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Degenerative effect of Ankaferd Blood Stopper® on mice peripheral sensory neurons *in vitro*

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Abstract

Ankaferd Blood Stopper® (ABS) is a licensed medicinal herbal extract that ensures effective hemostasis on external, internal, postoperative and dental bleeds. Dorsal root ganglia (DRG) harbor cell bodies of peripheral sensory neurons. DRG neurons receive peripheral information and regularly send projections to nuclei in the brainstem and the spinal cord. These neurons play critical roles in neural development. Neuronal dysfunctions were reported due to ABS use in surgical interventions. The purpose of this experiment was to investigate the degenerative effects of the ABS on mice DRG cells in vitro. DRG neurons were isolated from adult mice and cultured in vitro. The neurons were incubated with various concentrations of ABS for 24 h. At the end of 24 hours, under fluorescence microscopy, cell viability was determined with the fluorescent dye calcein-AM, and cell death was determined with the fluorescent dye propidium iodide. The behavior of the cells was displayed with time-lapse video microscopy for 12 hours from the time of treatment. ABS killed both neurons and non-neuronal cells via necrosis at a concentration of 25 µl/ml or more. ABS has the degenerative effect on mice peripheral sensory neurons, depending on the ABS level.

Key words: *DRG neurons, Ankaferd Blood Stopper, degeneration, cell death, in vitro.*

Introduction

Ankaferd Blood Stopper® (Immun Drug Cosmetic Co., Istanbul, Turkey) (ABS) is a topical hemostatic agent composed of five different herbal extracts [30]. In the 100 ml of the ABS solution, there are namely 9 mg of *Glycyrrhiza glabra* (dried leaf extract), 5 mg of *Thymus vulgaris* (dried grass extract), 7 mg of *Alpinia officinarum* (dried leaf extract), 6 mg of *Urtica dioica* (dried root extract), and 8 mg of *Vitis vinifera* (dried leaf extract) [8]. It has been used for centuries in Turkish traditional medicine as a hemostatic agent [4]. ABS has been approved by the Turkish Ministry of Health as a topical hemostatic agent for the management of dermal, external, internal, postoperative, and dental bleeding [14,26,31]. It has been manufactured in buffer, ampoule and spray forms since 2007 [5]. The mechanism of action of ABS is the formation of an encapsulated protein network providing focal points where vital erythrocytes aggregate [10]. ABS is used in tens of thousands of patients in many clinics across Turkey and all over the world. ABS is clinically used in gastrointestinal bleedings [17,23], urologic surgeries [15], tonsillectomy [29], acute anterior epistaxis [18], partial nephrectomy [14]. However, neu-

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ronal dysfunctions were reported due to ABS use in surgical interventions [25].

Dorsal root ganglia (DRG) is a major component in the spinal cord to relay neural signaling from peripheral sensory systems to the brain, thus playing critical roles in neural development, neuropathic pain, and neurodegeneration in human diseases [12,33]. Sensory neuropathies characterized by first involvement of sensory neurons in dorsal root ganglion are severe disorders [1].

Caspases are a family of intracellular cysteine proteases and are well known for their roles in regulating apoptosis and neurodegeneration. Among different caspases, caspase 6 is of particular interest because it is localized in axons and involved in axonal degeneration [11]. Caspase 6 is highly expressed in the neuronal axons of primary sensory neurons in DRG [21], suggesting a potential role for this caspase in pain control. Caspase 6 can adjust microglial activation and inflammatory pain hypersensitivity in male mice [2]. Caspase 3, together with the other effector caspases, break up into pieces cellular structures by cleaving particular substrates inducing cell death. Recent researches indicate that in addition to its critical role in neuronal death by apoptosis, caspase 3 carries through other functions, such as dendrite pruning and synaptic plasticity functions [27].

Peripheral nerves may be exposed to the degenerative risk of ABS. The purpose of this experiment is to investigate the degenerative effects of the ABS on mice DRG cells *in vitro*.

