Study of gender inequality in aluminium - induced alterations in lipid profiles and hepatic functions

THESIS

Submitted to

All India Institute of Medical Sciences, Jodhpur

In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE (M.Sc.)

PHYSIOLOGY

JUNE, 2023

AIIMS, JODHPUR

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Certificate

This is to certify that this thesis titled "Study of gender inequality in aluminium - induced alterations in lipid profiles and hepatic functions" is an original work of Ms. Priyanka Tanwar carried out under our direct supervision and guidance at Department of Physiology, All India Institute of Medical Sciences, Jodhpur.

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DECLARATION

I, hereby declare that the work reported in the thesis titled "Study of gender inequality in aluminium - induced alterations in lipid profiles and hepatic functions" embodies the result of original research work carried out by me in the Department of Physiology, All India Institute of Medical Sciences, Jodhpur.

I further state that no part of the thesis has been submitted either in part or in full for any other degree of All India Institute of Medical Sciences or any other institution/University.

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Acknowledgement

The success and final outcome of this dissertation work required a lot of guidance and assistance from many people and I am extremely fortunate to have got this all along the completion of my dissertation work. Whatever I have done is only due to such guidance and assistance and I would not forget to thank them.

First and foremost, I offer my sincere thanks to the almighty God for blessing me with the ability, skill and knowledge to carry out this research work. There is no word to express my humble gratitude towards the supreme power for the strength showered on me. The support of the almighty throughout my avenue enabled me to accomplish it effectively.

I owe deep sense of gratitude and sincere thanks to my guide, my advisor, my mentor Dr. Prasunpriya Nayak, Professor, Department of Physiology, AIIMS Jodhpur, who provided me an opportunity to work under his guidance. With his experience, management skill and knowledge, the direction throughout was a great help to complete this research. His encouragement and pertinent guidance along with exquisite and in-depth knowledge and mastery over the subject has been my constant source of inspiration. His pursuit to the science at the conceptualization and timely midterm corrections as-and-when required is amounted to the smooth completion of the project. I shall remain grateful to him forever.

I would like to express my profound gratitude to my co-guide, great advisor and truly wonderful person Dr. Abhinav Dixit, Professor and Head, Department of Physiology, AIIMS Jodhpur for his invaluable support, encouragement, guidance and help throughout my work. His vast knowledge, experience and suggestions have been a boon for completion of my study.

I would like to express my deep thanks to my co-guide Dr. Mithu Banerjee, Additional Professor and Head, Department of Biochemistry, AIIMS Jodhpur for her kind support and valuable time for my work. Her guidance and support in estimation of biochemical parameters and understanding their relevance helped me complete this aspect of the project.

I am extremely thankful to my co-guide Dr. Shival Srivastav, Associate Professor, Department of Physiology, AIIMS Jodhpur who played a pivotal role in this work with their unerring accuracy and ability to solve the problem. He always motivated and encouraged me beyond my capabilities and had faith in me throughout this journey. Also thanks for his continuous support, patient, enthusiasm and immense knowledge. I am greatly in debt to his

for devoting valuable time out of his busy schedule and being concerned for completion of this project. I could not have imagined a better advisor and mentor for my whole post-graduation.

I owe a great debt of gratitude to my co-guide, great adviser Dr. Hare Krishna, Associate Professor, Department of Anatomy, AIIMS Jodhpur for providing me with the opportunity to carry out study at their department. Words were not sufficient to express my thanks for constant support, encouragement, guidance and help throughout my work. I am lucky to find mentor like him.

I express my sincere gratitude to my faculty members Dr. Bharti Mehta, Professor, Dr. Om Lata Bhagat, Additional Professor, Dr. Pooja Ojha, Associate Professor and Dr. Mahesh Gadhvi, Assistant Professor, Department of Physiology, AIIMS Jodhpur for their invaluable advice, guidance, inspiration and encouragement from time to time.

I am highly grateful to all the senior residents Dr. Anant Kumar, Dr. Suresh Ravichandran, Dr. Trina Sengupta, Dr. Annu Ankita, Dr. Neelesh Gehlot, Dr. Anshika Baranwal, Dr. Jagdish Seoran, Dr. Sadhna Sharma and my seniors Dr. Dhirendra Tak, Dr. Mamta Shobhawat, Ms. Tripti Sharma, Ms. Deepshikha and Dr. Annie S Jose for their support, cooperation and help in successive stages of this research work.

I would like to thanks my friends and co-PG Dr. Ashwath Reddy, Dr. Sreeparvathy, Dr. V. Vani, Dr. Ritu Kumari with whom I worked closely. I am highly grateful for their support.

I am thankful to the Director, AIIMS, Jodhpur for thesis grant and permitting me to avail all the facilities available in the institute for conducting this study.

I am thankful to all the other staff of Department of Physiology, Mr. Shiv Kumar, Mr. Amit Kumar, Mr. Ajay Kumar, Mr. Martin Silvester, Mr. Gaje Singh Panwar, Mr. Surendra Kumar and Mr. Babu Ram for providing me the support whenever needed.

I would also like to acknowledge with much appreciation the crucial role of the IIT Jodhpur, giving a permission to use all the necessary tools in the laboratory.

Any amount of gratitude shall be less for the contribution of my parents, Mr. Mahesh Kumar Tanwar and Mrs. Meena Devi and my elder sister Mrs. Priti Gahlot and elder brother Mr. Ajay Tanwar for their support, love and blessings are the reason of my achievements.

Thank you one and all.

Ms. Priyanka Tanwar

Dedicated to

my family

and

Department of Physiology

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LIST OF ABBREVIATIONS

AC = Atherogenic coefficient.

AIP = Atherogenic index of plasma.

ALP = Alkaline phosphatase.

ALT = Alanine aminotransferase.

AST = Aspartate aminotransferase.

CRI-1 = Castelli risk index 1.

CRI-2 = Castelli risk index 2.

FAO/WHO = The Food and Agriculture Organization / World Health Organization.

HDL = High-density lipoprotein-cholesterol.

i.p. = Intraperitoneal.

ICMR-INDIAB = Indian Council of Medical Research–India Diabetes.

KW = Kruskal-Wallis test.

LCI = Lipoprotein combine index

LD = Lethal dose.

LDL = Low-density lipoprotein-cholesterol.

MW = Mann-Whitney U test.

nonHDL = non High-density lipoprotein-cholesterol

TC = Total cholesterol.

TG = Triglyceride.

VLDL = Very low-density lipoprotein-cholesterol.

INTRODUCTION

Gender inequality can significantly influence the treatment outcome (1). Sexual dimorphism was also reported in xenobiotic like aluminium metabolism because of dimorphous expression of some specific genes (2). Higher susceptibilities in males for liver pathologies was reported essentially when it faced challenges (3,4). In addition, liver of male and female animals were reported to express the receptors for estrogen as well as androgens (3). Liver being the major organ involved in the process of xenobiotic processing and it being a hormone sensitive organ, gender-dimorphism in its response towards aluminium handling and expressing the impact of aluminium toxicity is possible. Differential responses of xenobiotics with male and female hormones as well as their receptors were already suggested (5). Aluminium, a possible causative factor for dyslipidemia (9), is a metalloestrogen (6,7) whose effects could be dependent on gender and age (8).

Atherosclerotic cardiovascular diseases top the list of overall death worldwide (10) and India (11). Especially in younger population, Indians share nearly one-fifth of these deaths (11); while, in 2016, those accounted for more than one-fourth of total reported death in India (12). Among the 40-69 years age group, contribution of cardiovascular diseases on the total reported death was as high as 45% (12). Early age of onset of cardiovascular diseases in Indians, apart from fast progression and high mortality rate, was highlighted recently as point of concern (11,13). While listing the risk factors, hypertension, smoking, hypercholesterolemia, diabetes, sedentary lifestyle, obesity, family history and ethnic background were given primary importance (14). Other advised risk factors are progressive increase in the risk of cardiovascular diseases as individuals age over 50 years and higher risk of early onset in men (14). Interestingly, these orthodox risk issues were not enough to justify the reported higher rate of coronary artery disease in Indians (11). Dyslipidemia is a known and significant risk factor for cardiovascular diseases.

About dyslipidemia

Lipids [Fatty acids, Cholesterols, Triglycerides, Phospholipids], lipoproteins [Chylomicrons, Very low-density lipoproteins, Intermediate-density lipoproteins, Low-density lipoproteins, High-density lipoproteins, Lipoprotein-a] and apolipoproteins [Apo B structure, Apo-a homology to plasminogen] are present in plasma. Levels of representatives of plasma lipids are used as clinical predictors for numbers of diseases. The physiological range for these plasma lipid levels are well established. Deviations of plasma lipid levels from the accepted

standard range are clinically termed as dyslipidemia. Dyslipidemia and associated health problems are highly grievous and need serious attention. Though both hyperlipidemia and hypolipidemia are included in the dyslipidemic conditions (15), hyperlipidemia is more common. Hyperlipidemia or commonly dyslipidemia is a disorder of abnormal metabolism of lipoprotein which include increments in total cholesterol [TC], very low-density lipoprotein-cholesterol [VLDL], low-density lipoprotein-cholesterol [LDL] and triglyceride [TG], along with decrement of high-density lipoprotein-cholesterol [HDL] in plasma (16).

Of the total population of India, 15-30% are suffering from dyslipidemia with a higher percentage in urban population (17). Of late, dyslipidemia becomes more prevalent in younger adults, aged between 18-24 years (18). Similar observation is also supported in the National Health Portal, India. Higher prevalence of dyslipidemia is seen in the 30-40 years age bracket; nevertheless, the prevalence is very high in above 60 years age stratum (17). The ICMR-INDIAB study also indicated presence of dyslipidemia in young adults as alarming (19). Dyslipidemia in early adulthood is likely to increase the risk of atherosclerotic cardiovascular diseases by exposing individuals to cumulative high levels of atherogenic lipoproteins with aging (10).

Gender inequality in dyslipidemia

As per National Health Portal of India, the females are relatively less likely to have dyslipidemia; however, not excluded completely (17). This is in contrary to the earlier observation that female gender was positively associated with low HDL and females possessed significantly more lipid abnormalities compared to males (19). On the other hand, no significant association was found between gender and dyslipidemia in south Indian adults (20). In a recent study with Indian expatriates in Qatar demonstrated higher prevalence of atherogenic dyslipidemia in men than women (21). Higher prevalence of dyslipidemia in South Asian urban women were also reported recently (22).

For women, estrogen is a cardioprotective hormone. After menopause, level of estrogen decreases associated with high prevalence of dyslipidemia (23), this might make women at high risk of coronary heart disease (24). Thus, with menopause the risk of dyslipidemia and cardiovascular disease increases to a great extent (25,26). Even with variations with types, dyslipidemia is common in post-menopausal women (27) and the level of estrogen is an influencing factor for the lipid profile disturbances (28). In addition, reports of lower HDL and higher TG in men suggested the gender inequality (29,30).

Estrogen plays multitude of roles including reproductive as well as non-reproductive in both women and men (31). Apart from reproductive functions, functions of estrogen in different organs like liver, heart, brain, muscle, etc. are evident (32). It has been reviewed that excess estrogen after menopause increase the risk of cancers in female reproductive organs (33). On the other hand, low levels of estrogen function in postmenopausal women affects the non-reproductive organs and increase the risk of diseases like osteoporosis, coronary heart disease, Alzheimer's disease, etc. (33).

Estrogen can alter serum lipid profile along with other systemic effects (34) directly or indirectly influence the cardiovascular system functioning through both genomic and nongenomic pathways (35). While estrogen administration can correct the dyslipidemic alterations seen in postmenopausal women, the effects were reported to be mediated by its influence on the hepatic expression (34). Interestingly, these atheroprotective activities of estrogen could experimentally be demonstrated in normolipidemic and hypercholesterolemic animals as well as in castrated male animals and can be blocked by high dose of progesterone (34). These evidences suggest gender inequality in dyslipidemia with possible protective roles of estrogen mediated by estrogen receptor causing some impact on the hepatic functions.

Similarly, estrogen therapy was beneficial to correct the disorders in liver function and lipid profile in patients with aromatase deficiency (36). In addition, genes for LDL receptor is promoted by estrogen-estrogen receptor (32). Thus, estrogen plays a significant role in lipid metabolism both in males (31) and females (33). However, estrogen's antidyslipidemic property is suggested by the observation that the females of reproductive age group have lower LDL and higher HDL in comparison to their male counterparts (37).

Aluminium toxicity and gender issue

In the periodic table, aluminium is present as a Group IIIA element. It is a highly reactive metal and quite abundant in the earth's crust (38) and environment. Despite its abundance, it was believed to be unavailable to the biological world and even harmless (39). It also possesses some unique physico-chemical properties. Thus, the metal is not used in biological reactions (38,40) and the metal is essentially not required for humans or any other biological systems. In fact, its presence in the system produces toxic effects (38,39,41). Because of this so-called 'inertness' of aluminium towards the biological system, use of aluminium was thought to be safe.

Aluminium is a well-known neurotoxicant (42) and along with other toxicities, reproductive toxicity of aluminium in both male (43) and female (44) reproductive organs are also documented (45). In an interesting study with equivalent aluminium exposure to rats, the reproductive damages were more pronounce in male rats compared to that of female rats (2). Generalizing the concept of gender dimorphism in terms of reproductive toxicity of rats little further, compared to female, higher susceptibility of male rodents was suggested (45). Gender bias was also reported in aluminium-induced changes in neurotransmitters, ion channels, neurobehaviors as well as small molecule metabolism through a study in mice with Al_2O_3 nanoparticles (46). Towards evaluation of gender differences in the metal deposition and their toxicity, cadmium, nickel, lead, mercury, arsenic were reported to have gender-biasness in terms of deposition and reported toxicities; however, the route of exposure and time of insult can modify the extent and impact of the toxicity (47). In this line, possibility of gender-specific difference was suggested in the detoxification systems too (2). This led to suggestion of gender-dependence on the systemic handling of aluminium by the liver. Accordingly, gender susceptibility in aluminium toxicity was suggested (48).

Aluminium as metalloestrogen

Metalloestrogens are identified as a separate group of xenoestrogens in which metals in their ionic form could interfere with the estrogenic functions (49,50). Metalloestrogens includes cadmium, chromium, copper, cobalt, lead, nickel, mercury, tin, vanadate and aluminium (50). Considering the ease of exposure and non-availability of systemic removal process (51), aluminium could be a substantial threat to female health by interfering the estrogen functions

Interestingly, the influence of exogenous estrogen suggested on the lipid status and metabolism (30); therefore, there is possibility that the exposure to metalloestrogens could also influence the lipid profile. Industrialization and modernization are adding a significant number of pollutants in our life. This condition has led to a dreadful impression on human health (52). Aluminium, a metalloestrogen, is the most widely distributed metal naturally present in the Earth's crust [approximately 8%]. Besides air and water, this metal is also present in several eatables and commercial products like cookware, food storage materials and even in medicinal products (53). So aluminium exposure cannot be avoided. It can interfere with the estrogen function and can lead to dyslipidemia. However, the possibility of dyslipidemia from liver dysfunction should also be ruled out. Thus, the current proposal is planned to evaluate the influence of metalloestrogen like aluminium on the serum lipid profile; while, levels of serum

estrogen and hepatic lipids also be evaluated along with morphological and functional changes in liver, if any.

Earliest evidence of aluminium-induced reproductive toxicity is reported with reduced fertility with microscopic and macroscopic degeneration in ovaries of mice exposed to aluminium through bread with an approximate daily exposure of 85-240 μg/kg (45,54). Similarly, decreased ovarian enzyme activity was noted with reduced serum estradiol level in mice exposed to aluminium (55).

These multiple factors suggest that gender inequality could be possible in handling of aluminium burden in the body as well as aluminium-associated dyslipidemia in young adults.

REVIEW OF LITERATURE

Aluminium is a commonly disseminated metal naturally existing in the earth's crust about 8%. Aluminium is hardly found in its native form, it is distributed as bauxite ore (39). Till date, more than one billion tonnes of aluminium are produced for commercial use (56). Because of recyclability, approximately 75% of total usable aluminium is continuously being reclaimed and still in use. Therefore, nearly 25 million tonnes of aluminium are added into the environment by the process of mining, processing, and consumption. Interestingly, this recycled aluminium can meet only one-third of the global demand of aluminium (56). Thus, aluminium production continues to meet the remaining global demand. Eventually, 25% of this newly marketed aluminium will be adding up the environmental load of aluminium. Yearly consumption of aluminium has already increased staggeringly, from nearly 1.5 million tonnes in 1945 to 45 tonnes in 2013 (57). The increment is likely to continue and may cross 100 million tonnes in 2050 (57). With more and more uses of aluminium in modern life, this vicious cycle is likely to continue.

Exposure to aluminium

Even in the absence of its role in the life process, aluminium possesses significant biological interactions in its biogeochemical cycle. With anthropological activities and growing urbanisation, more and more aluminium are added to the environment. Other environmental pollutants (like acidic or alkaline fertilisers, acid rain) also enhance the availability of aluminium to the biosphere inclusive of terrestrial and aquatic (39). Many plants and aquatic small animals can face the toxic impacts of aluminium and indirectly increase the chance and repeated exposure to aluminium in humans (58). However, considering the bioavailability of aluminium, provisional tolerable weekly intake was set by FAO/WHO Expert Committee on Food Additives as 2 mg/kg of body weight /week (59) which is double the level set by European Food Safety Authority (60) in the year 2011.

As aluminium is naturally present in the soil, even the fresh cereals, fruits, vegetables, and natural water could be contaminated with it (61). Some edible parts of plants can act as aluminium accumulator (48,62). There are different types sources for aluminium.

Food and food ingredients: Naturally grown rice, cereals as well as fruits, legumes, yellow and green vegetables always contain some amount of aluminium in them (48,63). However, if grown in aluminium-rich soil, the level of aluminium increases. Processed food may be contaminated with extra aluminium during the processing in addition to their original aluminium content – it may happen with oil and fat (64), sweets, dairy products, processed

cheese, salt, herbs, spices, seasoned foods, meat, seafoods including fish and shellfish (65), pound cake, pastries, biscuits, dark chocolates, confections (41,61,66,67).

Food processing: Unintended addition of aluminium to the processed food also can come from cooking utensils (41,58,61) as well as during serving.

Food additives: Increase in aluminium content in the food materials or food servings can also be there with the deliberate addition of aluminium compounds as preservatives or as anticaking agent (58,61).

