A DISSERTATION ON

Assessment of emerging Kidney Toxicity Parameters in humans and Animals

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT FOR THE DEGREE
M.TECH
IN BIOTECHNOLOGY
BY
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M. Tech Biotechnology (IV Semester)
Roll No: 2001361015

UNDER THE SUPERVISION OF
Dr Vikas Srivastava
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DECLARATION FORM

I, **Sabiha Aaysha**, a student of **M.Tech Biotechnology (2nd year/4th semester)**, Integral University have completed my six months dissertation work entitled "**Assessment of kidney toxicity parameters in humans and Animals**" successfully from **Integral University** under the guidance of **Dr. Vikas Srivastava, Principal Scientist, CSIR-IITR, Lucknow**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

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डा. वी.पी. शर्मा
Dr V. P. Sharma
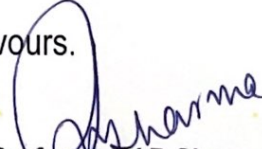मुख्य वैज्ञानिक एवं प्रोफेसर एसीएसआईआर
Chief Scientist & Prof. AcSIR

यह प्रमाणित किया जाता है कि **सुश्री सबीहा आयशा** [Enrollment No: 2000102449] **M.Tech (बायोटेक्नालॉजी)**, इंटीग्रल यूनिवर्सिटी, कुर्सी रोड, लखनऊ, 226026 उत्तर प्रदेश में अध्ययन कर रही है । इन्होने विषय Assessment of Kidney Toxicity Parameters in Human and Animals पर दिनांक 10/02/2022 से 09/08/2022 तक शोधकार्य का प्रशिक्षण डॉ विकास श्रीवास्तव प्रधान वैज्ञानिक, सीएसआईआर-आईआईटीआर लखनऊ, उत्तर प्रदेश के पर्यवेक्षण में प्राप्त किया है।

हम सभी **सुश्री सबीहा आयशा** के उज्जवल भविष्य की कामना करते है।

To Whomsoever it May Concern

This is to certify that **Ms Sabiha Aaysha** [Enrollment No: 2000102449] student of **M.Tech (Biotechnology)**, Integral University, Kursi Road, Lucknow, 226026 Uttar Pradesh, has undergone dissertation training on **Assessment of Kidney Toxicity Parameters in Humans and Animals** during **10/02/2022 to 09/08/2022** under **Dr. Vikas Srivastava**, Principal Scientist, CSIR - Indian Institute of Toxicology Research, Lucknow. Uttar Pradesh

We wish **Ms Sabiha Aaysha** success in her future endeavours.

Professor V P Sharma
Head,
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Place: Lucknow
Date: Oct 04th, 2022

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CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Sabiha Aaysha,** a student of **M.Tech Biotechnology** (2nd Year/4th Semester), Integral University has completed his six months dissertation work entitled **"Assessment of kidney Toxicity parameters in humans and Animals"** successfully. She has completed this work from Integral University under the guidance of **Er. Soban Ahmad Faridi**, Assistant Professor , Department of Bioengineering, Integral University, Lucknow. The dissertation was a compulsory part of her **M.Tech Biotechnology** degree**.**

I wish her good luck and bright future.

Er. Soban Ahmad Faridi

Assistant Professor

Department of Bioengineering

Faculty of Engineering

TO WHOM IT MAY CONCERN

This is to certify that **Sabiha Aaysha,** a student of **M.Tech Biotechnology** (2nd Year/ 4th Semester), Integral University has completed his six months dissertation work entitled **"*Assessment of kidney toxicity parameters in Humans and Animals"*** successfully. He has completed this work from Integral University under the guidance of Dr. Alvina Farooqui. The dissertation was a compulsory part of her **M.Tech Biotechnology** degree**.**

 I wish her good luck and bright future.

   Dr. Alvina Farooqui
Associate Professor and Head
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Integral University, Lucknow

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 DATE: **Sabiha Aaysha**

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LIST OF ABRREVIATIONS

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
WHO	World Health Organisation
IARC	International Agency for Research on Cancer
$MSMAs^V$	Monosodium methanearsonate
$DMAs^V$	Dimethylarsinic acid
CCA	Chromate copper arsenate
BPD	Bengal Delta Plains
ATO	Arsenic trioxide
AML	Acute Myeloid Leukaemia
PDH	Pyruvate dehydrogenase complex
ATP	Adenosine-5'- triphosphate
DPCs	DNA-protein cross-linking
GFs	Growth factors
CSF2	Colony stimulating factor-2
TGF	Transforming growth factor
MAPK	Mitogen-activated protein kinase
ERKs	Extracellular-regulated protein kinase
PBL	Peripheral blood leukocyte
JNKs	Jun N-terminal kinase pathway
ESRD	End -stage renal disease
LSD1	Lysine demethylase
MSG	Monosodium glutamate

ABSTRACT

In developing nations compared to developed nations, diabetes presents additional challenges. A developing foetus may suffer catastrophic effects from exposure to arsenic. Kidney disease and cardiovascular problems may occasionally result from it. About 25% of type 2 diabetes people get diabetic nephropathy, which is the main cause of end-stage renal disease (ESRD) in high-income nations. Clinical metabolomics frequently uses mass spectrometry (MS) to detect and quantify distinct groups of metabolites. The current study used an animal model to examine the expression of genes related to diabetic nephropathy in response to postnatal MSG-induced diabetes and low dosage prenatal arsenic exposure. The structural examination of the kidney employing hematoxylin and eosin stain and immunostaining was also detected in diabetic mouse models. The expression of genes linked to kidney injury and diabetes is increased by arsenic exposure. When compared to the non-exposed kidney, histopathology shows damage to the tubules and the podocytes. The entire kidney can be observed to have undergone glomerular alterations. This study reveals how kidney damage and diabetes are related. The developmental toxicity of arsenic in 38-week-old puppies is also demonstrated in this study. An international public health concern, arsenic exposure from tainted food, water, and other sources results in negative health effects like kidney impairment. The evidence points to serious kidney damage brought on by prolonged arsenic exposure. The genes Fabp4, Cebp-, Cebp-, Irs-1, ins-1, Sglt1 NgaL, Acta2, Col1a, and Fibronectin have been demonstrated to be activated by arsenic. Diabetes is brought on by this, which harms the kidneys.

Abbreviations: ESRD-End stage renal disease; LCMS- Liquid Chromatography mass Spectroscopy; MSG-monosodium glutamate

INTRODUCTION `

Over the last 20 years, the global diabetes population has more than doubled. Diabetes is one of the leading global pandemics and a threat to the health of humans. Diabetes presents many more challenges in developing countries than in developed countries. Sometimes, it leads to various kidney diseases and cardiovascular diseases. In 1998, King and his colleagues estimated it would be 300 million by the end of 2025. According to another study, 366 million individuals will have diabetes by 2030, up from 171 million in 2000. The three primary kinds of diabetes are Diabetes I, Diabetes II, and Gestational Diabetes. Type I diabetes is caused by an autoimmune response in which the body stops making insulin. With type II diabetes, the body cannot maintain blood glucose levels because it cannot adequately utilize insulin. With type II diabetes, the body cannot keep blood glucose levels because it cannot sufficiently use insulin. Gestational diabetes mellitus (GDM) is a condition that causes abnormal blood glucose levels in pregnant women who have never had diabetes. Genetic factors, diet, infections, lifestyle, Obesity, Puberty, and a variety of humoral factors are all relevant factors.

Diabetic nephropathy affects one-fourth of total T2D patients and is the leading cause of end-stage renal disease (ESRD) in developed countries. Elevated glucose levels, diabetes for a long time, high blood pressure, Obesity, and dyslipidemia are all risk factors for diabetic nephropathy development and progression. Use of statins, ACE inhibitors, or angiotensin receptor blockers slows the course of renal disease and decreases cardiovascular mobility in people with diabetic nephropathy. Diabetic Nephropathy can be detected in patients with type II diabetes. According to published data, approximately 7% of type II patients had microalbumin. In clinical metabolomics, mass spectrometry (M.S.) is commonly used to identify and quantify various classes of metabolites.

Arsenic is an environmental pollutant, which is present ubiquitously un earth crust, water, soil, and air and its contamination of the natural environment has both temporary and permanent adverse effects on human health. In ancient times, It was used for various purposes like like pesticides, homicidal agents, medicine, and many others. Exposure to arsenic is associated with the various health concern so there are various arsenic exposure routes, such as drinking water, soil, diet, and air. Exposure to As leads to acute and chronic disease such as skin cancer, metabolic syndrome, diabetic neuropathy and chronic kidney disease. For e.g Inadvertent insecticide/pesticide ingestion is the most common cause of acute poisoning. Furthermore, various reports have suggested inorganic arsenic is associated with acute and chronic kidney disease. Apart from adults studies, there is significant amount of research data, which suggest that exposure to arsenic during developmental period may make individuals susceptible for disease. So with this notion , we developed a low dose prenatal arsenic exposed mice model with monosodium glutamate (MSG) so treated the pups at 2 day with MSG by subcutaneously for dose at alternative days and we take care of animals till 30 week and climical parameters will be analysed.

REVIEW LITERATURE

Nephrotoxicity is a sharp decline in kidney function caused by the harmful effects of medications and chemical products. (Yang et al., 2018). Nephrotoxicity is caused by a variety of mechanisms, including renal tubular toxicity, inflammation, glomerular damage, crystal nephropathy, and thrombotic microangiopathy. Acute kidney injury (AKI) and chronic kidney disease(CKD) are examples of nephrotoxicity (CKD). Approximately 20% of nephrotoxicity is induced by the use of drugs (Peres et al., 2013). It is one of the leading global pandemics and a threat to the health of humans. Diabetes presents many more challenges in developing countries than in developed countries. Sometimes, it leads to various kidney diseases and cardiovascular diseases.

Diabetes is one of the most difficult health issues of the twenty-first century and is also considered a Cinderella Disease. This is high on the global health agenda and is on its way to becoming a worldwide epidemic (Zimmet et al. 2014). Diabetes was once thought to be a group of diseases linked to the retention or loss of essential water (lancet 2018). The disease's Greek term, "diabetes," which means "siphon," reflects Aretaeus of Cappadocia described by the statement "a dissolution of the body parts into urine" from the second century C.E." The Hindi term for diabetes, "madhumeha," which interpret as "honey-urine disease," reveals that one key symptom of the disease was recognized by ancient Indian medicine. (lancet 2018). The sweet taste of diabetic urine seems to have fled the attention of western physicians until 1679, when the English physician Thomas Willis used it to distinguish two types of the disease (lancet 2018). According to clinician and historian Robert Tattersall, diabetes was defined in the nineteenth century by its symptoms as "a fatal disease marked by polyuria, thirst, progressive weight loss, and debility." (lancet 2018). The food we eat is broken down into glucose (sugar) and released into the bloodstream. When glucose levels get high in the bloodstream, signals send to the pancreas to release insulin. Then the insulin released by the pancreas uses the blood glucose as an energy source. Diabetes occurs when the pancreas either does not produce sufficient insulin or does not utilize it properly.

The International Diabetes Federation and WHO have made great efforts since 1994, when the first global predictions of a significant rise in the number of persons with diabetes were seen, to update the figures. It was anticipated that 110 million people globally had diabetes in 1994, which was expected to rise to 239 million by 2010. In 1998, King and his colleagues estimated it would be 300 million by the end of 2025. According to another study, 366 million individuals will have diabetes by 2030, up from 171 million in 2000. But according to the WHO report 2022, it is much more than the expected patients with diabetes in all the previous records. It is found that approximately 422 million people have diabetes worldwide, and it is supposed that 1.5 million people may die because of diabetes.

TYPES OF DIABETES:

The three primary kinds of diabetes are Diabetes I, Diabetes II, and Gestational Diabetes.

Type I diabetes :

It is caused by an autoimmune response in which the body stops making insulin (Censi et al., 2018). Typically, both children and adults with this kind of diabetes are diagnosed. It is slightly more common in boys and men, whereas other autoimmune problems disproportionately affect women (Maahs et al., 2010). In the months or years preceding the onset of symptoms, there is also some seasonal synchronicity in the progression of type 1 diabetes-related autoimmunity, or the production of islet auto-antibodies (Taplin & Barker, 2008).

Cell-mediated and chronic autoimmune destruction of -cells generates immune-mediated type I diabetes.Various serological biomarkers can be used to diagnose Immune-mediated type 1 Diabetes (Burrack et al., 2017). The serological biomarkers include Islet cell antibodies (ICA), Autoantibodies against the B-cell zinc transporter 8 (ZnT8), glutamate decarboxylase (GAD65A), IA-2a and IA-2ß tyrosine phosphatase autoantibodies, Insulin autoantibodies (IAA) (for children and adolescents but not for adults). When at least one of these autoantibodies is recognized, Auto-immune 1 diabetes is analyzed. Somewhere around one of these autoantibodies is distinguishable in 85-90% of patients with hyperglycemia (Haak et al., 2019). Patients with idiopathic type 1 diabetes have perpetual insulin deficiency, frequent episodes of metabolic acidosis, and are autoantibody negative and there is no etiopathogenetic categorization of autoimmune type 1 diabetes. There is no link to the Human leukocyte antigen risk alleles. This type of type-1 diabetes is rare, inherited with high penetrance, and is more common in Asian or African patient populations. (Imagawa et al., 2000)]

STAGE 1
- Beta cell autoimmunity
- Normoglycemia
- Presymptomatic

STAGE 2
- Beta cell autoimmunity
- Dysglycemia
- Presymptomatic

STAGE 3
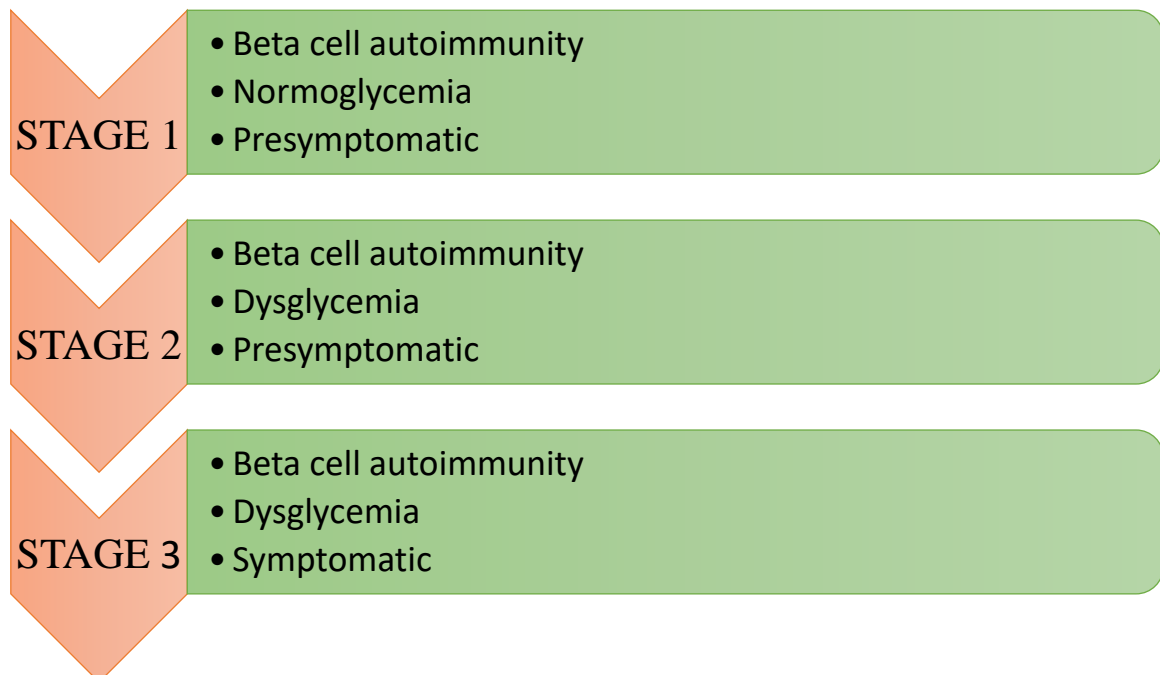- Beta cell autoimmunity
- Dysglycemia
- Symptomatic

FIGURE NO.1: Stages of type I diabetes according to Juvenile Diabetes Research Foundation (JDRF) and the American Diabetes Association(ADA) (Insel et al., 2015)

Prediction of Type 1 diabetes:

Various factors are linked with the risk of type 1 Diabetes. Some relevant factors are genetics, diet, Infections, and many humoral factors.

