BİR İLAÇ OLAN ANKAFERD BLOOD STOPPER'IN İNSAN UMBİLİKAL VEN ENDOTEL HÜCRE KÜLTÜRÜNE ETKİSİ

The Effect of Ankaferd Blood Stopper as a Drug On Human Umbilical Vein Endothelial Cell Culture(HUVEC)

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Geliş tarihi/Received: 11.10.2018
Kabul tarihi/Accepted: 25.07.2019
DOI: 10.16919/bozoktip.469448

Bozok Tıp Derg 2019;9(4):15-21
Bozok Med J 2019;9(4):15-21

ÖZET

Giriş: Ankaferd kanama durdurucu (ABS) tıbbi bitki ekstraksiyonudur. ABS Thymus vulgaris, Glicirhiza glabra, Vitis vinifera, Alpinia officinarum ve Urtica dioica bitkilerinin standardize edilmiş bir karışımıdır. Bu çalışmanın amacı in vitro endotel hücre çoğalmasının ABS'nin farklı zaman ve dozlarının in vitro apoptotik etkilerini araştırmaktır.

Gereç ve yöntem: Erciyes Üniversitesi Hastanesinde sezeryanla yapılan doğumlardan alınan göbek bağı toplardamarı PBS ile yıkandıktan sonra içine yaklaşık 10 mg/ml kollajenaz verildi ve 10 dakika 37ºC de inkübatörde bekletildi. Damar içerisindeki hücre ve kollajenaz karışımı tüp içine alındıktan ve 30 ml besi yeri ilave edildikten sonra 1000 rpm'de 10 dakika santrifüj edildi. Üstteki supernatant atıldı ve tüpün dip kısmında biriken hücreler üzerine 2 ml besi yeri ilave edilerek karıştırıldı ve 25 cm'lik flasklara ekildi. Thoma lamında sayılan hücreler gruplara ayrılarak her bir gruba ait kültür ortamına sırasıyla 5 µl, 25 µl and 50 µl ABS 24 saat uygulandı. Daha sonra hücrelerin canlılığı ve/veya proliferasyonu MTS testi ile tespit edildi.

Bulgular: Doza bağlı olarak deney grubu hücrelerinde bir artışın olduğu görüldü. Kontrol ve deney gruplarından elde edilen ortalama hücre sayısı sırasıyla 7,68x103 (±1,7), 5,56x103(±1,09) 4,12x103(±1,14) ve 2,43x103(±0,89) idi. Deney gruplarının kontrol grubu ile karşılaştırıldığında deney gruplarındaki azalma istatistiksel olarak anlamlıydı (p<0,05). Deney grupları karşılaştırıldığında yüksek dozda hücre sayısı azdı ve istatistiksel olarak anlamlı bulundu (p <0.05). Ayrıca, regresyon analizinin sonuçları hücre canlılığının doza bağlı olarak azaldığını, yani dozun artmasıyla hücre ölümünün arttığını gösterdi.

Sonuç: Çalışmamızda ABS'nin apoptotik etkisi HUVEC'de gösterildi. Apoptoz, bir hücrede gerçekleşen biyokimyasal, morfolojik ve moleküler değişikliklerden yararlanılarak çeşitli yöntemler aracılığı ile ölçülebilir. ABS'nin apoptotik etkisi, kanser hastalıklarının tedavisinde alternatif bir yol olabileceğini göstermektedir.

Anahtar sözcükler: *Ankaferd; Endotel; Umbilikal ven*

ABSTRACT

Introduction: Ankaferd Blood Stopper (ABS) is an herbal extract. ABS comprises of standardized mixture of herbs T. vulgaris, G. glabra, V. vinifera, A. officinarum and U. dioica. The aim of this study was to investigate the in vitro apoptotic effects of the different times and doses of ABS on in vitro endothelial cell proliferation.

Methods: Umbilical cord obtained at Caesarean sections from Erciyes University hospital. After washing PBS, the cord vein lumen was filled with PBS containing 10 mg/ml collagenase and incubated 10 minute at 37°C. The contents of the vein were flushed out with 30 ml medium collected in centrifuge tube then centrifugated at 1000 rpm for 10 minute. The cells were plated in 2 ml of medium at T-25 plastic flasks. The endothelial cells were incubated with only medium in control group and different concentrations of ABS (5 µl, 25 µl and 50 µl ABS) in the experimental groups for 24 h. Then the viability and/or proliferation of cells were detected by MTS assay.

