DAILY ARSENIC CONTAMINATION ASSOCIATED WITH GESTATIONAL DIABETES AND NEONATAL OUTCOME

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE MASTER OF SCIENCE IN BIOTECHNOLOGY

Submitted by-

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Biotechnology Program

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Dedicated

To

My beloved parents

DECLARATION

I hereby humbly declare that this thesis titled as **'Daily arsenic contamination associated with Gestational diabetes mellitus and neonatal outcome**' is based on works carried out by me. No part of it has been presented previously for any higher degree. The research work has been carried out in the Department of Biotechnology in BRAC University under the supervision of Professor Naiyyum Choudhury, Department of Mathematics and Natural Sciences, BRAC University and Prof Liaquat Ali, Department of Biochemistry and Cell Biology and Vice Chancellor, BUHS, Dhaka-1216, Bangladesh.

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ACKNOWLEDGEMENT

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I am overwhelmed to express my respect, sincere gratitude and heartfelt thanks to Prof Liaquat Ali, Vice-Chancellor, Bangladesh University of Health Sciences (BUHS) for his inspiration, constructive criticism, endearing company and specially for her scholastic guidance and his impeccable support to me in writing my thesis paper.

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BRAC University, October 2015.

ABBREVIATIONS

ADA	American Diabetic Association
ACOG	American College of Obstetricians and Gynecologists
ANC	Ante Natal Care
ASTDR	Agency for Toxic Substances And Diseases Registry
BHMT	Betaine--Homocysteine S-Methyltransferase
BIRDEM	Bangladesh Institute of Research and Rehabilitations in Diabetes, Endocrine and Metabolic Disorders
BMI	Body Mass Index
BMRG	Biomedical Research Group
CDA	Canadian Diabetes Association
DBP	Diastolic Blood Pressure
DM	Diabetes Mellitus
DMA	Dimethylamphetamine
FBG	Fasting Blood Glucose
FPG	Fasting Plasma Glucose
g	Gram
GCT	Glucose Challenge Test
GDM	Gestational Diabetes Mellitus
HAPO	Hyperglycemia and Adverse Pregnancy Outcome
HDL	High Density Lipoprotein,
hr	Hour
HTN	Hypertension
IARC	International Agency for Research on Cancer
LBW	Low Birth Weight
LDL	Low Density Lipoprotein

LGA	Large for Gestational Age
MBP	Mean Blood pressure
MCH	Maternal and Child Health
MDA	Malondialdehyde
NAP	National Academy Press
NAS	National Academy of Sciences
NHANES	National Health and Nutrition Examination Survey
NPV	Negative Predictive Value
NRC	National Research Council
OGTT	Oral Glucose Tolerance Test
PE	Preeclampsia
PEMT	Phosphatidylethanolamine N-Methyltransferase
PIH	Pregnancy Induced Hypertension
PPV	Positive Predictive Value
ROC	Receiver Operator Characteristics
SBP	Systolic Blood Pressure
SD	Standard Deviation
SES	Socioeconomic Status
SGA	Small for Gestational age
SPSS	Statistical Package of Social Sciences
STG	Serum Tri-Glyceride,
T Chol	Total Cholesterol,
TG	Triglycerides
U-As	Urinary Arsenic Level.

UNICEF	United Nations Children’s Fund
W-As	Water Arsenic Level;
WHO	World Health Organization
WHR	Waist hip ratio

