Reproductive and Histological Toxicity of Transdermal Exposure to Aluminum Chloride in Male Mice

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Abstract

Aluminum in its elemental form poses no health risks; however, the ionic form of aluminum (Al^{3+}) has been reported to be potentially toxic. Aluminum ions can enter the body by ingestion, inhalation, and penetration of intact skin, where it could bioaccumulate in soft body tissues. The aim of the present study was to investigate the reproductive and histological toxicity of transdermal exposure to Aluminum Chloride in male mice as well as the effectiveness of green tea extract (GTE) in alleviating the toxicity of $AlCl_3$. Male mice were randomly assigned to the following groups: control, $AlCl_3$, $AlCl_3$ + GTE, GTE, and deodorant. $AlCl_3$ or deodorant was daily transdermaly administrated by rubbing. Fresh GTE was prepared daily, and was fed orally (by drinking *ad libitum*). Three mice from each group were killed by cervical dislocation after 1, 3, 5, and 7 weeks of treatment period. The results show a statistically significant decrease in sperm count, testosterone levels, and percent of normal sperm morphology as well as histological lesions of the $AlCl_3$ and deodorant treated groups when compared to the control group. Therefore, prolonged transdermal exposure to Aluminum Chloride may induce reproductive failure and could be a cause of male infertility.

Key words: Aluminum Chloride, reproductive toxicity, green tea extract, sperm count, mice

Introduction

Aluminum is a silver-white flexible metal that naturally occurs as part of the earth's crust [1]. It has no known biological function [2], but has a vast number of uses. Aluminum is used for many products such as canned foods and beverages, cooking utensils, cosmetics, antiperspirants, deodorants, toothpaste, and drugs [3, 4, 5]. In medicine, Aluminum is used in vaccines, allergy testing, intravenous solutions, wound and antacid irrigation, ulcer treatment, blood oxygenation, bone or joint replacement and burn treatment [6]. Aluminum in its elemental form poses no health risks and is not harmful; however, the ionic form of aluminum (Al^{3+}) and compound forms of aluminum such as aluminum chloride ($AlCl_3$) and Aluminum sulfate ($Al_2 (SO4)_3$) [7] have been reported to be potentially toxic for humans [1, 5, 8] and may contribute to some diseases such as Alzheimer [9] and osteoporosis [10]. Aluminum ion (Al^{3+}) can enter the body by several routes including the gastrointestinal tract (ingestion), the respiratory tract (inhalation) [11], and the penetration of intact skin (dermal) [12] where it could bioaccumulate in all body tissues including kidney, liver, heart, blood, bone, brain [13], and testes. Aluminum ions alter properties and structure of cellular membranes, induce free radical mediated cytotoxicity [8], cause mineral imbalance [6] inhibit several oxidative enzymes such as catalase, superoxide dismutase [14].

Aluminum compounds are widely used in antiperspirants and deodorants without harmful effects to the skin [15]. However, once Aluminum compounds are dissolved in sweat they show all the chemical properties that their components show separately, the Aluminum frees and exhibits the action of Aluminum ions. Transdermal uptake of Aluminum is known to be more significant than Aluminum ingestion [12]. Therefore, it is important to conduct Aluminum reproductive toxicity studies, especially on sperm parameters (sperm count and sperm morphology) via transdermal route. Sperm count is the concentration of sperms in a given volume of seminal fluide, taken as an index of male infertility [16].

Green tea (*Camellia sinensis*) is one of the most commonly consumed beverages worldwide [17]. Green tea is considered to have beneficial effects on health due to its high content in polyphenols (e.g. epigallacathechin-3-gallate (EGCG)). EGCGs are known to possess strong antioxidative properties [18, 19] due to their radical scavening and metal chelating functions. Polyphenols found in green tea show 20 times more powerful antioxicant activity than vitamin C [20].

The present study was conducted to investigate the potential reproductive and histological toxicity of transdermal exposure to Aluminum Chloride in male mice by analysis of sperm parameters (total sperm count and sperm morphology) and histological examination of sections from testes as well as the effectiveness of green tea extract (GTE) in alleviating the toxicity of Aluminum Chloride.

