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The relationship between plasma aluminum content, lymphocyte DNA damage, and oxidative status in persons using aluminum containers and utensils daily

Hakim Celik [a], Necla Celik [b], Abdurrahim Kocyigit [b,*], Murat Dikilitas [c]

[a] Department of Physiology, Faculty of Medicine, Gaziantep University, Gaziantep, Turkey
[b] Department of Clinical Biochemistry, Faculty of Medicine, Harran University, Sanliurfa, Turkey
[c] Department of Plant Protection, Faculty of Agriculture, Harran University, 63200 Sanliurfa, Turkey

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ABSTRACT

Objectives: The aim of this study was to explore the *in vivo* effect of the plasma aluminum content on lymphocyte DNA damage, the plasma protein carbonyl (PC) content, and malondialdehyde (MDA) and total antioxidative capacity (TAC) levels in aluminum exposed and non-exposed humans.

Design and methods: Peripheral blood samples were collected from *in vivo* aluminum exposed and non-exposed humans and the above parameters were measured.

Results: The mean values of lymphocyte DNA damage, plasma MDA, PC levels, and aluminum concentrations were found to be significantly higher in the aluminum exposed group than within the control group ($p<0.01$). On the other hand, plasma TAC levels were found to be significantly lower in the aluminum exposed group than in the control group ($p<0.001$). Significant positive correlations were found to exist between lymphocyte DNA damage and the aluminum concentration ($r=0.643$, $p<0.001$), DNA damage and MDA ($r=0.491$, $p<0.001$), and DNA damage and PC ($r=0.548$, $p<0.01$). A negative correlation was found between TAC and DNA damage ($r=-0.600$ $p<0.001$) in the aluminum exposed group.

Conclusion: Findings from the study revealed that an increased plasma aluminum concentration was associated with increased oxidative stress and increased DNA damage in aluminum exposed humans.

Introduction

Aluminum is the most widely distributed element in the environment and is used extensively in our daily lives. Sources of aluminum in human organisms, in general, include foods and drinking water, as well as dialysis fluids for those undergoing renal treatment [1]. Aluminum is also present in many manufactured foods and is added to drinking water for purification purposes [2]. The most common foods containing aluminum are food additives, including those within some processed cheeses, baking powders, cake mixes, frozen dough, and pancake mixes [3]. The leaching of aluminum from beverage cans and cookware may also be an aluminum source. Approximately 20% of the daily intake of aluminum has been stated to come from cooking utensils (pans, pots, kettles, and trays) made of aluminum [4]. The migration of aluminum into food mainly occurs via acidic attack on plain aluminum materials. However, even the boiling of simple tap water releases considerable amounts of aluminum. Tomato, quince, citrus, and many fruits with a high content of organic acids readily dissolve aluminum from bare aluminum surfaces [5]. High amounts of aluminum have been reported to be mixed with mashed tomatoes and yogurt during processing from normal and non-coated aluminum pans as a result of acidity. Also, it has been demonstrated that the aluminum level in fresh yogurt fermented in aluminum containers was twenty-six fold higher in aluminum than that of yogurt fermented in steel and boron glass containers. Values obtained for a case of soured yogurt were twice that of fresh yogurt [6]. Muller et al. [5] stated that the aluminum content of tomato sauce was remarkably higher 60 min after cooking as compared to uncooked tomato sauce. The total body burden of aluminum in healthy human subjects is approximately 30–50 mg [7].

Although aluminum is not known to be essential for life processes, it is known to cause toxicity to a variety of organ systems including the brain, the bones, the kidneys, and blood [8,9]. High levels of aluminum have been linked to increased risk for a number of pathogenic disorders such as microcytic anemia and bone disturbances, in addition to neurodegenerative disorders such as Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis, and encephalopathy [10–12]. However, although many genotoxic and cytotoxic studies have been carried out [13–15], the mechanism of action is still poorly understood.

