Ankaferd hemostat induces DNA damage, apoptosis and cytotoxic activity by generating reactive oxygen species in melanoma and normal cell lines

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Abstract: Although, Ankaferd hemostat (Ankaferd Blood Stopper, ABS) could be utilized successfully as hemostatic agent, studies demonstrated that it has cytotoxic effects on cells. However, the mechanism(s) of this effect has not been elucidated yet. In this study, cytotoxic, genotoxic, apoptotic and reactive oxygen generating (ROS) activities of ABS were investigated in melanoma and normal cell lines. The cells were incubated with different concentrations of ABS (0.125 to 2%) for 24 h. The cell viability was assessed based on ATP cell viability assay. Intracellular accumulation of reactive oxygen species (ROS) was determined using the fluorescent probes 2',7'-dichloro-dihydrofluorescein-diacetate (H2DCF-DA). DNA damage was evaluated by alkaline single cell gel electrophoresis assay (Comet Assay) and, apoptosis induction was detected by Acridine Orange/Ethidium Bromide AO/EB double staining method. Our results demonstrated that ABS increases DNA damage, apoptosis and ROS levels in both melanoma and normal cell lines in a dose dependent manner, and all of these activities were significantly higher in melanoma cells than in normal cells. There was a statistically significant positive correlation between DNA damage, apoptosis and ROS levels in ABS treated cell lines. Our results revealed that although ABS commonly used as hemostatic agent, it causes DNA damage and apoptosis by generating ROS in a dose dependent manner. Therefore, it should be removed the unused ABS by cleaning once the hemostasis is achieved to minimize the postoperative side effects. These results could also contribute to the development of new treatment for cancer.

Keywords: Ankaferd, DNA damage, reactive oxygen species, apoptosis, cytotoxicity

Introduction

Ankaferd hemostat (Ankaferd Blood Stopper, ABS) is the first topical hemostatic agent regarding the red blood cell (RBC)-fibrinogen interactions tested in the clinical trials [1]. ABS is a standardized mixture of the plants Thymus vulgaris (dried leaf), Glycyrrhiza glabra (dried leaf), Vitis vinifera (dried leaf), Alpinia officinarum (dried leaf), and Urtica dioica (dried root) [2]. ABS-induced pharmacological modulation of essential erythroid proteins (ankyrin, spectrin, actin) can cause vital erythroid aggregation via acting on fibrinogen gamma [3]. The pleiotropic effects of ABS on vascular endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics and cellular mediators have been investigated [4-8]. The use of ABS in the gastrointestinal system (GI) hemorrhages to control bleeding and/or infected GI wounds is also evident [9]. Controlled clinical trials indicated the safety and efficacy of ABS for the control of clinical hemorrhages in a wide variety of settings [10-17]. Besides all of these activities, it has recently been demonstrated that ABS has also apoptotic [18], and cytotoxic [19] effects in some in vitro cell culture studies. However, the mechanism(s) of these effects has not been elucidated yet and, to the best of our knowledge, there are no studies investigating the relationship between cytotoxic, genotoxic, apoptotic and reactive oxygen species (ROS) generating effects of ABS on both cancer and normal cells. For this purpose, we designed a study to analyze the cytotoxic, genotoxic, apoptotic and ROS generating effects of ABS on
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B16F10 melanoma and L-929 normal fibroblast cell lines at different concentrations for 24 h.

Materials and methods

Chemicals

Fetal calf serum (FCS), Dulbecco's modified Eagle medium (DMEM), 2',7'-dichloro-dihydrofluorescein-diacetate (H2DCF-DA), Penicillin-streptomycin, Acridine Orange (AO) and ethidium bromide (EB), Low melting Agarose and Normal Melting Agarose were purchased from Sigma-Aldrich (Seelze, Germany). All other reagents used were of analytical grade unless otherwise stated.

Ingredients of ABS

An ampoule form of ABS (2 ml) was used in the experiments. Amount of active substances in ampoule form of ABS is as follows: T. vulgaris (0.05 mg/ml), G. glabra (0.09 mg/ml), V. vinifera (0.08 mg/ml), A. officinarum (0.07 mg/ml), and U. dioica (0.06 mg/ml).