Food storage: Sometimes processed food are stored by wrapping in commercially available aluminium foils. Depending on the chemical and physical nature of the food being stored and duration of storage, some amount of aluminium may be leached from these wrappers or containers and increase the content of aluminium in the stored food (41). The fact could be evidenced by higher level of aluminium in processed-stored-marketed milk in comparison to the raw milk at the source (68).

Water processing: As such, drinking water contributes relatively less amount of daily consumption of aluminium (69). However, if the drinking water is processed with special formulation or otherwise, the level of aluminium in the processed water may go abnormally high (67). Similarly, the uses of aluminium salts have been reported for sewage water treatment, water recycling and water purification (38,61,64).

Beverages: Apart from regular food, beverages also contribute significantly in the aluminium exposure burden, tea is a common customary drink consumed by many. In addition, coffee, soups, packaged drinks, soft drinks, wines, beer, distilled spirits also contribute, though not important (61,66).

Personal hygiene products: Major personal hygiene products like toothpaste, deodorants, antiperspirants, sun protection lotions, cosmetics are also rich in aluminium salts (41,58,67), because of some special properties of these salts which are utilized for specific purposes.

Medicaments: A large numbers of chemicals to be used in the form of medicine contains aluminium in them. Antacids, phosphate binders, buffered aspirins, astringents, vaccines, and allergen injections (38,41,48,58,67), dental crowns and dentures (39), dietary supplements (66) are few of them which are used for medical purposes.

Industrial uses: Construction industry (cement), automotive industry, aviation industry and electrical industry (39). Aluminium is also used as fuel additives, solid fuel rocket propellant, for manufacture of explosives and fireworks (39,58).

Substance abuse: Though not in common uses, the abusive substances also can be good source of aluminium exposure. Thus, glue sniffing (70), tobacco, marijuana, cocaine, heroin (66) can contribute to overall body aluminium burden, to some extent, in individuals consuming those regularly.

Occupational exposure: In some closed-door working setup a complex condition could arise where employees face exposure to aluminium through inhalable, thoracic and respirable particulates. Even after 95% urinary elimination, this aluminium could be crucial source of body aluminium burden depending on the level and duration of exposure. In some cases, as mining (bauxite), refining or welding, workers may face similar or more challenge of such exposures even though they are working in open air (66,71).

As it appears, common possible exposure to aluminium are through oral, nasal and dermal routes; oral being the dominant one (41,70). Overall, the unintentional exposure to aluminium may be as high as 110 mg per day (39).

This omnipresence of aluminium indicates that the exposure to aluminium is unavoidable; however, the level of exposure depends on habitat, food and personal habits, occupation, medication (if any).

Internalisation of aluminium

Even though aluminium is a dispensable element, on exposure, some amount of aluminium is collected and retained by the body. Both active and passive transport of aluminium across the small intestinal wall have been suggested. Likewise, aluminium can access either transcellular or paracellular pathways for internalisation (72). Therefore, multitudes of factors are likely to influence the absorption of aluminium through the gut. While, short chain carboxylic acids, including citric acid, favours the passive transport of aluminium, phosphates oppose the same. On the other hand, transferrin/1,25 DHCC mediated active transport is stimulated by parathyroid hormone (73,74), whereas Fe^{3+} can cause a competitive hindrance to the same (66). Interestingly, similar competition is not observed in serum (75), even though most of the serum aluminium is bound to transferrin (76). Nonetheless, ionised aluminium in soluble Al^{3+} form is the responsible one for the observed tissue damages and toxicity (66).

The total body aluminium burden ranges between 50-150 mg (39). Only a minute amount (<1%) of oral exposure is reported to be absorbed, while the majority [nearly 99 %] of the absorbed amount is excreted through urine (57,77). Upon acute exposure, aluminium remains in the human body only for a short while with $t_{1/2}$ of 8 hours (71). Therefore, the body could handle the acute aluminium insult quite efficiently. However, the efficiency of managing aluminium depends on the load of acute exposure. Single dose of 50 mg aluminium when given intraperitoneally, morphological changes in liver was observed including Glisson's capsule and subcapsular area and particularly in portal area and acini (71).

The process of body aluminium handling in case of chronic exposure is unusually different. With continuous exposure, aluminium slowly starts piling up in different organs.

Aluminium in the system

Even in absence of specific transport system, how aluminium enters the cells and system is still debatable. It is thus the opportunistic approach for the aluminium to gain access inside the cells through the carrier protein meant for other trivalent ions namely Fe^{3+}. With the same principle, Fe^{3+} binding capacity of transferrin is utilized by Al^{3+} for binding and transportation (78). As nearly 90% of the plasma aluminium is bound to transferrin (79), accessibility of aluminium to organs which are otherwise protected also increases via transferrin mediated transports.

Nearly, 60% of the body aluminium is present in bones with approximately 25% in the lungs and 10% in the muscles (79). Along with others, the liver is a target organ for aluminium accumulation in the body (41). However, the level of aluminium accumulation in the liver is relatively less and below the level of the bones and lungs. Considerable accumulation of aluminium in hepatocytes, particularly in lysosomes, has been reported (71). The body burden of aluminium is also shared by brain, kidneys, spleen, testes, heart, lymph nodes, lungs, intestine, and thyroid (38,66,71,80). The liver accounts to only 3%, while that is 1% for brain, of the total aluminium load of the body (79); however, the neurotoxicity and hepatotoxicity are studied maximally for the insults of aluminium.

Lack of specific transport system for aluminium rendered benefit in terms of minimum assimilation; nevertheless, the same mechanism provided some disadvantage too. By some means, once entered the cellular system, there is no mechanism to remove aluminium out of the system. On the other hand, the 'biological inertness' also could be a cause of cells not bothering to have specific remotion mechanism(s) for aluminium. Therefore, of the total

aluminium exposure, only a fraction gets systemized while most of it remain in the gastrointestinal tract as non-systemic aluminium and eliminated along with the fecal matters (72).

Natural removal of aluminium from system

The systemic aluminium can be removed from the body by urine, sweat, skin, hair, nails, sebum, and semen (72). More than 90% of systemized aluminium should be excreted through kidney function in a healthy individual (79), provided the exposure level of aluminium remains within normal limit. Alternative routes of removal of aluminium include perspiration (72) and biliary secretion (79). Overall, these alternative routes might be contributing in the removal of not more than 5% of systemic aluminium and they cannot compensate the urinary excretion of aluminium in case of compromised kidney function.

Though the major part of systemic aluminium is naturally removed, the time taken for this elimination process is variable and dependent on many other factors apart from efficiency of the removal system itself.

Aluminium hepatotoxicity

Even with natural bypassing and protection against aluminium, the exposure does occur. Many toxic effects of aluminium have already been suggested (51,62). Most of the studies are experimental in different animal species. Toxic impacts of aluminium on human are also reported (72) with noted health hazards (81) including neurodegenerative changes (42), skeletal diseases (82). Though much is not talked about hepatotoxicity in earlier reports, of late, hepatotoxicity caused by aluminium is receiving much attention. Liver is now considered as an important target organ of aluminium toxicity (83); however, not much was explored till now (84). Recently, hepatotoxicity by aluminium is gaining attention and being reported (41,85). Nevertheless, earlier it was perceived as unimportant (71).

Alterations in hepatic functions including cholestasis, impairments in organic ion transport, impaired synthesis of essential proteins, xenobiotics, etc. in response to aluminium exposure have already been reported (71).

Effect of aluminium on lipid metabolism

These reports indicate that metalloestrogens, like aluminium, may disrupt the lipid metabolism and can cause dyslipidemia in the exposed individuals. Reports about the effects

of aluminium exposure on serum lipid profile in rat are inconclusive. An acute exposure [14 days] to 38mg/kg body weight of $AlCl_3$ demonstrated significant decrease in serum TC and LDL levels (86). Corroborating these, Chary *et al*. found significant decreases in the levels of TC, HDL, VLDL and TG in rats exposed with 50 mg and 100 mg $AlCl_3$ per kg body weight (87). On the other hand, an exposure of $Al_2(SO_4)_3$ through drinking water for 60 days demonstrated significant increases in all the studied lipid profile parameters (53). Similar changes in LDL, TC and TG were observed with similar treatment of $AlCl_3$ (88). Single dose of $AlCl_3$ also caused increase in TC, LDL and TG in Wistar rats (89).

There are limited human studies showing direct relation between aluminium exposure and serum lipid profile. Workers occupationally exposed to aluminium demonstrated higher level of TC, LDL and TG with lower level of HDL in serum; however, the alterations were not significantly co related with serum aluminium level (90).

Lacunae found from Review of the Literature

- While it is well understood that chronic exposure to aluminium is toxic, little is known about the hepatotoxicity in case of short-term exposure to aluminium.
- Studies supported that aluminium exposure could be associated with dyslipidemia. Whether the hepatotoxicity is associated with dyslipidemia or not is yet defined.
- If hepatotoxicity and dyslipidemia could be there in young adults, aluminium can be ascribed one of the possible causes of dyslipidemia in young adults, as it was seen in recent reports. Possible role of aluminium in the growing dyslipidemia in young adults are not evaluated.
- Based on the antiatherogenic property of estrogen, there are possibility that young adult female may response differently in terms of dyslipidemia and/or hepatotoxicity, particularly when aluminium is a known metalloestrogen. There is no detailed study about the gender inequality in terms of aluminium-induced hepatotoxicity and dyslipidemia.

Accordingly, the **research question** of the study was formed as –

Is there any difference in aluminium-induced dyslipidemia between young adult male and female?

Thus, in this study, it was **hypothesised** that

Aluminium induces dyslipidemic differences among young adult male and female rats.

AIM

&

OBJECTIVES

Aim:

To evaluate the differences in aluminium-induced dyslipidemia and hepatic functions in young adult male rats and young adult female rats.

Objectives:

1. To compare the levels of TC, TG, HDL, LDL in serum and liver of aluminium-exposed and aluminium non-exposed young adult male and young adult female rats.
2. To compare the levels of calculated atherogenic indices between aluminium-exposed and aluminium non-exposed young adult male and young adult female rats.
3. To compare the status of liver by carrying out liver function tests and histology of aluminium-exposed and aluminium non-exposed young adult male and young adult female rats.

MATERIALS

&

METHODS

The present experimental protocol was adopted for the purpose of conducting the study of gender inequality in aluminium induced alterations in lipid profile and hepatic functions. The experiment was conducted on young adult male and female Wistar rats (*Rattus norvegicus*).

Animal maintenance

Calculation of sample size –

For the current study, two groups of animals were required – (a) without aluminium exposure group that served as control arm for the study, and (b) with aluminium exposure that served as experimental arm for the study. As gender inequality was also part of the hypothesis, there were two subgroups – male and female animals for each group mentioned above.

Considering the maximum number of animals required for the study, degree of freedom was taken as 20 and the total number of animals required for the study was calculated on the basis 'Resource Equation' approach.

n = (20/4) +1 = 6

Therefore, 6 animals were required in each subgroup. Having 4 subgroups in the study, the total number of rats required for the study was found to be 24.

Resource equation:

$$n = \frac{df}{k} + 1$$

Where,
n = number of animals per group,
df = degrees of freedom,
k = number of groups.

Animal maintenance and aluminium exposure –

For the present study, procured rats were housed in Central Animal Facility of All India Institute of Medical Sciences (AIIMS), Jodhpur. Twelve young adults male Wistar rats (Age: 3 months, Weight: 130-150g) and 12 young adults female Wistar rats (Age: 3 months, Weight: 130-150g) were used to perform the experiment. The current experimental protocol is approved by the Institutional Animal Ethics Committee (IAEC) of All India Institute of Medical Sciences, Jodhpur.

After procuring, the male and female animals were divided into two subgroups with the help of 'Random Allocation Software' (Version 1.0, May 2004) having six animals in each. Those six animals were caged together for rest of the period. After acclimatization of 10 days, the animals were divided as follows:

Male Al-0: Young adult male group exposed to intraperitoneal injection of vehicle solution (1% gum acacia) for 15 days.

Male Al-5: Young adult male group exposed to intraperitoneal injection of aluminium (5 mg Al /kg body weight) solution dissolved in vehicle for 15 days.

Female Al-0: Young adult female group exposed to intraperitoneal injection of vehicle solution (1% gum acacia) for 15 days.

Female Al-5: Young adult female group exposed to intraperitoneal injection of aluminium (5 mg Al /kg body weight) solution dissolved in vehicle for 15 days.

This dose of Al was chosen in accordance with the previous studies.

Throughout the procedure including acclimatization, rats were maintained in a well-ventilated room having 25 ± 2 °C temperature and 65 ± 5% humidity. The animals were maintained with 12:12 light-dark cycle.

A layer of husk was spread on the floor of the cages. Food and water were provided *ad libitum*; however, amount of leftover food and water were measured to approximate cage-wise daily consumption of food and water. They were fed on standard rat chow. The water bottles were regularly cleaned. The husk spread on the cage floors were cleaned every alternate day.

Animal sacrifice and blood collection –

After the completion of the 15 days of intraperitoneal exposure to the animals, the animals were sacrificed by cervical dislocation. Blood samples collected by cardiac puncture and allowed to coagulate in the room temperature. Sera collected were preserved in -40ºC till processing.

Tissue collection and processing –

Whole liver from each animal was collected immediately, washed with cold saline, bloated dry and weighed to note the total liver weight. Part of each liver sample was immediately fixed with 10% formalin and rest of the liver sample was immediately preserved in -40ºC till processing.

Approximately 0.5g liver tissue was homogenized in 2 mL of phosphate buffer saline using ultrasonic cell crusher system [Probe ϕ2 with ultrasonic power of 150W] by Intelligent Ultrasonic Processor [Ningbo Sjialab Equipment Co. Ltd. / Unigenetics Instruments Pvt. Ltd.] using Process time – 5 minutes, Pulse on – 10 seconds, Pulse off – 5 seconds, Power rate – 10%. The homogenized solution was centrifuged in 4ºC for 10 minutes at 15000 rpm (Remi C24plus). The supernatant was separated and used for biochemical estimations (91–93).

Estimation of biochemical parameters –

Levels of triglycerides, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total protein, albumin, globulin, and total conjugated bilirubin, activities of alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase of serum were estimated by autoanalyzer [Beckman Coulter, Model AU680] in the Department of Biochemistry, AIIMS, Jodhpur. Levels of triglycerides, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total protein, and total conjugated bilirubin, activities of alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase of hepatic tissue supernatant were also estimated.

Albumin-globulin ratio in serum, NonHDL, Atherogenic coefficient, Castelli Risk Index 1, Castelli Risk Index 2, Atherogenic Index of Plasma, Lipoprotein Combine Index were calculated from the above-mentioned biochemical parameters.

- NonHDL = TC-HDL (94)
- Atherogenic coefficient = NonHDL/HDL (95)
- Castelli risk index 1 = TC/HDL (95,96)
- Castelli risk index 2 = LDL/HDL (95,96)
- Atherogenic index of plasma = log[TG/HDL] (96,97)
- Lipoprotein combine index = [TC×TG×LDL]/HDL (94)

Tissue processing and histological slide preparation –

For histological study, the liver tissues were processed and slides were prepared following the standard methods described by Drury and Wallington (98). Small pieces of formalin-fixed tissues were carefully cut and placed in individual tissue processing cassettes and kept under running tap water for 3 hours, followed by washing in distilled water for 30 minutes. Tissue samples were then placed in 50% alcohol for 1 hour and kept for overnight in 70% alcohol. Next morning, dehydration of tissue samples was continued by placing in 90% alcohol for 1 hour followed by 100% alcohol for 1 hour twice. Then the clearing of tissue samples was done by placing twice in xylene for 15 minutes each. Before block preparation, samples were also processed sequentially in xylene-wax [50%-50%] mixture, wax 1 and wax 2 for 1½ hour each. After processing, tissues were embedded in paraffin blocks and preserved.

These blocks were used for sectioning. Sections of 5 μm thickness were cut on a microtome [Spencers, Model No. 1010-SMT-005] and placed on glass slides. Deparaffinization of these sections were initiated by heating those up to 60ºC on a regulated hotplate. Deparaffinization were continued by placing the slides sequentially in xylene-1, and xylene-2 for about 3 minutes each. Then the sections were placed into 100% alcohol, 90% alcohol, 70% alcohol and 50% alcohol for 2-3 minutes each. Then, the sections were washed with distilled water and dipped in hematoxylin solution for 10-15 minutes. After taking out from the hematoxylin solution, slides were placed in distilled water for 3 minutes followed by 2 dips in 1% acid alcohol solution for differentiation and then placed in running tap water [approximately for 5 minutes or as required]. The slides were placed again in tap water for 3 minutes after a single dip in 1% Eosin preparation. Then the slides are placed sequentially in 90% alcohol, 100% alcohol and xylene for one minute each. After drying, the slides are mounted with Canada balsam and preserved for microscopic evaluation. The stained sections were assessed for both qualitative and quantitative histological changes. Each sample of control and aluminium-treated groups were examined for the histopathological findings like cytoplasmic vacuolation, sinusoidal dilatation, degenerated hepatocytes with pyknotic nuclei and inflammatory cell infiltration around portal area/sinusoidal space.

Histological quantification –

The quantification of the histological observations in liver microslides was carried out following the method described elsewhere (99). An area of approximate 0.5 square mm area [Figure 1] was selected under microscope (400x magnification) and number of hepatocytes were counted. During the process of counting hepatocytes, cells were also evaluated for the presence or absence of cytoplasmic vacuolation. Similar 5 areas were randomly identified and the process was repeated for each histologically stained slide. The procedure was carried out under the supervision of blinded cytologists. The results of this quantitative evaluation are presented as numbers of hepatocytes per square millimeter.