CONTENTS

ABSTRACT

Association of chronic arsenic exposure with diabetes mellitus is fairly well established, but the association of arsenic toxicity with glucose intolerance in pregnancy and with neonatal outcome have yet not been studied adequately. The present study was undertaken to explore the association of chronic arsenic exposure with gestational diabetes mellitus (GDM) and neonatal outcome in a Bangladeshi population. Under an observational cross-sectional design a total of 263 pregnant women (age in yrs, M±SD, 21±3.7) residing in an arsenic affected area of Bangladesh, were subjected to a 2 sample OGTT at third trimester of gestation. Clinical and anthropometric measurements were done by standard techniques. Degree of chronic arsenic exposure was assessed by the level of As in the usable (drinking, cooking, washing and bathing) water at the respective households and total urinary arsenic level. GDM was diagnosed by WHO criteria and neonatal outcome was assessed using APGAR Score measured by a Specialist Obstetrician. Serum glucose was measured by Glucose Oxidase method and As level in water (WAs) was measured by ultraviolet/visible spectrophotometry. Out of the 263 pregnant women 73(28%) developed GDM. WAs was significantly higher in the GDM as compared to the Non-GDM group [WAs, µg/l, median (range), 62(34-354) vs 3.6(1.02-99), $p<0.001$]. Apger Score of the neonates from GDM mothers was significantly lower compared to the neonates from Non-GDM mothers [APGER Score, M±SD, 4.7±0.8 vs 6.4±0.7, $p<0.001$]. On Pearson's correlation analysis in GDM subjects, both fasting and postprandial serum glucose levels were found to have a significant positive correlation with WAs levels ($r=0.429$;$p=<0.001$) and water arsenic and also significant positive correlation with water arsenic ($r=0.234$; $p<0.001$). The APGER Score of the neonates were found to have a significant negative correlation ($r=-0.233$; $p=0.041$) with WAs level. Chronic arsenic exposure is associated with worsening of glucose intolerance during pregnancy and it also affects neonatal outcome in an adverse way.

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Association of chronic arsenic exposure with diabetes mellitus is fairly well established, but the association of arsenic toxicity with glucose intolerance in pregnancy and with neonatal outcome have yet not been studied adequately. The present study was undertaken to explore the association of chronic arsenic exposure with gestational diabetes mellitus (GDM) and neonatal outcome in a Bangladeshi population. Under an observational cross-sectional design a total of 263 pregnant women (age in yrs, M±SD, 21±3.7) residing in an arsenic affected area of Bangladesh, were subjected to a 2 sample OGTT at third trimester of gestation. Clinical and anthropometric measurements were done by standard techniques. Degree of chronic arsenic exposure was assessed by the level of As in the usable (drinking, cooking, washing and bathing) water at the respective households and total urinary arsenic level. GDM was diagnosed by WHO criteria and neonatal outcome was assessed using APGAR Score measured by a Specialist Obstetrician. Serum glucose was measured by Glucose Oxidase method and As level in water (WAs) was measured by ultraviolet/visible spectrophotometry. Out of the 263 pregnant women 73(28%) developed GDM. WAs was significantly higher in the GDM as compared to the Non-GDM group [WAs, µg/l, median (range), 62(34-354) vs 3.6(1.02-99), $p<0.001$]. Apger Score of the neonates from GDM mothers was significantly lower compared to the neonates from Non-GDM mothers [APGER Score, M±SD, 4.7±0.8 vs 6.4±0.7, $p<0.001$]. On Pearson's correlation analysis in GDM subjects, both fasting and postprandial serum glucose levels were found to have a significant positive correlation with WAs levels ($r=0.429$;$p=<0.001$) and water arsenic and also significant positive correlation with water arsenic ($r=0.234$; $p<0.001$). The APGER Score of the neonates were found to have a significant negative correlation ($r=-0.233$; $p=0.041$) with WAs level. Chronic arsenic exposure is associated with worsening of glucose intolerance during pregnancy and it also affects neonatal outcome in an adverse way.

1. INTRODUCTION

1.1 Background:

Arsenic exposure is a well-recognized public health problem. Millions of people world-wide are potentially exposed predominantly to inorganic arsenic from drinking water contaminated by naturally occurring sources (Tchounwou *et al,* 1999). Chronic exposure to arsenic is associated with a number of adverse health effects (Yoshida *et al,* 2004). Accumulating evidence has shown an increased risk of type 2 diabetes in general populations exposed to arsenic, but little is known about exposures during pregnancy and the association with gestational diabetes (Ettinger *et al*, 2009).

Chronic exposure to arsenic through drinking water has the potential to cause adverse pregnancy outcomes, the association between arsenic in drinking water and spontaneous abortion, stillbirth, low Apgar score and neonatal death has been noticed (Milton *et al*, 2005).

