

Comparative Biochemical Profiling of Aluminum Chloride and Sodium Azide Induced Neuroinflammation and Cardiometabolic Disturbance

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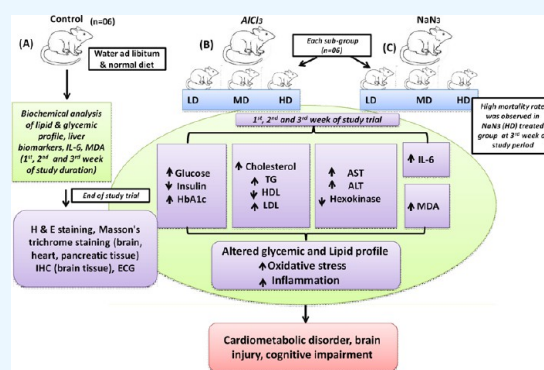
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ABSTRACT: Comorbidities in human beings signify the numerous risk factors that increase the incidences of neuro- and cardio-metabolic disorders. Experimental models depicting comorbidities are important to explore the molecular pathophysiology that can help suggest appropriate treatment strategies. Tissue-accumulating potential and pathological effects of aluminium chloride (AlCl_3) and sodium azide (NaN_3) are well recognized. Hence, in the current work, we have for the first time aimed to investigate the unexplored potential of graded dose effects of AlCl_3 and NaN_3 in inducing early inflammation and cardiometabolic toxicity *via* comparative biochemical analysis of AlCl_3 and/or NaN_3 . Rats were allocated into seven groups ($n = 6$). Group 1 was normal control. Remaining groups were given graded doses of AlCl_3 and/or NaN_3 , as LD- AlCl_3 (AlCl_3 40 mg), MD- AlCl_3 (AlCl_3 45 mg), and HD- AlCl_3 (AlCl_3 50 mg) representing low dose, medium dose, and high dose of AlCl_3 , respectively, and the remaining as LD- NaN_3 (NaN_3 13 mg), MD- NaN_3 (NaN_3 15 mg), and HD- NaN_3 (NaN_3 17 mg) representing low dose, medium dose, and high dose of NaN_3 , respectively. Serum levels of glucose, insulin, lipid profile, inflammatory mediators like IL-6 and oxidative stress marker, and malondialdehyde (MDA) were analyzed. Likewise, subacute toxicity parameters were analyzed. Immunohistochemistry (IHC) and histopathology (H&E/Masson's trichrome staining) of brain, heart, and pancreatic tissues were done. ECG pattern of all groups was observed. HD- AlCl_3 was associated with elevated levels of inflammatory biomarkers, MDA, and glycemic and lipid profiles, whereas it decreased the insulin levels. HD- NaN_3 also showed the similar effects of aggravated inflammatory biomarkers, impaired glycemic and lipid profiles, but depicted the maximum mortality rate as compared to HD- AlCl_3 . IHC showed prominent amyloid plaques and neurofibrillary tangle formation with MD- AlCl_3 and HD- AlCl_3 as compared to NaN_3 -treated groups. Likewise, in brain tissues, vacuolation of white matter, vascular congestion, and hemorrhage were seen in HD- AlCl_3 treated group, while HD- NaN_3 induced death in animals. AlCl_3 exposure resulted in an inverted QRS complex, while exposure to NaN_3 showed ST depression but with increased mortality. AlCl_3 has better controlled results as compared to NaN_3 for induction of comorbid experimental animal model depicting early neuroinflammation and cardiometabolic disruption. These determined efforts facilitate the researchers for the development of clinically effective treatment strategies using such experimental models.



INTRODUCTION

According to World Heart Federation, about 18.6 million people globally die because of cardiovascular diseases (CVDs), which is almost 31% of all deaths. Among this, 4 million deaths are from south-east Asia.¹ Likewise, according to International Diabetes Federation, about 463 million people globally are affected by diabetes mellitus (DM) which makes it 1 in every 11 person which can increase up to 700 million or more by 2045.² Correspondingly, these federations have also recommended that individuals with DM are 2–3 times more vulnerable to progress with CVDs.^{1,2} Nevertheless, new technologies like artificial neural network³ have emerged for better decision making by clinicians and for predicting the complications of disorders like CVDs.⁴ Likewise, hybrid

artificial neural network-based monarch butterfly optimization algorithm for better disease classification⁵ has been suggested by different scientists.

Cardiometabolic disorders (CMDs) are a cluster of diseases including impairment and cardiac/metabolic dysfunctions. CMDs are primarily characterized by impaired glucose tolerance, hypertension, insulin resistance, and dyslipidemia,

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where excess secretion and accumulation of free fatty acids are linked with insulin resistance *via* reduction in transport and muscular uptake of glucose. Among various factors responsible for the progression and development of CMDs and other ailments, lack of physical activity, family history, dietary habits, and environmental and genetic factors are the most critical.⁶ On the other hand, many traditional medicinal bioactive components have been evaluated against such ailments.⁷

As CMD is progressive in nature comprising of multiple clinical manifestations, studies have also supported their linkage with each other, where people with DM have been reported to be more susceptible to have CVDs.^{1,2} Numerous chemicals have been experimented for the purpose of induction of CMDs in experimental models to study either the molecular pathophysiology or treatment and management of these diseases. For instance, adult rats treated with monosodium glutamate have been observed to be affected by neurotoxicity which can lead to the loss of protective neurons,⁸ whereas in another study, dystrophic and necrotic changes were observed in rats' myocardium after exposure to adrenaline.⁹ Likewise, vecuronium bromide has been used in experimental work to induce cardiac arrest in the experimental model.¹⁰ Similarly, among such other chemical compounds, aluminium chloride (AlCl₃) and sodium azide (NaN₃) have been specifically experimented for the induction of neurological and other disease symptoms.^{11,12} AlCl₃ has been known to have a tendency to accumulate in various tissues of the body such as liver, pancreas, kidney, brain, and heart. It has been associated with hepatic dysfunction, nephron toxicity, neurotoxicity, and cardiotoxicity^{11,13,14} *via* generation of ROS species and induction of oxidative stress, mitochondrial dysfunction, and lipid peroxidation. Further, the molecular basis for the effect of AlCl₃ toxicity may include the reduction in neuronal plasticity and suppressed axon maintenance in the hippocampus.¹⁵ Nevertheless, being the underlying cause of many diseases, oxidative stress biomarkers vary depending on some factors like seasonal impact of the disease.¹⁶ However, long-term administration of AlCl₃ results in AlCl₃ getting predominantly stored in the hippocampus and cortex of the brain leading to compromised cognitive ability, memory, and learning skills.¹⁷ Likewise, researchers have reported that NaN₃ has a critical role in affecting and inhibiting the cytochrome oxidase activity, altering the hepatic hexokinase, declining the activity of lactate dehydrogenase, and stimulating the glycolytic pathway.¹⁸ Correspondingly, NaN₃ has been suggested to interfere with mitochondrial respiratory chain complex IV as an inhibitor, which indicates NaN₃ as a suitable chemical compound available for induction of cytotoxicity in cardiac myocytes, astrocytes, glial, hepatic, and pancreatic cells.¹⁹

In the context to recent literature previewed above, the tissue-accumulating potential and pathological effects of AlCl₃ and NaN₃ are well recognized. Hence, in the current work, we have aimed to investigate the unrevealed graded dose effects of AlCl₃ and NaN₃ on the induction of early inflammation along with a disturbed glucose and lipid profile in relation to tolerability and mortality. This could help in conducting future research relating to either diagnosis and/or treatment/management using the comorbid experimental animal model of metabolic dysfunction with inflammatory symptoms.

RESULTS

Effect of AlCl₃ and NaN₃ Exposure on Glycemic Profile. Rats were exposed to different doses of AlCl₃ and

NaN₃ to assess the effect of both compounds on glucose (Figure 1A), insulin (Figure 1B), and HbA1c (Figure 1C) at

