

# Effects of Ankaferd Hemostat on Retinal Apoptosis in an Experimental Rabbit Retinal Model

## DeneySEL TavşAN Retina Modelinde Ankaferd Hemostatın Retinal Apoptoza Etkisi

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**ABSTRACT Objective:** To assess ultrastructural alterations and cellular apoptotic effects of Ankaferd Blood Stopper® (ABS) in an experimental rabbit retinal model. Retinal changes following the intravitreal injection of ABS were investigated on ten retina tissues of five rabbits. **Material and Methods:** ABS was injected into the vitreous of right eye of five rabbits. The left eyes of rabbits received an intravitreal injection of balanced salt solution and were used as the control group. Oxidative damage, apoptosis, protein carbonylation and DNA fragmentation were studied. **Results:** In the ABS injected eyes, the results of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of a few protein bands were up-regulated whereas some other bands showed down-regulation. The apoptosis was delayed, indicated by the morphological analyses and caspase activity in the ABS injected eyes. Furthermore, ABS-induced protein carbonyl formation and DNA fragmentation were also demonstrated in the rabbit retina. **Conclusion:** ABS has de novo effects on apoptosis. ABS-induced down-regulations in the expressions of apoptotic molecules in the retinal microenvironment indicated that ABS might act as a topical biological response modifier. Intravitreal injection of ABS also has cytotoxic effects. Since ABS is currently being developed in basic and clinical grounds, those novel observations will guide future studies focusing on the pleiotropic effects of this unique hemostatic agent.

**Key Words:** Ankaferd blood stopper; apoptosis; DNA fragmentation; intravitreal injections; protein carbonylation; retina

**ÖZET Amaç:** Deneysel tavşan retina modelinde Ankaferd Kan Durdurucu® (ABS)'nin hücreSEL apoptotik etkisini ve neden olduğu ince yapısal değişiklikleri değerlendirmek. ABS'nin intravitreal enjeksiyonundan sonra beş tavşandaki on retina dokusu üzerinde meydana gelen değişiklikler incelendi. **Gereç ve Yöntemler:** Beş tavşanın sağ gözüne vitreus içine ABS enjekte edildi. Tavşanların sol gözleri kontrol grubu olarak kullanıldı ve vitreus içine dengeli tuz solüsyonu enjekte edildi. Oksidatif hasar, apoptoz, protein karbonilasyonu ve DNA fragmentasyonu incelendi. **Bulgular:** ABS enjekte edilen gözlerde, sodyum dodesil sülfat poliakrilamid jel elektroforezi (SDS-PAGE)'nde birkaç protein bandında artma ve bazı protein bantlarında azalma görüldü. ABS enjekte edilen gözlerde kaspaz aktivitesi ve morfolojik analizler ile tespit edilen apoptozda gecikme belirlendi. Ayrıca, tavşan retinalarında ABS'nin indüklediği protein karbonilasyonu oluşumu ve DNA fragmentasyonu da tespit edildi. **Sonuç:** ABS'nin apoptoz üzerine de-novo etkileri vardır. Retinal mikroçevrede ABS'nin indüklediği apoptotik moleküllerde aşağı doğru oluşan düzenlemeler, ABS'nin yerel bir biyolojik yanıt değiştirici olarak hareket edebileceğini göstermiştir. Aynı zamanda ABS'nin intravitreal enjeksiyonunun sitotoksik etkileri de vardır. ABS şu anda temel ve klinik alanda geliştirilmekte olduğundan, bu yeni gözlemler gelecekteki çalışmalarda bu eşsiz hemostatik ajanın pleiotropik etkileri konusundaki çalışmalara yön verecektir.

