

Effect of Reconstituted Apple Juice with Alkaline Electrolyzed Water on Cell Proliferation and Apoptosis of HT-29 Cells

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ABSTRACT

Apple juice, known as a good antioxidant source, may partially protect the body from oxidative stress. In this report, the effect of apple juice reconstituted with alkaline electrolyzed water (EO-apple juice) on HT-29 cells was investigated. Specifically, the total antioxidant capacity (ORAC and TEAC) of EO-apple juice, and the effects this EO-apple juice on cell proliferation (MTT assay), DNA fragmentation, and oxidative DNA damage (comet assay) of HT-29 cells were evaluated. The TEAC value of EO-apple juice was higher (2773 $\mu\text{M TE}$ vs. 1739 $\mu\text{M TE}$) than apple juice reconstituted with ultra pure water (Water-apple juice). EO-apple juice also had a higher ORAC value (15446 $\mu\text{M TE}$) than that Water-apple juice (13908 $\mu\text{M TE}$). After 72 h cell incubation, HT-29 cell proliferation was more effectively reduced by the EO-apple juice than the Water-apple juice. Induction of apoptosis was determined using a DNA fragmentation method, and apoptotic cells increased in a dose-dependent manner after treatment with both reconstituted apple juices. In conclusion, EO-apple juice had a stronger antioxidant effect than water-apple juice and can inhibit HT-29 cell proliferation plus induce apoptosis.

Key words: electrolyzed water, the total antioxidant capacity, cell proliferation, apoptosis, DNA damage

INTRODUCTION

Unlimited cell proliferation and evasion of apoptosis within a cell cycle can lead to the development of cancer; the initial stage is characterized by DNA damage and the need for cellular repair⁽¹⁾. A prolonged imbalance of cell proliferation and apoptosis may result in tumor development with continued DNA damage⁽²⁾.

Apples and apple juice are rich dietary sources of polyphenolic flavonoids⁽³⁾, and recent studies have focused on the capacity of flavonoids to act as cancer-preventing compounds. Flavonoids may act as antioxidants, which scavenge free radicals and reduce oxidative stress⁽⁴⁾; inhibit cell proliferation⁽⁵⁾; induce apoptosis⁽⁶⁾; and prevent DNA damage⁽⁴⁾. Some studies have also reported that complex mixtures of phytochemicals in fresh fruits have a greater preventive effect on colon cancer cells than the sum of individual ingredients when tested alone⁽⁵⁾.

Electrolysis of water produces two types of water: alkaline water at the cathode and acid water at the anode⁽⁷⁾. Recently, alkaline electrolyzed (EO) water has received attention due to its proposed health benefits. Alkaline EO water, which has a high pH, low oxidation-reduction potential, low dissolved oxygen, and high dissolved hydrogen, has been used for drinking water. In

addition, alkaline EO water enhanced the antioxidant effects of ascorbic acid⁽⁸⁾ and showed a protective effect against oxidative damage to DNA⁽⁹⁾.

MATERIALS AND METHODS

I. Materials, Chemicals, Cells, and Media

Commercial frozen concentrated apple juice (Kroger, Cincinnati, Ohio) was purchased from a grocery store in Athens, Georgia. McCoy's 5a medium, trypsin-EDTA, and other reagents were purchased from Sigma-Aldrich (Milwaukee, WI). The MTT kits, HT-29 cells, and fetal bovine serum (FBS) were obtained from America Type Culture Collection (ATCC: Manassas, VA). The ELISA kit was purchased from Roche (Indianapolis, IN). The comet assay kit was purchased from Trevigen[®] (Gaithersburg, MD). ABTS substrate (Southern Biotech, Birmingham, AL) was used to generate the ABTS radical.

II. Cell Culture and Preparation of Sample and Medium

pH and ORP values were determined using a pH/ion/conductivity meter (Accumet model 50, Fischer Scientific Co., Fair Lawn, NJ).