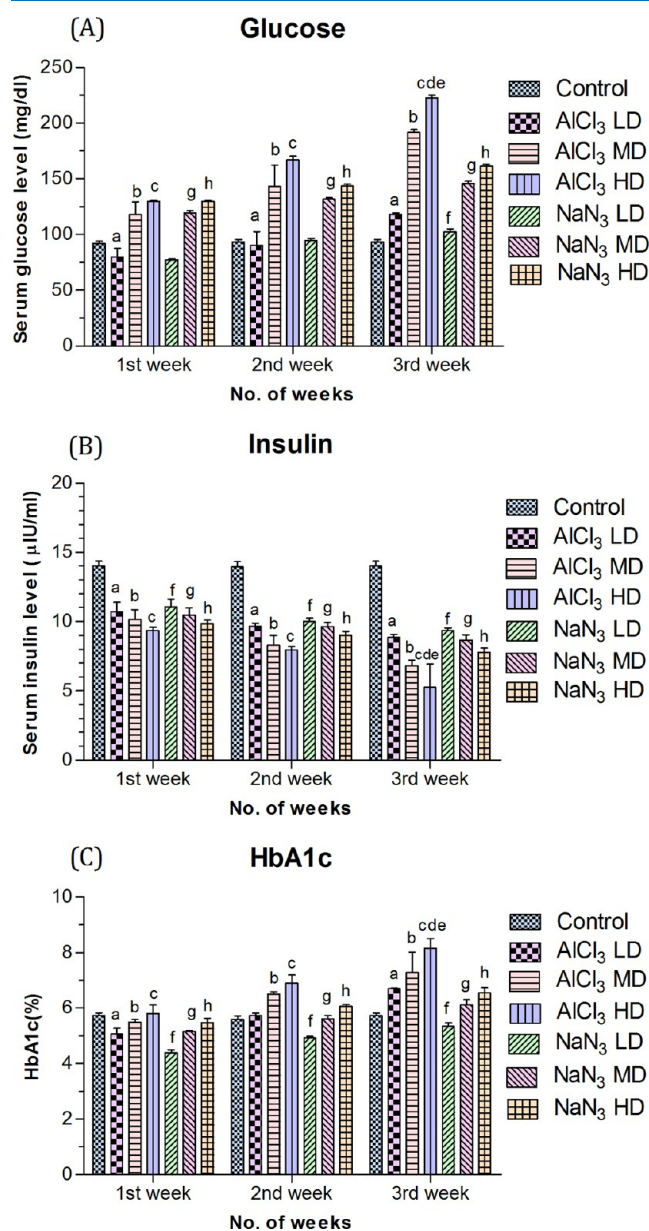


Figure 1. Effect of treatment on glucose (A), insulin (B), HbA1c (C). The effect of treatment on glycemia at 1st, 2nd, and 3rd weeks of the treatment period was measured. The level of significant difference was estimated by Bonferroni post-test using two-way ANOVA. (a) Represents $P < 0.01$ when compared AlCl₃ (LD) treated group with control group. (b) Represents $P < 0.01$ when compared AlCl₃ (MD) treated group with the AlCl₃ (LD) group. (c) represents $P < 0.01$ when compared AlCl₃ (HD) treated group with the control group, AlCl₃ (LD) and AlCl₃ (MD) treated groups. (d) Represents $P < 0.001$ when compared AlCl₃ (HD) alone treated group with AlCl₃ (LD) alone treated group. (e) Represents $P < 0.001$ when compared AlCl₃ (HD) treated group with AlCl₃ (MD) alone treated group. (f) Represents $p < 0.01$ when compared NaN₃ (LD) treated group with the AlCl₃ (LD, MD and HD) treated groups. (g) represents $P < 0.01$ when compared NaN₃ (MD) treated group with the NaN₃ (LD) treated group. (h) represents $P < 0.01$ when compared NaN₃ (HD) treated group with the control, NaN₃ (LD and MD) treated groups. LD: low dose, MD: medium dose, HD: high dose, AlCl₃: aluminum chloride, NaN₃: sodium azide.

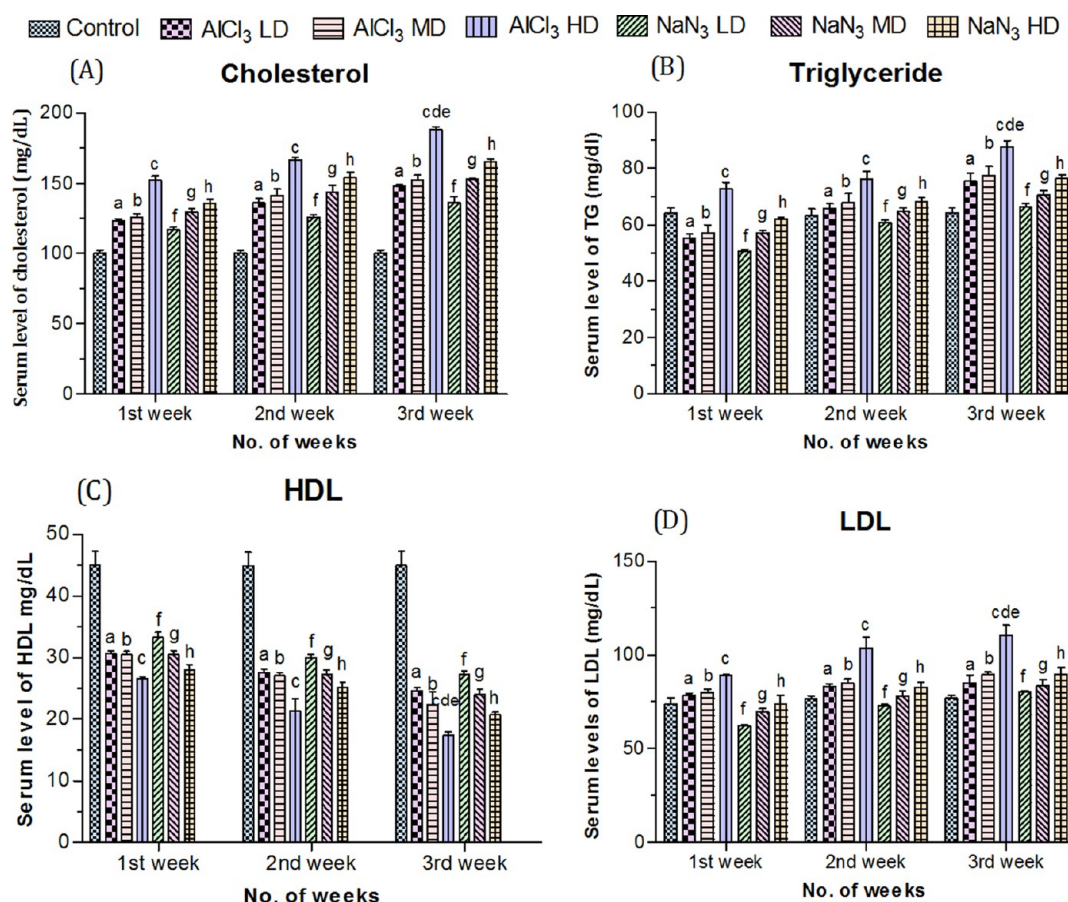


Figure 2. Effect of treatment on cholesterol (A), triglyceride (B), HDL (C) and LDL (D). The effect of AlCl₃ and NaN₃ on lipid profile at 1st, 2nd, and 3rd weeks of the treatment period was measured. The level of significant difference was estimated by Bonferroni post-test using two-way ANOVA. (a) Represents $P < 0.01$ when compared AlCl₃ (LD) treated group with control group. (b) Represents $P < 0.05$ when compared AlCl₃ (MD) treated group with the AlCl₃ (LD) group. (c) Represents $P < 0.001$ when compared AlCl₃ (HD) treated group with the control group, AlCl₃ (LD) and AlCl₃ (MD) treated groups. (d) represents $P < 0.001$ when compared AlCl₃ (HD) treated group with AlCl₃ (LD) alone treated group. (e) Represents $p < 0.001$ when compared AlCl₃ (HD) treated group with AlCl₃ (MD) alone treated group. (f) represents $p < 0.01$ when compared NaN₃ (LD) treated group with the AlCl₃ (LD, MD and HD) treated groups. (g) Represents $p < 0.01$ when compared NaN₃ (MD) treated group with the NaN₃ (LD) treated group. (h) Represents $P < 0.01$ when compared NaN₃ (HD) treated group with the control, NaN₃ (LD and MD) treated groups. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl₃: aluminum chloride, NaN₃: sodium azide.

first, second, and third weeks of the study duration. We observed that the serum levels of glucose and HbA1c were significantly increased ($P < 0.05$), and the insulin level was significantly decreased ($P < 0.05$) after exposure to AlCl₃ (40 mg/kg/day) and NaN₃ (13 mg/kg/day) at first, second, and third weeks of treatment. We found that at the second week of study period, the serum levels of glucose ($P < 0.01$) and HbA1c ($P < 0.01$) in AlCl₃ (45 mg/kg/day) treated groups were elevated, while the level of insulin ($P < 0.01$) was declined when compared with NaN₃ (15 and 17 mg/kg/day) treated groups. NaN₃ (17 mg/kg/day) treated group showed mortality at the third week of study period and less significant effect to decline the serum level of insulin ($P < 0.01$) and elevate the serum levels of glucose ($P < 0.01$) and HbA1c ($P < 0.01$) when compared with the AlCl₃ (50 mg/kg/day) treated group. However, AlCl₃ (50 mg/kg/day) treated group showed maximum effect in decreasing the serum level of insulin ($P < 0.001$) and increasing the serum levels of glucose ($P < 0.001$) and HbA1c ($P < 0.001$) at the third week of study period.

Effect of AlCl₃ and NaN₃ Exposure on Lipid Status.

Rats were exposed to different doses of AlCl₃ and NaN₃ to assess the effect of both compounds on cholesterol (Figure

2A), triglycerides (TGs) (Figure 2B), high-density lipoprotein (HDL) (Figure 2C), and low-density lipoprotein (LDL) (Figure 2D) at the first, second, and third weeks of treatment duration. We observed that AlCl₃ (40 and 45 mg/kg/day) treated groups showed less significant effect ($P < 0.05$) in declining the serum level of HDL and elevating the serum levels of cholesterol, TG, and LDL when compared with the AlCl₃ (50 mg/kg/day) treated group. At the second and third weeks of study duration, NaN₃ (13, 15 and 17 mg/kg/day) treated groups showed less significant effect in increasing the serum levels of cholesterol ($P < 0.05$), TG ($P < 0.05$), and LDL ($P < 0.05$) and decreasing the serum level of HDL ($P < 0.05$) when compared with the AlCl₃ (50 mg/kg/day) treated group. At the third week of treatment duration, we found that the AlCl₃ (50 mg/kg/day) treated group showed maximum effect and declined the serum level of HDL ($P < 0.001$) and elevated the serum levels of cholesterol ($P < 0.001$), LDL ($P < 0.001$), and TGs ($P < 0.001$).