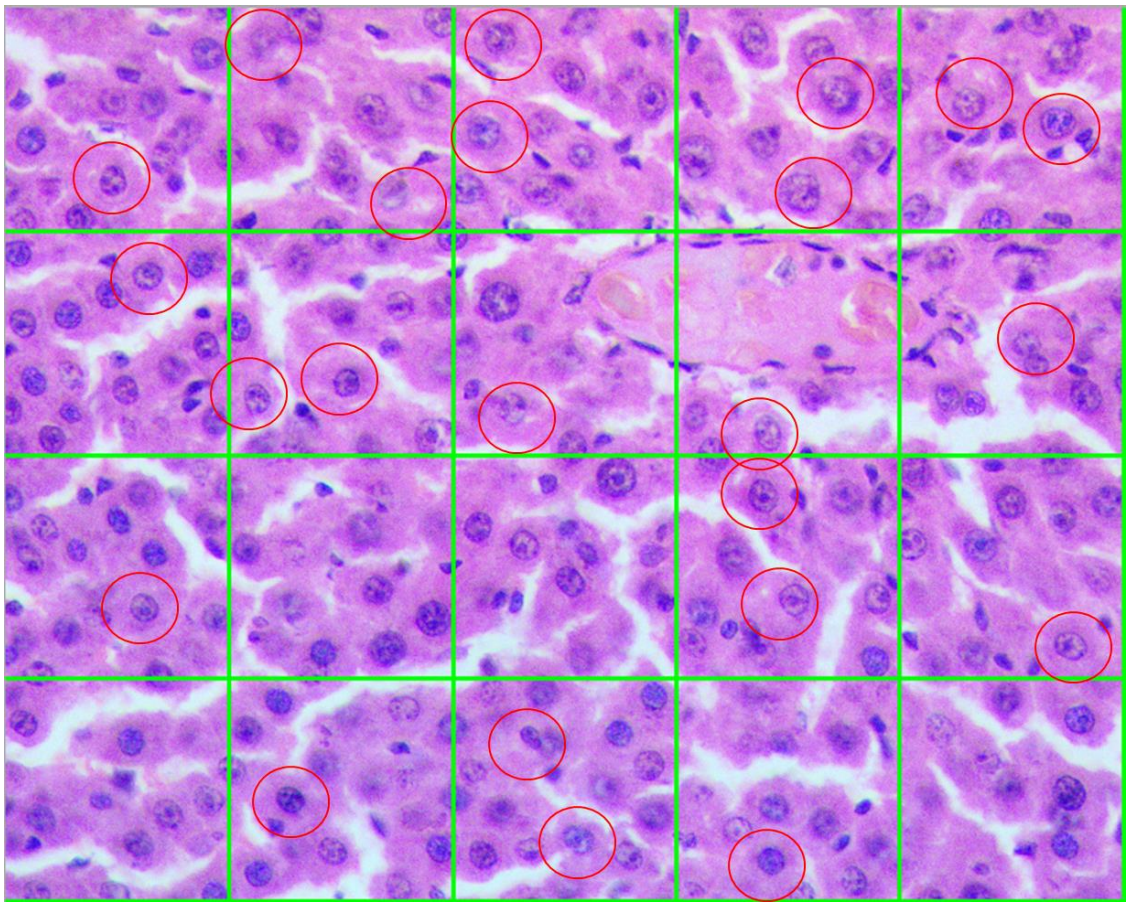

Figure 1. Sample snap of quantitative analysis of liver histology.

Statistical analyses –

After performing the normality tests [Kolmogorov-Smirnov test, Shapiro-Wilk test], it was found that the some of the obtained data were not following the normal distribution pattern. For tabulation, all values were expressed as mean ± standard deviation. Analysis of variance was carried out by Kruskal-Wallis [KW] test and the differences between the groups were analysed by Mann-Whitney U, post-hoc test accepting the probability of 5% or less as significant using PAST© [ver. 4.03] statistical software (100). Graphical representations of the data are done by columns, line diagrams and box-whisker plot. Column graph and line diagrams are prepared by Microsoft Excel version 2209 [Build 1562920208], and the box-whisker plots are prepared by R version 4.1.3 [2022-03-10] © 2022 The R Foundation for Statistical Computing (101).

RESULTS

Continuous monitoring of animals was done after their procurement. Animals were grouped and maintained as six animals in a cage. Body weights of individual rats were noted daily and the weekly averages are presented in the figure 2. During the acclimation phase, before starting the Al exposure, all the animals were maintaining their growth; however, during the phase of Al exposure, the Al-exposed groups demonstrated a retardation in their growth. Interestingly, on the last week, it appeared that the male Al-exposed animals were able to stabilize their growth; nevertheless, the female Al-exposed group continued to lose their weights. The weekly changes during acclimation phase and exposure phase are more prominent in terms of the percentage alterations of their body weights, as presented in figure 3.

The food and water consumed by each group were also recorded daily and averages for each cage are presented in the figure 4 as food intake per 100g body weight. As noted in figure 4A, both the Al-exposed groups reduced their food intake drastically on the 1st day of Al exposure. Both male and female groups consumed only ~ 31% in comparison to the respective average food intakes on the previous week. As shown in figure 4A, the male Al-5 group started to increase the food consumption from the next day and reached the comparable food intake on day 6 of Al exposure. Interestingly, female Al-5 group the increments in food intake was delayed by one day and continued at a slower pace in comparison to their male counterparts. Likewise, male Al-5 rats demonstrated excess food intake [per 100g body weight] during the second week of Al exposure compared to their average food intake before the starting of Al exposure [Figure 4A]. However, this type of change in food intake in female Al-5 rats were subtle and delayed [Figure 4A]. Daily record of average water intakes [per 100g body weight] for each group of animals are presented in figure 4B. Noticeable lessening in water intakes were seen in both male and female Al-exposed groups only for initial two days of Al exposure that were followed by some amount of excess water intake in both groups [Figure 4B].

Mean values and their standard deviations of serum lipid parameters of young adult male and female rats are shown in Table 1. Increases in mean TG levels of serum were found to be elevated in both the Al-5 groups of male and female rats with comparable raises [Male: 41% and Female: 46%]. KW test for equal medians showed that there is a significant difference between the sample medians [$\chi^2 = 9.47$, $p = 0.024$] for serum TG. MW tests showed that the median serum TG of female Al-5 was significantly different [$p = 0.005$] from that of female Al-0 group [Figure 5A].

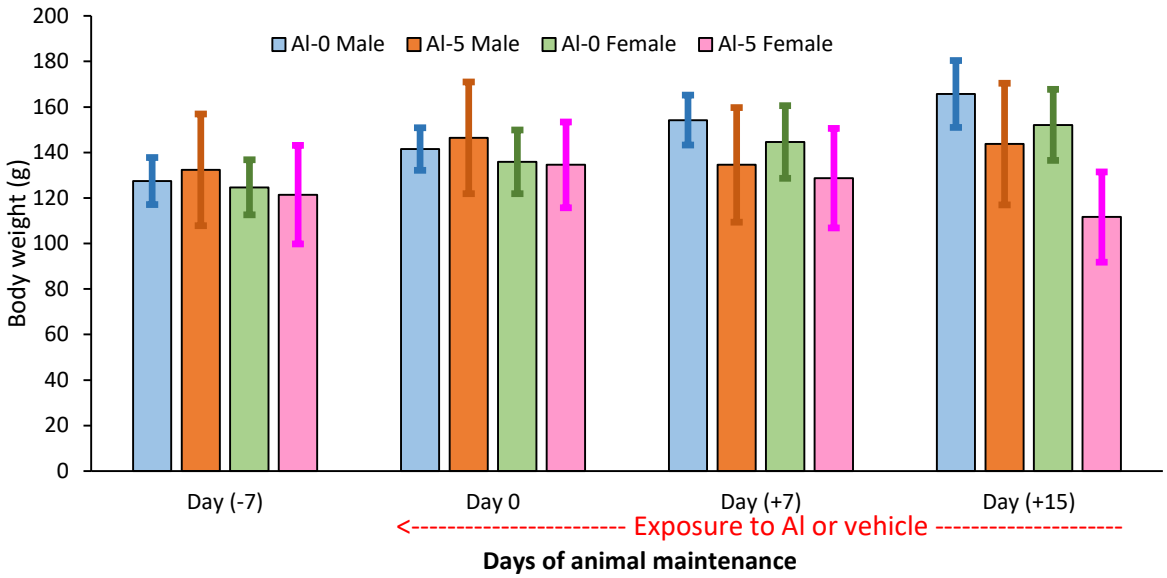

Figure 2. Column diagram of absolute body weights of rats of different groups during each week of animal maintenance. Each column represents mean of six observations ± standard deviation.

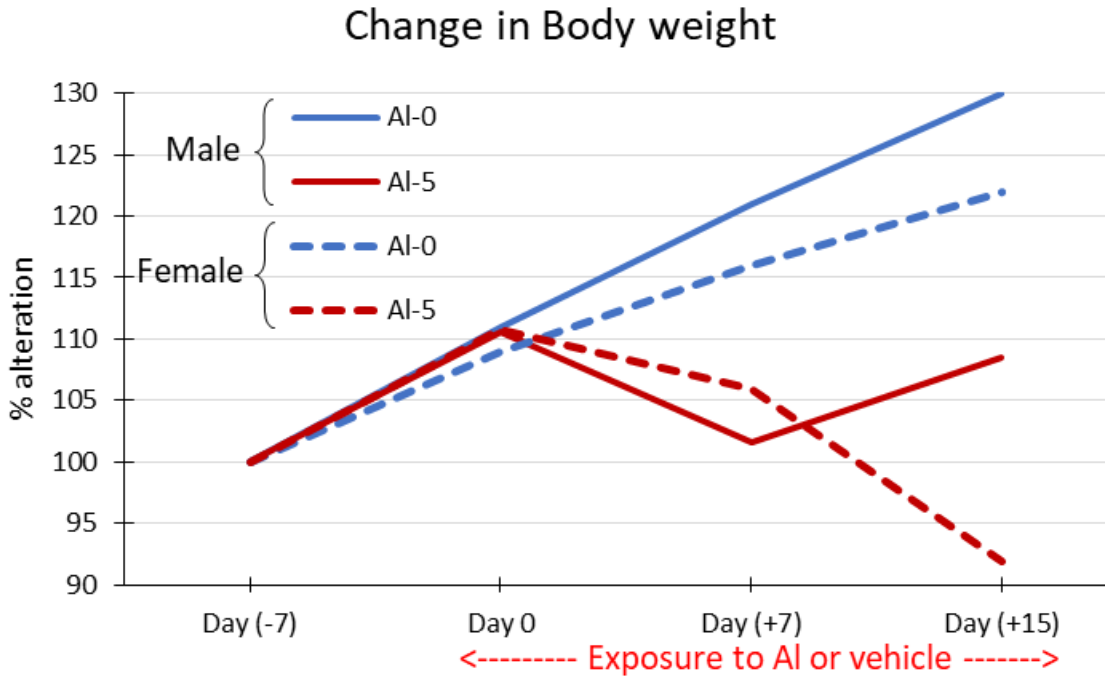

Figure 3. Line diagram of percentage alterations of body weights of rats of different groups during the period of aluminium exposure. Each column represents mean of six observations ± standard deviation.

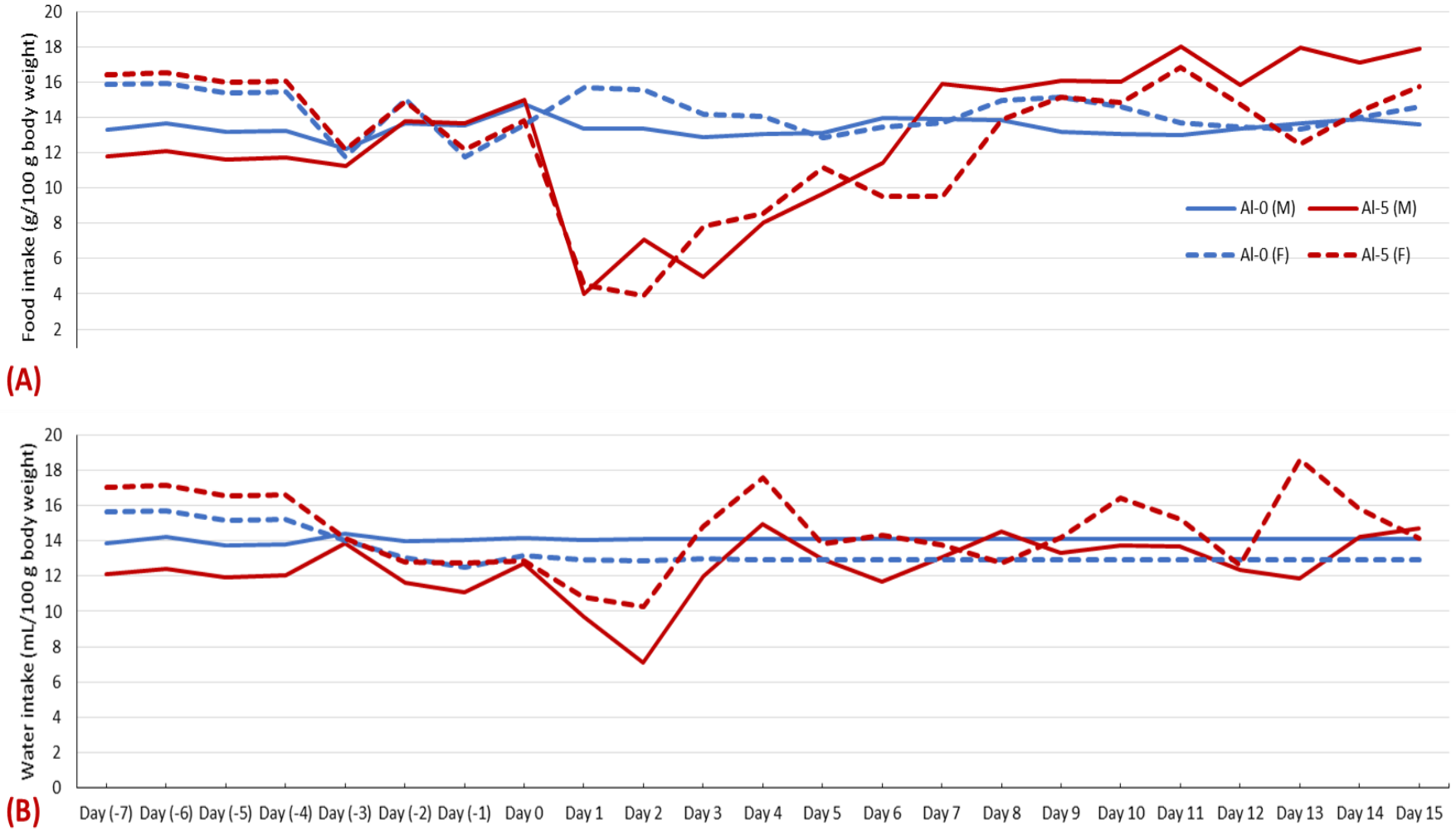

Figure 4. Line diagrams representing (A) average food intake and (B) average water intake by the rats of different groups during the period of aluminium exposure.

In case of mean serum TC levels, Al exposures demonstrated [Table 1] decreases in comparison to untreated groups. The decrement in mean serum TC of male Al-5 group [39%] was relatively higher than that of female group [18%]. By performing KW test for equal medians, a significant difference between the sample medians [$\chi^2 = 16.46$, $p = 9.12\times10^{-4}$] was observed. MW tests show that the median serum TC of both male and female Al-0 groups were significantly higher [p = 0.005 and 0.03, respectively] compared to Al-5 group of animals [Figure 5B].

Decrements in mean serum LDL level was seen [Table 1] in Al-5 group of male rats [45%]; while, Al-5 female rats demonstrated only 9% reduction. KW test for equal medians showed that there is a significant difference between the sample medians [$\chi^2 = 12.37$, p = 0.006]. MW tests showed that the differences between Al-0 and Al-5 were statistically significant [p = 0.015] in male rats; however, differences between other group of animals in terms of median values of serum LDL were only insignificant statistically [Figure 5C].

Like that of TC, mean serum HDL levels demonstrated decrements in Al-exposed group irrespective of genders [Table 1]. Male Al-5 group demonstrated relatively higher decrements in mean serum HDL levels [45%], compared to that of female rats [19%]. By applying KW test for equal medians, a significant difference between the sample medians [$\chi^2 = 17.59$, p = 0.0005] was identified. In case of male young adult rats, median serum HDL value of Al-0 group was significantly higher [p = 0.005] than that of Al-5 group. Similarly, the median serum HDL level of Al-exposed female young adult rats was significantly [p = 0.02] lower than that of Al-0 group [Figure 5D].

Table 2 depicts the mean and standard deviations of total protein levels, albumin levels, and globulin levels of serum of different groups of rats along with the albumin-globulin ratio. All the parameters demonstrated reduction in their levels or ratios in the Al-exposed group compared to the control group. The median values and data distributions of these parameters are presented as box and whisker plots in figure 6 with their statistical processing for significant differences. When compared with mean values of Al-0 group, the mean values of total proteins of Al-5 demonstrated 26% reductions in case of male rats and 10% reductions in case of female rats, respectively [Table 2]. Comparison of median values by KW test showed significant difference between the medians [$\chi^2 = 11.75$, p = 0.008] and MW test revealed that the afore-mentioned difference in male is statistically significant [Figure 6A]. The reductions in mean values of serum protein, serum albumin and serum globulin were relatively lesser in female

Table- 1: Serum lipid parameters of young adult male and female rats with different doses of intraperitoneal aluminium exposures for 15 days.