Genetic factors

Many studies and explorers have concluded that genetic component can account for up to 50 percent of the risk of type 1 diabetes (Steck & Rewers, 2011). Relatives of T1D patients are about 15-20 times more likely to develop T1D than the general population, where the risk is about 0.4 percent (Pociot & Lernmark, 2016). The human leukocyte antigen (HLA) complex is crucial in the development of T1D, accounting for approximately half of the genetic risk. On chromosome 6p21, the HLA region encodes genes for classes I, II, and III. The telomeric boundary of the locus contains HLA-A, HLA-B, and HLA-C genes, whereas the centromeric boundary contains HLA-DP, HLA-DQ, and HLA-DR genes. Class III is found in the centre of the HLA region. (Trowsdale & Knight, 2013). The HLA class II allele combinations have a strong impact on the likelihood of T1D. HLA-DRB1*04 in combination with DQA1*03:01-DQB1*03:02 (DR4-DQ8) raises the risk of T1D, whereas HLA DRB1*04 in combination with DQA1*03-DQB1*03:01 does not. (Nguyen et al., 2013).

T1D risk is increased not just by DR4-DQ8 haplotype, but also by the DR3-DQ2 (DRB103:01-DQA1 05:01-DQB102:01) haplotype. (Redondo et al., 2017)

Diet

The role of diet in the history of T1D is not comprehended, and the results are still conflicting.It has been proposed that cow's milk proteins trigger autoimmune reactions in genetically predisposed individuals, resulting in pancreatic beta cell destruction (Lamb et al., 2015)). Animal studies have suggested that the milk protein responsible for the development of diabetes is bovine serum albumin (BSA). DAISY, abbreviated as Diabetes Autoimmunity Study in the Young, discovered that a high risk of beta cell autoimmunity was associated with consumption of cow's milk protein only in low-/moderate-risk individuals with HLA-DR rather than in children with high-risk HLA-DR. (Lamb et al., 2015)

According to several studies, the supplementation of vitamin D can assist in treating some autoimmune diseases. All cells of the immune system have vit D receptors and can thus be controlled by calcitriol, according to research. (Prietl et al., 2013).

Infections

Childhood infections are undoubtedly among the most extensively researched factors. Epidemiological, serological, and histological studies all support the contribution of viral infections in the pathogenicity of type 1 diabetes (prediction and prevention). As described in explanations of childhood allergies, infections in early childhood may protect T1D, T1D can be caused by specific or combined infections that destroy pancreatic beta cells (Kondrashova & Hyöty, 2014). A link was discovered in both enterovirus infection and the emergence of the first autoantibody in the study of Diabetes Prediction and Prevention (DIPP). (Hyöty, 2002)(Lönnrot et al., 2000).

Type II Diabetes

Diabetes affects 415 million people worldwide, with an additional 193 million people living with undiagnosed diabetes. Type II diabetes was first recognized as a component of metabolic syndrome in 1988. Diabetes was thought to affect 415 million people in2015, with type 2 diabetes reporting for more than 90% of cases, and this figure is expected to rise to 642 million by 2040 (Chatterjee et al., 2017). Some studies suggest that vitamin D supplementation may help treat autoimmune diseases. Today, approximately 90-95% of people have type II diabetes. This is an asymptomatic kind of diabetes. An additional 318 million people have pre - clinical impaired glucose regulation, but focussed lifestyle changes, drug therapy, or both can reverse or delay the progression of type 2 diabetes. (Chatterjee et al., 2017). Type 2 diabetes patients have a 15% greater risk of all-cause deaths, which is twice the rate in young people. It is twice as high in those under the age of 55 and has a glycated haemoglobin (HbA1c) concentration of 69% (55 mmol/mol) sometimes less when compared to people without Diabetes. (Tancredi et al., 2015).

Risk Factors of Type II Diabetes

Insulin Resistance

T2DM results from an unbalance in insulin production and its activity. resistant to insulin is insufficient in T2DM to cause full-blown fasting hyperglycemia, which occurs only when ß-cell insulin secretion is insufficient. It has been argued for years if the main symptom in adult T2DM is resistance to insulin or less secretion of insulin.

Genetic factors

The disease has a clear hereditary component that is likely multigenic, which means that More than one gene defect within a single patient could be responsible for the diabetic phenotype's ultimate expression. Adult monozygotic twins have approximately 90% concordance with T2DM, and first-degree relatives have a 40% lifetime risk. (Rizkalla et al., 2004). When compared to families in which neither of the siblings has T2DM, the absolute risk of siblings of a patient with T2DM having the disease is 2-3, but this figure rises to 30 if two siblings have Type 2 diabetes mellitus (Hemminki et al., 2010). The chance of T2DM is higher when the mother has the disease compared to when the father has the disease(Groop et al., 1996). It has been tough to identify the genes responsible for complex polygenic diseases such as T2DM (DeFronzo et al., 2015). A single-nucleotide polymorphism (SNP) in TCF7L2 was discovered to be strongly linked with T2DM (Grant et al., 2006).

Obesity and lifestyle

A "global obesity epidemic" shows a rising number of nations face increasing threat to their populations' health. (Rao, 2015). In almost all populations, High body weight and an rised distribution of abdominal fat are primary risk factors for adult-onset T2DM. (Mellitus, 1974). Obesity has been shown in studies to harm insulin sensitivity and the pathogenesis of glucose metabolism disturbances in white and black children as early as childhood (Rao, 2015). Just like it is in adults, Obesity seems to be a major contributing factor in children for type 2 diabetes. Type II diabetes is an important risk factor that causes Obesity in children and adults (Dis-, 2000). From 1975 to1995, the rising

prevalence of T2DM in Japanese children appears to have paralleled the increasing prevalence of Obesity (Mellitus, 1974).

Rather a significant genetic link, the rising pervasiveness of obesity is broadly attributed to environmental factors that encourage expression of obesity. Increased animal fat and protein intake correlate with an increased incidence of T2DM in Japanese children(Mellitus, 1974). According to a recent longitudinal survey conducted in the United States, Body mass index and the probability of Obesity increased with each extra serving of sugar-sweetened beverage utilised. (Ludwig et al., 2001).

Puberty

The majority of adolescents with Type 2 diabetes mellitus present at the age of nearly 13-14 years, around early adolescence (Dabelea et al., 1999). There is compelling proof that puberty is associated with short-lived insulin resistance (Arslanian, 2000). Several studies, Using a wide range of scientific approaches, researchers discovered that insulin sensitivity in adolescents is 30% lower than in preadolescent children and adults (Hurst C Lawrence, 2009). when the normal pancreatic ß-cell function is present , the increased insulin secretion compensates for insulin resistance, resulting in peripheral hyperinsulinemia (Caprio et al., 1989). The reason of insulin resistance during puberty has been investigated. Both growth hormone (G.H.) and sex stimulants are viable choices. Many researchers have discovered that insulin-stimulated glucose metabolism correlates inversely with G.H. and insulin-like growth factor levels.(Ii-iv, 1994). Contrary to testosterone or dihydrotestosterone administration, giving G.H. to teenagers which are not insufficient in G.H. has been linked to a decrease in activity of insulin (Saad et al., 2001). Therefore, the development of insulin resistance during normal puberty is most likely caused by increased G.H. secretion, and both of these changes disappear after puberty is over.

GESTATIONAL DIABETES

Gestational diabetes mellitus (GDM) is a condition where women who have never had diabetes history and are pregnant have abnormal blood glucose levels. In a healthy pregnancy, prolactin and human placental lactogen stimulate pancreatic B-cell hyperplasia, which raises insulin levels. Some diabetogenic substances secreted by the placenta include growth hormone (GH), corticotropin-releasing hormone (CRH), placental lactogen, and progesterone. GDM results from B-cell hyperplasia and the failure to overcome the insulin resistance of pregnancy. Women with diabetes during the initial trimester would be classified as having type 2 diabetes rather than GDM, which is reserved for women whose diabetes was discovered in the second or third trimester but was not certain whether it was type 1 or type 2. Short-term complications that are more common in women with gestational diabetes include large for gestational age newborns and macrosomia, pre-eclampsia, polyhydramnios, stillbirth, and higher infant morbidity.. Beyond the postpartum and neonatal periods, gestational diabetes is connected with long-term consequences. These could signify the infant's higher risk of metabolic syndrome, reduced glucose tolerance, and childhood obesity.

DIABETES COMPLICATIONS

Acute and chronic diabetes problems are separated into two groups. Acute complication of diabetes includes hypoglycemia and comas resulting either from DKA or HHNS. At the same time, Chronic complications comprise microvascular and macrovascular complications. Many serious microvascular complications include Diabetic Retinopathy, Diabetic Neuropathy, and Diabetic Nephropathy. The most severe microvascular diabetes complications are eye complications. Patients with diabetes are highly encouraged to get frequent eye exams.

Hypertension

Hypertension is one of the biggest problems that is seen in diabetic patients. About 40% of type I and 70% of type II have elevated blood pressure (>140/90 mmHg). Blood pressure targets for the diabetic patient must be less than 130/80 mmHg. In a study, diastolic pressure should be less than 80 or 81 mmHg to reduce the chances of cardiovascular disease in diabetic patients.

Diabetic Retinopathy

Diabetic retinopathy is the most major reason for blindness in the employed population of Developed nations. The duration and intensity of hyperglycemia have been linked to an increased risk of evolving diabetic retinopathy or other micro - vascular complications of diabetes (Almdal, 2006). Having a thorough understanding of each group's peculiarities is essential. Small hemorrhages in the retina's intermediate layers are just one example of the characteristics of background retinopathy. They are frequently referred to as "dot hemorrhages" because they have the clinical appearance of dots. Proliferative retinopathy is characterised by the formation of fresh blood vessels on the surface of the retina, which results in vitreous haemorrhage (Watkins 2003).

Diabetic Neuropathy

After all potential causes have been ruled out, Diabetic neuropathy is defined by the American Diabetes Association (ADA) as "the occurrence of signs and/or indications of peripheral nerve damage in people with diabetes." (Diabetes Care 2007). The severity and length of hyperglycemia are inversely related to the probability of developing diabetic neuropathy, and some people may be genetically predisposed to such issues. Although the precise nature of hyperglycemia-induced peripheral nerve damage is unknown, it is most likely connected to pathways such as polyol buildup, AGE injury, and oxidative stress.. Diabetes-related peripheral neuropathy can appear in various ways, including sensory, focal/multifocal, and autonomic neuropathies. More than 80% of complications are performed as a result of foot wounds or ulcerations caused by diabetic neuropathy (Boulton et al. 2005).

Cardiovascular disease

Among diabetes patients, cardiovascular disease is a serious consequence and the number one factor in premature mortality (Merz et al. 2002). Diabetes patients are 2 to 6 times more likely to develop health problems such as cerebrovascular disease, peripheral vascular disease, and ischemic heart disease. Numerous investigations, starting with the Framingham research, have shown an association between diabetes and coronary heart disease, one of

the macrovascular consequences of Diabetes (Kannel et al. 1979). Smoking, high blood pressure, and hyperlipidemia—the three main cardiovascular risk factors in the non-diabetic population—all affect people with diabetes, but the condition increases the risks. Patients with diabetes typically live 7 to 10 years less than those without the disease. The growth of the ischemic disease, stroke, and death, even in the presence of other risk factors, is caused by Type II diabetes (Almdal et al. 2004). Diabetes, like coronary artery disease, is a significant early marker of the probability of a stroke and cerebrovascular disease (Lehto et al. 1996). Uncontrolled diabetes poses a risk for macrovascular disease by causing hyperlipidemia or developing on its own. A diabetes clinic will see about 25% of patients with increased lipid levels (Jacobson, 1985).

DIABETIC NEPHROPATHY

Diabetic Nephropathy occurs in approximately 25% of Type 2 Diabetes patients and is the major factor of end-stage renal disease (ESRD) in developed countries (Dias et al., 2019). Elevated blood sugars, diabetes for a long time, elevated blood pressure, overweight, and dyslipidemia are all risk factors for the progression of diabetic nephropathy. Statins, angiotensin-converting enzyme (ACE) inhibitors, or angiotensin receptor blockers slow the development of renal disease and reduce cardiovascular mobility in Diabetic Nephropathy patients. (Tziomalos & Athyros, 2015).

Risk factors for Diabetic Nephropathy (DN)

Elevated blood sugars, diabetes for an extended period of time, elevated blood pressure, overweight, and imbalance in lipid are all possible causes for the growth and cycle of Diabetic Nephropathy.

Increased Urinary Albumin Excretion (UAE)

Elevated urinary albumin excretion is a major cause for the development of diabetic nephropathy in both type 1 and type II diabetes (Viberti et al., 1982). High urinary albumin excretion, measured as 30-300 mg/g creatinine in a urine sample, is the first sign of diabetic nephropathy. Patients who develop heavily enhanced albuminuria, defined as more than 300 mg albumin/g creatinine in a spot urine sample (also known as macroalbuminuria or clinical albuminuria), are more likely to develop kidney function decline. (Milik & Hrynkiewicz, 2014).