Effect of AlCl₃ and NaN₃ Exposure on Liver Enzymes and Glycolysis. The effects of both compounds were assessed on aspartate aminotransferase (AST) (Figure 3A), alanine transaminase (ALT) (Figure 3B), and hexokinase (Figure 3C)

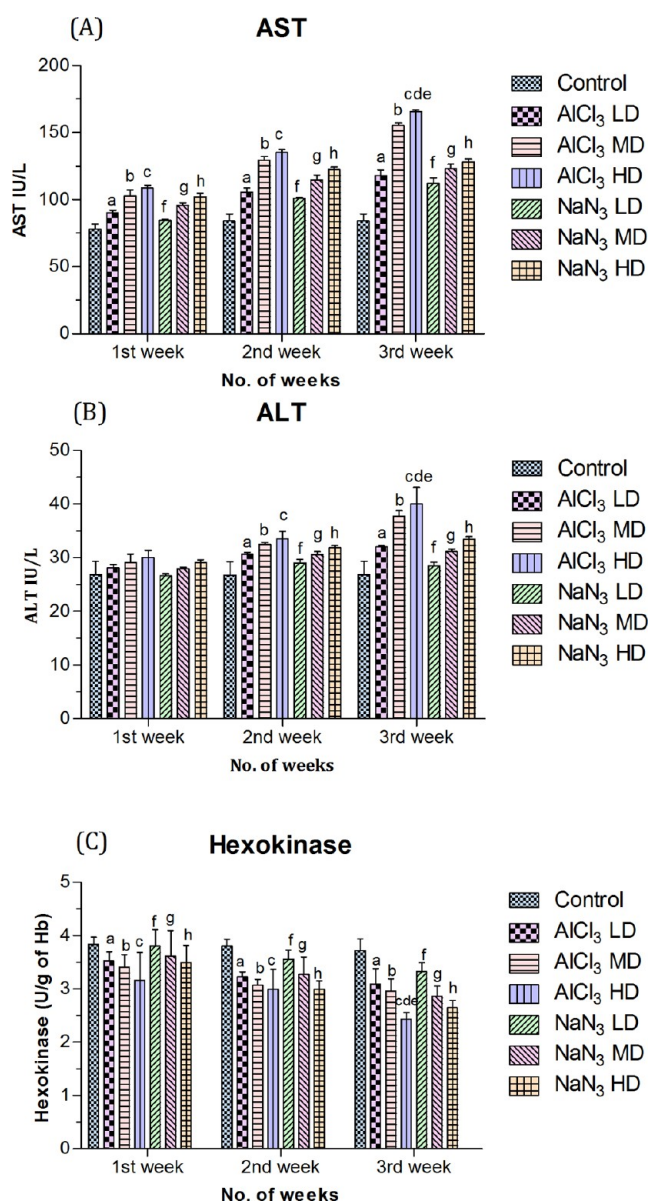


Figure 3. Effect of treatment on AST (A), ALT (B) and hexokinase (C). The effect of treatment on liver biomarkers at 1st, 2nd, and 3rd weeks of the treatment period was observed. The level of significant difference was estimated by Bonferroni post-test using two-way ANOVA. (a) Represents $P < 0.01$ when compared AlCl₃ (LD) treated group with control-group. (b) Represents $P < 0.01$ when compared AlCl₃ (MD) treated group with the AlCl₃ (LD) group. (c) Represents $P < 0.01$ when compared AlCl₃ (HD) treated group with the control groups, AlCl₃ (LD) and AlCl₃ (MD) treated group. (d) represents $P < 0.01$ when compared AlCl₃ (HD) treated group with AlCl₃ (LD) alone treated group. (e) represents $P < 0.001$ when compared AlCl₃ (HD) treated group with AlCl₃ (MD) alone treated group. (f) represents $P < 0.01$ when compared NaN₃ (LD) treated group with the AlCl₃ (LD, MD and HD) treated groups. (g) Represents $P < 0.01$ when compared NaN₃ (MD) treated group with the NaN₃ (LD) treated group. (h) Represents $P < 0.01$ when compared NaN₃ (HD) treated group with the control and NaN₃ (LD and MD) treated groups. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl₃: aluminum chloride, NaN₃: sodium azide.

at first, second, and third weeks of treatment duration. At the first week of the treatment period, a slight increase in the serum level of AST ($P < 0.05$) and a reduction in the serum

level of hexokinase ($P < 0.05$) were observed in AlCl₃ (40 and 45 mg/kg/day) treated groups. At the second and third weeks of the study period, the AlCl₃ (50 mg/kg/day) treated group showed a significant increase in the serum levels of AST ($P < 0.001$) and ALT ($P < 0.001$) and a decline in the serum level of hexokinase ($P < 0.001$) as compared to control and/or NaN₃ (17 mg/kg/day) treated groups.

Effect of AlCl₃ and NaN₃ Exposure on Inflammatory Biomarkers. The effect of different doses of AlCl₃ and NaN₃ on the serum level of inflammatory biomarker, for example, IL-6 (Figure 4), was measured at first, second, and third weeks of

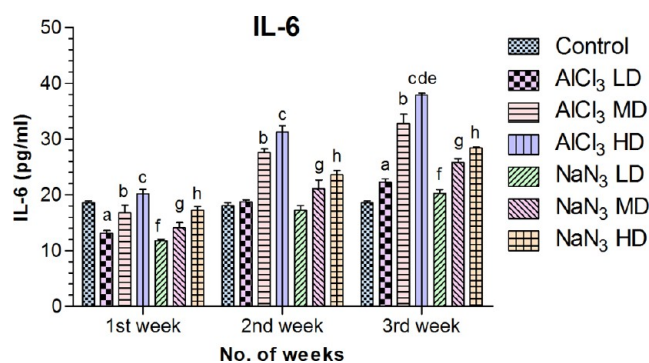


Figure 4. Effect of treatment on IL-6. The effect of treatment on inflammatory biomarker at 1st, 2nd, and 3rd weeks of the treatment period was observed. The level of significant difference was estimated by the Bonferroni post-test using two-way ANOVA. (a) Represents $P < 0.01$ when compared AlCl₃ (LD) treated group with control group. (b) Represents $P < 0.01$ when compared AlCl₃ (MD) treated group with the AlCl₃ (LD) group. (c) Represents $P < 0.01$ when compared AlCl₃ (HD) treated group with the control group, AlCl₃ (LD) and AlCl₃ (MD) treated groups. (d) Represents $P < 0.01$ when compared AlCl₃ (HD) treated group with AlCl₃ (LD) alone treated group. (e) Represents $P < 0.001$ when compared AlCl₃ (HD) treated group with AlCl₃ (MD) alone treated group. (f) Represents $P < 0.01$ when compared NaN₃ (LD) treated group with the AlCl₃ (LD, MD and HD) treated groups. (g) Represents $P < 0.01$ when compared NaN₃ (MD) treated group with the NaN₃ (LD) treated group. (h) Represents $P < 0.01$ when compared NaN₃ (HD) treated group with the control and NaN₃ (LD and MD) treated groups. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl₃: aluminum chloride, NaN₃: sodium azide.

study duration. We observed that the serum level of IL-6 was significantly increased ($P < 0.05$) after exposure to AlCl₃ (45 mg/kg/day) and NaN₃ (15 mg/kg/day) at the first and second weeks of the study period. However, it was also found that the AlCl₃ (50 mg/kg/day) treated group showed maximum effect to increase ($P < 0.001$) the serum level of IL-6 as compared to control and NaN₃ (17 mg/kg/day) treated groups at the end of the study period.

Effect of AlCl₃ and NaN₃ on Oxidative Stress. On the serum level of malondialdehyde (MDA), the effects of different doses of AlCl₃ and NaN₃ are shown in Figure 5 at the first, second, and third weeks of the treatment period. After exposure to AlCl₃ and NaN₃, we found that the AlCl₃ (50 mg/kg/day) treated group showed significantly increased ($P < 0.01$) level of MDA when compared with control and NaN₃ (15 and 17 mg/kg/day) treated groups at the second week of the study period. At the third week of treatment duration, we observed that the NaN₃ (17 mg/kg/day) treated group started showing mortality.

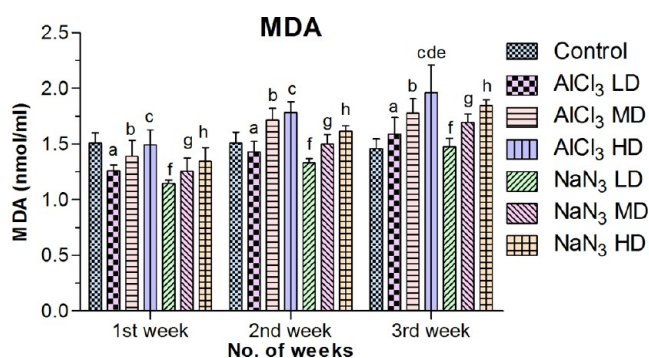


Figure 5. Treatment Effect on MDA in serum. The effect of treatment on serum level of MDA was measured at 1st, 2nd, and 3rd weeks of the treatment period. The level of significant difference was estimated by Bonferroni post-test using two-way ANOVA. (a) Represents $P < 0.01$ when compared AlCl₃ (LD) treated group with control group. (b) Represents $P < 0.01$ when compared AlCl₃ (MD) treated group with the AlCl₃ (LD) group. (c) Represents $P < 0.01$ when compared AlCl₃ (HD) treated group with the control group, AlCl₃ (LD) and AlCl₃ (MD) treated groups. (d) Represents $P < 0.01$ when compared AlCl₃ (HD) treated group with AlCl₃ (LD) alone treated group. (e) Represents $P < 0.001$ when compared AlCl₃ (HD) treated group with AlCl₃ (MD) alone treated group. (f) Represents $P < 0.01$ when compared NaN₃ (LD) treated group with the AlCl₃ (LD, MD and HD) treated groups. (g) Represents $P < 0.01$ when compared NaN₃ (MD) treated group with the NaN₃ (LD) treated group. (h) Represents $P < 0.01$ when compared NaN₃ (HD) treated group with the control and NaN₃ (LD and MD) treated groups. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl₃: aluminum chloride, NaN₃: sodium azide.