Materials and Methods

Reagents

- Aluminum chloride-6-hydrate ($AlCl_3.6H_2O$), analytical grade was purchased from Nur Al-Elmia Company (Tripoli-Libya).
- Green tea (Zohra brand, China made) was purchased from grocery store.

Ethical Approval

The experimental procedures in this study were performed according to the bioethical research guide established by the Libyan National Committee for Biosafety and Bioethics; which comply with the published guide "Principles of laboratory animal care" [22].

Animals

Swiss albino mice weighing between 23-35g were inbred in animal house of the Zoology Department, Faculty of Science, Tripoli University. The mice were housed in polypropylene cages containing wooden flakes or ships under standard husbandry condition, and maintained under natural light/dark photoperiod. Mice were fed a standard lab diet and given *ad libitum* access to food and water or food and green tea extract only (i.e. were not provided any water, mice drank green tea only in place of water). The animals were apparently health and free of any external parasites or skin disease. They received different doses of Aluminum Chloride and *ad libitum* doses of green tea extract.

Preparation of Aluminum Chloride Solution

Six grams of Aluminum Chloride ($AlCl_3$) was dissolved in 100 ml distilled water to prepare a stock solution (60 mg/ml). The solution was prepared weekly and kept in a dark bottle at room temperature. $AlCl_3$ was daily transdermaly administrated on the dorsal side to a 4 cm^2 of shaved area by rubbing 10 µL or 20 µL of $AlCl_3$ stock solution, which is approximately equivalent to 0.5 mg or 1.0 mg of $AlCl_3$ respectively.

Preparation of Green Tea Extract (antioxidant)

Green tea extract (GTE) was prepared by adding 20 gm of green tea to 500 mL of boiling water and steeped for 15 minutes. The extract was cooled to room temperature and then filtered. Fresh GTE was prepared daily, and was fed orally (by drinking *ad libitum*) to mice by replacing water bottles with GTE bottles.

Experimental Design

The experiments were performed on 72 adult Swiss albino male mice. Aluminum Chloride was dissolved in distilled water and given to mice via transdermal route daily by rubbing for 7 weeks. The experiments consisted of six groups. 12 male mice per group were randomly assigned to one of the following six groups:

Group 1: mice served as control (i.e. did not receive $AlCl_3$ or GTE)
Group 2: mice treated with 0.5 mg $AlCl_3$
Group 3: mice treated with 0.5 mg $AlCl_3$ and supplied with GTE bottles instead of water
Group 4: mice treated with 1.0 mg $AlCl_3$
Group 5: mice treated with 1.0 mg $AlCl_3$ and supplied with GTE bottles instead of water
Group 6: mice treated with 20 µL of roll on antiperspirant (deodorant)

The body weights of mice were recorded on the first day before dermal application of $AlCl_3$ (initial) and on the day of sacrifice (final). Three mice from each group were killed by cervical dislocation and they were processed after 1, 3, 5, and 7 weeks of treatment period. These weeks assay the differential sensitivity of spermatogonia cell types to $AlCl_3$. In week 1 the cell population treated is spermatozoa; week 3 the cell population treated is spermatids, week 5 the cell population treated is preleptotene late spermatogonium; week 7 the cell population treated is spermatogonium.

Sperm count determination
Sperm count was determined in five squares (four corners and the center, diagonal line) in the center grid of both sides of Neubauer hemocytometer following the method of [21]. Sperm samples were collected from vas deferens, the count was repeated at least three times to minimize error. Each vas deferens was gently squeezed and thoroughly stripped in a clean watch glass containing 1 mL of physiological normal saline (0.9 NaCl). The sperm suspension was incubated for 15 minutes at 37 °C to allow sperm separation, after incubation sperm suspension was diluted 1: 100. After thorough mixing with a fine pipette, a drop of the diluted sperm suspension was placed on counting chamber. The number of motile and nonmotile sperms were counted on the ruled area only and expressed as $\times 10^6$/mL.

