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Effect of selenium against doxorubicin-induced oxidative stress, inflammation, and apoptosis in the brain of rats: Role of TRPM2 channel

Kenan Yıldızhan[1]*, Zübeyir Huyut[2], Fikret Altındağ[3] & Adem Ahlatcı[4]

[1]Department of Biophysics, Faculty of Medicine; [2]Department of Biochemistry, Faculty of Medicine; [3]Department of Histology and Embryology, Faculty of Medicine; & [4]Vocational School of Health Services, Van Yuzuncu Yil University, Van-65090, Turkey

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Doxorubicin (DOX) is widely used as an anticancer drug in humans' various solid and haematological tumours. Although many studies on the toxic effect of DOX are used in different organs, its impact on brain tissue has yet to be adequately studied. This study investigated the protective effect of selenium (Se) and the role of transient receptor potential melastatin-2 (TRPM2) channel activation against brain damage caused by DOX administration. Sixty rats were randomly divided into the sham, dimethyl sulfoxide (DMSO), DOX, DOX + Se, DOX + N-(p-amylcinnamoyl) anthranilic acid (ACA), and DOX + Se + ACA groups. The reactive oxygen species (ROS), poly [ADP-ribose] polymerase 1 (PARP1), and TRPM2 channel levels in brain tissues were measured by ELISA. In addition, a histopathological examination was performed in the cerebral cortex and hippocampal areas, and the TRPM2 channel, NF-κB, and caspase-3 expression were determined immunohistochemically. The levels of ROS, PARP1 and TRPM2 channel in the DOX group were higher than in the sham and DMSO groups ($P < 0.05$). However, these parameters were decreased in the in DOX+Se and DOX+ACA groups by the treatments of Se and ACA ($P < 0.05$). Also, we determined that Se and ACA treatment decreased the NF-κB, caspase-3, and TRPM2 channel expression in the cerebral cortex and hippocampal areas in the DOX-induced rats. The data showed that Se and/or ACA administration together with DOX administration could be used as a protective agent against DOX-induced brain damage.

Keywords: Apoptosis, Doxorubicin, Oxidative stress, Selenium, TRPM2 channel

Doxorubicin (DOX) is an antibiotic and anticancer drug obtained from the cultures of various microorganisms from the alkylating anthracycline group[1]. DOX is used as a single agent or combination in treating many types of cancer, especially breast cancer[2]. Although different side effects are known depending on the dose of DOX, the most prominent side effect of DOX is the increase in oxidative stress (OS)[3]. Studies have shown that DOX cannot pass through the blood-brain barrier. However, clinical studies showed that chemotherapeutic drugs affected the focusing disorder in central nervous system cancers and caused disabilities in remembering and finishing an event or learning a new phenomenon[4-6]. Studies performed in rats determined that increased OS and cytokines caused neuroinflammation and neurotoxicity in the brain[7-9]. Kuzu *et al*. reported that induction of DOX in rats increased inflammatory and OS parameters in brain tissue. The same study determined that antioxidants decreased brain damage by inhibiting DOX-induced OS[10].

Selenium (Se) is an essential element for living organisms because it is found in the structure of many enzymes[11]. Se helps to defend the organism against free radicals and plays a vital role in the metabolism of thyroid hormones. In addition, many studies showed that Se-dependent enzymes inhibited OS by destroying reactive oxygen species (ROS)[12-14]. One of these enzymes is glutathione peroxidase (GSH-Px). GSH-Px catalyses the breakdown of hydrogen peroxide and other free hydrogen peroxides. Therefore, Se indirectly functions as an antioxidant[11]. Another study emphasized that Se and selenoproteins may be associated with many physiological functions such as neurotransmission, inflammation, ion channels (such as TRP channels), protein phosphorylation, calcium homeostasis, and brain cholesterol metabolism, thanks to their antioxidant properties[15]. Although Se's essential functions and positive effects on brain damage and neurotoxicity are known, the protective effects of Se against DOX-induced brain damage and the underlying mechanism are still not fully understood.