Histopathology Analysis. Effect of AlCl₃ and NaN₃ Exposure on Brain Tissue. The effects of AlCl₃ and NaN₃ on the brain hippocampus (Figure 6) and cortex (Figure 7) tissues were observed. Control group showed normal histology of the brain tissue. We observed that NaN₃ (15 and 17 mg/kg/day) treated groups showed reduce cellularity of purkinje cells and mild hemorrhage in the brain (hippocampus) tissue, while ischemic neurons were seen in NaN₃ (15 and 17 mg/kg/day) treated groups of the brain (cortex) tissue. However, we found that the AlCl₃ (50 mg/kg/day) treated group showed reduced cellularity, degradation of neurons, and hemorrhage in the brain (hippocampus) tissue, while granulovacuolar degeneration and ischemia were seen at the hematoxylin & eosin (H&E) stained section of the brain (cortex) tissue.

Effect of AlCl₃ and NaN₃ Exposure on Heart Tissue. The effect of AlCl₃ and NaN₃ on the heart tissue (Figure 8) was observed. Appearance of heart tissue in the control group showed a normal heart structure, and no lesions and disarrangement of cell nucleus were observed. However, we found that NaN₃ (15 and 17 mg/kg/day) treated groups showed cell disarrangement and mild necrosis, while the AlCl₃ (50 mg/kg/day) treated group showed marked morphological changes in the heart tissue such as necrosis and disarrangement of the muscle cell nucleus.

Effect of AlCl₃ and NaN₃ Exposure on Pancreatic Tissue. The effect of AlCl₃ and NaN₃ on the pancreatic tissue (Figure 9) was also observed. Appearance of pancreatic tissue in the control group showed normal islets of Langerhans. In this study, we found that AlCl₃ (40 mg/kg/day) and NaN₃ (13 mg/kg/day) treated groups showed mild inflammation, while the NaN₃ (17 mg/kg/day) treated group showed shrinkage of

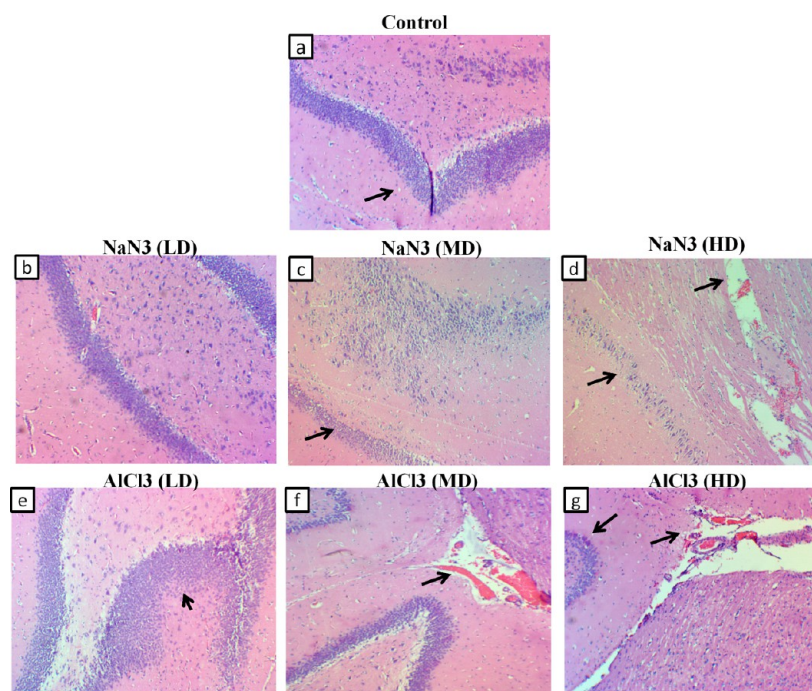


Figure 6. Effect of AlCl₃ and NaN₃ exposure on hippocampus. (a): Glial cells and neurons appeared normal in structure; Purkinje cells' cellularity and thickness were maintained. (b): degradation of neurons was seen in group which was treated with NaN₃ (LD). Reduce cellularity of purkinje cells and hemorrhage were observed in medium (c) and high (d) dose NaN₃ treated groups. (e): degradation of neurons was seen in group which was treated with AlCl₃ (LD). (f): hemorrhage and reduced cellularity of Purkinje cells were seen at few places in AlCl₃ (MD) treated groups (g): reduced cellularity, degradation of neurons and hemorrhage were seen in AlCl₃ (HD) treated group. Abbreviations—low dose (LD), medium dose (MD), high dose (HD), aluminum chloride (AlCl₃), sodium azide (NaN₃).

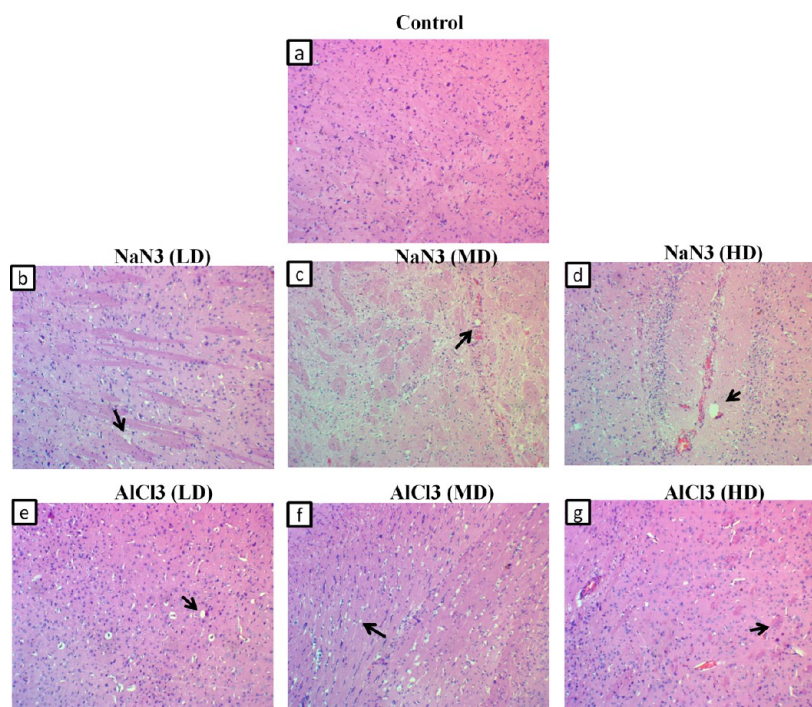


Figure 7. Effect of AlCl_3 and NaN_3 exposure on cortex. (a): Glial cells, neurons appeared normal in structure. (b–d): Ischemic neurons were seen in groups which were treated with low, medium and high doses of NaN_3 . (e,f): glial cells disarrangement and ischemic neurons were seen in the AlCl_3 (LD and MD) treated groups. (g) The appearance of granulovacuolar degeneration and ischemic neurons were observed in AlCl_3 (HD) treated group. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl_3 : aluminum chloride, NaN_3 : sodium azide.

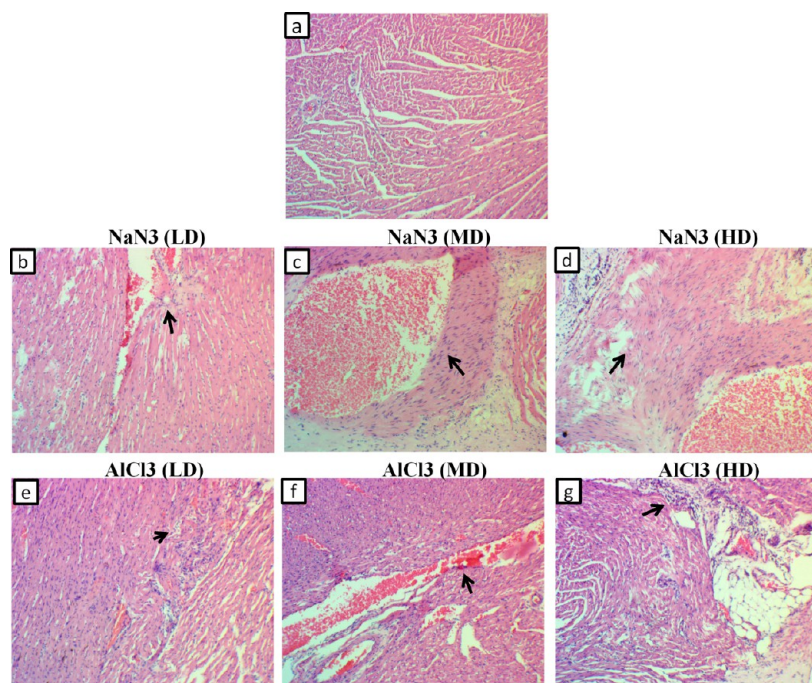


Figure 8. Effect of AlCl_3 and NaN_3 exposure on heart tissue. (a): Intercalated disk and muscle cell nucleus appeared normal in structure. (b): Necrosis of cardiomyocytes was seen in NaN_3 (LD) treated group. Cell disarrangement was observed in medium (c) and high (d) dose NaN_3 treated groups. (e,f) Mild necrosis and cell nucleus disarrangement was seen at few places in AlCl_3 (LD and MD) treated groups. (g): cell nucleus disarrangement, necrosis and inflammation were found in AlCl_3 (HD) treated group. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl_3 : aluminum chloride, NaN_3 : sodium azide.

islets of Langerhans and mild necrosis. Likewise, the AlCl_3 (50 mg/kg/day) treated group showed shrinkage of islets of Langerhans along with necrosis and inflammation.

