The Role of Reduced Glutathione on the Activity of Adenosine Deaminase, Antioxidative System, Aluminum and Zinc Levels in Experimental Aluminum Toxicity

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Abstract

Aluminum (Al) is the most common and widely used element in nature, which cause many health problems and toxic effects. The accumulation of aluminum in the brain, which easily crosses the blood-brain barrier, causes neurodegenerative diseases such as Alzheimer's and dementia, especially diseases associated with free radicals. Reduced glutathione (GSH) is an intracellular tripeptide with low molecular weight, hydrophilic properties, and strong antioxidant effects. This study aimed to investigate the role of reduced glutathione on the activity of adenosine deaminase, antioxidative system, aluminum and zinc levels in aluminum toxicity. In this study, Sprague Dawley rats (n=32) were used. The rats were divided into four equal groups. Group I received 0.5 mL intraperitoneal injection of 0.9 % saline solution (NaCl), Group II received single-dose $AlCl_3$, Group III was given GSH for seven days, Group IV was given $AlCl_3$ single dose and at the same time 100 mg/kg GSH was given for seven days At the end of the experimental period of seven days, blood samples were collected by cardiac puncture. Plasma TAS and Zn levels were lower than the control group. In contrast, plasma TOS and aluminum concentrations and ADA activity were higher in the aluminum-administered group than in the control group. It was determined that the changed parameters returned to their normal levels in the group given GSH together with aluminum. As a result, additional GSH administration has a protective effect on adenosine deaminase activity, antioxidant system, and Zn level in experimental aluminum toxicity.

Introduction

Aluminum (Al) is the third most common and widely used element. It has been reported to cause many health problems and toxic effects for living creatures due to its widespread use in food, cosmetics, and industry [1,2]. Low aluminum concentrations show genotoxic effects. It can also interact antagonistically with metals such as iron in the organism. This situation causes an increase in the amount of extracellular aluminum in particular. Additionally, aluminum disrupts the glutamate or glutamate-GABA system, forming a complex structure with L-glutamate [3]. Thus, the accumulation of aluminum in the brain, which easily crosses the blood-brain barrier, causes neurological diseases such as Alzheimer's and dementia[4–6]. Aluminum is defined as a pro-oxidant substance as it can trigger the Fenton reaction in the organism and is influential in the formation of superoxide radicals[4,7,8]. Due to these properties, many studies investigate aluminum’s different effects on the antioxidant system. Free radicals damage macromolecules such as DNA, lipids, and proteins due to their excessive reactivity; antioxidants are defined as molecules that destroy or reduce the effects of free radicals[9,10,11,12]. Free radicals are essential molecules in increasing oxidative stress, in the breakdown of redox systems involved in cell signaling and various metabolism processes, and oxidative stress formation. The thiol system is located at the center of the oxidant/antioxidant balance [13]. The redox states of thiol systems are sensitive to two-electron oxidants and controlled by the thioredoxins (Trx), glutathione (GSH), and cysteine (Cys). Trx and GSH systems are maintain under stable, but nonequilibrium conditions, due to continuous oxidation of cell thiols at a rate of about 0.5% of the total thiol pool per minute. Redox-sensitive thiols are critical for signal transduction, transcription factor binding to DNA, receptor activation, and other processes[13].

GSH is a low molecular weight, hydrophilic, highly concentrated intracellular tripeptide. The most focused place of GSH is the cell cytoplasm. In addition, the remaining part is found dispersed in other organelles[14,15]. Synthesized in all cells, especially in the liver, GSH is generated by two-step ATP-dependent reactions catalyzed by two cytosolic enzymes. In the first stage (rate-limiting step), γ-glutamylcysteine is formed from glutamate and cysteine. In the second stage, GSH synthase (E.C.6.3.2.3., GS) uses γ-glutamylcysteine as a substrate and completes the GSH synthesis [16–19]. The amount of GSH synthesized in almost all mammals in the presence of cysteine precursors is regulated by the negative feedback mechanism of GSH produced as the final product. While many peptides are metabolized by peptidase cleavage of the a-carbonyl bond at the N-terminal end, GSH is cleaved by glutamyl transferase (GGT), not peptidases[16–20]. GSH is oxidized to glutathione disulfide (GSSH) by non-enzymatic reactions by toxic oxygen products and free radicals with electrophilic properties [20]. Cysteine is a crucial GSH component that plays a role in detoxifying molecules with oxidant character caused by natural metabolic processes or by the processes in which metabolism occurs[21]. Oral intake is sufficient for GSH production. However, since it plays an essential role in many redox reactions, GSH is constantly oxidized, causing a decrease in its level. Therefore, the amount of GSH needed may vary depending on physiological processes[22]. In particular, low GSH levels in the blood may result from irregular diets, exposure to oxidants, drug use, and toxicity[23].