Results: The mean cell number were obtained from the control and experimental groups 7,68x103 (±1,7), 5,56x103(±1,09) 4,12x103(±1,14), and 2,43x103(±0,89) respectively. The cells of the experimental groups compared with the control group to the decrease in experimental groups was statistically significant (p<0,05). Experimental groups were showed a decreased cell number in HUVEC compared with the each other depended on high dose and was found to be statistically significant (p<0.05). Furthermore, the results of regression analysis showed that cell viability was decreased depending on the dose, that was, the cell death was increased as the dose was increased.

Conclusion: In our study, the apoptotic effect of ABS was investigated in HUVEC. The apoptosis can be measured using various of methods by taking advantage of the biochemical, morphological, and molecular changes undergoing in a cell during this process. The apoptotic effect of ABS shows that it may be an alternative way of treating cancer diseases.

Key words: *Ankaferd; Endothelial; Umbilical vein*

INTRODUCTION

Ankaferd Blood Stopper (ABS) is a herbal extract which has been used for centuries as a folk hemostatic agent in Turkish traditional medicine. ABS contains a standardized mixture of several plants including Urtica dioica, Vitis vinifera, Alpinia officinarum, Thymus vulgaris and Glycyrrhiza glabra. Each of these plants has an influence on angiogenesis, cellular proliferation, endothelium, blood cells, vascular dynamics and cell mediators (1). All of these plants affect the hemostatic system in various ways, but the certain mechanism of ABS is still unknown (2) Huri et al. were investigated the effect of ABS, on renal tubular apoptosis in a rat model. In this research the challenge of severe hemorrhage in the renal tubular cellular micro-environment causes ABS-induced down-regulations in the expressions of apoptotic molecules. Findings from this study were indicated that ABS may act as a topical biological response modifier (3). There are several hypotheses such as decreased angiogenesis, increased apoptosis, and interactions with tumor hemostasis that attempt to account for ABS's mechanism of action on tumor tissue (4,5).

The hemostatic action of ABS is correlated with a reduction in tumor neo-angiogenesis (6). There is important phenotypic variation among ECs from different sources, different sites within the same organ (7), and same vessel, and different vessel sizes (8). EC derived from different sources are known to exhibit different behaviors in terms of the angiogenic potential, molecular permeability, homeostasis, vascular tone and even immune tolerance (9). Therefore, cell source is an aspect that has to be accept critically. Human umbilical vein ECs (HUVECs) have been frequently used for the in vitro study of angiogenesis. These cells are, in fact, easily extractable from an available supply of discarded umbilical cords, and can be expanded to relatively large numbers. However, vessels obtained using HUVECs resulted often unstable. The transfection of HUVECs with human telomerase reverse transcriptase or with an anti-apoptotic protein (Bcl-2) allowed to support EC survival and in vivo formation of blood vessels to similar arterioles and venules (10-12). However, these genetic manipulations resulted associated with an oncogenic risk. In addition, the use of HUVECs do not resolve the problem of immune compatibility for a cellularized tissue implant. Using human microvascular ECs showed that adult ECs cultured on plastic or collagen gels and allow to spontaneous self-assembly into capillary-like structures. As a result, ECs can arrange into prevascular network within a tissue without the addition of growth or angiogenic factors (13-16).

ABS has also cytotoxic (17) and apoptotic [Mumcuoğlu,2015], effects in some in vitro cell culture studies. In addition there is one study investigating the relationship between cytotoxic, genotoxic, apoptotic and reactive oxygen species (ROS) generating effects of ABS on both cancer (B16F10 melanoma) and normal cells (L-929 normal fibroblast cell lines)(Koçyiğit,2017). For this purpose of this study was to investigate the apoptotic effects of ABS on human umbilical vein endothelial cell (HUVEC) proliferation at different concentrations for 24 h.

METHODS

Endothelial cell culture

The study was approved by the ethics committee of Erciyes University Medical Faculty. All study procedures were conducted in accordance with a protocol previously approved by the Institutional Review Board of Erciyes University. Human umbilical cord were obtained from the Department of Gynecology and Obstetrics in Erciyes University. Endothelial cells were obtained from human umbilical cord veins by an adaptation of the method of Jaffe (26). A sterile technique was utilized in all manipulations of the cord. The cord was severed from the placenta soon after birth, placed in a sterile container filled with Hanks Balanced Salt Solution (HANKS, pH 7.4), and held at 40C until processing. The cord was inspected, and all areas with clamp marks were cut off. The umbilical vein was cannulated with feeding catheter of 8 french (Fr), was secured by clamping the cord over the feeding catheter with an umbilical cord tie. The vein was perfused with 50 ml of Phosfate buffer saline (PBS) to wash out the blood and allowed to drain. The other end of the umbilical vein was then cannulated with a blunt, 12 gauge needle shaft over which was slipped a 4 cm length of tubing. 10 ml of 0.2% collagenase (PAA, Cat.no:K21-240) was infused into the umbilical

