

Effects of Aerobic Training and Pumpkin Seed Extract Consumption on the Heart and Aorta Oxidative Stress Biomarkers: A Case of Rats Exposed With Arsenic

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ABSTRACT

Introduction: It has been proposed that regular aerobic exercise training and medicinal plants are able to reduce oxidative damage resulted from toxin metal poisoning such as arsenic which is a significant risk factor in the heart diseases. The purpose of this research is to evaluate the simultaneous effect of regular aerobic exercise training and pumpkin extract on oxidative stress of the heart and endothelial cells of the aorta in rats poisoned with arsenic.

Materials and Methods: 56 male Wistar rats were divided into seven groups including TC (toxic control); AE (aerobic exercise training); TAP1 (toxic aerobic exercise training + 300 mg/kg/day pumpkin seed extract consumption); TAP2 (toxic aerobic exercise training + 600 mg/kg/day pumpkin seed extract consumption); TP1 (300 mg/kg/day toxic pumpkin seed extract consumption); TP2 (600 mg/kg/day toxic pumpkin seed extract consumption), and HC (healthy control). The intervention was performed based on the groups' categorization. 24 hours after the last intervention the samples were collected. Statistical analyses were performed using one and two-way analysis of variance (ANOVA).

Results: The results showed that exercise training increased GSH ($p=0.0001$) and GSSG ($p=0.0001$) and declined MDA levels ($p=0.0001$) in the aortic endothelial cells and heart tissue. Also, pumpkin seed extract consumption induced a significant increase in GSH ($p=0.0001$) and GSSG ($p=0.0001$) and decreased MDA levels ($p=0.0001$) in the aortic endothelial cells and heart. On the other hand, interaction of exercise training and pumpkin seed extract consumption increased GSH ($p=0.0001$) and GSSG ($p=0.0001$) and decreased MDA levels ($p=0.0001$) in the aortic endothelial cells and heart.

Conclusion: It is concluded that concurrent of pumpkin seed extract consumption and aerobic exercise training could intensify mutual antioxidant effects in oxidative stresses such as cardiac disorders caused by arsenic poisoning.

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Introduction:

Today, due to population explosion and the need to food and other living facilities, humans have changed the environment to meet their needs. Factories and roads use

further surface and underground water resources and mining has made the environment contaminated with all kinds of toxic metals and other pesticides. Among the chemical pollutants, arsenic is a significant

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element existing in drinking and underground waters which happens to be dissolved in different layers of soil. One of the most important global health problems is the concentration of arsenic in underground and drinking waters (1). Inorganic arsenic exists in the form of arsenic and arsenate in drinking water. Unfortunately, arsenic, as a carcinogen, can be absorbed through skin, respiratory and digestive systems, and has appeared as a threat to humans and animals health. Poisoning by arsenic would happen in both acute and chronic forms. The main attributes of acute arsenic poisoning include serious damage to the digestive system and cardiovascular disorders (2).

Notably, arsenic is able to inhibit about 200 synthesizing and modifying enzymes involved in cellular energy metabolization. Mineral arsenic or its metabolites may induce oxidative stress, altering the gene expression interfering with the cellular signaling. Arsenic leads to the formation of reactive oxygen species and induction of oxidative stress through affecting antioxidant system (3). Oxidative stress is an imbalance between the production of free oxygen radicals and the body's antioxidant capacity, which plays an important role in the pathophysiology of the heart (4). The antioxidant system plays an important role in damages caused by free radicals. Antioxidant defense system contains non-enzymatic and key enzymatic antioxidants agents including superoxide dismutase enzyme (SOD), catalase enzyme (CAT) and glutathione (5). Glutathione is one of the non-enzymatic antioxidants found in cytosol and mitochondria which involves in detoxification of the cell, antioxidant defense and modulation of cell proliferation. Glutathione could be found in both forms of GSH and GSSG (glutathione-disulfide), so that the excessive release of free radicals can lead to reduced levels of GSH or accumulation of GSSG in the cell (5).

