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Assessment of the cytostatic, cytotoxic and antibacterial potential of aluminium (III) chloride hexahydrate

Original Article

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Abstract:

Even though aluminium does not participate in any known biochemical process in living organisms, it is omnipresent in various products intended for human consumption. This study aimed to evaluate the cytostatic and cytotoxic effects of aluminium (III) chloride hexahydrate ($AlCl_3$) in cultured human peripheral blood lymphocytes, as well as its antimicrobial potential using agar diffusion method. Our results showed a significant deviation in the mitotic index (MI) values between 2.20, 2.40 and 2.50% of $AlCl_3$ and control group. MI value decreased with an increase in the concentration of $AlCl_3$. An increased frequency of apoptotic cells at all concentrations of the $AlCl_3$ was detected, whereby the significantly higher frequency of apoptotic cells was detected between 2.20 and 2.30% $AlCl_3$ and the control group. *Staphylococcus aureus* and *Escherichia coli* were resistant to the tested compound. Inhibition zones detected in *Bacillus subtilis* and *Pseudomonas aeruginosa* were 13.67±0.58 mm at 1 mg/ml, 12.00±0.00 mm at 10 mg/ml; and 13.00±0.00 mm at 1 mg/ml, and 14.67±0.58 mm et 10 mg/ml, respectively. Our results indicate that $AlCl_3$ possesses antiproliferative and apoptosis-inducing potential. Discrete antibacterial effects were demonstrated. Further studies are needed to strengthen these findings.

Key words:
aluminium (III) chloride hexahydrate, human lymphocytes, toxicological effects, antibacterial activity, agar diffusion method

Apstrakt:

Procena citostatskog, citotoksičnog i antibakterijskog potencijala aluminijum (III) hlorid heksahidrata

Iako aluminijum ne učestvuje ni u jednom poznatom biohemijskom procesu u živim organizmima, on je sveprisutan u raznim proizvodima namenjenim ljudskoj upotrebi. Ova studija je imala za cilj da proceni citostatske i citotoksične efekte aluminijum (III) hlorid heksahidrata ($AlCl_3$) u kultivisanim limfocitima periferne krvi čoveka, kao i njegov antimikrobni potencijal metodom agar difuzije. Naši rezultati su pokazali značajno odstupanje vrednosti mitotičkog indeksa (MI) između 2.20, 2.40 i 2.50% $AlCl_3$ i kontrolne grupe. Vrednost MI se smanjivala sa povećanjem koncentracije $AlCl_3$. Utvrđena je povećana učestalost apoptotičkih ćelija pri svim koncentracijama $AlCl_3$, pri čemu je značajno veća učestalost apoptotičkih ćelija između 2.20 i 2.30% $AlCl_3$ i kontrolne grupe. *Staphylococcus aureus* i *Escherichia coli* su bili otporni na testirano jedinjenje. Zone inhibicije uočene kod *Bacillus subtilis* i *Pseudomonas aeruginosa* bile su 13.67±0.58 mm pri 1 mg/ml, 12.00±0.00 mm pri 10 mg/ml; i 13.00±0.00 mm pri 1 mg/ml, i 14.67±0.58 mm pri 10 mg/ml, respektivno. Naši rezultati pokazuju da $AlCl_3$ poseduje antiproliferativni potencijal i potencijal indukcije apoptoze. Pokazani su diskretni antibakterijski efekti. Potrebne su dalje studije kako bi se ovi nalazi ojačali.