Cell culture and maintenance

L-929 cell line (as a standard cell line originated from mouse fibroblast cells) and B16-F10 cell line as a standard cell line originated from mouse (Mus musculus) skin melanoma cells were obtained from American Type Cell Culture Collection (ATCC). All cells were cultured in DMEM equilibrated with 5% CO2 atmosphere at 37°C. The medium was supplemented with 10% FCS, 100 U/ml of penicillin and 100 ng/ml of streptomycin. The number of viable cells was estimated by trypan blue exclusion test.

Cytotoxicity assay

Cytotoxic activities of ABS on L-929 and B16-F10 cells were determined by ATP levels using a luminescence test (Cell-Titer-Glo Luminescent Cell Viability Assay, Promega). Cells were seeded onto 96-well plates at a density of 5×10^3 cells per well and incubated overnight at 37°C in 5% CO2. The medium was then replaced with fresh complete medium containing various concentrations of ABS (0.125 to 2% in phosphate buffered saline (PBS) were added to the cells and incubated for another 24 hours at 37°C. PBS was used as control. After incubation, the cells were washed with phosphate buffered saline (PBS), harvested using trypsin/EDTA and collected for centrifugation at 400×g for 5 min at 4°C. The supernatant was discharged and the cell density was adjusted to 2×10^5 cells/ml using cold PBS.

Ten µL cell suspension was mixed with 90 µL of 0.6% low melting agarose (LMA) and added to the slides pre-coated with 1% normal melting agarose (NMA). After solidification of the agarose, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h at 4°C. After removing the slides from lysis solution, they were washed with cold PBS and placed in a horizontal electrophoresis tank side by side. DNA was allowed to unwind for 40 minutes in freshly prepared alkaline electrophoresis buffer containing 300 mM NaOH and 10 mM Na2EDTA (pH 13.0). After unwinding, electrophoresis was run at 0.72 V/cm (25 V, 300 mA) for 25 min at 4°C under minimal illumina-
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Figure 1. Morphological changes were observed in B16F10 cells after exposure to different concentrations of ABS for 24 h. Untreated cells appeared normal in shape, with about 95-100% confluency, while loss of cell adhesion, decreased cell density along with incorporation of ABS into cells was visualized in the treated sample (magnification 100×).

Apoptosis measurement

Morphological changes in cells were studied by Acridine Orange/Ethidium Bromide (AO/EB) double staining as described by McGahon et al. [22]. Using this technique, the cells undergoing apoptosis are distinguished from the viable cells by the morphological changes of apoptotic nuclei. EB and AO are DNA intercalating dyes. AO is taken up by both viable and dead cells and stains double-stranded (ds) and single-stranded (ss) nucleic acids. When AO diffuses into dsDNA, it emits green fluorescence upon excitation at 480-490 nm from viable cells. EB is taken up by dead cells and stains DNA orange. Briefly, the cells were cultured in six-well plates (2×10^5 cells/well) and incubated overnight. Then, the cells were treated with ABS under doses of IC_{50} determined by cytotoxicity assay for 24 hours at 37°C. DMSO (1%) was used as a negative control. The cells were then harvested and washed twice with PBS. Finally, AO/EB solution was added to the cell suspension and the nuclear morphology was evaluated by fluorescence microscopy (Leica DM 1000, Solms, Germany). Multiple photos were taken at randomly-selected areas and a minimum of 100 cells were counted. According to the method, the live cells have normal green nuclei, apoptotic cells have green nuclei with fragmented chromatin, and dead cells have orange/red nuclei. Tests were done in triplicate.

Measurement of ROS generation

Generation of ROS was assessed by using a cell-permeable fluorescent signal (H2DCF-DA)
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**Figure 2.** Effect of ABS on cell viability. B16 F10 and L-929, cells were treated with different concentrations of ABS or vehicle control PBS for 24 h. ATP assay was used to determine cell proliferation. The percent cell proliferation was calculated by normalizing with a control panel. Data are representative of three independent trials and are expressed as the mean ± SD. * and ** denote statistical differences compared to controls when \( P < 0.05 \) and \( P < 0.01 \), respectively. Significant differences between cancer and normal cells were indicated by \( P < 0.05 \) and \( P < 0.01 \).