1.2 Prevalence of arsenic contamination

Arsenic (As) contamination of ground water has been reported from different parts of the world and is gradually evolving in to a global problem (Rahman *et al*, 2001; Tseng *et al*, 2005; Foy *et al*, 1992; Cebrian *et al*, 1983; Borzsonyi M *et al*, 1992; Hopenhayn-Rich *et al,* 1998). More than one hundred million people worldwide are at risk of elevated arsenic exposure. The situation is particularly problematic in Southeast Asia, where a large fraction of the many hand-pumped wells yield drinking water with arsenic concentrations above 10µg/L (Br Geol Survey, 2001), the drinking water guideline value of the World Health Organization (WHO, 2004). Millions of people worldwide rely on drinking water sources containing arsenic; in the U S, about 13 million people live where arsenic levels in public drinking water supplies exceed the U.S. Environmental Protection Agency's standard (Navas-Acien *et al,* 2008). Bangladesh and West Bengal, a province of India, are the two most affected regions in the world (Smith *et al*, 2000; Mandal *et al*, 1996)

Until recently groundwater has been the principal source of drinking water for more than 80% of the Bangladeshi population [United Nations Children's Fund (UNICEF), 1998]. It is estimated that 35 to 77 million people in Bangladesh are exposed to As mainly through the drinking of contaminated water (Argos M *et al*, 2010). Since 1993, World Health Organization has recommended the provisional guideline value for As concentration in drinking water to be 10 µg/L. (WHO, 2011).

The arsenic contamination problem in Bangladesh is rapidly emerging. In December, 1995 it was estimated that only 10 million people were at risk of arsenic exposure through tube-well water. Until 1995, the arsenic contamination situation in West Bengal, India, was believed to be the greatest arsenic disaster in the world (Ahmad *et al*, 1999). However, in the following years, arsenic contamination in groundwater in Bangladesh became apparent, and the situation is now considered as the largest in the world (Ahmad *et al*, 2000).

Chronic exposure to arsenic may affect all of the organs and systems of the human body. Arsenic readily crosses the placental barrier and thus affects fetal development. Reproductive and developmental effects of inorganic arsenic on humans and on animal species have been reported (Hood *et al*, 1988; Gerver *et al*, 1988; Concha *et al*, 1982; Zierler *et al*, 1998). In contrast, there are few reports about effects of arsenic in drinking water on human pregnancy outcomes (Borzsonyi *et al*, 1992, Aschengrau *et al*, 1989). Higher spontaneous abortions (69.57/1,000 live births) and stillbirths (7.68/1,000 live births) were observed in the high arsenic area (where drinking water arsenic > 0.1 mg/L), compared to the control area (where drinking water arsenic < 0.1 mg/L); among controls, the rates for spontaneous abortions and stillbirths were 51.14/1000 live births and 2.84/1,000 live births, respectively (Borzsonyi *et al*, 1992). Gestational diabetes has an estimated prevalence of 1–14% of all pregnancies depending on race or ethnicity and diagnostic criteria used [American Diabetes Association (ADA) 2004; Ferrara *et al*, 2007].

1.3 Arsenic exposure and health effect

Exposure during pregnancy was associated with increased risks of morbidity (e.g. diarrhea) among pregnant women; (Ahmad SA *et al*, 2001) pregnancy complications (Raqib *et al*, 2009; Von Ehrenstein *et al*, 2006; Milton *et al*, 2005) as well as infant mortality (Raqib *et al*, 2009; Rahman *et al*, 2007) and morbidity (e.g. respiratory infection) (Ahmad *et al*. 2001, Rahman *et al*. 2011). The uncovering of increasing number of adverse effects due to maternal As exposure shows that it is an emerging public health problem in Bangladesh (Ser *et al*, 2015).

Health effects associated with long-term consumption of arsenic-contaminated water include cancers of the bladder, kidney, lung, and skin (Borzsonyi, Bereczky and Rudnai ,1992; Hopenhayn-Rich , Biggs and Smith ,1998; Br. Geol. Surv. 2001; World Health Organization 2004), as well as chronic non-malignant conditions, the most frequently observed being characteristic skin lesions (Haque , Mazumder and Samanta,2003;National Academy Press, 2001). Although recently more attention has been focused on the reproductive health effects of arsenic, the findings are still inconclusive (Ahmad , Sayed and Barua ,2001; Hopenhayn-Rich, Browning and Hertz-Picciotto ,2000; Borzsonyi, Bereczky and Rudnai ,1992; Yang , Chang and Tsai ,2003;Aschengrau , Zierler and Cohen ,1989; Ihrig , Shalat and Baynes ,1998).