Ključne reči:
aluminijum (III) hlorid heksahidrat, humani limfociti, toksikološki efekti, antibakterijska aktivnost, metod agar difuzije

Introduction

Metal ions are present in more than one-third of all cellular proteins, and are pivotal for the activity of these proteins (Martinez-Finley, 2012). Aluminium represents the most abundant metal, which does not participate in any known biochemical process in living organisms (Tenan et al., 2021). Despite this,

aluminium is nowadays a ubiquitous component of human life, since it is added to water, food, medicines, vaccines, cosmetic and personal care products intended for human use (Tenan et al., 2021; Rahimzadeh et al., 2022). Extraneous metals can disturb the delicate equilibrium that allows the correct course of cellular activities, and thus exhibit toxic and/or carcinogenic effects (Beyersmann & Hartwig, 2008; Jan et al., 2015). Aluminium chloride represents an inorganic compound with the formula $AlCl_3$. It forms a hexahydrate containing six water molecules of hydration. The anhydrous form as well as the hexahydrate are colourless crystals. The anhydrous form is commercially important since it possesses a low melting and boiling point (Helmboldt et al., 2007).

Prolo et al. (2007) demonstrated that aluminium reduced the proliferative response of normal human peripheral blood mononuclear cells to phytohemagglutinin stimulation. Furthermore, it has been shown that aluminium inhibited the proliferation of the HT-29 cells (Yu et al., 2019), as well as that aluminium trichloride inhibited the proliferation of osteoblasts (Huang et al., 2017). Considering its large presence in products that people consume daily, as a result of continuous exposure aluminium accumulates in various organs including bone, brain, kidney, liver, lung, and mammary gland (Exley, 2013; Darbre, 2016). High levels of aluminium are associated with dialysis, encephalopathy, osteomalacia, and Parkinson's disease (Alfrey et al., 1976; Ellis et al., 1979; Hirsch et al., 1991). Aluminium has also been described as a potential human carcinogen involved in the etiology of breast cancer, based on the experimental evidence that daily application of antiperspirants, especially those rich in aluminium salts, contribute to an increased incidence of breast cancers (Darbre, 2001; McGrath, 2003). In support of this, Linhart et al. (2017) demonstrated that frequent use of underarm cosmetic products may lead to an accumulation of aluminium in breast tissue, suggesting a possible association between breast cancer risk and frequent use of these products. Earlier investigations discussed the antiseptic and antibacterial activity of some aluminium salts (Cromwell & Leffler, 1942; Shelley et al., 1953; Aguilar et al., 1956), as well as the validity of using the aluminium salts in cosmetic preparations for their antibacterial potential (Blank & Dawes, 1960). The antimicrobial activity of aluminium is investigated by Londono et al. (2017) and the study suggests a potential synergism with other metals to achieve antibacterial effects. Through evolution, microorganisms found ways to avoid environmental stress, and one of such mechanisms is illustrated via the production of siderophores. Siderophores could be defined as low-molecular-weight molecules with a high and specific affinity to chelate metals, such as iron (Hider & Kong, 2010). Timofeeva et al. (2022) investigated siderophores involved in iron uptake and mentioned about 20 different bacterial genera that are known for siderophore production. The research of Farh et al. (2017) suggests that the production of siderophores is related to bacterial resistance to aluminium toxicity. Therefore, the present study aimed to assess the cytostatic, cytotoxic, and antibacterial potential of aluminium (III) chloride hexahydrate ($AlCl_3$).

Materials and Methods

Peripheral blood lymphocyte culture

For this research, whole blood from two healthy female donors, aged 24, who were non-smokers was used. Blood is collected into sterile vials with 68 IU of sodium heparin per milliliter of blood (BD VACUTAINER VR, Becton, Dickinson and Co., NJ, USA). Lymphocyte cultures were prepared according to Moorhead et al. (1960) with some modifications. In that sense, 0.5 mL of whole blood was added to 5 mL RPMI-based complete medium containing fetal bovine serum (FBS), L-glutamine and phytohemagglutinin (PHA) (GIBCO™ PB-MAX™ Karyotyping Medium, Invitrogen, CA, USA). Lymphocyte cultures were incubated in total for 72 hours at 37 °C. After 48 hours, cultures were treated with $AlCl_3$ in four test concentrations (2.20, 2.30, 2.40 and 2.50%) determined according to SCCS (2020), and incubated for another 24 hours. In addition to cultures with four concentrations of the test substance ($AlCl_3$), cultures containing untreated lymphocytes (control group), were incubated. Two replicates for each test concentration and control group were made. Two hours before the end of the incubation period, COLCEMID™ (Sigma-Aldrich, St. Louis, MO, USA) was added to the cultures at a final concentration of 0.2 µg/mL to block cell division in metaphase. After the end of the incubation period, the cultures were treated with fresh hypotonic solution (0.075 M KCl) to lyse the cells. Afterward, cultures were fixed with methanol/glacial acetic acid (3:1 v/v) fixative. The lymphocytes were then spread onto clean, cooled glass slides that were left to air-dry, and then stained with 10% Giemsa solution.