Material and methods

Drugs and chemicals

Ankaferd Blood Stopper was purchased from Immun Drug Cosmetic Co. Ltd (Istanbul, Turkey). Ketamine Hydrochloride Injection was purchased from Pfizer (İstanbul, Turkey). Neuro Basal A Medium (NBA), B27 supplement, Glutamax, Calcein AM was obtained from Life Technologies (Grand Island, NY, USA). Antibiotic Antimycotic Solution, RPMI-1640 Medium, Fetal Bovine Serum, Hanks' Balanced Salt solution, Poly-L-lysine hydrobromide, Laminin, Trypsin solution, Trypsin inhibitor, Collagenase, Deoxyribonuclease (DNAz), Propidium iodide, Dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental animals

All animal protocols were approved by the local animal experiment ethics commission, in accordance with the European Community Council Directive 86/609/ECC for the care and use of laboratory animals. Experiments were performed on six- to eight-week-old Male Balb-C mice purchased from the Yüzüncü Yıl University Experimental Animal Production and Research Center. Every efforts were made to minimize the suffering of animals and to reduce the number of animals used. Mice were killed by cervical transection under deep anesthesia after an intraperitoneal injection of ketamine (100 mg/kg, Ketalar, Pfizer).

Preparation of primary culture of mice DRG neurons

DRGs (thoracic and lumbar, 15-20 per mice) were obtained from mice. DRG neurons were then dissociated enzymatically and mechanically by trituration as previously reported [24]. Briefly, DRGs were transferred to the Neuro Basal-A medium supplemented with 2% B27 (Invitrogen) containing 2 mM Glutamax-I (Invitrogen), 100 units of penicillin, 100 mg streptomycin and 250 ng/ml amphotericin-B (Sigma) and 100 U/ml collagenase (Sigma). They were incubated at 37°C, 5% CO_2. The neurons were seeded on 35 mm in diameter glass-bottomed petri dishes (WPI, Sarasota, USA) which had been previously coated with poly-l lysine (Sigma) (1.8 mg/cm^2, 3 h at room temperature) and then laminin (Sigma) (40 ng/mm^2, one-night at 37°C). The preparations were saved in the incubator for 2 h to let the neurons attach to the bottom of the dish, after which they were gently washed with warm NBA-B27 to remove unattached cells and finally filled up NBA-B27 and placed to the incubator. Then DRG neurons were maintained at 37°C in 100% humidity and supplied with 5% CO_2 for 48 h [24].

Verification of cell viability

Primary mice DRG cultures consist of a heterogeneous population of cells that includes sensory neuronal cells, proliferative supporting cells and non-neuronal cells. Cultured neurons started to regenerate axons within hours of incubation. After 48 h, the cells were viewed on a computer-controlled inverted microscope with a stage incubator that pro-

vides a physiological atmosphere (Cell Observer, Carl-Zeiss, Oberkochen, Germany), their coordinates were recorded, and corresponding marks were put with a pen on the lid of the dish to use as references for the following procedures. To make sure that only viable cells were evaluated, propidium iodide dye (Sigma) was added to the medium (7.5 µM) and absence of nuclear staining in neurons was confirmed [24].

ABS preparing and treatment to cell culture

In the experiments, 1 milliliter ampoule forms of ABS were used. If ABS is directly added to neuron culture in petri dishes; the medium will be blurred, and it will be difficult to see the cells under microscopy. Therefore, ABS diluted with the culture medium to final concentrations of 100 µl/ml, 50 µl/ml, 25 µl/ml, and 10 µl/ml. The ABS diluted was vortexed for 30 s, filtered with Whatman filter (0.2 µm pore size) to remove solid particulates. Cell culture medium was replaced with ABS diluted at concentrations of 100 µl/ml, 50 µl/ml, 25 µl/ml, and 10 µl/ml, and the cells were incubated with diluted ABS for 24 hours.

