Cytotoxicity and genotoxicity effects of water boiled in aluminum vessels on Allium cepa root tip cells

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Abstract
Cookwares made from aluminum (Al) are supposed to be a potential source of Al contamination of food. In this study, the cytotoxicity and genotoxicity effects of water boiled in aluminum cookwares on the dividing cells of onion root tip were examined using Allium cepa assay. Three used aluminum cookwares from different sources were selected. Distilled water was gently boiled in each pot and then used for growing onions. The cells of root tip were analyzed for mitotic and phase indexes as well as aberrations appeared in the interphase and mitotic phase. One way analysis of variance and post-hoc Tukey HSD test were applied for comparison between experimental groups. The results showed that the mitotic index in one of the treated groups increased significantly compared to the control. Also the frequency of prophase in two of treated groups increased significantly compared to that of the control. There was a borderline significant increase in the frequency average of total aberrations from three treated groups compared to that of the control ($p$ value = 0.063). Also, a significant increase was observed in the frequency average of disturbed mitosis from three treated groups compared to that of the control ($p$ value = 0.04). The findings of this preliminary study supported a possible health hazard of aluminum cookwares. Further investigation with larger sample and food with various compositions is needed to reach a full conclusion about the health effect of aluminum cookwares.

Keywords Allium cepa assay · Aluminum cookware · Mitotic index · Chromosome aberration

Introduction
Pottery is one of the oldest cooking wares known to man. Currently, various materials used to make pots, pans and other cookwares, but not all pottery is safe for cooking. On the other hand, harmful vessels can be a source of contamination and disease. Materials applied for pottery can enter the food during cooking and thus, care should be taken, as there are potential risks in some of those materials. It has been reported that when pottery is not properly treated, lead can leach into food products during cooking or storage [1, 2]. Leached lead could be absorbed through the gut and can cause lead poisoning with the most deleterious effects on the hemopoietic, nervous, and reproductive systems and the urinary tract [3]. One of the materials widely used in pottery and manufacture of food canning or packaging is aluminum (Al). Cookwares made from aluminum are supposed to be a potential source of Al contamination of food. Recent investigation on environmental toxicology suggested that aluminum may be a major threat for living organisms as it interferes with many crucial biological reactions [4]. Al is believed to be absorbed by the intestines and could be accumulated in some tissues such as bone and brain, and may persist for very long time before it is excreted in the urine [5, 6]. Exposure to Al has been suggested to be associated with various diseases such as aluminum bone disease, encephalopathy in dialysis patients, microcytic anemia, lung problems, and impaired iron absorption. Other disorders associated with Al toxicity are nervous system problems such as loss of memory and coordination, and neurodegenerative disorders such as Alzheimer’s disease [4, 5, 7]. There is increasing evidence that the use of aluminum foil and cookwares lead to increase the amount of Al in food [8–13].

Many studies investigated the biological effect of heavy metals in the forms of ionic or nanoparticles, but very little effort has been made to assess the potential health risks or benefits of metals in the form of alloy used for making cookwares. If they are not properly manufactured, cookwares could be one of the potential sources of exposure of man to the...
heavy metals. Aluminum cookwares such as pans, pots, pressure cookers, and kettles are widely used in Iran for cooking food, boiling liquids and preserving edibles. Considering the possible toxicity of Al as well as the above mentioned association of excessive aluminum intake with some diseases, this kind of pots need to be paid more attention as a likely serious public health threat. The present study was designed to investigate the ability of water boiled in aluminum vessels to induce DNA damage or cell cycle disturbance in proliferating cells of onion root tip.

**Materials and methods**

In this study, the cytotoxicity and genotoxicity effects of water boiled in aluminum cookwares on the dividing cells of onion root tip were examined using *Allium cepa* assay. *A. cepa* assay has been regarded as a favorable test to evaluating the genotoxicity and cytotoxicity effects of chemical agents present in different environments. It is considered as a short-term, low cost and easy handling bioassay to measure different genetic endpoints such as mitotic index, chromosome aberrations, nuclear abnormalities, and micronucleus. The mitotic index (MI) indicates the frequency of cell division, and has been regarded as an important parameter to examine the cytotoxicity of agents. Decreased MI which may reflect a disturbance in growth could be indicative of the presence of cytotoxic agents in the environment. Chromosome aberration (CA) is referred to the atypical number of chromosomes or changes in the chromosomal structure in the cells exposed to physical or chemical agents. Different types of CA such as chromosome bridges and breaks, losses, delays, adherence, multipolarity and C-mitosis can be evaluated using *A. cepa* test. Nuclear abnormalities which characterized by morphological alterations in the interphase nuclei, are also detectable in *A. cepa* test as lobulated nuclei, nuclei carrying nuclear buds, and micronuclei. Micronuclei is round or oval with similar structure as the main nucleus, but in a reduced size, which result from the development of some CA [14].