Sperm Morphology Examination
Sperm morphology examination was done by making sperm smears from the sperm suspension. One drop of sperm suspension was placed on a clean microscopic slide and a sperm smear was made, allowed to air dry, and then stained with 1.0% eosin Y in water for ten minutes. These smears were observed at 400× magnification using a standard light microscope and the number of normal and abnormal sperms was determined. The criteria for abnormal sperm morphology include the following: the shape of sperm head, the presence or absence of hook, and the shape of tail.

Determination of serum testosterone levels
Testosterone levels were determined by enzyme-linked immunosorbent assay commercial kit, following the procedures outlined by the manufacturer (BioChek). Clotted blood samples were centrifuged for 15 minutes at 3,000 rpm to separate the serum and were stored at −20°C until measurement of testosterone hormone.

Histological examination
Testes were fixed in 10% buffered formalin, passed through ascending series of ethanol and then through xylene and embedded in paraffin wax. Tissues were sectioned at the thickness of 7-8 µm and stained with hematoxylin and eosin (H&E), and microscopically analyzed and photographed.

Statistical analysis
The results were expressed as mean ± standard error (SE). The data was analyzed statistically using one way analysis of variance (Anova) to test for any differences between mean values of all groups. Anova was followed by post hoc Tukey test used for multiple comparisons (i.e. the mean values of the treated groups were compared with those of the control group). A value of $P \leq 0.05$ was considered as statistically significant. These statistics were done using IBM SPSS package version 20.

Results

The results of this study revealed that there was a statistically significant decrease in sperm count and an increased percentage in abnormal sperm morphology as well as histological lesions of the $AlCl_3$ and deodorant treated groups when compared to the control group. The observed decrease in sperm count was statistically significant ($P < 0.05$) for all studied weeks (i.e. weeks 1, 3, 5, and 7). The results of sperm count, testosterone levels, perecent of abnormal sperm morphology, and abnormal sperm shapes are shown in figures 1, 2, 3,and 4, respectively. The histological lesions of the testes are shown in figures 6, 7, and 8. Overall observations revealed marked damage in histoarchitecture of seminiferous tubules of $AlCl_3$ and deordant treated mice. The lesions include congestion of semineferous tubules which led to loose tunica albicana, distortion of geminal epithelial cells, especially the spermatogonia and spermatocytes, reduction in spermatozooa, presence of multinucleated giant cells and hyperemic blood vessels.

Sperm count

The sperm count in weeks 1, 3, 5, and 7 was decreased in all of the groups treated with $AlCl_3$ plus GTE in comparison with the control group (Fig. 1). In the first and third week group 0.5 mg $AlCl_3$ + GTE, 1.0 mg $AlCl_3$, and 1.0 mg $AlCl_3$ + GTE the sperm count was significantly reduced ($P < 0.05$) in comparison with control and deodorant group (Fig. 1); the mean value of sperm count $x10^6$/ml were 0.080 ± 0.000, 0.040 ± 0.00, 0.06 ± 0.60, respectively. Whereas groups 0.5 mg $AlCl_3$ and deodorant sperm count was only slightly decreased. In the fifth week the deodorant treated group had the lowest sperm count with a mean value of 0.060 ± 0.000, while the other treated groups showed an increase in sperm count indicating a slight recovery of the testicular toxic effect of $AlCl_3$. The seventh week sperm count showed a significant reduction in all groups, especially 0.5 mg $AlCl_3$ + GTE and 1.0 mg $AlCl_3$ + GTE; while the deodorant group showed nonsignificant increase in sperm count in comparison to control group (Fig. 1).

Testosterone level

Testosterone levels were significantly reduced in Aluminum Chloride ($AlCl_3$) and Aluminum Chloride ($AlCl_3$) plus green tea extract (GTE) treated groups in a time dependent manner (i.e. gradual decrease from week 1 to week 7) when compared to normal control (Fig. 2). However, there was a significant steady increase of testosterone levels in green tea treated group in all weeks (Fig. 2).