**Figure 3.** Reactive oxygen species (ROS) generating effect of ABS on B16F10 cancer cells and L-929 normal cells. ABS induced ROS generation was measured using fluorescent dye DCF-DA by fluorimeter. Data are representative of three independent trials and are expressed as the mean ± SD. * and ** denote statistical differences compared to controls when \( P < 0.05 \) and \( P < 0.01 \), respectively. Significant differences between cancer and normal cells were indicated by \( P < 0.05 \) and \( P < 0.01 \).

indicator for ROS [23]. As described previously, H2DCF-DA is oxidized to a highly green fluorescent DCF (2,7-dichlorofluorescein) by the generation of ROS. L-929 cell lines were pretreated with various concentrations of ABS for 24 h. After 24 h incubation period, the cells were washed with cold PBS and incubated with 100 mM H2DCF-DA for another 30 min at 37°C. DCF fluorescence intensity was measured using the fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, MA) at Ex./Em.=488/525 nm. The estimations were carried out thrice in triplicate, ensuring each time that the number of cells per treatment group were the same to ensure reproducibility. The values were expressed as % relative fluorescence compared to the control.

**Statistical analysis**

The results are presented as the mean ± standard deviation of tree replicates. Data in all experiments were analyzed for statistical significance using analyses of variance (One-Vay ANOVA). \( IC_{50} \) values of ABS over the cell lines were calculated by nonlinear regression analysis. Associations between ROS generation and cell viability parameters were analyzed by Pearson correlation coefficient. The \( P \) value <0.05 was considered as statistically significant. All statistical analyses were performed using SPSS package program for Windows (Version 20, Chicago, IL).

**Results**

**Cytotoxicity of ABS toward cancer and normal cells**

Concentration response assays were performed with B16F10 and L-929 cell lines for 24 h to evaluate the effect of ABS on cell growth. The representative image of morphological changes observed under phase contrast inverted microscopy in these cells after exposure to different concentrations of ABS for 24 h is shown in **Figure 1**. Cytotoxic effect of ABS was determined by the ATP cell viability assay. The cell viability in B16-F10 and L-929 cell cultures were greater than 95% before all experiments. On the addition of ABS, cell viability was significantly reduced \( (P < 0.001) \), PBS was used as the control. According to the control the percentage of anti-proliferative activity progressively increased significantly \( (P < 0.001) \) in a concentra-
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Figure 4. Genotoxic effect of different concentrations of ABS on B16-F10 melanoma cells after 24-hour incubation. Comet formation pattern showed that ABS induces DNA damage in both cancer and normal cells in a dose dependent manner but, genotoxic effect significantly higher in cancer cells than those of normal cells.

Figure 5. ABS induces DNA damage in cell lines were treated with different doses of ABS for 24 h. Results are expressed as the mean ± SD. +, ++ and +++ denote statistical differences compared to controls when $P<0.0$ and $P<0.01$, $P<0.001$ respectively. Significant differences between cancer and normal cell lines were indicated by *, $P<0.05$ and ***, $P<0.01$.

The IC$_{50}$ value of ABS for B16F10 cells and L-929 cells at 24 h were calculated concentration-response curve. IC$_{50}$ doses of ABS were 1.1% for B16F10 cells and 2% for L-929 cells. These data indicate that ABS at lower concentrations can selectively inhibit the growth of melanoma cells without affecting normal cells. Further studies were performed assessing apoptosis and genotoxicity of ABS using under IC$_{50}$ values.

Effects of ABS on ROS generation in the cells

ROS generation was detected by fluorescent probe H2DCF-DA. ROS production increased significantly after ABS exposure on cancer and normal cells ($P<0.001$), and it could efficiently induce ROS generation in cancer cells than in normal cells over doses of 1% ABS in a concentration dependent manner (Figure 3).

There was a statistically strong negative relationship between B16-F10 and L-929 cells via-
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Genotoxic effect of ABS on B16-F10 and L-929 cell lines detected by Comet assay

ABS induced DNA damage was determined by using the Comet assay, a specific assay for detection of genotoxicity [24]. To analyze genotoxic activity, cells were treated with different dosages of ABS for 24 h and DNA damage was detected by the comet assay. Nuclei with damaged DNA have a comet feature with a bright head and a tail, whereas nuclei with undamaged DNA appear round with no tail. Each figure represents a typical comet tail of the observed cells (at least 100 cells) from two slides in each experiment. Typical micrographs of comet assays are shown in Figure 4.