*Correspondence:
Phone: + 90 4322150471
Fax: + 90 04322168519
E-mail: kenanyldzhan@gmail.com

The transient receptor potential (TRP) melastatin-2 (TRPM2) channel, a member of the TRP family, is a voltage-independent and non-selective cation channel sensitive to oxidant molecules. TRPM2 channel is known for its expression in many organs, and its abundant expression has been detected, especially in the brain[16]. TRPM2 channel triggers inflammation and apoptosis by causing an increase in intracellular calcium due to the rise in extracellular OS and acts as a sensor for OS. With the activation of this pathway, it has been emphasized that TRPM2 plays a vital role in cell damage or cell death[17].

Although DOX cannot cross the blood-brain barrier, it is important to investigate how it, widely used in different types of cancer, will affect brain tissue because it increases OS and inflammation in various tissues of our body. In our literature review, we did not find any studies on the role of the TRPM2 channel and the protective effect of Se against brain damage caused by DOX. Therefore, we aimed to investigate the role of the TRPM2 channel and the protective effect of Se against damage in the cortex and hippocampus of DOX-applied rats.

Material and Methods

Drugs and chemicals

Doxorubicin (Cat no: T1020) and N-(p-amylcinnamoyl) anthranilic acid (Cat no: T5454) were purchased from TargetMol (Target Molecule Corp., USA) and dissolved in dimethyl sulfoxide (DMSO). Sodium selenite (Cat no: 214485) was purchased from Sigma Aldrich (St. Louis, MO, USA) and dissolved in saline. All other chemical compounds were of a high purity grade. ROS (Cat no: SG-20415), PARP1 (Cat no: SG-21060), and TRPM2 (Catno: MBS9395911) levels were determined using commercially available ELISA kits according to the manufacturer's instructions (Sinogeneclon Biotech Co., and MyBioSource Inc., respectively).

Ethics statement

In the study, 60 Albino Wistar rats weighing between 200-300 g and aged between 2 and 3 months were used. An illumination system was configured to provide a light/dark photoperiod of 12:12. The rats were given a daily standard pellet diet as ad libitum. This study was conducted at the Van Yuzuncu Yil University Experimental Medicine Research and Application Center after the approval of the Van Yuzuncu Yil University Animal Experiments Local Ethics Committee (decision number: 2022/09-02 and approval date: 29.09.2022).

Experimental design

The dose and duration of the DOX were applied according to the amount and duration previously reported[3,18]. Stock DOX solution was prepared in DMSO at 2.5 mg/mL concentration. Se's effective dose and duration were applied at 0.5 mg/kg every day for 14 days, taking into account the study of Cengiz *et al.*[19]. Stock Se solution was prepared in saline at a concentration of 0.5 mg/mL. The effective dose and duration for ACA were applied to the amount and time used by Cakir *et al.*[20]. Stock ACA solution was prepared in DMSO at a concentration of 25 mg/mL and injected intraperitoneally, according to the weight of the rats during the study.

Rats were randomly divided into six groups, with ten rats each.

Sham group: This group received isotonic solution (100 µL) intraperitoneally (*i.p.*) for 14 days (every day).

DMSO group: This group received DMSO (100 µL) *i.p.* for 14 days (every other day).

DOX group: This group received DOX (2.5 mg/kg) *i.p.* for 14 days (every other day).

DOX+ACA group: This group received DOX (2.5 mg/kg) *i.p.* (every other day), and ACA (25 mg/kg) *i.p.* for 14 days (every day).

DOX+Se group: This group received DOX (2.5 mg/kg) *i.p.* (every other day), and Se (0.5 mg/kg) *i.p.* for 14 days (every day).

DOX+Se+ACA group: This group received DOX (2.5 mg/kg) *i.p.* (every other day), and Se (0.5 mg/kg) *i.p.* for 14 days (every day) and, ACA (25 mg/kg) *i.p.* for 14 days (every day).

At the end of the study, the abdominal regions of the rats were opened under anaesthesia with ketamine (50 mg/kg) + xylazine (20 mg/kg), and the blood of the rats was taken intracardially. After, blood samples were born into a dry biochemistry tube and centrifuged at 3500 × g for 10 min[21]. The left brain was taken and placed in the freezer for biochemical analysis. The right brain was left in a 10% formaldehyde solution for histopathological and immunohistochemical examinations.

Homogenization of brain tissue and production of supernatant

After washing the left-brain tissues with 0.9% NaCl for biochemical analysis, they were frozen at –80°C. On the study day, 2.25 mL of 50 mM phosphate buffer (pH 7.4) was added to 250 mg of brain tissue (1:10 wt-vol), and the mixture was homogenized using a homogenizer (Ultra Turrax-T25). The samples were centrifuged at 3000 × g and 4°C for 20 min.