Elevated Glucose level

Inappropriate glucose and insulin control is a major contributing factor for the development of diabetic nephropathy. Major HbA1c levels are linked to a higher risk of developing Nephropathy in both Type 1 diabetes and Type 2 diabetes patients (Hovind et al., 2004). Empirical research discovered a significant reduction in the risk of diabetic nephropathy in both T1D and T2D patient populations who improved their glycemic control (Kenzie et al., 1994). Patients with medium albuminuria (microalbuminuria) but lesser HbA1c levels were less likely to progress to extreme albuminuria (macroalbuminuria) or end-stage renal disease (ESRD), and this is according to the study of DCCT/EDIC (Gosmanov & Gosmanova, 2011). Furthermore, Strict blood glucose control reduces the possibility of proceeding from severe albuminuria to decreased GFR or ESRD. (Nathan et al., 1993).

Elevated Blood Pressure

Another significant independent risk factor for Nephropathy is high blood pressure (Hovind et al., 2004). Reduced blood pressure was linked to a reduced likelihood of advancing from moderate to severe albuminuria or ESRD., according to the DCCT/EDIC study (Gosmanov & Gosmanova, 2011). Furthermore, in T2D patients, reduced albumin from microalbuminuria to normoalbuminuria was linked to decreased blood pressure (Araki et al., 2005).

Dyslipidemia

Dyslipidemia appears to be involved in the pathogenesis of diabetic Nephropathy as well. Decreased levels of low-density lipoprotein cholesterol (LDL-C) and triglyceride (T.G.) were linked to a reduced risk of progression from moderate to severe albuminuria or ESRD in the study of DCCT/EDIC (Gosmanov & Gosmanova, 2011). Elevated total cholesterol (T.C.) levels in T2D patients are also linked to a higher risk of developing moderately and severely elevated Urinary Albumin Excretion (Tziomalos & Athyros, 2015). Furthermore, in T2D, low levels of T.C. and T.G. are linked with regression from microalbuminuria to normoalbuminuria (Maritz, 2005).

Obesity

Diabetes nephropathy is also associated with an increased incidence of Obesity. Obesity appears to be a risk factor for diabetes and chronic kidney disease. As an indicator of obesity, a high body mass index (BMI) has been linked to diabetic nephropathy (DN) and end-stage renal disease (ESRD) (Hsu et al., 2006). Weight loss was widely assumed to reduce the risk of renal injury associated with Obesity and Diabetes. Loss of weight lowered urinary protein by 51% after 24 months in subjects with obesity-related glomerulopathy, according to a study (Shen et al., 2010).

Saiki et al. reported that weight loss using a formula diet had a protective effect on renal function in obese patients with D.N. (Saiki et al., 2005). The DCCT found that excess body weight, as measured by waist circumference, was associated with a higher occurrence of albuminuria but did not indicate a decline in GFR (De Boer et al., 2007). At the same time, Weight reduction lowers urinary albumin excretion and keeps GFR from dropping. (Morales et al., 2003).

Smoking

Including both T1D and T2D patients, smoking is associated with higher albuminuria and a decrease in GFR. Smoking cigarettes contributes to diabetic Nephropathy in Type 1 diabetes (T1DM) (Orth & Hallan, 2008). A few studies suggest that it may also be a risk factor in Type 2 diabetes (T2DM). Actual smokers had a higher prevalence of microalbuminuria and proteinuric disease than non-and former smokers. So, Nephropathy progression was less common in nonsmokers and former smokers than in smokers (Orth & Hallan, 2008).

Screening of Diabetic Nephropathy:

Albumin measurement in a spot urine sample is the first step in the screening and diagnosis of diabetic nephropathy, which can be taken at the start of the day or at random, such as during a hospital visit (Merker et al., 2019). This method is precise, simple, and

suggested by the American Diabetes Association (ADA). Urinary albumin concentration (mg/l) or urinary albumin-to-creatinine ratio (mg/g or mg/mmol) are the outcomes or results of albumin measurements in spot collections. Although albumin concentration results may be influenced by urine sample dilution/concentration, this option is still more precise and less costly than albumin-to-creatinine ratio results. When a 24-hour timed urine collection was used as the benchmark, a cut-off value of 17 mg/l in a random urine specimen had a sensitivity of 100% and a precision of 80% for the diagnosis of microalbuminuria. That value is comparable to the European Diabetes Policy Group's recommended cut-off value of 20 mg/l. Screening should not be done if you have a urinary tract infection, haematuria, acute febrile illness, strenuous exercise, short-term pronounced hyperglycemia, uncontrolled hypertension, or heart failure.

Immunoassays commonly used for albumin measurements have sufficient diagnostic sensitivity for detecting diabetic nephropathy. Recently, it was shown that traditional immunochemical-based techniques failed to identify an unreactive component of albuminuria, underestimating UAE.

METABOLOMICS:

Unlike genomic and proteomic methods, metabolomics presents a significant analytical challenge because it seeks to quantify molecules with varying physical properties (e.g., ranging in polarity from very water-soluble organic acids to very nonpolar lipids). Consequently, comprehensive metabolomic platforms frequently break the metabolome into small sets of metabolites (mainly based on compound polarity, common functional groups, or structural similarity) and design specific preparation and analytical procedures to optimize for each.

We are analyzing metabolites (such as sugars, amino acids, organic acids, nucleotides, bile acids, acylcarnitine, and lipids) in a biological sample systematically and thoroughly like Urine, Serum, etc., which is known as metabolomics has been identified as a powerful tool to identify the biomarker in disease and discovery field (Zhang et al., 2020).

Several methods, including nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and capillary electrophoresis mass spectrometry (CE-MS), are now used in metabolomics (Nagana Gowda & Djukovic, 2014).

Since kidney function significantly impacts circulating metabolite levels and metabolites may have functional roles in the pathophysiology of DKD and its consequences, metabolomics methods are particularly intriguing in terms of nephrology research. (Xia et al., 2009).

Metabolomics has been classified as targeted or untargeted. A targeted approach entails identifying a specific set of metabolites, typically focusing on a pathway of interest or metabolites previously linked to the disease under investigation. This is typically assumption, but it can also serve as a means of invention if numerous classes of molecules or biochemical pathways are investigated. An untargeted approach, which is typically used only for discovery, offers a thorough examination of all quantifiable chemical compounds in a sample without partiality. These terms have some overlap, which has caused some confusion in the field. At the moment, the term untargeted may also refer to

a collection of molecules distinguished by their retention time and mass/charge ratios. Nonetheless, metabolites are not typically quantified for the sake of throughput and cost.

To find biomarkers for DKD, targeted profiling has been utilized, which involves detecting just a few particular sets of metabolites.

Advancement in the technologies like NMR, GC-MS, and LC-MS offers improved high sensitivity and specificity, and it has also enhanced the utility of the targeted metabolites. These technologies have advantages and disadvantages too.

NMR spectroscopy:

This is a quantitative technique in which minimum sample preparation is required. This technique is ideal for biofluids like urine or serum because no separation or derivatization is required during sample processing (Beckonert et al., 2007). There are two types of profiling 1H NMR spectroscopy and 2D NMR spectroscopy (Pan & Raftery, 2007). For metabolic profiling, 1H NMR spectroscopy is commonly used, whereas 2D NMR is used for structural assignment (Pan & Raftery, 2007). There as various advantages of NMR, like there is no ionization involved and no chromatographic separation is there. The data acquisition rapidly in NMR (2-3 per sample). On the other hand, it is a less sensitive technique than Mass spectroscopy. The instrument cost is very high (Darshi & Espen, 2016).

Mass Spectroscopy:

Mass spectrometry (M.S.) is commonly used in clinical metabolomics to identify and quantify various classes of metabolites. In a nutshell, M.S. involves the generation of vapour ions, which are then identified and evaluated based on their mass and charge. (Murray et al., 2013). A mass spectrometer is made up of several components, including a specimen inlet, an ion supply, a mass analyzer, and a detector. The specimen inlet feeds the specimen into the mass spectrometer, the ion source uses an ionisation method to generate gas-phase ions, the mass analyzer distinguishes the ions relying on their mass-to-charge ratio (m/z), and the detector produces an electric current roughly equivalent to the abundances of the incident ions. (Cannella, 2018). Mass spectrometers can be used singly or in conjunction. Tandem mass spectrometry (MS/MS) refers to the variety of two mass analyzers of the same type or two different mass analyzers (hybrid instruments). In MS/MS, Ions arriving at the first mass analyzer (precursor ions) are separated, disintegrated, and finally differentiated by m/z in a second mass analyzer and then detected (Makarov & Scigelova, 2010).

Gas Chromatography-Mass Spectroscopy

Mass spectroscopy in combination with gas chromatography (GC-MS) and liquid chromatography (LC-MS) provides increased reactivity and a wide range of metabolite detection. In GCMS, a gas mixture is used to separate samples through the section, after which the fragments are ionised either by electron or chemical ionisation and detected by the mass spectrometer. While Gas Chromatography (GC-MS) is a susceptible and accurate method, Because the separation takes place at extended temperatures, the metabolites must be instable and thermally non-volatile. (Darshi & Espen, 2016).

Drawbacks

- GC-MS is the more time-consuming technique required for derivatization, which can take up to two days.
- Because of the multiple steps and incomplete derivatization or adduct formation, there are frequently minor unaccounted variations in sample preparation.
- It is useful for compounds with a low molecular mass (molecular ion mass-to-charge ratio, m/z,800), but not for compounds with a low or medium polarity.
- Since the molecular ion is regularly destroyed during E.I., so identifying the metabolite is complicated when the spectrum does not exist in the library. (Darshi & Espen, 2016).

Liquid Chromatography- Mass Spectroscopy

Liquid chromatography (L.C.) is the most commonly used separation method in metabolomics, owing to its flexibility, i.e., the ability to separate various types of compounds ranging from really polar to quite nonpolar. This adaptability may be attributed to the availability of numerous chromatographic sections with different stationary phases (Kuehnbaum & Britz-McKibbin, 2013). Some properties of the compounds, such as hydrophobic nature, molecular mass, and polarisation, are primarily responsible for separation in the chromatographic system. Compounds are separated in a chromatographic column of a stationary phase with polar or nonpolar properties. The solvent which is used to remove the molecules from the stationary phase to the mobile phase has a higher polarity than the stationary phase in chromatography using polar stationary phase columns, known as normal-phase liquid chromatography (NPLC). In reversed-phase liquid chromatography (RPLC), the mobile step is less polar than the stationary phase in chromatography using nonpolar stationary phase columns. Then, in NPLC, nonpolar compounds like lipids elute first, while polar compounds like amino acids elute first in RPLC (*M 7.7.2:* 1997). Polar solvent compounds (amino acids) and non - polar compounds (phospholipids) are found in clinical samples. If the study's goal is targeted metabolomics, The stationary phase can be selected according to the component classes of choice. Moreover, many type of column is required if the goal is to gather as much data as possible (untargeted metabolomics). (Rainville et al., 2014).

Advantages

- LC-MS has significant advantages in that it requires little sample processing and can be used to evaluate metabolites that GC-MS may not separate/fragment due to their high molecular mass or polarity.
- Produces a molecular ion used for metabolite identification, making it more suitable for discovery-based approaches.
- Matrix effects in LC-MS are more pronounced than in GC-MS, resulting in ion reduction and interruption with spectral resolution.

ARSENIC

Introduction

Arsenic (As) belongs to group 15, or nitrogen family element, which is placed in the fourth period at the 33rd position in the Periodic table. It occurs both in free crystalline form as well as in Sulphur and other metals (Varadwaj et al., 2022).

Physical characteristics

Ar, Arsenic classified as a metalloid and has properties of both metals and non-metals. In the context of toxicology-based studies, Arsenic is considered as one a heavy metal (Jaishankar, Tseten, Anbalagan, Mathew, & Beeregowda, 2014). Arsenic has three allotropic forms in a metallic state α (yellow), β (black), and γ (grey) (Jomova et al., 2011).

Chemical properties

Arsenic can mimic the structure of phosphate and form covalent bonds with non-metals, influencing biological processes because Arsenic and phosphorus have similar electro-negativity and ionization energy. Arsenic can form organic and inorganic compounds from its many forms of ions, the most common of which are (+3), (+5), and (-3). It can form inorganic and organic arsenic compounds combined with O, S, Cl, C, and H. Both pentavalent and trivalent As are toxic. However, the trivalent forms are more so due to their higher reactivity with S-containing compounds. Arsenic can form Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). Arsenic has a strong affinity for thiol groups, especially dithiols. The influence of the lone pair of electrons causes Arsenic to be pyramidal in the +3 oxidation state. The toxicity of pentavalent compounds is driven primarily by their transformation to trivalent form.

PRESENCE OF ARSENIC IN THE ENVIRONMENT

Prevalence of As in the Nature

Arsenic (As) is found in the crystal lattices of minerals found in the environment and traces adsorbed onto the surfaces of other minerals as inner or outer sphere complexes. (Nikolopoulos D, 2015). Trace amounts of As can also be found in the air, water bodies, ground, flora, and fauna as they can enter the environment through various natural processes, including weathering, erosion, volcanic eruptions, and forest fires. Weathering is the most critical factor in the conversion of As sulfides (found in ores) into highly reactive forms of As [As+5 and As+3], which enter the As cycle as dust or through solvation in water(Maity et al., 2011).

Presence of As in Soil

Arsenic (As) concentrations in soil are much higher than in rock, so the risk of contamination increases. (Bundschuh et al., 2013). This is found in inorganic and organic forms, which are more common inorganic forms. Substratum, climate, biotic and abiotic soil constituents, redox potential, and all environmental factors influence soil concentrations (Kar et al., 2010).

Occurrence of As in Mineral Forms

In the lithosphere, Arsenic (As) is a common element that may be found in igneous and sedimentary rocks and different kinds of sediments (Maity et al., 2011). Even though it is widely distributed in the earth, water, and atmosphere, approximately 99% of it is found integrated into rocks and minerals. (Jaishankar, Tseten, Anbalagan, Mathew, Beeregowda, et al., 2014)). Sedimentary rocks may be co-deposited with iron oxides and sulfides. As is abundant in iron ores, deposits, and manganese nodules. (Maity et al., 2011)

Occurrence of As in Water

The occurrence of As in water has recently gained prominence due to the ongoing contamination crisis. (Shaji et al., 2021). Inorganic As species are the most abundant in aqueous environments. The mobility of arsenic minerals in water systems is influenced by factors such as pH, redox potential (Eh), temperature, dissolved humus, microbial activity, speciation, ionic strength of water, and the availability of absorbents such as Fe(III), Al(III), Mn(III), Mn(IV), silicic components, and clay minerals (Klump et al., 2006). As per WHO, the safe limit of As in water meant for consumption is ten $\mu g \ l^{-1}$ (World Health Organization, 2020). As per BIS 2012 (I.S. 10500:2012),the acceptable level of As is 10 $\mu g \ L^{-1}$ and the adequate level in the absence of a safe alternative water source is 50 $\mu g \ L^{-1}$ (Ministry of Jal Shakti, 2021).