Live cell observer microscopy

Cell cultures were put down on a computer-controlled Cell Observer microscopy system (Zeiss) where multiple positions of the preparation could be imaged at indicated time points while physiological conditions could be composed of the neurons with an integrated stage-top incubator. The bright field pictures of the neurons were digitally captured every 5 minutes for 12 hours. After 24 hours, to observe the death and survival of the neurons, propidium iodide (7.5 µM) and calcein AM (1 µM) dyes, respectively, were added to the culture medium. The bright field and fluorescence images of cells were observed. Axiovision 3.0 software was used during these microscopic works.

Study groups

Before designating the study groups, preliminary research was done. To determine the smallest toxic dose, different concentrations of ABS (100 µl/ml, 50 µl/ml, 25 µl/ml, 10 µl/ml) were assayed on the cells. Each concentration was tested on at least 175 cells. Finally, study groups were created taking into account the 25 µl/ml ABS dosage.

In the control group, physiological saline (PS) (25 µl/ml) was added to the neuron culture medium.

In the ABS experiment group, 25 µl/ml ABS was added to the neuron culture.

In the ABS+Caspase-3 inhibitor (C3I) experiment group, firstly 100 µM C3I, one hour later 25 µl/ml ABS were added to the neuron culture.

In the ABS+Caspase-6 inhibitor (C6I) experiment group, firstly 100 µM C6I, one hour later 25 µl/ml ABS were added to the neuron culture.

Statistical analysis

Statistical significance was presented as count and percentages of living and dead cell populations. Z test was used for comparison of two groups. The statistical significance level was considered as 5%, and MINITAB (ver. 14) statistical program was used for the statistical computations.

Results

Preliminary studies

Neuronal cell mortalities were determined by the red fluorescence stained nucleus by propidium iodide uptake test (Figs. 1 and 2). Neuronal cell viability was determined by the green fluorescent staining by Calcein-AM uptake assay (Figs. 2.I.c-d).

In the 100 µl/ml ABS treatment, before treatment, there were 208 live neurons. After 24 hours of treatment, it was determined that all the cells had died and disintegrated. In the 50 µl/ml ABS treatment, before treatment, there were 193 live neurons. After 24 hours of treatment, all the cells died and lost their neurite extensions via disintegration. In the 25 µl/ml ABS treatment, initially there were 216 cells, all cells died, but the cells did not lose their integrity and extensions. 10 µl/ml ABS treatment of the cells resulted in approximately 33% cell death (88 neurons from 264). The difference of 4 different densities of the ABS from the PS group was significant ($p < 0.05$).

Control (Physiological Saline Treatment) group

Before applying PS, there were 249 neurons in this group. After applying PS, it was observed that 5 cells become dead and 244 cells become alive (Fig. 2.I.b-c). A video of the cells was recorded for 12 h. The neurons maintained their viability and neurite outgrowth (Suppl. Video 1).