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Apple juice from concentrate was reconstituted using either alkaline EO water or ultra pure water with 3 parts water to 1 part frozen juice concentrate. Juice samples were diluted to final concentrations (0.1, 0.2, 0.3, 0.4, 0.5 $\mu\text{L}/\text{mL}$) with fresh medium for MTT, apoptosis, and DNA damage assay.

McCoy's 5a medium was prepared with ultra pure water or alkaline EO water (pH 10.59) and then adjusted to the same pH as regular media by adding diluted HCl. The HT-29 cells were cultivated in 75 cm^2 flasks with the medium containing 10% FBS in an incubator with 5% CO_2 at 37°C. The medium was replaced every 2 - 3 days.

III. Cell Viability

HT-29 cells (1.0×10^4) were seeded in 96 well plates and then incubated with control (McCoy's 5A medium) or samples as described above for 72 h at 37°C. After incubation, the MTT reduction assay was used to measure cell viability according to the modified procedures⁽¹⁰⁾.

IV. DNA Fragmentation

To detect apoptosis, HT-29 cells (1.5×10^5) incubated for overnight. The cells were replaced with the medium containing apple juice and then incubated for 72 h at 37°C. After incubation, the DNA fragmentation was measured using a Cell Death detection ELISA^{plus} kit (Boehringer Mannheim, Roche) with a microplate reader (EL \times 800TM, Bio-Tek) at a test wavelength of 405 nm and a reference wavelength of 490 nm. Results were recalculated as the relative induction apoptosis of cells treated with H_2O_2 as described in Kern *et al.*⁽⁶⁾.

V. Alkaline Single Cell Gel Electrophoresis (Comet Assay)

For measurement of DNA damage, HT-29 cells (1.5×10^5) incubated for 24 h and the medium was replaced with medium containing apple juice with the same concentration as described above for 24 h at 37°C. After treatment, the comet assay was conducted according to the modified procedure⁽⁴⁾ using the comet assay kit (Trevigen[®], Gaithersburg, MD). Electrophoresis was carried out at 25 V and 295 mA for 30 minutes. After electrophoresis, the slides were placed in 70% ethanol for 5 min, then dried, and stained using a silver staining kit (Trevigen[®], Gaithersburg, MD). The DNA of each cell migrated according to the amount of DNA damage resulting in a head (original location of DNA) and tail (migration of damaged DNA). The slides were then photographed and analyzed using the CometScore software (TriTek, Sumerduck, VA) with at least 50 - 100 cells per slide. DNA damage was calculated as mean % DNA in the tail, which corresponds to percentage of DNA damage, as described in Schefer *et al.*⁽⁴⁾.

VI. Antioxidant Capacity (ORAC and TEAC Assay)

The ORAC-FL assay was based on a modified procedure⁽¹¹⁾. Reconstituted apple juice was diluted 700 fold in phosphate buffer (pH 7.4) prior to analysis. Briefly, 20 μL of diluted reconstituted apple juices, 75 mM phosphate buffer (as blank; pH 7.4), or Trolox (as standard calibration solution) were placed in the 96-well plates, respectively. After 30 min, AAPH was added and the decrease of fluorescence was monitored for 154 min (ex/em 485/520 nm) using a FLUOstar OPTIMA plate reader (BMG Lab Technologies, Durham, NC). The area under curve and ORAC values were calculated as described in Prior *et al.*⁽¹¹⁾.

TEAC assay was conducted using a modified method described in⁽¹²⁾. Briefly, a 40 μL of samples (ethanol as blank, reconstituted apple juice, alkaline EO water, and Trolox as the standard) was mixed with 1960 μL ABTS⁺⁺ solution (absorbance adjusted to 0.70 ± 0.1) and incubated for 6 min to read absorbance at 734 nm using a spectrophotometer (DU@520 Ceneral Purpose UV/V is Spectrophotometer, Beckman). Antioxidant capacity was calculated based on the Trolox standard curve as Trolox equivalents (TE) per liter of apple juice ($\mu\text{M TE}$).

VII. Statistical Analysis

Results are expressed as the mean \pm standard error. The difference between the control and each experimental test condition was determined using general linear models (GLM), with Fisher's least significant difference (LSD). A value of $p < 0.05$ was considered to be statistically significant. Statistical analysis was conducted using the statistical analysis system (SAS).

