



Transient receptor potential channel stimulation induced oxidative stress and apoptosis in the colon of mice with colitis-associated colon cancer: modulator role of *Sambucus ebulus* L.

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Abstract

Background Increased Ca^{2+} entry causes an increase in tumor cell proliferation, apoptosis, cytosolic reactive free oxygen species (cyROS), and mitochondrial ROS (miROS) in tumor cells. The cyROS and miROS stimulate the cation channels, including the TRPA1, TRPM2, and TRPV1. *Sambucus ebulus* L (SEB) (Dwarf Elder) induced both antioxidant and anti-cancer effects in the human hepatocarcinoma and human colon carcinoma cancer cell lines. We investigated the etiology of colorectal cancer and the impact of three channels, as well as the protective effects of SEB on apoptosis, cyROS, and miROS in the colon of mice with colitis-associated colon cancer (AOM/DSS).

Methods A total 28 mice were equally divided into four groups as control, SEB (100 mg/kg/day for 14 days), AOM/DSS, and SEB + AOM/DSS. Azoxymethane/dextran sulfate sodium-induced colon cancer associated with colitis was induced in the AOM/DSS groups within 10 weeks. At the end of the experiments, the colon samples were removed from the mice.

Results The protein bands of caspase – 3, TRPA1, TRPM2, and TRPV1 were increased by the treatments of AOM/DSS. The levels of apoptosis, cyROS, cleaved caspase – 3, and cleaved caspase – 9, as well as the depolarization of the mitochondrial membrane, all increased in the AOM/DSS group. Although they were reduced in the SEB and AOM/DSS + SEB groups by the treatments of SEB, TRPA1 (AP18), TRPM2 (ACA), and TRPV1 (capsazepine) antagonists, the apoptotic and oxidant values were further elevated in the AOM/DSS group by the treatments of TRPA1 (cinnamaldehyde), TRPM2 (H_2O_2), and TRPV1 (capsaicin) agonists.

Conclusion The activations of TRPA1, TRPM2, and TRPV1 channels induced the increase of apoptotic and oxidant actions in the colon cancer cells, although their inhibition via SEB treatment decreased the actions. Hence, TRPA1, TRPM2, and TRPV1 activations could be used as effective agents in the treatment of colon tumors.

Keywords Apoptosis · Colon cancer · Oxidative stress · *Sambucus ebulus* L. · TRP channels

Abbreviations

ACA	<i>N</i> -(<i>p</i> -amylcinnamoyl)anthranilic acid
ADPR	ADP-ribose
Ca^{2+}	Calcium ion
CAP	Capsaicin
CASP/3	Cleaved caspase – 3
CASP/9	Cleaved caspase – 9
CiNN	Cinnamaldehyde
CPZ	Capsazepine
cyROS	Cytosolic reactive oxygen species
miPOT	Mitochondrial membrane potential
miROS	Mitochondrial reactive oxygen species
TRP	Transient receptor potential
TRPA1	Transient receptor ankyrin 1
TRPM2	Transient receptor potential melastatin 2
TRPV1	Transient receptor potential vanilloid 1

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Introduction

Colorectal cancer is the third most common cancer in men and women worldwide [1]. Despite numerous investigations and remarkable developments over the past decades, colorectal cancer still remains one of the leading causes of death worldwide. According to recent statistics, the death rate was 13.4 per 100,000 men and women per year [2]. Chemotherapy, radiation, and surgery are used for the treatment of colorectal cancer. However, these strategies don't just applicable to the management of colon cancer [3]. In the development of colorectal cancer, the excessive generation of reactive oxygen species (ROS) and the induction of apoptosis play important roles [1, 3]. However, the treatments of chemotherapeutic agents induce the generation of apoptosis and ROS for killing the tumor cells. Many researchers have discovered that using two or three treatments, including antioxidants in combination frequently produces better and more effective effects than using just one [4, 5]. In the meantime, healthy cells frequently also die after the treatment of chemotherapeutic agents, and the patient may have adverse effects. Therefore, the investigations of novel antioxidant and apoptotic therapies for colorectal cancer control are crucial.

Metabolites with high oxidative capacities, which are associated with oxidative stress, are implicated in a number of intracellular signal transductions, including Ca^{2+} signaling [6]. Because ROS are known to damage double-stranded DNA and induce an excessive amount of ADP-ribose (ADPR) synthesis, they have been associated with the emergence of cancer [1, 3, 6]. Cancer cells already have elevated levels of cyROS as a result of their reliance on aerobic glycolysis for energy production (the Warburg effect), as well as the fact that oncogenic activation makes them vulnerable to oxidative stress [7]. In addition to ROS, it has been demonstrated that increased Ca^{2+} influx through activated cation channels, particularly those belonging to the transient receptor potential (TRP) superfamily, is linked to the proliferation of cancer cells [8]. One of the main sources of ROS formation in tumor cells is an increase in mitochondrial membrane depolarization (miDEP), which is caused by the loss of mitochondrial membrane potential in colorectal cancer cells [1, 3, 5]. Inducing mitochondrial ROS (miROS) and apoptosis in colorectal cells is caused by the accumulation of Ca^{2+} into mitochondria through the activation of cation channels [9, 10]. TRP channels have not yet been fully understood as to how cyROS and miROS cause apoptosis in the colorectal cancer tissue and cells.

The TRP superfamily contains 28 members in the mammalian cells, and some members of the superfamily such as TRP ankryn 1 (TRPA1), TRP melastatin 2 (TRPM2),

and TRP vanilloid 1 (TRPV1) are activated in normal and tumor cells by miROS [11–13]. In addition to the miROS, cinnamaldehyde (CiNN) activates TRPA1, whereas AP18 inhibits it [14, 15]. Hydrogen peroxide (H_2O_2) and cytosolic ADPR both increase TRPM2 stimulation [16, 17]. TRPM2 is inhibited by N-(p-aminocinnamoyl)anthranilic acid (ACA) [18]. TRPV1 is activated by capsaicin (CAP) and inhibited by capsazepine (CPZ) correspondingly [19, 20]. In various cancer cell types as well as normal cell types, activation of TRPA1, TRPM2, and TRPV1 channels induced apoptosis levels by increasing cleaved caspase – 3 (CASP/3) and cleaved caspase – 9 (CASP/9) activities [21–24]. Thus, in cancer treatments for glioblastoma, breast cancer, and endometrial tumors [22, 24, 25], the channels are targets of therapeutic agents to reduce tumor growth and increase tumor death. Additionally, although there are conflicting findings on the matter [7, 22], antioxidant plant extracts have been shown to enhance the anticancer effects of TRP channels in the colorectal cancer cell lines [5, 13].

One of the most prevalent species that grows in the *Sambucus ebulus* L. (SEB) (dwarf elder) is native to Southern and Central Europe, Northwest Africa, and Southwest Asia [26]. Various substances, including ribosome-inactivating proteins (ebulin), which have been exploited to induce anti-cancer immunotoxins, are present in SEB [27]. The flower also had analgesic, hemorrhagic, and anti-rheumatic activities in experimental animals and humans [26]. In chicken brain extract, antioxidant activity was induced by the SEB treatment [28]. However, the treatment of SEB induced toxic action in the lungs and intestines of mice [29]. Despite various claims of antioxidant activity, anticancer action, and systematic research on SEB's antitumor effect in the tissues of mice with colon cancer, the anticancer effect of SEB has not been clarified in the colon with cancer yet.