vein, and the polyethylene tubing was clamped shut with a hemostat. The umbilical cord, suspended by its ends, was placed in petri dishes containing HANKS and incubated at 370C for 20 min. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 30 ml of PBS. The effluent was collected in a sterile 50 ml conical centrifuge tube (Falcon Plastics) containing 10 ml of Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Cat. No: SH30003.03) with 20% fetal calf serum (FCS). The cells were mixed and sedimented at 1000 rpm for 10 minute. The supernatant was aspired using sterile pasteur pipet and added 100 ml whole culture medium (80 ml DMEM, 20 ml FCS and 1 ml penisilin/ streptomisin). The cell solution mixed and sedimented at 1000 rpm for 10 minute again. After sedimentitation supernatant was aspired using sterile pasteur pipet then added 2-2.5 whole culture medium. The cell suspension was added 25 cm2 Petri dishes (Falcon Plastics). The dishes were incubated at 370C under 5% C02. The cells were controlled under microscope and washed with PBS three times tomorrow (Figure 1). The cells were fed twice a day with a complete change of whole culture medium. The base of the container is full of the endothelial cells at the end of the first week of culture were washed with PBS before the cells was observed. Cells were then detached with a 0.05% trypsin-0.02% EDTA solution and subcultured to the second passage until confluence.

Occuring the experimental groups

Confluent cells at the end of 2 passage was trypsinized and viable cells were counted with hemocytometer under the light microscope. Then the groups were divided into 1 control and 3 experimental groups. Forming groups of each well were added to 5x104 cells per ml. After the cells are plated on culture dishes in the incubator overnight the wells were washed twice with PBS then added fresh medium. The endothelial cells were incubated with only medium in control group and different concentrations of ABS (5 µl, 25 µl and 50 µl ABS) in the experimental groups for 24 h. After 24 hours the cells were then detached with tripsin/EDTA solution. Cells were stained with trypan blue and viable cells were counted with hemocytometer under the light microscope then photographs were taken of the process steps.

Figure 1:1-Preparation of the umbilical cord for endothelial isolation in sterile conditions (Laminate-air flow), 2-Placement of the umbilical vein feeding probe, 3-Treatment of umbilical vein with collagenase,4- Disintegration of endothelial cells from collagenase, 5-Addition of the cell suspension to 25 cm2 Petri dishes after isolation 6-A The morphology of endothelial cell after 24 hr incubation 6-B: Confluent cells 6-C: HUVECs adhered to each other, within seconds, just after the treatment of ABS 6-D: Reversible vital endothelial cell adherence/aggregation in HUVECs 24 h after treatment of ABS.

Cell viability and proliferation/growth assays

The viability and/or proliferation of cells were detected by MTS assay (Promega, Madison, WI, USA), after cells treatment, to measure cell growth. Cells were counted using a hemocytometer and viable cells were identified by trypan blue exclusion. Viable cells were seeded in 96-well plates (1.5×103 cells/well), and transfected with indicated 5 µl, 25 µl and 50 µl ABS. After 72 h of treatment, a solution containing MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and PMS (phenazine methosulfate) (20 1 v/v) was added to the cells. After 2-3 h of incubation at 37°C, the viable growing cells were estimated by monitoring the absorption of the product at 490 nm, based on the generation of formazan via live cells. All experiments were performed in triplicate

and the results were reported as mean of absorption ± standard deviation.

Statistical analysis

All tests were performed using Sigma Stat 3.5. The distributions of all parameters were determined by using the Kolmogonov-Smirnov test. The comparisons between the groups were determined by using the Kruskal-Wallis test. Multiple comparisons were determined by using the Tukey's test. p-value of <0.05 was accepted as statistically significant.

RESULTS

HUVECs were grown to confluence in between four and seven days after plating on 25 cm2 culture flasks, and incubating in DMEM with 10 % FBS at 37ºC. The confluence time was dependent upon the number of cells seeded in the 25 cm2 flask. Some cell colonies were seen within 24 hours after plating (Figure 1: 6A). The cells appeared flat, had polygonal shapes, and were closely apposed to one another. The second 24 hours showed a rapid migration and proliferation period. Large cell colonies were seen during this period, and the cells exhibited the cobblestone appearance characteristic of endothelial cells. Migration and proliferation continued as the cells became confluent (Figure 1: 6B). This was replicated in the typical morphological appearance of endothelial cells in vitro culture medium.

