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ORIGINAL ARTICLE

# Mitochondrial oxidative stress-induced brain and hippocampus apoptosis decrease through modulation of caspase activity, $Ca^{2+}$ influx and inflammatory cytokine molecular pathways in the docetaxel-treated mice by melatonin and selenium treatments

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## Abstract

Docetaxel (DOCE) is widely used to treat several types of glioblastoma. Adverse effects DOCE seriously limit its clinical use in several tissues. Its side effects on brain cortex and hippocampus have not been clarified yet. Limited data indicated a protective effect of melatonin (MLT) and selenium (SELEN) on DOCE-induced apoptosis, $Ca^{2+}$ influx and mitochondrial reactive oxygen species (ROS) in several tissues except brain and hippocampus. The purpose of this study is to discover the protective effect of MLT and SELEN on DOCE-induced brain and hippocampus oxidative toxicity in mice. MLT and SELEN pretreatments significantly ameliorated acute DOCE-induced mitochondrial ROS production in the hippocampus and brain tissues by reducing levels of lipid peroxidation, intracellular ROS production and mitochondrial membrane depolarization, while increasing levels of total antioxidant status, glutathione, glutathione peroxidase, MLT, α-tocopherol, γ-tocopherol, vitamin A, vitamin C and β-carotene in the tissues. Furthermore, MLT and SELEN pretreatments increased cell viability and TRPM2 channel activation in the hippocampus and brain followed by decreased activations of TNF-α, IL-1β, IL-6, and caspase −3 and − 9, suggesting a suppression of calcium ion influx, apoptosis and inflammation responses. However, modulator role of SELEN on the values in the tissues is more significant than in the MLT treatment. MLT and SELEN prevent DOCE-induced hippocampus and brain injury by inhibiting mitochondrial ROS and cellular apoptosis through regulating caspase −3 and − 9 activation signaling pathways. MLT and SELEN may serve as potential therapeutic targets against DOCE-induced toxicity in the hippocampus and brain.

**Keywords** Apoptosis · Docetaxel · Hippocampus · Inflammation · Melatonin · Mitochondrial oxidative cytotoxicity

## Abbreviations

| | |
|---|---|
| 2-APB | 2-aminoethyl diphenylborinate |
| ADPR | ADP-ribose |
| CPx | cumene hydroperoxide |
| DCF | 2′,7′-dichlorofluorescein |
| DCFH-DA | 2′,7′-dichlorodihydrofluorescin diacetate |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DOCE | docetaxel |
| ELISA | enzyme-linked immunosorbent assay |
| GSH | reduced glutathione |
| GSH-Px | glutathione peroxidase |
| HEK293 | human embryonic kidney 293 |
| IL | interleukin |
| IL-1β | interleukin 1beta |
| LPx | lipid peroxidation |
| MLT | melatonin |
| MMP | mitochondrial membrane potential |
| PARP-1 | poly (ADP-ribose) polymerase-1 |
| PUFA | polyunsaturated fatty acid |
| ROS | reactive oxygen species |

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| SELEN | selenium |
| TAS | total antioxidant status |
| TNF-α | tumor necrosis factor alpha |
| TRP | transient receptor potential |
| TRPM2 | transient receptor potential 2 |

## Introduction

Taxane group chemotherapeutic agents induce apoptosis by inhibition of microtubules in the tumors (Ghoochani et al. 2016). A member of Taxane group is docetaxel (DOCE) and it is a common chemotherapeutic drug in the treatment of patients with several cancers such as prostate, breast and brain due to its high efficiency and easy administration (Ju et al. 2018). It has been also used in the treatment of brain metastasis (Ghoochani et al. 2016). However, DOCE treatment has a serious dose-limiting side effects including brain toxicity in human and experimental animals (Yamamoto et al. 2012; Fushida et al. 2016; Tabaczar et al. 2017). Therefore, reducing normal brain and hippocampus injuries in DOCE-treated cancer patients are urgent subjects and the pathogenesis of DOCE should be clarified to develop a new drug to extenuate the DOCE-caused brain toxicity. To date, although the pathophysiological basis of DOCE-induced toxicity in brain (Tabaczar et al. 2017), kidney (Baş and Nazıroğlu 2018; Mohri et al. 2018) and testis (Altintas et al. 2015) has been investigated in recent years, the molecular mechanism of DOCE-induced brain injury has not been investigated yet. There are evidences though that excessive $Ca^{2+}$ entry, oxidative stress, inflammation, and apoptosis may play crucial roles in DOCE-induced normal tissue toxicity (Altintas et al. 2015; Nazıroğlu and Braidy 2017; Tabaczar et al. 2017; Yilmaz et al. 2017; Baş and Nazıroğlu 2018; Mohri et al. 2018).

A recent report suggests that DOCE induces mitochondrial dysfunction and accumulated reactive oxygen species (ROS) products in the human embryonic kidney 293 (HEK293) cells, causing the rapid generation of reactive oxygen species (ROS) and activates the oxidative metabolism system (Tabaczar et al. 2017). The main target of ROS is a polyunsaturated fatty acid (PUFAs) in cellular structures. In addition to the low antioxidant defense system, brain is containing rich PUFA contents and has high oxygen consumption (Nazıroğlu 2009). In brain and kidney, cisplatin-induced excessive ROS production over insufficient antioxidant system results in an elevation in lipid peroxidation (LPx) formation and attenuation of enzymatic antioxidants such as glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) and non-enzymatic antioxidants such as reduced glutathione (GSH), vitamin A, β-carotene, vitamin C and vitamin E as well as the elevations of inflammation factors such as TNF-α, IL-1β, and IL-6 (Nazıroğlu 2007; Nazıroğlu and Braidy 2017; Mi et al. 2018; Waseem et al. 2018). The generation of proinflammatory cytokines activates the signal pathway of apoptosis (Waseem et al. 2018). In tumor-bearing mice with muscle atrophy, DOCE supplementation-induced increases of IL-6 and TNF-α level was decreased by antioxidant selenium (SELEN) treatment (Wang et al. 2015). This evidence highlights the critical role of antioxidants on DOCE-induced TNF- , IL-β and IL-6 productions in normal cells. It is well known that chemotherapeutic agents such as cisplatin and DOCE induce generation of ROS and activation of pro-apoptotic factors such as caspase −3 and − 9 (Lanza-Jacoby and Cheng 2018).

Melatonin (MLT) and SELEN have strong antioxidant actions in several tissues through upregulation of antioxidant enzymes (Ekmekcioglu 2014; Carrasco et al. 2015; Sakallı Çetin et al. 2017). They have also modulator roles of chemotherapeutic agent-induced cytokine production in neuronal cells (Yakubov et al. 2014; Nopparat et al. 2017). In the brain and kidney, modulator roles of MLT and SELEN on cisplatin-induced oxidative toxicity (Surendran et al. 2012; Demir et al. 2015; Karavelioglu et al. 2015) and inflammation markers (Nopparat et al. 2017; Demir et al. 2015; Mi et al. 2018) were recently reported. More recently, MLT and SELEN treatments reduced $Ca^{2+}$ entry, oxidative toxicity and apoptosis in hippocampus of rats (Kahya et al. 2017). Therefore, we hypothesize that MLT and SELEN treatments may be capable to protect DOCE-induced acute brain and hippocampus injuries through upregulation of antioxidants but down regulation of $Ca^{2+}$ entry, apoptosis and inflammation.