**Anahtar Kelimeler:** Ankaferd kan durdurucu; apoptoz; DNA fragmentasyonu; intravitreal enjeksiyon; protein karbonilasyonu; retina

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Ankaferd is an herbal extract, which has been used historically as a hemostatic agent in traditional Turkish medicine for centuries. The medicine comprised the standardized mixture of herbs *Thymus vul-*

*garis*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum* and *Urtica dioica*. Each one of these herbs is effective on endothelium, blood cells, angiogenesis, cell proliferation, vascular dynamics, and cellular mediators.<sup>1</sup> *Glycyrrhiza glabra* inhibits angiogenesis, decreases vascular endothelial growth factor production, and cytokine induced neovascularisation.<sup>2</sup> *Thymus vulgaris* has been shown to exhibit varying levels of antioxidant activity, which may help to prevent in vivo oxidative damage, such as lipid peroxidation associated with atherosclerosis.<sup>3</sup> *Vitis vinifera* has an anti-atherosclerotic effect.<sup>1</sup> *Alpinia officinarum* inhibits nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages.<sup>4</sup> *Urtica dioica* produces hypotensive responses through a vasorelaxation effect mediated by the release of endothelial nitric oxide and the opening of potassium channels, and through a negative inotropic action.<sup>5</sup> Since ABS is a relatively novel hemostatic agent, the experience regarding its use in distinct hemorrhagic states is expanding.<sup>6-22</sup>

ABS-induced formation of the protein network with vital erythroid aggregation covers the entire physiological hemostatic process.<sup>6,22-24</sup> Mainly, there are distinct important components of the ABS-induced hemostatic network. Vital erythroid aggregation takes place with the spectrin and ankrin receptors on the surface of red blood cells. Those proteins and the required adenosine triphosphate (ATP) bioenergy are included in the ABS protein library.<sup>25,26</sup> ABS also upregulates GATA/Friend of GATA (FOG) transcription system affecting erythroid functions. Urotensin II is also an essential component of ABS and represents the link between injured vascular endothelium, adhesive proteins, and active erythroid cells. Those concepts have been developed via matrix assisted laser desorption/ionization (MALDI) time-of-flight (TOF) proteomic molecular analyses, cytometric arrays, transcription analysis, and scanning electron microscopy (SEM) ultrastructural examinations as well as numerous investigations interacting with *in vitro* and *in vivo* research settings.<sup>8,22,27,28</sup>

ABS has been shown to affect renal tubular apoptosis based on the level of hemorrhage in a

previous study.<sup>22</sup> It decreases apoptosis in renal tubular cells.<sup>22</sup> Therefore, ABS modulates the cellular apoptotic responses to hemorrhagic stress as well as its hemostatic hemodynamic activity. The finding of ABS-induced protease activated receptor (PAR)-1 down-regulation gives an additional clue on the possible mechanism of ABS associated apoptosis modulation at the tissue level.<sup>29</sup> Preliminary findings focusing on *in vitro* anti-neoplastic effects of ABS also prompt to start investigating the ABS effects at the cellular level.<sup>30,31</sup> The aim of this study was to assess ultrastructural changes in retina following intravitreal injection of ABS and to focus on the effect of ABS on retinal apoptosis. The clinical perspectives with the study hypothesis were that ABS could be used in vitreoretinal surgery for intraoperative bleedings and the anti-angiogenic effects of the drug might aid the management of choroidal and retinal neovascularization.

## MATERIAL AND METHODS

### ANIMALS

This study was performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) resolution for the use of animals in Ophthalmic and Vision Research. The study was approved by the Ethical Committee of Harran University; the tenets of the 2<sup>nd</sup> Declaration of Helsinki were followed. The animal care protocol was approved by the Institutional Animal Care and Use Committee of Harran University. Five adult rabbits were used for the study. All procedures were performed under sterile conditions. An intramuscular injection of 25 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride was used to anesthetize the rabbits. The pupils were dilated with 5% topical phenylephrine hydrochloride and tropicamide eye drops. Ocular region massage was given to reduce intraocular pressure and to minimize drug reflux after injection. Intravitreal injections were given 2 mm posterior to the limbus into the mid-vitreous cavity at the superotemporal quadrant with a 30-gauge needle. Doses of 0.1 mL of ABS were prepared and were injected into the right eye of 5 rabbits (for each dose). The same volume of sterile balanced salt solution (BSS) was injected into

the left eye of each rabbit since the injection by itself or the additional volume injected into the eye may alter the outcome of parameters. The salt injected eyes served as an experimental control group as in other ophthalmology trials. Binocular indirect ophthalmoscopy was performed before and after each injection. Lens damage following intravitreal injections was not observed. After the injections, animals were returned to their cages. No endophthalmitis developed in any of the 10 eyes during the follow-up period.