Human exposure to As

Arsenic (As) is an environmental pollutant, and contamination of the natural environment has negative prolong-term effects on the health of human (Smedley & Kinniburgh, 2002).International Agency for Research on Cancer (IARC) is a specialized, autonomous agency of the World Health Organisation (WHO) to promote international collaboration in cancer research. Its functions are coordination of international studies on human cancer causes,mechanisms of carcinogenesis, and strategies for cancer prevention, with a greater focus on thecountries where such research is lacking. Arsenic and arsenic substances have been categorised as carcinogenic to humans by the International Agency for Research on Cancer (IARC). Arsenic in drinking water has also been declared carcinogenic to humans. Long-term use of inorganic Arsenic may cause cardiovascular illness, Diabetes, pulmonary disease, and developmental consequences. Myocardial infarction caused by Arsenic, in particular, can be a significant cause of excess mortality.

USES OF ARSENIC

Arsenic as Pesticide

Paris Green served as an insecticide for about 30 years because it effectively controlled the Colorado Potato beetle and mosquito population (Thomas, 2009). Lead arsenate pesticide was introduced to the market in the late 1800s and quickly became popular in apple and cherry orchards, which remained until the early 1900s. It was a powerful pesticide that was less harmful to plants than Paris Green (Schooley, 2006). However, it was completely phased out by 1960 after it caused illness in orchard workers and raised concerns about the fruits absorbing the Arsenic. (Nelson, 1973.). It was effectively

prohibited in the United States by 1988 (Schooley, 2006). Until the early 1900s, studies on As toxicity was limited to the effects of acute As exposure.

Arsenic as Homicidal Agent

it is readily available, and it was widely used as a homicidal and suicidal poison until the mid-1850s. It also lacks odor and taste, making it undetectable in food and beverages. (Vahidnia et al., 2007).

Arsenic used in medicine

Arsenic was previously used as a therapeutic agent. It has been used as a therapeutic agent since around 2000 BCE. (Doyle, 2009) .Arsenic was used as a balm by Hippocrates to treat ulcers and abscesses. (Riethmiller, 2005). Arsenic has been used in many traditional Chinese, Tibetan, and Indian medicines fortified with Arsenic in organic and inorganic forms. (Martena MJ, 2010.).Thomas Fowler discovered Fowler's solution in 1786. It is a 1% potassium arsenite (AsKO2) solution which was initially utilised to cure malaria, syphilis, asthma, cholera, eczema, psoriasis, and later, leukemia. (Sambu & Wilson, 2008)

Arsenic is used as a raw material for pigment and other products.

In the 1800s, an As-based pigment called Paris Green or copper acetoarsenite was thought to be the leading cause of accidental As poisoning. Paris Green was used in various products like clothing, but the use of it in wallpaper was blamed for prevalent illness. (Scheindlin, 2005.).Bartolomeo Gosia demonstrated the volatilization of Arsenic from As-based compounds, including Paris Green, in 1893. (Hughes et al., 2011).

MAJOR ROUTES OF ARSENIC EXPOSURE

As in our Drinking Water

Contamination is prevalent in India's Ganga-Brahmaputra fluvial plains. (*Geol evolution of Ganga Plains - an overview.pdf*, n.d.).As poisoning has been reported in the Ganges flood plains (Uttar Pradesh, Bihar, West Bengal, and Jharkhand), the Brahmaputra alluvial plains (Assam and Manipur), the Imphal river, and the Rajnangaon village of Chhattisgarh. (*Geol evolution of Ganga Plains - an overview.pdf*, n.d.). According to the Indian Standard Drinking Water Specification 2012, the permissible As-level for developing countries (such as India) is 50 µg/ml. Although As levels of up to 500-1000 µg/ml have been reported in West Bengal. According to extensive research, As has numerous adverse health effects on people. Ingestion has been associated to surged cases of cancers of the kidneys, lungs, skin, liver, and even the breast and prostate. A number of non-cancerous effects have been observed, including lesions in the skin, kidney disease, cardiovascular disease, neurological effects, and diabetes. (ATSDR., 2007(b).).

Arsenic in our Diet

Inorganic As was intentionally added to food during the late 1800s and early 1900s. This practice has been discontinued (Buck, 1904.).Dietary forms such as seafood, rice, mushrooms, and poultry are the largest sources of both inorganic and organic forms of As(Hughes et al., 2011). There is increasing evidence of As levels increasing in rice grains grown in West Bengal, where paddy fields are irrigated with As-rich water, whereas As levels in rice grains of areas having low amounts of As has found to be very low

(Pattnayak, 2018). Contamination has also been observed in soils irrigated with As contaminated water (Technology, 2018). Food also contains low toxicity organic As compounds like monomethylarsonic acid (MMAs), dimethyl arsenic acid (DMAs), arsenocholines, arsenobetaines, arsenosugars, and arsenolipids. DMAs or MMAs are usually found in low concentrations in different kinds of seafood. Arsenobetaine is the most commonly encountered organic As compound in marine animals and is nontoxic upon human consumption. Arsenobetaine does mutagenic,immunotoxic, or embryotoxic properties(Borak & Hosgood, 2007).

As in Soil

The soil in India is found in littoral depositions of Bengal Delta Plains (BPD), which form the fertile plains of Ganga, Brahmaputra, and Meghna. Regional vegetation and climate broadly impact the sediment depositions in these regions. Therefore, it can be saidthat the sources of origin and diffusion patterns of As in these regions are modulated by determinants such as humus distribution, oxic/anoxic circumstances, and native microbiota(Ghosh et al., 2014). Industrial contaminants cause the most significant deposition of As in soil and sediments, with concentrations reaching upwards of several thousands of milligrams per kilogram (Chandra et al., 2011).

Arsenic in Air

The level of As exposure from the air is about 20 ng/m^3, which is primarily due to inorganic As, which is much lower than that due to food and water (European Commission., 2000.).Events like volcanic eruptions, aerial mobilization, marine aerosols, and industrialexhausts introduce particulates into the atmosphere (Hughes et al., 2011). Arsinine, a highly toxic As-containing gas, is formed in a highly reducing and alkaline environment (Chauhan et al., 2008).

Arsenic in the treatment of various diseases

Arsenic trioxide (ATO) drug is widely utilized for treating Acute Promyelocytic Leukaemia (APL), an M3 variant of Acute Myeloid Leukaemia (AML) (Mathews & Chendamarai, 2011).ATO is used to treat non-APL-related hematologic malignancies like myelodysplastic syndrome (MDS), lymphomas, lymphoid leukemia, and multiple myeloma (MM) (Kwong, 2004).

Biomarkers of Arsenic

Biomarkers are indicators of changes in biomolecules, cells, or other biological changes in the body, thus their increasing usage in epidemiological case studies for various experiments ("IARC publications. Regarding the assessment of the human carcinogenic risk posed by chemicals, "1977). Biomarkers indicate exposure, its effects, responses induced by it in cells, tissues, and ultimately organism, individual susceptibility, and inference of a mechanism. Biological indicators of As exposure are nails, hairs, skin, blood, and urine, in which the level of As is measured (Abernathy & Ohanian, 1992). As it is broken down and assimilated from the blood within a few hours, thus the As-level measurement in the blood is a poor indicator of long-term (chronic) As exposure (Tam et al., 1979). The usual range of As concentration in the blood of unexposed people is ≤ 1 µg l^{-1},while As levels in cases of acute toxicity and potentially fatality may be ≤ 1000 µg l^{-1}

(Conway et al., 2021). Organs like lungs, gastrointestinal tract, etc. absorb most of the As entering the body, which is then excreted out via the urinary system within 1–2 days. Thus urinary As levels arethe most reliable biomarkers in the context of acute As exposure (Polissar et al., 1990).

MODE OF ARSENIC TOXICITY

Interactions with Sulphur

Studies of *in-vitro* and *in-vivo* As-exposure have demonstrated that certain sulfhydryl tripeptides like glutathione and other similar compounds (e.g., thioglycollate, cysteine) boost the trypanocidal effect of arsenoxide. It was discovered that only As^{3+} binds tofixed sulfhydryl group-bearing proteins of tissues (Publications, 1970).

Interactions with Phosphate

Arsenate affects in-vitro phosphate turnover (Abbas et al., 2018). Arsenic and phosphorus have similar physiochemical properties and fully protonated forms; H_3AsO_4 and H_3PO_4 have comparable structures and acid dissociation constants. This enables the substitution of phosphate for arsenate in many biochemical reactions (Dixon, 1996). Although both As and P can form ester bonds, the P-O bond is 10% shorter than the As-O bond. As a result, the As-O bond is unstable and easily broken. Both As and P can form ester linkages, but the P-O bond is 10% shorter than the As-O bond Thu (Dixon, 1996).

Formation of Reactive Oxygen Species

Arsenite can induce stress proteins *"in vitro."*(Polissar et al., 1990)In mammals, DMA^vs can cause DNA strand breaks (Kitchin, 2001). As-exposure in both *in vitro* and *in vivo* experiments led to the formation of ROS (e.g., superoxide anion, $O^{.H.-}$, H_2O_2, As centered and As peroxyl radicals), which can be detected by checking the peroxidation of lipids, expression of stress response genes, oxidative damage, (8- hydroxy-2'-deoxyguanosine), weakening/losing antioxidant defense (e.g., glutathione), and heat shock inducing proteins or by using techniques like electron spin resonance (ESR) and fluorescence spectroscopy (Shi et al., 2004)

Genotoxicity

Biological toxicity *Bacillus subtilis* recombination deficient (Rec-) and wild type (Rec+) culture were used in the Rec Assay to discover the first chemical. Growth was demonstrated to be inhibited more by arsenite and, to a lesser extent, arsenate in Rec than Rec+, suggesting that As damages DNA. (Nishioka, 1975). It is co-mutagenic, which means that it does not act as a DNA mutagen in and of itself but rather enhances the ability of other mutagens to induce mutations (Basu et al., 2001). Arsenite, for example, can boost the mutagenic effects of ultraviolet (U.V.) light, methyl methanesulfonate, and N-methyl-N-nitrosourea. (Li & Rossman, 1991). Even though it is not a DNA mutagen, As is genotoxic and causes defects such as gene deletion mutation, DNA oxidation, DNA strand breakage, dyad exchange, chromosomal aberrations such as aneuploidy and micronuclei, gene amplification, transforming activity, and unstable genome. These As-related genotoxic effects have been observed in both Vitro and Vivo studies. (Rossman et. al, 2003).

Altered DNA Repair

The inhibition of DNA repair by As in both mammalian and bacterial cells causes the co-genotoxic effect of As in conjunction with N-methyl-N-nitrosourea and UV rays. (Rossman, 2003) .In human cell nuclear extract-based studies, the induction of ROS or alteration in the signalling of cell caused changes in expression of gene , inhibiting DNA repair and resulting from collateral damage caused by As at very low concentrations. (Hu et al., 1998) .Trivalent arsenicals interfere with the zinc finger motifs (ZNF) of the proteins involved in two types of DNA repair systems i.e Base Excision Repair System and Nucleotide Excision Repair System(Ding et al., 2009) .Arsenical carcinogenicity is mediated by As inhibition of DNA repair. The main features of As-induced carcinogenicity include oxidative stress-induced DNA repair inhibition, inflammation, and cell proliferation signaling, which leads to uninhibited mitosis without maintaining cellular and DNA integrity. (Moura & Houten, 2010).

Altered DNA Methylation

Since DNA methylation regulates gene expression, epigenetic processes (changes in DNA methylation) also contribute to As-toxicity and carcinogenicity. In-vitro exposure resulted in both hypo- and hypermethylation of DNA. Incubating rat liver epithelial cell line (TRL 1215) with arsenic compound for 18 weeks resulted in hypomethylation of the DNA, erratic expression of the gene, and liver epithelial cell malignancy. The human lung adenocarcinoma A549 cell line treated with arsenite showed hypermethylation in certain regions of the cell line's p53 promoter region (Mass & Wang, 1997). Human cell when exposed to Arsenic in vitro it was observed that hypo and hypermethylation of DNA happens (Zhong & Mass, 2001) .

Signal Transduction

When As alters signal transduction, it causes erratic activation/inhibition of DNA-binding transcription factors and regulatory proteins, further disrupting gene transcription regulation. (Druwe & Vaillancourt, 2010).In vitro arsenite exposure activated all three kinase cascade families of the mitogen-activated protein kinase (MAPK) pathway, namely the p38 pathway, the extracellular-regulated protein kinase (ERKs), and the c-Jun N-terminal kinase pathway (JNKs) (Bode & Dong, 2002).

Cellular Proliferation

As can cause the over-expression of growth factors, resulting in skin neoplasia or hyperkeratosis, the most visible symptom of arsenic toxicity in humans. When primary human keratinocytes were exposed to arsenite, they overexpressed growth factors (G.F.s) such as colony-stimulating factor-2 (CSF2), transforming growth factor- (TGF-), and the proinflammatory cytokine TNF-.(Germolec et al., 1997) Cells in the body begin to proliferate due to mitogen stimulation or regeneration to compensate for cells lost due to cytotoxicity and cell death (Cohen & Ellwein, 1990). Surged cytotoxicity and proliferation in the cells were detected in rat bladder urothelium when exposed to DMAsV via water or diet. Both studies revealed an increase in the bromodeoxyuridine labeling index, a cell proliferation indicator. (Nascimento et al., 2008).In vitro studies on the urothelial cells of rat proposed that trivalent arsenical-induced cytotoxicity could be one of the causes of oxidative damage. (Wei et al., 2005).

Diseases associated with exposure

Clinical symptoms caused due to As are directly proportional to the amount of ingested As.Depending on the duration and the concentration to which an individual is/was exposed, As-based poisoning has been classified into two categories: -

Acute As exposure

Poisoning caused by acute exposure is mostly caused by inadvertent insecticide/pesticide ingestion and only rarely by suicide attempts (Ratnaike, 2003). Low doses (5 mg) cause vomiting and diarrhea, but they usually go away on their own after 12 hours without Treatment (Kingston et al., 1993) as they have a lethal dosage concentration ranging from 100 mg to 300 mg (Schoolmeester, 1980.). According to the Risk Assessment Information System database, 0.6 mg/kg per day of iAs exposure is classified as an acute lethal dosage. (Kingston et al., 1993). Clinical manifestations of acute poisoning arise in almost all body parts (Ratnaike, 2003). Diarrhoea is caused by increased blood vessel permeability. The voluminous watery diarrhoea is referred to as "choleroid diarrhoea." In the case of cholera, the stools are referred to as "rice water," whereas acute arsenic poisoning is referred to as "bloody rice water" diarrhoea due to blood loss from the gastrointestinal tract. Massive fluid loss from the gastrointestinal tract causes extreme dehydration, followed by a huge drop in blood volume and, eventually, a breakdown of the circulatory system, resulting in death. Hepatic steatosis, oesophagitis, and gastritis were also discovered during the autopsy. (Ghariani et al., 1991). Several hematological abnormalities have been reported, including basophilic stippling, intravascular coagulation, severe pancytopenia, hemoglobinuria, intravascular coagulation, and normocytic anemia. (Greenberg et al., 1979).