Clean and healthy bulbs of *A. cepa* were chosen and then the outer scales were removed without damaging the primordia of the root. Three used aluminum cookwares from different sources were selected. Distilled water was gently boiled in each pot with small flame for about 2 h and after getting cold the water was used for growing onions. Three bulbs were used for each water sample. The onion disc was exposed to the prepared boiled water and kept in dark at room temperature for three days. The water sample was changed every day. Three buls were treated with unboiled distilled water as a control group. In the third day of treatment at 13:00 pm, 12 healthy root tips were excised from each bulb and fixed in ethanol/glacial acetic acid (3:1) fixative for 2 h. After fixation, the roots were hydrolyzed in 5 N HCl for 30 min at room temperature, and then were stained with Schiff’s reagent for 90 min in the dark. Six roots were transferred from dye to a microscope slide. The root tips were cut into 1-2 mm pieces and covered with a cover-glass and subsequently, gently squashed by knocking with a blunt end of a pen. The cells were analyzed under a total magnification of 400× using the Olympus microscope. In each root meristem more than 200 cells were counted. Totally more than 1800 cells were evaluated for each onion bulb. The percentage of MI and phase indexes (PIs) was determined with the following formulas [15]:

\[
\text{MI} (%) = \frac{\text{number of cells in mitosis} \times 100}{\text{total cell number}}
\]

\[
\text{PI} (%) = \frac{\text{number of cells in each mitotic phase} \times 100}{\text{total cell number}}
\]

Aberrations appeared in the mitotic phase and interphase were determined together with the MI study. The percentage of the aberrations was measured as the ratio of number of aberrant cells × 100 to total cell number.

**Statistical analysis**

All the results (MI, PIs, and abnormalities in the interphase and mitotic phase) were expressed as the mean and standard deviation of three replicates per each water sample. Analysis of variance (One-way ANOVA) was used to find the significant different in various parameters between control and treated groups. Comparison between control and each of treated groups were done using the post-hoc Tukey HSD tests. The ANOVA contrasts was applied to compare between control and average of treated groups. Statistical analysis was performed using the SPSS version 22 and significance was accepted at \( P < 0.05 \). The power analysis was performed using of G-Power 3.0.10 computer software.

**Results**

The data of mitotic and phase indexes in *A. cepa* root tip cells exposed to each of water sample have been presented in Table 1. Water sample from all three pots induced increase in the mitotic index compared to the water sample of the control; however this change was statistically significant just for water sample from pot 2. Increase of MI% in treated groups was also confirmed by comparison of this index in control group with the average of that from three treated groups using ANOVA contrasts (\( p \) value = 0.005). In the case of MI, which is the most important index of the current study, a post hoc calculations of statistical power using G-Power showed that the total sample used for MI% analysis had sufficient statistical power (\( 1 - \beta > 95\% \)). Analysis of various stages of the mitotic phase revealed that the treatment of *A. cepa* cells by water boiled in pot 2 and 3 significantly
increased the prophase index (ProI) compared to the control. The water sample from pot 1 also increased the ProI, although the difference from the control did not reach to significant levels ($p$ value = 0.096). Increase of ProI% in treated groups was also confirmed by comparison of this index in control group with the average of that from three treated groups using ANOVA contrasts ($p$ value = 0.000). Other phase indexes including metaphase, anaphase, and telophase indexes in the cells treated with water sample from any of three pots did not change significantly compared to that of the control.

The types of appeared abnormalities in interphase and mitotic phase which are analyzed in this study were sticky chromosomes, anaphase bridge, going ahead chromosome, c-mitosis, chromosome breaks, vagrant chromosomes, disturbed mitosis, micronuclei and binucleated cells. Some of these abnormalities were depicted in Fig. 1. Cells with any of the above abnormalities were analyzed and the data have been presented in Table 2. The most frequent observed aberration in the cells treated with any of water sample including control was disturbed mitosis. The frequency of total aberrations in the cells treated with water sample from each of three pots does not differ from that of the control. However when the average of total aberrations in treated groups was compared to that of the control, a borderline significant increase was observed in treated groups ($p$ value = 0.063). No any substantial difference in each kind of the above aberrations was observed between the control and any of the treated groups. Also, ANOVA contrasts analysis confirmed these negative results except for disturbed mitosis. On the other hand, the frequency average of disturbed mitosis from three treated groups increased significantly compared to that of the control ($p$ value = 0.04).

**Discussion**

In this work the effect of aluminum cookwares on the mitotic index as well as appearance of chromosomal aberrations was investigated. It has been reported that the ionic and nanoparticulate form of the metals used in the cookwares such as aluminum, chromium, and copper change the mitotic index.

| Treatment | MI ± SD (%) | PIs ± SD (%) |
|-----------|-------------|--------------|
|           | ProI MetI AnaI TelI  |
| Control   | 6.57 ± 1.02 3.40 ± 0.19 0.91 ± 0.33 0.53 ± 0.29 1.74 ± 0.64 |
| Pot 1     | 7.64 ± 0.14 3.95 ± 0.17 1.35 ± 0.14 0.61 ± 0.09 1.72 ± 0.10 |
| Pot 2     | 10.08 ± 1.02 5.44 ± 0.38* 1.59 ± 0.05 0.62 ± 0.25 2.43 ± 0.58 |
| Pot 3     | 7.87 ± 0.56 4.44 ± 0.19* 1.19 ± 0.41 0.40 ± 0.10 1.84 ± 0.13 |