IHC of Brain. Control group showed normal histology of the brain tissue. Formation of neurofibrillary tangles in NaN_3 (15 mg/kg/day) treated group was observed, while the formation of amyloid plaques and neurofibrillary tangles was

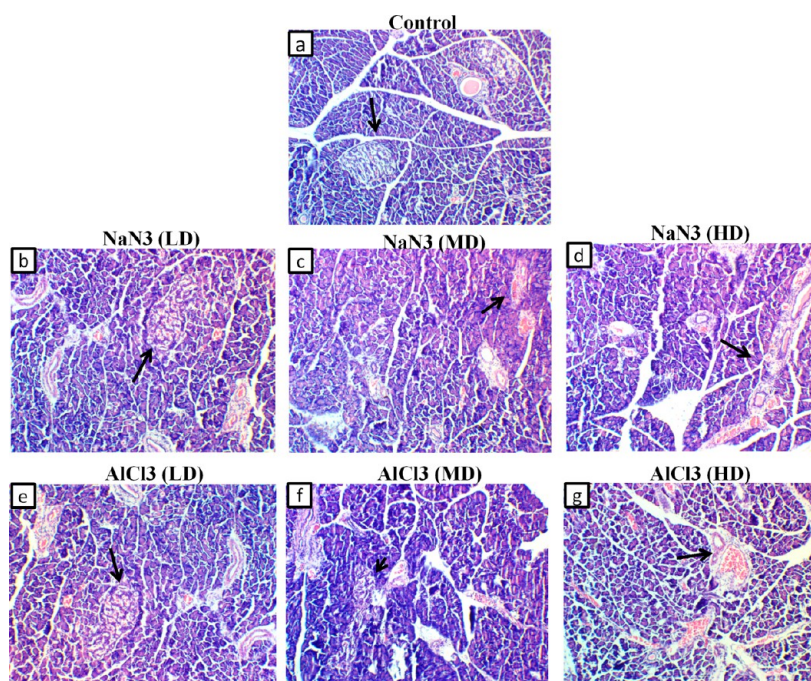


Figure 9. Effect of AlCl_3 and NaN_3 exposure on pancreatic tissue. (a): Islet of Langerhans and acinar cells were appeared normal in structure. (b): Shrinkage of islets of Langerhans was seen in a group which was treated with NaN_3 (LD). Necrosis was found in medium (c) and high (d) dose of NaN_3 treated groups. (e,f): Shrinkage of islets of Langerhans and mild necrosis were seen at a few places in AlCl_3 (LD and MD) treated groups. (g): Necrosis, cell swelling, and inflammation were observed in AlCl_3 (HD) treated group. Abbreviations—low dose (LD), medium dose (MD), high dose (HD), aluminum chloride (AlCl_3), sodium azide (NaN_3).

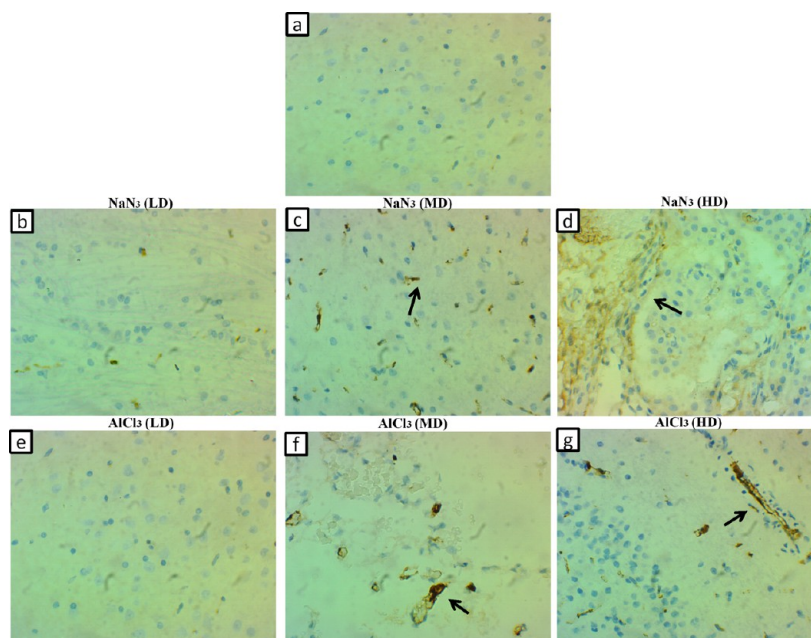


Figure 10. Immunohistochemistry (IHC) of brain tissue. (a) Control group showed normal histology of the brain tissue (b): few neurofibrillary tangles formation was seen in a NaN_3 (LD) treated group. Amyloid plaques and neurofibrillary tangles formation were observed in medium (c) and high (d) dose treated groups. (e,f): plaques formation was observed in AlCl_3 (LD & MD) treated groups. (g): amyloid plaques and neurofibrillary tangles were seen in AlCl_3 (HD) treated group. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl_3 : aluminum chloride, NaN_3 : sodium azide.

seen in the NaN_3 (17 mg/kg/day) treated group. However, marked formation of plaques and neurofibrillary tangle formation were observed at immunohistochemistry (IHC) stained section of the brain tissue in the AlCl_3 (50 mg/kg/day) treated group (Figure 10).

Analysis of Fibrosis in Brain Tissue. Figure 11 shows the fibrotic effect of AlCl_3 and NaN_3 on the brain tissue. Control group revealed normal morphology of the neurons supported by glial cells. We observed that the NaN_3 (17 mg/kg/day) treated group showed vacuolation and hemorrhage in the white matter, while vacuolation, vascular congestion, and

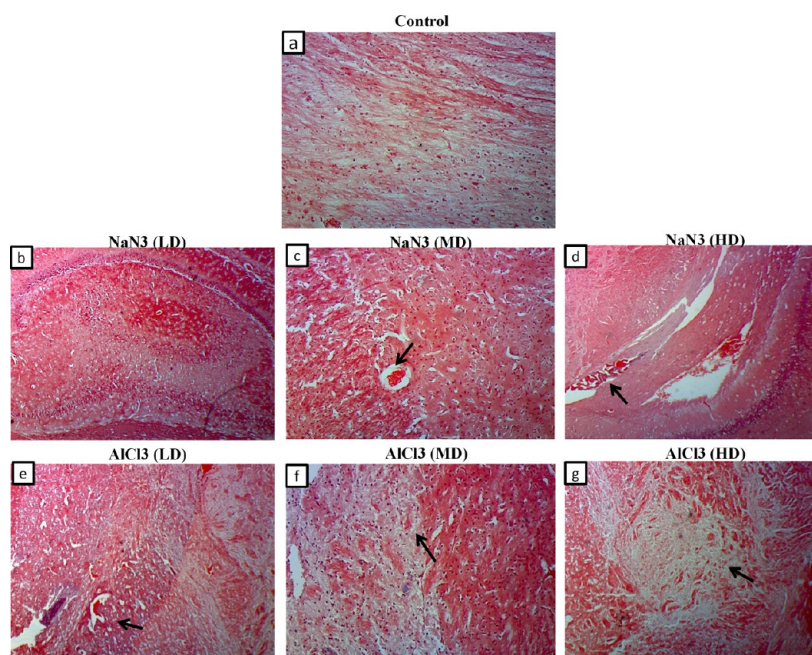


Figure 11. Fibrosis analysis of brain tissue. (a): Control group revealed normal morphology of the neurons supported by glial cells. (b,c,e,f): Mild vacuolation of white matter was seen in groups which were treated with NaN_3 (LD & MD) and AlCl_3 (LD & MD) groups. NaN_3 (HD) treated group (d) showed vacuolation of white matter. (g) Vacuolation, vascular congestion and hemorrhage were seen in AlCl_3 (HD) treated group. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl_3 : aluminum chloride, NaN_3 : sodium azide.

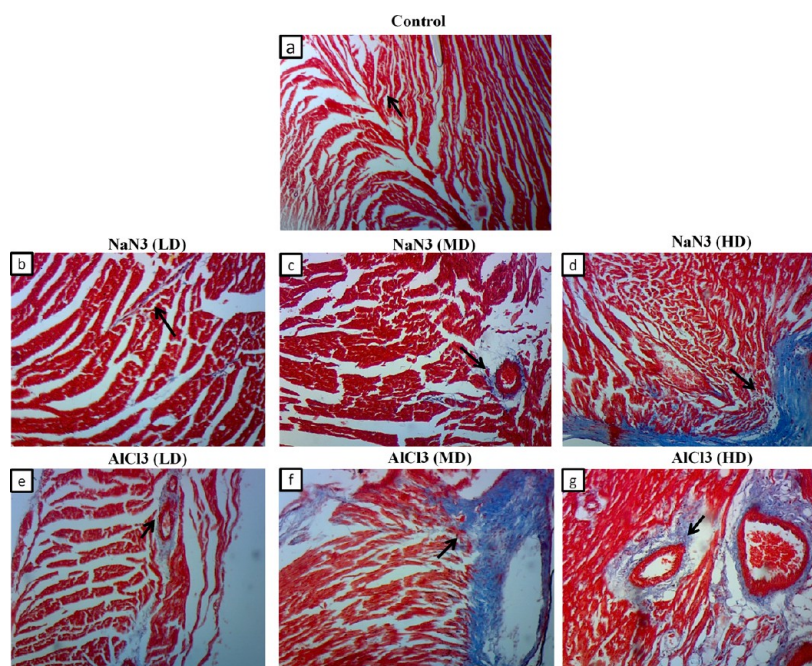


Figure 12. Fibrosis analysis of heart tissue. (a): In control group cardiac tissue appeared normal in structure. (b): mild collagen disposition was seen in a group which was treated with NaN_3 (LD). Collagen deposition was seen in medium (c) dose NaN_3 treated groups. NaN_3 (HD) treated group (d) showed development of fibrotic tissue. (e): Collagen deposition around the blood vessels was seen in group which was treated with AlCl_3 (LD). (f): Fibrotic tissue development was seen in AlCl_3 (MD) treated group. (g): Fibrotic tissues with necrosis around blood vessels were seen in AlCl_3 (HD) treated group. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl_3 : aluminum chloride, NaN_3 : sodium azide.

hemorrhage were clearly seen in the AlCl_3 (50 mg/kg/day) treated group after staining with Masson trichrome.