Arsenic induces oxidative stress by increasing malondialdehyde (MDA). Malondialdehyde is a tri-carbon aldehyde and an important product of unsaturated fatty acids degradation. Malondialdehyde can also be produced from membrane lipids peroxidation and is a marker for oxidative heart damage (3). Myocardium is one of the

most vulnerable tissues of chronic oxygen-induced oxidative damage due to continuous activity. Oxygen-reactive species (ROS) include superoxide, peroxide and hydroxyl as the main cause of the heart muscle damage and cause many changes such as weakening of contractile function, arrhythmias and changes in gene expression. It has also been shown that ROS can interfere with the integrity of endothelial cells. Increased oxidative stress in the heart failure has been observed to cause inflammation and endothelial dysfunction doing an important role in the development of congestive heart failure (CHF) (6). ROS-induced dysfunction of endothelial function includes activation of the complement system and production of proinflammatory cytokines, prostacyclin, thromboxane A2 and platelet factors, as well as increased expression of adhesion molecules (7).

Myocardium is highly defenseless to free radicals due to high oxygen consumption and weak antioxidant systems (8). Recent studies have demonstrated that performing regular aerobic exercises promotes compensation and protection responses to oxidative stress injuries by increasing the expression of antioxidant-enzyme genes (9), so that aerobic activity increases the size of the heart, blood and plasma volume and level of anti-oxidative enzymes, and subsequently these factors lead to optimum oxygen consumption. Furthermore, aerobic exercise performance makes people less likely suffer from oxygen constraints and, and consequently, do a certain activity with lower oxygen consumption. Aerobic exercises have created some kind of compatibilities with antioxidant and repair systems, which results in a significant reduction of oxidative stresses (10). One of the most important sources of antioxidant compounds are plants which have a wide range of scavenging molecules of free radicals. Interestingly, natural antioxidants such as plants, increase the strength of body antioxidants defense and also reduce the risk of cancer and cardiovascular disease (11).

Pumpkinseeds (*Cucurbitapepo L.*) is an annual and crawling plant from the *Cucurbitaceae* family. The nutritional value of pumpkin is very high and its fruit is rich in antioxidants such as beta carotene (a

major source of vitamin A), vitamins E and C, and salts and minerals including selenium, calcium, manganese, magnesium, potassium, iron, zinc, as well as omega-3 and omega-6 unsaturated fatty acids like linoleic acid, oleic acid and many fibers (12). Pumpkin has potent antioxidant activity due to phenolic compounds, tocopherols and zinc. Many reports have displayed that pumpkin extract is a useful compound for heart health, lowering blood pressure and lowering blood lipids because of its essential fatty acids, proteins, salts and vitamins, as well as antioxidants and phytoestrogens (13). Importantly, unsaturated fatty acids such as linoleic acid and oleic acid in pumpkin have been observed to be associated with a significant reduction of cardiotoxicity by decreasing the production of cyclooxygenase enzyme in animal models (14).

Regarding protective potential of pumpkin and the effect of this plant on inflammation and vascular factors, as well as advantages of regular aerobic exercises on the cardiovascular system, this study aimed to examine the effect of simultaneous regular aerobic exercise training and pumpkin seed extract consumption on the oxidative stress of the heart and aorta endothelial cells in rats infected with arsenic.

Materials and Methods

Animals and Ethics

In this study, 56 male Wistar rats (6-8 weeks old and weighing 220-240 g) were used. They were kept in an animal room at 19-22°C in 12:12h light-dark cycle. Animals were fed with standard rodent laboratory diet (crude protein 19.50–20.50%, fat 3.5–4.5%, fibre 4–4.5%, calcium 0.95–1%, phosphorus 0.65–0.7%, salt 0.5–0.55%, lysine 1.15%, methionine 0.33%, threonine 0.72%, tryptophan 0.25%, energy 16.16–17 mJ/kg) and tap water. All animal experimentations were approved by the Ethical Committee of Arak Branch, Islamic Azad University (Ethic code: IR.IAU.ARAK.REC.1398.012) and were in accordance with National Institutes of Health (NIH) guidelines as well.

Experimental Design

Rats were randomly divided into seven groups of eight animals, and based on receiving regular aerobic exercise and/or pumpkin seed extract consumption were categorized as: 1) TC (toxic control); 2) AE (aerobic exercise training); 3) TAP1 (toxic aerobic exercise training + 300 mg/kg/day pumpkin seed extract consumption); 4) TAP2 (toxic aerobic exercise training + 600 mg/kg/day pumpkin seed extract consumption); 5) TP1 (300 mg/kg/day toxic pumpkin seed extract consumption); 6) TP2 (600 mg/kg/day toxic pumpkin seed extract consumption), and 7) HC (healthy control) groups.