After 24 h ABS treatment, cancer cells showed different size, fragmentation and comet structures with increased % tail intensity when compared to the normal cells. The results indicated that ABS induced DNA damage in a dose-dependent manner (P<0.001), and there were significantly changes in the tail % of DNA between the cancer and normal cells (Figure 5).

Acridine orange/ethidium bromide (AO/EB) staining

Defects in apoptosis are critical in tumorigenesis and resistance to therapy. To clarify whether ABS induced cell apoptosis in cancer and normal cells, AO/EB double staining was performed. To confirm the morphological characteristics of apoptosis, B16-F10 and L-929 cells were exposed to different doses of ABS for 24 h, stained with AO/EB staining and observed under fluorescence microscopy (Figure 6).

As the concentration of ABS increased, the number of uniformly green viable cells decreased and yellow-orange apoptotic cells increased significantly (P<0.001) after 24 h. in both cancer and normal cells and, higher doses of ABS (2%) resulted in greater apoptosis in cancer cells than that of normal cells (P<0.001) (Figure 7).

Discussion

Although, ABS could be used successfully as hemostatic agents for the management of clini-
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Figure 7. Apoptotic activity of ABS on B16-F10 and L-929 cell lines. Cells were treated with different concentrations of ABS (0.25 to 2%) for 24 h, AO/PI double staining and measured by fluorimeter. Data presented were Mean ± SD (n=3). *, ** and *** denote statistical differences compared to controls when P<0.0, P<0.01 and P<0.001, respectively. Abbreviations: Viable cells (VC), Apoptotic cells (AC), Necrotic cells (NC).

In the present study, we identified effective concentrations for the cytotoxicity of ABS using the most sensitive luminometric ATP cell viability assay and we found that ABS treatment dramatically decreased the proliferation of melanoma cancer cells and, the percentage of proliferative activity progressively increased in a dose-dependent manner and, this effect was higher in cancer cells than in normal cells.

The cytotoxic effects of ABS were first investigated on Saos-2 osteosarcoma cell survival and growth [1]. Saos-2, an osteosarcoma cell line often employed in drug resistance studies, were cultured in RPMI media containing 10% FCS, 1% pen/strep, and 1% Na-pyruvate. Following the cultivation, cells were transferred into 12-well tissue plates, in which 2, 4, 6, 8, 10 µL/mL concentrations of ABS solution was added to the growth medium. Control group was cultured in growth media without ABS. Growth of Saos-2 cells was monitored for 17 days during which yellow and opaque-looking aggregates were reported in cultures growing with the presence of ABS. There was a dose-dependent cytotoxic activity and a marked cytotoxicity was observed in the survival of Saos-2 cells. Aggregate formation increased with higher doses of ABS and dose-dependent inhibition was observed in cell invasion. ABS treated Saos-2 osteosarcoma cells were determined to lose adhesion in vitro [27]. Following to this initial study, successful cytotoxic effects of ABS on colon cancer were further demonstrated. Two types of cells were placed separately in 12-well tissue culture containers and ABS solutions with 2, 4, 6, 8 and 10 µL/mL concentrations were added to the culture medium. The cells cultivated in culture medium without exposure to ABS were used as control. The growth of CaCo-2 and Saos-2 cells was monitored for 16 days. ABS application to culture medium resulted in yellow and cloudy aggregates, increasing in parallel with ABS concentration. Therefore, it was concluded that the inhibition of cellular reproduction and decrease in viability of human colon CaCo-2 cells were correlated with applied ABS concentration in vitro. The results have shown that the invasion of cells was also inhibited in a dose-dependent manner. The inhibitor effect of ABS on CaCo-2 cells was observed at 2 µL/mL level and become more prominent at 10 µL/ml concentr-
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It was also noted that CaCo-2 cells that were exposed to ABS, lost their adhesive characteristics in vitro and significant viability loss was observed [28]. In another study Odabas et al. [25], the cytotoxicity of ABS was investigated on human pulp fibroblasts in vitro. ABS was eluted with fresh Dulbecco’s modified Eagle’s medium (DMEM) without serum for 72 h and kept at 37°C. The results have shown that ABS is cytotoxic to human pulp fibroblasts by modified 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, depending on the concentrations of applied ABS. Increased dilutions exhibited fewer cytotoxic characteristics compared to the more concentrated forms on human pulp fibroblasts. However, the mechanism(s) of cytotoxicity of ABS was not clarified yet. One of the mechanisms is its ROS generating activity as a pro-oxidant. We know that ABS is composed of five different plants and, these plants contain polyphenolic compounds [29-31]. Although, polyphenolic compounds are potent antioxidants which can protect the cell from the oxidative stress, i.e., neutralize the damaging effect of ROS [32], they can also show cytotoxic effects at high concentrations through their pro-oxidant activity [33]. Therefore, we studied firstly genotoxic and ROS generating activities of ABS together with apoptotic activity to elucidate the mechanism(s) of cytotoxicity.