Chronic As exposure

Chronic exposure causes cutaneous lesions (the most common), melanosis (hyperpigmentation), keratosis, and leukomelanosis (hypo-pigmentation)(Das & Sengupta, 2008). As is also a carcinogen, causing epithelial, liver, kidney, bladder, and lung cancers. (Martinez et al., 2011). Several other health issues include carotid atherosclerosis, ischemic heart complications, and reduced motor functions and cognitive abilities. (Huang et al., 2009) As also interferes with the regulatory functions of hormones such as retinoic acid, thyroid hormone, and estrogen. (Barr et al., 2009).As Induces free radicals and oxidative stress pathways in cells. This activates the oxidative-sensitive signaling pathway, resulting in cell damage and death. (De Vizcaya-Ruiz et al., 2009).Since Arsenic have also been associated to cardiovascular disease, diabetes, and neurological disorders (Navas-Acien et al., 2005) based on the organ system affected, illnesses caused by chronic As exposure are grouped into several categories:

Dermal Disease

Chronic exposure causes skin lesions such as hyperkeratosis and hyperpigmentation, which are diagnostic features of arsenicosis. (McCarty et al., 2007). Palmar and solar keratosis is another common chronic As exposure-related diagnostic trait that can appear on the skin as a uniform thickening or discrete nodules. (Nickson et al., 1998).

Respiratory Diseases

A case study from West Bengal, India, demonstrates that patients with characteristic skin lesions associated with chronic As toxicity have the lung-based respiratory disease of both restrictive and obstructive types. (Guha Mazumder & Dasgupta, 2011). In Chile and Taiwan, researchers discovered a link between skin manifestations and lung disease (S. M. Tsai et al., 1999).

Cardiovascular diseases

As inhalation may also lead to cardiovascular system complications (Navas-Acien et al., 2005), The clinical manifestations of the heart from long-term As exposure are cardiac arrhythmias and altered myocardial depolarizations(Mumford et al., 2007). Low or moderate levels of chronic As exposure can cause mild vascular system damage,while in acute exposure, ventricular walls associated with severe hypertrophy were observed (Quatrehomme, 1992.).Areas with As-contaminated drinking water (of conc. >20 $\mu g\, l^{-1}$) reported increased deaths from cardiovascular complications like arteriosclerosis, aneurysm, and other related cardiac diseases (Engel & Smith, 1994).

Gastrointestinal diseases

Common clinical symptoms in both acute and chronic exposure cases are gastrointestinal irritation along with nausea, vomiting, diarrhoea, and abdominal pain (Vantroyen et al., 2004). As exposure-related animal studies reported hemorrhagic gastrointestinal lesions (Heywood & Sortwell, 1979)

Liver diseases

Daily oral exposure to a low dosage (0.01–0.1 mg kg^{-1}) of iAs in humans causes symptoms of hepatic injury (confirmed by clinical examination and blood analysis, which reveals higher hepatic enzyme levels in the bloodstream). Liver histology shows several portal tract fibrosis. More frequent exposure leads to liver cirrhosis, which is collateral damagefrom hepatic blood vessel injuries (Mazumder et al., 2005). Rats which are given only one oral dose of 10 mg kg^{-1} sodium arsenite showed elevated Zn^{2+} as well as Cu^{2+} concentrations in the liver. A dosage of 200 mg kg−1 of GaAs leads to decreased hepatic concentrations of malondialdehyde and glutathione (Road, 1998). A dosage of 0.02 mg kg−1 /day for continuous 60 days via drinking water contaminated with 2.5 mg of sodium arsenite led to an increased showing of peroxidation markers in rats(Bashir et al., 2006). Diffuse inflammation and mild hepatocellular degeneration were observed upon liver histology of rabbits repeatedly treated with MMA(Jaghabir & Orleans, 1989). Rats treated with 72.4 mgMMA kg^{-1} per day for 104 weeks decreased absolute liver weight. The rats were revealed to DMA (Siewicki, 1981). however, they did not report any issues. However, the mice exhibited decreased levels of glutathione and cytochrome P-450 as well as declined the activity of serum ornithine decarboxylase in the liver (Ahmad et al., 1999).

Renal diseases

Exposure to As through consumption or inhalation has been linked to severe kidney problems. Various human and animal reports around the world indicate the adverse effect of As on the structure and function of kidneys of the exposed population. Reports of human studies based in India, Bangladesh, Taiwan, China, Pakistan, Chile, and the Czech Republicreported decreased kidney function, Decreased GFR rate, and increased serum creatinine and urinary albumin. Apart from these, As also causes complications like renal tubule damage, increased ROS, oxidative stress, and kidney fibrosis, as it does not cause any significant renal injury in humans. However, exposure cases have been reported with higher than normal levels of bilirubin and creatinine (Khairul et al., 2017).

Epigenetics and kidney diseases

Diabetes or hypertension are linked to greater than 70% of end-stage renal disease (ESRD). Diabetic Nephropathy is observed in diabetic patients despite normal blood glucose levels, implying that the hyperglycemic environment induces Nephropathy through permanent changes in the gene expression patterns of the kidney. This process has been dubbed "metabolic memory," It entails permanent epigenetic modifications of regulatory domains responsible for the activation/inactivation of critical inflammatory gene transcription. (Reddy et al., 2013). Many diabetic-related experiments have revealed that epigenetic changes are linked to metabolic memory-related gene expression variations. Streptozocin kills insulin-producing -cells in Zebrafish, resulting in hyperglycemia and impaired fin regeneration and wound healing. Even after withdrawal and recovery, followed by -cell regeneration and re-establishment of euglycemic state, fin regeneration and wound healing was still impaired. (Jr et al., 2013).MiRNAs are responsible for the progression of renal fibrosis, particularly miRNA-192, which is thought to regulate pro-fibrotic gene expression. (Putta et al., 2012). Mutations in the H3K4 methyltransferase cofactor PTIP of podocyte cells cause glomerular sclerosis as well as mesangial expansion, though epigenetic pathway mutations were not identified in patients. (Lefevre et al., 2010). Whether epigenetic changes are instructive, predetermined, or simply a result/indicator of disease progression is unknown.

Neurological diseases

When inhaled or consumed, it causes severe neurological damage. Neurotransmitter levels in rats treated with sodium arsenite fluctuated (e.g., norepinephrine, dopamine, and 5-hydroxytryptamine)(Kannan et al., 2001). As infants are more susceptible to the neurological effects of iAs than adults, they are used to develop an estimate for the safe human arsenic exposure limit. Drinking water, for example, has been linked to abnormal neurobehavioral changes in children (poor performance in attention and memory-related tests as compared to unexposed controls) (S. Y. Tsai et al., 2003).In the long run, due to the exposure (2 mg kg-1 per day), encephalopathy develops, along with headache, mental confusion, seizures,even in extreme cases, coma. (Bartolomé et al., 1999) .Chronic exposure of low As levels (0.03-0.1 mg kg1 per day) causes symmetrical peripheral neuropathy. (Chakraborti et al., 2003). Degeneration of sensory and motor neurons occurs, followed by muscular weakness (Goebel, 1990.).

Reproductive health-based diseases

Animal-based studies demonstrated the ineffectiveness of As^{3+} dosage given from 14 days before mating(Holson et al., 1999). Three generations of mice were given. As a result of drinking water, there was an increase in the population size of small litter, and additionally, in all three generations of the treated group, there was a tendency toward fewer pups in each litter. (Schroeder & Mitchener, 1971).

Endocrine and hematological systems

Exposure to high As-concentrations leads increased risk of diabetes mellitus (S. M. Tsai et al., 1999). Chronic arsenic toxicity also causes neutropenia (Poklis, 1990.).

Malignancy and cancer

Under certain conditions, it is also a potential co-carcinogen and tumorigenic promoter, though these mechanisms have yet to be fully elucidated. High As levels can also be a teratogen. (Hood & Vedel-Macrander, 1984). Exposure has been linked to skin, lung, liver, kidney, and bladder cancers in Bangladesh and India. (Rahman et al., 2001) (Rahman M. C., 2001.). As-mediated cancers of the epidermis, lungs, liver, kidneys, and gallbladder have also been reported in other countries. Taiwanese researchers also said skin, nasal cavities, lungs, larynx, stomach, lymphoma, liver, colon, bladder, kidney, and bone cancers. (S. M. Tsai et al., 1999). Trivalent arsenicals are more toxic than their pentavalent counterparts, but they also have a high affinity for the Fe found in the Fe-storage protein (ferritin)(Salnikow & Zhitkovich, 2008).

Methylation is the body's natural detoxification and excretion of iAs. However, methylation also results in carcinogenic trivalent methylated arsenicals (MMA III and DMA III). (Cohen & Ellwein, 1990) . MA^V does not have any prospective carcinogenic effect (Cohen et al., 2006) DMA^V at high doses can potentially induce bladder tumors in rats (Cohen et al., 2006). DMAV and MMAV are stored in the bladder lumen, so it is more vulnerable to malignant transformation than the liver and kidney. (Kitchin, 2001). DMA^V acts as a carcinogen when co-administered with other tumorigeniccompounds (Wanibuchi et al., 2004). DM^{AV} increases the probability of developing cancers of the liver, thyroid, and kidney (Yamamoto et al., 1995). DM^{AV} promotes skin tumor formation in mice (Morikawa et al., 2000). DM^{AIII}, an unstable metabolite, stabilizes itself by forming a DMA ^{III}– GSH conjugate, which contributes to its toxic activity (M. et al., 2000). DMAIII's genotoxic activity is caused by indirect interaction with DNA via the formation of ROS, which activates transcription factors and causes hypersecretion of cytokines related to inflammation and growth, followed by increased cell proliferation and carcinogenesis. (Kitchin, 2001).

MONOSODIUM GLUTAMATE

One of nature's most abundant amino acids is monosodium glutamate (MSG). It was found to be utilised as a taste enhancer in various kinds of foods and was used as an additive (E621) in purified monosodium salt or hydrolyzed protein (2011's Assessment & Standards). Previously, MSG and glutamic acid were extracted, which was a time-consuming and expensive process. (Kazmi et al., 2017). It became available in the United States for the first time in the late 1940s. Later (1956), fermentation was successfully

used on a large scale to produce monosodium glutamate and glutamic acid. Since 1957, MSG has been manufactured in the United States via genetically altered bacteria fermentation that secretes glutamic acid through their cell walls. (Kazmi et al., 2017).

Uses of MSG worldwide

As per one report from2014, Asia was the world's top producer of monosodium glutamate, with approximately 94% of global MSG production capability (Kazmi et al., 2017). Monosodium glutamate was the world's leading producer (65%), the consumer (55%), and exporter (44%). Indonesia was the second-highest contributor to export of MSG i.e 16%. According to reports, MSG consumption was 4% in the Middle East and Africa, 3% in Europe, 2% in North America, and 2% in Central and South America. (Kazmi et al., 2017).

A side effect of MSG on humans and Animals

Monosodium Glutamate has been shown to be toxic to humans as well as laboratory animals (Tawfik & Al-Badr, 2012). Side effects reported by various studies include the appearance of metabolic/digestive, respiratory, circulatory, and nervous system anomalies. (Geha et al., 2000).

Induction of Obesity and Diabetes by the use of MSG

According to one study, MSG may be one of the suitable reagents to induce Obesity and Diabetes. (Kazmi et al., 2017). It was reported that newly born male and female mice were administered with (2 mg/g) doses of MSG, giving rise in glycosuria and other clinical signs by the age of approximately 29 weeks. (Nagata et al., 2006) MSG-treated mice had higher sugar levels, insulin, and lipid than in the control mice(Nagata et al., 2006). Male and female mice's pancreatic islets demonstrates hypertrophy, that was thought to be the progression of diabetes mellitus. Diabetes induced by MSG in mice was remarkably identical to type 2 diabetes mellitus in humans. As a consequence, these mice were considered not only suitable for diabetes research, but also for testing the possible side effects of monosodium glutamate treatment in animal studies (Nagata et al. 2006).

AIM AND OBJECTIVES

Assessment of low prenatal dose arsenic exposure effect in diabetic kidney disease after MSG induction.

OBJECTIVES
1. **Analysis of urinary metabolites in Diabetic nephropathy patients' urine by LC-MS**
2. **Early kidney injury biomarker analysis in urine samples of Diabetic nephropathy patients**
3. **Assessment of Kidney injury at 38 weeks old prenatally arsenic exposed Mice Kidney**

MATERIAL AND METHOD

Sample collection and analysis:

Urine samples of diabetic patients from AIIMS Jodhpur were collected and transferred to frozen tubes and stored at –80 °C until processing. At normal temperature, frozen urine samples were defrosted. And Urine samples (1ml) were taken from the stock and centrifuged at 12000g 15,000 rpm at 4°C for 15-20 min. After centrifugation, 400µL of supernatant was transferred into fresh tubes and blended with 1600 µL 0.1% Formic acid solution made in distilled water. Finally, the whole 2000 µL of this mixture was ready to use. Finally, samples were filtered with a 0.2-micron syringe filter, and 1ml sample was used for analysis (Lopes et al., n.d.).

LUMINEX ASSAY

Detection of kidney Toxicity

- Chronic kidney injury (CKI), Diabetic Nephropathy(D.N.), End Stage Renal Disease (ESRD) originate from chronic illnesses including diabetes, hypertension, or an inherited ailment.

- Diabetic Nephropathy is detected earlier, and proper treatment regimens are critical in preventing further loss in functions of kidney.

- The "Bio-Plex Pro^{TM} RBM kidney toxicity assays" comprise a group of biomarkers that are very useful for the early identification and characterization of kidney toxicity or damage and are detected in urine.

- The "Bio-Plex Pro RBM kidney toxicity" tests offer a method to find biomarkers that could quickly show harm, enabling effective drug testing in preclinical and clinical research settings. Days or weeks after kidney damage, conventional indicators such as serum creatinine (SCr) and blood urea nitrogen (BUN) can be seen.

Principle

- The three fundamental components of xMAP technology

- form the foundation of the Bio-Plex® multiplex system:

- Magnetic microspheres (also known as beads) that have been fluorescently colored and are each assigned a unique color code or spectral address to allow for the differentiation of various tests within a multiplex suspension.

- A 96-well microplate using a "Bio-Plex® 3D system", up to 100 kinds of molecules using a Bio-Plex® 200 system, and up to 50 kinds of molecules using a "Bio-Plex® MAGPIX^{TM} system".