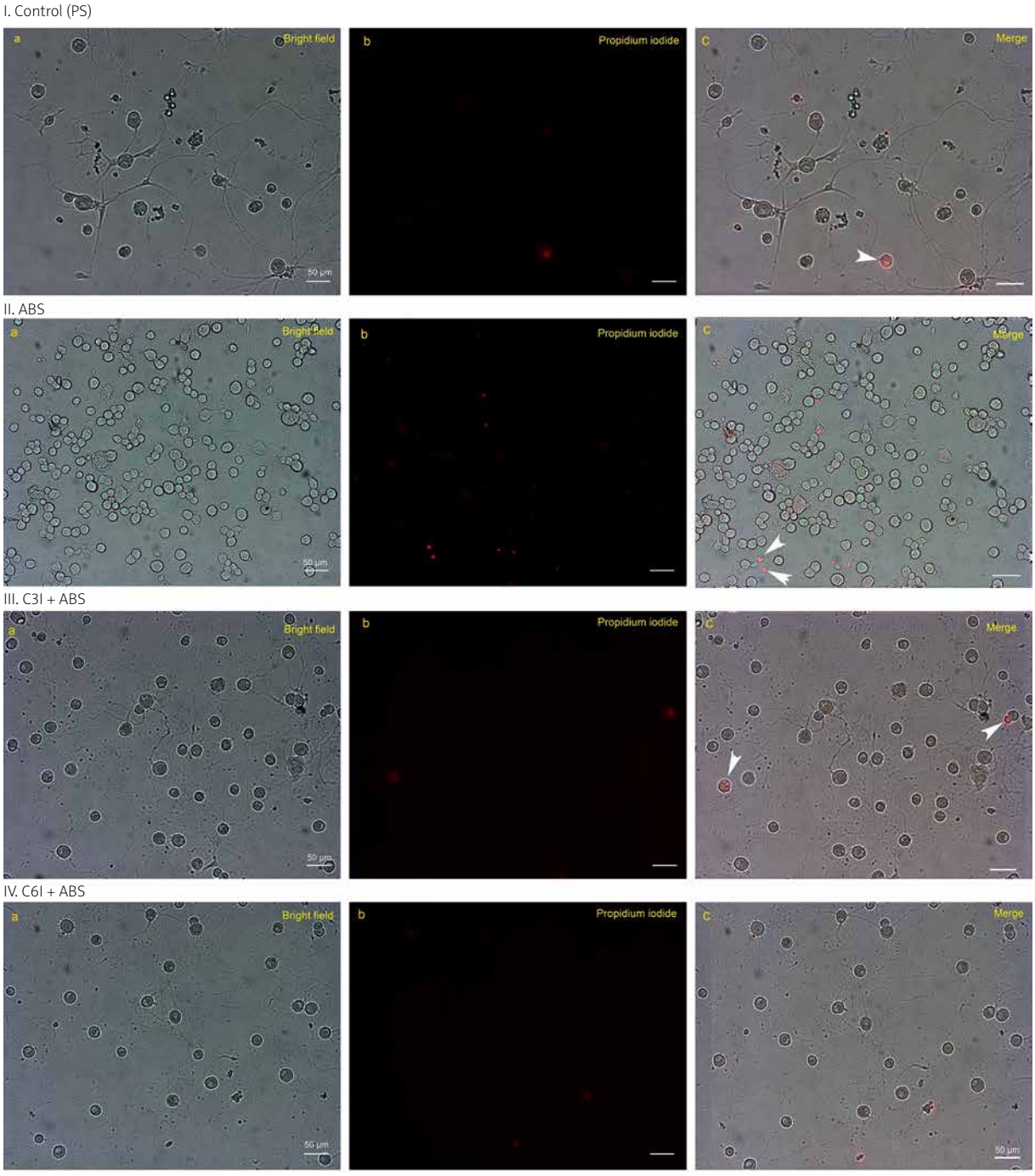

Fig. 1. Bright field, fluorescent and merged microscopic images of DRG neurons before treatment. The cells with red fluorescence image (shown by the white arrow) are dead cells. It was confirmed that other cells that did not receive the propidium iodide dye were alive. Scale bar = 50 µm.

Ankaferd Blood Stopper treatment group

Before ABS treatment, there were 243 live neurons. The neurons which were detected alive before the ABS treatment died completely after 24 hours of treatment (Fig. 2.II.b-d). A video of the cells was recorded for 12 h. It was observed that the cells were quickly killed via necrosis by the ABS (Suppl. Video 2).

