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Nutrition

# Apple juice attenuates genotoxicity and oxidative stress induced by cadmium exposure in multiple organs of rats

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ABSTRACT

The aim of this study was to evaluate the health benefits associated with apple consumption following cadmium exposure. A total of 15 Wistar rats were distributed into three groups ($n = 5$), as follows: control group (non-treated group, CTRL); cadmium group (Cd) and apple juice group (Cd + AJ). The results showed a decrease in the frequency micronucleated cells in bone marrow and hepatocytes in the group exposed to cadmium and treated with apple juice. Apple juice was also able to reduce the 8OHdG levels and to decrease genetic damage in liver and peripheral blood cells. Catalase (CAT) was decreased following apple juice intake. Taken together, our results demonstrate that apple juice seems to be able to prevent genotoxicity and oxidative stress induced by cadmium exposure in multiple organs of Wistar rats.


## Introduction

Over recent years, concern about environmental pollution as well as its impact on human health has been the subject of several studies so far [1–3]. It is well established that exposure to environmental pollutants is related with the etiology and pathogenesis of many chronic degenerative diseases [4].

Cadmium is one of the most toxic environmental and industrial pollutants, present in contaminated soil, water, air, foodstuffs and in the smoke released by cigarettes [5,6]. The metal is highly toxic, accumulating in various human tissues [7], and it induces oxidative stress, lipid peroxidation and DNA damage. Moreover, it is responsible for several pathologies such as renal dysfunction, skeletal disorders, cardiovascular diseases, lung, kidney and gastrointestinal damage, as well as liver and salivary glands malfunction and cancer [8–13].

In view of the human exposure to cadmium due to environmental, occupational, dietary and smoking contamination, the search for methods able to modulate the influence of this metal on the development of diseases is extremely important [4]. The current knowledge on the mechanisms of action of cadmium exposure in our body leads us to believe that the consumption of antioxidant compounds might be able to mitigate the toxic effects caused by the metal, either by scavenging of reactive oxygen species (ROS) or by the chelating action [14]. Therefore, it has been recommended the regular consumption of functional foods prevents some chronic degenerative diseases [15].

Apple and its derivatives are a great source of nutrients due to high levels of bioactive compounds such as polyphenols and phytochemicals [16]. Studies show that the consumption of apples is associated with decreased risk of developing chronic diseases [17]. According to Ribeiro et al. [18] and Soyalan et al. [19], apple and their compounds act as an antioxidant agent, improving the production of oxidative stress enzymes.

This work aimed to know the benefits of apple consumption following cadmium exposure as a result of its biological effects on genotoxicity and oxidative stress by gene expression of Copper-Zinc Superoxide Dismutase (CuZn-SOD), Manganese Superoxide Dismutase (Mn-SOD) and Catalase (CAT).

## Materials and methods

### *Animals and experimental design*

All experimental protocols involving animals are conformed to procedures described in the Principles for the Use of Laboratory

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**Table 1**
Mean initial, and final body weight (±S.D.) of rats intoxicated with cadmium and treated with apple juice.

| | CTRL | Cd | Cd + AJ |
|---|---|---|---|
| Initial body weight (g) | 288.2 ± 10.6 | 272.2 ± 22.7 | 282.6 ± 9.9 |
| Final body weight (g) | 399.5 ± 1.9 | 363.4 ± 46.7 | 338.2 ± 27.1 |

$p > 0.05$. CTRL, control group; Cd, cadmium group; Cd + AJ, cadmium associated with apple juice group. $n = 5$ per group.

Animals Guidelines. The study was approved by the Animal Ethics Committee of Federal University of Sao Paulo, UNIFESP, SP, Brazil (Protocol: CEUA number 484411).

A total of 15 Wistar rats weighing ~250 g, 8 weeks age were distributed into three groups ($n = 5$), as follows: Control group (non-treated group, CTRL); Cadmium group (Cd) and Apple Juice group (Cd + AJ). All animals were provided from Development Center of Experimental Models for Medicine and Biology (CEDEME) of Federal University of São Paulo, SP, Brazil, and they were maintained under controlled conditions of temperature (23 ± 1 °C), light–dark periods of 12 h and free access to water and diet.

Animals from CTRL group received a single intraperitoneal (ip) water injection while those from the groups Cd and Cd + AJ received a single dose of 1.2 mg/kg body weight (BW) intraperitoneally (CdCl2, Nuclear®), diluted in water according to the method described by Predes et al. [20]. After 15 days, Cd + AJ group received apple juice for 15 days, by gavage (1 mL, per day). CTRL and Cd groups were treated by gavage containing drinking water during the same experimental period. All animals were checked daily in order to evaluate individual behavior and general health conditions. The body mass was recorded weekly. No significant statistically differences ($p > 0.05$) in body weight were detected among groups after experimental design. Such data are demonstrated in Table 1.

At the end of the experimental period, all animals were anesthetized with inhalational anesthetic halothane (Tanohalo™, Cristália™, SP, Brazil) and euthanatized for colleting tissues/organs to be used in this study. Previous studies have demonstrated that anesthesia does not interfere with redox status or cytokine profile levels [21,22].