- A special flowcytometer with two lasers and related optics for measuring the various

Component	Quantity	Volume	Volume After Reconstitution or Dilution
Capture beads (1x)	1 tube	1.4 ml	
Detection antibodies	1 vial	Lyophilized	4.8 ml
Standards mix	1 vial	Lyophilized	150 µl
Control 1 (high)	1 vial	Lyophilized	100 µl
Control 2 (low)	1 vial	Lyophilized	100 µl
Blocking buffer	1 vial	Lyophilized	1.5 ml
Standard diluent	1 vial	Lyophilized	1.0 ml
Sample dilution buffer	1 bottle	100 ml	
Assay buffer (10x)	1 bottle	60 ml	600 ml
Streptavidin-PE (10x)	1 tube	250 µl	2.5 ml
Assay plate (96-well flat bottom)	1 plate		
Plate seals	1 pack of 3		
Assay quick guide	1 sheet		
Product data sheet	1 sheet		

compounds bonded to the surface of the beads on the "Bio-Plex 200" and "Bio-Plex

- 3D systems.

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- The Bio-Plex MAGPIX uses LED and CCD technology to photograph the beads after injecting the complete sample load volume into the chamber.
- A quick digital signal processor that effectively controls fluorescence data.

Assay Format

- Sandwich ELISA assay works on a similar concept.
- The desired biomarker-specific capture antibodies are linked to the beads. Combined beads react with the sample-containing target biomarker.
- A sandwich complex is made by adding a biotinylated detection antibody after a series of washing steps to remove unattached protein.
- The ultimate detection complex is formed by the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin is a fluorescent indicator or reporter.

Components of Kidney Toxicity Panel

Plate Layout

- Columns 1 and 2 should be assigned standards, with row A receiving the greatest concentration and row H
- receiving the lowest.
- Put the space in wells which is mentioned as A3 and A4. The blank should only contain standard diluent and be handled in the same manner as the sample and control wells. The blank (B) MFI value was automatically subtracted from all other test wells by Bio-Plex Manager.
- Wells in columns 3 and 4 are given user-specific controls, and the quality controls are included in thekits.
- Samples of the remaining portion of the plate are available.

Reagent Preparation

- Before using, reconstitute the lyophilized reagents listed below in dH_2O following the table:

Reagent	dH_2O Volume
Standards mix	150 µl

Control 1	100 µl
Control 2	100 µl
Blocking buffer	1.5 ml
Standard diluent	1.0 ml
Detection antibodies	4.8 ml

- Ideally, the vial should be left at ambient temperature for 30 minutes.

- Vortexing the mixture in a moderate setting.

- Bring the 10x assay buffer to room temperature (R.T.).

- Stir by inversion to ensure that all salts are present in the sample.

- Make 1x assay buffer by dilution with nine parts dH20 of 1 part 10x assay buffer.

Sample Preparation:

- To remove particulates from urine samples, centrifuge them for 5 minutes at 500 g.

- Place the cleared samples in new aliquot tubes.

- For immediate use, place on ice; otherwise, Keep at -70 °C in single-use aliquots. Avoid multiple freeze-thaw cycles.

- Before dilution, completely thaw frozen samples.

- Prepare sample dilutions in polypropylene tubes as directed below within 1 hour of thawing. The dilution scenarios provided are adequate to run each sample twice.

- Just before starting the assay, bring diluted samples to room temperature. Diluted samples should not be frozen.

Assay Protocol

- Pour 10 µl of blocking buffer into each well of the plate.

- Fill the appropriate well of the plate with 30 µl of the standard, blank, control, or sample.

- Vortex the capture beads at a medium speed for 10 to 20 seconds. Each well on the plate should have 10 µl of beads in it.

- To block the light, wrap the plate in aluminum foil and seal it. Shake for one hour at R.T. while incubating at 850 ± 50 rpm

- With 100 µl of 1x assay buffer, wash the plate three times.

- For 10–20 seconds, vortex the reconstituted detection antibodies at medium speed. Give each well 40 µl.

- Cover and incubate at 850-900 rpm for one hour at room temperature, as in Step 4.

- Make the necessary 1:10 ratio of streptavidin-PE (SA-PE) dilution.

- 20 µl of diluted SA-PE should be added to each needed plate well.

- As in Step 4, cover and incubate at 850 50 rpm for 30 minutes at room temperature With 100 µl of 1x assay buffer wash the plate three times.

- Resuspend the beads in 100 µl of assay buffer after the last wash. Cover the plate as in Step 4, then shake it for 30 seconds at 850 50 rpm.

- Remove the plate seal and read the plate using the default settings.

Materials

Chemicals and reagents:
- Arsenite: Sodium arsenite NaAsO2 (arsenic);
- MSG (monosodium glutamate)
- Hematoxylin
- Eosin

Buffer
10% Neutral Buffered Formalin:

- 100ml Formalin (37-40% stock solution)
- 900ml Water
- 4g/L NaH2PO4 (monobasic)
- 6.5g/L Na2HPO4 (dibasic/anhydrous)

PBS (1X, pH 7.4, 1 Litre)

Component	Amount	Concentration
NaCl (MW: 58.4 g/mol)	8 g	0.137 M
KCl (MW: 74.551 g/mol)	200 mg	0.0027 M
Na_2HPO_4 (MW: 141.96 g/mol)	1.44 g	0.01 M
KH_2PO_4 (MW: 136.086 g/mol)	245 mg	0.0018 M

Animal housing and treatment: -

6 weeks old Female and male Balb/C mice (*Mus musculus*) were obtained from the animal house of the IIT R (Indian Institute of Toxicology Research) Guru Campus. Animals were quarantined for a week in a polypropylene cage and then housed in 12 hrs dark and light cycle at 25º With libitum food and water availability. The arsenic dose was prepared in distilled water, in the concentration of 0.04ppm As and 0.4 ppm As. Female mice were grouped into 6 groups like control, 0.04 ppm As. 0.4ppm, control MSG, 0.04 As+MSG, and 0.4 ppm As+MSG. After that, an oral dose of Arsenic was given to the mice for 15 days before mating till gestation day (G.D.) 21.Then two days, pups were treated with (write dose anMSG through the subcutaneous route. Body weight, food, and water were recorded weekly, and parameters for diabetes were also recorded periodically.

Methods:
Arsenic:
The arsenic dose was prepared in sterile distilled water. Female mice were grouped into 6 groups:

- Group-1 (control)

- Group-2 (0.04 ppm As)

- Group-3 (0.4 ppm As)

- Group -4 (Only MSG)

- Group-5 (0.04 ppm As+MSG)

- Group-6 (0.4 ppm As+ MSG)

Observation:
We observed animals from gestation day 2 to till 38 weeks and recorded body weight parameters. Also, during this period, we have done the Oral Glucose Tolerance Test(OGTT) and Insulin Tolerance Test (ITT).

After 38th week, the mice were sacrificed, their blood samples were taken into Eppendorf tubes, organs werecollected, and snap froze into liquid nitrogen and stored in a -80 deep freezer for further process.

mRNA isolation.

Procedure

Homogenization.
- Take a small fragment of the tissue and rinse it with sterile water.
- Take about 50 mg of tissue for 500 ml of TRIzol.
- Homogenize with a handheld homogenizer at 2000 RPM for ~60 seconds.
- Incubate at room temperature for 5-10 minutes.

Phase separation

- Add 100 ml chilled chloroform and agitate for ~30 seconds.
- Again,put it for incubation at room temperature for 5-15 minutes.
- Then , Centrifugation will be at 12000g for 15 minutes at 4 ℃. Transfer the aqueous phase containing the dissolved RNA to a new tube with care.

RNA precipitation and washing

- Add 250 µl of chilled isopropyl alcohol and put for incubation at room temperature for 10minutes.
- Centrifugation will be done at 12000g for 8 minutes at 4 ℃.
- Wash the pellets with500 µl 75% ethanol.
- Centrifugation will be done at 12000g for 5 minutes at 4 ℃.
- Wash once more with 75% ethanol.
- Resuspend RNA pellet in 20-30 µl DPEG-TE.

RNA quantification.

The quality and quantity of the RNA extracted were tested by inspecting the absorptionratio at 260 nm and 280 nm using the technique U.V. spectrophotometry. High-quality RNA gives A260/A280 values in the 1.9-2.1.

cDNA conversion.

- Take out the RNA from the -80 ℃ freezer and thaw them.
- 1 µg of total RNA is used per 20 µl reaction of cDNA.

- Primer/RNA mixture:
  - ✓ Total RNA- 5 µg
  - ✓ Random hexamers (50 ng/ul)- 3 µl
  - ✓ 10 mM dNTP mixture- 1 µl
  - ✓ NSFW H_2O- to make up the volume to 10 µl
  - ✓ Incubate at 70 ℃ for 5-10 minutes.
- Prepare buffer/enzyme master mixture:

  - ✓ 10×RT buffer- 2 µl
  - ✓ 25 mM $MgCl_2$- 4 µl
  - ✓ DTT- 2 µl
  - ✓ µl RNAase

- Mix the master mixture into the RNA/primer mixture, then set it aside at room temperature for 2 minutes.
- Add 1 µl SuperScript R.T. to each tube then mix it properly and then incubate at 25 ℃ for 10 minutes.
- Incubate the tubes at 42 ℃ for 60 minutes, heat inactivates them at 70 ℃ for nearly 10-15 minutes,and then chill it on ice.
- Add 1 µl RNAase H and then again incubate the tubes at 37 ℃ for 20 minutes.
- Finally , Store the 1st strand cDNA at -20 ℃ until used that cDNA for real-time PCR.

RT-PCR (qPCR)

- Using SYBR Green PCR Master Mix, relative quantitative-RT-PCR was performed ina thermocycler in a total volume of 20 µL that contains 0.5 µM primers.

- The primers used in the case of the 38-week-old kidney are for Obesity, and Diabetes gene transcripts are as follows:
  - ✓ Fabp 4
  - ✓ Cebu-α
  - ✓ Cebu-δ
  - ✓ Irs-1
  - ✓ Ins-1
- The primers used for 38week old mice for Kidney Damage are as follows:

  - ✓ Salt-1
  - ✓ Ngai
  - ✓ Acta2
  - ✓ Col1a
  - ✓ Fibronectin

CRYO BLOCKS PREPARATION AND SECTIONING
- Kidney Tissue was taken and put on the piece of cork. After that small amount of cryo matrix was added to the sample of kidney tissue.
- Put the samples for cryo-sectioning.
- After paraffin block formation, blocks were stored in a -20 ℃ freezer. Then after the section, cutting was done using a microtome with 4 μ thickness.

HISTOPATHOLOGY

Hematoxylin and Eosin staining-
When the cryo-block section was done, sections were stained with hematoxylin and eosin to look at structural changes. Staining was done by the following protocol.

Xylene 1	10 min
Xylene 2	10 min
Xylene: ethanol, 50:50	5 min
100% ethanol	5 min
90% ethanol	5 min
70% ethanol	5 min
50% ethanol	5 min
Distilled water	2 min
Hematoxylin	1.5 min
Tap water	8-10 min
90% ethanol	10-15 dip
Eosin	45 seconds
50% ethanol	2 min

70% ethanol	2 min
90% ethanol	2 min
100% ethanol	2 min
Xylene: ethanol, 50:50	5 min
Xylene 3	10 min
Xylene 4	10 min
Slides mounting	With DPX mount

IMMUNOFLUORESCENCE (IF) STAINING

Principle

The technique of immunofluorescence (IF) staining is used to study protein various cellular localization in fixed tissue specimens.Immunofluorescence uses the specificity of antibodies with fluorescent dyes to recognize their antigen, allowing visualization of the target molecule distribution using fluorescent dyes and a fluorescence microscope.

Materials and reagents

- Xylene
- EtOH
- TBST
- Citrate Buffer
- Antibodies (Podocin & KIM-1)
- DAPI
- Cy3 dye

Initial Rinsing and Permeabilization Steps
- Take out the Coplin jar after 45 min.
- Cool down at R.T.
- Wash the slides with TBST for 15 min. on the Rocker.

Blocking Step
- Add blocking agent, i.e., (3% BSA), in the jar.
- Keep the slides in the pot.
- Allow it to incubate for 1 hour.

Incubation With Primary Antibody Step
- Drain off the excess fluid room the slides with the help of tissue.
- Use a hydrophobic marker on the slides for using multiple antibodies on a single drop.

- Add 2 different primary antibodies on each slide in the sequence:
  - ✓ KIM-1
  - ✓ Podocin
- Incubate the primary antibody for 2 hours or overnight at 4C.

Second Day

Second Rinsing Step
- Wash primary antibody with TBST, 2x 5 minutes each.
- Drain off the excess fluid with the help of tissue.
- Add secondary antibody, i.e., cy3 dye (Fluorescent dye).
- Put the slides in the flat surface box.
- Incubate it for 1-2 hours in the dark.

Final Rinsing and Mounting Steps
- Wash secondary antibody with TBST, 2x 5 minutes each on Rocker/ Shaker.
- Mount the tissue with DAPI by adding a drop to it.
- The slides are covered with a coverslip,which is to be guided by forceps to avoid air bubbles.
- Keep the slides in suitable position for drying in the dark for 24 h at R.T. before imaging.

Imaging Step
- Photos of the IF slides are obtained 24 hours after mounting.
- Using a NIKON microscope, fluorescent pictures were captured.
- All images were processed.