The aim of this study was to investigate if the treatment of SEB could stimulate TRPA1, TRPM2, and TRPV1 channels-caused oxidative stress, apoptosis, and cell death in the colon of mice with colorectal cancer. The increase of channel stimulation in the colon tissue may represent a significant pathway for stimulating colorectal tumor cell death.

Materials and methods

Preparation of SEB extract

Between the cities of Kars and Ardahan in the East Anatolia region of Türkiye (41° 15' 02.0" N 42° 21' 34.4" E, 41° 12' 02.5" N 42° 31' 42.7" E), SEB was collected. It was divided into smaller pieces and dried for four days at 40 °C in the shade [28, 30], and then 30 g of the SEB was dissolved in 250 ml of distilled water. The mixture was boiled for

150 min, and then the molecules of steam air were collected in a cooler. Until the treatments, the extract was kept in sterile microtubes at 4 °C in a refrigerator [31].

Animals

In the animal house of the Kafkas University (KAU) Experimental Animal Research Center in Kars, Türkiye, a total of 28 ten-week-old female Balb/c mice weighing 20 ± 2 g were housed at 20 ± 2 °C and 60–70% relative humidity with a 12-hour light/dark cycle. A standard value of food (Bayramoğlu Feed Inc., Erzurum, Türkiye) and water were also available to the animals during the trial. At every stage of the investigation, the intended ethical regulations were upheld (KAU-HADYEK 2022/138), and all animals were handled in accordance with the standards of the National Experimental Animal Research Council of KAU.

Study groups

The mice were equally divided into four groups. The first of which was the control (CNT) group with no treatment but with normal saline gavage. Mice in the second received SEB (100 mg/kg/day) via gastric gavage once a day for two weeks [4, 32]. The azoxymethane (AOM)/dextran sulfate sodium (DSS) combination was applied for the groups of third and fourth [33, 34]. Regarding the fourth group, AOM/DSS-administrated animals received 100 mg/kg/day of SEB for 14 days.

To establish the cancer model, mice received an intraperitoneal injection of AOM (10 mg/kg) dissolved in physiological saline [33, 34]. After 7 days, the animals were provided with drinking water containing 2.5% dextran sulfate sodium (DSS) for 7 days, followed by drinking water for 14 days, and exposed to two more 2.5% (w/v) DSS treatment cycles. The CNT group was given equivalent normal physiologic saline solution.

Fourteen days after the SEB injection, the mice were euthanized. A mixture of 10 mg/kg ketamine (10%) and 80 mg/kg xylazine (2%) was administered intraperitoneally to all animals before their tissues were sampled. After that, half of the colon tissue samples were removed and stored at -80 °C in a freezer for Western blot and oxidant/antioxidant studies. Using an ultrasonic homogenizer, the remaining tissue samples were homogenized before being employed in plate reader assays. Before the agonist activations (20 μ M CiNN, 1 mM H₂O₂, and 10 μ M CAP) of the channels in the plate reader tests, the tissue homogenates of the colon were incubated with the TRPA1 (10 μ M AP18), TRPM2 (25 μ M ACA), and TRPV1 (100 μ M CPZ) antagonists for 30 min [21, 23, 25].

Assays of apoptosis level, CASP/3 and CASP/9 activities

We employed a commercial kit (ApoPercentage, Biocolor Ltd. Co Antrim, United Kingdom) for the apoptosis analysis, which we bought from (Northern Ireland). In the white wells of a microplate reader (Infinite 200 PRO, Tecan Life Sci., Austria GmbH, Groedig, Austria), the ApoPercentage absorbance change as released intracellular dye was detected at 550 nm [35].

With a few minor adjustments, a previously described approach was used for the CASP/3 and CASP/9 activity analyses [36, 37]. Cleavages of the substrates (N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin for CASP/3 and N-Acetyl-Leu-Glu-His-Asp-7-amino-4-Trifluor for CASP/9) were determined using the microplate reader (Infinite PRO 200). The excitation and emission nanometers were kept at 380 and 460 nm, respectively.

For the apoptosis, CASP/3, and CASP/9 assays, we performed a total of 3 trials ($n = 3$). The results of the apoptosis, CASP/3, and CASP/9 experiments were presented as percentage (%) changes following determination of fluorescence units per mg protein.

Cytosolic ROS (cyROS) level detections

A cyROS indicator, the DCFH-DA (ThermoFisher Scientific, Germany) is an uncharged, green-fluorescent dye. DCFH-DA builds up in the cells and is then changed into fluorescent form (DCF) [36, 37]. Colon tissue cell homogenate was treated with 1 μ M DCFH-DA for 15–20 min. The fluorescence variations of DCF were examined in the microplate reader after washing the cells with 1xPBS. Changes in percentage (%) terms were used to express the DCF results.

Measuring the potential of the mitochondrial membrane (miPOT)

The JC-1 (Abcam, Istanbul, Türkiye) is a cell permeable fluorescent dye of miPOT [38]. As indicated in previous reports [36, 37], the concentration of miPOT was determined in the cell homogenate of colon tissue by using the JC-1 dye. The cells were exposed to 2 μ M JC-1 for 15–20 min at 37 °C. They were examined in the plate reader after being washed of the JC-1 (Infinite 200 PRO). Although JC-1 monomers were measured in the cells at 525 nm, the fluorescence intensity of JC-1 aggregates was detected at 590 nm after the cells were excited (488 nm). The JC-1 dye results were reported as a percentage (%) of the control using the emission ratios (590/525) as the source.

Analyses for the levels of lipid peroxidation (LPO), reduced glutathione (GSH), and glutathione peroxidase (GSH/Px)

The levels of GSH and GSH/Px in the colon tissue homogenate of mice were spectrophotometrically (UV-1800, Shimadzu, Kyoto, Japan) measured at 412 nm [14, 21]. A wavelength (532 nm) of the spectrophotometer was used to measure the LPO concentration in the cells. GSH/Px activity is shown as IU/g protein, whereas GSH and LPO levels are displayed as $\mu\text{M/g}$ protein. The total protein test in the cells was performed using Lowry's reagent and a spectrophotometer at 595 nm (Shimadzu UV-1800).

Immunoblotting in colon samples for TRP channels and CASP/3

The Western blot analyses were performed in the frozen mouse colon tissues according to the standard protocols [35]. While the polyclonal antibodies for CASP/3 (a p17-specific polyclonal antibody) (Cat #: sc-7272; and 1: 200) was obtained from Santa Cruz (Istanbul, Türkiye), the antibodies for β -actin (Cat #: mAbcam 8226 and 1: 500), TRPA1 (Cat #: ab68847 and 1: 200), TRPM2 (Cat #: ab11168 and 1: 500), and TRPV1 (an anti-VR1 antibody) (Cat #: ab10296 and 1: 200) were purchased from Abcam (Istanbul, Türkiye). They obtained their secondary antibodies (donkey whole anti-A, rabbit IgG, and HRP-linked) from GE Healthcare (Amersham, UK). The concentration of proteins added was internally controlled using rabbit anti-actin (1:2000). Using ECL Western HRP Substrate, the protein band intensities of β -actin, TRPA1, TRPM2, TRPV1, and CASP/3 were recorded in the G:Box Gel Imagination System (Syngene, UK). The data were reported as relative density.

Statistical analyses

The data were presented as mean and standard deviation (SD) values. The LSD test in the SPSS 22.0 program was used to determine whether there were any individual statistical differences among the groups (IBM, New York, NY, USA). A Student's T test was used to assess the presence and degree of statistical significances, and a presence level of $p \leq 0.05$ was considered to be significant.