In the current study, the pretreatment of MLT and SELEN effectively prevented DOCE-induced brain and hippocampus injuries. It was demonstrated that MLT and SELEN treatments inhibited the oxidative stress, inflammation and apoptosis in DOCE-induced brain and hippocampus oxidative injury, through regulation of mitochondria-dependent ROS, caspase and inflammation signaling pathways.

## Materials and methods

### Animals

A total of 32 adult male C57BL/6j black mice (6–8 weeks old) were purchased by the Burdur Mehmet Akif University (BMAU), Burdur, Turkey. Every effort was made to reduce the number and suffering of animals. All experimental procedures (22–23 °C, 60–70% relative humidity and 12-h light-dark cycles throughout the experiment) were in accordance with the Guidelines for the Laboratory Animals of BMAU and approved by the BMAU Ethical Committee for Laboratory Animals (Permit Number: 60. Application Date: 10.12.2018).

## Study groups

The mice were randomly divided into four groups with 8 animals each and treated for seven days. The first group was used as a normal control and the group received intraperitoneal physiological saline (0.9% and *w*/*v*) via for 7 days. The second group was administered with a single injection of DOCE (30 mg/kg body weight and intraperitoneal) (Kim et al. 2017). The third (DOCE+MLT) group received a combination as a single dose of intraperitoneal DOCE and 7 doses of MLT (10 mg/kg body weight and each day) for 7 days (Kahya et al. 2017). Mice in the DOCE+SELEN group received a combination as a single dose of intraperitoneal DOCE and 3 doses of Se (1.5 mg/kg body weight and over a day) for 7 days (Kahya et al. 2015). Mice were anesthetized with carbon dioxide at 6 h after last MLT injection.

MLT and SELEN were prepared in DMSO and sterile physiological saline solution (0.9% NaCl), respectively. They were diluted with saline solution as described in a previous study (Kahya et al. 2017). After 10 h of last MLT and SELEN treatment, all mice were sacrificed and the hippocampus and brain samples were obtained.

## Preparation of brain and hippocampal neurons

Details of isolations of neurons from the total cortex brain and hippocampus were given in previous studies (Akpınar et al. 2016; Kahya et al. 2017). Briefly, the brain and hippocampus tissues were minced and incubated with enzymes, including trysin and 0.5 mg/ml collagenase in 5 ml DMEM at 37 °C in a shaking bath for 40 min after removing the attached nerves and surrounding connective tissues. The isolated neurons were transferred into a 35-mm culture dish and kept still for at least 30 min.

A part of the total brain and hippocampal neurons were used in the plate reader and ELISA analyses within 2 h. The remaining brain and hippocampal neurons were counted in the CASY Model TTC Cell Counter and Analyzer (Roche Diagnostics Corporation, Istanbul, Turkey), and they were split to sterile cell culture flasks (Greiner Bio-one, Istanbul, Turkey) at a density of $1 \times 10^6$ neurons/ml for the laser confocal microscope analyses. Some parts of the remaining whole brain samples in small plastic boxes were stored at −85 °C for the oxidant and antioxidant analyses.

## Cell viability analyses

Cell viability analyses were performed on the hippocampus and brain samples in plate wells by using a 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). The absorbance of MTT was measured at 490 nm using a microplate reader (Infinite pro200; Tecan Austria GmbH, Groedig, Austria) (Oz et al. 2017). The MTT values were expressed as % increase (experimental/control).

## Apoptosis and caspase assays

For apoptosis measurement was measured according to the protocol provided in the microplate reader (Infinite pro200) kit (Biocolor Ltd., Northern Ireland) as described in a previous study (Kahya et al. 2017; Oz et al. 2017). First we calculated the data as fluorescence units/mg protein. Then, we observed 8.12 ± 1.23% apoptosis in the control cells and the apoptosis levels in the samples were presented as % increase.

Caspase −3 and − 9 activations are two reliable markers of apoptosis. The caspase −3 an caspase-9 activity was monitored the microplate reader (Infinite pro200) by measuring the degradation of the fluorometric substrate AcDEVD-AMC and Ac-LEHD-AMC as previously described (Uğuz et al. 2009; Sakallı Çetin et al. 2017). The data were calculated as fluorescence units/mg protein and presented as % increase.

## Detection of intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (MMP)

The cell-permeable fluorescence probe DCFH-DA was used to measure the levels of intracellular ROS (Wang and Roper 2014). The probe was freshly prepared in DMSO and added to the cells and they were incubated with the probe for 30 min at 37 °C. The samples were washed twice with Hank's balanced saline solution (HBS) and re-suspended in the HBS within the black plates for the ROS measurement. After excitation at 488 nm, the fluorescence emission was registered in an automatic microplate reader (Infinite pro200) at 525 nm. The data were calculated as fluorescence units/mg protein and presented as % increase.

The membrane-permanent JC-1 dye is widely used in apoptosis studies to monitor mitochondrial health (Joshi and Bakowska 2011). The neurons ($1 \times 10^6$) were incubated for 30 min at 37 °C with the fresh DMEM medium containing 3 μg/ml of JC-1. After centrifugation at 1000 g for 5 min, the neurons were washed once with DMEM. The green (excitation; 485 nm and emission; 535 nm) and red (excitation; 540 nm and emission; 590 nm) JC-1 signals were measured in the hippocampus and brain cells as described in previous studies (Uguz et al. 2012; Oz et al. 2017). Fluorescence changes in the black plates were analyzed using the microplate reader (Infinite Pro200). The data were calculated as fluorescence units/mg protein and presented as % increase.

## Brain homogenate preparation

Cortex brain tissues were washed twice with cold saline solution, placed into glass bottles, labeled and stored in a deep freeze (−85 °C) until processing (maximum 4 weeks). After

weighing, half of the cortex was placed on ice, cut into small pieces, using scissors, and homogenized (2 min at 5000 rpm) in a five volumes (1:5, *w*/*v*) of ice-cold Tris-HCl buffer (50 mM, pH 7.4), by using an ultrasonic homogenizer (Bandelin-2070, BANDELIN electronic, Berlin, Germany). All preparation procedures were performed on ice.

## Lipid peroxidation (LPx) measurements

LPx levels in the brain homogenate and hippocampal neuron ($1 \times 10^6$) were performed according to the method of Botsoglou et al. (1994) with minor modifications. The absorbance changes of the supernatant brain samples (0.25 ml) and hippocampal neuron ($1 \times 10^6$) were spectrophotometrically (Cary 60 UV-Vis Spectrophotometer, Agilent, İzmir, Turkey) measured at 532 nm. 1,1,3,3-Tetraethoxypropane-1,3-d2 was used as a standard. The values of LPx in the samples were expressed as μmol/g protein.

## Analyses of reduced glutathione (GSH), glutathione peroxidase (GSH-Px), MLT and total antioxidant status (TAS) and total tissue protein

The GSH levels of the brain homogenate and hippocampal neuron ($1 \times 10^6$) were spectrophotometrically (Cary 60 UV) measured at 412 nm using the method of Saxena et al. (1992). This method utilizes the enzymatic conjugation of glutathione to 1-chloro-2,4-dinitrobenzene through reaction catalyzed by glutathione S-transferase. GSH level in the samples was expressed as μmol/g protein.

GSH-Px activities of the brain samples and hippocampal neuron ($1 \times 10^6$) were measured spectrophotometrically (Cary 60 UV) at 37 °C according to the method of Lawrence and Burk (1976). GSH-Px catalyzes the oxidation of glutathione by cumene hydroperoxide (CPx). The decrease of GSH-Px in absorbance at 412 nm against blank was measured spectrophotometrically. GSH-Px activity was expressed as IU/ g protein.

