin, another chemotherapeutic agent, was applied to breast cancer cells and TRPV1 channels were found to play an important role in the apoptotic activity of the drug and melatonin induced this effect (29).

In many studies, selenium, which has been reported to be associated with breast cancer, was found to exert chemo-protective and anti-cancer activity (9). Besides the antioxidant effects of selenium, which is an essential element, effects such as reproduction, toxicity, antiaging and DNA reproduction have also been reported. Furthermore, selenium has been reported to be effective in preventing infection and has an important role in anti-inflammatory processes, cardiovascular and neurological disorders (30). In case-control studies on selenium, which has a controversial relationship with breast cancer, no significant association was found between the amount of selenium in the toenails and breast cancer, however a significant relationship was found between serum concentrations and breast cancer (31). In addition, selenium is an important component of glutathione peroxidase enzyme and this enzyme has been reported to have an important role in intracellular ROS level and oxidation-reduction (redox) balance. Oxidative stress associated with ROS level has been reported to be pivotal in cellular damage, carcinogenesis, tumor suppressor gene mutation and tumor angiogenesis (32). In a study examining the relationship between selenium and chemoterapeutic agents on breast cancers it has been

reported that when selenium is administered in nontoxic doses, it increases the apoptotic activity of fluorouracil in breast and colon cancer cells (33). In our study, when paclitaxel and selenium were administered together to breast cancer cells, it affected the apoptotic pathway by causing an increase in intracellular  $\text{Ca}^{2+}$  level, ROS and mitochondrial depolarization and it was also concluded that paclitaxel increased TRPM2 mediated apoptotic activity.

## CONCLUSION

In conclusion, in our study, apoptotic effects of paclitaxel, which is an agent used in breast cancer treatment, on tumor cell were found to be directly related with increased oxidative stress through TRPM2 channels. In addition, it has been shown that TRPM2 channels play an important role in the whole molecular pathway of apoptosis by causing increased intracellular  $\text{Ca}^{2+}$  level and enhanced mitochondrial depolarization. Furthermore, it was found that the application of selenium in cell culture medium in non-toxic doses increased the TRPM2 mediated apoptotic activity of paclitaxel.

*Competing interests:* The authors declare that they have no competing interest.

*Financial Disclosure:* There are no financial supports.

*Ethical approval:* This research is cell culture study.

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