Microscopic analysis

The cytogenetic analysis included the examination of two microscope slides (two replicates) for each of the tested concentrations of $AlCl_3$ and the control group. The analysis of the mitotic index (MI) value, as well as the frequency of apoptotic cells (cytotoxic biomarkers), was performed in 1000 cells per slide

(a total of 2000 cells per concentration of the test substance and the control group). The MI was calculated as the ratio between the number of cells in the division and the total number of cells analyzed neglecting broken cells, clumped cells and cellular debris.

Microbiological analysis

To test the antimicrobial activity of $AlCl_3$, a solid substance was dissolved in dimethyl sulfoxide, DMSO (Sigma Aldrich, St. Louis, MO, USA) to the final concentrations of 1 mg/mL and 10 mg/mL. As the positive control, 10 µg/disk of ampicillin (OXOID ™, Watham, MA, USA) was used. The antibacterial activity of $AlCl_3$ was evaluated using a total of four bacteria: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 8739. Tested bacteria were obtained from the American Type Culture Collection (ATCC) and inoculums were prepared according to EUCAST (2017). Overnight cultures were dissolved in sterile saline solution to achieve the final turbidity of 0.5 McFarland standard, which represents the bacterial cell concentration of 1.5×10^8 CFU/mL.

Agar diffusion method

The agar diffusion method is performed according to Balouiri et al. (2016). Inoculation of the agar plates was performed using the sterile swabs soaked in a suspension of test bacteria, and the seeded plates were left at room temperature for 15 minutes to allow the better absorption of the applied inoculum into the substrate. Plates were then drilled to make wells with 8 mm in diameter. The amount of 50 µL of $AlCl_3$ was transferred into the wells and plates were incubated for 16 to 18 hours at 37 °C. Both concentrations of $AlCl_3$ were triple tested. The antibacterial activity was evaluated based on the diameter of inhibition zones, after the $AlCl_3$ diffusion in the medium.

Statistical analysis

All descriptive statistical computations were conducted by use of MICROSOFT EXCEL 2019 (Microsoft Corporation, Redmond, USA). One-way ANOVA with the post hoc multiple comparison tests (LSD) in terms of determining significant differences between the test compound ($AlCl_3$) and the control for all analyzed parameters was performed using IBM SPSS Statistics for Windows, version 20.0 (Armonk, NY, USA) for the results of cytogenetic analysis, and STATISTICA 10 (StatSoft.Inc., Tulsa, OK, USA) for the results of microbiological assay. Differences were considered statistically significant at the $p<0.05$ level.

Results and discussion

Cytostatic and cytotoxic effects of AlCl3

The results of $AlCl_3$ effects on cell proliferation are summarized in **Tab. 1**. Mitotic index (MI) values varied depending on the applied concentration of the test substance. In that sense, a significant deviation of the MI values in mutual comparisons between the groups treated with $AlCl_3$ at concentrations of 2.20, 2.40 and 2.50% $AlCl_3$ and the control group was detected. The highest MI value was observed at a concentration 2.20% of $AlCl_3$, with a tendency for MI value to decrease with an increase in the concentration of the test substance. As for the cytotoxic effects (**Tab. 1**), the results revealed that the frequency of apoptosis was elevated at all four tested concentrations of $AlCl_3$ as compared to the control group. However, a significantly higher frequency of apoptotic cells was observed between 2.20% and 2.30% of $AlCl_3$ and the control group.