All values were expressed as the mean of three replicates per water sample

$ProI$ prophase index, $MetI$ metaphase index, $AnaI$ anaphase index, $Tell$ telophase index

*$p < 0.01$ indicates significance level compared with the control

![Fig. 1 Mitotic phase abnormalities.](image)

- (a), going ahead chromosome; (b), disturbed mitosis; (c), c-mitosis; (d), chromosome breaks; (e), sticky chromosomes; (f), vagrant chromosomes; (g), anaphase bridge
Hexavalent chromium decreased the mitotic index in *A. cepa* root cells in a dose dependent manner [16]. Addition of AlCl₃ to whole blood culture and exposing cooper nanoparticle to onion root tips change the mitotic capacity in a dose/exposure time dependent manner; that is, the mitotic index increased at low metal concentration or short term of exposure, but decreased as the metal concentration or exposure time increased [17, 18]. Al treatment on barley root cells significantly reduced the mitotic activity and it also induced micronuclei and damaged nuclei [19]. According to the result of this study, one of the treated groups, pot 2, significantly increased the mitotic index compared to the control. Also analyzing of various stages of the mitotic phase showed that the treatment of *A. cepa* cells by water boiled in pot 2 and 3 significantly increased the MII compared to the control. It should be noted that increase in MI and MII by water sample from the examined pots was also observed when these indexes in control were compared to the average of that from treated groups. These increases in MI and MII which could be indicative of a positive effect of Al on mitosis, is in agreement with previous studies showed mitotic stimulatory effect of Al at low concentration and short exposure time on cultured human blood and root tip cells of *Helianthus annuus* [17, 20]. In spite of evidences which support the possible health toxicity of Al, this metal has been shown to be likely nutritionally essential, and its deprivation lead to depressed growth in animals; although a possible required amount for Al in animal deficiency experiments have not been exactly revealed [21]. In this study the concentration of Al in boiled water was not measured, however, previous studies have revealed that boiling water or neutral solutions in Al pots did not result in much Al release [8, 13]. The above observations, therefore, may reflect the fact that not much Al was leached from the examined pots during water boiling.

There are accumulated evidences that Al induced apoptosis, DNA damage and disturbance in cell cycle [22–25]. The rate of DNA damage, micronuclei and apoptotic cells have been shown to increase in the cultured human lymphocytes treated with AlCl₃ [22, 23]. Aluminum increased DNA damage and ploidy modifications as well as apoptosis and disturbances of the cell cycle progression in the lymphocytes of carp [24]. Al₂O₃ nanoparticles decreased the mitotic index and increased the number of various chromosomal aberrations in onion root tip cells [25]. There is increasing evidence that aluminum foils and cookwares increase the amount of Al in the foods cooked or preserved in, especially when the pH of the food is acidic [8–13]. It has been shown that significant amounts of lead, aluminum and cadmium can release from aluminum cookwares contacting with dilute acetic acid at boiling and ambient temperatures, in the level exceeding recommended public health guidelines [2, 13]. Base on the data of this study, there was no any significant difference in the frequency of total abnormalities between the control and any of the treated groups. Although it should be mentioned that the average of total abnormalities from treated groups was higher compared to that of the control at a borderline significant level. Moreover the frequency average of disturbed mitosis from three treated groups significantly increased compared to that of the control. These observations together with the result of MI indicated that although the concentration of Al in water sample used for treated groups is possibly low, it could be suspected to be at the threshold level to exert genotoxicity effects. On the other hand, supplemental information concerning Al concentration or applying more sensitive genotoxicity assay is needed to reach a reasonable conclusion.

This study investigated for the first time the cytotoxic and genotoxic potency of the aluminum cooking vessels using *A. cepa* assay. Although the association findings support a possible health hazard of aluminum cookwares, some limitations affecting firm conclusion need to be addressed. In the present study the effect of distilled water was examined. It has been shown that boiling water or neutral solutions in aluminum cookware result in negligible Al release [8, 13]. On the other hand, many factors such as pH and composition of food, duration and temperature of heating, and presence of salt and other ions have been proposed to affect the Al leak from cookwares [6]. This is thus a preliminary study and further investigation with larger sample while regarding the following points need to achieve a firm conclusion: i) measuring the amount of leached Al and its uptake by the cells, ii) examining the effect of different kind of food especially in regard of...
composition and pH on induction of Al toxicity using animal models, iii) evaluation of the biological adverse effects of Al using various genotoxicity and cytotoxicity tests.

Conclusion

Overall, the result of this study approved the potential detrimental effects of water boiled in aluminum cookwares on the cytological parameters of A. cepa root tip cells. As evidence on aluminum leaching from cookwares has been increased, studies regarding the health effect of this kind of cookwares seem valuable.

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Authors’ contributions Zahra Zendehboodi was responsible for study concept and design, collection, analysis and interpretation of data, drafting and revising the manuscript and approving the final manuscript.

Compliance with ethical standards

Conflict of interest No conflicts of interests are declared by the author.