Analysis of Fibrosis in Heart Tissue. The effect of AlCl_3 and NaN_3 on the heart tissue fibrosis was observed. Appearance of heart tissue in the control group (Figure 12) showed normal heart morphology, and no fibrotic tissue was

seen. We observed collagen deposition in the NaN_3 (15 and 17 mg/kg/day) treated groups. However, the AlCl_3 (50 mg/kg/day) treated group showed morphological changes in the heart tissue, that is, formation of fibrotic tissues with necrosis around the blood vessels.

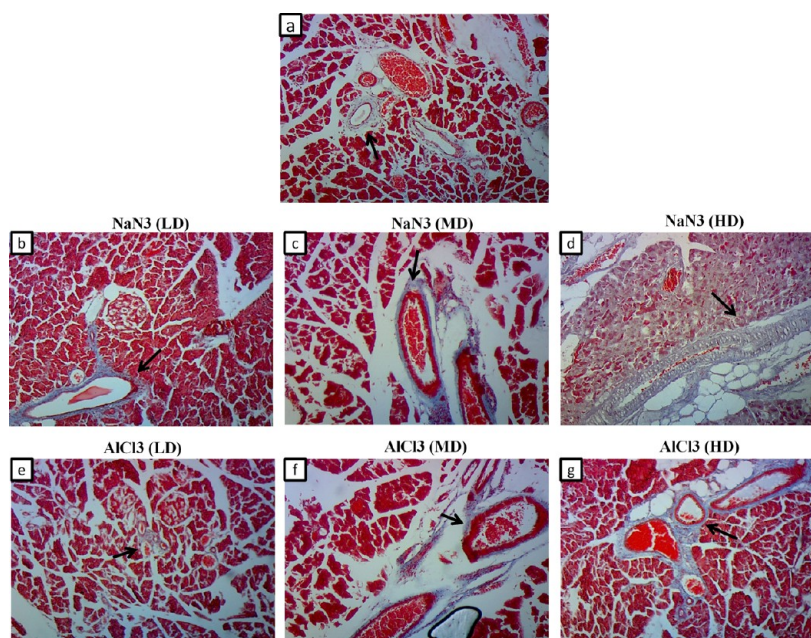


Figure 13. Fibrosis analysis of pancreatic tissue. (a): In control group, pancreatic tissue showed normal appearance of islet of Langerhans. (b): Mild collagen deposition around the islet of Langerhans was seen in group which was treated with NaN_3 (LD). Mild fibrotic tissues development around the islet of Langerhans and fibrotic tissues between acinar cells were seen in NaN_3 medium (c) and high (d) dose treated groups. (e): Collagen deposition around the islet of Langerhans was seen in a group treated with AlCl_3 (LD). (f): Fibrotic tissue development around the islet of Langerhans was seen in AlCl_3 (MD) treated group. (g): Fibrotic tissues between acinar cells and around islets of Langerhans were seen in AlCl_3 (HD) treated group. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl_3 : aluminum chloride, NaN_3 : sodium azide.

Analysis of Fibrosis in Pancreatic Tissue. Likewise, appearance of pancreatic tissue in the control group showed normal islets of Langerhans (Figure 13) with no fibrotic appearance. We found mild collagen deposition around the islets of Langerhans of the pancreatic tissue in AlCl_3 (40 mg/kg/day) and NaN_3 (13 mg/kg/day) treated groups. However, pancreatic tissues of the NaN_3 (15 and 17 mg/kg/day) treated group after Masson's trichrome staining showed mild fibrotic tissue development around the islet of Langerhans and between the acinar cells, while the AlCl_3 (50 mg/kg/day) treated group showed fibrotic tissues between acinar cells and around the islet of Langerhans.

Analysis of ECG Pattern. Electrocardiogram (ECG) was also recorded for each group with the help of power-lab to evaluate the effect of different doses of AlCl_3 and NaN_3 on the heart's electrical activity (Figure 14). Control group rats showed a normal ECG pattern. However, the NaN_3 (13, 15 and 17 mg/kg/day) treated groups showed ST depression, while we found inverted QRS complex in the AlCl_3 (50 mg/kg/day) treated group.

DISCUSSION

AlCl_3 -induced neurotoxicity in animals has been proven that has also been involved in the etiology of neurodegenerative disorders, for example, Alzheimer's disease (AD). AlCl_3 can induce physiological, biochemical, and morphological deformities through the generation of ROS.^{13,20}

In the current study, we examined the concentration of glucose, insulin, and HbA1c in rats that were exposed to different doses of AlCl_3 and NaN_3 . In this study, we found that exposure to AlCl_3 (50 mg/kg/day) significantly increased the levels of glucose and HbA1c in serum when compared with control and AlCl_3 (45 mg/kg/day) treated groups, while the serum level of insulin was reduced. It is reported in the

literature that NaN_3 is a reversible inhibitor of mitochondrial respiration; it plays an important role to block glucose-induced electrical activity and insulin secretion in pancreatic β cells. The effects of NaN_3 on ATP-sensitive K^+ channels have been attributed to the reduction in ATP levels. ATP-sensitive K^+ channels play a key role in the insulin secretory mechanism of pancreatic β cells. Closure of these channels results in depolarization of the cell membrane and Ca^{2+} influx via voltage-gated Ca^{2+} channels.²¹ Likewise, in this study, we also found that a gradual increase in the doses of NaN_3 can raise the levels of glucose and HbA1c when compared to the control group, but at the end of the trial, a high rate of mortality in experimental animals exposed to 17 mg/kg/day of NaN_3 (Figure 1) indicates a fairly lethal effect of this compound at a moderate dose.

The current work also revealed that AlCl_3 (50 mg/kg/day) significantly elevated the serum levels of cholesterol, TGs, and LDL with a reduced serum level of HDL (Figure 2), while at a high dose, NaN_3 (17 mg/kg/day) caused mortality. Investigations have shown that NaN_3 can cause sudden lowering of blood pressure, tachycardia, cardiac arrhythmia, and cardiac toxicity; moreover, late onset of hypotension presents an ominous sign of death.²² Any of these effects reported earlier could be a cause of sudden death in the experimental animals of the current study exposed to NaN_3 (17 mg/kg/day).

Moreover, the present study was also aimed to investigate the serum levels of liver biomarkers in the experimental model that was exposed to different doses of AlCl_3 and NaN_3 . According to available literature, aluminum compound exposure can induce the toxic effects on respiratory, hematopoietic, neurologic, and immunologic systems.²³ Accumulation of aluminum in liver causes bile acid deposition, apoptosis of hepatocytes, antioxidant imbalance, microsomal cytochrome P₄₅₀ enzyme system inhibition, and activation of