When the viable cells were counted with hemocytometer, in experimental groups which were significantly decreased by control dependent on doses (Table l). The mean cell number were obtained from the control and experimental groups 7,68x103 (±1,7), 5,56x103(±1,09) 4,12x103(±1,14), and 2,43x103(±0,89) respectively (Table 1 and Figure 2). The cells of the experimental groups compared with the control group to the decrease in experimental groups was statistically significant (p<0,05).

Experimental groups (5 µl, 25 µl and 50 µl ABS) were showed a decreased cell number in HUVEC compared with the each other depended on high dose and was found to be statistically significant (p<0.05).

Furthermore, the results of regression analysis showed that cell viability was decreased depending on the dose, that was, the cell death was increased as the dose was increased. (Figure 2,3,4).

Table 1: Effect of different doses of ABS on endothelial cell proliferation in vitro

Groups	N	Mean (±SD)
Control	16	7,68X103 (±1,7)
5 µl ABS	16	5,56X103(±1,09)
25 µl ABS	16	4,12X103(±1,14)
50 µl ABS	16	2,43X104(±0,89)

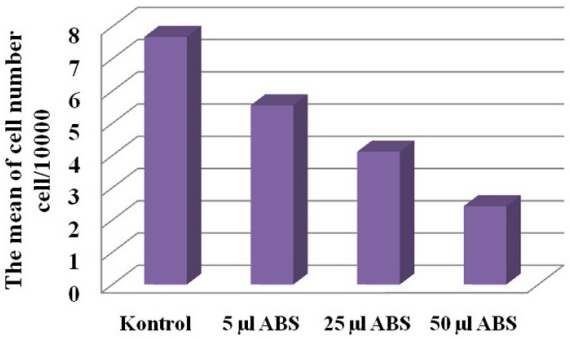

Figure 2: Cell distribution between groups

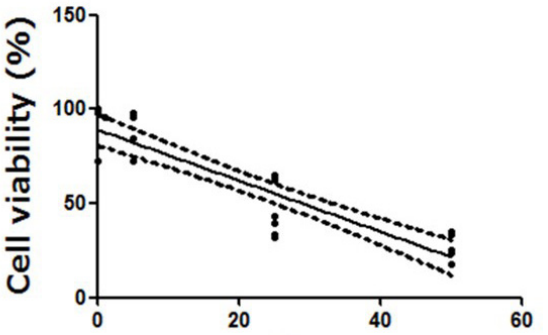

Figure 3: Regression analysis

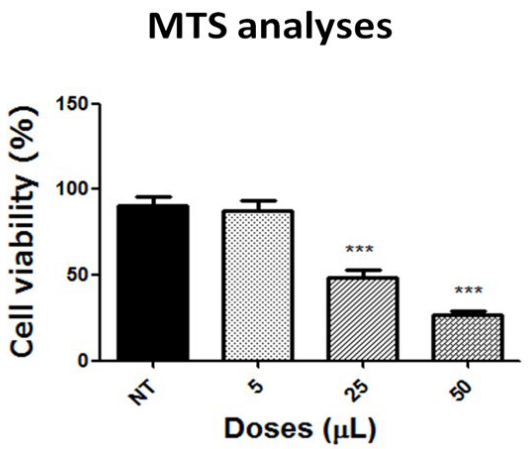

Figure 4: The graph of cell viability

DISCUSSION

ABS, which has long been used as a traditional medicine, represents an alternative treatment modality for many kinds of bleeding that are resistant to conventional methods. The ability of ABS to induce formation of a protein network not only makes it an effective hemostatic agent, but also confers anti-infective, antineoplastic, and healing modulator properties to the extract. Future controlled studies on these effects are warranted (2).

Turhan et al showed that ABS administration to bleeding gastrointestinal carcinomas decreases tumor vascularization (18). Lower gastrointestinal bleeding due to radiation colitis is a rare but important cause of emergency admissions. Despite novel endoscopic approaches like argon plasma coagulation, it is still a major cause of morbidity and mortality worldwide. ABS has been found to be effective in gastrointestinal bleeding due to radiation colitis in several reports (19,20).

Yılmaz et al. to investigate the effects of ABS on endothelium, and possible transcription factor changes in HUVEC and the erythrocyte membrane profile. 5 µL and 50 µL ABS was administered to HUVEC for 5 and 15 min. At the end of the work ABS caused significant increases in the level of activation of the following transcription factors;AP2, AR, CRE/ATF1, CREB, E2F1-5, E2F6, EGR, GATA, HNF-1, ISRE, Myc-Max, NF-1, NFkB,p53, PPAR, SMAD 2/3, SP1, TRE/AP1, and YY1. Consequently they demonstrated that the cellular effects of ABS could be related to different intracellular biological pathways (4).