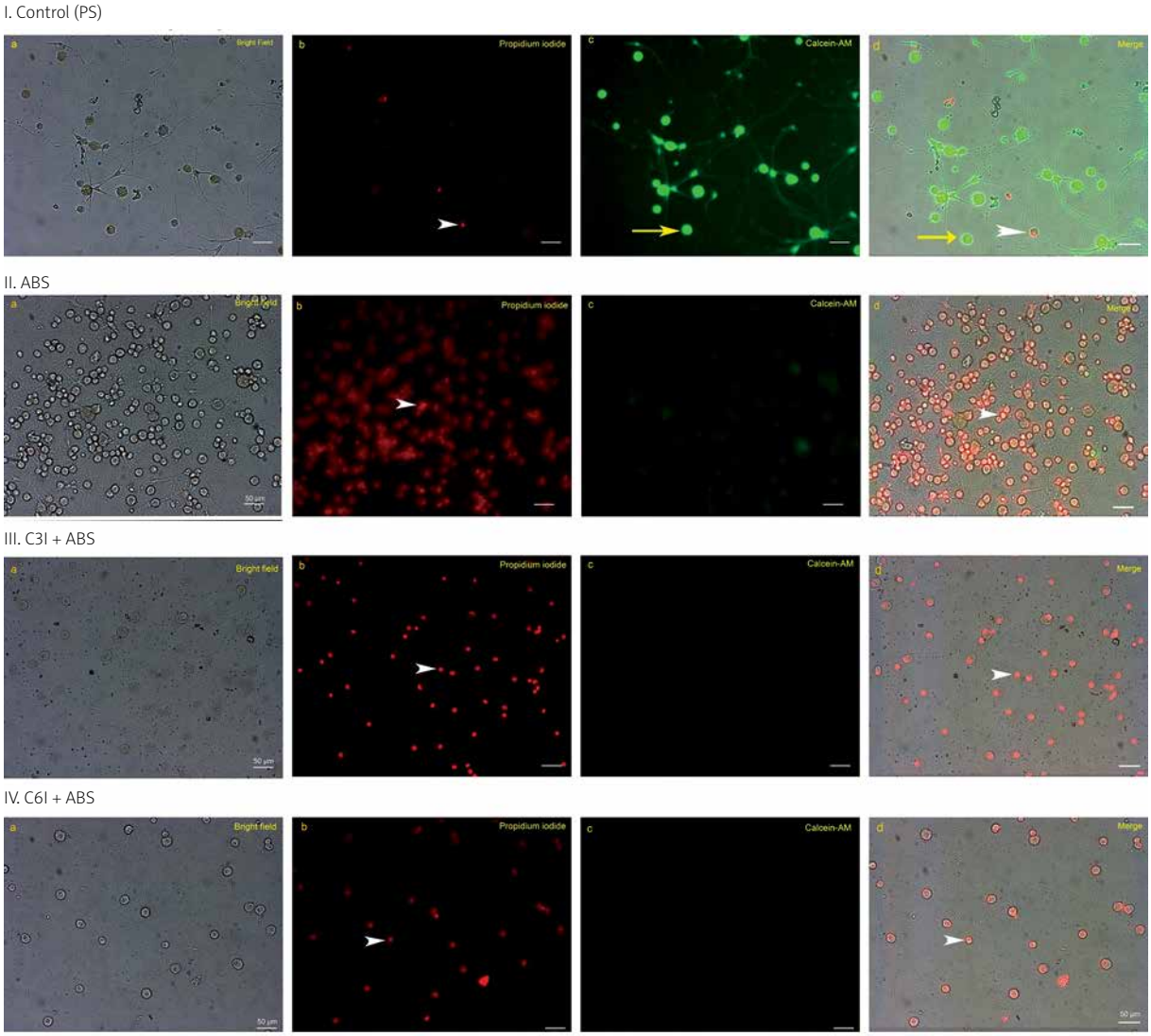

Fig. 2. Bright field, fluorescent and merged microscopic images of DRG neurons after treatment. The cells with red fluorescence image (shown by the white arrow) are dead cells. The cells with green fluorescence image (shown by the yellow arrow) are live cells. Scale bar = 50 µm.

C3I and ABS treatment group

Before treatment, there were 231 live neurons. After 24 hours of treatment, all the neurons and the non-neuronal cells were observed dead completely (Fig. 2.III.b-d).

C6I and ABS treatment group

Before treatment, there were 175 neurons. After treatment, all the cells detected as alive before treatment were observed dead completely after 24 hours (Fig. 2.IV.b-d).

Discussion

Hemostasis is compulsory to protect a clean operative field and to avert blood loss and postoperative hemorrhage. Intraoperative hemostasis in the course of neurosurgical procedures is one of the most important features of intracranial surgery [9]. It is a challenging task to provide hemostasis in clinical bleeds. It requires extensive effort to stanch bleeding in difficult, traumatic injuries [13]. As an adjunct to bleeding control, topical hemostatic agents have long been used in all surgical disciplines. The ideal hemostatic agents also should be free of cytotoxicity [32].