Table 1. Cytostatic and cytotoxic effects of $AlCl_3$ in human lymphocytes

$AlCl_3$ concentration (%)	Mitotic index	Apoptotic cells
2.50	0.25±0.07*	6.50±0.07
2.40	0.87±0.40*	7.50±2.12
2.30	3.00±0.56	17.00±2.82*
2.20	5.10±1.27*	15.50±3.53*
Control group	2.90±0.07	3.50±2.12

Results are presented as mean ± SD; The mitotic index is presented as a percentage; *Statistically significant differences as compared to the control group ($p<0.05$)

It is known that aluminium, even though is the most abundant metal, is not involved in any known biochemical process in living organisms. However, due to its mobilization from minerals which is encouraged by various human activities, aluminium is nowadays an omnipresent component of human life. Aluminium is an active ingredient present in many products for human use such as vaccines, drugs, antiperspirants, cosmetics, agricultural pesticides and foods (Sakhawoth et al., 2021; Tenan et al., 2021). This study aimed to evaluate the possible cytostatic and cytotoxic effects of $AlCl_3$ in human peripheral lymphocytes.

By analyzing the mitotic index, we observed a significant dose-dependent reduction of the mitotic index values, which provides evidence that aluminium is directly affecting the inhibition of cell proliferation. These findings confirm those obtained

by Prolo et al. (2007) whose study showed that aluminium diminished the proliferative response of normal human peripheral blood mononuclear cells to phytohemagglutinin stimulation. Furthermore, our results are in concordance with those which demonstrated that aluminium inhibited the proliferation of the HT-29 cells (Yu et al., 2019), as well as those which showed that aluminium trichloride inhibited osteoblastic proliferation (Huang et al., 2017).

The analysis of cytotoxicity markers showed a significantly higher frequency of apoptosis between $AlCl_3$ (2.20% and 2.30%) and control. Our results are supportive of those obtained by Yu et al. (2019) who showed that aluminium induced cellular apoptosis in HT-29, a human colon cancer cell line. Similarly, Prolo et al. (2007) demonstrated that aluminium increases apoptosis of cultured human cells (SaOs-2). Tenan et al. (2021) demonstrated that aluminium at low doses did not significantly affect cell viability, but did significantly increase chromosome double-strand breaks. This could be if not the most dangerous situation, then certainly one of the most dangerous, since these damaged cells have the potential to survive and disseminate karyotypic abnormalities. This suggests that low chronic doses of aluminium have the potential to be more damaging than higher doses, making them more likely to cause cell elimination by cytotoxicity. Cytotoxicity or cellular transformation might be dependent on several factors such as dose and duration of exposure to the foreign metal, with chronic low-dose exposure possibly favoring carcinogenesis (Zhao et al., 2014), which is by the reality of human exposure to low doses absorbed from various sources daily. DNA double-strand breaks represent the most cytotoxic lesions of those occurring in the DNA, which can lead to cell death, and/or to genome mutagenesis and chromosomal instability. Even though most of these rearrangements exhibit harmful effects on cellular survival, single events can ensure clonal advantage and result in abnormal cellular proliferation and cancer (Gómez-Herreros, 2019). Apoptosis and the genes involved in its control have a significant impact on the malignant phenotype (Katavic et al., 2023). Disruption of apoptosis can promote tumor formation by promoting cell proliferation through the expansion of selected clones (Labi & Erlacher, 2015). The link between the increase in the rate of apoptosis and the development of tumors was demonstrated during the study of the development of breast cancer in mice, highlighting that the apoptosis inducer CD95 promoted cancer growth (Wang et al., 2013).