To induce oxidative stress in the rats, all groups except HC were exposed to 25 ppm arsenic as sodium arsenite per day in drinking water for 16 weeks (15).

Regular aerobic exercise training protocol

Firstly, all rats were familiarized with running on the treadmill with 5% incline at a speed of 5-10 m/min and 10 min/day for one week. Aerobic exercise training began with warm-up and terminated with cool-down both of which consisted of 2 min running at 10 m/min. Then the main running started with 10 m/min, with 5% incline for 10 min/day, so that the speed and time were gradually increased up to 15 m/min for 20 min/day for two weeks. In the last 2 sessions, the intensity of aerobic activity reached 25 m/min for 30 min/day (16).

Pumpkin seed preparation (crude extract)

Iranian oil-free pumpkin seed (cold-pressed oil) was purchased from PAKAN BAZR Co., Isfahan, Iran. In the first step, 10 g of grinded seeds was mixed with 70% alcohol in a blender and incubated for 72 h at room temperature. Then ethanol liquid was separated with Whatman® Anotop® 10 syringe filters 0.02 µm paper. The pumpkin seed extract was fed to rats by specific gavage at a dose of 300 and 600 mg/kg/day, so that rats in the supplemental groups (groups 3-6) received 300 mg/kg/day and 600 mg/kg/day of pumpkin seed extract for 8 weeks by oral gavage.

Autopsy Procedures

24 hours after the last intervention during which rats were fasting for 10–12 hours,

they were anesthetized by intraperitoneal injection of a combination of Ketamine (90 mg / kg) and Xylazine (10 mg / kg). The chest was then opened and the heart and aorta were immediately separated and frozen using liquid nitrogen in order to send to lab. Next, the tissues were defrosted and homogenized at 4° C, so that 50 mg of ventricle was homogenized on ice via dissolving in 1 ml of cell lysis buffer. Then they were centrifuged at 4° C for 1 minute at 1000 rpm and the supernatants were separated and then stored in a nitrogen tank at -80° C in the refrigerator for long-term use.

Measurement of lipid peroxidation

Malondialdehyde (MDA), as a marker of lipid peroxidation and oxidative stress, was determined by measuring the thiobarbituric acid-reactive substances (TBARS). In this method, MDA was measured by its reactivity with TBA in acidic conditions to generate a pink colored chromophore, which was read spectrophotometrically. In brief, the samples were mixed with 1 mL 10% trichloroacetic acid and 1 mL 0.67% thiobarbituric acid. Then, the samples were heated in boiling water for 15 min, and next, N-butanol (2:1, v: v) was added to the solution. After centrifugation (at 900g for 5 min), TBARS were assessed by solution absorbance at 532 nm (17).

Glutathione cycle components

Since GSSG is considered as an indicator of oxidative stress and GSH as an indicator of cell antioxidant capacity, GSSG to GSH ratio is recognized as a useful indicator for determining cellular oxidative state. To investigate the glutathione reduction, glutathione oxide was used and measured from a specific tissue kit of Zell Bio Company (ZB-MDA₉₆, Germany) using ELISA technique (18).

Statistically analyses

Statistical analyses were performed using SPSS software 22, USA. The normal distribution of variables was confirmed using the Shapiro-Wilk test. All data were reported as means ± standard deviation (SD) which were analyzed using One-way ANOVA to determine the difference between the healthy-control and toxic-control groups. Moreover, the main effect of aerobic exercise training, main effect of

pumpkin extract consumption, and interaction of exercise training and the supplement consumption (exercise supplement) was revealed by two-way ANOVA. Meaningful effects were followed by the least significant difference *post hoc* test, so that the effect size was calculated. The statistical significance was considered $P \leq 0.05$ for all the statistical calculations.

Results

The results of one-way ANOVA indicated that there was a significant difference between the healthy-control and toxic-control groups regarding the present study variables (Table 1).