The brain homogenate TAS levels were measured calorimetrically using the TAS commercial kit (Mega Tıp Inc., Gaziantep, Turkey). The results in the mucosa were expressed in μmol $H_2O_2$ equivalent/g protein (μmol $H_2O_2$ equiv./ g prot.).

MLT levels in the brain samples were measured using a manufacturer's enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (DRG Inc., Marburg, Germany). The absorbance of MLT was read in the microplate reader (Infinite pro200) at 405 nm. The results were expressed as % increase.

Protein content was measured in the spectrophotometer (Cary 60 UV) by using the method of Lowry using bovine albumin as standard.

## Measurements of retinol, β-carotene, α- and γ-tocopherol concentrations in the brain samples

Extraction procedures of retinol, β-carotene, α- and γ-tocopherol from the brain samples were described in a previous study (Desai 1984). Briefly, the brain samples (0.2 mg) were homogenized in 1.0 ml absolute ethanol containing 1% pyrogallol by an ultrasonic homogenizer (Bandelin-2070). After mixing and heating (at 70 °C for 30 min for saponification), the tubes were cooled under faucet water. Distilled water (1.0 ml) and hexane (1.0 ml) was added to the homogenate samples. The top hexane layer was transferred to a clean glass vial in a water bath (at 70 °C) after drying down under nitrogen gas. The dried sample was reconstituted in 0.1 ml of methanol. The methanol samples were used the vitamin A (retinol), β-carotene, α- and γ-tocopherol analyses.

For the tocopherol analysis, 5 μl aliquots were analyzed by reverse-phase HPLC with fluorescence detection on a 1260 Infinity II fluorescence detector (Agilent Inc., Izmir, Turkey) with excitation at 292 nm and emission at 340 nm (Desai 1984). Tocopherol in the samples and internal standards (Tocopherol Set, Callbiochem-Merck Millipore, Darmstadt, Germany), were eluted with 100% methanol on a C18, 250 × 4.6 mm, 3-mm particle size column (HIC-ACE 121-2546). Mobile phase of the column was methanol and distilled water (98:2). The retention times of α- and γ-tocopherol were 8.05 and 9.4 min, respectively (Desai 1984).

The retinol and β-carotene concentrations in the methanol extract were spectrophotometrically measured at 325 and 453 nm, respectively (Suzuki and Katoh 1990). Calibration was performed using standard solutions of all-trans retinol and β-carotene in methanol. The fat soluble antioxidant vitamin analyses and preparation of standards were performed in a dark room.

Quantification of ascorbic acid in the brain samples was performed according to the method of Jagota and Dani (1982). The absorbance of the samples was measured spectrophotometrically at 760 nm.

## Cytokine measurements

The levels of cytokines TNF-α, IL-1β and IL-6 in the brain samples were measured by using commercial the enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols (R&D Systems, Istanbul, Turkey) as described in a recent study (Deveci et al. 2019). The data were expressed as ng/mg protein.

### Measurement of intracellular $Ca^{2+}$ fluorescence intensity through TRPM2 activation

Transient receptor potential (TRP) super family channels are cation permeable channels in several neurons (Nazıroğlu and Braidy 2017). One member of the TRP superfamily is TRP melastatin 2 (TRPM2). The TRPM2 channel is activated in hippocampus by oxidative stress (Nazıroğlu 2007; Nazıroğlu 2009), although the channel is inhibited by several chemicals, including 2-aminoethyl diphenylborinate (2-APB). After observation an increase of ROS and LPx in the hippocampus neurons, we want to investigate DOCE-induced oxidative stress dependent activation of the TRPM2 channel in the hippocampus neurons by laser confocal microscope analyses.

Intracellular changes in the $Ca^{2+}$ fluorescence concentration in the hippocampus neurons were monitored by using 1 μM florescent dye (Fluo-3, Calbiochem, Darmstadt, Germany). The Fluo-3 is a single wavelength excitation and emission dye that excited by a 488 nm argon laser from the confocal microscope (LSM 800, Zeiss, Ankara, Turkey) (Deveci et al. 2019). The results of Fluo-3 in 15 $\mu m^2$ of cytosol were expressed as the mean fluorescence intensity as arbitrary unit per cell. Intracellular fluorescence intensities of 20 cells were analyzed in the confocal microscope by ZEN program.

### Statistical analysis

Results were expressed as mean ± standard deviation (SD). For significant values a Mann-Whitney Test was utilized. Statistically significant values were those with a $P$ value <0.05. Statistics were conducted using SPSS Statistical program (17.0, SPSS Inc. Chicago, Illinois, USA).

## Results

### MLT and SELEN treatments decreased DOCE-induced apoptosis levels but increased cell viability (MTT) values

As shown in Fig. 1a, apoptosis levels were markedly ($p < 0.05$) increased in the DOCE group as compared to control group, although its level was decreased in DOCE+MLT and DOCE+SELEN groups by MLT and SELEN treatments ($p < 0.05$). As shown in Fig. 1b, DOCE treatment decreased the levels of cell viability level in DOCE groups, but the MLT and SELEN treatments increased the cell viability levels in the DOCE+MLT and DOCE+SELEN groups ($p < 0.05$). Importantly, we found that MLT and SELEN treatments reduced the level of apoptosis but increased the MTT levels in the brain and hippocampus neurons. Our data suggested that DOCE-induced decrease of cell viability, but the decrease of MTT could be inhibited by MLT and SELEN treatments through inhibition of apoptosis.

### MLT and SELEN treatments decreased DOCE-induced increase of caspase −3 and − 9 activities

Caspases are constitutively synthesized as inactive proenzymes in body cells. Processes of apoptosis contain an activation of caspases such as caspase −3 and − 9 (Uğuz et al. 2009; González et al. 2010). In this study, activations of caspase-3 and caspase-9 were detected in the brain and hippocampus during DOCE-induced apoptosis.

The results of caspase −3 and − 9 activities in the four groups are shown in Fig. 2a, b, respectively. The caspase −3 and − 9 activities were markedly ($p < 0.001$) higher in the DOCE group than in the control group. The increased caspase

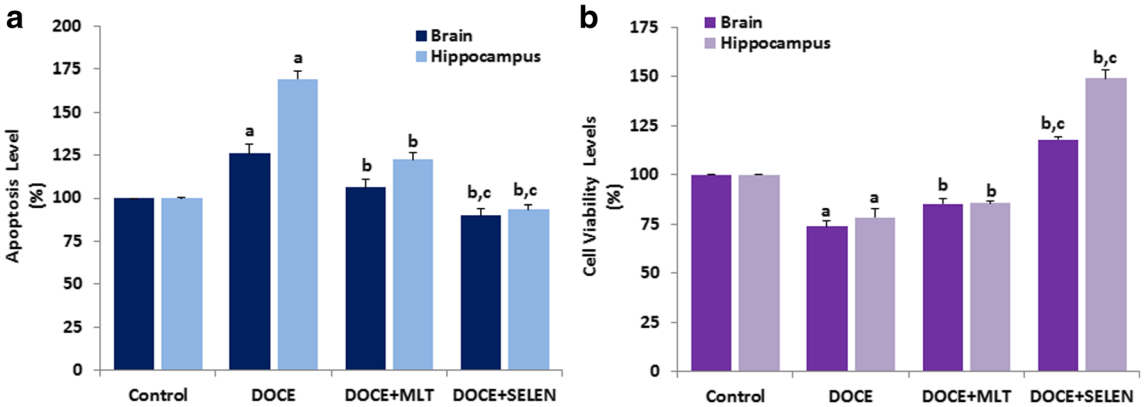


**Fig. 1** The melatonin (MLT) and selenium (SELEN) treatments decreased docetaxel (DOCE)-induced apoptosis (**a**), but increased cell viability (**b**) in brain and hippocampus of mice. The apoptosis levels were measured by using a commercial kit. For the cell viability analyses, MTT was used. Both analyses were performed in the microplate reader. All data are expressed as mean ± SD (n = 8 in each group). ([a]$p < 0.05$ versus control group. [b]$p < 0.05$ versus DOCE group)