### PREPARATION OF TISSUE SAMPLES

After 24 hours, the animals were killed with an intracardiac injection of sodium pentobarbital (50 mg/kg, Pentothal Sodium<sup>R</sup>, Abbott). The eyes were enucleated and dissection was performed quickly under the microscope. The eyeball was cut posteriorly to the limbus and after excision of the anterior segment. The vitreous was removed and then the retina was carefully dissected from the sclera. The wet weight of the retina was measured as soon as possible. The isolated retina was placed on dry ice immediately and was kept frozen at -80°C until the subsequent biochemical analyses.

### PROTEIN ISOLATION

Protein oxidation is defined as the covalent modification of a protein induced by reactive oxygen species products of oxidative stress. The most common products of protein oxidation in biological samples are the protein carbonyl derivatives of Pro, Arg, Lys, and Thr amino acids. These derivatives are chemically stable and serve as markers of oxidative stress for most types of reactive oxygen species. Approximately 50 mg of retina tissues were diluted at 1:20 in extraction buffer [20 mM Tris, 150 mM NaCl, 0.5% Triton, Protease-inhibitor Cocktail and 1 mM phenylmethanesulfonylfluoride (PMSF)] using Lysing Matrix D tubes and was homogenized using a FastPrep-24 Instrument (MP Biomedical, USA) for 40 seconds.

### IMMUNOBLOTTING AND APOPTOSIS TEST

All homogenized samples were centrifuged at 13 000 rpm for 10 minutes at 4°C. Protein concentration of collected supernatant was determined

and then samples were mixed with 2x sample buffer. Equal amounts of the samples were subjected to a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli with the Mini Protean II system (Bio-Rad, USA).<sup>32</sup> The separated proteins were transferred electrophoretically on a polyvinylidene difluoride membrane using the iBlot<sup>®</sup> Dry Blotting System (Invitrogen, USA) for about 7 min. Nonspecific binding sites on the polyvinylidene difluoride filter were blocked by incubation with 5% skimmed milk in phosphate buffered saline (PBS) (50 mM sodium phosphate, pH:7.4, 150 NaCl) containing Triton for one hour at room temperature. The filter was then incubated with the anti-active caspase-3 primer antibody (react only with 17 kDa subunit, Chemicon, AB3623) for two hours at room temperature. After washing 3 times with PBS-T, the filter was incubated with secondary goat anti-rabbit IgG antibody conjugated with alkaline phosphatase for one hour at room temperature. After washing with PBS-T (3x) and once with PBS only, color reaction was performed in the dark with nitro blue tetrazolium chloride (NBT)/5-Bromo-4-chloro-3-indolyl phosphate (BCIP) alkaline phosphatase substrate tablets (Sigma Fast tablet) with agitation for 10 to 30 min until black-purple color development. Reaction was stopped by washing in several changes of distilled water and was air dried and stored in a plastic sleeve in a cool dark place.