The independent effect of exercise training, independent effect of pumpkin seed extract consumption, and combined effect of exercise training and pumpkin seed extract consumption were analysed using two-way ANOVA. Consequently, the results showed that exercise training increased GSH ($F_{1,44}=8.43$, $p= 0.0001$, $\eta^2=0.38$) and GSSG ($F_{1,44}=7.01$, $p= 0.0001$, $\eta^2=0.28$) and declined MDA ($F_{1,44}=6.016$, $p= 0.0001$, $\eta^2=0.30$) level in the aortic endothelial cells (Figure 1). Also, pumpkin seed extract consumption induced a significant increase in GSH ($F_{1,44}=8.43$, $p= 0.0001$, $\eta^2=0.38$) and GSSG ($F_{1,44}=8.01$, $p= 0.0001$, $\eta^2=0.41$) and decreased MDA ($F_{1,44}=7.86$, $p= 0.0001$, $\eta^2=0.32$) levels in the aortic endothelial cells (Figure 1). Interaction of exercise training and pumpkin seed extract consumption increased GSH ($F_{1,44}=7.45$, $p= 0.0001$, $\eta^2=0.39$) and GSSG ($F_{1,44}=7.89$, $p= 0.0001$, $\eta^2=0.34$) and decreased MDA ($F=6.95$, $p= 0.0001$, $\eta^2=0.37$) levels in the aortic endothelial cells (Figure 1). Moreover, data on the heart tissue cells indicated that exercise training augmented GSH ($F_{1,44}=7.29$, $p= 0.0001$, $\eta^2=0.32$) and GSSG ($F_{1,44}=7.59$, $p= 0.0001$, $\eta^2=0.37$) and diminished MDA ($F_{1,44}=7.22$, $p= 0.0001$, $\eta^2=0.33$) (Figure 2). Also, pumpkin seed extract consumption triggered a considerable increase in GSH ($F_{1,44}=8.02$, $p= 0.0001$, $\eta^2=0.35$) and GSSG ($F=7.88$, $p= 0.0001$, $\eta^2=0.34$) and decreased MDA ($F_{1,44}=8.11$, $p= 0.0001$, $\eta^2=0.33$) (Figure 2). Eventually, simultaneous exercise training and pumpkin seed extract consumption heightened significantly the levels of GSH ($F_{1,44}=7.12$, $p= 0.0001$, $\eta^2=0.29$) and GSSG ($F_{1,44}=7.54$, $p= 0.0001$,

eta=0.32) and decreased MDA ($F_{1,44}=7.01, p=0.0001, \eta=0.30$) (Figure 2).

Table 1: Difference in the variables between the healthy-control and toxic-control groups.

Variables (pmol/ml)	Healthy-control	Toxic-control	F value	P value	Eta	
Endothelial Aortic cells	GSH	581.9 ± 4.021	119 ± 3.445	254.0	0.0001	0.99
	GSSG	1456 ± 61.24	430.1 ± 24.92	101.7	0.0001	0.98
	MDA	124.2 ± 9.96	1063 ± 80.48	50.18	0.0001	0.95
Heart tissue cells	GSH	571 ± 67.43	107.9 ± 6.858	115.3	0.0001	0.99
	GSSG	1458 ± 121.21	429.5 ± 43.6	97.49	0.0001	0.97
	MDA	390 ± 33.15	985.7 ± 54.55	50.73	0.0001	0.95

Data are analyzed using one-way ANOVA and presented as mean ± SD. (n= 8 in each group).