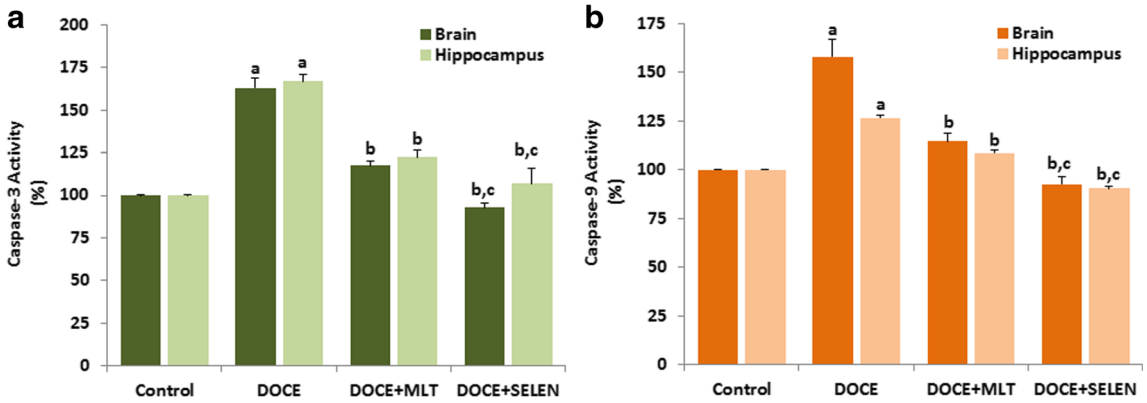


**Fig. 2** Docetaxel (DOCE)-induced increases of caspase-3 (**a**) and − 9 (**b**) activities decrease in the brain and hippocampus of mice by the melatonin (MLT) and selenium (SELEN) treatments. The caspase −3 and − 9 substrates were used in the microplate reader for the caspase activity analyses. All data are expressed as mean ± SD (n = 8 in each group). ([a]p < 0.001 versus control group. [b]p < 0.001 versus DOCE group. [c]p < 0.001 versus DOCE+MLT group)

−3 and − 9 activities were markedly (p < 0.001) decreased in the DOCE+MLT and DOCE+SELEN groups by the MLT and SELEN treatments.

## MLT and SELEN modulated lipid peroxidation (LPx), intracellular ROS production and mitochondrial membrane potential (MMP) induced by DOCE treatment

The electron transport system of mitochondria induces loss of MMP in the mitochondria (Joshi and Bakowska 2011). For this reason, MMP is an important parameter of mitochondrial function and has been used as an indicator of normal cells. As oxidative stress played a key role on DOCE-evoked brain and hippocampus toxicity in mice, we investigated whether MLT and SELEN treatments could improve DOCE-evoked oxidative stress. As indicated in Fig. 3a, b, c, d, DOCE dramatically increased brain MMP, ROS, LPx and hippocampus LPx levels, but its treatment decreased the TAS levels in the brain. The elevations in the brain and hippocampus were indicated excessive ROS production in the DOCE-treated brain and hippocampus, indicating oxidative stress ($p < 0.001$). In contrast, administration with MLT and SELEN in the brain and hippocampus improved the attenuated the levels of MMP, ROS and LPx, which rehabilitated the TAS as elaborated by the improvement of α-tocopherol, γ-tocopherol (Fig. 4a, b), MLT ($p < 0.001$) (Fig. 4c) levels, GSH-Px activity ($p < 0.05$ or $p < 0.001$) (Fig. 4d) and GSH concentrations (Table 1) significantly ($p < 0.05$).

## MLT and SELEN treatments increased antioxidant vitamin responses after DOCE treatment

In addition to the enzymatic antioxidants such as GSH-Px and SOD, the mitochondrial ROS are also scavenged by non-enzymatic antioxidants such as α-tocopherol, γ-tocopherol, β-carotene, retinol, ascorbic acid and MLT (Jagota and Dani 1982; Desai 1984; Suzuki and Katoh 1990; Nazıroğlu 2007). The positive anticancer role of α-tocopherol and ascorbic acid with the chemotherapeutic agent combination was reported on progression of tumor in lung carcinoma-induced mice (Yam et al. 2001). After the increases observed in the ROS and LPx values in the brain, we investigated whether MLT and SELEN pretreatments could improve DOCE-induced oxidative stress through upregulation of α-tocopherol, γ-tocopherol, β-carotene, retinol, ascorbic acid and MLT. As indicated in Fig. 4a, b, c, d and Table 1, DOCE treatment diminished the brain α-tocopherol, γ-tocopherol, β-carotene, retinol, and ascorbic acid and MLT concentrations ($p < 0.05$ and $p < 0.001$). The diminishes in the brain were further indicating excessive ROS production in the DOCE-treated brain, indicating oxidative stress. In contrast, administration with MLT and SELEN improved the decreased concentrations of α-tocopherol, γ-tocopherol, β-carotene, retinol, ascorbic acid and MLT ($p < 0.05$ or $p < 0.001$). However, improvement of SELEN on the antioxidant concentrations in the brain is more important than in MLT ($p < 0.05$).

## MLT and SELEN suppress inflammation response in the brain after DOCE treatment

To verify whether DOCE-induced brain injury was associated with the onset of inflammation, we measured the contents of inflammatory cytokines and mediators by the ELISA assay kits (R&D Systems), such as TNF-α, IL-1β and IL-6. The results of Fig. 5 showed that the activities of IL-1β (a), IL-6 (b) and TNF-α (c) were significantly increased in the brain in DOCE treated mice ($p < 0.001$). In contrast, pretreatment with MLT and SELEN significantly suppressed the activities of TNF-α, IL-1β and IL-6 ($p < 0.001$).