Adenosine deaminase (ADA) is a metabolic enzyme produced in all cells and is mainly involved in purine metabolism and the immune system[24]. ADA catalyzes its substrates adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively, by deamination reactions. ADA also catalyzes the deamination of methylated adenosines. Adenosine, the substrate of ADA, is an anti-inflammatory molecule involved in regulating the immune system. The increase in ADA activity is also effective in increasing markers of inflammation (26–28). Adenosine, 2'-deoxyadenosine, and dATP, whose levels increase due to ADA deficiency, show an immunosuppressive effect [25,26].

In addition, the decrease in ADA activity causes the accumulation of 2'-deoxyadenosine and consequently the inhibition of S-adenosyl-L-homocysteine (SAH) hydroxylase. SAH hydroxylase is vital for methylation in the formation of lymphocytes. 2'-deoxyadenosine is also an essential component of the DNA molecule. As a result of the increase in the level of 2'-deoxyadenosine, intracellular phosphorylation increases and causes an increase in the amount of dATP. This situation acts as an inhibitor on dATP ribonucleotide reductase, disrupting DNA synthesis, repair, or replication[27,28]. Adenosine is one of the extracellular purines involved in the signaling step of processes such as immunity and inflammation, especially under metabolic stress[29]. In case of the increased immune response, ADA levels may increase up to 100 times in inflammatory and hypoxia cases such as immunodeficiency syndromes, atherosclerosis, cancer, infectious diseases, diabetes, obesity, and autocoid adenosine production[30,31]. Increasing the amount of adenosine rapidly increases ADA activation. Thus, the rapid increase of ADA activity is considered a marker of oxidative stress[32]. The amount of adenosine that increases with the excessive decrease of ADA activity may have toxic effects. Its over-activation limits the formation of the immune response, especially the production of B and T lymphocytes. It has been shown in many studies that ADA activity increases due to ROS increase and that there is a decrease in ADA activity due to the presence of

antioxidant molecules[33,34]. In light of this information, the study aimed to investigate the role of reduced glutathione on adenosine deaminase activity, antioxidant system, aluminum and zinc levels in aluminum toxicity.

Materials And Methods

This study purchased 32 Sprague Dawley rats (200-230 g) at 2-3 months of age from The Animal Breeding Laboratories of The Experimental Research and Application Center (Elazığ, Turkey). Before the experimental procedure, permission for the use of laboratory animals was obtained from Kafkas University Animal Experimentation Ethics Board (KAU-HADYEK 2017-034). Animals were kept at room temperature (25°C) and relative humidity (50-55%) in a 12- hour light and dark cycle. Animals were fed a regular pelleted diet (Bayramoğlu-Erzurum), and drinking water was provided *ad libitum*.

Experimental design

Rats were treated as follows:

Group I: Served as control and received 0.5 mL intraperitoneal injection of 0.9 % saline solution (NaCl).

Group II: Treated single dose $AlCl_3$ (34 mg/kg) intraperitoneal injection. $AlCl_3$ was dissolved in 0.9 % saline.

Group III: Reduced glutathione (100 mg/kg) was dissolved in 0.9 saline and given intraperitoneally for seven days

Group IV: $AlCl_3$ was dissolved in 0.9 % saline and treated single dose $AlCl_3$ (34 mg/kg) intraperitoneal injection. At the same time, 100 mg/kg GSH (Merck) was given intraperitoneally for seven days. At the end of the experimental period of 7 days, blood samples were collected by cardiac puncture. The serum were separated by centrifugation at 3000 rpm for 10 min and stored -45°C until analysis. Aluminum and zinc levels in serum were assayed by ICP-MS (CPMS Nexion 300X). The serum TAS and TOS were determined colorimetrically (PowerWave XS, BioTek, Instruments, USA) with a commercial kit (Rel Assay Diagnostic, Gaziantep, Turkey). Serum ADA activity was assayed by a colorimetric method.

Determination of ADA activity in serum

Serum ADA activity was assayed by a colorimetric method described by Giusti and Galanti. It is based on the measurement of ammonia in a Berthelot reaction. Ammonia is generated when ADA reacts with adenosine (substrate). The chemical definition of one unit of total ADA is the amount of ADA necessary to release one µmol of ammonia per minute from the substrate under standard experimental conditions. The ADA activities of the groups were expressed as U/L [35].