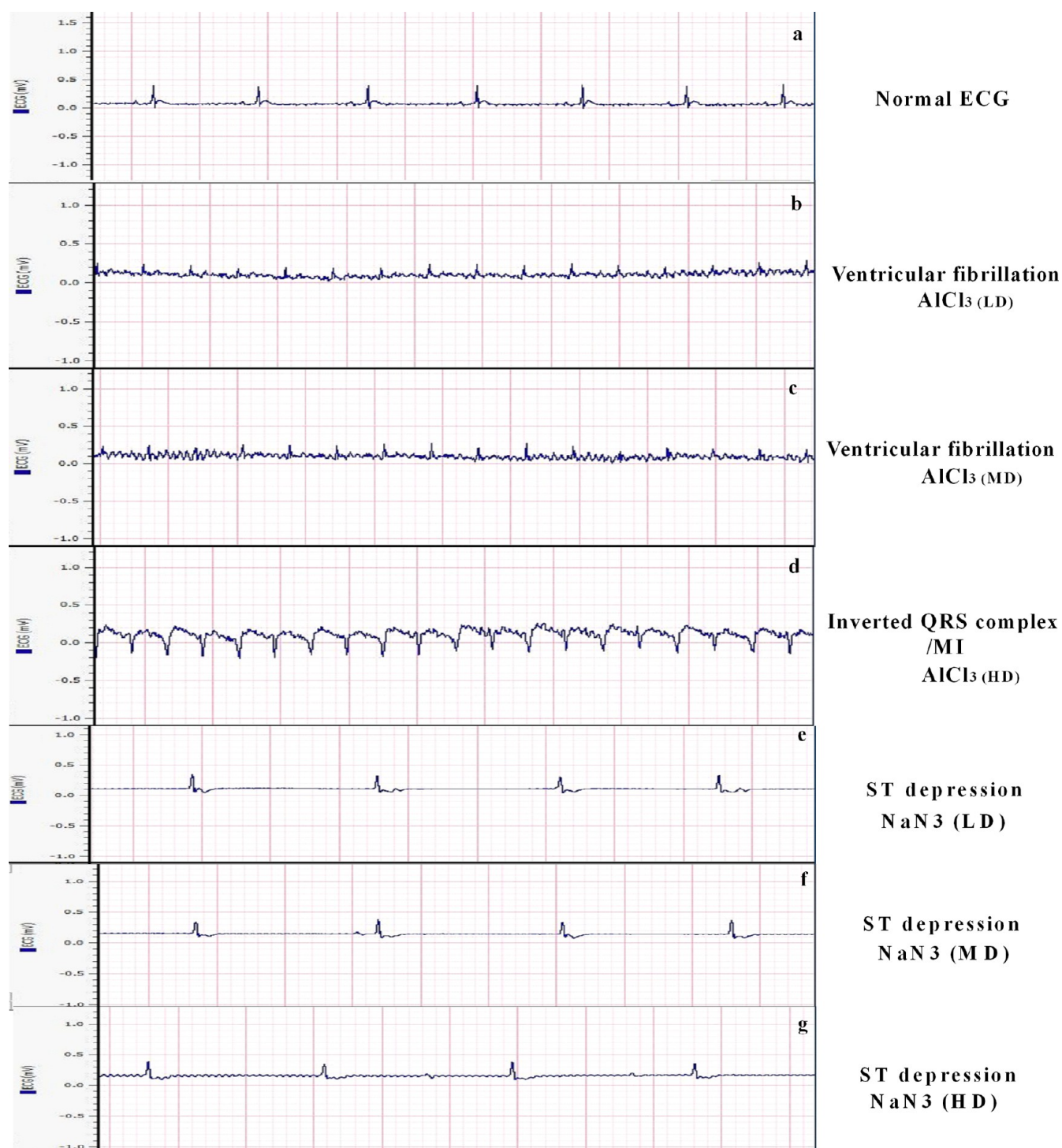


Figure 14. ECG fluctuations after the exposure of different doses of AlCl_3 and NaN_3 . Control group “a” showed normal ECG pattern. Group “b, c” showed ventricular fibrillation and group “d” showed inverted QRS complex after being treated with LD, MD and HD AlCl_3 ; respectively. While group “e, f and g” showed ST depression after treated with NaN_3 (LD, MD and HD). Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl_3 : aluminum chloride, NaN_3 : sodium azide.

inflammatory reactions, leading to liver dysfunction in rats.²⁴ In this study, we found a significant increase in the serum levels of ALT and AST and a decrease in the level of hexokinase in AlCl_3 (50 mg/kg/day) treated group. Very recently, increased permeability and necrosis of hepatocytes have also been reported in the rat model after exposure to AlCl_3 .²⁵ As a result of hepatic toxicity, the levels of liver enzymes are known to increase in blood.²⁶ Previous investigation has also revealed

that azide-induced hepatotoxicity is recognized by marked elevation in the levels of liver enzymes.²⁷ The current study also found that NaN_3 (15 and 17 mg/kg/day) treated groups have increased level of liver enzymes.

Correspondingly, AlCl_3 (50 mg/kg/day) treated group showed an increased serum level of inflammatory biomarker, that is, IL-6 when compared with NaN_3 (17 mg/kg/day) treated group (Figure 4). Accumulation of aluminum in the

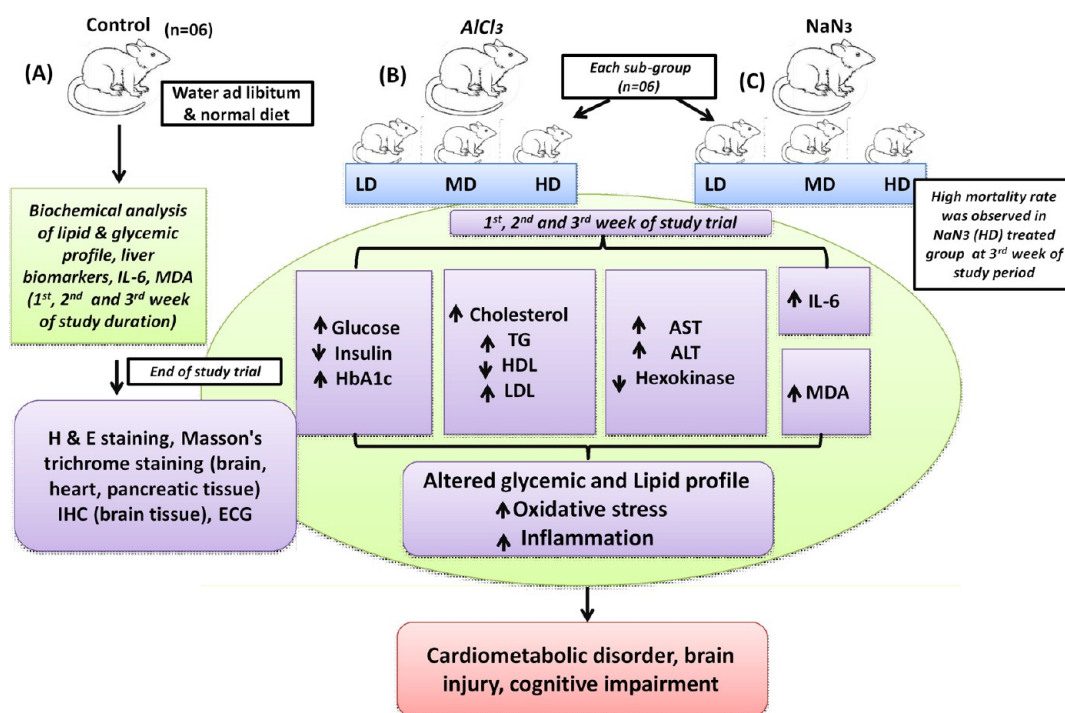


Figure 15. This diagram illustrates that adult Wistar rats were divided into 3 groups. Group (A) was named as control group that received normal diet and water *ad libitum*. Other groups AlCl_3 (B) and NaN_3 (C) were further subdivided and received different doses (LD, MD, and HD) of AlCl_3 and NaN_3 intraperitoneally. AlCl_3 and NaN_3 treated groups effectively enhances the inflammatory response, altered the glycemic & lipid profile and oxidative stress at 3rd week of study period, consequently; aluminum chloride (HD) treated rats showed higher risk of cardiometabolic disorder, brain injury and cognitive impairment, while; high mortality rate was observed in NaN_3 (HD) treated group at the end of study trial. Moreover, H&E staining, Masson's trichrome staining of brain, heart, pancreatic tissue, IHC (brain tissue) and ECG were also performed at the end of study period. Abbreviations—LD: low dose, MD: medium dose, HD: high dose, AlCl_3 : aluminum chloride, NaN_3 : sodium azide.

heart and brain sites can lead to the inflammation through the activation of immune system.²⁸ Oxidative stress triggered by ROS generation is detrimental and one of the main etiological factors of cardiometabolic and neurological disorders.²⁹ The toxic effects of NaN_3 exposure mainly arise from its ability to inhibit the oxidative phosphorylation *via* inhibition of cytochrome oxidase, with the resultant overproduction of ROS. Consequently, there is a reduction in the ability of cells to utilize oxygen, as well as reduced generation of ATP leads to cell death.³⁰ In this study, we also found that the NaN_3 (17 mg/kg/day) treated group showed more mortality rate when compared with AlCl_3 (45 and 50 mg/kg/day) treated groups.

Furthermore, MDA is a biomarker of lipid peroxidation where increased lipid peroxidation leads to ROS formation and oxidative stress that can eventually persuade the risk of neuroinflammation, myocarditis, and insulin resistance.³¹ This study found that AlCl_3 (50 mg/kg/day) significantly increased the MDA level in serum as compared to NaN_3 (15 and 17 mg/kg/day) treated groups, and hence the risk of IR, AD, and CVD development was increased in the AlCl_3 (50 mg/kg/day) treated group (Figure 5).

Aluminum has also been reported to induce the memory loss and neurological changes; experimentally, it has been proven that exposure to AlCl_3 can cause the neurological modifications which mimic the development and progression of neurodegeneration and ischemia in brain.³² In this study, we found that exposure to AlCl_3 (50 mg/kg/day) may induce hemorrhage and reduce cellularity, degradation of neurons, and ischemia in the brain tissue; likewise, hemorrhage and ischemic neurons were also observed in the NaN_3 (17 mg/kg/day) treated group (Figures 6 and 7). The NaN_3 -induced neuronal

oxidative stress has been shown to involve in the inhibition of cytochrome C oxidase which may interfere with numerous complexes in the electron transport chain, with the resultant ROS production. Over-production of ROS may often lead to neuronal cell death.³³

NaN_3 is a highly toxic compound, and exposure to NaN_3 poses a risk of death.¹² Researchers have proven that a high dose of NaN_3 can potentially cause the toxic damage to numerous tissues and organs.³⁴ This study also found that exposure to NaN_3 (17 mg/kg/day) can cause cardiac cell disarrangement, necrosis, and shrinkage of islets of Langerhans after H&E staining of heart and pancreatic tissues, respectively (Figures 8 and 9). Previous study has reported abnormal histological sign, that is, a decrease in the pancreatic islet numbers, size, and necrotic changes of pancreatic islets in the pancreatic tissue after exposure to AlCl_3 .³⁵ The current study also found that the AlCl_3 (50 mg/kg/day) treated group showed shrinkage of islets of Langerhans, necrosis, and inflammation.