Animal Groups		Al-0	Al-5
Triglycerides [mg/dL]	Males	68.31 ± 16.38	96.57 ± 60.04
	Females	61.79 ± 11.27	90.00 ± 8.72
Total cholesterol [mg/dL]	Males	90.08 ± 6.44	54.57 ± 16.27
	Females	84.17 ± 10.52	69.17 ± 5.00
Low density lipoprotein cholesterol [mg/dL]	Males	33.96 ± 19.10	19.71 ± 6.26
	Females	24.17 ± 3.24	23.00 ± 1.79
High density lipoprotein cholesterol [mg/dL]	Males	55.46 ± 4.99	30.71 ± 9.57
	Females	51.25 ± 6.34	41.50 ± 3.08

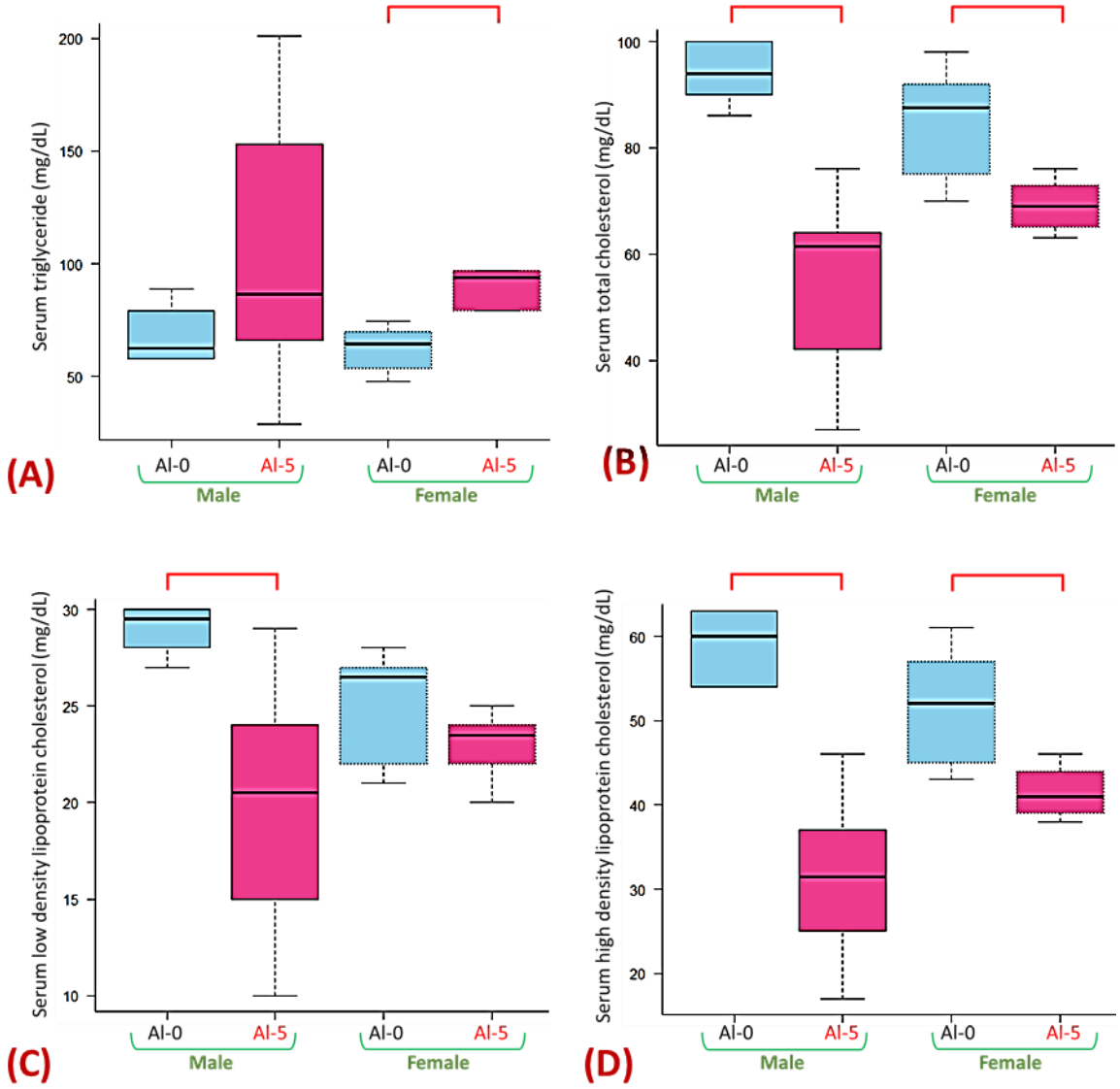

Figure 5. Box and whisker presentation of data distribution of (A) serum triglyceride, (B) serum total cholesterol, (C) serum LDL cholesterol and (D) serum HDL cholesterol. Red lines indicate significant ($p < 0.05$) differences between the study groups.

groups; however, the reductions in albumin-globulin ratio were comparable between male and female groups.

The mean serum albumin levels were decreased in male Al-5 [31%] group in comparison to Al-0 group [Table 2] with significant difference between their medians [Figure 6B]. In case of female, serum albumin level was slightly less [17%] in Al-5 group [Table 2]. The differences between median serum albumin values of the female groups were also found to be statistically significant [p = 0.005 and 0.013, respectively]. By applying KW test for equal medians, significant difference between the sample medians was also noted [$\chi^2 = 16.42$, $p = 9.29\times10^{-4}$].

The observed differences between the medians of serum globulin were statistically insignificant [$\chi^2 = 5.93$, $p = 0.11$] through the KW test for equal medians. The median differences of serum globulin levels of Al-5 group were insignificant from that of Al-0 group in both male and female rats [Figure 6C]. The mean values of serum globulin levels were showed, 22% and 4% reductions in Al-5 group in comparison to Al-0 group for male and female young adult rats, respectively [Table 2].

As shown in Table 2, the mean values of albumin-globulin ratio were reduced by 12% and 11% in Al-5 group in male and female rats, respectively. As depicted in Figure 6D, the difference between Al-5 and Al-0 in terms of albumin-globulin ratios were statistically [MW test] significant [p = 0.016] only for male young adult rats. The KW test for equal medians also demonstrated a significant χ^2 value [10.68] with a p value of 0.014.

In case of mean serum total conjugated bilirubin levels, male Al-5 group demonstrated 13% decrements and female rats registered 34% increment [Table 3]. The KW test showed insignificant differences between the median total conjugated bilirubin values of different groups of rats [$\chi^2 = 7.77$, $p = 0.05$].

In case of serum ALP, the mean values show decrements in male Al-5 [48%] group while in female Al-5 [29%] group in comparison to their respective Al-0 groups [Table 3]. However, the levels of serum ALP activities of male and female Al-0 groups were also noticeably different. The KW test for differences in medians recorded significant χ^2 value [15.21] with a probability of 0.002. The MW test demonstrated significant differences in medians of serum ALP activities between Al-0 and Al-5 groups of males [p = 0.013] and females [p = 0.020] rats [Figure 7B].

Table- 2: Serum protein details of young adult male and female rats with different doses of intraperitoneal aluminium exposures for 15 days.

Animal Groups		Al-0	Al-5
Total protein level [g/dL]	Males	6.69 ± 0.21	4.93 ± 1.40
	Females	6.88 ± 0.51	6.17 ± 0.64
Albumin level [g/dL]	Males	3.52 ± 0.14	2.44 ± 0.67
	Females	3.61 ± 0.23	3.01 ± 0.22
Globulin level [g/dL]	Males	3.17 ± 0.16	2.48 ± 0.76
	Females	3.28 ± 0.34	3.16 ± 0.44
Albumin – Globulin ratio	Males	1.13 ± 0.07	1.00 ± 0.10
	Females	1.08 ± 0.09	0.96 ± 0.08

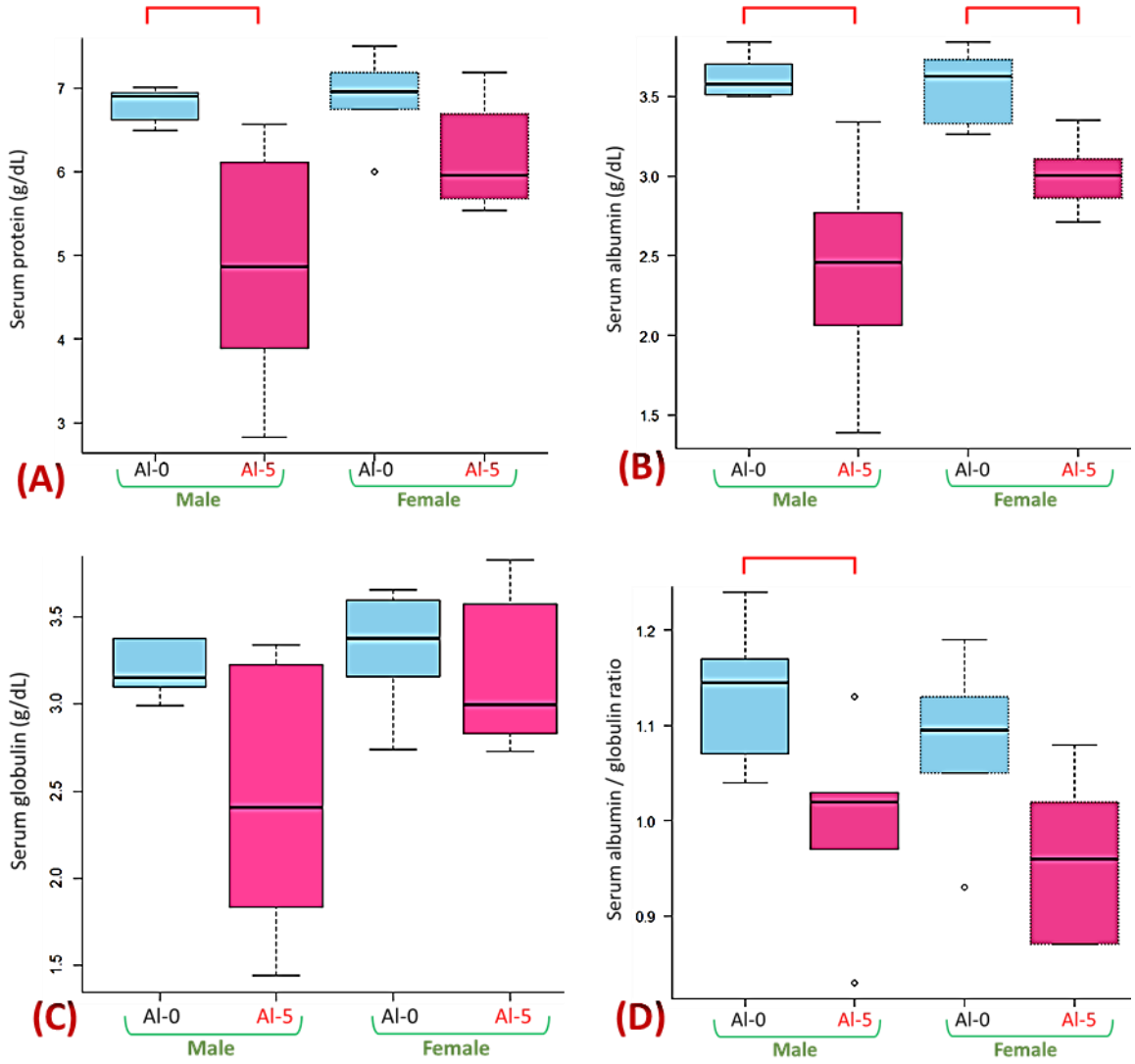

Figure 6. Box and whisker presentation of data distribution of (A) serum total protein, (B) serum albumin level, (C) serum globulin level and (D) serum albumin globulin ratio. Red lines indicate significant ($p < 0.05$) differences between the study groups.

Table-3: Serum levels of hepatic enzymes of young adult male and female rats with different doses of intraperitoneal aluminium exposures for 15 days.

	Animal Groups	Al-0	Al-5
Total conjugated bilirubin [mg/dL]	Males	0.11 ± 0.03	0.10 ± 0.02
	Females	0.11 ± 0.01	0.15 ± 0.04
Serum alkaline phosphatase [U/L]	Males	381.88 ± 52.28	198.86 ± 72.42
	Females	385.50 ± 40.24	272.67 ± 60.58
Serum alanine aminotransferase [U/L]	Males	31.38 ± 3.48	31.40 ± 22.14
	Females	28.43 ± 11.27	38.10 ± 20.62
Serum aspartate aminotransferase [U/L]	Males	187.82 ± 34.13	127.53 ± 50.86
	Females	207.58 ± 20.04	165.73 ± 53.23

Values are presented as mean ± standard deviation

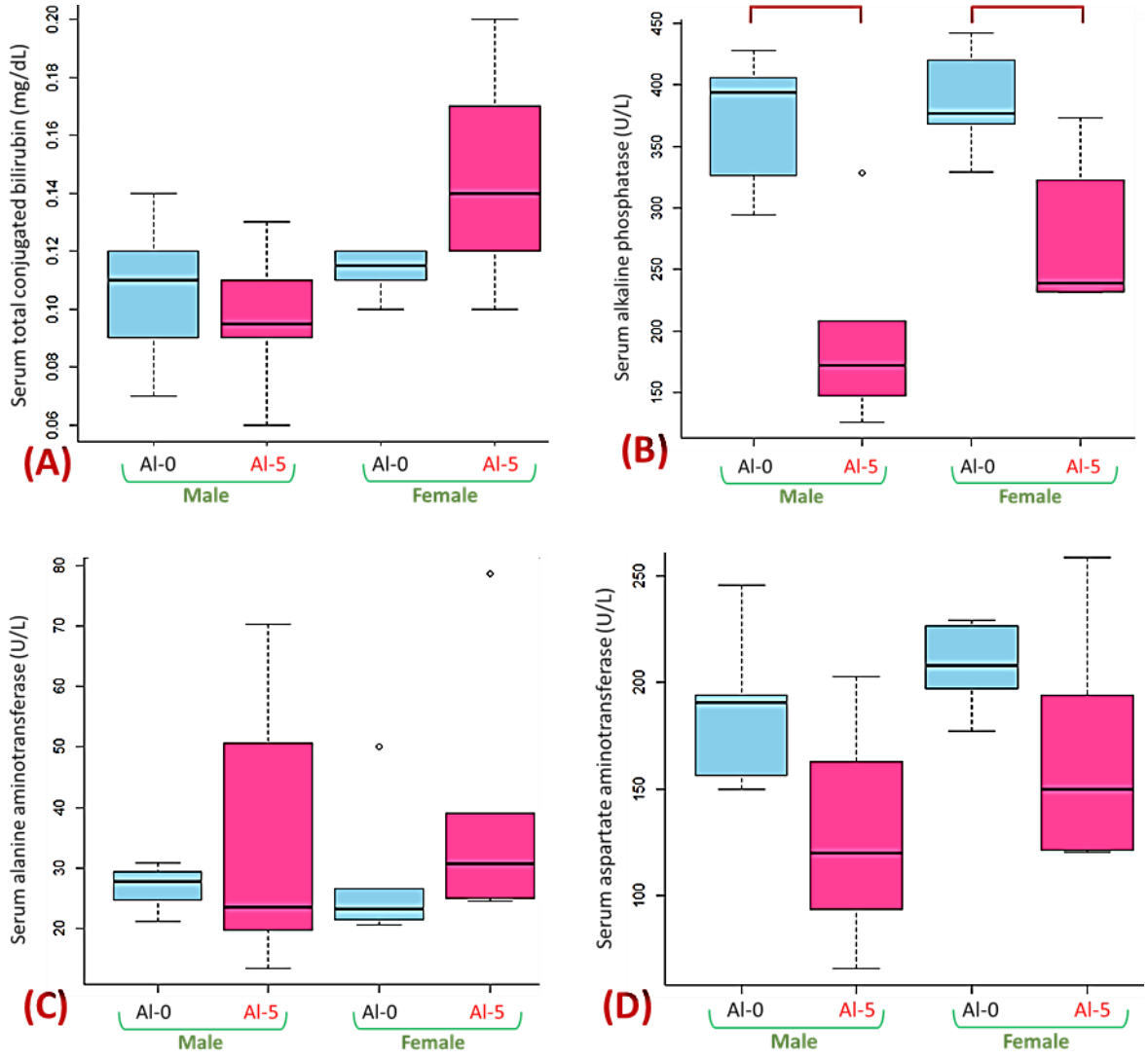

Figure 7. Box and whisker presentation of data distribution of (A) serum conjugated bilirubin levels, (B) serum ALP activity, (C) serum ALT activity and (D) serum AST activity. Red lines indicate significant ($p < 0.05$) differences between the study groups.

The KW test for serum ALT activities registered an insignificant [p = 0.32] value for the χ^2 [3.53]. Interestingly, the mean serum ALT activities of male Al-5 remained unaltered in comparison to their Al-0 counterparts [Table 3]. On the other hand, female Al-5 group of rats

Notable decrements in mean serum AST activities were noted in male [32%] and female [20%] Al-5 groups of rats in comparison to their respective control groups [Table 4]. However, when compared the median values of the same through MW tests, male and female groups demonstrated statistically insignificant decreases only [Figure 7D]; however, KW test recorded significant differences [$\chi^2 = 8.05$, p = 0.045] between the medians.

Table- 4: Atherogenic indices of young adult male and female rats with different doses of intraperitoneal aluminium exposures for 15 days.

	Animal Groups	Al-0	Al-5
NonHDL [mg/dL]	Males	34.62 ± 2.00	23.86 ± 9.53
	Females	32.92 ± 3.93	27.67 ± 2.16
Atherogenic Coefficient	Males	0.63 ± 0.04	0.79 ± 0.29
	Females	0.64 ± 0.04	0.67 ± 0.03
Castelli Risk Index 1	Males	1.63 ± 0.04	1.79 ± 0.29
	Females	1.64 ± 0.04	1.67 ± 0.03
Castelli Risk Index 2	Males	0.60 ± 0.42	0.66 ± 0.21
	Females	0.47 ± 0.02	0.56 ± 0.05
Lipoprotein Combine Index [$\times 10^3$]	Males	3.65 ± 2.37	4.27 ± 4.87
	Females	2.45 ± 0.37	3.44 ± 0.34
Atherogenic Index of Plasma	Males	0.08 ± 0.09	0.44 ± 0.22
	Females	0.08 ± 0.12	0.33 ± 0.06

Values are presented as mean ± standard deviation

Table 4 displays the mean values and their standard deviation of atherogenic indices of young adult male and adult female rats. NonHDL were reduced in Al-5 [31%] group of males with corresponding Al-0 group. Similarly, female group demonstrated reduction in NonHDL levels of Al-5 group [16%]. KW test for equal medians showed that there is a significant difference between the sample medians [$\chi^2 = 10.69$, p = 0.013]. MW tests showed that the median NonHDL level of both male and female Al-5 groups were significantly different [p = 0.043 and 0.035, respectively] from their Al-0 counterparts [Figure 8A].