Arsenic-induced diabetes may occur through induction of insulin resistance and beta-cell dysfunction by arsenic (or its methylated metabolites) via induction of oxidative stress or interferences in signal transduction or gene expression (Tseng 2004). Individual factors (e.g., nutritional status, genes) may also influence arsenic toxicity (Vahter *et al*, 2007).

Inorganic arsenic is an established potent human carcinogen, causing cancer in skin, lungs, urinary bladder, and kidney, and possibly also in liver, prostate, and ovaries (Intl. Agency Res. Cancer. 2004;Waalkes, Liu and Diwan 2007). Even drinking water concentrations around 10 µg/L, which is the standard in many countries, are associated with an appreciable cancer risk, on the order of 0.1%–0.3% (Natl. Res. Counc. 2001). In

addition, chronic arsenic exposure is associated with increased risk of numerous non cancer effects, e.g. hyperkeratosis; pigmentation changes; cardiovascular diseases including hypertension; respiratory effects; neurological, liver, and kidney disorders; and diabetes mellitus (Intl. Agency Res. Cancer. 2004; Natl. Res. Counc. 2001;World Health Org./Intl. Prog. Chem. Safety. 2001).

All these modes of action are highly relevant for maternal and fetal health. Still, the arsenic-related health effects have mostly been documented in adult populations in general, and little is known about variation insusceptibility depending on gender and age (Vahter *et al*, 2007). Usually, the developing organism is particularly vulnerable to toxic insult, because of rapid cell division and differentiation, especially in the brain (Grandjean and Landrigan, 2006). Because arsenic easily passes the placenta (Concha *et al* ,1998), exposure during pregnancy may be critical. In the following, the state of the art concerning the consequences of arsenic exposure for maternal health and early-life development is discussed.

1.4 Arsenic Exposure and Maternal Health

Despite the high prevalence of elevated arsenic exposure worldwide and the documented toxicity of arsenic, very few studies have investigated the effects on maternal health.

1.4.1 Hypertension: In a large cross-sectional study in Inner Mongolia, even fairly low water arsenic concentrations (20–50 μg/L) were associated with increased systolic blood pressure in women six weeks post partum (Kwok *et al*,2007). It was suggested that the cardio vascular challenge in pregnancy increased the susceptibility to arsenic.

1.4.2 Anemia: There is also certain evidence that arsenic may cause anemia, probably by destabilizing membranes (Biswas 2008) and decreasing the delta-aminolevulinic acid dehydratase activity (Kannan *et al*,2001), especially during pregnancy. Exposure to moderately elevated arsenic concentrations in drinking water (40 μg/L)in Antofagasta, northern Chile, was found to be associated with a higher rate of anemia during

pregnancy compared to that in Valparaiso, at own with essentially no arsenic in the drinking water (Hopenhayn *et al*,2006). Prevalence of anemia increased more sharply among the exposed women. This may be a serious effect of arsenic as iron deficiency in women is a common phenomenon in late pregnancy and may result in complications for both mother and child (Kumar *et al* ,2008; Mahajan *et al* ,2008). Interestingly, a recent study from Bangladesh showed a negative association between individual measures of arsenic exposure (urine concentrations) and hemoglobin concentrations in men but not in women (Heck *et al* ,2008). Only in the small fraction of women with hemoglobin concentrations below 100 g/L was elevated arsenic exposure (more than 100 µg/L in urine) associated with decreased hemoglobin concentrations.