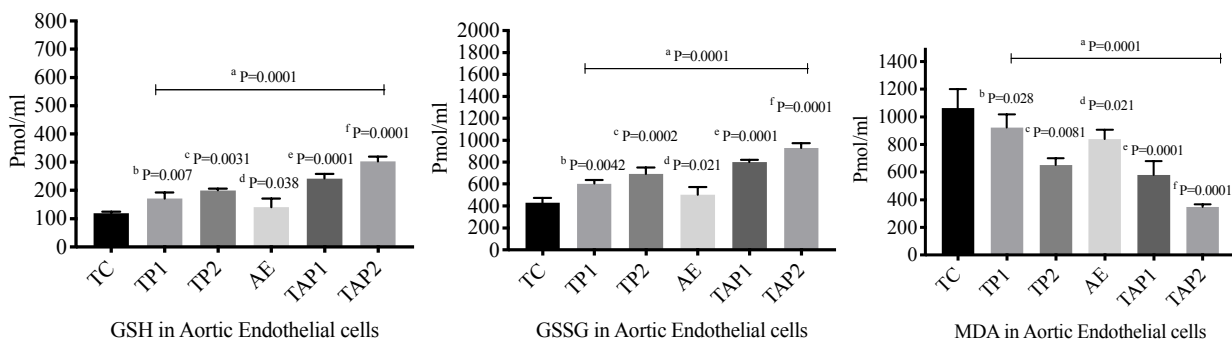


Figure 1. Aortic endothelial cells GSH, GSSG and MDA concentration in different groups. Each group is consisted of 8 rats and data are expressed as mean ± SD. ^a Significant effect of Pumpkin (PKs) extract and aerobic exercise vs control group. ^b TP1 vs TC. ^c TP2 vs TC. ^d TAP1 vs TC. ^e TAP2 vs TC. ^f AE vs TC.

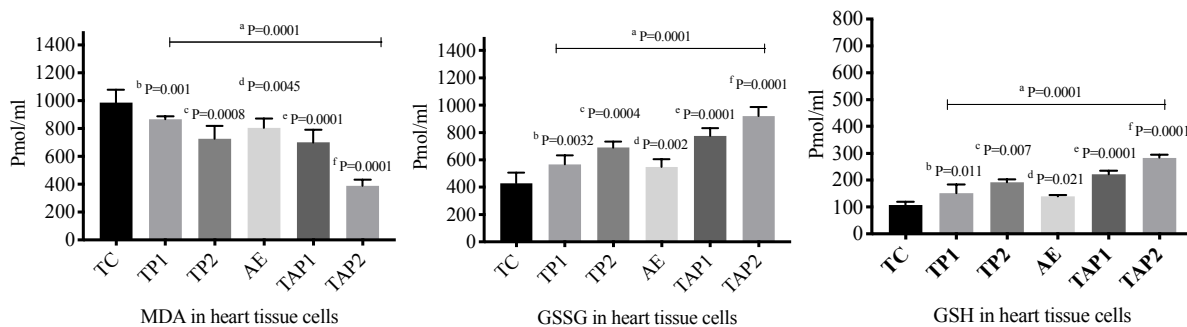


Figure 2. Heart tissue cells GSH, GSSG and MDA concentration in different groups. Each group is consisted of 8 rats and data are expressed as mean ± SD. ^a Significant effect of Pumpkin (PKs) extract and aerobic exercise vs control group. ^b TP1 vs TC. ^c TP2 vs TC. ^d TAP1 vs TC. ^e TAP2 vs TC. ^f AE vs TC.

Discussion

Studies have reported that arsenic disrupts cellular oxidation and resuscitation balance through various mechanisms. Arsenic causes oxidative damage to tissues by disrupting glutathione consumption. Arsenic has high toxicity that is naturally

found in the environment and, as a well-known toxic metal, can lead to damage and even death in various species (19). Generally, the toxic effects of arsenic are due to impaired cellular respiration and inhibition of mitochondrial enzymes and oxidative phosphorylation, as oxidative

stress and free radicals play a pivotal role in the pathophysiological mechanisms of cardiac disorders (20). Direct and indirect observations have also shown that ROS is the main cause of damage to the myocardium and further evidence suggests ROS involvement in cardiac dysfunction, for instance, Bugger et al. noted that ROS plays a significant role in Ischemia-Reperfusion (IR) and it also increases significantly after several minutes of reperfusion and causes many changes, including weakening of contractile reactivity, arrhythmias, and expression of genes (21). Furthermore, reactive oxygen species and calcium homeostasis disorders strongly trigger myocardial stunning, as ROS production in conditions such as arsenic poisoning, increases intracellular calcium by releasing calcium from sarcoplasmic reticulum, opening ryanodine receptors and damaging the calcium dehydration system in cardiomyocyte as well as disruption of endothelial connective tissue integration (22).