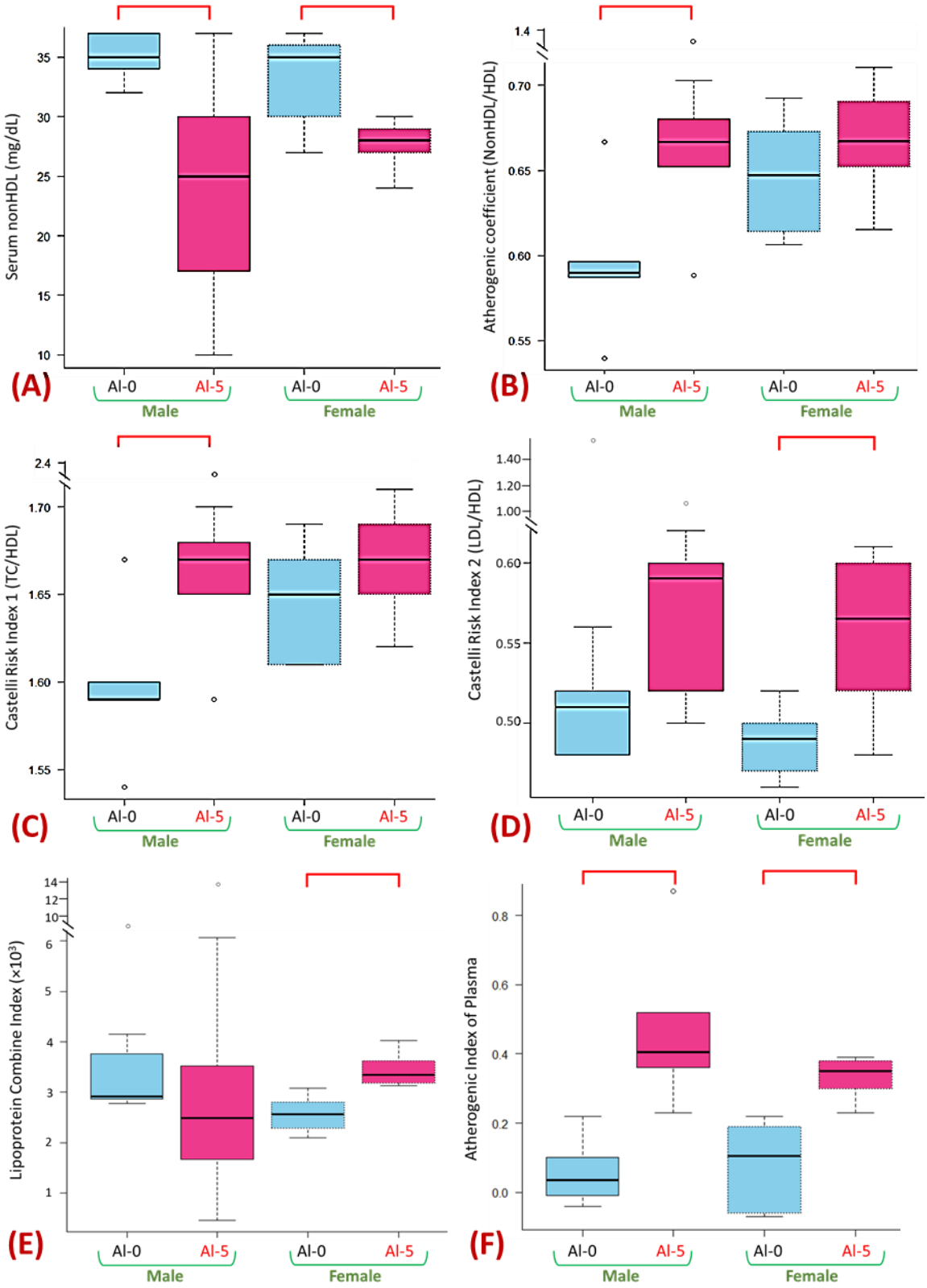

Figure 8. Box and whisker presentation of data distribution of (A) NonHDL, (B) Atherogenic Coefficient, (C) Castelli Risk Index 1, (D) Castelli Risk Index 2, (E) Atherogenic Index of Plasma and (F) Lipoprotein Combine Index. Red lines indicate significant ($p < 0.05$) differences between the study groups.

Mean values of Atherogenic Coefficient [AC] were increased in both Al-5 male [25%] and female [4%] group. The KW test for equal medians in case of AC demonstrated significant differences [χ^2 = 8.713, p = 0.033] while MW test demonstrated significant difference [p = 0.041] between male Al-0 and Al-5 group only. From the figure [8B], there was a no significant difference [p = 0.334] in terms of AC among the Al-0 and Al-5 groups of female rats.

Increments in mean values of Castelli Risk Index 1 was seen in Al-exposed groups of males [10%] and female [2%] rats. Significant differences between the medians of CRI 1 was also observed [χ^2 = 8.71, p = 0.033] through the KW test for equal medians. The CRI 1 of Al-0 group shows significant difference [p = 0.041] with the Al-5 group of male rats. As can be seen from figure 8C, there is a no significant difference between Al-0 and Al-5 group of females.

A similar increasing pattern has been noted in Castelli Risk Index 2 [CRI 2] of both male and female Al-exposed rats. The CRI 2 mean values of were increased in Al-5 male [9%] and female [18%] groups. KW test for equal medians identified significant difference between the sample medians [χ^2 = 8.371, p = 0.039] for CRI 2. In female rats, the difference between Al-0 group and Al-5 group was statistically significant [MW test], while male group demonstrated statistically insignificant increase [Figure 8D].

Increases in mean Lipoprotein Combine Index [LCI] were noted in both the male and female groups. Higher level of increase in mean LCI was noticed in female Al-5 [40%] group compared to the increment [17%] noticed in male group. By performing KW test for equal medians, insignificant differences were observed between the sample medians [χ^2 = 6.55, p = 0.087]. From the figure 8[E], there is significant [p = 0.005] difference between the Al-0 and Al-5 rats in female group. On the other hand, comparison of medians between male groups of rats showed insignificant [p = 0.572] decrement of LCI in Al-5 group of rats.

Both male and female of Al-5 groups demonstrated more than two-fold increments in mean values of Atherogenic Index of Plasma [AIP]. Significant differences between the medians of AIP were also observed [χ^2 = 17.93, p = 4.55×10^{-4}] through the KW test for equal medians. AIP in both Al-exposed groups were significantly [p = 0.005] higher from that of Al-0 counterparts [Figure 8F].

Mean values and their standard deviations of hepatic lipid parameters of young adult male and female rats are shown in Table 5. When compared with Al-0 group, female rats demonstrated noticeable raise in mean hepatic TG levels in Al-5 group; however, the increment in male group was subtle. KW test for equal medians showed that there is a significant were

difference between the sample medians [χ^2 = 8.594, p = 0.035] for hepatic TG levels. Applied MW test identified that the median values of hepatic TG levels of Al-0 group was significantly different from that of Al-5 group in female [p 0.005] rats, but not in male [p 0.378] rats, as indicated in figure 9A.

In comparison to Al-0 rats, Al-5 rats demonstrated notable lowered level of hepatic TC [Table 5] in both male and female groups while their mean values were reduced by 20% and 42%, respectively. The KW test for differences in medians recorded significant χ^2 value [9.852] with a probability of 0.020. The difference between median TC levels of Al-5 and Al-0 groups of male rats was found to be statistically insignificant [p = 0.377] by the MW test. On the other hand, female group demonstrated statistically significant [p = 0.012] decrease in the hepatic TC level in Al-5 rats [Figure 9B].

The KW test for equal medians in case of hepatic LDL levels demonstrated significant differences [χ^2 = 8.996, p = 0.029] while MW test demonstrated significant [p = 0.011] difference between only female Al-0 and Al-5 groups. As shown in figure 9C, there was no significant [p = 0.125] difference in male study groups in terms of hepatic LDL. Nevertheless, both male and female Al-5 groups demonstrated considerable decrements in mean hepatic LDL levels, 35% and 41%, respectively.

Mean hepatic HDL values were found to be reduced by 43% and 56% in male and female Al-5 group, respectively, when compared with respective Al-0 group. When the median values of hepatic HDL levels were compared between the groups, KW test found significant difference [χ^2 = 16.37, p = 9.54×10^{-4}] and MW test found significant differences in Al-5 male [p = 0.018] and female [p = 0.005] in comparison to their respective Al-0 group [Figure 9D].

Table 6 depicts the mean and standard deviations of hepatic total protein levels, ALP, ALT and AST of different groups of rats. The mean values of hepatic protein decreased by 26% in male Al-5 group and by 17% in female Al-5 group, in comparison to their respective Al-0 groups of rats. Significant differences between the median values were noted through KW test [χ^2 = 19.14, p = 2.55×10^{-4}]. Likewise, MW tests also reported significant decrements in hepatic proteins in both male [p = 0.005] and female [p = 0.004] groups of Al-exposed rats in comparison to their Al-unexposed control rats [Figure 10A].

Activities of hepatic ALP also demonstrated only insignificant differences between the study groups as per KW test [χ^2 = 7.61, p = 0.055]. Accordingly, MW test also did not find any significant difference between the groups in both male and female rats [Figure 10B]. However,

Table- 5: Hepatic lipid parameters of young adult male and female rats with different doses of intraperitoneal aluminium exposures for 15 days.

Animal Groups		Al-0	Al-5
Hepatic Triglycerides [µg/ mg liver tissue]	Males	20.16 ± 0.38	21.24 ± 5.22
	Females	16.77 ± 0.64	21.42 ± 2.82
Hepatic total cholesterol [µg/ 100 mg liver tissue]	Males	173.33 ± 29.36	138.02 ± 64.06
	Females	205.33 ± 31.05	119.13 ± 46.53
Hepatic low density lipoprotein cholesterol [µg/ 100 mg liver tissue]	Males	149.33 ± 64.53	97.50 ± 42.85
	Females	122.67 ± 8.26	71.78 ± 38.26
Hepatic high density lipoprotein cholesterol [µg/ 100 mg liver tissue]	Males	48.00 ± 17.53	27.58 ± 10.84
	Females	47.87 ± 4.08	20.86 ± 7.64

Values are presented as mean ± standard deviation

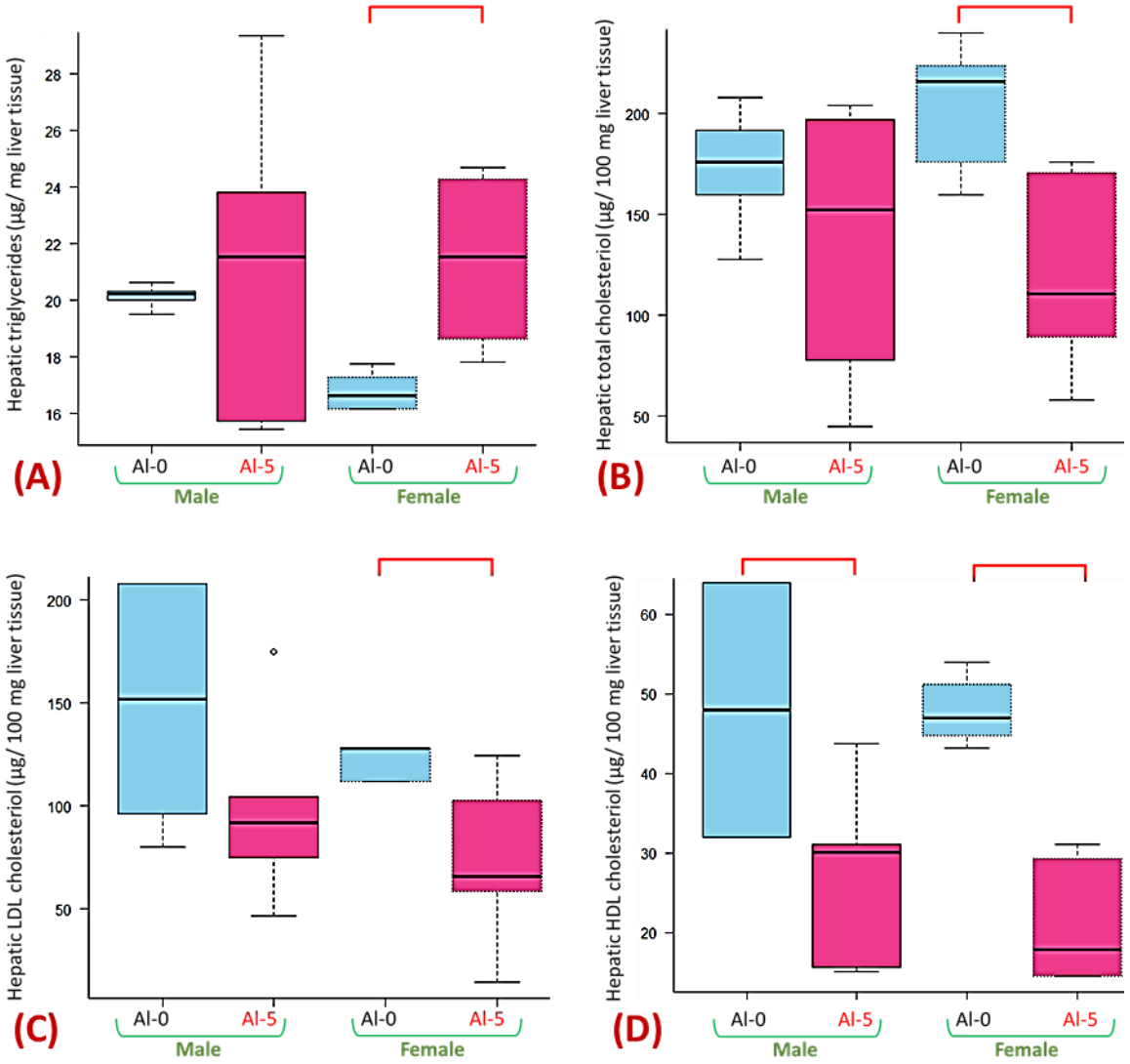

Figure 9. Box and whisker presentation of data distribution of (A) hepatic triglycerides, (B) hepatic total cholesterol, (C) hepatic low density lipoprotein cholesterol, and (D) hepatic high density lipoprotein cholesterol. Red lines indicate significant ($p < 0.05$) differences between the study groups.

Table- 6: Hepatic protein and enzyme levels young adult male and female rats with different doses of intraperitoneal aluminium exposures for 15 days.

Animal Groups		Al-0	Al-5
Total protein [mg/100 mg liver tissue supernatant]	Males	26.35 ± 2.42	19.38 ± 1.79
	Females	23.44 ± 0.41	19.52 ± 1.36
Hepatic alkaline phosphatase [U/L liver tissue supernatant]	Males	3.25 ± 1.36	4.46 ± 0.93
	Females	3.62 ± 0.14	2.40 ± 0.81
Hepatic alanine aminotransferase [U/L liver tissue supernatant]	Males	445.84 ± 6.74	187.70 ± 41.41
	Females	352.68 ± 3.83	252.68 ± 46.79
Hepatic aspartate aminotransferase [U/L liver tissue supernatant]	Males	232.57 ± 78.78	56.32 ± 10.23
	Females	225.36 ± 33.13	61.57 ± 11.76

Values are presented as mean ± standard deviation

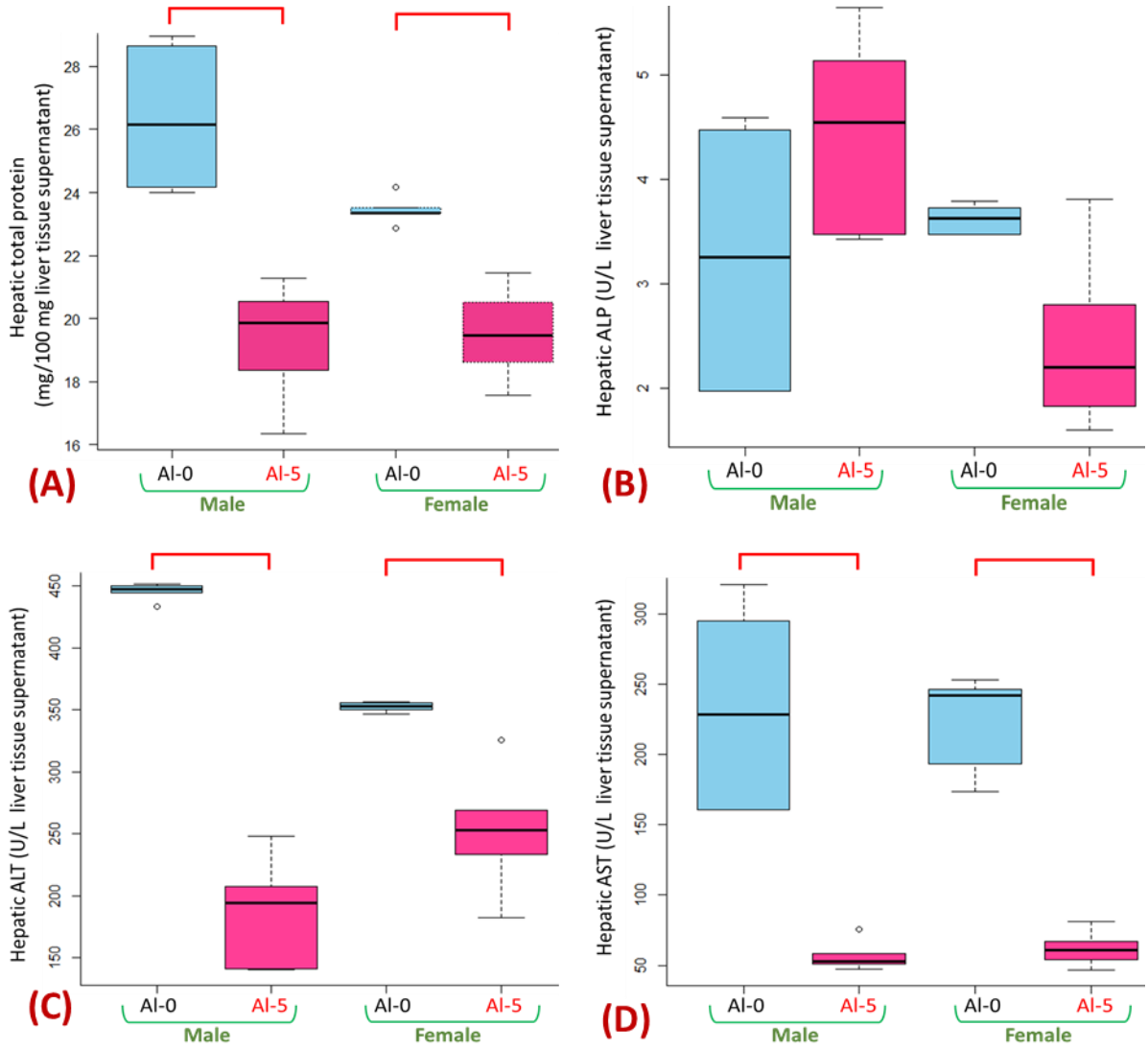

Figure 10. Box and whisker presentation of data distribution of (A) total protein level, (B) hepatic alkaline phosphatase activity, (C) hepatic alanine aminotransferase activity, and (D) hepatic aspartate aminotransferase activity in the obtained supernatant. Red lines indicate significant ($p < 0.05$) differences between the study groups.

means of hepatic ALP activities showed an increment in male groups while a decrement in female groups of Al-5 rats [Table 6].