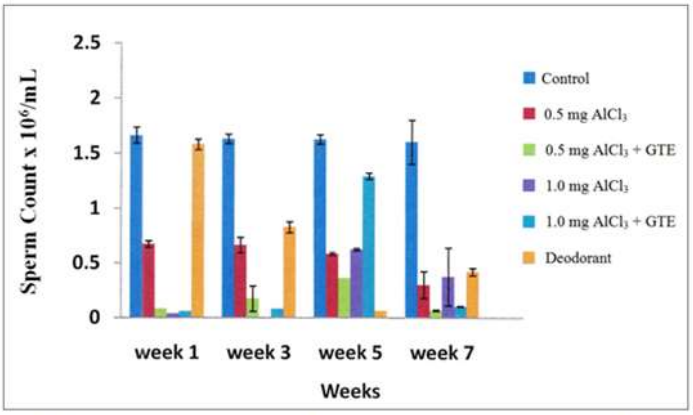

Figure 1. Sperm count for weeks 1, 3, 5, and 7 of control, Aluminum Chloride ($AlCl_3$), and green tea extract (GTE) treated groups.

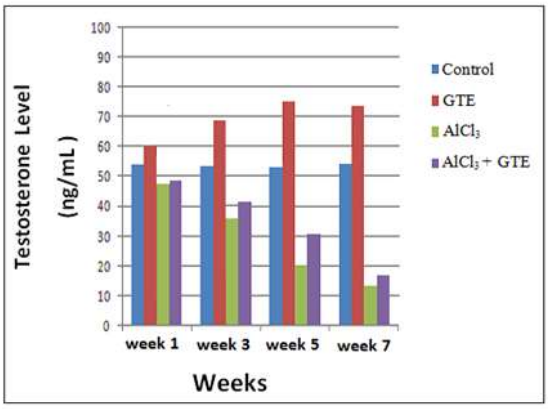

Figure 2. Testosterone levels for weeks 1, 3, 5, and 7 of control, Aluminum Chloride ($AlCl_3$), and green tea extract (GTE) treated groups.

Sperm Morphology

There was a significant ($P < 0.05$) reduction in the mean percentage of normal sperm morphology of all $AlCl_3$ treated mice in comparison with control group (Fig. 3). There was a gradual increase of abnormal sperm shapes from week 1 to week 7. The highest level of sperm shape abnormalities was observed in week 7 in groups 1.0 mg $AlCl_3$ (96.6%) and deodorant (88%); followed by week 5 seen in 1.0 mg $AlCl_3$ +

GTE group (88%), week 1 in 1.0 mg $AlCl_3$ + GTE group (81.8%), and week 3 in deodorant group (74.6%) (Fig. 3). The morphological different forms of sperm shape abnormalities include bannana head,fused head, kink tail, ring tail, and tight tail (Fig. 4). Kink tail was the most common morphological sperm abnormality.

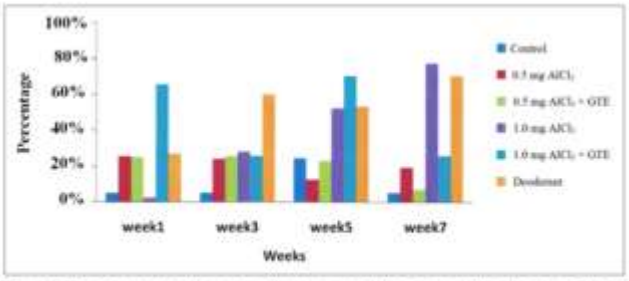

Figure 3. The percentage of abnormal sperm morphology in control, Aluminum Chloride ($AlCl_3$), and green tea extract (GTE) for weeks 1, 3, 5, and 7.

Figure 4. Photomicrograph of sperm smear of $AlCl_3$ treated mice showing the morphological different forms of sperm abnormalities which include: kink tail (a), bannana head (b), tight tail (c). 1.0% eosin stain.