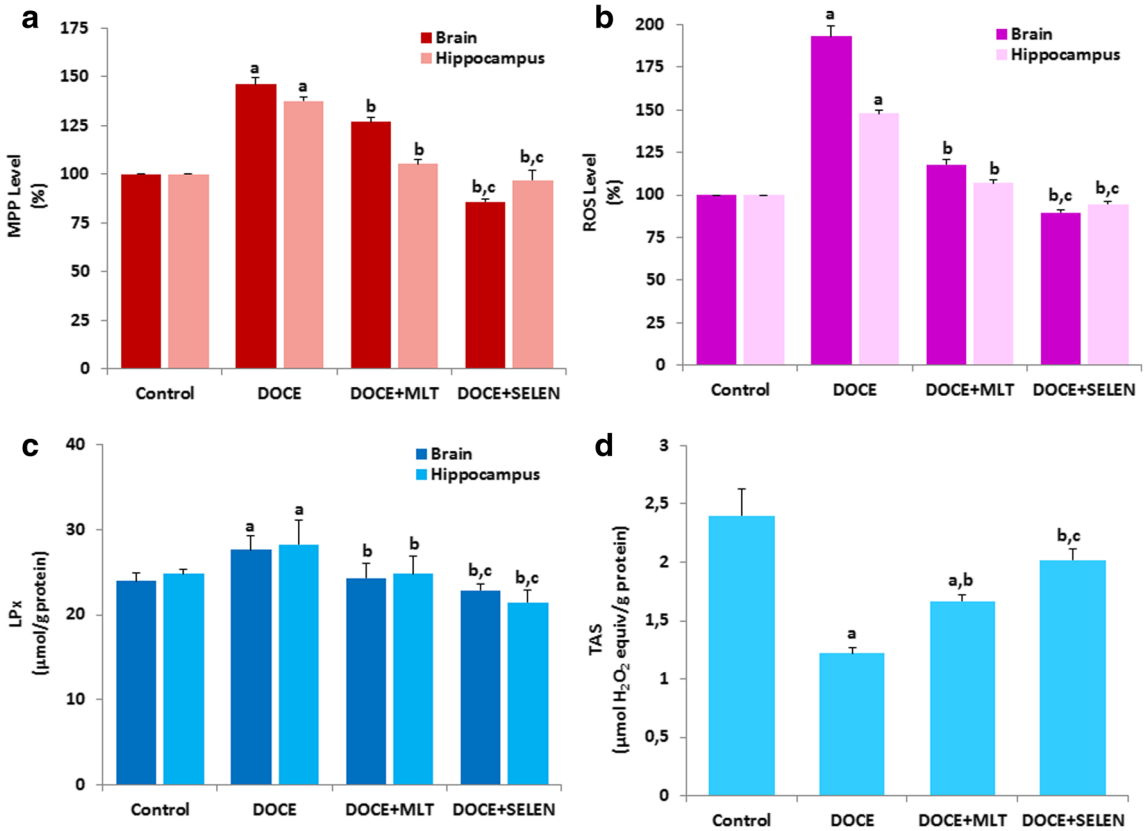


**Fig. 3** Modulator role of melatonin (MLT) and selenium (SELEN) on docetaxel (DOCE)-induced mitochondrial membrane potential (MPP) level (**a**), intracellular ROS production (**b**), lipid peroxidation (LPx) (**c**) and total antioxidant status (TAS) (**d**) changes in the brain and hippocampal neurons. The fluorescence dyes as JC-1 and DCFH-DA used in the microplate reader (Infinite Pro200) for the MPP and ROS analyses, respectively. LPx analyses in the brain and hippocampus samples were spectrophotometrically performed by using the manual technique, although TAS analysis in the brain samples was performed in the spectrophotometer (Cary 60 UV) by using a commercial kit. All data are expressed as mean ± SD (n = 8 in each group). ($^{a}p < 0.001$ versus control group. $^{b}p < 0.001$ versus DOCE group. $^{c}p < 0.05$ versus DOCE+MLT group)

## MLT and SELEN suppress $Ca^{2+}$ response through inhibition of TRPM2 channels in the hippocampus after DOCE treatment

It is well known that calcium signaling triggers MMP in the mitochondria. In turn, the increase of MMP in the mitochondria induces an increase of caspase activations and ROS production. In previous studies, we observed oxidative stress-dependent activation of the TRPM2 channel in neurons, such as hipokampus and dorsal rood ganglion (Akpınar et al. 2016; Kahya et al. 2017). After observation of an increase of caspase activations and ROS, we decided to investigate overload $Ca^{2+}$ entry through TRPM2 channel activation in the hippocampal neurons.

Intracellular $Ca^{2+}$ fluorescence intensity through TRPM2 channel activation was increased in DOCE group as compared to control group ($p < 0.001$) (Fig. 6). In contrast, administration with MLT and SELEN improved the increased $Ca^{2+}$ fluorescence intensity ($p < 0.001$). However, improvement of SELEN on the $Ca^{2+}$ fluorescence intensity in the hippocampus is more important than in MLT ($p < 0.05$).

## Discussion

Taxane group chemotherapeutic agents have been commonly used treatment for neuronal tumors and brain metastasis (Ghoochani et al. 2016; Ju et al. 2018). However, the use of these chemotherapeutics is limited in the brain by the adverse effects after a single dose of DOCE (Tabaczar et al. 2017). Although adverse effects of DOCE have been reported for several years, the protective effect of MLT and SELEN on DOCE-induced acute brain and hippocampus injury and their molecular mechanism have not been investigated yet. In the current study, we investigated the potential protective effect of MLT and SELEN against DOCE-induced brain and hippocampus injury using mouse models. Simultaneously, the possible molecular pathways underlying the neuroprotective effect were explored by investigating oxidative stress markers, inflammatory mediators, calcium signaling as well as cell apoptosis. It has been demonstrated that MLT and SELEN exert a major protective effect on the DOCE-induced acute brain and hippocampus injury and the underlying molecular mechanisms may be through TRPM2 channel activation and