Determination of Aluminum and Zinc levels in serum by ICP-MS

All glassware, containers, tubes, and pipette tips must be soaked in 10% TraceMetal Grade HNO_3 for at least 24 h. Then they were washed with double distilled water several times before use. Metal-free (Al ≤1 ng/g) labware was used in all sample processing and analysis procedures to prevent possible Al contamination.

Standards & chemicals

Al and rhodium (Rh) reference standard solutions and tuning solutions were purchased from Agilent Technologies (CA, USA). High purity (99.999%) argon gas used for ICP–MS analysis was obtained from HABAŞ (Antalya, Turkey). Ultrapure water (18.2 M × cm) was produced via ELGA DI Polishing System (High Wycombe, UK). HNO_3 (TraceMetal Grade) was purchased from Sigma.

Calibration standards and quality controls

For comparative analysis of the trace element concentrations of Zn and Al in serum, a serial dilution of the control samples and calibration in decreasing amounts (10, 50, 100, 200, 450, 700, and 1000 ng/ml) as calibration standards had been prepared via diluting the stock solution. The calibration standards were designed daily in the surrogate matrix.

We selected Zn 66 and Al 27 as the isotope of our targeted analytes. The matrix-matched protocol was used to eliminate mass matrix interference. The surrogate matrix used in this study was 2% (v/v) HNO_3 solution prepared in ultrapure H_2O. The kinetic energy discrimination mode was used for Al to eliminate polyatomic ion interference. To prevent pollution with atmospheric particulates, sample preparation and dilution of standards were performed under a clean hood.

Sample preparation for ICP–MS analysis

Before analysis, 50 µL of the blood samples were placed in metal-free plastic containers pre-cleaned with 2% (v/v) HNO_3 for digesting with 8 ml 65% HNO_3 and 1 ml 30% H_2O_2 in a microwave oven (MilestoneStard-D) in two steps. The samples were heated to 180C for 10 min in the first step n. Following this, the heating was continued for another 15 min at 180°C. After digestion, they were placed in the autosampler for analysis. Inductively coupledplasmamasss spectrometer,y an ICP-MS Nexion 300X system equipped with software was used to determine Zn and Al in blood samples. The operational parameters of ICP-MS are listed in Table 1.

Sample uptake tubing was 0.38 mm id (green/orange). The PVC drain tubing was 1.30 mm id (gray, gray) and the autosampler tubes were metal-free conical tubes, all of which were prewashed with 2% (v/v) HNO_3 for sterilization. The ICP–MS was operated via RF power of 1600 W with helium flow of 4.5 $L.min^{-1}$ in the gas cell. The nebulizer flow rate, the auxiliary gas flow rate, and the plasma gas flow rate were 0.98, 1.2, and 15 $L.min^{-1}$, respectively. The integration time was 0.1 s per point to obtain one point per mass. The autosampler was also equipped with a programmable rinse function. The assay was blindly run in triplicate for each sample, and the mean of three readings was used for further statistical analyses.

Table 1. ICP-MS Operating parameters

Parameter	Value
Plasma Conditions	1600 W
Plasma gas flow	15 $L.min^{-1}$
Carrier gas flow	0.75 $L.min^{-1}$
Dilution gas flow	1 $L.min^{-1}$
He gas flow	4.5 $L.min^{-1}$

Determination of TAS and TOS in serum by commercially

Serum TAS and TOS levels were measured by commercially available test kits (Rel Assay Diagnostics, Turkey). The TAS levels were measured by a novel method based on automated colorimetric measurement developed by Erel (2004). In this method, the antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The results are expressed as μmol Trolox Eq/L[36].

In the serum samples, TOS levels were measured by a novel method based on automated colorimetric measurement developed by Erel (2005). This reaction is based on the oxidation of ferrous to ferric ions in the presence of various oxidative species in acidic medium and the measurement of the ferric ion by xylenol orange. The colour intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide (H_2O_2), and the results are expressed in terms of micromolar hydrogen peroxide equivalent per litre (μmol H_2O_2 Eq/L).

Statistical Analysis

The data obtained from the analyses were expressed as mean ± SEM. Statistical analyses of data were performed using SPSS 16.0. Data were initially tested for normality by the Kolmogorov Smirnov test. Then the data were tested with ANOVA, followed by the posthoc Tukey test. *P* values less than 0.05 ($p < 0.05$) were considered statistically significant.