Quantitative protein determination

According to the method used by Zubeyir *et al.*, protein amounts in brain tissue homogenates were measured[22]. 10 μL homogenate of the brain was added to 0.499 mL Coomassie-Brillant Blue solution and incubated at room temperature and in the dark for 10 min. Then, data was read at 595 nm in the spectrophotometer (Shimadzu UV Mini 1240 from Japan). A mixture of 0.1 mL of distilled water and 4.9 L of Coomassie-Brillant Blue solution was used as the blank. Protein amounts were calculated by the standard curve.

Measurement of biochemical in brain homogenates

TRPM2, PARP1, and ROS levels were measured on the BioTek EL & 800 instrument at 450 nm with commercial ELISA kits following the instructions in the kit procedure. After adding 10 μL of antibodies to the 40 μL sample excluding the standards, 50 μL streptavidin HRP was added to all wells. After incubation at +37°C for 60 min, it was washed three times with a washing solution. 50 μL of chromogen A and B were added to the wells and incubated at +37°C for 10 min. Then the reaction was stopped by adding 50 μL of stop solution and the reading was performed at 450 nm. Concentrations were calculated by comparing the obtained absorbance values with the standard curve. Data were presented as a fold change (experimental/sham).

Histopathological and Immunohistochemical analysis

For histopathological examination, the right hemisphere of the brain from rats was fixed in 10% buffered formaldehyde and embedded in paraffin. 4 micrometer slices taken from paraffin blocks were stained with hematoxylin and eosin (H&E). Histopathological examination was performed in randomly 10 areas in each stained brain section and photographed in the light microscope (Olympus BX53, Japan). For immunohistochemistry (IHC) analysis, 4 micrometer sections were deparaffinized and rehydrated. Slides were deparaffinized, rehydrated, and then performed Heat-Induced Antigen Retrieval. The slides were incubated with TRPM2, NF-κB, and caspase-3 primary antibodies overnight at 4°C, followed by incubation with secondary antibodies at room temperature. Diaminobenzidine was used to visualize the reaction as the chromogen, and hematoxylin was used as the counterstain. The slides were scored as negative (0), mild reaction (1), moderate reaction (2), and severe reaction (3) according to their immunostaining.

Statistical analysis

The SPSS package program was used for biochemical analyses (version 21). Descriptive statistics were given as mean and standard deviation. The Shapiro-Wilk test was used to determine whether the data were normally distributed. Since the data were normally distributed, Duncan's test was performed following One-Way ANOVA. Results with a $P < 0.05$ were considered significant.

Results

Histopathological findings in the cortex and hippocampus of DOX-induced rats

Rats in sham and DMSO groups had normal histological cortex (Fig. 1Aa & Ab) and hippocampus (Fig. 1Ba & Bb). Cells with pycnotic nuclei were observed in the cortex of the rats in the DOX group (Fig. 1Ac), and cells with karyolytic nuclei were observed in the hippocampal area (Fig. 1Bc). The cortex and hippocampus of the subjects in the DOX+ACA (Fig. 1Ad and Bd), DOX+Se (Fig. 1Ae and Be), and DOX+Se+ACA (Fig. 1Af and Bf) groups were similar to the sham group.

Effect of Se and ACA on ROS, PARP1, and TRPM2 channel levels in the brain of DOX-induced rats

Although TRPM2 is inhibited by a non-specific inhibitor (ACA), TRPM2 is known to be activated by ADP-ribose (ADPR) caused by PARP1 activation and DNA damage caused by ROS[23,24]. Therefore, after observing the histopathological change in the cortex and hippocampus in DOX-induced brain tissue, we wanted to examine how ROS, PARP1, and TRPM2 channel levels changed in DOX-induced brain tissue.

The ROS (Fig. 2A), PARP1 (Fig. 2B), and TRPM2 (Fig. 2C) levels of sham and DMSO groups in brain tissue were similar ($P > 0.05$). The DOX group's ROS, PARP1, and TRPM2 levels were the highest compared to all the groups ($P < 0.05$). In the DOX+ACA and DOX+Se groups, although ROS, PARP1, and TRPM2 levels were significantly lower than the DOX group, ROS, PARP1, and TRPM2 levels in the DOX+Se+ACA group were the lowest compared to the other groups with DOX ($P < 0.05$).