Results

The treatment of SEB reduced the AOM/DSS-induced upregulation of TRP channels and CASP/3 expressions

The induction of cyROS and apoptosis, as well as the growth of both normal and malignant cells, depend on the

Ca^{2+} concentration [1, 4, 7, 11]. Ca^{2+} permeable cation channels include the TRPA1, TRPM2, and TRPV1 [21]. In both normal and tumor cells, the activity change of CASP/3 is a key modulator on the induction of apoptosis and the cell proliferation [14, 21, 24]. According to reports [13, 24, 25, 39], there has been an increase in the expression of TRPA1, TRPM2, TRPV1, and CASP/3 in tumor cell lines, including colorectal malignancies. However, their levels of expression in colon samples from mice with colon cancer have not been studied. The first objective of current study was to look into the levels of protein bands (Supp. Figure 1).

In Fig. 1a, the representative bands of the channels were shown. In comparison to the CNT and SEB groups, the protein band relative density concentrations of TRPA1 (Fig. 1b), TRPM2 (Fig. 1c), TRPV1 (Fig. 1d), and CASP/3 (Fig. 1e) were considerably ($p \leq 0.05$) higher in the AOM/DSS group. The TRP channel and CASP/3 expression concentrations, however, were reduced in the SEB + AOM/DSS group following SEB therapy. The channel and CASP/3 band expression concentrations were also lower in the health colon (SEB group) than in the control group.

The injection of SEB reduced the levels of stimulation-induced apoptosis caused by TRPA1, TRPM2, and TRPV1

In many cancer cells, with the exception of colon malignancies, the relationship between three channels and apoptosis was well studied [14, 24, 25]. In order to further our understanding of apoptosis (as second aim of the current study), we investigated the colon cells of mice that had colorectal cancer.

The levels of apoptosis were higher in the AOM/DSS group, while they were lower in the AOM/DSS + SEB group as a result of the SEB therapy ($p \leq 0.05$). In addition, the levels of apoptosis were further elevated in the four groups by the administration of TRPA1 (20 μM CiNN) (Fig. 2a), TRPM2 (1 mM H_2O_2) (Fig. 2c), and TRPV1 (10 μM CAP) (Fig. 2e) agonists, even though these levels were further decreased by the administration of TRPA1 (10 μM AP18) (Fig. 2b), TRPM2 (25 μM ACA) (Fig. 2d), and TRPV1 (100 μM CPZ) (Fig. 2f) antagonists.

The TRPA1, TRPM2, and TRPV1 stimulation-induced CASP/3 and CASP/9 activations were decreased in the colon of mice with colon cancer by the administration of SEB

The decrease of tumor cell numbers is induced by the stimulation of apoptosis. The increases of caspase activities, such as the active CASP/3 and CASP/9, are crucial mediators

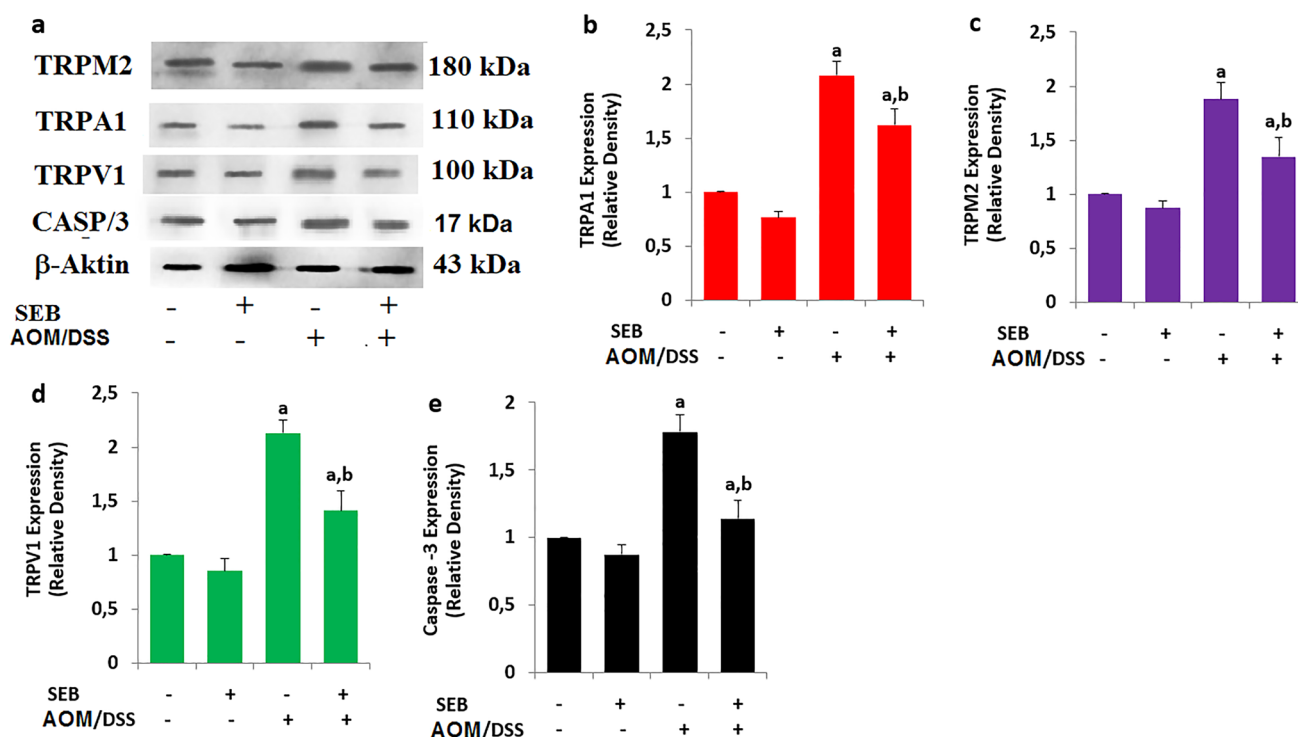


Fig. 1 The administration of SEB (100 mg/kg/day) modulated the AOM/DSS-induced increase of TRP channel and caspase –3 (CASP/3) expressions in the colon of the mice with colorectal cancer. (Mean $\pm$ SD and $n=3$). **a.** The analyses of protein expression levels were visualized in the G:Box Gel Imagination System by using the specific antibodies and antigens, and their representative bands were

indicated in the Fig. 1a. The β -actin protein bands were employed as a control for the identical protein loading. The protein band intensities of TRPA1 (**b**), TRPM2 (**c**), TRPV1 (**d**), and CASP/3 (**e**) were presented in the colon samples by using the columns. ($^a p \leq 0.05$ vs. CNT and SEB groups. $^b p \leq 0.05$ vs. AOM/DSS group)

in the extrinsic and intrinsic apoptosis pathways of tumor cells [12, 14, 40]. After observing the increase of apoptosis in the AOM/DSS groups, we suspected the changes of the CASP/3 and CASP/9 activities in the colon cells after the AOM/DSS administration and the SEB treatments. As third aim of the current study, the CASP/3 (Fig. 3) and CASP/9 (Fig. 4) activities were assayed in the plate reader by using the caspase substrates.

The CASP/3 activities were increased in the AOM/DSS group, although their activities were decreased the AOM/DSS + SEB group by the treatment of SEB. In addition, the CASP/3 activities were further increased in the four groups by the treatments of TRPA1 (CiNN) (Fig. 3a), TRPM2 (H_2O_2) (Fig. 3c), and TRPV1 (CAP) (Fig. 3e) agonists, although their activities were further decreased by the treatments of TRPA1 (10 μ M AP18) (Fig. 3b), TRPM2 (25 μ M ACA) (Fig. 3d), and TRPV1 (100 μ M CPZ) (Fig. 3f) agonists ($p \leq 0.05$).