Histopathology of Testes

Light microscope examination in the control group showed normal histological structure of the testes (Fig. 5) with normal pattern of seminiferous tubules, orderly arranged spermatogenic cells, Leydig cells and intact tunica albuginea. However, mice testes treated with Aluminum Chloride (Fig. 6), deodorant (Fig. 7), and $AlCl_3$ + GTE (Fig. 8) revealed many histopathological lesions of testes post treatment. The figures show marked damage in spermatogonia of seminiferous tubules as well as shrinkage in seminiferous tubules which led to loose tunica albuginea, wide lumen, irregular distribution of epithelial lining of seminiferous tubules, large interstitial spaces, and lack of Leydig cells around basement membranes (Fig. 6,7, and 8).

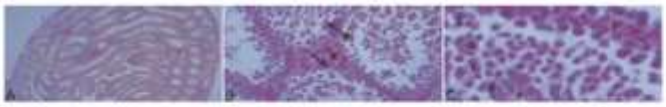

Figure 5. Photomicrograph of testicular tissue in male mice of the control group showing: **(A)** normal patterns of seminiferous tubules with narrow lumen, stratified epithelial lining, little interstitial space, and intact tunica albuginea; **(B)** seminiferous tubules lined by spermatogonia (double head arrow) and normal number of Leydig interstitial cells (arrow); **(C)** seminiferous tubules lined by spermatogonia (a), primary spermatocyte (b), secondary spermatocyte (c), spermatids (d), spermatozoa (e) and a basement membrane (f). H&E, A:x50, B: x400, and C: x1000.

Figure 6. Photomicrograph of testicular tissue in male mice treated with 1.0 mg of $AlCl_3$ showing: **(A)** shrinkage of seminiferous tubules and loose tunica albuginea; **(B)** hyperemic blood vessel (yellow arrow), interstitial edema (blue arrow), and Gaint cell (red arrow); **(C)** interstitial edema (orange arrows). H&E, A:x50, B: x400, and C: x1000.

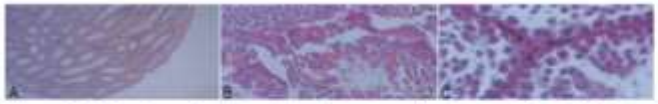

Figure 7. Photomicrograph of testicular tissue in male mice treated with 20 µL of deordant showing: **(A)** contraction of seminiferous tubules with wide lumin and loose tunica albuginea; **(B)** disorganized structure of seminiferous tubules that have lost the normal distribution of epithelial lining (arrows); **(C)** sloughing of epithelial germ cells and shrinkage of interstitial connective tissue (arrows). H&E, A:x50, B: x400, and C: x1000.

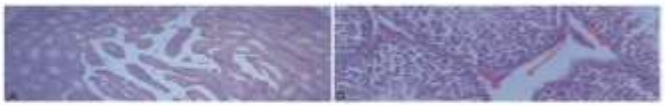

Figure 8. Photomicrograph of testicular tissue in male mice treated with 1.0 mg $AlCl_3$ + GTE showing: (A) irregular distorted seminiferous tubules which attained different shapes and increased interstitial space; (B) interstitial edema (arrow). H&E, A:x50, B: x400.

Discussion

Most of experimental studies on Aluminum Chloride toxicity in animals the chosen routes of Aluminum administration are oral, intraperitoneal, and intravenous; however, these routes do not represent the main ways by which humans are exposed to Aluminum. The penetration of chemicals through living intact skin is an important route of exposure in toxicological and pharmacological studies. Since no attention has previously been given to the possibility that Aluminum accumulation in the testes via transdermal exposure could lead to reproductive toxicity; it was interesting to investigate the reproductive toxicity of $AlCl_3$ in adult male mice through transdermal route. Furthermore, the effect of oral green tea extract (GTE) on $AlCl_3$ testicular mediated toxicity was not examined in previous studies. Therefore, it was also interesting to

investigate the protective effects of GTE against $AlCl_3$ induced histopathological changes in testes tissue of mice.