ABS has been used as a hemostatic agent in surgical clinics since 2007. In the prospectus of the bulb form, if pure ABS is used topically in the bleeding zone, it is declared that there will be no adverse effects [5].

In a preliminary study, in the 100 µl/ml ABS treatment, all the cells disintegrated and disappeared from the eye. In the 50 µl/ml ABS treatment, all cells died, their neurite extensions disintegrated and disappeared. In the 25 µl/ml ABS treatment, all cells died, but the cells did not lose their neurite extensions. In the 10 µl/ml ABS treatment, there were 264 live neurons before applying ABS. After applying ABS, it was observed that 88 cells became dead and 176 cells became alive. As the ABS concentration increased, the toxicity on the cell increased, and as the ABS concentration decreased, the toxicity on the cell decreased.

In the control (PS) group, 244 out of 249 neurons survived. In the video display, neurons maintained neurites outgrowth. The cells maintained their vitality and their physiological functions. It was seen that the cells lived on the 24th hour of fluorescent microscopic imaging.

In the ABS test group, after ABS treatment, initially, the neurons did not extend their neurites due to cytotoxic stress. Neuron deaths began in 2 hours, and was completed in 6 hours. Fluorescence microscopic imaging performed 24 hours after ABS treatment showed that all cells (sensory neurons, fibroblast, Schwann cells, and satellite cells) died. The toxic effect of ABS on neuroglia such as Schwann cells and satellite cells was observed to be higher and faster when compared with neurons. This difference was found to be statistically significant ($p < 0.05$).

Similar results to the ABS group were obtained in the caspase-3 inhibitor + ABS test group and caspase-6 inhibitor + ABS test group. All the cells died after 24-hour treatment as determined by microscopic imaging. In this research, we used caspase 3 inhibitor in the third group and caspase 6 inhibitor in the fourth group. However, the caspase inhibitors could not prevent cell deaths. It was detected that cells were not killed by caspase-3 or caspase-6 dependent apoptotic cell death. The difference between all the experiment groups and control group was found to be statistically significant ($p < 0.05$).

Mihmanli *et al.* studied that the cytotoxic effects of different doses of ABS on human lymphocytes were tested at 24 and 48 h. All the ABS concentrations except its 1% dilution caused the releasing of LDH and cytotoxicity. Increases in the level of LDH were observed after treatment with 50%, 25%, 12.5%, and 5% dilutions of ABS when compared with negative control groups. This increase was found to be statistically significant ($p < 0.05$) [19]. In our study, the cytotoxic effect was observed after treatment with 10 µl/ml (1%), 25 µl/ml (2.5%), 50 µl/ml (5%), and 100 µl/ml (10%) ABS.

Odabas *et al.* reported that ABS was cytotoxic to human pulp fibroblasts by MTT assay. The sensitivity of cytotoxicity to human pulp fibroblasts depended on the concentration of ABS. More diluted concentrations exhibited less cytotoxic characteristics when compared with more concentrated forms [22]. In our research, we obtained similar results. The toxicity increases at high density (100 µl/ml, 50 µl/ml and 25 µl/ml), and decreases at low density (10 µl/ml).

Mumcuoglu *et al.* noted that in K-562 cells, a small dose of ABS triggers apoptosis at 1 h. But at a high dose, profoundly higher apoptosis was observed in 6 and 24 h samples. Moreover, in Jurkat cells, a small dose of ABS generated the same amount of apoptosis at all-time points. However, this was true at a high dose, 6 and 24 h. Samples showed higher levels of apoptosis as compared to other time points. To analyze the apoptotic mechanism generated by ABS treatment in leukemic cells, critical genes that are located in apoptotic pathways were investigated. Bax, Bcl-2, and p21genes were analyzed by qRT-PCR at four different time points. Bax and Bcl-2 expression results did not show any changes upon ABS treatment. This finding supports the idea that there is a link between increases in PAR1 and p21 expression and apoptosis that was observed in Jurkat cells [20]. In our study, at higher doses, neuronal deaths started immediately after ABS treatment, the cells and their neurites disintegrated. ABS killed neuronal and non-neuronal cells via inducing necrosis which may be caused by anoikis. Literature information and the results of our study suggest that ABS causes cell death via apoptosis in low density, cell death via necrosis in high density.