We observed similar results on the CASP/9 activities in the four groups. The CASP/9 activities were increased in the AOM/DSS group, although their activities were decreased in the AOM/DSS + SEB group by the treatment of SEB. In addition, the CASP/9 activities were further increased in the

four groups by the treatments of TRPA1 (CiNN) (Fig. 4a), TRPM2 (H_2O_2) (Fig. 4c), and TRPV1 (CAP) (Fig. 4e) agonists, although their activities were further decreased by the treatments of AP18 (Fig. 4b), ACA (Fig. 4d), and CPZ antagonists (Fig. 4f) ($p \leq 0.05$).

The treatment of TRP agonists stimulated AOM/DSS-induced increase of miPOT and cyROS: modulator role of SEB

Increasing evidence suggests that the excessive TRP channel activation-induced mitPOT stimulates mitochondria-dependent extrinsic and intrinsic apoptotic pathways in tumor cells [12, 14, 25]. In the glioblastoma and breast cancer cells, the activation of TRPA1, TRPM2, and TRPV1 was found to enhance the levels of mitPOT and cyROS [12, 14, 25]. The matter has not been clarified in colon cancer cells yet. We chose to assess the levels of miPOT and cyROS in the colon cancer cells as the fourth aim of the current investigation after seeing increases in apoptosis and caspase activity. The plate reader assays were used to measure the levels of miPOT (Fig. 5) and cyROS (Fig. 6) using the JC-1 and DCFH-DA dyes, respectively.

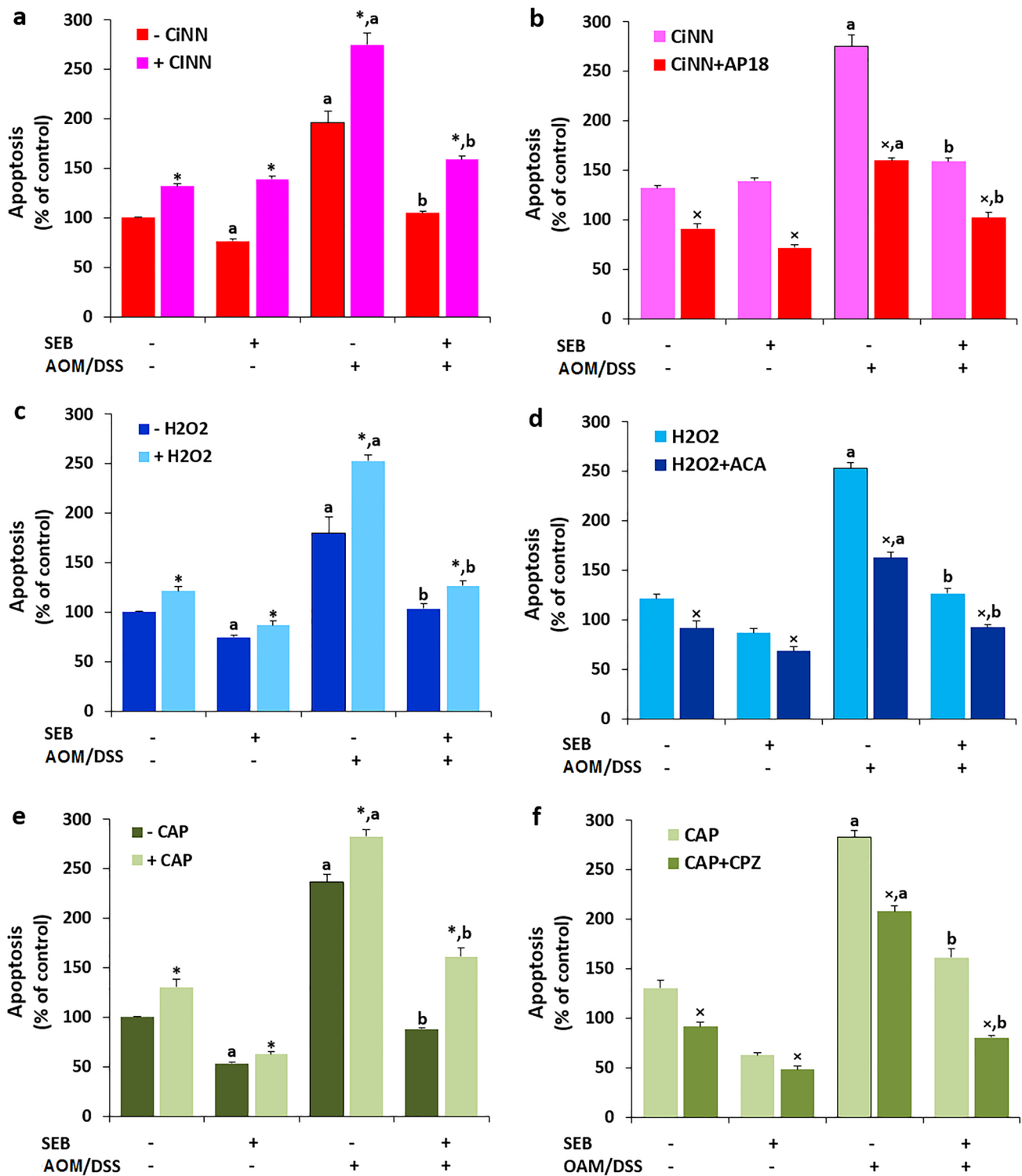


Fig. 2 The TRPA1, TRPM2, and TRPV1 stimulation-induced apoptosis levels were decreased in the colon of mice with colon cancer by the administration of SEB (100 mg/kg/day). (Mean $\pm$ SD and $n=3$). The apoptosis analyses were performed in the colon cells by using the commercial kit (APOpercentage). The apoptosis levels were increased in the AOM/DSS group, although their levels were decreased the AOM/DSS+SEB group by the treatment of SEB. In addition, the apoptosis levels were further increased in the four

groups by the treatments of TRPA1 (CiNN) (a), TRPM2 (H_2O_2) (c), and TRPV1 (CAP) (e) agonists, although their levels were further decreased by the treatment of TRPA1 (AP18) (b), TRPM2 (ACA) (d), and TRPV1 (CPZ) (f) antagonists. ($^a p \leq 0.05$ vs. CNT and SEB groups. $^b p \leq 0.05$ vs. AOM/DSS group. $^* p \leq 0.05$ vs. (-CiNN), (- H_2O_2), and (-CAP) groups. $^x p \leq 0.05$ vs. CiNN, H_2O_2 , and CAP groups