Experimental antineoplastic activities of ABS have been demonstrated in lymphoid and leukemic neoplastic cell lines (21). ABS could alter proteinase-activated receptor 1 (PAR1) and endothelial protein C receptor (EPCR) expression in K562 and Jurkat cells in a time- and dose dependent manner and treatment had induced apoptosis in leukemia cells. Possible involvement of PAR1 and p21 in the apoptotic process was observed in Jurkat cells (22). In a study by Odabas et al., the cytotoxicity of ABS was evaluated on human pulp fibroblasts in vitro. The results of Odabas' study had shown that ABS is cytotoxic to human pulp fibroblasts which depended on the concentration of ABS applied (23).

ABS affects the levels of different critical factors and proteins such as androgen receptor (AR), protein 2 (AP2), cyclic adenosine monophosphate (AMP) response element, cyclic AMP response element binding protein(CREB), E2F1-5, E2F6, , Myc-Max, nuclear factor-1 (NF-1), interferon (IFN)-stimulated response element (ISRE), protein 53 or tumor protein 53 (p53), peroxisome proliferator-activated receptor (PPAR), and Yin Yang 1 (YY1) transcription factors (4).
These regulator molecules affect different stages of cellular proliferation such as cell vascular hemostasis, signal transduction, angiogenesis, immunity, inflammation, apoptosis, acute phase reaction, and several metabolic molecular pathways. Fort his reason, the protein content and their effects on the transcriptomics may be active in the cellular action of ABS (4,24).

In a study by Turk et al.,to determine the effect of Ankaferd hemostat on viability of melanoma cell lines. They were used dissimilar melanoma cell lines and primary cells. These cells were treated with different concentrations of Ankaferd hemostat to assess the impact of different dosages of the drug. All cells treated with different concentrations were incubated for different time intervals. They demonstrated in this

study that cells treated with Ankaferd hemostat showed a significant decrease in cell viability compared to control groups. The cells showed different resistances against Ankaferd hemostat which depended on the dosage applied and the time treated cells had been incubated. They also demonstrated an inverse relationship between the concentration of the drug and the incubation time on one hand and the viability of the cells on the other hand, that is, increasing the concentration of the drug and the incubation time had a negative impact on cell viability. Consequently their findings indicated that ABS can induce apoptosis, regulate cellular proliferation, and decrease tumor vascularization. Experimental antineoplastic activities of ABS have been shown in leukemic and lymphoid neoplastic cell lines (25).

Koçyiğit et al. reported that ABS is generally used as a hemostatic agent, it can cause apoptosis, DNA damage and cytotoxicity by generating ROS activity. Fort this reason, it should be removed the unused ABS by cleaning once the hemostasis is achieved to minimize the postoperative side effects and demonstrated that ABS may be a useful therapeutic intervention for cancer (26).

Although ABS is a medical product used to stop minor and major bleeding after traumatic incisions, dental operations, spontaneous or surgical interventions, various studies demonstrated that it has genotoxic, cytotoxic and apoptotic effects on cells (22, 26, 27).
Natural products are widely preferred for the production of anticancer drugs as the required property of an anticancer drug is to have the ability to kill the cancer cells and cause no damage to normal human cells. ABS can decrease the viability of the cells significantly and is reported to have no cytotoxic properties (28).
Provides feeding through diffusion as the tumor develops. However, after the size of the tumor reaches 2 mm2, it stimulates the endothelial cells by releasing various factors in order to continue feeding. Then the endothelial cells come together and begin to form the vascular structure. Turhan et. al.(29) were performed by the rectal and cancer tissues when the application of ABS showed reduced vascularization of the tumor.

In our study, the apoptotic effect of ABS was investigated in HUVEC. The apoptosis can be measured using various of methods by taking advantage of the biochemical, morphological, and molecular changes undergoing in a cell during this process. We estimated apoptotic and live cells by using MTS assay method. The results of this study showed that ABS treated endothelial cells clearly display the apoptotic events with a significant decrease in cell viability depending on the dose. These observations are in agreement with the other in vitro and in vivo studies that showed the treatment of cancer cell with ABS results in induction of apoptosis. The apoptotic effect of ABS shows that it may be an alternative way of treating cancer diseases. Also these finding represent a starting point to search the effects of ABS on cancer in future basic research and controlled clinical trials.

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