In this study, we measured genotoxic effects of ABS on cancer and normal cell lines with alkaline comet assay, using under IC_{50} concentrations of this compound. The comet assay is a sensitive method for detecting DNA strand breakage at the level of an individual cell [34]. We observed that DNA damage increased significantly treated with ABS in a dose dependent manner and, cancer cells was more affected than in normal cells. There was also a positive correlation between DNA damage and ROS generation in ABS exposed melanoma cancer and normal cells. However, there is no available report about ABS and its genotoxic effect. It has been known that the induction of DNA damage in dividing cells results in the activation of cell cycle checkpoints which halts the proliferating cell in its cell cycle progression in order to give time to the DNA damage repair machinery to repair the DNA damage. Eventually, when repair is completed, the cell may proceed into its cell cycle. Alternatively, if the repair process fails, the cell cycle can be blocked permanently, leading to cell senescence or apoptosis [35]. Thus, we found a close positive relationship between DNA damage and apoptosis in ABC treated cells.

Induction of apoptosis is the major mechanism for cytotoxicity [36]. Apoptosis can be measured by a number of methods by taking advantage of the morphological, biochemical, and molecular changes undergoing in a cell during this process. We evaluated apoptotic, necrotic and live cells by using AO/EB fluorescence staining method. After staining, living cells are distinguished with a normal green nucleus; Apoptotic cells show orange-stained nuclei with chromatin condensation or fragmentations, while necrotic cells are clearly observed with uniformly orange-stained cell nuclei with no condensed chromatin. The results of present work revealed that ABC treated cancer cells clearly exhibit more apoptotic events (chromatin condensation and nuclear fragmentation) with a significant decrease in cell viability than normal cells in a dose dependent manner. 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 [18, 37]. It is has been reported that the anticancer drugs kill the cancer cells by stimulating the apoptotic pathways [38]. Phenolic compounds can affect the cellular redox status because of their pro-oxidant properties. This may result cytotoxic effect, apoptosis and/or necrosis following DNA damage [39].

The increased production of ROS in cancer cells was observed in vitro and in vivo [40, 41]. In the present study, it was observed that the cancer cells (B16-F10) generated more ROS than normal cells (L-929). As expected, ABS also caused significantly high ROS generation in B16-F10 cells in a dose dependent manner. Therefore, cumulative effect of ROS in cancer cells can cause higher cytotoxicity than those of normal cells. The strong negative relationship between cell viability and ROS generating activity in melanoma cancer and normal cells demonstrated that antiproliferative effect of ABS might be depending on its pro-oxidant activity. This is the first report showing ROS generating activity of ABS in cancer and normal cells in a dose dependent manner. Indeed, under physiological conditions, the mainte-
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nance of an appropriate level of intracellular ROS is important in keeping redox balance and cell proliferation [42]. Even a modest increase in ROS levels can stimulate cell growth and proliferation [43]. However, high levels of ROS can be an important mechanism of cell death [44]. Overproduction of ROS and free radicals lead to serious damage to lipids, proteins, and DNA, and regulate the process involved in the initiation of apoptotic signaling [45]. Cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents [46]. Based on the results of our present study, we hypothesized that the cytotoxic effects of ABS in the cell lines may be due to the reactive oxygen species (ROS) mechanism inside the cells. There was a strong negative correlation between cell viability and ROS generating activity and, positive correlations between ROS and DNA damage and apoptosis in ABS exposed cancer and normal cells.

Although ABS is commonly used as a hemostatic agent, it can cause DNA damage, apoptosis and cytotoxicity by generating ROS activity. Therefore, it should be removed the unused ABS by cleaning once the hemostasis is achieved to minimize the postoperative side effects. Moreover, our results demonstrated that ABS may be a useful therapeutic intervention for cancer.

Disclosure of conflict of interest

None.

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