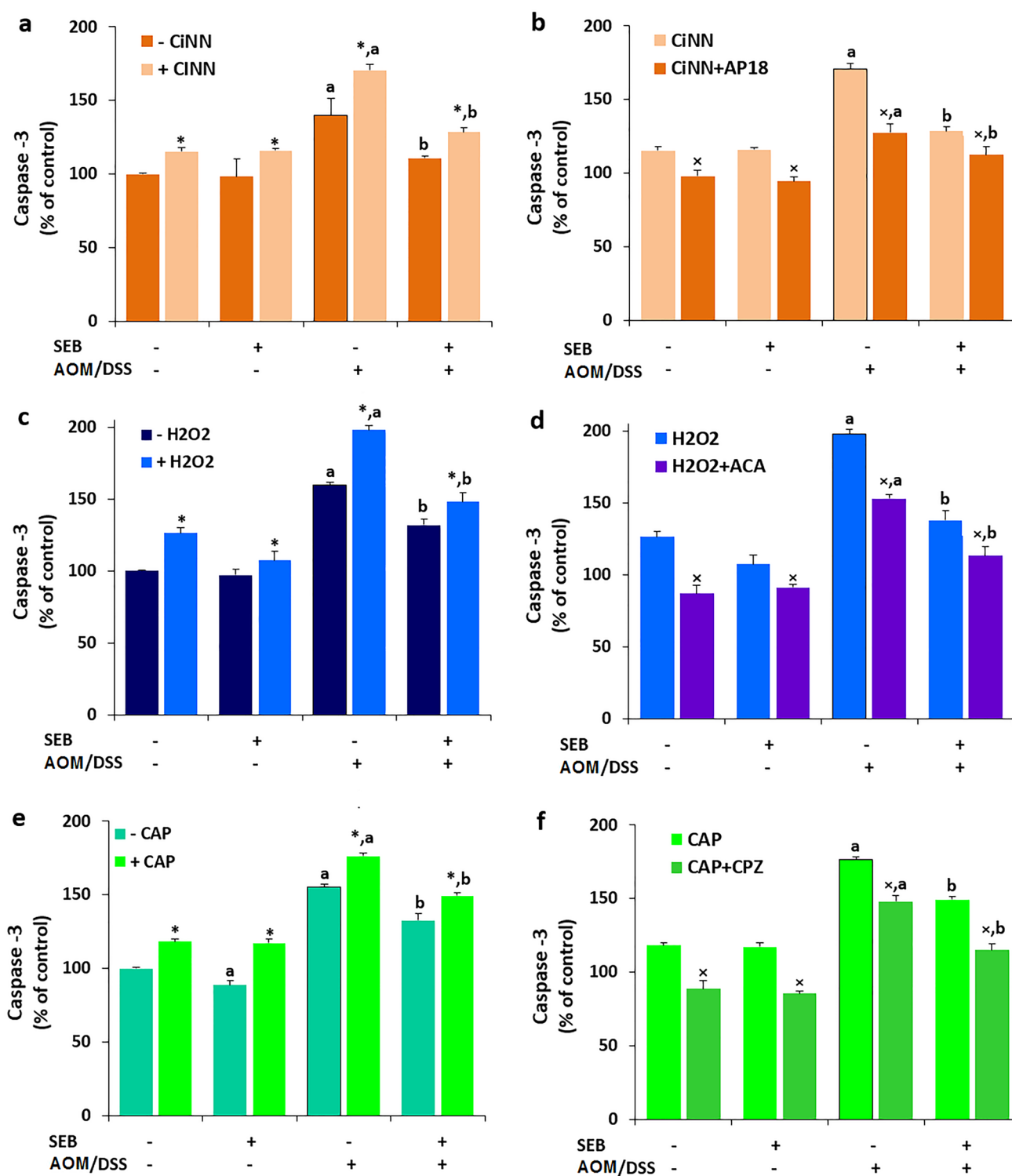


Fig. 3 The TRPA1, TRPM2, and TRPV1 stimulation-induced cleaved caspase – 3 activations were decreased in the colon of mice with colon cancer by the administration of SEB (100 mg/kg/day). (Mean $\pm$ SD and $n=3$). The cleaved caspase – 3 analyses were performed in the plate reader (PRO 200) by using the caspase – 3 substrate. The caspase – 3 activities were increased in the AOM/DSS group, although their activities were decreased the AOM/DSS+SEB group by the treatment of SEB. In addition, the caspase – 3 activi-

ties were further increased in the four groups by the treatments of TRPA1 (CiNN) (a), TRPM2 (H₂O₂) (c), and TRPV1 (CAP) (e) agonists, although their activities were further decreased by the treatment of TRPA1 (AP18) (b), TRPM2 (ACA) (d), and TRPV1 (CPZ) (f) antagonists. (^a $p \leq 0.05$ vs. CNT and SEB groups. ^b $p \leq 0.05$ vs. AOM/DSS group. ^{*} $p \leq 0.05$ vs. (–CiNN), (–H₂O₂), and (–CAP) groups. ^x $p \leq 0.05$ vs. CiNN, H₂O₂, and CAP groups