Recently, a series of *in vitro* and *in vivo* experimental studies have been performed to determine the detrimental effects of aluminum

Abbreviations: PC, protein carbonyl; PO, protein oxidation; MDA, malondialdehyde; TAC, total antioxidative capacity; ROS, reactive oxygen species; LPx, lipid peroxidation; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

* Corresponding author at: Harran University, Medical Faculty, Department of Biochemistry, Research Hospital, 63100 Sanliurfa, Turkey. Fax: +90 414 3151181.
E-mail address: abdurrahimkocyigit@yahoo.com (A. Kocyigit).

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[16–19]. Such studies have demonstrated that aluminum exposure is associated with the impairment of mitochondrial functions, *in vitro*. Banasik et al. [17] demonstrated an increase in the rate of micronuclei and the number of apoptotic cells induced by aluminum in cultured human lymphocytes. Kumar et al. [18] also demonstrated that aluminum induced oxidative DNA damage and cell-cycle disruptions in different regions of rat brains. However, human studies for evaluating the relationship between DNA damage and oxidative status with aluminum are scarce.

Due to poor socio-economic conditions and an unawareness of the dangers associated with aluminum toxicity, the use of aluminum containers and utensils in the fermentation of yogurt and in the cooking and preservation of foods such as tomato sauce has been common in southeast Anatolia in Turkey. Therefore, we investigated the relationship of the plasma aluminum content on DNA damage and oxidative status in people that used aluminum containers and utensils daily for cooking and storing food. Results obtained from the aluminum impacted group were compared to those from the group that claimed they avoided using aluminum containers and utensils.

Materials and methods

Subjects

The study was performed in order to test the toxicity of aluminum in two groups. The first group, defined as the aluminum exposed group, consisted of twenty eight healthy subjects (with a mean age of 36 ± 6 years) that claimed to use aluminum containers and utensils daily for the fermentation, cooking, and storage of food, especially yogurt and tomato sauce. The second group, defined as the control group, consisted of twenty eight healthy subjects (with a mean age of 38 ± 2 years) that claimed to avoid using aluminum containers and utensils.

The study's protocol was carried out in accordance with the Helsinki Declaration as revised in 1989, and approved by the local human institutional review committee. All subjects were informed regarding the study's protocol and written consents were obtained from all participants. People taking supplemental vitamins, aluminum containing antacids and adjuvants, as well as those with a history of diabetes mellitus, coronary artery disease, rheumatoid arthritis, malignancy, systemic or local infections, hypertension, acute-chronic liver disease, renal dysfunction, anemia, and a smoking habit were excluded from the study.

Sample preparation

Following an overnight fasting, peripheral blood samples (a total of 6 mL) were collected from an antecubital vein into heparinized tubes, stored at 2–4 °C, kept in the dark to prevent DNA damage, and processed within 2 h. Mononuclear leukocyte isolation for the comet assay was performed using the Histopaque 1077 (Sigma). An amount of 1 mL of heparinized blood was carefully layered over 1 mL of Histopaque and centrifuged for 35 min at $500 \times g$ at 25 °C. Interface bands containing mononuclear leukocytes were washed with phosphate buffered saline (PBS), then collected with 15 min of centrifugation at $400 \times g$. Resulting pellets were resuspended in PBS, and cells were counted using an automatic cell counter (Abbott 3700, USA). Membrane integrity was assessed using a Trypan Blue exclusion method. The remaining blood was centrifuged at $1500 \times g$ for 10 min in order to obtain the plasma. Separated plasma was then stored at −80 °C until an analysis for MDA, PC, and TAC levels could be performed.