Expression of TRPM2 channel, NF-κB, and Caspase-3 in the cortex and hippocampus of DOX-induced rats

Hippocampus and cortical expressions of TRPM2 (Fig. 3), NF-κB (Fig. 4), and caspase-3 (Fig. 5) were evaluated by the immunohistochemical method. DOX administration significantly increased hippocampal and cortical expressions of TRPM2 and NF-κB (in the

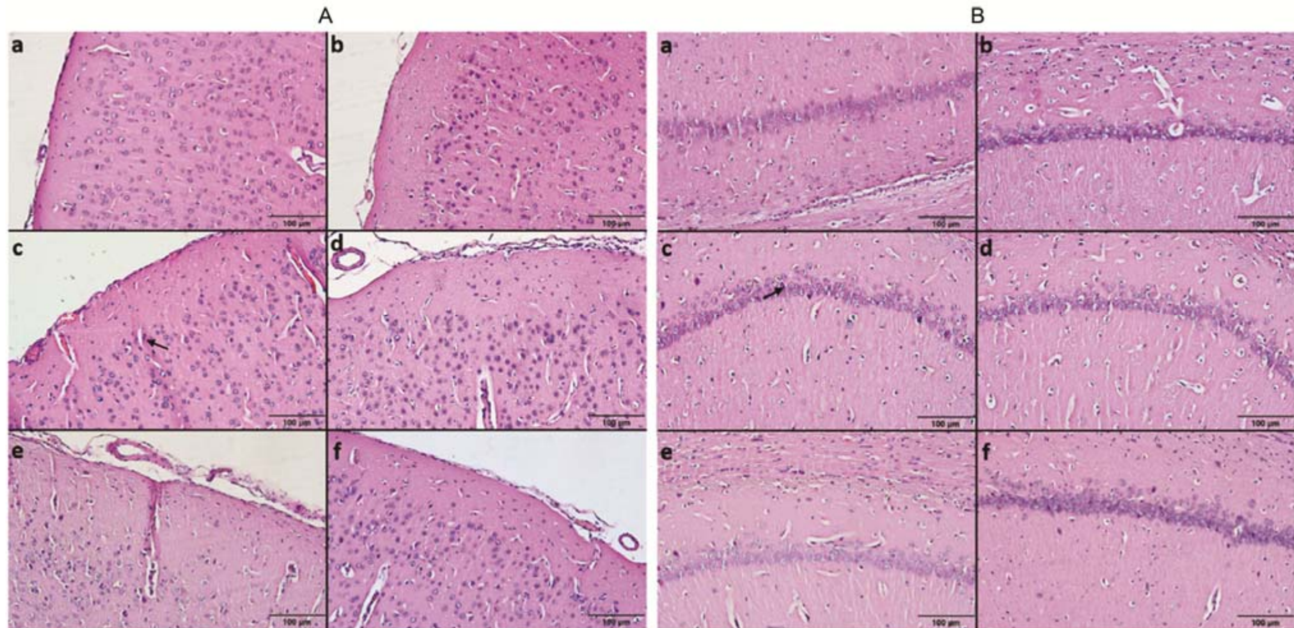

Fig. 1 — Effect of Se and TRPM2 antagonist (ACA) in the DOX-induced rats' brain. For histopathological examination, light microscopic images of the (A) cortex; and (B) hippocampus. (a) sham; (b) DMSO; (c) DOX; (d) DOX+ACA; (e) DOX+Se; and (f) DOX+Se+ACA. Rats in the sham and DMSO groups had normal histological cortex (Aa and Ab) and hippocampus (Ba and Bb). Cells with pycnotic nuclei (arrow) in the cortex in the DOX group (Ac) and cells with karyolytic nuclei (arrow) were observed in the hippocampal area (Bc). H&E. Images were evaluated semi-quantitatively based on observation. Scale Bar: 100 micrometer