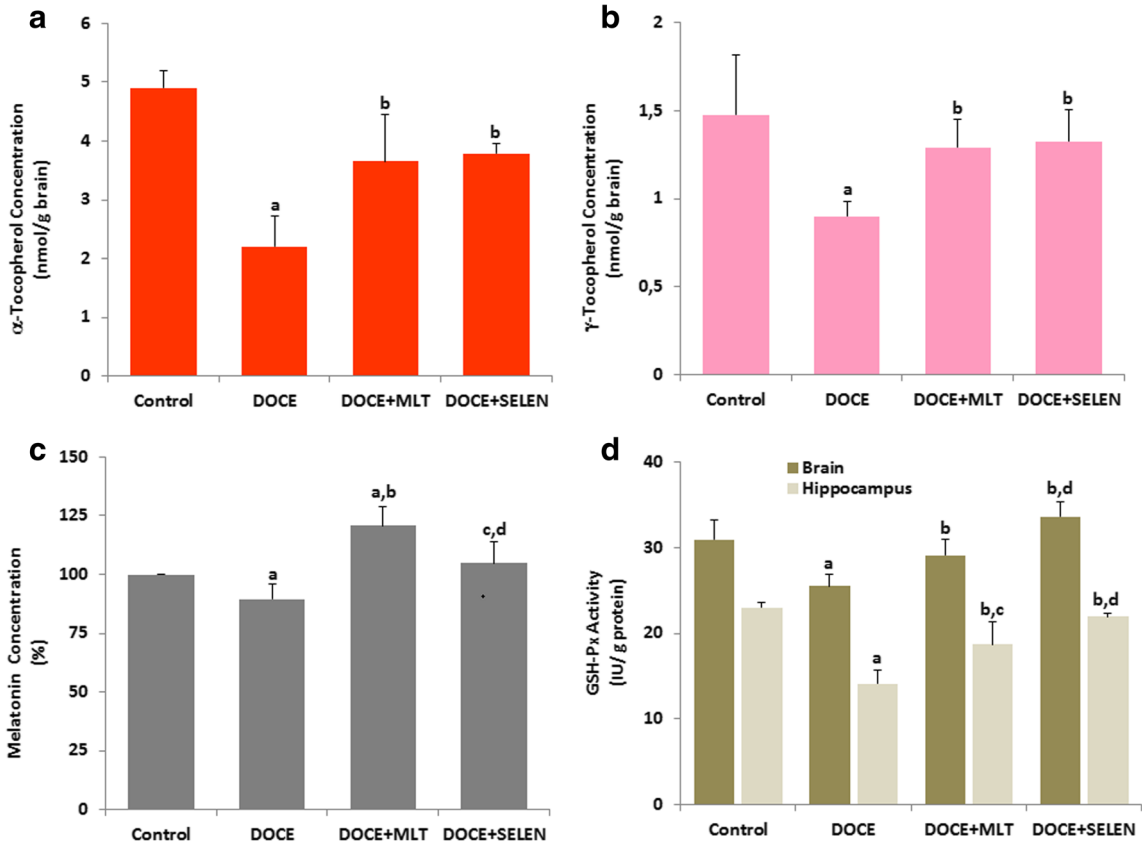


**Fig. 4** Melatonin (MLT) and selenium (SELEN) modulated docetaxel (DOCE)-induced decrease of α-tocopherol (**a**), γ-tocopherol (**b**), MLT (**c**) concentrations and GSH-Px activity (**d**) in the brain and hippocampus samples. The α- and γ-tocopherol analyses in the brain were performed by the HPLC (Agilent). Melatonin concentrations in the brain were measured in the samples by the commercial kits, although GSH-Px activity in the brain and hippocampus was measured by using a manual spectrophotometric (Cary 60 UV) method. All data are expressed as mean ± SD (n = 8 in each group). ($^{a}p < 0.001$ and $^{c}p < 0.05$ versus control group. $^{b}p < 0.001$ versus DOCE group. $^{d}p < 0.001$ versus DOCE+MLT group)

mitochondria-related oxidative stress and caspase signaling pathways.

In addition to a poor enzymatic antioxidant defense system, brain and hippocampus have a high rate of oxygen consumption and a rich content of PUFAs. Hence, the brain and hippocampus exhibit increased vulnerability to chemotherapeutic agent-induced oxidative stress (Yakubov et al. 2014; Nazıroğlu and Braidy 2017). Excessive ROS production has been known as an important factor to promote chemotherapeutic agent-induced oxidative tissue toxicity and apoptosis through increasing the accumulation of intracellular ROS (Mi et al. 2018). The chemotherapeutic agent-induced ROS are scavenged by enzymatic antioxidants such as GSH-Px and SOD and non-enzymatic antioxidants such as retinol, β-

**Table 1** The effects of melatonin (MLT) and selenium (SELEN) on brain reduced glutathione (GSH) and antioxidant vitamin concentrations in docetaxel (DOCE)-induced brain and hippocampus injury in the mouse (mean ± SD and $n = 8$)

| Parameters | Control | DOCE | DOCE+MLT | DOCE+SELEN |
|---|---|---|---|---|
| Brain GSH (μmol/g protein) | 14.40 ± 0.74 | 12.90 ± 1.07[a] | 14.40 ± 0.67[c] | 15.00 ± 0.91[c] |
| Hippocampus GSH (μmol/g protein) | 12.30 ± 0.39 | 10.20 ± 0.60[a] | 11.72 ± 0.73[c] | 12.70 ± 0.47[c,e] |
| Retinol (μmol/g brain) | 3.55 ± 0.80 | 2.49 ± 0.52[b] | 3.36 ± 0.98[d] | 3.47 ± 0.61[d] |
| β-Carotene (μmol/g brain) | 1.85 ± 0.28 | 0.86 ± 0.20[b] | 1.88 ± 0.16[d] | 1.58 ± 0.28[d,e] |
| Ascorbic acid (μmol/g brain) | 90.84 ± 9.27 | 76.65 ± 10.87[a] | 85.66 ± 14.66[c] | 93.68 ± 8.03[c] |

[a] $p < 0.05$ and [b] $p < 0.001$ versus control. [c] $p < 0.05$ and [d] $p < 0.001$ versus DOCE group. [e] $p < 0.05$ versus DOCE+MLT group

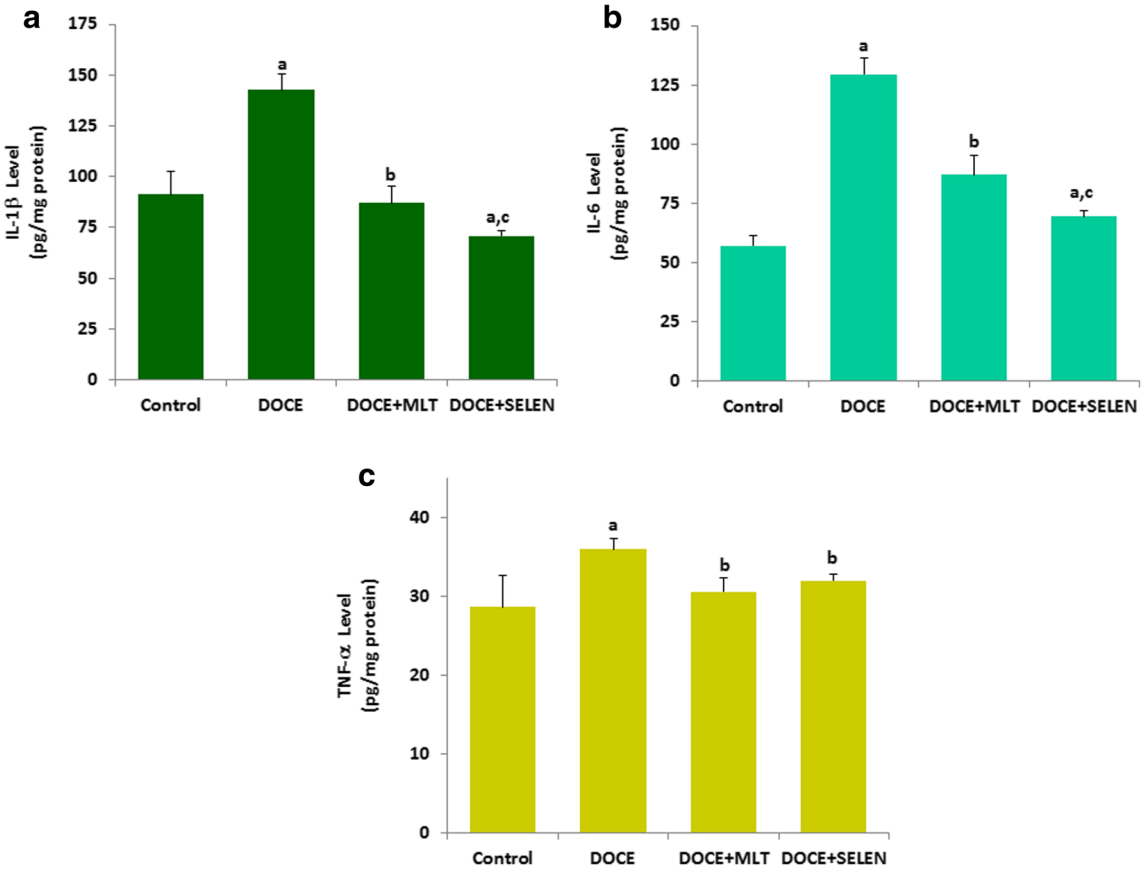


**Fig. 5** Effects of melatonin (MLT) and selenium (SELEN) against docetaxel (DOCE)-induced cytokine production in the mouse brain. The IL-1β (**a**), IL-6 (**b**) and TNF-α (**c**) levels in the supernatant of the brain homogenate were measured by ELISA. Quantification of protein levels was performed by the spectrophotometer (Cary 60 UV). All data are expressed as mean ± SD (n = 8 in each group). ($^{a}p < 0.001$ versus control group. $^{b}p < 0.001$ versus DOCE group. $^{c}p < 0.001$ versus DOCE+MLT group)