Reproductive and histopathological studies were performed on the testes of adult male mice after dermal application of $AlCl_3$ solution (at a concentration of 0.5 and 1.0 mg) or deodorant (20 μL) over a period of 7 weeks. The effect of oral administration of antioxidant green tea extract on $AlCl_3$ testicular mediated toxicity was also examined. The results of this study showed that there was statistically significant decline in sperm count and serum testosterone levels of the Aluminum Chloride treated groups when compared with control group and the observed decrease was statistically significant ($P < 0.05$). $AlCl_3$ treatment led to impaired sperm parameters (sperm count and abnormal sperm morphology), this is in agreement with previous studies [3, 16, 23, 24, 25, 26]. But with the difference that they have conducted their experiments on rats and they used the route of administration through oral intubation, but the present study used transdermal route by rubbing. The reduced sperm count observed in Aluminum treated groups could be associated with reduced gonadotropins and testosterone, since these hormones are essential for spermatogenesis [16] or it could be associated with the possible mechanism of how Aluminum induces reduced sperm count may be due to the possible interference of Aluminum with maturation and storage of sperm in the epididymis or an interference with the production of sperm in the testes [16].

There was a significant increase in the percentage of sperm morphological abnormality in Aluminum treated groups as compared with the control groups, this agrees with previous study [23, 25] these changes may be attributed to impairment of sperm maturation and secretory functions of epididymal cells which might be due to oxidative stress or to insufficiency of androgen [23, 25].

Reduced sperm count and abnormal sperm morphology indicate that there must be a certain interruption in the process of spermatogenesis as it represents the results of all the stages of meiosis. Testicular tissues are vulnerable to oxidative injury [14] and defective sperm function is associated with an increase in lipid peroxidation derived free radicals and impaired antioxidant defense [27]. The histological lesions emphasize the positive correlation between cytogenetic damage and abnormal sperm parameters. Each time interval post treatment represents sperms treated at a different stage of spermatogenesis: week 1 post treatment represents spermatozoa, week 3 post treatment repersents spermatids, week 5 post treatment repersents sperms that were treated at pre leptotene and late spermatogonia, week 7 post treatment repersents sperms treated at spermatogonia. The highest observed damage in all treated groups was found in week 1 (spermarozoa) and week 5 (pre leptotene) and week 7 (spermatogonia). Therefore, there is an agreement with low sperm count seen in weeks 1, 5, and 7 after transdermal application of $AlCl_3$. Furthermore, drinking green tea extract did not antagonize the harmful effects of $AlCl_3$. In fact it has elevated $AlCl_3$ reproductive toxicity. This result was also reported by Vieira et al [28] who founded that green tea extract does not improve the spermatic parameters of Wistar rats submitted to testicular heat shock. Chandra et al [29] have reported that GTE at relatively high dose may cause impairment of both morphological and normal functional status of testis in rodents and thus green tea consumption at relatively high doses raises concern on male reproductive function in spite of its other beneficial effects. The number of normal sperm count is a measure of male fertility. Reduced sperm count might reduce the chance of sucssessful fertilization; since a low sperm count in humans is correlated with a decrease in fertility [16].

Light microscopic examination of histological sections of the AlCl3 treated groups showed disorganization of the seminiferous tubules and degeneration of spermatogenic cells, few spermatozoa in the lumen, large interstitial spaces and decreased number of Leydig cells around basement membranes. These results are in agreement with those reported by Moselhy et al [30] who exposed rats to a daily dose of 34 mg/kg of AlCl3 for 60 days, histopathological examination of rat testis revealed degenerative changes in seminiferous tubules with necrosed spermatogenic cells.

Conclusion

The results of the present study showed that aluminum chloride ($AlCl_3$) caused significant testicular damage, decline in sperm count and testosterone levels, and an increase in morphological sperm abnormalities. Therefore, prolonged transdermal exposure to Aluminum Chloride may induce reproductive failure and could be a cause of male infertility. While, the oral administration of natural antioxidant green tea extract did not play a protective role as an antioxidant, but it increased the reduction in sperm count and abnormal sperm morphology induced by $AlCl_3$. Consequently, drinking relatively large amounts of green tea could be harmful to human males and might be a cause of infertility. Further studies are needed to increase our knowledge about the toxic effects of Aluminum on male fertility and a need to decrease or prevent the use of Aluminum on such a large scale as it exists now days.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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