The chemical characterization of apple juice (pulp wash juice) was performed in a previous study conducted by our research group [18]. The major components of interest with $m/z$ between 300 and 500 identified in it was caffeoylquinic acid, cyanidin-3-O-glucoside, 1-dodecanoyl-glycero-3-phospho-(10-sn-glycerol) and heptacosanoic acid. Several studies have calculated and estimated the daily amount of flavonoids that should be consumed by individuals. According Kahle et al. [23] and van der Sluis et al. [24], it is estimated that the daily amount of flavonoid intake should be between 0.15 and 1 g of total phenols. Under previous analysis, the apple juice used in this work has 4.3 mg/mL of total phenols. In this regard, it was administered 1 mL of apple juice daily.

*Micronucleus test*

After completing the experimental period, the micronucleus test was performed in bone marrow and liver tissue. The micronucleus test using bone marrow cells was performed according to Ribeiro et al. [25]. For this purpose, femoral bones were collected and stored in sodium chloride at 0.9%. The proximal epiphyses of bones were removed and 1 mL of fetal bovine serum (FBS; Cultilab™, Campinas, São Paulo, Brazil) was injected into the medullar canal. A smear on glass slide was performed with the suspension formed by the bone marrow and fetal bone serum. After drying the slides, they were stained with Giemsa (Merck™, Darmstadt, Germany). For liver, the micronucleus test was performed using paraffin sections (3 μm) stained by Feulgen and counterstained with Fast Green (Sigma Aldrich™, USA). A total of one thousand polychromatic erythrocytes or hepatocytes were analyzed per animal. Slides were scored blindly using a light microscope with a 100× immersion objective.

*Single cell gel (Comet) assay*

The protocol used for peripheral blood and liver cells followed the guidelines outlined by Tice et al. [26]. Peripheral blood was collected by cardiac puncture and liver cells were obtained by liver tissue maceration with PBS. Cells were transferred to individual plastic tubes, containing 1 mL of cold phosphate buffer solution (PBS, $Ca^{+2}$, $Mg^{+2}$ free, pH 7.3), and centrifuged for 5 min, 1000 rpm, at room temperature. The supernatant was removed and the cell suspensions (~10 μL) were used for single cell gel (Comet) assay. A volume of 10 μL of cellular suspension was added to 120 μL of 0.5% low-melting point agarose at 37 °C, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and the slides immersed in lysis solution (2.5 mol $L^{-1}$ NaCl, 100 mmol $L^{-1}$ EDTA–Merck™, Darmstadt, Germany; 10 mmol $L^{-1}$ Tris–HCl buffer, pH 10 – Sigma Aldrich™, St Louis, MO, EUA; 1% sodium sarcosinate – Sigma™, St Louis, MO, EUA; with 1% Triton X-100 – Sigma™, St Louis, MO, EUA; 10% dimethyl sulphoxide – Merck™, Darmstadt, Germany) for about 1 h. Afterwards, the slides were washed in ice-cold PBS ($Ca^{+2}$, $Mg^{+2}$ free, pH 7.3) for 5 min, left in electrophoresis buffer (0.3 m mol $L^{-1}$ NaOH and 1 m mol $L^{-1}$ EDTA–Merck™, Darmstadt, Germany, pH > 13) for DNA unwinding during 20 min, and electrophoresed in the same buffer for 20 min at 25 V (0.86 V/cm) and 300 mA. Following electrophoresis, slides were neutralized in 0.4 mol $L^{-1}$ Tris–HCl (pH 7.5, Sigma Aldrich™, St Louis, MO, EUA), fixed in absolute ethanol and stored at room temperature until analysis in a fluorescence microscope at 400× magnification. All steps were performed under reduced light.

A total of 50 randomly captured comets per animal (25 cells from each slide) were examined blindly by one expert observer at 400× magnification using a fluorescent microscope (Olympus™, Orangeburg, NY, USA). The microscope was connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments™, Suffolk, Haverhill, UK) calibrated previously according to the manufacturer's instructions. To measure DNA damage, we used the tail moment defined as the product of the tail length and the fraction of DNA in the Comet tail [26].

*Challenge assay*

For this purpose, one aliquot (20 μL) from liver cells was treated with 4-nitroquinoline 1-oxide (4NQO, 0.05 m mol $L^{-1}$) for 15 min or $H_2O_2$ (0.6 m mol $L^{-1}$) for 5 min. Then, agarose was added and smears were made and left to lysis solution (2.5 mol $L^{-1}$ NaCl, 100 m mol $L^{-1}$ EDTA, 10 mmol $L^{-1}$ Tris–HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100, and 10% DMSO) for about 1 h. All the slides prepared were immersed into alkaline buffer for 20 min and electrophoresis was conducted for another 20 min, at 300 mA and 25 V. After that, the slides were neutralized using Tris solution (Sigma Aldrich™, EUA) and fixed at ethanol and stored at room temperature until analysis in a fluorescence microscope at 400× magnification. The analysis was the same described previously.

*8-OHdG immunohistochemistry*

Liver serial sections of 4 μm were desparafinized in xylene and rehydrated in graded ethanol (99.5%), then pretreated in a