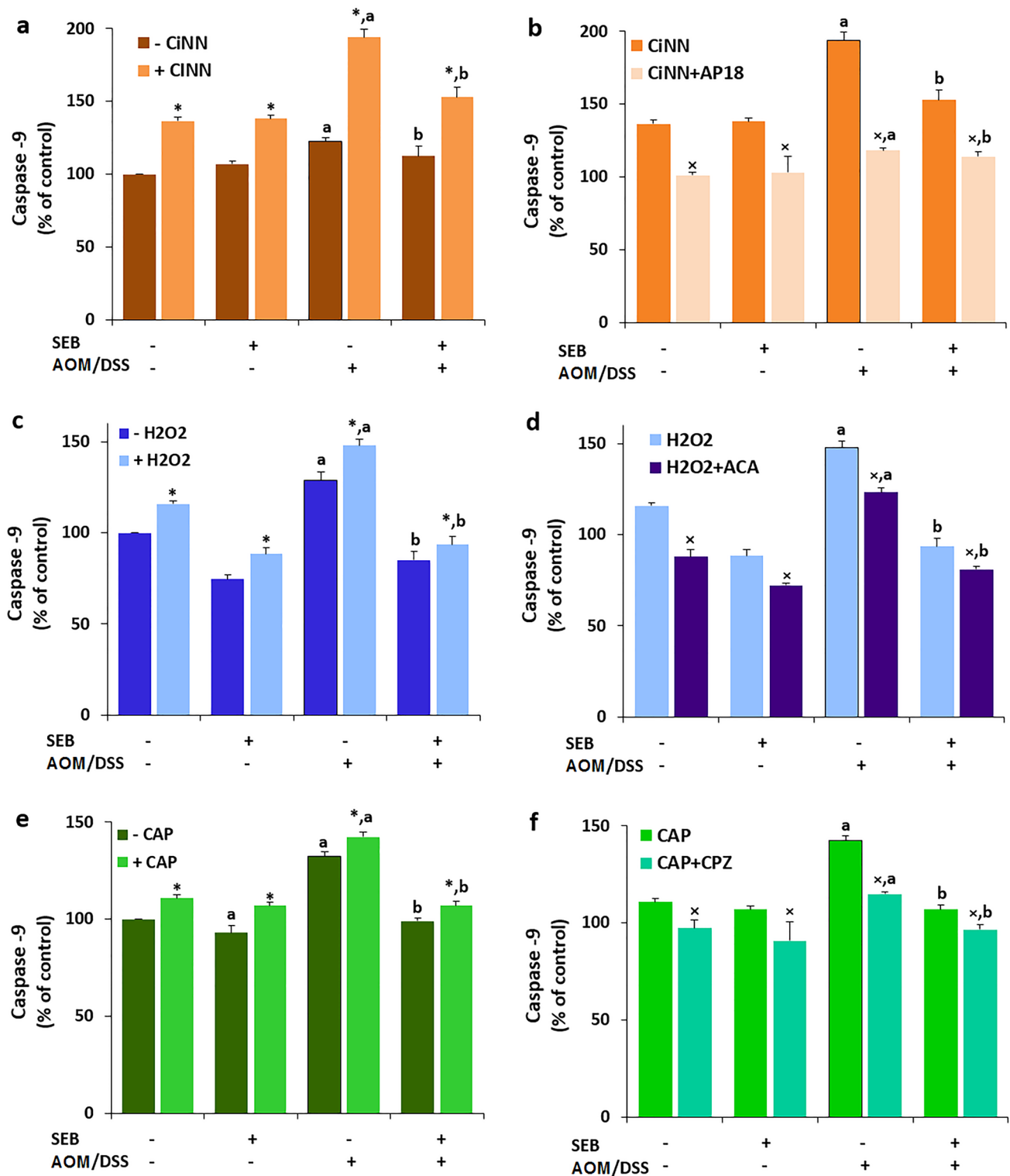


Fig. 4 The TRPA1, TRPM2, and TRPV1 stimulation-induced cleaved caspase -9 activations were decreased in the colon of mice with colon cancer by the administration of SEB (100 mg/kg/day). (Mean $\pm$ SD and $n=3$). The cleaved caspase -9 analyses were performed in the plate reader (PRO 200) by using the caspase -9 substrate. The caspase -9 activities were increased in the AOM/DSS group, although their activities were decreased the AOM/DSS+SEB group by the treatment of SEB. In addition, the caspase -9 activi-

ties were further increased in the four groups by the treatments of TRPA1 (CiNN) (a), TRPM2 (H₂O₂) (c), and TRPV1 (CAP) (e) agonists, although their activities were further decreased by the treatment of TRPA1 (CiNN) (b), TRPM2 (H₂O₂) (d), and TRPV1 (CPZ) (f) antagonists. (^a $p \leq 0.05$ vs. CNT and SEB groups. ^b $p \leq 0.05$ vs. AOM/DSS group. ^{*} $p \leq 0.05$ vs. (-CiNN), (-H₂O₂), and (-CAP) groups. ^x $p \leq 0.05$ vs. CiNN, H₂O₂, and CAP groups

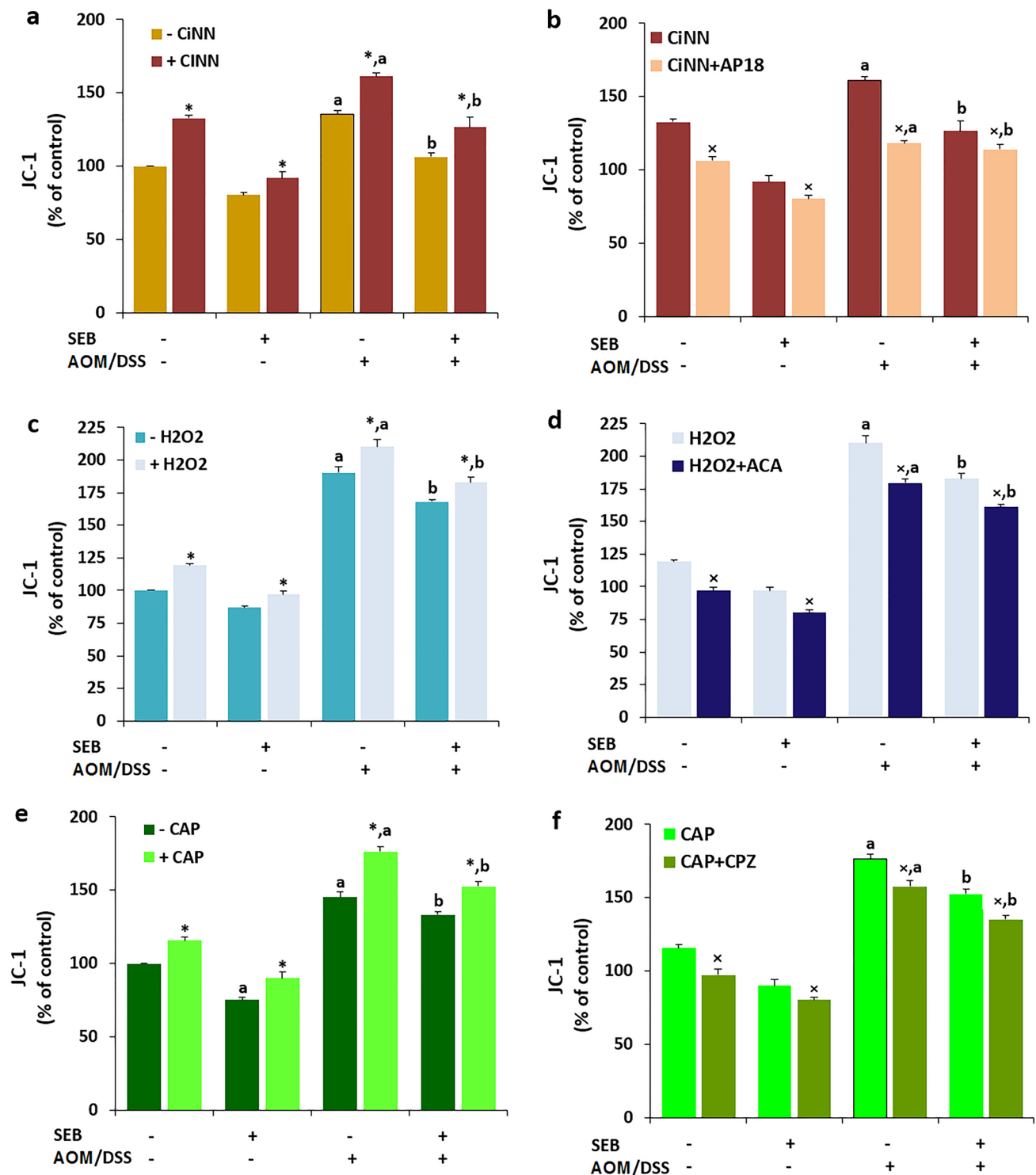


Fig. 5 The treatment of TRP agonists stimulated AOM/DSS-induced increase of mitochondrial membrane potential (miPOT) in the colon cells of mice with colorectal cancer: Modulator role of SEB (100 mg/kg/day). (Mean $\pm$ SD and $n=3$). The miPOT analyses were performed in the plate reader (PRO 200) by using the JC-1 probe. The miPOT levels were increased in the AOM/DSS group, although their levels were decreased the AOM/DSS+SEB group by the treatment of SEB. In addition, the miPOT levels were further increased in the four

groups by the treatments of TRPA1 (CiNN) (a), TRPM2 (H₂O₂) (c), and TRPV1 (CAP) (e) agonists, although their levels were further decreased by the treatment of TRPA1 (CiNN) (b), TRPM2 (H₂O₂) (d), and TRPV1 (CPZ) (f) antagonists. ^a $p \leq 0.05$ vs. CNT and SEB groups. ^b $p \leq 0.05$ vs. AOM/DSS group. ^{*} $p \leq 0.05$ vs. (-CiNN), (-H₂O₂), and (-CAP) groups. ^x $p \leq 0.05$ vs. CiNN, H₂O₂, and CAP groups