Experimental studies indicate that the long-term use of aluminium may correlate with breast cancer development and progression, due to its possible estrogen-like activities (Gorgogietas et al., 2018). In another study, $AlCl_3$ was used, and loss of contact inhibition, as well as anchorage-independent growth in MCF-10A human mammary epithelial cells, were observed. These processes were preceded by increased DNA synthesis, the occurrence of double-strand breaks, and accelerated aging in proliferating cultures, as well as in proliferating primary human mammary epithelial cells (Sappino et al., 2012). The foregoing undoubtedly suggests that $AlCl_3$, by causing an increased frequency of apoptosis, could affect the malignant transformation of cells in humans.

Antibacterial activity

The obtained results of the antibacterial activity of investigated aluminium salt showed susceptibility in the case of *Bacillus subtilis* and *Pseudomonas aeruginosa*, while *Staphylococcus aureus* and *Escherichia coli* were resistant to the investigated compound in both tested concentrations of 1 mg/mL and 10 mg/mL (**Tab. 2**). The concentration of 1 mg/mL of $AlCl_3$ caused an inhibition zone of 13.67±0.58 mm in *B. subtilis*, and 13.00±0.00 mm in *P. aeruginosa*, while the concentration of 10 mg/mL of tested substance generated inhibition zones of 12.00±0.00 mm and 14.67±0.58 mm in *B. subtilis* and *P. aeruginosa* respectively. Antibiotic ampicillin performed strong antibacterial activity, with the more extensive growth inhibition of Gram-positive bacteria in comparison to Gram-negative strains (**Tab. 2**). Solvent control showed that DMSO did not perform antibacterial action.

Our investigation regarding the antibacterial properties of tested $AlCl_3$ provided interesting results, bearing in mind that a particular compound is used in cosmetic products due to its antiseptic potential. *Staphylococcus aureus* and *Escherichia coli* used in this study were completely resistant to both concentrations of the $AlCl_3$, while in *Bacillus subtilis* and *Pseudomonas aeruginosa* discrete inhibition zones have been detected. Impact of the aluminium on the bacterial cell could be discussed at several levels. Aluminium can bind with DNA, bacterial cell membranes, and the cell wall. Also, the presence of the aluminium ion in the cell causes competition of iron and magnesium uptake which changes enzymatic reactions (Piña & Cervantes, 1996). This could be responsible for the decline in the overall growth rate of bacteria (Maier, 2009).

The research of Londono et al. (2017) noted that aluminium alone is not responsible for bactericidal effects, but rather is the combination with other metals. That kind of chemical interaction could be related to the formation of a toxic environment for the bacteria

Table 2. Observed zones of inhibition (mm) due to the activity of $AlCl_3$

Tested bacteria	Investigated substance			
	$AlCl_3$		Ampicillin (10 µg)	DMSO (≥99.7%)
	1 mg/mL	10 mg/mL		
***Bacillus subtilis* ATCC 6633**	13.67±0.58	12.00±0.00	48.13±0.32	NI
***Staphylococcus aureus* ATCC 6538**	NI	NI	32.97±0.25	NI
***Pseudomonas aeruginosa* ATCC 27853**	13.00±0.00	14.67±0.58	13.10±0.21	NI
***Escherichia coli* ATCC 8739**	NI	NI	8.93±0.31	NI

Results are presented as mean ± SD; All values differ significantly at $p<0.05$ after performing the post hoc Fisher's LSD test