DNA damage measurements

The comet assay was performed as described by Singh et al. [20] using the following modifications. Ten μL of fresh mononuclear leukocyte cell suspension (around 20,000 cells) was mixed with 80 μL of a 0.7% low-melting agarose in a phosphate buffered saline (PBS) at 37 °C. Subsequently, 80 μL of the mixture was layered on a slide precoated with thin layers of a 1% normal melting point agarose (NMA), and immediately covered with a coverslip. The slides were left for 5 min at 4 °C to allow the agarose to solidify. After removing the coverslips, slides were then submersed in a freshly prepared cold (4 °C) lysing solution (2.5 M NaCl, 100 mM Na_2EDTA; 10 mM Tris–HCl, pH 10–10.5; 1% Triton X-100 and 10% DMSO added just prior to use) for at least 1 h. The slides were then immersed in a freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH, and 1 mmol/L Na_2EDTA, pH > 13) at 4 °C for unwinding (40 min), then electrophoresed (25 V/300 mA, 25 min). All of the steps were carried out with minimal illumination. Following electrophoresis, slides were neutralized (0.4 M Tris–HCl, pH 7.5) for 5 min. Dried microscope slides were stained with ethidium bromide (2 μg/mL in distilled H_2O; 70 μL/slide), covered with a coverslip, and analyzed using a fluorescence microscope (Olympus BX51, Japan) at a 400× magnification with epiflourescence and equipped with a rhodamine filter (with an excitation wavelength of 546 nm; and a barrier of 580 nm). Fifty cells were randomly scored by eye in each sample, on a scale of 0–4, based on fluorescence beyond the nucleus, as described by Kobayashi et al. [21]. The following scale was utilized: 0 = no comet; 1 = comet <0.5 times the width of the nucleus; 2 = comet equal to the width of the nucleus; 3 = comet greater than the width of the nucleus; 4 = comet > twice the width of the nucleus. Scoring cells in this manner has been shown to be as accurate and precise as using computer image analyses, and has been shown to be time-efficient [21,22]. Individual scoring of slides was blinded for demographic or biochemical aspects of the blood sample. The visual score for each class was calculated by multiplying the percentage of cells in the appropriate comet class by the value of the class. The total visual comet score, which characterized the degree of DNA damage in the examined patient groups, was the sum of the scores in the five comet classes. Therefore, the total visual score could range from 0 [all undamaged] to 400 [all maximally damaged] arbitrary units (AU). The visual scoring method was suggested by Collins et al. [23]. All of the procedures were completed using the same biochemistry staff, and DNA damage was detected using a single observer that was not aware of the subject's status. Comets were scored independently by two investigators.

Measurements of the plasma aluminum content

Determinations for the concentrations of plasma aluminum were performed using graphite furnace atomic absorption spectrometry (Varian GTA 96, Australia); a widely used technique for determining aluminum in plasma due to its ability to provide reliable results at the concentrations observed in non-exposed healthy groups [24]. Aluminum was measured as described by Valkonen and Aitio [24]. Determinations were performed using a direct comparison with standard solutions prepared in 0.1 M nitric acid.

Measurements of the plasma lipid peroxidation

Plasma lipid peroxidation was evaluated with the fluorometric method based on the reaction between malondialdehyde (MDA) and thiobarbutiric acid [25]. Briefly, 50 μL of plasma was added to 1 mL of a 10 mmol/L diethylthiobarbutiric acid (DETBA) reagent in phosphate buffer (0.1 mol/L, pH 3). The solution was mixed for 5 s and incubated for 60 min at 95 °C. Samples were then placed in ice for 5 min, and then 5 mL of butanol was added. The mixture was shaken for 1 min to extract the DETBA-MDA adduct, and then centrifuged at $1500 \times g$ for 10 min at 4 °C. The fluorescence of the butanol extract was measured at the excitation wavelength of 539 and at an emission wavelength of 553. A solution of 1,1,3,3, tetraethoxypropane (Sigma) was used as a standard. The values are presented as μmol/L.

Measurements of the plasma protein carbonyl content

The PC of plasma was measured according to Reznick and Parker [26]. Fifteen μL of the plasma was treated using 500 μL of 10 mmol/l 2,4-dinitrophenylhydrazine (DNPH), dissolved in 2 mol/L HCl. The samples were then incubated for 1 h at room temperature in the dark, precipitated with 10% trichloracetic acid (TCA), and centrifuged at 13,000 *g* for 5 min. The resulting pellets were washed three times with 1 mL of ethanol/ethyl acetate - 1/1 (v/v), and redissolved in 1 mL of 6 mol guanidine in 10 mmol/L of phosphate buffer/trifluoroacetic acid (pH 2.3). The difference in absorbance between DNPH-treated and HCl-treated samples was determined at 366 nm. The results are expressed as nmol of carbonyl groups per mg of protein using the extinction coefficient of 22,000 M^{-1} cm^{-1} for aliphatic hydrazones.