Reduced ALT activities in hepatic supernatant were noted in Al-5 groups of male rats [58%] and female rats [28%]. KW test for comparing medians also supported the observation with significant difference [$\chi^2 = 20.57$, $p = 1.29\times10^{-4}$] along with significant differences [p = 0.005 as per MW test] between the Al-0 and Al-5 rats of both male and female groups [Figure 10C].

Similarly, MW test recorded significant differences [p = 0.005] between the Al-0 and Al-5 rats of both male and female groups in terms of hepatic AST activities [Figure 10D]. The analysis of variance by KW test also found significant difference between the medians [$\chi^2 = 17.52$, $p = 5.52\times10^{-4}$] of hepatic AST activities of the studied groups of rats. Their mean values were showing 76% and 73% decrement in male and female, respectively, rats [Table 6].

Quantitative analyses of histological evaluation of liver tissues from different groups of rats are presented in table 7. Numbers of hepatocytes without prominent cytoplasmic vacuolation per square millimetre were less in Al-5 groups of males [6%] and females [6%] rats in comparison to their Al-0 counterparts. No significant difference between the medians was observed as per the KW test [$\chi^2 = 6.01$, p = 0.111] and MW test [p = 0.128 for male and 0.149 for female].

Significant differences between the Al-0 and Al-5 groups in terms of numbers of hepatocytes with prominent cytoplasmic vacuolation were observed in both male and female study groups [Figure 11B, MW test p = 0.005]. Accordingly, male Al-5 group showed 59% increment in degenerating cells and female Al-5 group showed 38% increment in the same [Table 7]. The analysis of variance by KW test also recorded significant [$\chi^2 = 19.3$, $p = 2.37\times10^{-4}$] differences between medians.

Even though there were differences in number of hepatocytes with or without prominent cytoplasmic vacuolation, the total number of hepatocytes per square millimeter area were comparable between all the groups of study animals [Table 7 and Figure 11C]. Interestingly, percentages [17.18 % in male and 14.52% in female] of cells having cytoplasmic vacuolation were higher in the Al-5 groups compared to their Al-0 counterparts [10.96 % in male and 10.37% in female]. The observed differences were found to be statistically significant [MW test; p = 0.005 for male and 0.008 for female]. Analysis of variance through KW test also found the significant [$\chi^2 = 17.53$, $p = 5.49\times10^{-4}$] differences within medians.

Mean values along with standard deviation for numbers of hepatocytes per square millimetre area of four study groups of rats are presented in table 7. Both male and female groups demonstrated 6% decrement in the number of hepatocytes without cytoplasmic vacuolation in Al-5 groups in comparison to their respective Al-0 group. However, KW and MW tests did not report any significance of these observed differences [Figure 11A]. On the other hand, male Al-5 group demonstrated 59% increase in number of hepatocytes with cytoplasmic vacuolation, while female Al-5 group demonstrated 38% increase in the same, when compared with the respective Al-0 groups [Table 7]. Analysis of variance through KW test also found the significant [$\chi^2 = 19.3$, $p = 2.37\times10^{-4}$] differences within medians of the hepatocytes with cytoplasmic vacuolations. The observed differences between the experimental and control groups were also found to be statistically significant [MW test; $p = 0.005$] for both male and female study groups [Figure 11B]. Very similar observation was noticed when the ratio of cells with cytoplasmic vacuolation and cells without cytoplasmic vacuolation [presented as percentage] were compared. Analysis of variance through KW test also found the significant [$\chi^2 = 17.53$, $p = 5.49\times10^{-4}$] differences within medians of the hepatocytes with cytoplasmic vacuolations. The observed differences between the experimental and control groups were also found to be statistically significant for both male [$p = 0.005$] and female [$p = 0.008$] study groups [Figure 11D]. Nevertheless, there was no noticeable changes in total number of hepatocytes counted per unit area in either of the study groups [Table 7, Figure 11C].

The liver tissue from Al-0 group showed normal liver plate architecture in both male and female animals. All the slides prepared from liver samples from both male and female Al-5 groups showed derangement of hepatic cytoarchitecture [Figure 12] and cytoplasmic vacuolar degeneration [Figure 14]. Mild sinusoidal dilatation and central vein dilatation were also observed in liver slide of both male and female Al-5 groups [Figure 13].

Table- 7: Quantification of hepatocytes with and without cytoplasmic vacuolation in young adult male and female rats with different doses of intraperitoneal aluminium exposures for 15 days

Animal Groups		Al-0	Al-5
Hepatocytes without prominent cytoplasmic vacuolation [number / sq.mm. area]	Males	299.67 ± 15.43	282.80 ± 20.28
	Females	306.47 ± 13.56	288.13 ± 17.94
Hepatocytes with prominent cytoplasmic vacuolation [number / sq.mm. area]	Males	36.53 ± 3.47	57.93 ± 5.80
	Females	35.20 ± 3.15	48.60 ± 2.80
Total number cells counted per sq.mm. area	Males	336.20 ± 14.27	340.73 ± 16.97
	Females	341.67 ± 11.59	336.73 ± 16.28

Values are presented as mean ± standard deviation

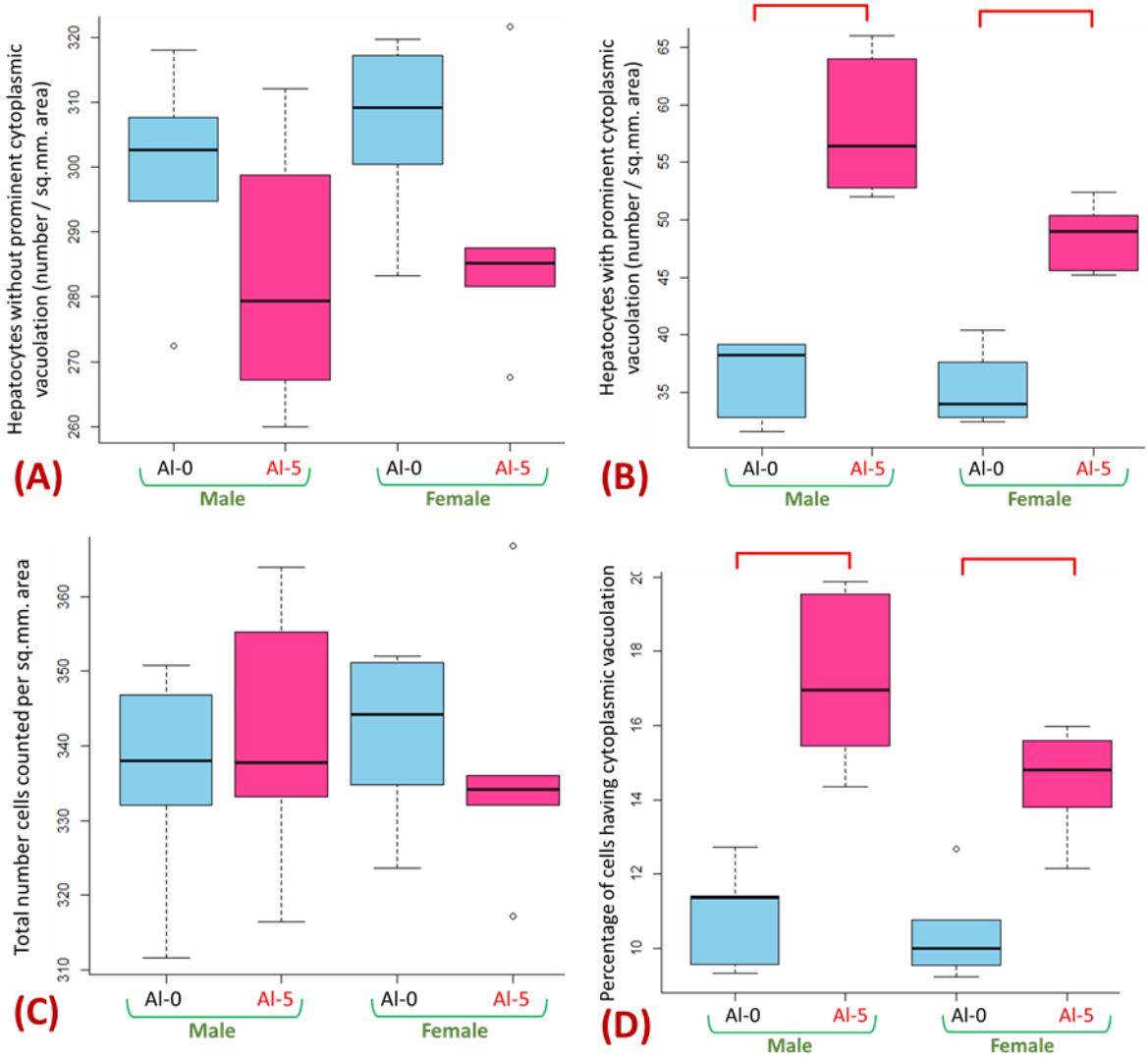

Figure 11. Box and whisker presentation of data distribution of (A) hepatocytes without prominent vacuolation per unit area, (B) hepatocytes with prominent vacuolation per unit area, (C) total number cells counted per unit area, and (D) percentage of hepatocytes having prominent cytoplasmic vacuolations. Red lines indicate significant ($p < 0.05$) differences between the study groups.

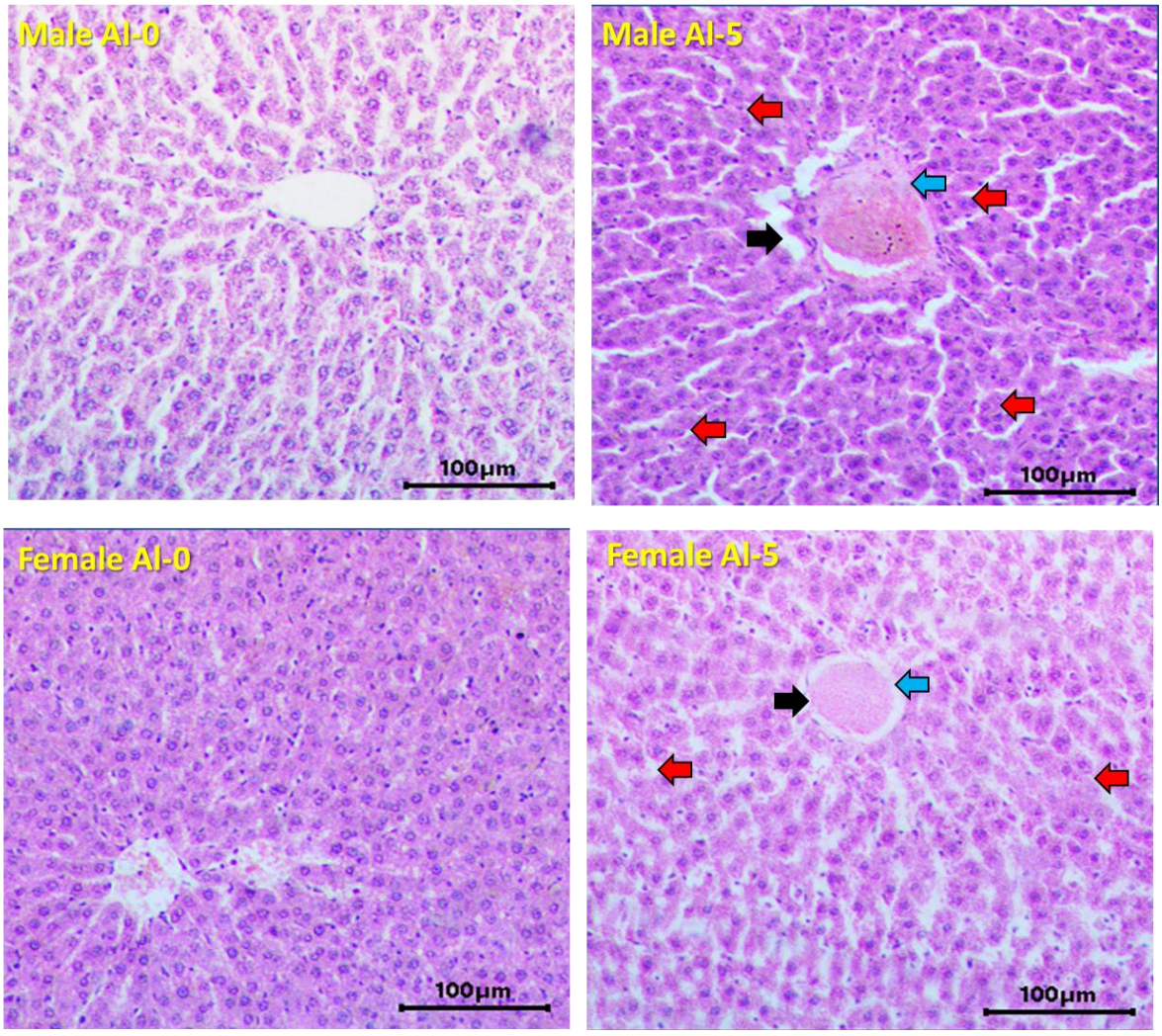

Figure 12. Sample photomicrographs (Magnification 100×) of liver histology from rats of different study groups. ⇦ indicates derangement of hepatic architecture, ➡ indicates sinusoidal dilatation, ⇦ indicates central vein congestion with dilatation.

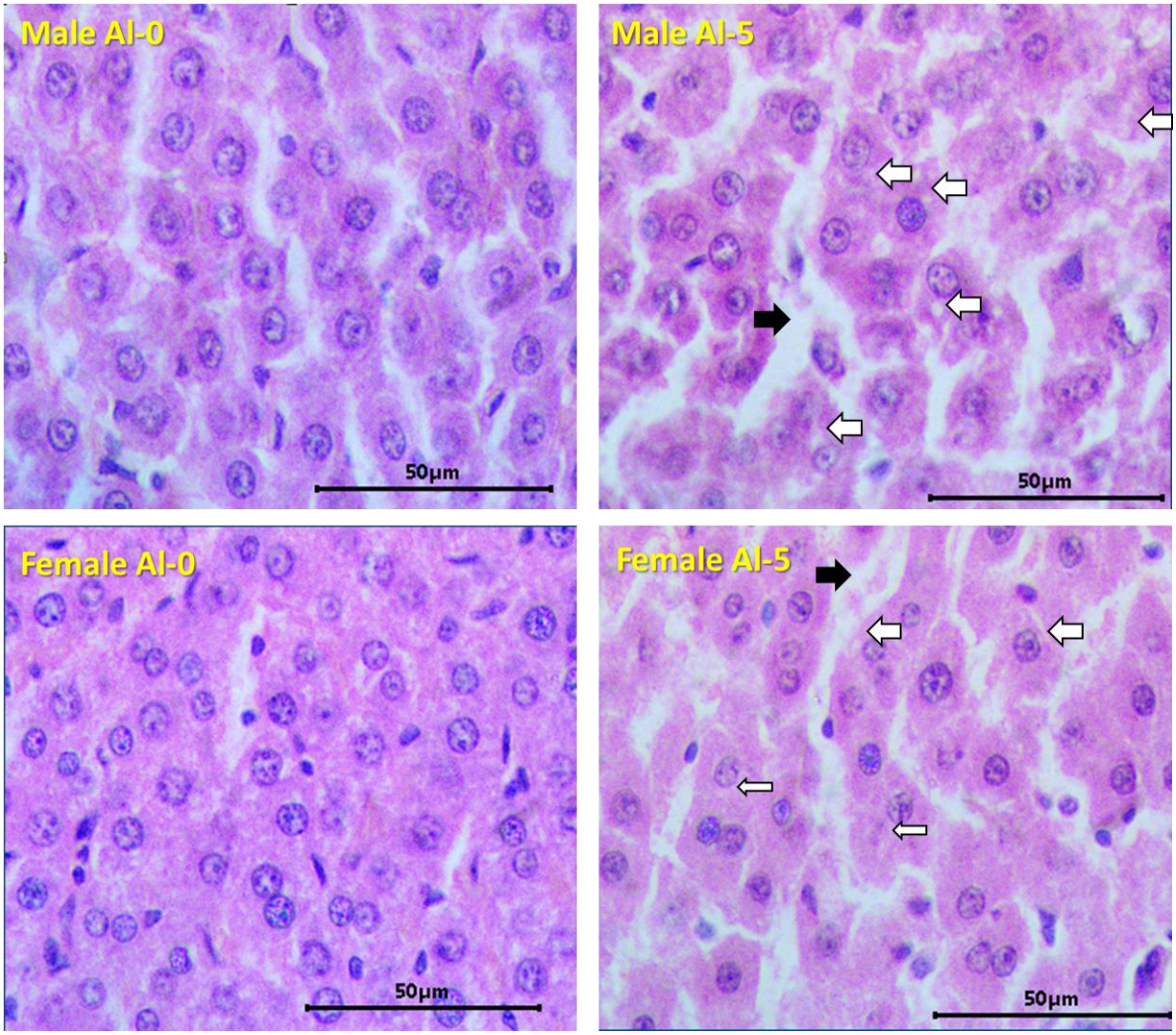

Figure 13. Sample photomicrographs (Magnification 400×) of liver histology from rats of different study groups. ➡ indicates sinusoidal dilatation, ⇦ indicates cytoplasmic vacuolar degeneration.