### DNA FRAGMENTATION ASSAY

The retina tissues were lysed in 30 µl lysis buffer [10 mM Tris, pH:7.4; 100 mM NaCl; 25 mM ethylenediaminetetraacetic acid (EDTA); 1% N-Lauroyl-sarcosine or 1% SDS) at 1:10 dilution; by gentle vortexing 4 µl of proteinase K (10 µg/µl) was added, and was homogenated using a glass dounce homogenate system. The tissue lysates were incubated at 45°C for 1-2 h. Then 2 µl of RNase (10 µg/µl) was added and the cell lysate was incubated for another 1 h at room temperature. Then, 4 µl of 6x DNA sample dye (50% glycerol; 1 mM EDTA, pH:8.0; 0.25% bromophenol blue; 0.25% xylene cyanol) was mixed in the lysate (the final volume of

each sample was about 40 µl) and the sample was ready for electrophoresis. The gel running buffer, 1x tris-acetate-EDTA (TAE) was added to the gel box until the buffer reached the surface level of the agarose gel. The DNA marker used was the 1 kb DNA ladder. Genomic DNA was electrophoresed in 2% agarose gel with ethidium bromide (0.2 µg/mL) at 7 V/cm.<sup>33</sup>

### PROTEIN CARBOXYLATION

Protein samples were diluted to 10 µg/ml with PBS by reading with Quant-iT Protein Assay Kit using Qubit fluorimeter (Invitrogen). Then 100 µl of 10 µg/ml protein samples including reduced/oxidized bovine serum albumin standards were added to the 96-well protein binding plate and the plate was incubated at 37°C for 2 hours. After washing the plate three times with PBS, 100 µl of dinitrophenylhydrazine working solution was added and the plate was and incubated for 45 minutes at room temperature in the dark. Plate wells were washed with PBS/Ethanol (1:1, v/v) with incubation on an orbital shaker for 5 minutes. This was repeated three more times and was washed twice only with PBS. Following this, 200 µl of blocking solution was added to each well and was incubated for two hours at room temperature on an orbital shaker. After washing the plate three times with wash buffer, 100 µl of anti-dinitrophenol (DNP) antibody was added and was incubated for one hour at room temperature on an orbital shaker. The plate wells were washed again with wash buffer as above and then 100 µl of substrate solution was added and was incubated for 20 minutes. The reaction was stopped by adding 100 µl of stop solution. The absorbance of each well (blank, standards and samples) was read on a plate reader (FluoStar Omega) at 450 nm. The protein carbonyl level of each sample was determined according to the standard. Protein carbonyl in each sample was calculated by comparing its absorbance with that of a known reduced/oxidized Bovine Serum Albumin standard curve. The concentration of protein carbonyls was expressed as nmol/mg protein.

Statistical analysis was performed using SPSS 15.0 software package. Differences in measured pa-

rameters between two groups were analyzed by Wilcoxon signed rank test. A statistically significant difference was defined as a p value <0.05.

## RESULTS

Gross examination of eye specimens showed no evidence of retinal detachment, retinal hemorrhages or signs of infection in any ABS injected or BSS injected eyes in rabbits. Gross examination showed that the vitreous of ABS injected eyes had a hazy appearance, rather dense, yellow-gray in color. Whereas, the vitreous of BSS injected eyes was optically clear and white in color.

The samples were separated by SDS-PAGE, and the dried gels were photographed. The multiresolution algorithms for the identification of relative protein bands in SDS-PAGE images were used. The SDS-PAGE pattern of retina proteins of ABS injected eyes showed polymorphism based on difference in protein intensity. The presence or absence of protein bands has also been detected. On the other hand, some bands in the ABS group were not observed in BSS injected eyes. ABS was suggested to affect protein expression in the cell. Compared to BSS injected eyes, the expression of protein was significantly increased by ABS injection.

The caspase activity corresponded to the appearance of immunofluorescence using the caspase-3 antibody. The presence of caspase-3, indicative of apoptosis, was detected in BSS injected eyes. The apoptosis was delayed, and this was apparent by caspase activity in ABS injected eyes, measured by immunofluorescence.