Measurements of the plasma total antioxidant capacity

The TAC of plasma was measured using an automated analyzer (Abbott, Aeroset, IL, USA) with a TAC measurement kit developed by Erel [27]. In this assay, a standardized solution of $Fe^{2}+-o$-dianisidine complex reacts with a standardized solution of hydrogen peroxide via a Fenton-type reaction, producing hydroxyl radicals. At a low pH, these potent reactive oxygen species (ROS) oxidize reduced colorless *o*-dianisidine molecules to yellow–brown colored dianisidyl radicals. Oxidation reactions progress among the dianisidyl radicals and further oxidation reactions occur. Color formations are increased with additional oxidation reactions. Antioxidants in the sample suppress oxidation reactions and color formation. In the past, the assay has obtained a precision of less than 3% CV. The results are expressed as mmol of Trolox equivalent/L.

The statistical analysis

All of the analyses were conducted using the SPSS 11.5 statistical program (SPSS for Windows 11.5, Chicago, IL, USA). In this paper, the data are expressed as the mean ± the standard deviation. The normality of the distributions was evaluated using the Kolmogorov–Smirnov test. Comparisons of the parameters were performed using a Student's *t-test* and the correlation analyses were performed using a Pearson's correlation test. A χ^2 test was used to compare gender distributions within the controls and the aluminum group. All of the statistical tests were two-sided; a *p* value less than 0.05 was accepted as significant.

Results

The demographic, clinical, and laboratory data of the subjects are presented in Table 1. No statistically significant differences between the groups were determined in respect to age, gender, and body mass index (BMI) (all $p>0.05$).

Since it has several advantages over other genotoxicity methods, the alkaline version of the comet assay was used to measure lymphocyte DNA damage. The assay is able to detect very short-lived primary DNA lesions such as single strand breaks in the DNA of individual cells [28]. Lymphocyte DNA damage values, and plasma aluminum, MDA, and PC levels were found to be significantly higher in the aluminum exposed groups than those not exposed ($p<0.01$, $p<0.001$ $p<0.05$ and $p<0.05$, respectively). The aluminum exposed group also displayed lower TAC levels than those of the control group ($p<0.001$) (Table 2). A negative significant correlation was found between TAC and DNA damage ($r=-0.600$, $p<0.001$); while a positive significant correlation was determined between DNA damage and aluminum content ($r=0.643$, $p<0.001$) (Fig. 1), DNA damage and PC ($r=0.548$, $p<0.01$), and DNA damage and MDA levels within the aluminum group ($r=0.491$ $p<0.01$) (Table 3).

Table 1
The demographic and clinical parameters in the control and aluminum exposed groups.

Parameters	Control group ($n=26$)	Aluminum exposed group ($n=28$)	*p*
Age (years)	36 ± 6	38 ± 2	*ns*
Sex (female/male)	11/15	13/15	*ns*
BMI (kg/m^2)	21.1 ± 1.5	20.2 ± 1.2	*ns*

BMI: body mass index, the values provided are the mean ± SD.

Discussion

The goal of this study was to evaluate the effects of the plasma aluminum content on DNA damage and oxidative status in people exposed daily to aluminum toxicity. We found that plasma aluminum concentrations and lymphocyte DNA damage levels were significantly higher and that TAC levels were significantly lower within the aluminum exposed group; and that there was a close positive relationship between aluminum levels and lymphocyte DNA damage, and aluminum levels and plasma MDA levels. A negative relationship between the plasma aluminum content and TAC levels was also evident in the aluminum exposed group.

We determined that plasma aluminum concentrations were much higher in the aluminum exposed group. However, the control group also displayed a higher aluminum content according to the reference ranges. In general, plasma aluminum levels represented a wide variation in reference values [29–31]. The exposure rate of aluminum in humans could be different according to lifestyle and living conditions. Since aluminum is used in many areas, including in construction, automobiles, and various industrial areas, as well as in the food and cosmetic sectors, it is almost impossible to avoid aluminum toxicity. However, aluminum toxicity can be worse if people are unaware of its dangers. In our study, both groups were selected from the same area and for similar living conditions, where the control group claimed to avoid using aluminum utensils and cookware. However, the group selected as a control also faced aluminum toxicity due to the habits and lifestyles of a city in which restaurants, bakeries, and other businesses and community areas ignore the toxicity of aluminum (Table 2).