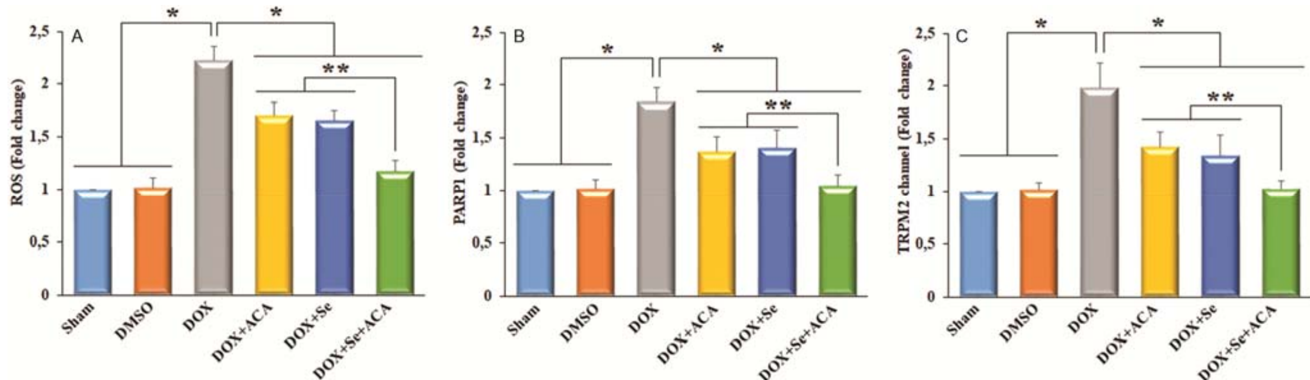

Fig. 2 — Comparison of (A) ROS; (B) PARP1; and (C) TRPM2 channel levels in the DOX-induced rats' brains. (Values were given as mean ± SD and n=10). (*P <0.05 *vs* the DOX group, **P <0.05 *vs* the DOX+Se+ACA group)

DOX group). However, hippocampal and cortical expressions of TRPM2 and NF-κB were reduced considerably in the DOX+Se and DOX+ACA groups compared to the DOX group. However, caspase-3 expression was intense in the cortices of the DOX group, while low caspase-3 expression was found in the hippocampus. The cortical expression of caspase-3 was minimal in the DOX+Se group, while caspase-3 expression was not found in the cortices of the DOX+ACA and DOX+Se+ACA groups. However, caspase-3 expression in the hippocampus of all the groups was not observed.

Discussion

DOX is widely used in treating various types of solid and hematological tumours in humans and animals. While there are many studies on the toxic effect of DOX use in multiple organs, its impact on brain tissue has not been adequately studied[25,26]. Although the first studies mentioned the poor penetration of DOX through the blood-brain barrier (BBB), many studies have shown that it has adverse side effects on the brain tissue after using DOX[8,26]. The most important mechanism of action of DOX is explained by its intercalation to DNA, inhibition of