The protein carbonyl level was 2.13 nmol/mg, 1.97 nmol/mg, 1.73 nmol/mg, 2.08 nmol/mg, and 1.89 nmol/mg of ABS injected eyes; whereas the concentration of free carbonyls in proteins was 0.88 nmol/mg, 1.34 nmol/mg, 0.98 nmol/mg, 0.96 nmol/mg, and 1.04 nmol/mg in BSS injected eyes, respectively. After oxidation, the carbonyl concentration activity in ABS injected eyes was significantly higher than in the BSS injected eyes. Figure 1 shows that the concentration of free carbonyls in proteins is higher in activity in ABS injected eyes at

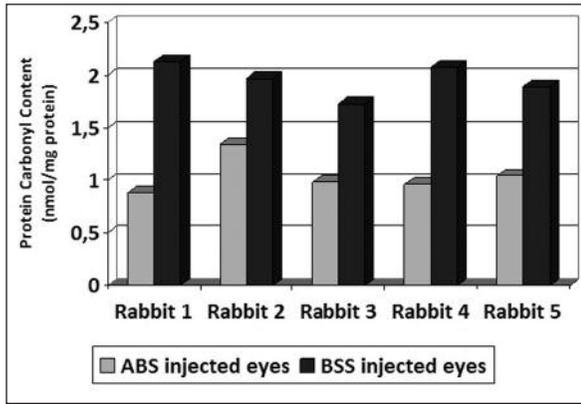


FIGURE 1: Relative levels of protein expression of five rabbits.

all stages than in activity in BSS injected eyes. Intravitreal injection of ABS caused a significant increase in protein carbonyls, an index of protein oxidation. The median concentration of protein carbonyls was 1.97 nmol/mg in the retina of ABS injected eyes (1.73-2.13 nmol/mg) and 0.98 nmol/mg in the retina of BSS injected eyes (0.88-1.34 nmol/mg); this difference was significant ( $p=0.043$ ).

DNA fragmentation was also evaluated by agarose gel electrophoresis. DNA from the ABS injected eyes showed obvious DNA fragmentation in a step-ladder fashion. The typical ladder pattern of oligonucleosomal fragments was observed in ABS injected eyes. However, BSS injected eyes had no DNA fragmentation. Electrophoretic separation then revealed fragments corresponding to multiples of major band populations and a number of minor band populations were observed.

## DISCUSSION

ABS is currently topically used in spontaneous bleedings or bleeding secondary to body hurts, traumas, minor or major surgical interventions.<sup>6-22</sup> It affects not only hemostasis but also wound healing with some antibacterial properties.<sup>7-8,16-19,34-39</sup> Clinical conditions of ABS use are increased in course of time as hemorrhagic problems persist despite available anti-hemorrhagic interventions.

Bilgili et al. showed that acute mucosal toxicity, hematotoxicity, hepatotoxicity, nephrotoxicity, and biochemical toxicity did not develop after

oral ABS administration in rabbits.<sup>40</sup> Besides the initial network, preliminary study has demonstrated antiangiogenic properties of the ABS, measured as microvessel density.<sup>39</sup> The controls of retinal and choroidal angiogenesis are of critical importance for the preservation of vision. Retinal and choroidal neovascularization characterizes proliferative diabetic retinopathy and age-related macular degeneration. Vitreous levels of angiogenic growth factors have been shown to be directly associated with the degree of retinal and choroidal angiogenesis.<sup>41</sup> We suggest that the ABS may affect the process of angiogenesis and we may use the antiangiogenic effect of this drug. Evidence supporting this hypothesis will be established from several lines of experimental investigations. In mice treated with *Glycyrrhiza glabra*, the levels of the cytokine vascular endothelial growth factor (VEGF), the density of microvessel in the peritoneum, the VEGF formation of the plant extract and the neovascularization induced by cytokine decreased.<sup>2</sup>

Pars plana vitrectomy leads to several problems such as defective healing, inflammation, suture related problems, cataract, astigmatism, high intraocular pressure, and bleeding of the eye during surgery.<sup>42</sup> The intraoperative bleeding during vitrectomy is a major cause of surgical failure. Reproliferation is likely to occur in the presence of significant preretinal bleeding. Resected vascular tissue, sclerotomy wounds, retinal breaks, iris neovascularization, and retinal neovascularization are the most frequent causes of intraoperative hemorrhage. It is crucial to reduce intra and postoperative hemorrhage in complicated vitrectomy.<sup>43</sup> With the thought to use ABS in vitreoretinal surgery complicated with intraoperative hemorrhage we ran this study to investigate the effect of ABS on the rabbit retina.