RESULTS

We have examined that some metabolites are present in normoalbuminuric samples. The metabolites such as 3,5,6 Trichloro-2-pyridinyl (ppb), Fipronil sulfone (ppb), Dimethyl Phosphate (ppb), 3-Phenoxybenzoic acid(ppb) are found in approximately every sample. So, we can conclude that they might cause Diabetic Nephropathy. And on the other hand, from the Luminex assay of these urine samples, we can say that there might be an association between biomarkers to these metabolites.
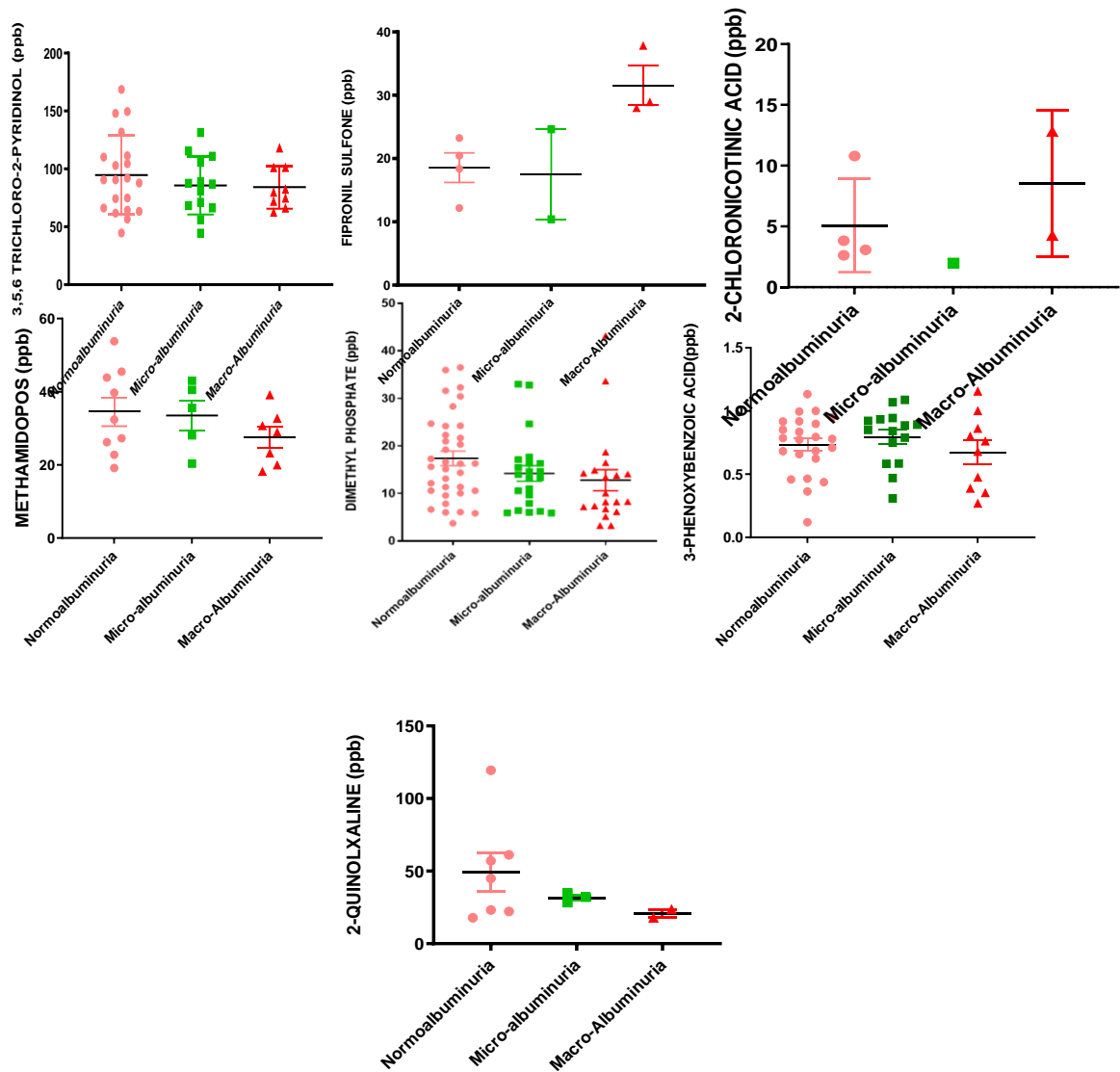

FIG NO.2: This shows the concentration of different metabolites in Urine Samples.
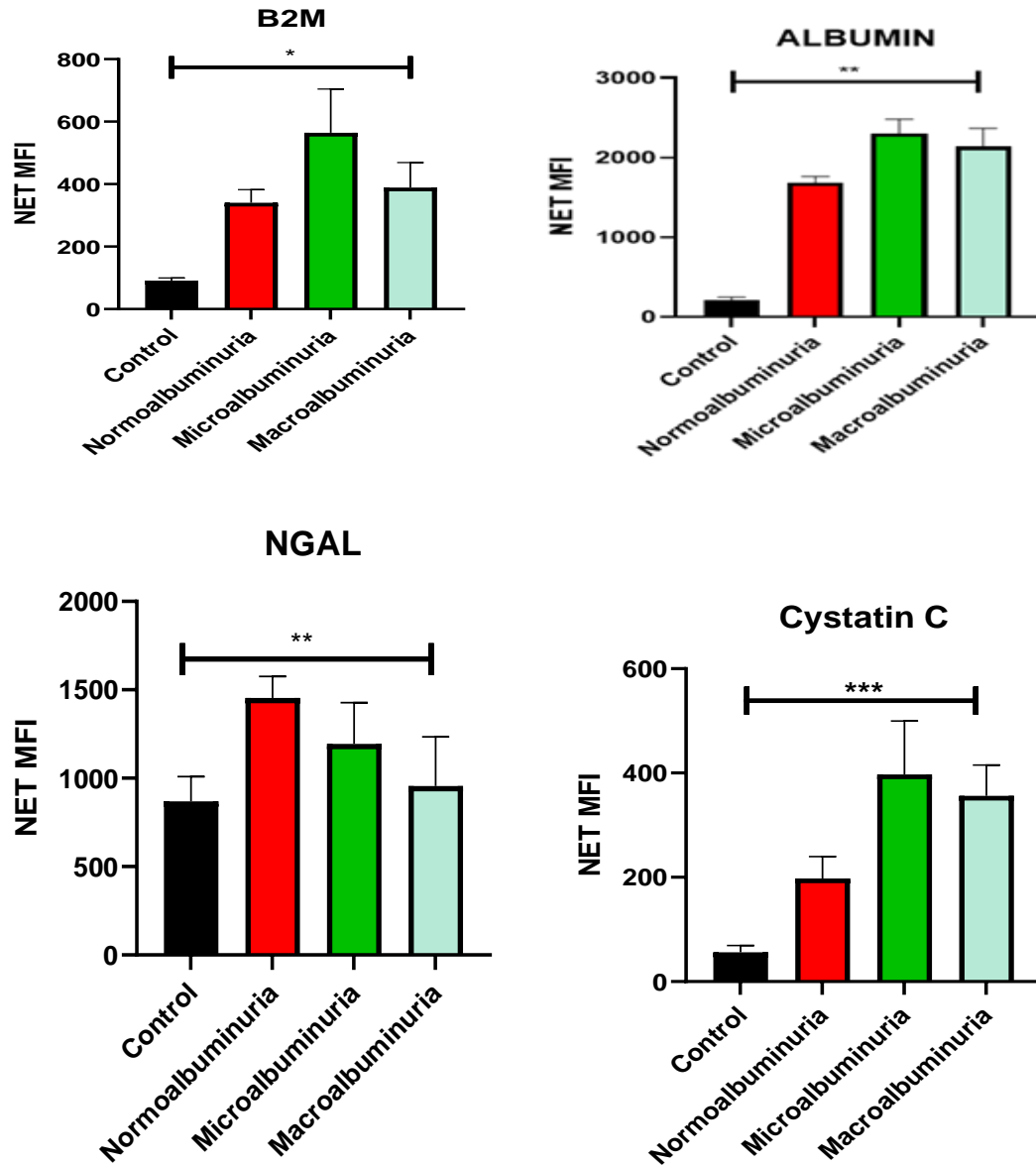
FIG NO. 1: This shows the NET MFI of different Biomarkers in urine samples.
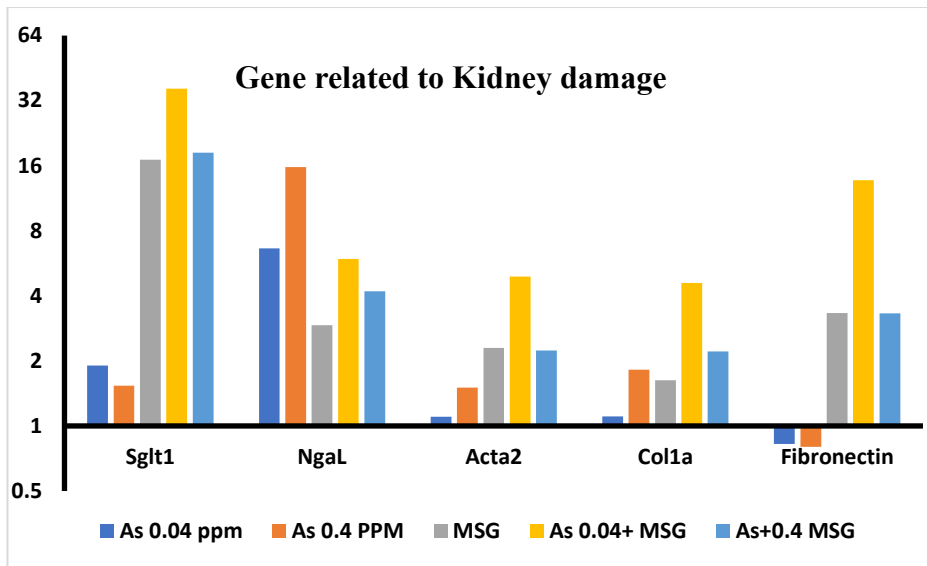
FIGURE NO.3:The arsenic treatment induces the expression of kidney injury markers and Kidney inflammation markers. Kidney injury gene including sglt1, Ngal and fibrosis genes including Acta2, col1a and Fibronectin are showing higher expression.
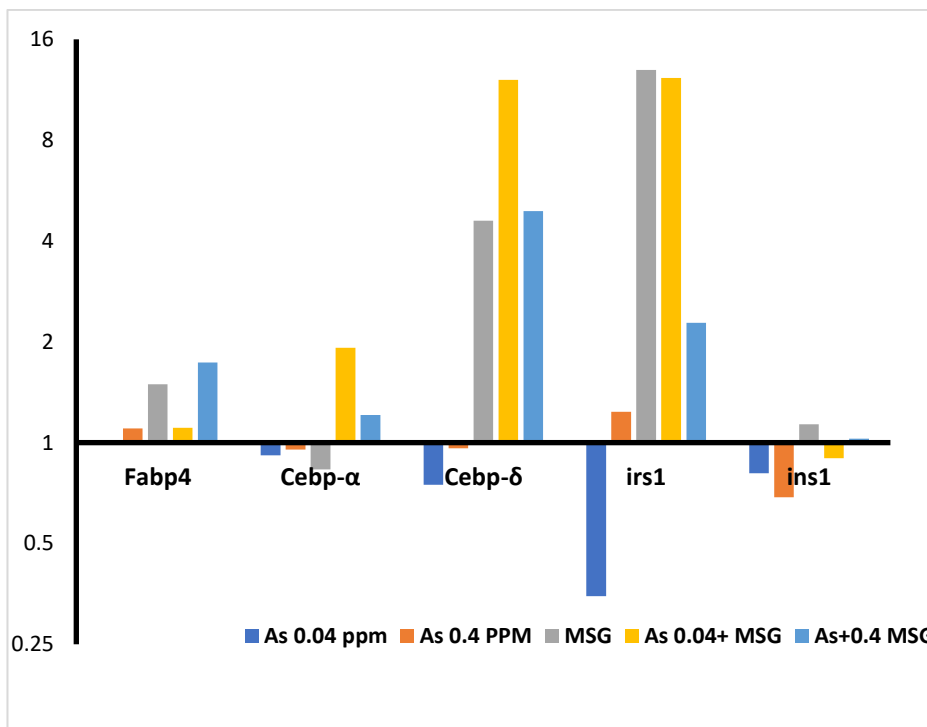

FIGURE. NO. 4: Prenatal arsenic also induces the expression of obesity related genes like Fabp4, Cebp-alpha, Cebp-delta, irs-1,ins-1

The arsenic treatment causes degeneration of kidney cells
Histopathology of the kidney samples revealed that arsenic treatment could cause damage to mouse kidney. Microscopic examination reveals the damage done to the

DCT, PCT, Bowman's capsule, glomerulus, and Podocyte. In group containing 0.04ppm As, we can observe increased urinary space, basement membrane gets thickened here and tubular damage can also be observed. In 0.4 ppm As, severe damage in glomerulus is observed and tubular dilation with endocapillary and tubular immune hyperfilteration has been detected. Futhermore, the group containing only MSG, there is damage in tubular region but 0.04 ppm As+MSG and 0.4ppm As+MSG, shows severe damage as compare to the group that contain only MSG. 0.4ppm+MSG group shows severe glomerulus damage with tubular damage (necrotic tubules)