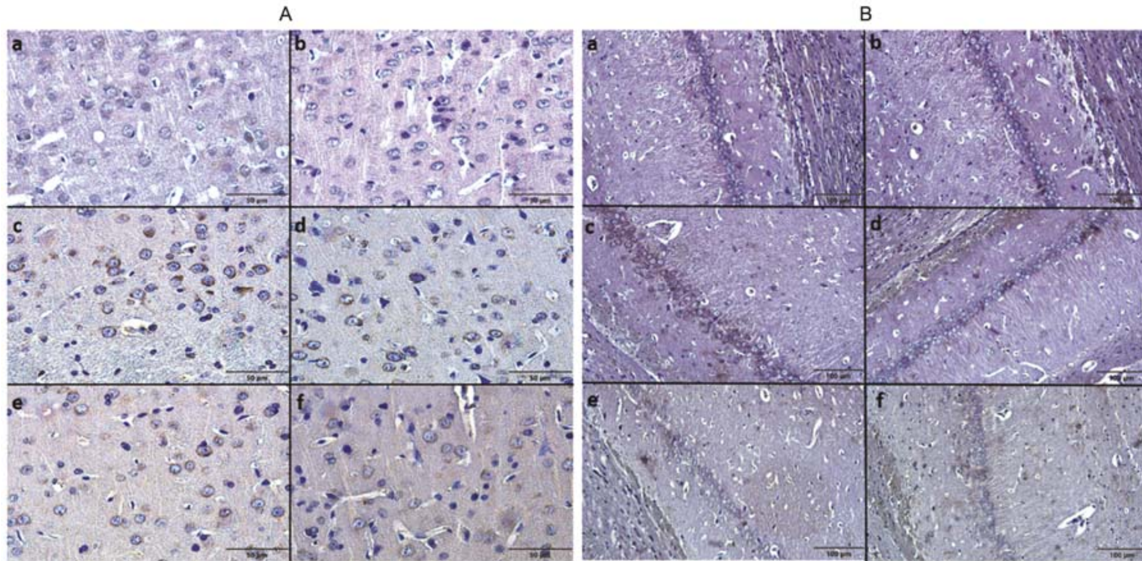

Fig. 3 — Immunohistochemical staining of the (A) cortex; and (B) hippocampus by TRPM2 antibody. (a) sham; (b) DMSO; (c) DOX; (d) DOX+ACA; (e) DOX+Se; and (f) DOX+Se+ACA. Images were evaluated semi-quantitatively based on observation. Scale Bar: 50 micrometer for the cortex and 100 micrometer for the hippocampus

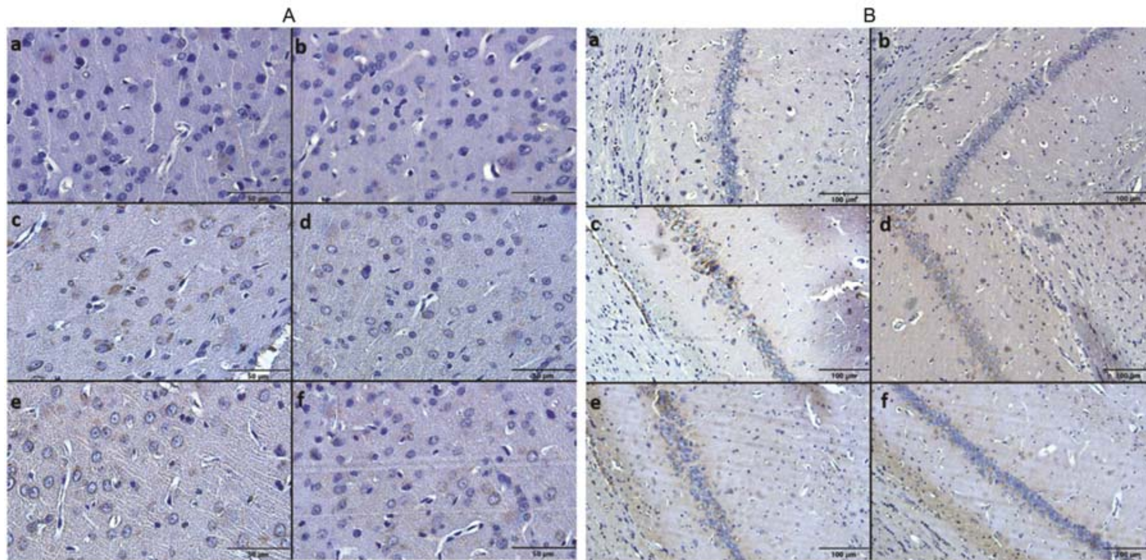

Fig. 4 — Immunohistochemical staining of the (A) cortex; and (B) hippocampus by NF-κB antibody. (a) sham; (b) DMSO; (c) DOX; (d) DOX+ACA; (e) DOX+Se; and (f) DOX+Se+ACA. Images were evaluated semi-quantitatively based on observation. Scale Bar: 50 micrometer for the cortex and 100 micrometer for the hippocampus

topoisomerase-II activity and overproduction of ROS[27]. In addition, the increase in OS stands out at the beginning of DOX-induced damage mechanisms in many other organs[1,6,7].

This study examined the damage in the brains of rats after DOX administration. Histopathologically, after using DOX, we observed cells with pycnotic nuclei in the cortex of the rats and cells with karyolytic nuclei in the hippocampal area. Our results showed that Se treatment and inactivation of the TRPM2 channel could reduce the DOX-induced damage in rats' cerebral cortex and hippocampal areas.

Kaymak *et al*. emphasised that although DOX is a crucial anticancer drug in many cancer treatments, DOX causes neurotoxicity. They observed pycnotic nuclei, necrotic cells and haemorrhage in the brain histopathological examination after DOX use in their experimental study[28]. Zhou *et al*. determined that MDA, caspase-3 and Bax activation were increased in the hippocampal CA1 region of DOX-induced rats.