Exercise and physical activity are believed to be one of the most known factors in prevention and treatment of cardiovascular disease. Aerobic exercises have direct beneficial effects on cardiovascular and coronary arteries, including the amount of oxygen needed for the myocardium, endothelial function, coagulation factors, and inflammatory markers (23). Aerobic exercise increases the ability of the heart for glycolysis which leads to increased ATP concentration in order to compensate the reduction of oxidative phosphorylation in myocardial ischemia (24). Another reason justifying cardio-protective influence of aerobic exercise is its ability to increase the cyclooxygenase-2 enzyme (Cox-2). Cox-2 is one of the most important enzymes in the synthesis of prostaglandins and emergence of inflammation in humans (25). Aerobic exercise induces the processes of angiogenesis and increases the blood vessel diameter in the skeletal and cardiac muscles and, as a result, improves blood flow in the organs and improves vascular endothelial function and also augments the release of nitric oxide (NO) which boosts arterial vasodilatation (26). Recent studies have displayed that performing regular aerobic

exercises can stimulate expression of antioxidant enzymes, providing compromise and protection responses against oxidative stress (9). Carvalho et al. (2011) reported that aerobic exercises improved endothelial function and decreased myocardial ischemia. It seems that during the aerobic exercises, an increment in average shear stress on arterial walls leads to improved endothelial function which is associated with the facilitation of synthesis, release and durability of nitric oxide activity (27). Kanter et al. (2017) examined the effect of low-intensity aerobic exercises in rats. Their results showed that aerobic exercise significantly reduced oxidative stress and cellular apoptosis and also enhanced the activity of antioxidants in the heart (28). To treat poisoning or prevent harmful effects, the use of different antioxidant compounds with natural origins is expanding rapidly. Pumpkin is a high-nutritional plant with potent antioxidant activity due to phenolic compounds, tocopherols and unsaturated fatty acids (29). Seif et al. (2014) in an experimental study of rats implied that the use of pumpkin extract escalated the antioxidant enzymes such as superoxide dismutase and glutamate peroxidase and reduced Malondialdehyde (MDA) in serum of rats with cancer (14). In this study, we examined the effect of regular aerobic exercise training and pumpkin seed extract consumption on the oxidative stress of the heart and aortic endothelial cells. The results showed that regular aerobic exercise training and pumpkin seed extract led to reduction in MDA levels in the heart tissue and aortic endothelial cells as well as an increase in the levels of GSH and GSSG in the heart tissue and aortic endothelial cells.

Badalzadeh et al. reviewed the effect of cinnamon and aerobic exercise on oxidative stress. The results revealed that they separately increased lactate dehydrogenases (LDH) but their simultaneous effect was stronger and statistically significant. Exercise with cinnamon also led to abatement in almond levels and raise of superoxide dismutase and glutathione peroxidase enzymes (30). Parallel to our data, Yoon et al. investigated the effect of exercise and antioxidant activity of soy isoflavone on the oxidative stress and antioxidant enzyme activity in mice. The

results showed that oxidative stress declined and the activity of the superoxide dismutase (SOD) and catalase enzymes increased(31). In accordance, the results of the study by Soleimani et al. who examined the effect of aerobic exercise for two weeks with *Tribulus terrestris* supplement exhibited that the *Tribulus terrestris* supplement in combination with aerobic exercise reduced the degradative effects of free radicals and had positive significant effect on antioxidant capacity (32). Tofighi et al. also evaluated the effects of resveratrol supplementation and aerobic exercise on cardiac tissue changes in rats with acute myocardial infarction. The results showed that concurrent existence of resveratrol and exercise training protects the heart through reduction of superoxide production, fat peroxide and activation of antioxidant defense system against oxidative stress after ischemia (33).

Conclusion

Eventually, the current study concluded that arsenic-poisoning can lead to oxidative stresses in cardiac tissue and endothelial cells, which could be remarkably attenuated via regular aerobic exercise and herbal supplements. In this study, it was found that though regular aerobic exercise has positive effects on antioxidant system, when herbal supplements such as pumpkin seed extract is administered simultaneously, the effectiveness of both is boosted manifold. Hopingly, these therapeutic approaches could be used in future treatments not only in metal-poisoning but in all other oxidative stress-related cardiac diseases, though more studies are still needed in this regard.

Author Contributions

BA conceived and designed the study. BA and MB analyzed the data. MB and SRA wrote and revised the manuscript, respectively. All authors read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

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