Loss of memory and cognitive dysfunction have been reported after exposure to AlCl_3 .³⁶ Another study has revealed that the rats treated with AlCl_3 showed severe toxicity in the brain, leading to memory impairment and cognitive dysfunction which may ultimately lead to the development of neurological disorders.³⁷ In this study, IHC was done to determine the effect of different doses of AlCl_3 on the brain. Formation of neurofibrillary tangles and amyloid plaques was seen in the AlCl_3 (50 mg/kg/day) treated group. However, the NaN_3 (17 mg/kg/day) treated group showed formation of neurofibrillary tangles (Figure 10). Long-term exposure to NaN_3 may also be responsible for overproduction of ROS

through the impairment of mitochondria energy mechanism resulting in neuronal cell death which may often lead to neurological disorders.³⁸

Heart contains a network of collagen that plays a role in cardiac muscle contractility and also provides cardiac strength. Investigation has found that aluminum toxicities may include fibrosis, myocarditis, thrombosis, ischemic stroke, AD, dementia, pancreatitis, pancreatic necrosis, and DM.²³ Numerous reports have revealed that NaN_3 poisoning causes severe hypoxemia, myocardial fibrosis, and extensive damage in the nervous and cardiac systems.²² In this study, fibrosis of the brain, heart, and pancreatic tissue, after exposure to different doses of AlCl_3 and NaN_3 , was analyzed *via* Masson's trichrome staining (Figures 11, 12, 13). Researchers have found that chronic exposure to AlCl_3 can influence the cardiac rhythm.³⁹ One study has reported that toxicity of NaN_3 affects the cardiac activity to the point of death.¹² Previous investigation has revealed that low blood pressure (hypotension), CNS depression, chest discomfort, slow or rapid heart rate (bradycardia or tachycardia), abnormal or disordered heart rhythms (atrial and ventricular dysrhythmias), difficulty in breathing, coma, and death were recorded due to its toxic effects.^{12,22} In this study, we found that NaN_3 treated groups showed ST depression, while ventricular fibrillation and irregular cardiac activities were seen in AlCl_3 treated groups (Figure 14). Some studies have suggested therapeutic effects against effects of aluminum compounds.⁴⁰

In general, disease induction in experimental models is a keystone of the drug development process. Our study represents the development of experimental model with a comorbid disorder of vital organs like pancreas and heart, expressing cardiometabolic disturbance induced through the uses of different doses/concentrations of AlCl_3 and NaN_3 (Figure 15). Because the impact of diseases on the experimental model is to closely mimic the effects of disease in humans, comorbidity of diseases in the animal model is basically a scientific tool for the challenges and development of new drugs.

MATERIALS AND METHODS

Drugs Used. Aluminum chloride (AlCl_3) was purchased from ICN Biomedicals, and sodium azide (NaN_3) was purchased from Fluka Chemika.

AlCl_3 - and NaN_3 -Induced Experimental Animal Model. About 42 adult Wistar albino rats weighing approximately 150–200 g were purchased locally and kept in the animal room ($25 \pm 5^\circ\text{C}$) of University of Agriculture Faisalabad (UAF), Pakistan. Rats were fed with water *ad libitum* with a normal diet. They were allowed to acclimatize for 1 week before the start of the experimental trial. All experimental protocols were followed according to approved guidelines of animal biosafety and rules of Institutional Biosafety committee of UAF (no. 2875/ORIC). The body weight of all rats was noted earlier at the start of the study and on a weekly basis during the duration of the experiment. Following acclimatization, the rats were allocated into 7 groups ($n = 6$). Group 1 was termed as normal control that received only vehicle along with a normal diet without any treatment during the experimental trial. Remaining groups were given graded doses of AlCl_3 and/or NaN_3 , where groups 2, 3, and 4 were designated as LD- AlCl_3 (AlCl_3 40 mg), MD- AlCl_3 (AlCl_3 45 mg), and HD- AlCl_3 (AlCl_3 50 mg) representing low dose, medium dose, and high dose of AlCl_3 , respectively, while

groups 5, 6, and 7 were designated as LD- NaN_3 (NaN_3 13 mg), MD- NaN_3 (NaN_3 15 mg), HD- NaN_3 (NaN_3 17 mg) representing low dose, medium dose, and high dose of NaN_3 , respectively.

Blood and Tissue Sampling. For the biochemical analysis of relevant biomarkers, about 1.5–2.5 mL of blood samples were taken at the first, second, and third weeks and at the end of treatment duration from each rat. After collection, the blood samples were centrifuged for 20 min at 3000 rpm for serum separation and stored at -20°C until further analysis.

Body and Organ Weight. Body weight was measured every week throughout the study period. On the last day of trial, rats were fasted throughout the night and after collection of the blood samples, heart, brain and pancreatic tissues were excised from sacrificed rats followed by washing with normal saline and weighed individually.

Biochemical Analysis. The serum was used to estimate the biochemical parameters including glycemic profile (glucose, insulin, and HbA1c), lipid profile (HDL, LDL, cholesterol, TGs), lipid peroxidation analysis (MDA), and inflammatory.

Assessment of Glycemic Status. For each group, the serum level of glucose was observed using the glucose assay kit (ref no. 1004) through a Microlab 300 chemistry analyzer (ELITech Group) to estimate the effects of AlCl_3 and NaN_3 on the glycemic status. HbA1c and insulin levels in serum were also measured using their respective Elisa kits, insulin ELISA kit (cat no. INS 5275, Elabscience), and HbA1c ELISA kit (cat no. SG10984, Elabscience) through a microplate ELISA reader (BIOBASE-EL 10A).

Assessment of Lipid Profile. The serum level of lipids was measured from the collected animal samples. Cholesterol (ref no. 1011), TGs (cat no. 090618), HDL (cat no. 6011668), and LDL (cat no. 6011668) concentrations in serum were measured *via* a Microlab 300 chemistry analyzer.

Assessment of Liver Function Enzymes. The serum levels of AST, ALT, and hexokinase were measured using the AST assay kit (cat no. BD088918), ALT assay kit (cat no. BD088918) through Microlab 300, and hexokinase (cat No. E-EL-M146, Elabscience) through a microplate ELISA reader.

Assessment of Inflammatory Biomarkers. The serum level of IL-6 was measured by using an IL-6 ELISA kit (E-EL-R0015, Elabscience) through a microplate ELISA reader.

Assessment of Oxidative Stress Biomarker. MDA, an end product of lipid-peroxidation and a recognized oxidative stress biomarker, was also evaluated. We evaluated the effect of AlCl_3 and NaN_3 in the experimental model during the first, second, and third weeks of study period by using an MDA ELISA kit (cat no. E-EL 0060, Elabscience) through a microplate ELISA reader.

Analysis of ECG Pattern. The animals from each experimental groups were placed flat on the working slab after being anaesthetized. Each arm was connected to a separate electrode. The standard leads were placed on the palm of left and right limbs of the animal, while the grounded lead was attached to the right hind foot. After plugging the Bio Amp cable into the Bio Amp input, electric signals were generated and recordings were obtained by a device attached to the PowerLab (AD Instruments).

Histopathological Examination of Heart, Brain, and Pancreatic Tissues. Masson's Trichrome Tissue Staining. Masson trichrome staining was performed for the detection of collagen fibers in the brain, heart, and pancreatic tissues. The

samples were preserved in formalin, and paraffin-embedded sections were stored. All tissue slides were deparaffinized by heating and rehydrated through 70, 95, and 100% alcohol. This was followed by washing with distilled water. Later, the tissue slides were treated with Bouin's solution and heated at 56 °C for 1 min. The slides were allowed to stand for 15 min and washed with water for 5 min. Tissue slides were stained with Weigert's iron hematoxylin solution for 10 min and rinsed with warm water for 10 min. After washing with distilled water, the slides were immersed and stained in Biebrich scarlet acid fuchsin for 15 min. Then, the tissue slides were washed with distilled water and immersed in phosphomolybdic/phosphotungstic acid solution for 15 min. Further, the slides were stained with aniline blue solution for 10 min and rinsed with distilled water. The slides were placed in 1% acetic acid solution for 2 min followed by washing with distilled water. Finally, the slides were dehydrated by placing in 95% ethanol followed by absolute alcohol and cleared in xylene, followed by mounting.

H&E Tissue Staining. To examine the effects of AlCl₃ and NaN₃ on the brain, heart and pancreatic tissue morphology, the rats were killed by cervical dislocation. The skull and abdomen were dissected, and the brain, heart, and pancreas were separated for histopathological examination. Tissue samples were subjected to the process of fixation, embedding, sectioning, mounting, and staining according to the protocol. Briefly, slices of brain, heart, and pancreatic tissues were preserved in 10% formalin solution. The tissue sections were washed and rehydrated through alcohol. Then, all tissue sections were transferred to the xylene solution. Afterward, the sections were submerged in liquid paraffin for approximately 2 h and allowed to fix in paraffin wax. Later, the slides were stained with H&E stain and covered with a glass coverslip for analysis.

Immunohistochemistry. About 3–5 micron paraffin embedded brain tissue was cut and mounted onto a microscopic slide. The slides were air dried for about 2 h at 58 °C followed by deparaffinization and rehydration. The tissue slides were then subjected to heat-induced epitope retrieval solutions, that is, the immuno-DNA retriever with citrate. The slides were placed in a staining dish that contained the immuno-DNA retriever with citrate. Then, the slides were placed on a trivet in the pressure cooker. About 1–2 inches of distilled water was added to the pressure cooker, and heat was turned on to high. All slides were incubated for about 15 min followed by opening of the pressure cooker. Immediately, the slides were allowed to sit at room temperature. This was followed by mounting using biodegradable permanent mounting media [X Green PermaMounter (BB0169-0174)].

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Notes

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