(Preston et al., 2000). According to Schoonen & Lanzirotti (2006), the pH strongly influences metal toxicity. This study used DMSO as a solvent, which is an alkaline reagent, and this might be connected to the observed subtle bactericidal effects. Besides the already mentioned, microorganisms have developed numerous mechanisms to cope with a variety of toxic metals to survive (Mittal et al., 2003). Aluminium-resistant bacteria from different genera have been previously reported, and according to Farh et al. (2017), the resistance to aluminium toxicity is mainly correlated to the production of siderophores. Timofeeva et al. (2022) gave a summarized list of the siderophore-producing bacteria, that could be classified into the genera: *Azotobacter*, *Azospirillum*, *Bacillus*, *Dickeya*, *Enterobacter*, *Klebsiella*, *Kosakonia*, *Methylobacterium*, *Nocardia*, *Pantoea*, *Paenibacillus*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Streptomyces,* etc. The study of Schalk et al. (2011) highlighted that other metals besides iron can activate the production of siderophores, as well as that some siderophores except for their preference for iron can chelate other elements and, in that way, maintain the homeostasis of metal in the bacterial cell. Aluminium easily forms complexes with siderophores, based on the similarity in size and charge between the aluminium and iron ions, and besides the passive aluminium uptake, siderophore-mediated uptake of this metal is recognized among the microorganisms (Bojić et al., 2002).

According to their chemical nature, siderophores are classified into several groups, where the hydroxamate siderophores characteristically form a bidentate ligand with Fe ions (Ustiatik et al., 2021; Timofeeva et al., 2022). It is shown that *Bacillus megaterium* produces two hydroxamate siderophores (Byers et al., 1967), that are capable of chelating aluminium. The siderophore transport receptor absorbs the aluminium which provides an additional pathway for the aluminium accumulation. This process is closely related to the overall implications of metal toxicity (Hu & Boyer, 1996). *Bacillus cereus* also stays viable in the increased concentration of aluminium (Ji et al., 2016). Furthermore, the study of Hazarika et al. (2023) confirmed that mitigation of Al-toxicity through the activity of *B. subtilis* is connected to siderophore production. Cornelis (2008) noted that pyoverdine found in *P. aeruginosa* can complex aluminium with high affinity, which implies that is involved in the sequestration of toxic metals. Some other species of the genus *Pseudomonas* are also able to tolerate aluminium-induced stress (Appanna et al., 1994; Singh et al., 2005; Purwanti et al., 2019; Mozumder et al., 2022).

The literature is generally scarce regarding the tolerance of aluminium toxicity in *S. aureus*. Ghssein & Ezzedine (2022) emphasize the presence of high-affinity metal import pathways, including metal ions acquisition, recruitment, and metal-chelate complex import. Therefore, siderophores together with metallophores increase the infectious properties of *S. aureus*. These findings are in accordance with the investigation of Aras et al. (2023) where the impact of aluminium chlorohydrate on the development of antibiotic resistance in *Staphylococcus epidermidis* is highlighted. Guida et al. (1991) discuss the aluminium toxicity to *E. coli* and suggest pH as one of the crucial factors in that term. Also, the same study observed that aluminium binding is happening intracellularly as well as at the bacterial cell surface, while intracellular binding sites involve iron transport pathways. Bojić et al. (2002) observed that aluminium toxicity to *E. coli* depends on the type of growing medium, mainly because of the different uptake of the metal in different media. Al-Khikani et al. (2023) investigated the effects of aluminium potassium sulphate on *E. coli* and the results were concentration-dependent, where the higher concentrations of the substance were effective, while lower concentrations inhibited bacterial growth only in combination with antibiotics, probably due to the synergistic activity. The strain of *E. coli* used in our experiment performed low susceptibility to stand-

ard antibiotic ampicillin. The study of Khazaal et al. (2022) confirmed a positive correlation between antibiotic resistance and siderophore production in different *E. coli* strains.

Conclusion

The analysis of cytostatic and cytotoxic properties of $AlCl_3$ in human lymphocytes revealed significant alterations in cell proliferation and cell death. Microbiological assays in this investigation revealed resistance of Gram-positive and Gram-negative bacteria to $AlCl_3$ which potentially raises the question of justification of the use of this substance in a variety of commercial products. Our results suggest that special consideration should be given to the long-term repercussions of regular low-dose exposure for human tumorigenesis.

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