|	20X	40X
MSG only		
0.04ppm+ MSG		
0.4 ppm + MSG		
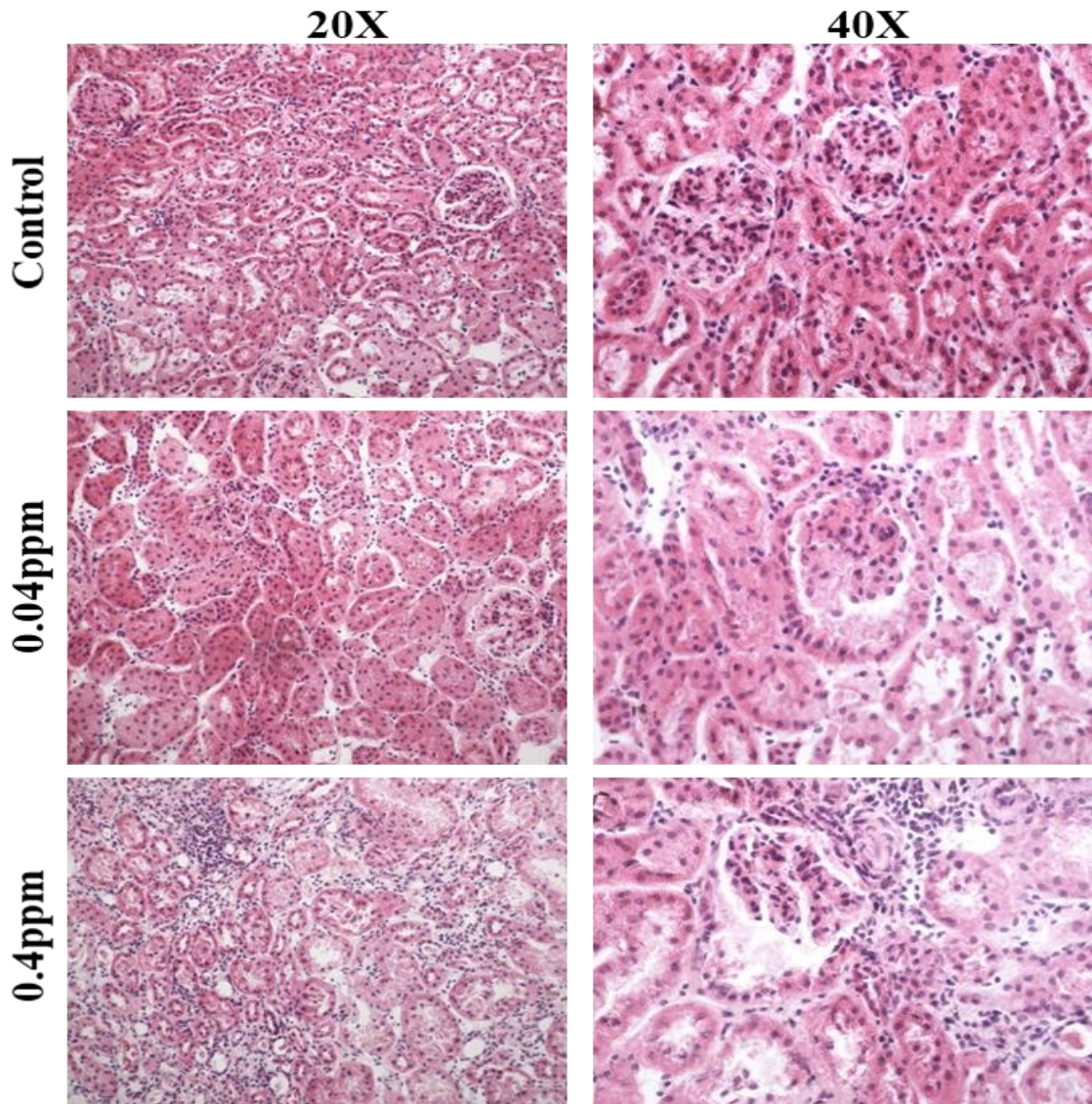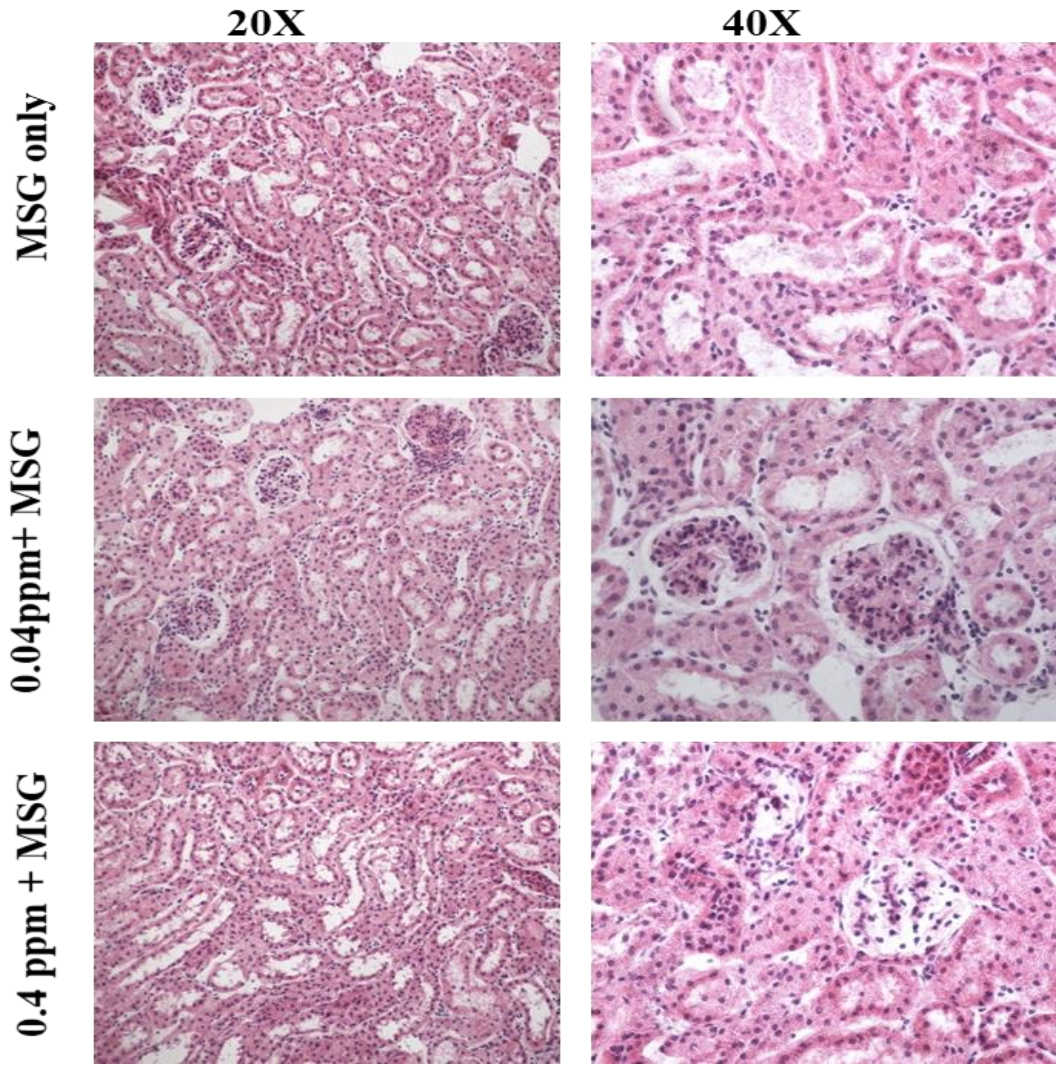
Podocyte Injury induced by prenatal arsenic exposure is detected by podocin Staining.

Immunofluorescence staining for podocin is showing the glomerulus damage. Podocin is the main component of podocyte cells, which are the main filtration unit of nephron. In prenatally As exposed animals are showing the decreased expression of Podocin when comparing with Control group. So, it is indicating that low dose prenatal arsenic is responsible for decreased expression of podocin in the nephrons as well as podocyte damage. The expression of podocin get decreased, when the kidney damage occurs. Absence of podocin leads to fast progression of kidney disease which may categorized by syndrome of nephron. When the podocin is absent, the absorption rate gets decreases. Due to which, albumin get excrete out in urine.

	Podocin	DAPI	Merged

MSG only

0.04ppm+ MSG

0.4 ppm + MSG
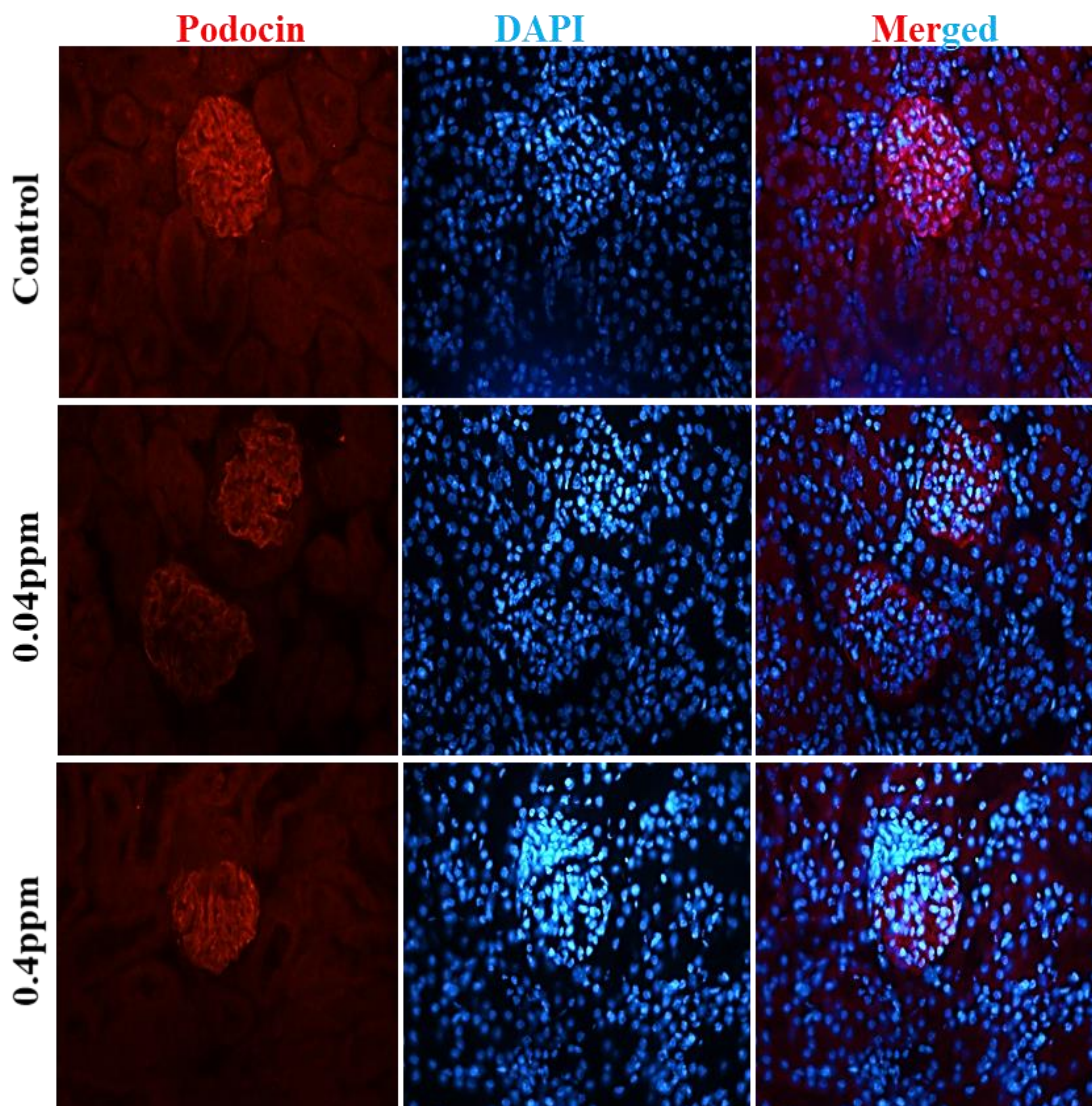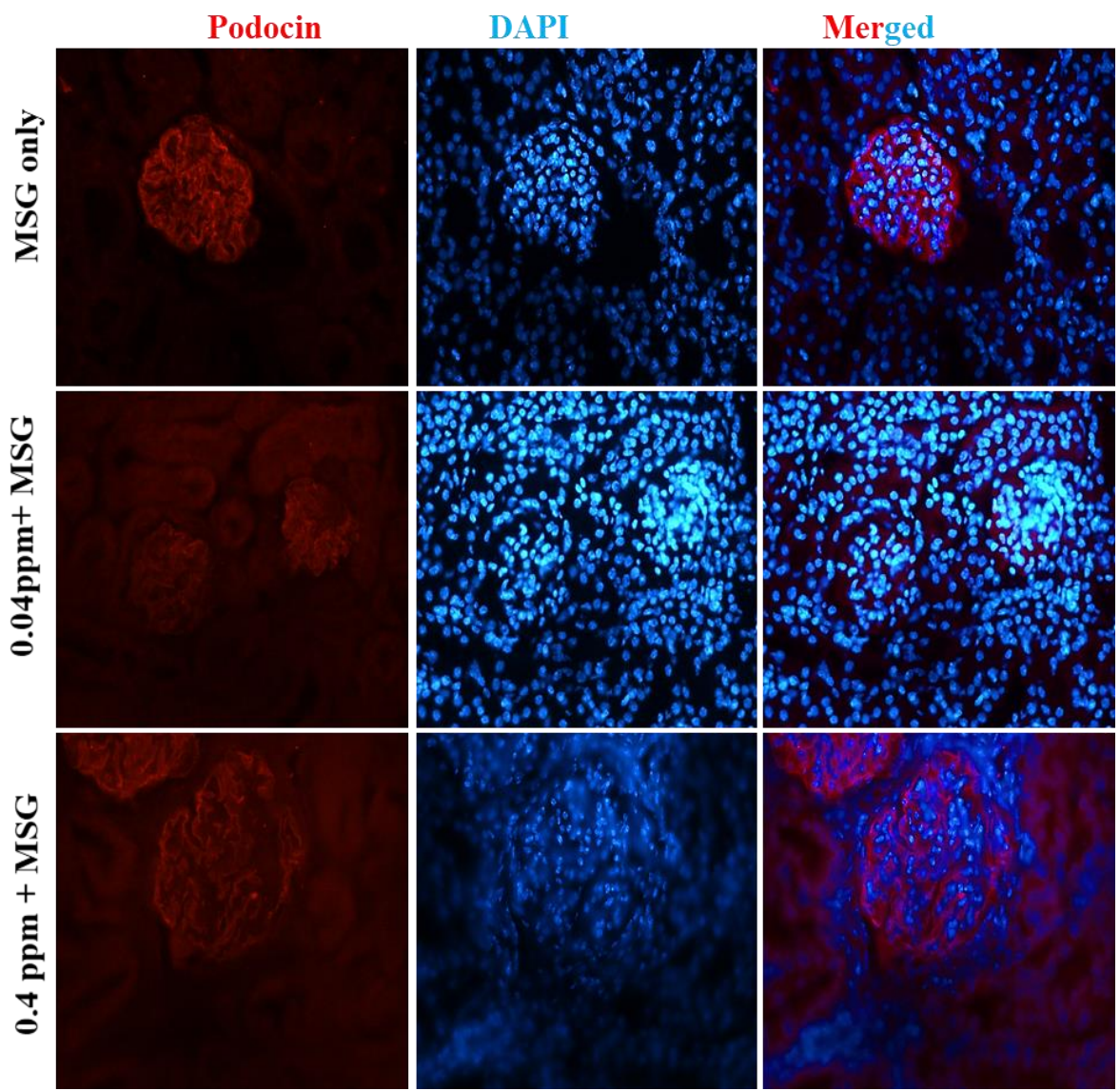
DISCUSSION

About 25% of type 2 diabetes people get diabetic nephropathy, which is the main cause of end-stage renal disease (ESRD) in developed nations. Nearly 30-40% of diabetes mellitus leads to Diabetic nephropathy (Rabkin R. Diabetic nephropathy. Clin Cornerstone. 2003;5(2):1-11. [PubMed]). Clinical metabolomics frequently uses mass spectrometry (MS) to detect and quantify distinct groups of metabolites. So, In this study, we had tried to find out the any marker or metabolites that can be present at early stage. During the experimental study , we found many pesticides in different samples of urine, which is categorised on the basis of Albumin. There are seven pesticides and insecticides that are present in different categories of samples, but a pesticide i.e 3, 5, 6 trichloro 2-pyrinidol is present in the abundant amount in all categories of patient i.e normoalbumin, microalbumin and microalbumin. Again, comparing to the control patient, this pesticide is absent. So, it can be use as early biomarker for the detection of diabetic nephropathy. Diabetes-related structural and functional abnormalities in the kidney can lead to proteinuria, hypertension, and a progressive decline in kidney function, which are the characteristics of diabetic nephropathy. The current study used in an animal model to examine the expression of genes related to diabetic nephropathy in response to postnatal MSG-induced diabetes and low dosage prenatal arsenic exposure leads to kidney toxicity. The structural examination of the kidney employing hematoxylin and eosin stain and immunostaining was also detected in diabetic mouse models. The expression of genes linked to kidney injury and diabetes is increased by arsenic exposure. When compared to the non-exposed kidney, histopathology shows damage to the tubules and the podocytes. The entire kidney can be observed to have undergone glomerular alterations. This study reveals how kidney damage and diabetes are related. The developmental toxicity of arsenic in 38-week-old puppies is also demonstrated in this study. The evidence points to serious kidney damage brought on by prolonged arsenic exposure. The genes Fabp4, , Cebp-α, Cebp-δ, Irs-1, ins-1, Sglt1 NgaL, Acta2, Col1a, and Fibronectin have been demonstrated to be activated by arsenic. Diabetes is brought on by this, which harms the kidney

CONCLUSION

As discussed, now we can say that a pesticide 3,5,6 trichloro 2-pyrinidol can be used as early biomarker detection of diabetic nephropathy. And from the animal study ,exposure of As due to food and water contamination etc., leads to global public health epidemic causing adverse health effects like kidney damage. A developing foetus may suffer catastrophic effects from exposure to arsenic. Kidney disease and cardiovascular problems may occasionally result from it.

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