Results

At the end of the seven-day experimental period, plasma TOS concentration in the aluminum-given group was significantly (P<0.05) higher than in the control and other experimental groups. In contrast to the data on TOS concentration, plasma TAS concentration in the aluminum-given group was found significantly (P<0.001) lower than in the control and other experimental groups.

Serum ADA activity in the aluminum-given group was significantly higher (P<0.05) than in the control and reduced glutathione-given groups. At the same time, no statistical difference was found in the Aluminum+ Reduced Glutathione-given group.

The highest serum Al level was detected in the group given Al and the group given Al+GSH compared with the control group. The aluminum levels detected in the group given reduced glutathione were found statistically (P<0.001) lower when compared to the control group. Serum zinc concentration in the aluminum-treated group was found significantly (P<0.05) lower than in the control group and other experimental groups (**Table II)**.

Table II. Aluminum, Zinc, TAC, TOC levels, and ADA activity in experimental groups.

Parameters	Group I	Group II	Group III	Group IV	P Values
Aluminum (PPM)	0.58± 0.09^{bc}	2.82 ± 0.195^{a}	0.46 ±0.061^{c}	0.87 ±0.010^{b}	0.000
Zinc (PPM)	*0.91±0.051^{a}*	*0.41 ± 0.083^{b}*	*0.91 ± 0.135^{a}*	*0.64 ± 0.12^{ba}*	*0.003*
TAC (mmol Trolox Eq/L)	*1.10± 0.053 ^{a}*	*0.79± 0.081^{b}*	*1.13± 0.084 ^{a}*	*1.10± 0.084^{a}*	*0.015*
TOC (µmol H2O2 Eq/L)	*12.35± 0.76 ^{b}*	*19.01±1.35 ^{a}*	*10.48 ±1.46 ^{b}*	*13.32 ±0.71 ^{b}*	*0.000*
ADA (U/L)	*10.33± 0.72^{b}*	*15.78 ± 1.81^{a}*	*10.20 ± 0.79^{b}*	*12.83 ± 1.75^{ab}*	*0.015*

Group I: Control, **Group II**: Aluminum, **Group III**: Reduced Glutathione, **Group IV**: Aluminum+ Reduced Glutathione. $^{a.b}$ The groups in the same line labeled in different letters are statistically significant.

Discussion And Conclusion

It has been observed in many studies that heavy metal accumulation increases ROS production in many tissues and disrupts the oxidative balance. Today, Al exposure is almost impossible to avoid, so it is crucial to minimize the damage caused by Al. The use of molecules with antioxidant properties is considered an effective method to eliminate oxidative stress[37–39].

Our study aimed to investigate the effect of reduced glutathione on experimental aluminum toxicity on adenosine deaminase activity, antioxidative system, aluminum, and zinc levels. Exposure to Al causes an increase in mitochondrial activity and deterioration of outer membrane permeability. This increases the number of cytochromes in the extracellular region and the activation of signaling pathways such as caspase-3 and Phosphoinositide-3 Kinase (PI3K). As a result, an increase in ROS and oxidized GSH can be observed [40,41]. Indeed, Orihuela et al. (2005) showed that in one study; At the end of oral administration of aluminum chloride hexahydrate ($AlCl_3.6H_2O$), there was an increase in the number of substances that react with thiobarbituric acid (TBARS), which is an essential indicator of lipid

peroxidation, and a significant decrease in the amount of GSH. for seven days on rats[9] Similarly, in a study investigating the effect of oral $AlCl_3$ administration to rats for 30 days; They found that it caused a significant decrease in SOD, CAT, GPx, GR activity and GSH amount and an increase in TBARS levels[42].

It has been observed in many studies that heavy metal accumulation in tissues increases ROS production and disrupts oxidative balance [37–39]. Nowadays, it is almost impossible to avoid Al exposure, and therefore it is essential to reduce the damage caused by Al to low levels. Therefore, using molecules with antioxidant characteristics is considered an effective method. Antioxidant substances such as curcumin, bromelain, selenium, and vitamin E contributed to the normalization of antioxidant molecules, which decreased due to aluminum toxicity[10,43–45]. Especially in cases where oxidant molecules increase administration of GSH itself or its esters, such as N-acetyl cysteine is one of the preferred methods in improving the antioxidant capacity by increasing the amount of GSH[46,47]. Although it is known that glutathione supplementation is effective in increasing antioxidant capacity, there are differences in results depending on the way GSH is given to living things. In the study in which oral GSH was preferred, it was reported that there was no difference in the total amount of GSH. This situation is thought to be caused by its breakdown by intestinal and hepatic γ-glutamyltransferase[14,48].