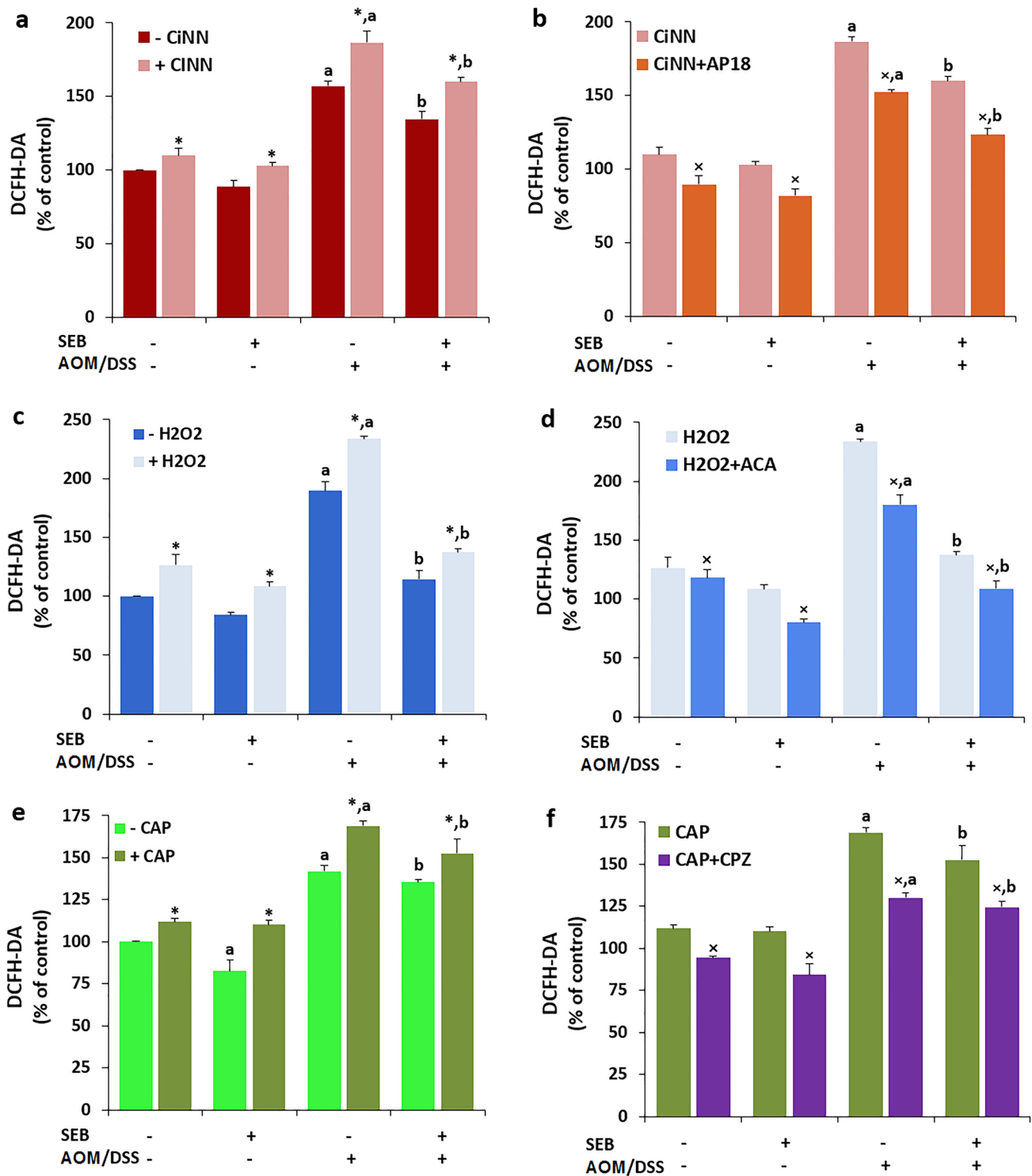


Fig. 6 The treatment of TRP agonists stimulated AOM/DSS-induced increase of cytosolic ROS (cyROS) production in the colon cells of mice with colorectal cancer: Modulator role of SEB (100 mg/kg/day). (Mean $\pm$ SD and $n=3$). The cyROS analyses were performed in the plate reader (PRO 200) by using the DCFH-DA probe. The cyROS levels were increased in the AOM/DSS group, although their levels were decreased the AOM/DSS+SEB group by the treatment of SEB. In addition, the cyROS levels were further increased in the four

groups by the treatments of TRPA1 (CiNN) (a), TRPM2 (H₂O₂) (c), and TRPV1 (CAP) (e) agonists, although their levels were further decreased by the treatment of TRPA1 (CiNN) (b), TRPM2 (H₂O₂) (d), and TRPV1 (CPZ) (f) antagonists. (^a $p \leq 0.05$ vs. CNT and SEB groups. ^b $p \leq 0.05$ vs. AOM/DSS group. ^{*} $p \leq 0.05$ vs. (-CiNN), (-H₂O₂), and (-CAP) groups. ^x $p \leq 0.05$ vs. CiNN, H₂O₂, and CAP groups

Although their levels were decreased in the AOM/DSS + SEB group due to the treatment of SEB, miPOT levels were enhanced in the AOM/DSS group. Although their levels were further decreased by the treatment of AP18 (Fig. 5b), ACA (Fig. 5d), and CPZ (Fig. 5f) agonists, the levels of miPOT were further elevated in the four groups by the treatments of CiNN (Fig. 5a), H₂O₂ (Fig. 5c), and CAP (Fig. 5e) ($p \leq 0.05$).

The levels of cyROS (DCFH-DA) in the four groups showed similar results. The cyROS levels were higher in the AOM/DSS group, whereas the SEB and AOM/DSS + SEB groups' levels were lower due to the SEB treatment ($p \leq 0.05$). Although their levels were further decreased by the treatment of AP18 (Fig. 6b), ACA (Fig. 6d), and CPZ (Fig. 6f) agonists, the levels of cyROS were further elevated in the four groups by the treatments of CiNN (Fig. 6a), H₂O₂ (Fig. 6c), and CAP (Fig. 6e) ($p \leq 0.05$).

Treatment with SEB decreased the AOM/DSS-induced rise in LPO by increasing GSH levels and GSH/Px activity

Increased oxidative stress has been noted in the colon tumor tissues of both humans and laboratory animals [1, 3]. By measuring LPO, the impact of oxidative stress on the oxidation of proteins and lipids within body cells was investigated [6]. The LPO concentration in the CNT, SEB, AOM/DSS, and AOM/DSS + SEB groups was therefore investigated (Table 1). When compared to the CNT and SEB groups, the LPO concentration of AOM/DSS group was higher ($p \leq 0.05$). In the AOM/DSS + SEB group compared to the AOM/DSS group, the concentration of LPO was lower ($p \leq 0.05$). The LPO data provided additional support for SEB's antioxidant effects in the colon sample.

The antioxidants scavenge the cyROS [6]. Mammalian cells contain large amounts of the antioxidant GSH in their cytoplasm. H₂O₂ is changed into water by the catalytic activity of GSH/Px, a powerful enzyme-based antioxidant [6, 35].

Recently, it was shown that GSH depletion and a reduction in GSH/Px affected the activations of TRPA1, TRPM2, and TRPV1 in several cells [35, 41, 42]. We suspected a decrease in the GSH concentration and GSH/Px activity in the colon tissue after noticing an increase in the concentration of LPO in the AOM/DSS group. When compared to the CNT and SEB groups, the GSH and GSH/Px concentrations in the AOM/DSS group were considerably ($p \leq 0.05$) lower. Compared to the AOM/DSS group, the GSH levels and GSH/Px activity were higher in the AOM/DSS + SEB group ($p \leq 0.05$). It appears that the decrease of GSH and GSH/Px in the colon of colon cancer-induced animals was the cause of the AOM/DSS-induced elevation in the levels of LPO.