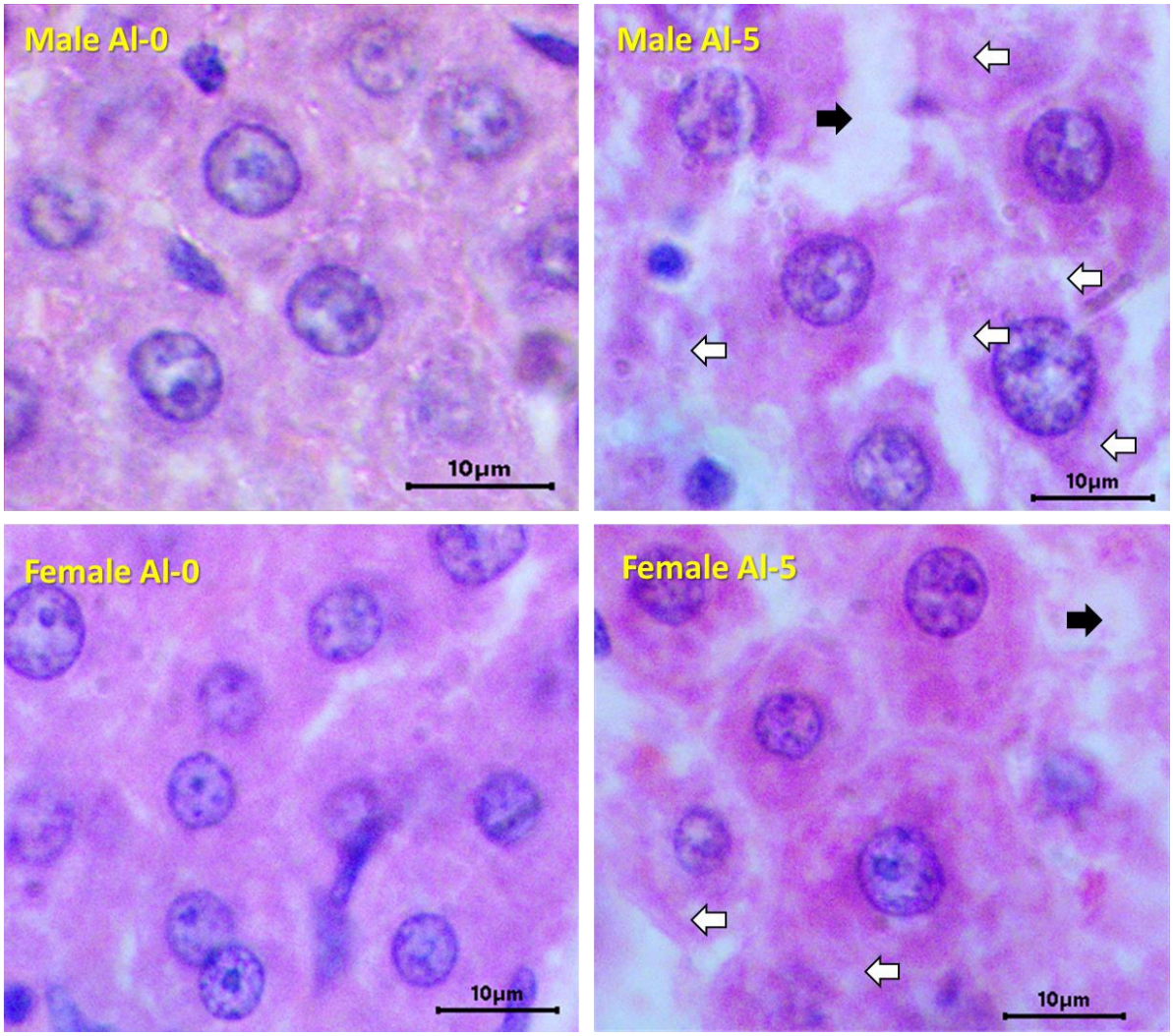

Figure 14. Sample photomicrographs (Magnification 1000×) of liver histology from rats of different study groups. ➡ indicates sinusoidal dilatation, ⇦ indicates cytoplasmic vacuolar degeneration.

DISCUSSION

Aluminium is an omnipresent metal without any reported essentiality for life process. On the other hand, from the toxico-pathological viewpoint, aluminium was never given due importance mostly because of poor intestinal absorption. However, with anthropological and man-made upthrust of aluminium uses, slowly the other routes of aluminium internalization also started contributing towards the total load of body aluminium burden. With the knowledge of metalloestrogenic property of aluminium, many disregarded roles of aluminium started coming into the limelight. In this context, the current study was taken up to evaluate the possible gender inequality in terms of alteration in lipid profile and hepatotoxicity of adult rat exposed to aluminium [*i.p.*] for 15 days.

In this study male and female young adult rats were exposed to aluminium for a short span of 15 days only and their changes in body weight, food intake and water intake were recorded throughout the study period. At the end of the study, collected serum and liver tissue from the sacrificed animals were studied for total protein, albumin, globulin, TG, TC, LDL, HDL, ALP, AST, ALT, bilirubin [as applicable] and their ratios [as applicable] were calculated.

As the rats were in their growing age, there was a continuous increase in their body weight [Figure 2]. However, changes in growth rate were observed even within this short span of aluminium exposure [Figure 3]. Likewise, significant impact of *i.p.* exposure of $AlCl_3$ for 14 days demonstrated significant impact of aluminium on the body weight gain in male adult rats (102). In terms of absolute body weight, male aluminium-exposed rats were not statistically different from their aluminium-unexposed counterparts. However, female rats demonstrated significant difference between the aluminium-exposed rats and control rats at the end of the study, though those were not different at the onset of study. In comparison to control group, no significant difference in change in body weight of mice treated with exposed to aluminium nanoparticles intraperitoneally for 14 days (103). Chronic exposure to aluminium nitrate through drinking water could produce significant difference in body weight only on 12^{th} or 13^{th} week in comparison to their control group (104). On the other hand, only insignificant change in body weight at the end of four weeks was observed with *i.p.* exposure to $AlCl_3$ for 28 days (105) or dietary exposure to $Al(OH)_3$ or $AlK(SO_4)_2$ for 67 days (106). Similarly, single dose oral exposure to aluminium also could not produce any change in body weight till 45^{th} day (89). Nevertheless, when male rats were orally exposed for 45 days with LD_{25}, they have lost 9% body weight while their control counterpart gained body weight by 38% (107). Therefore, aluminium was unlikely to produce any impact on the growth or body weight within the current short duration of *i.p.* exposure. However, the current study observed significant reductions in

absolute body weight and noticeable change in growth rate of young adult female rats, but not in their male counterparts. In study of developing brain pups from aluminium-exposed mothers showed significant reduction in their growth rate (108). Therefore, age and gender both are important to counter the aluminium-induced changes in growth rate.

Food and water balance contribute significantly in the maintenance of normal physiology and growth rate. Intraperitoneal administration of aluminium has demonstrated to reduce the food intake appreciably within two weeks of exposure in a study of four weeks (109). Similarly, another study of 4 weeks aluminium exposure through drinking water also reported decrease in food intake (110). Corroborating these observations, decrease in food intake was observed immediately with the initiation of aluminium exposure in both male and female groups. Present study was observed that after a day of treatment both groups shortened their food intake [Figure 4A]. Male group increased their food consumption on the 6^{th} day of treatment while female group fail to compensate their food consumption as compared to their male counterparts. Similar significant decrease in food intake was observed on the very first day of aluminium exposure (102) in male adult rats. However, the present study demonstrated substantial reduction in average food intake of female group that might have influenced the growth rate of young adult female group [Figure 4A].

Levels of serum TG were higher in aluminium-exposed male and female animals in comparison to their respective control in the current study [Figure 5A]. However, the differences between the control and study group were statistically significant only in female groups. Similar rises in serum TG were observed when male Wistar rats were orally exposed for 28 days to $AlCl_3$ with a dose of 100 mg / kg body weight (111) or 20 mg / kg body weight (112). High dose of oral $AlCl_3$ [175 mg / kg body weight] exposure for 60 days also significantly enhanced the serum TG level in comparison to the control and vehicle-treated groups (88). Other studies of 60 days exposure to $AlCl_3$ and $Al_2(SO_4)_3$ with 100 and 98 mg/kg body weight doses, respectively, demonstrated increments in serum TG levels of male rats compared to their respective control animals (53,113). A study of long-term exposure [5 months] to $AlCl_3$ through drinking water also noticed enhancement in serum TG levels of female Wistar rats in comparison to their control counterparts (114). Intraperitoneal exposure to 70 mg $AlCl_3$ / kg body weight for 8 weeks also raised the serum TG level significantly (115). Even single dose of $AlCl_3$ [100 mg / kg body weight] could significantly increase the serum TG level after 45 days of exposure (89). On the other hand, oral exposure of 50 and 100 mg $AlCl_3$ per kg body weight in male Wistar rats demonstrated significant decrease in serum TG

after 28 days, even though the basal levels of experimental groups were more than 50% higher than the basal TG level of the control group (87). An earlier study also demonstrated significant decrease in serum TG levels with dietary $AlK(SO_4)_2$ exposure for 67 days while same regimen with $Al(OH)_3$ showed insignificant decrease in both young and adult rats (106). Every alternate day *i.p.* $AlCl_3$ exposure [8.3 mg/kg body weight] for 30 days also showed only insignificant alteration in serum TG of male Wistar rats (116). Increase in serum TG level was also observed in an eight weeks study with 10 mg $AlCl_3$/kg body weight of Wistar rats (117). Reported evidences suggest that aluminium could produce hypertriglyceridemia or hypotriglyceridemia in male rats. The observed increase in serum TG levels could also be ascribed to suggested hypoactivity of TG decomposing lipoprotein lipase in blood vessel (118).

Most of the available reports of aluminium-induced alterations in lipid profile demonstrated similar trends in all the lipid profile parameters. Increases in serum TG upon aluminium insult was associated with increases in serum TC (53,88,89,111–115,117) with a suggestion of increased cholesterol synthesis in liver of aluminium-exposed animals (119). Contrary to these, the current study demonstrated statistically significant decreases in serum TC level with *i.p.* exposure to $AlCl_3$ for 15 days in both male and female animals against their counterparts in control groups [Figure 5B]. Similar decrease in serum TC level was also noticed in an experiment of oral exposure to $AlCl_3$ for 14 days, insignificantly in lower dose and significantly in higher dose (86) in male rats only. In addition, insignificant alterations in serum TC were also seen in both young and adult animal groups exposed to dietary $Al(OH)_3$ and $AlK(SO_4)_2$ for 67 days (106). Similarly, insignificant alteration of serum TC was also observed in male Wistar rats exposed to $AlCl_3$ [8.3 mg/kg body weight, *i.p.*] on every alternate day for 30 days (116). Therefore, the current study is not in corroboration with good number of earlier reports of aluminium-induced hypercholesterolemic effects; however, this type of hypocholesterolemic impact of aluminium is also not an unprecedented one. Even though, both genders are compeer in terms of overall bearing of aluminium on serum TC levels, the impact is more detected in the male group of study animals [Figure 5B]. It was suggested that increased serum levels of TG and TC indicate functional abnormality of liver (118), the current study with aluminium-induced increase in serum TG and decrease in TC suggest some degree of compromise in hepatic functionality.

Likewise, decrements in serum LDL levels were also observed in aluminium-exposed groups of both genders; nevertheless, the difference between the experimental and control group was not statistically significant in female animals [Figure 5C]. Thus, the observed LDL-

lowering effect of cholesterol was less prominent in female study groups. Like that of serum TG and TC levels, available reports indicated equivocal responses of serum LDL in response to aluminium exposure in rats. Even though, available reports are based on mostly male rats; decrease in serum LDL after 21 days of exposure to $AlCl_3$ through drinking water in female rats was also available (119). One long term study with exposure to $AlCl_3$ through drinking water for 5 months also reported significant increase in serum LDL levels compared to control group (114); while, an eight months study demonstrated no significant impact of $AlCl_3$ on serum LDL level (117). Current study indicates that gender is an important factor in terms of serum LDL level. It is worth mentioning here that use of alum as adjuvant decreased the oxidized LDL level and provided the 'atheroprotection as side effect' in vaccination (120,121).

Interesting to note that only study with female Wistar rats showed insignificant alteration in serum HDL level even after 5 months long exposure to $AlCl_3$ (114). Similarly, a single exposure study with oral $AlCl_3$ also demonstrated insignificant alterations in serum HDL after 45 days of the exposure (89). All the other available reports evidenced significant decrease in serum HDL level after exposure to different types of aluminium salts through different roots for various durations (53,88,89,111–115,119,122). Highlighting the opposite response of LDL and HDL for the same aluminium insult in the same animals, abnormal activities of lipase was suggested for the observed aluminium-induced hypercholesterolemia (119). Present study with 15 days of *i.p.* exposure to $AlCl_3$ also demonstrated significant decline in serum HDL levels in both male and female study groups in comparison to their respective control groups [Figure 5D]. Noticeably, the decrements in serum HDL were more pronounced in male study groups.

By measuring total serum protein, an approximation about the functional changes in liver can be made, while it could provide a rough estimation protein status (113). While, the levels of serum protein, serum albumin and serum globulin indicate the functional [metabolic] status of liver (123). In the present study, aluminium-exposed groups demonstrated decrease in serum levels of total protein, though statistical significance was observed only in male adult groups [Figure 6A]. Significant decline in total serum protein level was also observed in male Wistar rats orally exposed to $AlCl_3$ for 21 days at a dose of 40mg/kg body weight (124). Similarly, oral exposures to $AlCl_3$ [34mg/kg body weight] for 30 days (91) and $AlCl_3$ [50mg/kg body weight] for 4 weeks (93) caused significant reductions in serum total protein levels. Aluminium-induced decreases in serum protein were also reported in chicken (123), mice (125) and rats (102). Thus, the decreasing effect of aluminium exposures were observed in different species and the impact was also passed to the next generation from aluminium-exposed adult

males (126). On the contrary, oral exposure to $AlCl_3$ [100 mg/kg body weight] for 60 days reported to increase the serum protein level significantly (113). Similarly, oral exposure to $AlCl_3$ [40mg/kg body weight] for a month demonstrated increased serum protein levels in matured male rat (127). On the other hand, oral exposure to 10 mg $AlCl_3$/kg body weight for 8 weeks (117) and 70 mg $AlCl_3$/kg body weight for 4 weeks (128) to male Wistar rats did not document any significant alteration in serum protein level. Even though, serum total protein level is an important parameter, variations in its response to aluminium exposure were documented. In the current study, decrement in serum protein was obvious in male aluminium-exposed groups; however, not in females. This protection in females from the aluminium-induced declining in serum protein level also support the gender inequality in terms of aluminium-induced toxicity. The observation of serum protein level could agree with the suggested mechanism of possible inhibitory effect of $AlCl_3$ (85). Additionally, decreased food intake could also be associated with the observed decrement in observed serum total protein levels (85,129). The possibility of proteinuria because of aluminium exposure also could not be ruled out as underlying cause of the observed decrement in serum protein level (117).

In the same line, there were reports showing no change in serum protein, albumin, globulin and A/G ratio (128), increase (113,125,127,130) and decrease (91,93,117,123) in serum albumin level, increase (113,130) and decrease (91) in serum globulin level, and increase (130) in serum A/G ratio. Diminished total protein levels along with albumin levels in serum observed in the current study [Figure 6B] might indicate towards the compromised liver functions as declined serum albumin level was suggested as an indicator of functional [anabolic] anomaly of liver (118). Changes in serum albumin and serum globulin levels were ascribed to direct toxic effects of aluminium on protein metabolic demand following the stress of aluminium exposure (130). Both undernutrition as indicated by comparatively slower growth rate and possible drop in anabolic [protein] activity of liver were attributed as suggested cause for the lowered serum albumin level (129,131). Unaltered serum globulin level could be because of compensation of loss in globulin by partial increment of it as consequence of reticuloendothelial inflammatory response what happens in hepatocellular damages (130). In accordance to these reports, the present study demonstrated unaltered serum globulin levels in the aluminium-exposed groups; nevertheless, decreasing trends in serum globulin levels could be noticed [Figure 6C]. Calculation of A/G ratio from the reported mean values also suggested possible decrements in its value in the aluminium-exposed groups (91,113).

Increments in total bilirubin level of serum were found to be associated with the various types of experimental aluminium insults (38,91,93,117,123,132,133) to various species. These observed rises in serum bilirubin levels were directly linked with the 'damaging effect' of aluminium on liver cells (131). Compromised functional status of liver including uptake and conjugation was suggested possible mechanism of such aluminium-induced rise in serum total bilirubin levels (131,134). At the same time, raised level of oxidative stress (135) and periportal necrosis (131) were also ascribed as possible reason for elevated total bilirubin level in aluminium toxicity. Significantly higher levels of total and direct bilirubin levels were observed in workers who were exposed to aluminium dust and fumes for many years (71). They have also recorded an insignificant alteration in indirect bilirubin level of serum in the aluminium-exposed workers along with insignificant changes in bile acids (71) and suggested the impact of aluminium on the biliary secretory function. Aluminium was also found to be dose-dependently associated with canalicular microvilli blunting and hamper the biliary excretion (136). In the present study, the changes in serum total conjugated bilirubin levels were only altered insignificantly in both male and female study groups in comparison to their respective control animals [Figure 7A]. This observation corroborates the earlier report of insignificant alteration in serum total bilirubin level (113). However, in female groups of the present study, there was noticeable increase in the serum total conjugated bilirubin level of aluminium-exposed group which could not attain the level of statistical significance [$p = 0.08$]. It has been suggested that increased serum bilirubin levels indicate possible involvement of haemolytic conditions, incomplete metabolism of bilirubin by hepatocytes or biliary obstruction (123). Nevertheless, possible contribution of decreased albumin level in serum was also suggested for the observed increase in serum bilirubin and concurrent hepatotoxicity and other toxicities (123). Overall, the current observation possibility indicated towards initiation of aluminium-induced hepatotoxicity.

Contrary to the available reports of increased activities of alkaline phosphatase [ALP], aspartate aminotransferase [AST] and alanine aminotransferase [ALT] in serum in response to aluminium exposure (38,91,93,111,113,127,133,137–139), the current study demonstrated significant reductions serum ALP activities [Figure 7B], insignificant reductions in serum AST activities [Figure 7C] and no noticeable change in serum ALT activities [Figure 7D]. These changes in serum enzyme activities, commonly considered as marker of hepatic status, were corroborated with the observations of decreased serum ALP activities in rats with *i.p.* exposure to $AlCl_3$ [10mg/kg body weight] for 8 weeks and insignificant alterations in serum ALT and

AST activities of rats exposed to aluminium through drinking water [80mg $AlCl_3.6H_2O$/L] for 3 months (140) and in *Oreochromis niloticus* with low dose exposure to alumina nanoparticles [2 mg/L Al_2O_3] for 7 days (141). Differing from these reports, with a dose of 20% LD_{50}, oral gavage of $AlCl_3$ demonstrated significant increments in ALT and AST activities of serum within 5 days (83). The study also noted gradual increments in these enzyme activities with longer durations (83). Therefore, the current results indicated that the dose of aluminium, even though the exposure was given *i.p.*, was not sufficient to produce such level of hepatotoxicity that the marker enzyme activities could be elevated in serum, within 15 days of exposure.