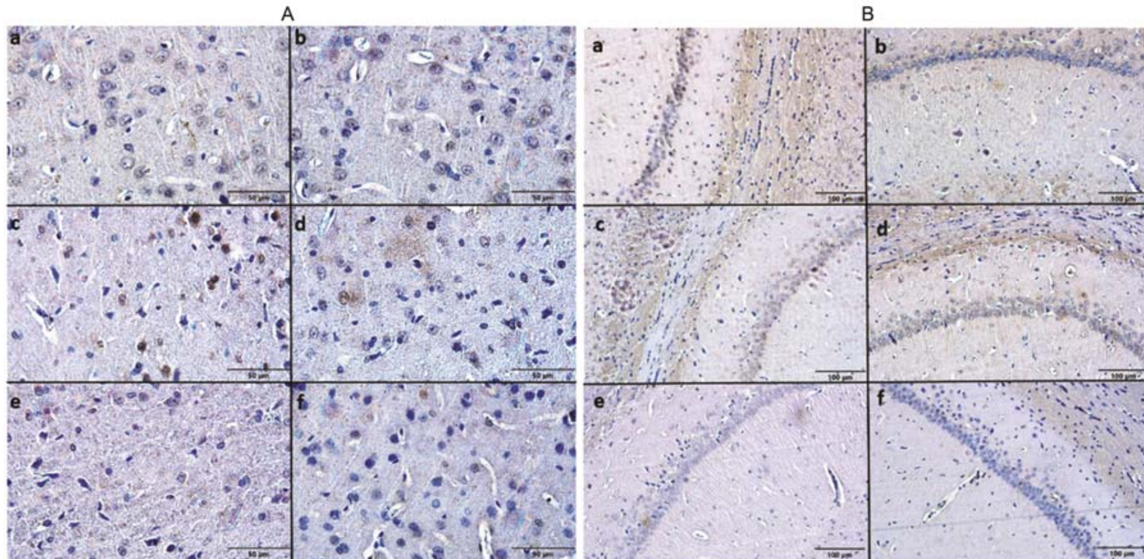

Fig. 5 — Immunohistochemical staining of the (A) cortex; and (B) hippocampus by the caspase-3 antibody. (a) sham; (b) DMSO; (c) DOX; (d) DOX+ACA; (e) DOX+Se; and (f) DOX+Se+ACA. Images were evaluated semi-quantitatively based on observation. Scale Bar: 50 micrometer for the cortex and 100 micrometer for the hippocampus

This study argued that specific doses of DOX (2.5 mg/kg DOX every other day for a total of seven injections) cause neurotoxicity and this damage can reduce by autophagy inhibitors[29]. Kuzu *et al*. (40 mg/kg single dose) showed atrophy, degeneration in neurons, and severe hyperemia in the meninges and paranasal veins in histopathological examination of the DOX-induced rats' brain tissues[10]. Consistent with the literature, our study observed cells with pycnotic nuclei in the cortex and cells with karyolytic nuclei in the hippocampal area of DOX-induced rats in the histopathological examination. In rats treated with Se and ACA (DOX+Se, DOX+ACA and DOX+Se+ACA), the cortex and hippocampus were similar in histological structure to the sham group (Fig. 1).

The most striking feature in the formation of a histopathological process is the disruption of intracellular ion homeostasis due to the disorder of the ionic mechanism in the cells. The most crucial ion in this mechanism is Ca^{2+} ion[16,17]. Mammalian cells have multiple ion channels that are important for maintaining and regulating intracellular ionic homeostasis. The TRPM2 is also expressed in neurons, a member of the TRP channel family responsible for intracellular Ca^{2+} flux. TRPM2 is highly sensitive to OS and is activated by intracellular ADPR formed by PARP1 activation depending on the increase in OS[30,31]. Also, increasing OS in brain tissue after DOX administration has been reported in the literature[7,8]. The protective effects of Se against the side effects that occur in different organs due to the use of DOX were reported in previous studies[3,10,32]. Se helps to defend the organism against free radicals and participates in the metabolism of thyroid hormones. Se-dependent enzymes inhibited OS by destroying ROS[12-14]. Studies have shown that OS-induced TRPM2 channel activation can be suppressed by Se, known for antioxidant properties, by maintaining the oxidant/antioxidant balance[3,11,33]. Consistent with the literature, our study determined a significant increase in the level of ROS in the rats' brain tissue after DOX administration. In addition, we determined the change in PARP1 and TRPM2 channel levels in the brain tissue after the DOX application and found an increase in the level of PARP1 and TRPM2 channels in the brain tissue together with ROS after the DOX application (Fig. 2). The levels of DOX-induced ROS, PARP1, and TRPM2 in the brain tissue of rats were decreased after the suppression of the TRPM2 channel by Se treatment (Fig. 2).