In this study we also evaluated the DNA-damaging effects of aluminum using the Comet assay in peripheral blood lymphocytes. The assay has been widely used to detect single- and double-strand DNA breaks. The high sensitivity of this test is due to the fact that it detects DNA breaks, alkali-labile lesions, and genomic lesions that are subject to repair [20]. We found remarkably high levels of DNA damage in the aluminum exposed group when a comparison was made to the control group. Aluminum, in general, has been shown to exert its effects by disrupting lipid membrane fluidity; and perturbing iron, magnesium, and calcium homeostasis, causing oxidative stress and DNA damage [32]. Thus far, quite a few *in vitro* studies have been undertaken in order to determine the genotoxic and cytotoxic potentials of aluminum on DNA damage [33]. These studies have demonstrated that aluminum toxicity decreases the repair capacity of cells, indicating that aluminum inhibits DNA repair [34]. On the other hand, increased DNA fragmentations resulting from aluminum toxicity, and as determined by an

Table 2
Aluminum concentrations, DNA damage, protein oxidation (PO), MDA, and TAC in the control and aluminum exposed groups.

Parameters	Control group $n=26$ Mean ± *SD*	Aluminum exposed group $n=28$ Mean ± *SD*	*p*
Aluminum (μg/L)	48.2 ± 16.4	99.7 ± 23.7	<0.001
DNA damage (AU)	26.5 ± 10.8	35.3 ± 11.4	0.006
MDA (μmol/L)	308.7 ± 86.2	368.7 ± 110.4	0.031
PO (PC content) (nmol/mg protein)	2.55 ± 0.74	3.06 ± 0.76	0.018
Total antioxidant capacity (mmol Trolox Eq./L)	1.79 ± 0.43	1.46 ± 0.13	<0.001

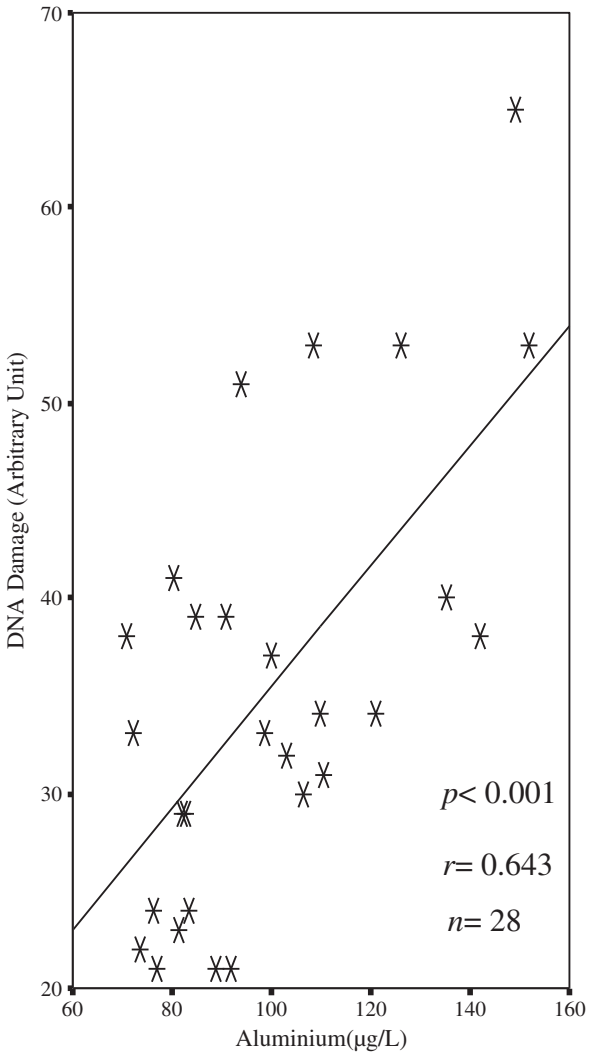

Fig. 1. The relationship between DNA damage and aluminum concentrations in the aluminum exposed group.

increase in the appearance of comets, have also been reported in studies as a consequence of aluminum exposure [33]. However, the genotoxic effects of aluminum exposure on human beings are not yet known. The work presented here is the first study aimed at determining the genotoxic effects of aluminum exposure on humans. We determined a close positive relationship for the plasma aluminum content, and DNA damage and oxidative stress markers. Our findings and those of others indicate that DNA damage may depend on the toxicity of aluminum, with the ability to promote the generation of oxidative stress.