RESULTS AND DISCUSSION

I. Characteristics of Alkaline EO Water and Total Antioxidant Capacity

Alkaline EO water used in this study had the following physical properties: pH 10.59 and oxidation reduction potential (ORP) of -139.2 mV.

The total antioxidant capacities of reconstituted apple juice are shown in Table 1. The TEAC value of EO-apple juice was significantly higher than that of

Table 1. Total antioxidant capacity of reconstituted apple juice

	TEAC value ($\mu\text{M TE}/\text{L}$)	ORAC value ($\mu\text{M TE}/\text{L}$)
Water-apple juice	1793 ± 105^b	13908 ± 2102^a
EO-apple juice	2773 ± 88^a	15446 ± 2723^a

Data are means \pm standard error of three replicates. The same letter after each mean value in the same column indicate not significantly different at p values < 0.05 .

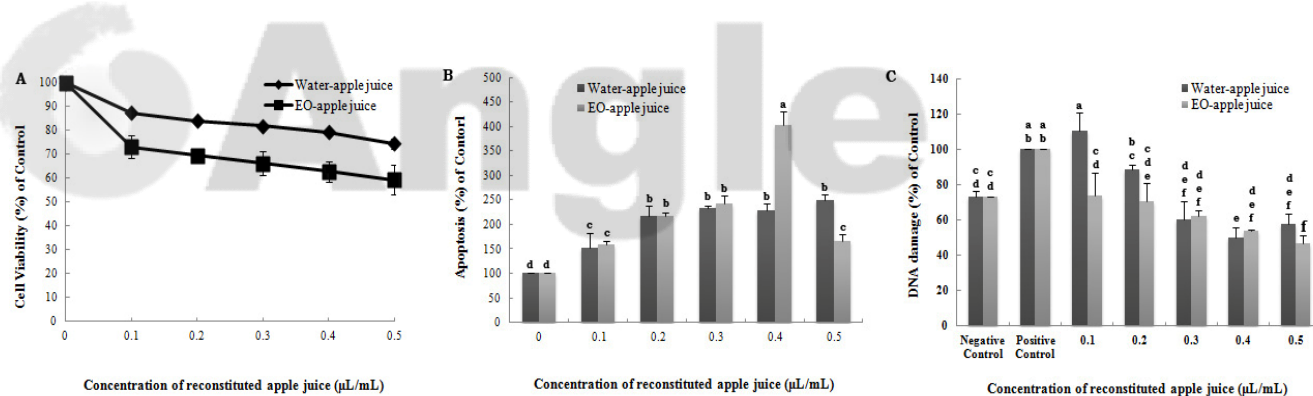


Figure 1. Effect of reconstituted apple juice on HT-29 cell viability, apoptosis, and DNA damage. Data are means \pm standard error of three replicates. Water-apple juice is apple juice reconstituted with ultra pure water. EO-apple juice is apple juice reconstituted with alkaline EO water. The same letter indicate not significantly different at p values $<$ 0.05.

water-apple juice (2773 μ M TE vs. 1793 μ M TE) ($p <$.0001). In contrast, EO-apple juice was only slightly higher than water-apple juice for ORAC value (15446 vs. 13980 μ M TE) but they were not significantly different ($p <$ 0.67). These results demonstrate that alkaline EO water may enhance the antioxidant properties of apple juice based on TEAC assay.

It has been theorized that active atomic hydrogen in alkaline EO water can contribute to ROS (reactive oxygen species) scavenging activity and may also be engaged in the redox regulation of cellular function⁽¹³⁾. In addition, the higher dissociation activity of alkaline EO water might increase the dissociation activity of antioxidant substances such as vitamin C with relatively lower dissociation activity and hence enhance their antioxidant capacity⁽⁸⁾. Therefore, our results showing an enhancement of TEAC value on EO-apple juice could provide support that alkaline EO water can enhance antioxidant capacity of other antioxidants.