Mentioning as a membrane-bound enzyme, increased serum ALP activities was ascribed as an index of membrane damage in $AlCl_3$ intoxication (111). Chronic exposure to aluminium was suggested to produce hepatocellular necrosis and raise the serum ALP activities (113); however, it was agreed upon that the observed change in serum ALP could be because of contribution form organs other than liver, as well. Interestingly, it has been reported that cholestasis could be one reason where serum ALP can be raised as liver ALP localizes in endothelial cells of the central and portal veins as in sinusoids and bile canaliculi (139,142). Which is corroborating the suggestions made based on alterations in serum total bilirubin level. On the other hand, increase in serum ALT and AST are commonly considered as consequence of deterioration of liver functions (111,113). Since, insignificant alterations of these enzymes advised that this low concentration and/or short duration of aluminium exposure might not be able to damage the hepatocyte cell membrane (141). Therefore, from the current results, aluminium-induced hepatotoxicity was not indicated. This could be because of short span of aluminium exposure [15 days] with very less dose [5mg/kg body weight], even though the current mode of exposure [*i.p.*] bypassed the natural barriers of exclusion. This view is also supported by the observation that the De Ritis ratio [AST/ALT], a marker of chronic hepatic fibrosis or cirrhosis (143), were decreased in both aluminium-exposed groups in comparison to their control counterparts. Thus, one side ruinous influence of $AlCl_3$ is suggested by observed decrease in serum total protein, albumin levels and A/G ratio; on the other side, no change in serum globulin levels and insignificant changes in serum transaminases along with significant alterations in ALP suggested that the current aluminium exposure could initiate the membrane damage only.

The potential of atherosclerosis occurrence possibilities, represented as atherogenic coefficient [AC] or atherogenic risk index (96) or atherogenic index (119), is calculated as the ratio of NonHDL over HDL (95), while the level of serum NonHDL is determined by

subtracting HDL from TC (94). It is a measure of fraction of cholesterol associated with all atherogenic lipoproteins with respect to fraction of cholesterol associated with lipoprotein that is considered to be nonatherogenic (144). AC was found to be increased in hyperlipidemic rats (145) and rats with diabetes mellitus showing hyperlipidemia (96). On the other hand, hypolipidemia was associated with decrease in the atherogenic coefficient (146) in rats treated with D-ribose-L-cysteine with prediction of decreased atherogenic lipoprotein status that, in turn, would decrease the atherogenic factors and coronary events. Significant rise in AC was noted along with dyslipidemia and cardiotoxicity in female Wistar rats exposed to 50 mg $AlCl_3$ through drinking water for 21 days (119). Therefore, the AC and other atherogenic indices could be used in rat study also. In the present study, significant increase in AC of male rats with aluminium exposure was noted while in female the increment was not statistically significant [Figure 8B]. The rise in AC was observed despite there was aluminium-induced hypolipidemic effects in terms of TC, LDL and HDL of serum [Figure 7]. Thus, current study demonstrated that even though the used dose and duration of aluminium exposure caused hypolipidemic effects, it increased the risk of atherogenesis and cardiovascular dysfunction.

Mere absence of dyslipidemia should not be the criteria to exclude the possibility of cardiovascular problems (144). To estimate the cardiovascular risk, commonly ratios of TC, LDL and HDL levels of serum are used in conjunction with AC. Ratios of TC/HDL and LDL/HDL are referred as Castelli risk index 1 [CRI-1] and Castelli risk index 2 [CRI-2], respectively. Increase in CRI-2 is also known as atherogenic dyslipidemia (96). In recent years, these atherogenic indices played more significant role than the original serum levels of cholesterols attached to different sizes of lipoprotein (144,147). Considering LDL/HDL and TC/LDL ratios as ‘pertinent indices for cardiovascular risk incidence [atherosclerotic index]’, rise in those ratios in female Wistar rats exposed to 50 mg $AlCl_3$/kg body weight for 21 days were reported along with rise in serum TG, TC, LDL and fall in serum HDL levels in comparison to control animals (119).

Most of the available reports that studied serum lipid profile with diversity of aluminium exposure for diverse durations have not reported any change in atherogenic indices. When the atherogenic indices were calculated from the values of lipid parameters available from these reports, most of them indicated changes in AC towards higher side (53,88,89,111–115,122). While the study showed aluminium-induced decrements in serum lipid parameters, calculation of the available data demonstrated either decrease or no-change in most of the indices except

CRI-2 (87). Similarly, deductions from data of the study which found no significant alteration in serum lipid parameters also demonstrated either fall or no-change in all the calculated lipid indices (116). Interestingly, the current study documented significant or insignificant rise in all the parameters except serum NonHDL level [Figure 8]. A decreasing trend in lipoprotein combine index [LCI] was also noticed in male rats exposed to aluminium of the current study; nevertheless, without statistical significance [Figure 8E]. Therefore, the current study presented a condition of higher atherogenicity associated with hypocholesterolemia and hypertriglyceridemia caused by 15 days of intraperitoneal administration of aluminium in young adult rats with subtle differences between genders.

With transmission electron microscopy, accumulation of aluminium was reported in the liver lysosomes in a study with *i.p.* exposure to aluminium for two weeks (148). Thus, aluminium is likely to be accumulated in liver tissues in the present study also. Echoing the observed trends in changes of lipid parameters in serum, the given dose and duration of aluminium exposure caused very similar changes in hepatic lipid parameters. Like that of serum triglyceride levels, hepatic TG also found to be raised significantly in females while insignificantly in males [Figure 9A]. Only report of aluminium-induced changes in hepatic TG documented a decrease in young animals (106). All the other tested lipid parameters in liver tissue were found to be decreased, as observed in serum, in response to *i.p.* aluminium exposure to rats. However, the levels of statistical significances were different [Figures 5 and 9]. Interestingly, the changes were statistically significant in the female study group, while only hepatic HDL demonstrated statistically significant difference in male group also [Figure 9].

Corroborating the decrease in serum protein level, hepatic total protein level was also found to be reduced in aluminium-exposed animals of both male and female study groups [Figure 10A]. Decrease in hepatic total protein in rats with oral $AlCl_3$ exposure for [1/25 LD_{50} every alternate day, for 30 days] was also demonstrated earlier (91). The same study also documented significant fall in hepatic ALP activity in the aluminium-exposed Sprague-Dawley rats compared to the control rats and used the suggestion of enhanced membrane permeability or cellular necrosis to explain the observed decline in hepatic ALP (91). Similarly, in rabbit, aluminium exposure caused decrease in the hepatic ALP activity (132) and suggested the same. On the contrary, Sprague-Dawley rats exposed orally to 100 mg $AlCl_3$ /kg body weight for 2 months demonstrated increased hepatic ALP activity and Kupffer cell activation as well as aluminium-induced cholestasis were suggested to be responsible for such increment (92). Only insignificant alterations in hepatic ALP activity were noted [Figure 10B] in the current study.

It is noteworthy that despite statistical insignificance the changes in hepatic ALP activities in male and female groups were in reverse directions [Figure 10B]. Thus, the current data indicated insufficiency of aluminium insult, either in dose or in duration; however, gender might be a deciding factor in the manifestation of the response to aluminium exposure.

Decreases in hepatic transaminases were reported in response to aluminium exposure to rats (91,92) and rabbit (132). In agreement with those reports, male and female rats exposed to aluminium demonstrated significant falls in hepatic AST and ALT activities in the present study [Figures 10C and 10D]. The decrease in AST and ALT activities were suggested to be consequence of cellular degeneration in liver which might lead to pathological lesions (92). Thus, irrespective of gender, the current aluminium insult produced some hepatocellular destruction without membrane damage and leakage of the enzymes into the serum.

Light microscopy of formalin-fixed liver tissues with H-E staining showed identifiable hepatic lobules. Surrounding a central vein, radiating arrays of hepatic plates form a nearly hexagonal appearance. The lobules are interspersed by interlobular septa and traversing portal veins. The nicely arranged hepatocytes had clear boundaries with granular cytoplasm and round nuclei.

Aluminium exposure in different doses [generally toxic] and for different duration [generally more than 21 days] could cause dilatation of sinusoids (93,107,118,128,137,149,150) that were accompanied by hepatocyte columns (151) with hepatocyte cord disruption (92,118). Apart from disorganized appearance (83,84,141,152) hepatocytes also demonstrated cytoplasmic alterations (151) with inclusion of vacuoles (84,107,141) in cells with higher severity in the periportal regions (92) along with diffuse ballooning (128) degeneration / necrosis (84,93,107,117,127,141). In addition, cellular damage was also seen with pyknotic nuclei (83,107,118,127,141) and cell membrane disruption (118). Aluminium intoxication also reported appearance of scattered RBCs in the hepatic sinuses (83,117,141) leading to congestion sometime (149). Central vein congestion with dilatation (93,107,128,137,141,150) was also found to be associated with aluminium insult. Inflammatory [mononuclear] cell infiltration (83,93,150) were also reported in severe aluminium toxicity. However, in the present study, derangement of hepatic architecture, sinusoidal dilatation and central vein congestion with dilatation were noticed in the aluminium-exposed male as well as female groups [Figure 12]. Hepatocytic degeneration with cytoplasmic vacuolation were also notice in both the aluminium-exposed groups [Figures 13 and 14]. Nevertheless, the numbers of hepatocytes with cytoplasmic vacuolation were relatively less in

the female aluminium study group, despite both the study groups demonstrated significant increase in that [Figure 11C].

Supporting the observations from biochemical studies, the qualitative and quantitative histopathological study suggested that the current dose of aluminium exposure [5 mg/kg body weight, *i.p.*] for short duration [15 days] could initiate the hepatic toxicity in young adult rats; however, the severity of hepatotoxicity was insidious with some degree of gender biasness.

Interesting to note from the present study that the current dose and duration of aluminium exposure could produce dyslipidemia with hypertriglyceridemia and hypocholesterolemia in young adult rats without much gender difference. Surprisingly, the hypocholesterolemia in these aluminium-exposed animals worsen the atherogenic indices suggesting higher risk of cardiovascular problems.

SUMMARY

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CONCLUSION

Aluminium is an omnipresent metal without any defined beneficial role in biology. Because of wide and extensive uses, exposure to aluminium is unavoidable. Normally, because of restricted entry and efficient elimination procedure, the level of aluminium internalization is quite less and the impacts of aluminium exposure remain only covert. Aluminium is a known neurotoxin even though the brain shares a small portion of total body aluminium burden. Relatively, larger level of aluminium accumulates in liver. Nevertheless, aluminium-induced hepatotoxicity is acknowledged and studied only recently. There are reports indicating possibilities of impaired lipid metabolism and dyslipidemia associated with aluminium toxicity. Thus, the present study was taken up to study the aluminium-induced alterations in lipid profile and hepatic functions.

Male and female Wistar rats were exposed intraperitoneally to $AlCl_3$ [5mg/kg body weight] for 15 days with an intention to produce a systemic load of aluminium which might cause some degree of hepatotoxicity. Four groups of rats, with six animals each, were maintained in standard laboratory condition with exposure to aluminium in Al-5 [Experimental] groups and without exposure to aluminium in Al-0 [Control] groups. After 15 days, blood and tissue samples were collected after sacrifice. With the available reports of aluminium as metalloestrogen, gender inequality for aluminium-induced dyslipidemia and atherogenic indices were also evaluated in the present experimental study.

The aluminium exposure protocol could produce a transient phase of decrease in food and water intakes with the initiation of *i.p.* injections that were not seen in control groups. There were some differences in the pattern of resuming food and water intakes between the male and female experimental groups. Even though statistically significant difference in final absolute body weights of studied animal groups were not observed, noticeable difference between the control and experimental groups were observed in terms of their growth rate during the study period.

Increase in serum triglyceride level in female rats only and decrease in serum low density lipoprotein cholesterol in male rats only were statistically significant, while both male rats and female rats demonstrated statistically significant decreases in serum total cholesterol and serum high density lipoprotein cholesterol, when Al-5 groups were compared with respective Al-0 groups. Thus, gender differences could be identified in the impacts of short-term aluminium exposure in terms of serum lipid profile; however, even in noted insignificant changes the trend of alterations were like that of significant alterations.

Similarly, animal groups of both genders demonstrated identical trends in the alterations of serum total protein, serum globulin and serum albumin / globulin ratio. However, evaluation of statistical tests demonstrated dissimilarities between the genders with statistical significance in males but not in females. On the other hand, no dissimilarities between genders were noticed in serum total conjugated bilirubin levels as well as activities of alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase in serum.

The current aluminium exposure produced significant decrease in nonHDL level of serum in both genders of rats. All other calculated atherogenic indices were found to be raised in response to current dose of short-term aluminium exposure. However, there were gender inequality in terms of statistical significance in these calculated indices. Male rats showed significant alterations in atherogenic coefficient, Castelli risk index 1 and atherogenic index of plasma, while female rats showed significant alterations in Castelli risk index 2, lipoprotein combine index and atherogenic index of plasma. Hence, the gender inequality was seen in the alterations of atherogenic indices in response to current aluminium insult in rats.

Female rats showed significantly higher hepatic triglyceride level along with significantly lower hepatic total cholesterol, hepatic low-density lipoprotein cholesterol and hepatic high-density lipoprotein cholesterol in aluminium-exposed rats when compared with the control groups of rats. Contrary to these, male experimental group demonstrated significant decrease only in hepatic high-density lipoprotein cholesterol in comparison to male control group of rats. These observations of hepatic lipid profile also evinced the gender dimorphism in response to aluminium exposure in the current study.

On the contrary hepatic total protein level, hepatic alanine aminotransferase activity and hepatic aspartate aminotransferase activity were evenly altered in male and female rats in response to aluminium exposure in the present study. All these parameters demonstrated significant reduction in their levels or activities in the experimental animals in comparison to the control animals. In addition, hepatic alkaline phosphatase activity was found to be altered statistically insignificantly irrespective of gender group, in the same study. Histological studies also could not support difference between the genders in terms of cytosolic vacuolation; however, there were some gender variations in the extent of damages in hepatocytes.

The study presented here, therefore, confirm the hepatotoxicity and alterations lipid profile by the low-dose short-term aluminium exposure. The study also evidenced gender inequality in some parameters, but it was surely not a generalized phenomenon.

STRENGTHS

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LIMITATIONS

Strengths of the study

- Current study of short-term low-dose intraperitoneal aluminium chloride exposure demonstrated aluminium-induced alterations in serum lipid profile and worsening of the atherogenic indices in young adult rats.
- Present study also demonstrated aluminium-induced hepatotoxicity with initiation of hepatocyte degeneration by this short-term low-dose aluminium exposure in young adult rats.
- The study also indicated gender inequality in lipid profile and atherogenic indices and to a lesser extent in hepatotoxicity.

Limitations of the study

- Estimation of aluminium levels in serum and liver could have provided more specific results.
- Specific aluminium staining along with histological staining would have provided better understanding about the association between aluminium exposure and hepatotoxicity.
- Variable doses and durations of aluminium exposures could have provided better evidence for the support of the current observations.

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ANNEXURES

Institutional Animal Ethics Committee
All India Institute of Medical Sciences, Jodhpur

Certificate

This is to certify that the project proposed no. **AIIMSJ/IAEC/2022/2** entitled **Study of gender inequality in aluminium-induced alterations in lipid profiles and hepatic functions submitted** by **Ms. Priyanka Tanwar** has been approved by the IAEC of Central Animal Facility, AIIMS Jodhpur in the meeting held on 13-08-2022 and **24** Wistar rats have been sanctioned under this proposal for a duration of next **6** months.

Authorized by	Name	Signature	Date
Chairman:	**Dr. Sneha Ambwani**	Sneha	13.08.2022
Member Secretary:	**Dr. Jaykaran Charan**	Jaykaran	13.08.2022
Main Nominee of CPCSEA:	**Dr. Kamal Purohit**	Kamal	13.08.2022

68th Annual National Conference of Association of Physiologists and Pharmacologists of India

Under the aegis of Association of Physiologists & Pharmacologists of India : Chandigarh Chapter

CERTIFICATE

This is to certify that Dr./Mr./Ms. PRIYANKA TANWAR

has participated as Invited Speaker / Resource Faculty / Chairperson / Judge / Organiser / Delegate and presented a paper titled short term aluminium exposure worsen the atherogenic indices even in absence of hyperlipidemia in rats.

in the conference held from 12th-15th December 2022 at PGIMER, Chandigarh.

Accredited 11 hours by the Punjab Medical Council (PMC/CME/2022/5650, dated 23.11.2022)

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Punjab Medical Council

Dr. Anita S. Malhotra *Organizing Chairperson*	**Dr. Anil K. Pandey** *General Secretary, APPI*	**Dr. Vijay Kumar** *Member, PMC*
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APPICON 2022 CHANDIGARH

68th Annual National Conference of
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Translational Medicine: Molecules to Individual

Department of Physiology & Pharmacology, GMCH, Chandigarh, Department of Pharmacology, PGIMER
13th – 15th December, 2022

Abstract

Short term aluminium exposure worsens atherogenic indices even in absence of hyperlipidemia in rats

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Introduction : Hyperlipidemia and associated atherosclerosis are common causative factors for cardiovascular diseases. These can also influence the development and outcome of stroke. Serum levels of lipid parameters ar used to predict the risk of these problems. However, in some cases, conventional lipid parameters fail to explai the Indian scenario. In a recent study, the impact of short-term aluminium (Al) exposure on the serum lipid profile in adolescent rats has been observed

Methods : Male and female Wistar rats were exposed to Al (5 and 10 mg/kg bw; i.p.) for 15 days. Levels of seru triglycerides (TG), cholesterol (TC), low-density lipoprotein cholesterol (LDL), and high-density lipoprotein cholesterol (HDL) were measured. Comprehensive lipid indices – NonHDL (NH), Atherogenic Coefficient (AC), Castelli Risk Index 1 (CRI1), Castelli Risk Index 2 (CRI2), Atherogenic Index of Plasma (AIP), Lipoprotein Combine Index (LCI), were calculated and compared with control values (Al-0).

Results : This short-term Al exposure significantly reduced the serum TC, HDL and LDL; however, serum TG was significantly elevated only in female groups. The levels of NH are reduced significantly in Al-5 groups but insignificantly in Al-10 groups for both genders. Al-10 demonstrated significantly raised CRI1 and CRI2 compared to respective Al-0 groups; while, the effects on Al-5 groups are gender specific. Similar changes in A are also noted. In the case of LCI, male groups did not show any significant alterations, while Al exposure raisec the LCI in female rats. Nevertheless, both male and female groups demonstrated a significant rise in AIP in response to both doses of Al exposures.

Conclusion : Thus, the current results demonstrated the lack of hyperlipidemia, except hypertriglyceridemia, in response to short-term Al exposures; however, we observed significant alterations in lipid indices. The protecti against atherogenicity was dependent on the dose of short-term Al exposure as well as gender-specific with males being affected more.

Annotations : Animal -Rat ; Cardiovascular ; Environment ;

website: appi.org.in, appicon2022.in