Measurement of total antioxidant capacity (TAC) in samples gives cumulative information about oxidative stress. It can help us infer by obtaining a cumulative value instead of the sum of the measurements of many enzymatic or non-enzymatic (such as GSH, Vitamin E, C) and antioxidant parameters such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase. TAC measurement is considered an important criterion, especially to evaluate the effects of antioxidant-rich supplements[49,50]. In experimental studies, it has been reported that glutathione supplementation increases the amount of TAC[51,52]. It has been reported that foods rich in sulphurous amino acids increase GSH expression, increase the activity of antioxidant enzymes such as superoxide dismutase and catalase, and have an antioxidant effect[53].

In the study, the TAC level of the group given GSH supplementation was found to be higher than the control group. At the same time, TAC level, which decreased in the group given aluminum compared to the control group, was higher in the group given GSH with aluminum. These results show the efficacy of GSH in antioxidant defense against Al toxicity. Parameters such as malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OH-dG), lipid peroxides, isoprostane, and total oxidant capacity (TOC) provide information about the oxidative damage that the body is exposed to[54]. It is an essential parameter in determining the level of peroxidation of MDA lipids, which is one of the oxidative stress markers [55]. It has been reported that GSH supplementation reduces serum MDA level; thus it may be effective in regulating oxidative stress[56–58]. In our study, the TOC level was high in the group given aluminum, while the TOC level was found to be statistically low in the group given GSH with aluminum. Our results show that GSH has a significant effect on heavy metal toxicity.

Studies investigating the effect of heavy metals on ADA activity are limited. Senger et al. showed that mercury chloride ($HgCl_2$) has an inhibitory effect on ADA activity in zebrafish[59]. Low concentrations of

+2 precious metal ions such as cadmium (Cd^{+2}) and cobalt (Co^{+2}) show a robust inhibitory effect on ADA activity. It was noted that Calcium (Ca^{+2}) and Iron (Fe^{+2}) ions showed weak inhibition[60]. In a study investigating the effect of Al toxicity on ADA activity, It has been reported that ADA activity increases during Al toxicity[61]. Increased reactive oxygen species cause a weakening of the antioxidant system. In a study conducted, It has been noted that there is an increase in ADA activity in parallel with the increase in oxidative stress[62]. In a survey of cancer patients; ADA activity is increased in cancer patients. In addition, it was stated that the activity of enzymatic antioxidants such as catalase and SOD decreased in these patients, and there was a negative correlation between ADA activity and antioxidant enzyme activities[63]. It has been shown that plants with antioxidant effects such as turmeric and ginger, which are polyphenolic foods, and taurine, carotene, and omega fatty acids significantly reduce the ADA activity of foods[46,64–66]. In a study in which rabbits were given GSH supplementation, it was reported that GSH supplementation did not cause any change in ADA activity[58]. In our research; According to the control group, While ADA activity was high in the group given aluminum, no difference was found in the group given GSH. The data we obtained are similar to the studies showing that ADA activity is positively correlated with increased oxidative stress.

Essential elements (including zinc, copper, calcium, and iron) are micro-nutrients present in blood and tissue. Zinc is intimately involved in protein, RNA, and DNA synthesis. The mechanism of aluminum-induced toxicity and zinc levels in sera has not yet been defined. This study was investigated the possible changes in zinc to exposed to Al. In the study, the zinc level was statistically low in the group given aluminum[67]. There is no satisfactory explanation for reducing serum concentrations of zinc associated with Al exposure. It was speculated that high plasma Al binds essential elements, especially copper and zinc, and which also induces metallothionein, which binds many heavy metals[68,69]. According to the results we obtained from the study, the zn level was found to be low in the AL given group. This indicates an inverse relationship between Al concentration and Zn concentration. As a result, additional GSH administration in experimental aluminum toxicity has a protective effect on adenosine deaminase activity, antioxidative system, and Zn level.

Declarations

Author Contributions O.A, E.A, K.Y.D and C.A. conceived the original idea and build. O.A, D.K, T.T.Z and C.G. supervised the research. Experiments were performed by O.A, K.YD, T.K, R.E. D E.A and C.G. All authors read and approved the final manuscript written by O.A, E.A and D.K. There are no conflicts to declare.

Data Availability The datas analyzed during the current study are not publicly available due to individual privacy but are available from the corresponding author on reasonable request.

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Conflict of Interest The authors declare no competing interests.

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