carotene, ascorbic acid, MLT and tocopherol (Nazıroğlu and Braidy 2017; Mi et al. 2018; Waseem et al. 2018). Previous reports have displayed that chemotherapeutic agents such as DOCE and cisplatin decrease antioxidant mechanisms followed an apparent reduction in the levels of GSH-Px, GSH, retinol, β-carotene, ascorbic acid, MLT and tocopherol, but the elevation in intracellular ROS and LPx contents (Nazıroğlu et al. 2004; Yamamoto et al. 2012; Altintas et al. 2015; Fushida et al. 2016; Tabaczar et al. 2017; Baş and Nazıroğlu 2018; Mohri et al. 2018). In rodents and human, protective effects of the MLT, SELEN, ascorbic acid and tocopherol on the cisplatin-induced adverse effects through upregulation of the enzymatic and non-enzymatic antioxidants in the several tissues including brain were reported (Nazıroğlu et al. 2004; Yamamoto et al. 2012; Zanini et al. 2014; Altintas et al. 2015; Fushida et al. 2016; Tabaczar et al. 2017; Baş and Nazıroğlu 2018; Mohri et al. 2018). In the present report, we observed for the first time that supplementation with MLT and SELEN significantly inhibited the elevation of ROS and LPx through upregulation of GSH-Px activity, GSH, retinol, β-carotene, ascorbic acid, MLT, α and -γ tocopherol concentrations in DOCE-treated hippocampus and brain. In addition, MLT and SELEN pretreatments evidently modulated the increase in intracellular ROS production. Hence, MLT and SELEN induced antioxidant action in the DOCE-induced oxidative toxicity in the hippocampus and brain. Similarly, decreased level of ascorbic acid and α-tocopherol but increased level of LPx in plasma and brain of cisplatin treated patients with lung cancer were reported (Zanini et al. 2014). DOCE-induced increase of LPx and ROS levels were more recently reported in brain (Tabaczar et al. 2017) and testis (Altintas et al. 2015) of rats. More recently, protective effect of SELEN on the DOCE-induced increase of ROS levels were also reported in the HEK293 cell line (Baş and Nazıroğlu 2018).

As it was mentioned above, several reports have confirmed that ROS-induced oxidative stress injury and activation of apoptotic signaling pathways are involved in the pathogenesis of acute brain and hippocampus injury. In this study, we identified the importance of oxidative stress in the improvement of DOCE neurotoxicity by MLT and SELEN. Interestingly, these phenomena were effectively reversed by MLT and SELEN pretreatment for seven days. In addition to oxidative stress, inflammation plays an important part in the pathogenesis of DOCE-induced acute tissue injury and it is an unclear

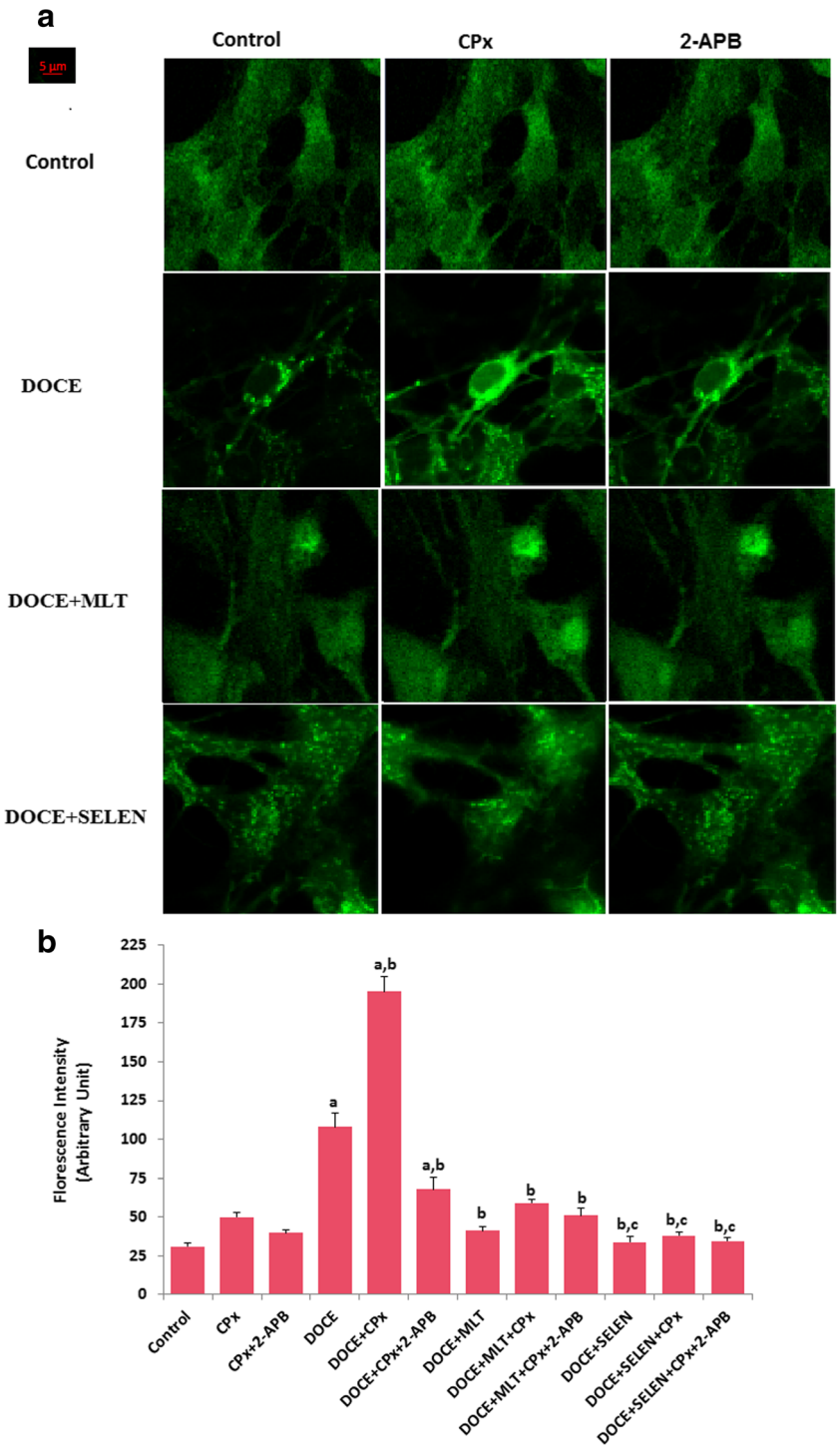


**Fig. 6** Melatonin (MLT) and selenium (SELEN) reduced docetaxel (DOCE)-induced $Ca^{2+}$ fluorescence intensity through inhibition of TRPM2 channel in the hippocampus neurons (mean ± SD). The cells were stained with Fluo-3 calcium dye and mean ± SD of fluorescence in 15 $mm^2$ of the neuron as arbitrary unit are presented; $n = 20$ independent experiments. The cells were treated with TRPM2 antagonist (2-APB, and 0.1 mM) to inhibit $Ca^{2+}$ entry before stimulation of TRPM2 (CPx and 1 mM). The neurons were analyzed at 515 nm by the laser confocal microscopy fitted with a 40x oil objectives (**a**). Changes of the $Ca^{2+}$ fluorescence intensity were shown by columns (**b**). ($^{a}p < 0.001$ versus control groups. $^{b}p < 0.001$ versus DOCE groups. $^{c}p < 0.001$ versus DOCE+MLT groups)