Emes *et al.* studied the primary fibroblast culture with ABS. Cells were isolated from the hindfoot extensor tendons of Sprague-Dawley rats. Emes *et al.* declared that cell viability was significantly higher in

the control group when compared to the ABS group. At the end of 24 hours, scanning electron microscopy (SEM) analysis showed that most of the cells degenerated in the ABS group. DNA synthesis was higher in the control group than in all of the experimental groups in the first 24 hours. At 72 hours, the control group only had a higher DNA synthesis than the ABS-treated group. Emes *et al.* have observed that cell proliferation was significantly greater in the control group than the ABS group on the first day. Their findings of a low cell number, cell viability, and cell proliferation in the ABS-treated group showed that the ABS has adverse effects on tissue healing to a certain extent [6]. In our study, the number of live cells in the ABS group was found to be minuscule when compared to the control group. It has been determined that survivors of the neurons in the ABS group cannot perform their physiological functions due to toxic stress.

Siironen *et al.* using the immunohistochemistry method, revealed the formation of type I collagen in nerve regeneration. Collagen formation and accumulation takes place in the damaged tissue in response to a traumatic incident. Fibroblasts aggregate in the damaged area and produce collagen after a severe nerve trauma [28]. In our study, we have observed that ABS killed not only neuronal cells but also non-neuronal cells such as Schwann cell and fibroblasts. Thus, ABS inhibits peripheral nerve regeneration due to fibroblast and Schwann cell toxicity.

Evren *et al.* studied ABS in the cartilaginous tissue. They observed a significantly increased incidence of fibrosis and necrosis in the auricular cartilage following ABS administration. Besides, histopathological analysis showed a statistically significant difference in the rate of fibrosis, necrosis, foreign body reaction, and cartilage degeneration between the groups ($p < 0.05$). This difference can be attributed to the fact that ABS remained in the cartilage tissue without draining for an extended period [7]. These results supported the necrotic effect of ABS on sensory neurons culture in our research.

Kaya *et al.* enlightened that ABS had toxic results on knee cartilage. The adverse effects increased with the come together of hemarthrosis and ABS. Consequently, ABS had harmful effects on experimental hemarthrosis [16].

Bilgili *et al.* performed biochemistry analysis on the first day and fourth day of ABS administration during seven-day follow-up. The authors claimed no mucosal toxicity, hematotoxicity, hepatotoxicity, nephrotoxicity, or biochemical toxicity after ABS administration [3]. These results are not consistent with our research and other researchers. Because in the study of Bilgili *et al.*, ABS was administered orally at low density (1.39 ml/1000 gr). On the other hand, ABS may have lost its efficacy with salivary, gastric, hepatic and pancreatic fluids in the digestive tract. As a result, Bilgili *et al.* were unable to detect the toxic effect of ABS. Whereas, ABS is used purely without dilution in the clinic. If they had used ABS in high doses, they would see toxic effects.

Conclusions

ABS has a degenerative effect on DRG neuron culture at 10 µl/ml and higher density. The degeneration occurred through necrosis in high ABS density. Necrotic cells killed both themselves and the surrounding cells. The degenerative effect of ABS is exerted both on sensory neurons and non-neuronal cells. Caspase 3 inhibitor and caspase 6 inhibitor could not prevent cell deaths. To the best of our knowledge, this study is the first in which ABS was administered in the primer peripheral sensory neuron culture. Further experimental and clinical studies are required to establish degenerative effects of ABS on peripheral sensory neurons.

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Disclosure

The authors report no conflict of interest.

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