Discussion

Although there are conflicting results, the antioxidant plant extracts have significant potential in the adjuvant therapy of numerous malignancies via the activating TRP channels [4, 39]. The fact that many antioxidant plant extracts have fewer serious adverse side effects than conventional chemotherapy drugs, in addition to the substantial experimental evidence pointing to a direct antitumor impact of the plant extract via the TRP channel stimulations, significantly supports their use [13, 14]. The inhibitory effect of the channel activations on colon cancer cell survivals is associated to miPOT (potential of the mitochondrial membrane) increase and cyROS overproduction, and is reversed by SEB treatment, TRPA1, TRPM2, and TRPV1 antagonists. Thus, the apoptotic and oxidative responses in the colon sample were diminished via the suppressions of TRPA1, TRPM2, and TRPV1 by the treatments of the SEB and channel antagonists. Therefore, by upregulating the cyROS and LPO while decreasing the antioxidants (GSH and GSH/Px) with the use of channel blockers and SEB, the activation of TRPA1, TRPM2, and TRPV1 will contribute to colon cancer apoptosis.

Table 1 The effects of treatments of SEB (100 mg/kg/day) on the AOM/DSS-induced changes of lipid peroxidation (LPO), reduced glutathione (GSH) levels, and glutathione peroxidase (GSH/Px) activity in the colon tissue of mice with colorectal cancer cells

Parameters	CNT	SEB	AOM/DSS	AOM/DSS + SEB
LPO ($\mu\text{mol/g protein}$)	19.60 $\pm$ 1.26	18.50 $\pm$ 1.43	24.10 $\pm$ 0.29 ^a	20.20 $\pm$ 1.26 ^b
GSH ($\mu\text{mol/g protein}$)	9.43 $\pm$ 0.38	10.00 $\pm$ 1.14	6.83 $\pm$ 0.69 ^a	8.56 $\pm$ 0.46 ^{a,b}
GSH/Px (IU/g protein)	21.30 $\pm$ 0.22	21.80 $\pm$ 0.77	14.80 $\pm$ 1.91 ^a	18.40 $\pm$ 0.99 ^{a,b}

^a $p \leq 0.05$ vs. CNT and SEB

^b $p \leq 0.05$ vs. AOM/DSS

(Mean $\pm$ STD and $n = 7$)

We first investigated the potential involvement of the SEB treatment on the channels in the colon cancer by utilizing the antagonists of TRPA1 (AP18), TRPM2 (ACA), and TRPV1 (CPZ). Three channels play a role in the control of cell division and apoptosis [21–24], and they are currently thought to be attractive cancer therapeutic targets, particularly for colon cancer. The channel expressions in several cell lines, including colon cancer cells were reported [13, 43]. The expression of the TRPA1, TRPM2, and TRPV1 protein bands in the colon of mice with colon cancer has been described here for the first time. More notably, we indicated that, despite being duplicated by the treatments of AP18, H₂O₂, and CAP, the effect of TRPA1, TRPM2, and TRPV1 antagonists on apoptosis was decreased by these substances. These findings taken together imply that the activation of TRPA1, TRPM2, and TRPV1 may play a role in AOM/DSS-induced cell live.

The normal generations of cyROS and miROS are physiological processes such as cell proliferation and differentiation in normal cells. However, high generations of cyROS and miROS via the excessive Ca²⁺ influx stimulate a series of mitochondria-associated events leading to apoptosis [7, 11]. The Warburg effect and the vulnerability of cancer cells to oxidative stress make it interesting to note that cancer cells already have elevated levels of cyROS as a result of their reliance on aerobic glycolysis for energy production [7]. The observation that many chemotherapeutic agents may be specifically hazardous to tumor cells due to increased oxidative stress and enhanced cyROS and miROS generation, which in turn induces the apoptosis of cancer cells, has further highlighted the connection between cyROS and cancer [12–14]. In the current study, we have shown that TRPA1, TRPM2, and TRPV1 stimulators were able to exert pro-apoptotic effects selectively increased miPOT, cyROS, CASP/3, and CASP/9 productions in AOM/DSS cells, thus suggesting that cyROS overproduction might be implicated in three channel-caused apoptosis. Interestingly, the effects of the channel activations on miPOT, cyROS, and apoptosis were abolished in colon cells by the treatments of TRPA1, TRPM2, and TRPV1 antagonists, and the results are suggestive of involvements of such channels in the pro-apoptotic action of mitochondrial Ca²⁺ influx. However, the apoptosis, miPOT, and cytROS levels were decreased in the SEB and AOM/DSS + SEB groups by the treatment of SEB. Hence, the SEB treatment protected the apoptotic and oxidant actions of the channels in the healthy cells (SEB group), but also in the AOM/DSS group. It seems that SEB acted adverse action via the inhibition of three channels on apoptosis in the AOM/DSS group. Similarly, the apoptotic and caspase activator roles of TRPA1, TRPM2, and TRPV1 were reported in the colorectal and glioblastoma cancer cell lines [12, 14]. The treatments of antioxidant plant extracts

decreased the miPOT, apoptotic and caspase activator roles of three channels in the cancer cells, including colorectal cancers [44–46].

The cytROS and mitROS generations are physiological products necessary for many physiological processes, such as phagocytic activity and mitochondrial function, but if their generations are not strictly controlled by the enzymatic and non-enzymatic antioxidant, they can become harmful to normal cells [6, 11]. Two components of the antioxidant redox system, GSH and GSH/Px, have a significant antioxidant role [6, 47]. The main scavenging antioxidants for the elimination of H₂O₂ and hydroxyl radicals from cells, including colon cells, are non-enzymatic antioxidant GSH and enzymatic antioxidant GSH/Px [47, 48]. Recent reports [35, 41, 42] discuss the effects of GSH and GSH/Px depletions on the activations of TRPA1, TRPM2, and TRPV1 in various cells. The SEB was treated, which resulted in an increase in the GSH concentration and GSH/Px activity. This may be explained by an increase of cyROS production brought on by cancer in the mitochondria, which may have an impact on the production of H₂O₂ during colon oxidative stress.

Conclusion

Our data indicated that the activations of TRPA1, TRPM2, and TRPV1 inhibit the live of mice colon cancer cells mainly through the stimulations of pro-apoptotic mechanisms such as CASP/3 and CASP/9. Increases in miPOT and cyROS are linked to the inhibitory effects of channel activations on the apoptosis of tumor cells. However, they are mimicked by the treatments of SEB, TRPA1, TRPM2, and TRPV1 antagonists, thus suggesting that such channels might be, at least in part, involved in their actions. In view of the channel stimulator actions, we hypothesize that the channel stimulators may be promising anti-cancer therapeutic agents, both for prevention and as a curative medicine. However, the treatment of SEB decreased the oxidant and apoptotic actions of the channel blockers via upregulating the LPO and cyROS but downregulating the GSH concentration and GSH/Px activity in the normal colon and colon of mice with colon cancer. It seems that the treatment of SEB protected the healthy colon from oxidative stress and apoptosis but it acted adverse action on the cancer in the colon.

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Author contributions MMK: Animal experiments, Methodology, Formal analysis, Data curation, Resources, Funding acquisition, Project administration. İK: Animal experiments, Methodology, Formal analysis, Data curation, Resources, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration. MN: Methodology, Formal analysis, Writing – review & editing, Supervision.

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Data availability The data used to support the findings of present study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval The study was approved by Kafkas University (KAU) Experimental Animal Research Center in Kars, Türkiye (Protocol number: KAU-HADYEK 2022/138), and all animals were handled in accordance with the standards of the National Experimental Animal Research Council of KAU.

Informed consent Not applicable.

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