phenomenon involving several cellular and molecular pathways (Yamamoto et al. 2012; Fushida et al. 2016; Baş and Nazıroğlu 2018; Mohri et al. 2018). TNF-α is a prototypical inflammatory mediator, which promotes cytokines generation and inflammation secretion by stimulating neutrophils and macrophages, ultimately resulting in cellular necrosis or apoptosis (Yamamoto et al. 2012; Fushida et al. 2016; Baş and Nazıroğlu 2018; Mohri et al. 2018). Similarly, IL-1β promotes the inflammation, causes fever, as well as stimulates the development and differentiation of the immune system (Waseem et al. 2018). In our previous studies, MLT and SELEN were reported to inhibit the overproduction of TNF-α, IL-1β and IL-6 in diabetes-induced brain injury in rats (Nazıroğlu et al. 2004). In addition, the modulator role of MLT and SELEN on antitumor activity, TNF-α and ROS levels in cancer-induced animals (Kisková et al. 2012; Li et al.

2018) and cell line (Uguz et al. 2012) were reported. In the current study, MLT and SELEN effectively inhibited the se cretion of these three cytokines. Thus, the reduced effect induced by MLT and SELEN may contribute to the inhibition of the inflammation associated with DOCE-induced loss of brain and hippocampus functions.

In the brain and hippocampus samples, DOCE-induced increases of MPP, apoptosis, caspase −3, and caspase −9 were decreased by MLT and SELEN treatments. Accumulated evidences have indicated that DOCE-induced acute adverse tissue injuries in kidney were associated with cellular apoptosis (Baş and Nazıroğlu 2018; Mohri et al. 2018). The current results showed that the apoptosis level, caspase −3 and − 9 activities were significantly increased after DOCE, but this phenomenon was reversed by the pretreatment of MLT and SELEN, consistent with the previous kidney reports (Baş and Nazıroğlu 2018). The findings in this work suggest that MLT and SELEN may act as an anti- apoptosis effective agent through restoring the inhibiting caspase −3 and − 9 activities.

$Ca^{2+}$ passes the cell membrane through well-known channels such as voltage gated calcium channels and chemical gated channels. Apart from the well-known channels, TRP channel superfamily with 30 members was recently discovered as $Ca^{2+}$ transporters (Nazıroğlu 2007; Nazıroğlu and Braidy 2017). One of the TRP superfamily is TRPM2 channel. The channel is independently activated in the Nudix domain by ADP-Ribose (ADPR) and oxidative stress (Nazıroğlu and Lückhoff 2008). Oxidative stress induces ADPR through poly (ADPR) polymerase-1 (PARP-1) activation (Nazıroğlu 2007). DOCE-induced PARP-1 activation in rat brain was reported (Tabaczar et al. 2017). In the current study, we observed increased levels of $Ca^{2+}$ fluorescence intensity through oxidative stress (CPx) stimulation, but a decrease of the $Ca^{2+}$ fluorescence intensity through inhibition of TRPM2 channel blocker (2-APB), MLT and SELEN. To our knowledge, there is no report on DOCE-induced TRPM2 channel activation but the modulator roles of MLT and SELEN on the TRPM2 channel and PARP-1 activation were reported in the hippocampus of diabetic rats (Tabaczar et al. 2017). The protective effect of MLT through inhibition of TRPM2 channel on oxidative stress in inflammation induced guinea pig brains by particulate matter 2.5 (Ji et al. 2018).

In summary, this study provides a new foresight into the potential neuroprotective molecular mechanisms of MLT and SELEN against DOCE-induced hippocampus and brain injury. MLT and SELEN pretreatment markedly suppressed DOCE-induced apoptosis, oxidative stress, cytokine productions, mitochondrial dysfunction, caspase −3 and − 9 activation as well as TRPM2 channel inhibition via the inhibition of ROS but stimulation of GSH and vitamin antioxidant redox systems (Fig. 7). Administration of MLT and SELEN or related compounds may be considered as a therapeutic strategy to prevent DOCE-induced acute hippocampus and brain oxidative toxicity.

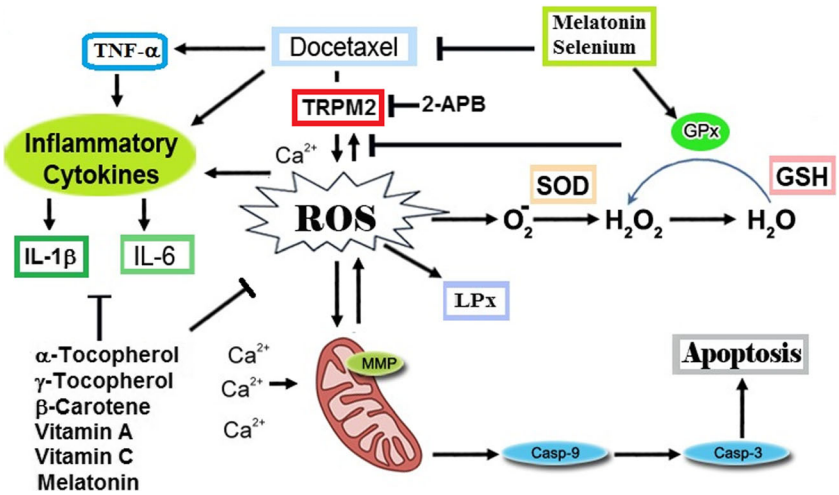


**Fig. 7** Summary of pathways involved in docetaxel-induced apoptosis, inflammation and reactive oxygen species (ROS) production and clearance by melatonin and selenium. Docetaxel induces excessive productions of several ROS from superoxide ($^{\cdot}O_2^-$). The main mechanism in the anti-apoptotic effect of melatonin and selenium is mediated by inhibition of ROS-mediated TRPM2 channel and caspase −3 and − 9 activations. Increase of mitochondrial membrane potential (MMP) through increase of cytosolic free $Ca^{2+}$ concentration activates apoptosis factors and ROS from the mitochondria. In response, melatonin and selenium in the hippocampus and brain stimulates antioxidant responses and inhibition of TRPM2 that facilitates the neutralization of ROS and lipid peroxidation (LPx) into less harmful products by supporting enzymatic antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and non-enzymatic antioxidants such as vitamin A (retinol), vitamin C (ascorbic acid), vitamin E (α- and γ–tocopherol) and β-carotene. The activations of cytokines through docetaxel treatment in the brain and hippocampus lead to production of TNF- . Extremely high level of ROS causes cell death while sustained low levels of oxidative insult leads to cell cycle alteration and an increase in pro-inflammatory cytokines. Hence, blockade of the docetaxel-induced TNF- activity by melatonin and selenium decreased the TNF- , IL-1β and IL-6 productions

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**Authors' contributions** ZSA and MN formulated the present hypothesis. MN was responsible for writing the report. MN and KE were responsible analyzing the spectrophotometer and plate reader. ZSA and KE made also critical revision for the manuscript.

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## Compliance with ethical standards

**Disclosures** None of the authors have any to disclose. All authors approved the final manuscript.

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