II. Cytotoxicity of Reconstituted Apple Juice and Alkaline EO Water on HT-29 Cells

The effects of reconstituted apple juice on the cell proliferation of HT-29 cells are shown in Figure 1A. Cell proliferation decreased with increasing concentrations of apple juice. The cells treated with EO-apple juice resulted in lower cell viability than that with water-apple juice (p value = 0.0005). The HT-29 cells treated with 0.5 μ L/mL EO-apple juice had the greatest reduction (Cell viability %: 58.57 ± 5.07) on cell population.

In previous studies, apple juice has been shown to have effective antioxidant activity and antiproliferation effects upon cancer cells^(4,5). Also, cancer cell proliferation was inhibited by increased concentration of antioxidant compounds including vitamin C⁽¹⁴⁾. Our study also demonstrated alkaline EO water reconstituted apple juice enhanced antioxidant capacity and inhibition of cell proliferation of HT-29 cells.

III. Apoptosis

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of apoptosis⁽²⁾. DNA fragmentation in HT-29 cells treated with reconstituted apple juice shows apoptosis was dependent on whether apple juice was reconstituted with alkaline EO water ($p <$ 0.0001) (Figure 1B). Apple juice at all concentrations showed a higher induction of apoptosis than control (treated with H₂O₂ alone and no apple juices). In addition, apple juice mixed with ultra pure or EO water showed 50% greater induction of apoptosis than control at an apple juice concentration of 0.1 μ L/mL. EO-apple juice had significant higher DNA fragmentation than water-apple juice only at 0.4 μ L/mL (p value = 0.0006) and EO-apple juice also had the greatest induction of apoptosis at 0.4 μ L/mL (Figure 1B).

Our finding on the effect of apple juice on apoptosis induction is in agreement with several previous studies. Kern and others⁽⁶⁾ reported that fragmented DNA of HT-29 cells was increased at a sudden point (high concentration of apple extract) and then decreased with further increase of concentration of apple extract for a prolonged treatment time. Prolonged incubation time may induce more apoptotic cells but also induced necrotic cells. Thus, at a concentration of 0.5 μ L/mL, apoptosis may decrease due to increased necrotic cells (Figure 1B).

IV. Oxidative DNA Damage Prevention of Reconstituted Apple Juice and Alkaline EO Water

The effects of reconstituted apple juice on H₂O₂-induced oxidative DNA damage in HT-29 cells are shown in Figure 1C. A significant decrease in H₂O₂-induced DNA damage was observed with apple juice (Figure 1C). DNA damage was reduced by 47% when HT-29 cells were pre-incubated with apple juice at concentration of 0.3 to 0.5 μ L/mL. Throughout the concentration range (0.1 to 0.5 μ L/mL), treatment with apple juice reduced DNA damage below that of the positive control suggesting an anti-oxidant effect on DNA damage. Cells treated with EO-apple juice had less DNA

damage than those were treated with water-apple juice at 0.1 $\mu\text{L}/\text{mL}$.

Oxidative damage is linked to the formation of tumors through several mechanisms. Cancer induced by oxidative damage might be prevented or limited with dietary antioxidants found in fruits and vegetables⁽¹⁵⁾. Previous studies also demonstrated that menadione-induced (oxidative) DNA damage was more effectively reduced by reconstituted mixtures of cider and table apples compared to the original extracts⁽⁴⁾ and that alkaline EO water had a protective effect on H_2O_2 induced DNA damage⁽¹⁶⁾. In the current study, EO-apple juice reduced DNA damage more than water-apple juice at 0.1 $\mu\text{L}/\text{mL}$ levels.

CONCLUSIONS

In conclusion, EO-apple juice showed higher antioxidant capacity (TEAC), and enhanced the effect of apple juice on HT-29 cell proliferation, cell apoptosis, and DNA damage at some, but not all concentrations of apple juice. In part, some effects are related to its antioxidant capacity to prevent oxidative DNA damage. In addition, alkaline EO water and apple juice reconstituted with alkaline EO water may affect other mechanisms leading to the inhibition of cell proliferation and induction of apoptosis.

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