Vitreous opacities appear secondary to cellular infiltrates, protein exudation, mineral and lipid deposits or congenital defects.<sup>44</sup> Gross examination in our study revealed that the translucency of the vitreous in ABS injected eyes were lost. The vitreous of ABS injected eyes had a rather dense, yellow-gray color whereas the vitreous of BSS injected

eyes was optically clear and white. This may be attributed to the degradation of the structural protein and collagen of vitreous body.

Polyacrylamide Gel Electrophoresis is one of the most common techniques in protein research. Denaturing SDS-PAGE is a significant method used for the separation of proteins based on the migration of negatively charged proteins depending on their molecular weight in an electrical field. Its advantage is that proteins can be visualized as well as being separated. SDS-PAGE technique provides information about protein concentrations of the protein mixture applied on a certain lane of the PAGE and the degree of purity of a particular protein in this mixture during protein purification process. Each band in the SDS-PAGE image represents a different protein band.<sup>45</sup> The protein extracts obtained from the rabbit retina materials were analyzed by SDS-PAGE in the present study. The soluble and the reduced fractions of the rabbit retina presented different profiles of separate protein bands on the gel and a high number of protein bands detected in ABS injected eyes were not present in BSS injected eyes. This suggests a change in the expression of some protein in the ABS injected eyes.

Apoptosis is a pathobiological process that is responsible from the programmed cell death, indicating tissue damage. It is a complex process that involves a variety of different signaling pathways and results in a multitude of changes in the dying cell. Many of the events that occur during apoptosis are mediated by a family of cysteine proteases called caspases.<sup>46</sup> Caspases show their action at several levels of signaling during apoptosis, ranging from responding to external factors at the transmembrane receptor to proteolytic breakdown of cellular components. Their proteolytic activity results ultimately in the demise of the cell and has led to caspases being called the central executioners of apoptosis. Among the group of 14 caspases identified to date in humans, caspase-3 has been recognized as a central player in mediating apoptosis and is the most widely studied.<sup>46</sup> In our study retinal cells were analyzed by Western blot using a previously described method to investigate the ef-

fect of caspase-3, key elements of the intrinsic mitochondrial pathway of apoptosis. Western blotting results showed that the ABS inhibited the spontaneous apoptosis-associated caspase-3 activation and delayed apoptosis in retinal cells.

The carbonyl assay is widely used to study oxidative protein damage in tissues.<sup>47</sup> It is based on the ability of several reactive oxygen species to attack amino acid residues (particularly histidine, arginine, lysine, and proline) to produce carbonyl groups that can be measured spectrophotometrically after reaction with 2,4-dinitrophenylhydrazine. Species able to generate carbonyls include free radicals generated by metal ion-dependent reactions and reactive oxygen species produced in other ways.<sup>47</sup> In the current study, we measured protein carbonyl formation as an index of oxidative damage in the retina based on the idea that formation of reactive carbonyl groups represented a major manifestation of oxidative modifications of proteins and reflected cellular damage induced by multiple forms of reactive oxygen species. We described in this study for the first time the time course of protein carbonylation in the retina, in response to intravitreal ABS injection in rabbits.