Aluminum is considered to be a pro-oxidant agent. Diverse studies have shown that exposure to high concentrations of aluminum can induce oxidative stress, thereby stimulating reactive oxygen species (ROS) production in cells [35,36] inducing lipid peroxidation (LPx); and altering the activity of diverse antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and promoting protein oxidation [37–39]. We determined that plasma PC and MDA levels were higher, that TAC levels were lower, and that aluminum concentrations were strongly and positively correlated with the content of oxidation products such as MDA and PC ($r=0.512$, $p=0.005$, $r=0.565$, $p=0.002$, respectively) in the aluminum exposed group, suggesting that aluminum is associated with oxidative stress. In a similar study, Guo and Wang [30] also determined that the aluminum concentration was associated with increased oxidative stress in hemodialysis patients.

Several mechanisms have been suggested regarding aluminum and oxidative stress. Various studies have shown that aluminum is able to displace Fe in several biomolecules and to increase intracellular Fe concentrations, thereby promoting Fenton reactions [40–42]. The metal can also directly damage the mitochondrion and impact electron transport in the respiratory chain [43]. In both cases, there is increased ROS production, which explains the increase of LPx. Additionally, aluminum could interact directly with enzymes such as SOD and GPx that are known to protect cells [44]. We found decreased levels of TAC in the aluminum exposed group that may be considered to result from the interaction between aluminum and antioxidative enzymes.

The increased generation of ROS is known to result in DNA damage. DNA damage, as a result of aluminum toxicity, can lead to gene modifications in cells that may be cytotoxic, mutagenic, or carcinogenic. In addition to DNA damage, a decrease in DNA repair and a susceptibility to apoptosis are also important factors with an emerging role in cancer studies [45]. Although there is a lack of information related to the possible impact of aluminum on carcinogenesis, epidemiological studies have shown that increased risks of lung and bladder cancer are associated with aluminum exposure [46,47]. Our *in vivo* study and additional *in vitro* and experimental studies have shown that aluminum induces DNA damage in a dose-dependent manner [18,34]. Results have revealed that high aluminum levels lead to the formation of oxidized purines and pyrimidines and cause DNA damage. Both oxidative stress and DNA damage may be responsible for neurodegenerative and carcinogenic disorders [16,48].

In conclusion, we suggest that a high plasma aluminum content leads to inductive oxidative stress and DNA damage. Further studies are needed to clarify the mechanisms of DNA damage and the relationship between DNA damage and clinical end-points that may be associated with aluminum toxicity. Additionally, antioxidant therapy may be beneficial for preventing the formation of products leading to oxidative damage and the accumulation of free radicals. In order to clarify aluminum genotoxicty, it is also beneficial to determine if there is a correlation between DNA repair capacity and reduced aluminum intake.

Table 3
Relationships among DNA damage values, aluminum concentrations, MDA, PO, and TAC in the control and aluminum exposed groups.

			DNA damage	TAC	PO	MDA
Control group	Aluminum	*r*	0.589	−0.336	−0.173	0.004
		p	0.002	0.093	0.399	0.984
	DNA damage	*r*		−0.435	−0.110	0.194
		p		0.026	0.593	0.342
	TAC	*r*			−0.042	0.089
		p			0.840	0.666
	PO	*r*				0.397
		p				0.044
Aluminum group	Aluminum	*r*	0.643	−0.565	0.409	0.512
		p	<0.001	0.002	0.031	0.005
	DNA damage	*r*		−0.600	0.548	0.491
		p		0.001	0.003	0.008
	TAC	*r*			−0.457	−0.477
		p			0.014	0.010
	PO	*r*				0.596
		p				0.001

r: regression, *p*: probability.

Conflict of interest

The authors declare that there are no conflicts of interest.

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