Western blot studies have shown that in addition to the increased activation of the TRPM2 channel by OS, the expression of TRPM2 is also increased[34,35]. Vaidya *et al*. showed that TRPM2 channels play a vital role in other neurodegenerative disorders associated with OS, and TRPM2 channel expression may be altered in MPTP-induced Parkinson's disease model in rats[36]. Different studies have shown that TRPM2 channel expression and activation play a critical role in neuronal damage, especially after

increasing OS in neuronal cells[37,38]. In this study, biochemical and immunohistochemical results observed a rise in TRPM2 channel expression in rat brains after DOX administration. This suggests that DOX may lead to TRPM2 channel activation and expression by increasing oxidative stress. Despite the DOX application, we determined a significant decrease in TRPM2 channel expression in groups with Se and ACA treatments compared with the DOX group (Fig. 3). This result may be due to the antioxidant properties or the TRPM2 channel inhibitory effect of ACA.

Fouad *et al*. determined that antioxidant capacity decreased and lipid peroxidation increased in the DOX-induced toxicity model in the rat brain. In addition, this study revealed that DOX caused neuroinflammation by increasing the level of pro-inflammatory mediator glial fibrillary acid protein (GFAP), NF-κB, and caspase-3 in the brain tissue[7]. Ali *et al*. investigated the effect of caffeic acid phenethyl ester against DOX-induced chemo-brain in rats. They found that treatment with DOX increased glial fibrillary acid protein levels and proinflammatory mediators (COX-II/TNF-α). In addition, they identified an increase in NF-κB nuclear translocation and neuroinflammation[39]. Cardoso *et al.* determined that although DOX did not pass through the BBB after doxorubicin administration in rats, a systemic proinflammatory response emerged with the increase of proinflammatory cytokines, which may be involved in the induction of oxidative molecules and proinflammatory cytokines caused by this drug[40]. In this study, we also evaluated the expression levels of NF-κB and caspase-3 by the immunohistochemical method in the hippocampal and cortical regions of the rats' brain tissue. We found that NF-κB significantly increased both hippocampal and cortical expressions after DOX treatment. However, Se and ACA treatment significantly reduced NF-κB expression in hippocampal and cortical expressions in rats' brains (Fig. 4). In addition, caspase-3 expression was observed in the cortices of the animals treated with DOX, while caspase-3 expression was not found in the hippocampus. Also, the cortical expression of caspase-3 was minimal in the DOX+Se group, while caspase-3 expression was not found in the cortices of animals treated with DOX+ACA and DOX+Se+ACA (Fig. 5). These results indicated that Se and ACA could suppress caspase-3 expressions in the rat brain.

Drugs can lead to oxidative stress by increasing reactive oxygen species such as singlet oxygen, hydrogen peroxide, hydroxyl radical, and superoxide[41,42]. In addition, OS plays an important role in the etiology of many diseases[43,44]. For this reason, we think that this study has made significant contributions to the literature since it investigates the relationship between ROS, NF-κB, and the apoptosis process.

Conclusion

Although DOX does not cross the BBB in rats, this study showed that DOX increased NF-κB and caspase-3 by increasing OS in the brain. Also, this study revealed that DOX could induce pro-inflammation and apoptosis and increase the levels of ROS, PARP1 and TRPM2 channels in the rats' brains. In addition to this damage mechanism, our study showed that combining Se and ACA reduced DOX-induced brain damage. However, apoptosis markers, antioxidant parameters, and other cytokines or signalling pathways related to TRPM2 activation should be investigated molecularly with new studies on brain damage to better understand the effects of DOX on the brain and to investigate the suppressive effects of the combination of Se treatment and TRPM2 channel blockade.

Conflicts of interest

All authors declare no conflicts of interest.

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