Fragmentation of genomic DNA is one of the hallmarks of apoptosis. A characteristic feature of apoptosis is activation of an endogenous endonuclease, which generates numerous DNA strand breaks in chromatin.<sup>48</sup> Apoptosis can be detected in somatic cells by electrophoresis of DNA fragments to produce a characteristic 'ladder' pattern due to endonuclease cuts in the linker section of the helix.<sup>48</sup> The appearance of the ladder of nucleosomal DNA fragments in agarose gels became the hallmark of apoptosis. Gel electrophoresis of DNA seems to provide a qualitative answer as to whether the apoptosis process is taking place or not. DNA fragmentation is our best available criterion, but this does not imply that *in vivo* it proceeds all the way to the formation of a nucleosomal-size ladder of DNA. In fact, our results demonstrated simultaneous occurrence of nuclear fragmentation and apoptotic cell death in rabbit retinal tissues after intravitreal injection of ABS. Apoptosis has been characterized biochemically based on DNA frag-

mentation. These results indicate that the death of retinal cell is accompanied by degradation of the chromosomal DNA, which is the most characteristic biochemical feature of apoptosis.

Angiogenesis is a multi-factorial process that involves different cell types and a number of cytokines and growth factors. Physiological angiogenesis is characterized by the existence of a delicate balance between pro-angiogenic and anti-angiogenic factors. Over-expression of angiogenesis-activating factors may cause hypervascularization. However, deficiency or disarray in the expression of anti-angiogenic factors may result in leaky vessels, unstable capillaries, and formation of dysfunctional neovascular tufts as seen in retinopathy of prematurity, diabetic retinopathy, or other conditions of retinal neovascularization.<sup>49</sup> Turhan et al. demonstrated significant decreases in bleeding gastrointestinal tumor microvessel density measurements in the tissue exposed to ABS, compared with microvessel densities from biopsy specimens before ABS administration and from deeper (unexposed) neoplastic tissue.<sup>39</sup> They suggest the presence of a secondary, more sustained mechanism of hemostasis in addition to the initial protein network. We suggest that intravitreal ABS injection led to retinal vascular regression. Regression of the vascular structure of retina may cause these undesirable effects.

There is a wide range of toxic effects that might be caused by intravitreal injections of drugs. Retinal toxicity can be evaluated by histopathologic changes, electron micrographic changes, electrophysiologic responses, and degraded products of cellular injury. We reported here the results of our investigations, which were designed to assess the retinal toxicity of the ABS after intravitreal administration in a rabbit animal model. We decided to consider the level of oxidative damage, apoptosis, protein carbonylation and DNA fragmentation as the indicator of ABS-induced retina toxicity in this study. The results of our study showed that the toxicity of ABS was evident. Thus, its use in the

clinic for hemostasis may not be safe.

ABS has numerous effects on the cellular microenvironment. The effects of ABS on two important endothelial hemostatic molecules, Endothelial Protein C Receptor (EPCR) and plasminogen activator inhibitor-1 (PAI-1) were previously examined.<sup>29</sup> ABS has dual diverse dynamic reversible actions on EPCR and PAI-1 inside vascular endothelial cells in the model of Human Umbilical Vein Endothelial Cells (HUVEC).<sup>50</sup> Immediate enhanced expression of pro-hemostatic PAI-1 and down-regulated expression of anti-coagulant EPCR upon exposure to ABS were compatible with the sudden anti-hemorrhagic efficacy of ABS. Lipopolysaccharides (LPS) are large molecules acting as endotoxins and elicit strong immune responses. LPS application to the HUVEC cells had enhanced ABS-induced up-regulations in the expressions of EPCR and PAI-1. Thus, ABS could act as a topical biological response modifier.<sup>50</sup> EPCR and PAI-1 molecules are considered the associates of PAR-1. ABS caused dose-dependent reversible PAR-1 down-regulation in the HUVEC cellular model. "LPS challenge" to HUVEC enhanced ABS-induced sustained down-regulations in the expressions of PAR-1.<sup>29</sup> ABS is therefore considered a topical biological response modifier. ABS has de novo effects on apoptosis.<sup>8</sup> ABS-induced down-regulations in the expressions of apoptotic molecules in the retinal microenvironment in our present study also indicated that ABS might act as a topical biological response modifier.

In conclusion, our results extend the range of biological effects of the ABS and provide new insight to the role of this drug in the modulation of the angiogenesis response by the retina. Further studies are needed to determine the full scope of the cellular effects of ABS including within the ophthalmic tissues.

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