1.4.3 Hormonal disturbance: Given that arsenic interacts with steroid hormones and estrogen in particular, it seems likely that arsenic exposure may have additional adverse effects on maternal health and child development as well as on women's health more generally. Indeed, recent studies indicate increased age at menarche in Indian girls exposed to arsenic through drinking water (Sen and Chaudhuri, 2007; Sengupta 2004). Exposure of female rats to arsenite in drinking water over seven estrous cycles caused a significant reduction in plasma levels of leutinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol, along with a significant follicular and uterine cell degeneration and decreases in ovarian steroid-metabolizing enzymes and ovarian and uterine glutathione peroxidase (Chattopadhyay 2003). Selenium supplementation restored the plasma hormone levels and prevented the arsenic-induced histopathological changes in ovaries and uterus.

1.4.4 Tumors: In utero exposure of mice to fairly high arsenic concentrations (42 or 85 mg/L in drinking water during gestational days 8–18) showed a marked increase in ovarian and lung tumors as well as uterine and oviduct hyperplasia in the females at adult age (Waalkes , Liu and Diwan 2007). This indicates that arsenic-related effects on women's health may be induced prenatally.

1.4.5 Skin diseases: One population-based study on arsenic-induced health effects in Bangladesh, which is one of the few designed to evaluate gender differences in arsenic-related health effects (Rahman 2006), showed that the lower risk of arsenic related skin lesions in women largely is explained by the more efficient metabolism of arsenic in women compared to men (Lindberg 2008). On the other hand, epidemiological studies that have reported data for men and women separately, although not directly comparable, indicate that women may be more sensitive to certain arsenic-related toxic effects (Vahter 2007). Obviously, more studies designed to evaluate these issues are needed.

1.4.6 Oxidative stress: Arsenic could influence diabetes development by mechanisms involving oxidative stress, inflammation, or programmed cell death (apoptosis) (Navas-Acien *et al*, 2006). Rat pancreatic beta cells treated with arsenic showed impaired insulin secretion and function (Diaz-Villasenor *et al*, 2006). This impaired insulin secretion (even by low level arsenic exposure) may disturb beta cell function via a mechanism involving oxidative stress (Fu *et al*. 2010). Other experimental studies showed that arsenic can affect beta cells, increasing beta cell apoptosis (programmed cell death) by causing oxidative stress. Arsenic can decrease insulin secretion as well as beta cell viability (Lu *et al*, 2011).

Arsenic exposure during pregnancy has been found to affect the immune cells in the placenta and umbilical cord blood, via inflammation and oxidative stress. Prenatal exposure to arsenic, then may affect the function of the immune system of the baby, and have consequences for diseases later in life (Ahmed *et al*, 2010). Arsenic is an endocrine (hormone) disruptor. In animals and cell cultures, arsenic can disrupt hormonal (endocrine) processes, including glucocorticoid, estrogen, androgen, progesterone, and thyroid receptors, as well as gene expression, at low doses similar to those found in the environment (Davey *et al*, 2008).

1.4.7 Gestational diabetes: There is also evidence that arsenic exposure may increase the risk of gestational diabetes. A study has found that pregnant women who had higher

arsenic levels also had higher blood glucose levels after a glucose tolerance test. This finding implies that arsenic may impair glucose tolerance, and may be associated with an increased risk of gestational diabetes. The women in this study lived near a hazardous waste site, and had arsenic levels higher than those in unexposed people, but their exposures were still "relatively low" (Ettinger *et al*, 2009).

1.5 Mechanisms Consideration

Pregnancy implies considerable stress on maternal metabolism. This is particularly true for one-carbon metabolism because methyl groups, as well as folate and choline, are critical for placental and fetal development. Elevated levels of Hcy in maternal plasma have been associated with pre-eclampsia, placental abruption, adverse pregnancy outcomes, and infertility (Braekke *et al*,2007;Forges *et al*, 2007; Refsum *et al*,2006)). To support the remethylation of produced Hcy and to meet the fetal demand of choline for brain development, PEMT is up-regulated in pregnancy to increase the de novo synthesis of choline in maternal liver (Zeisel 2006). Betaine, the oxidation product of choline and the form delivering the methyl group to Hcy, becomes increasingly important during pregnancy for adjusting the Hcy level (Velzing-Aarts 2006), especially in women with low serum concentrations of folate and methionine (Wallace 2008). Obviously, this betaine-dependent remethylation of Hcy by BHMT is particularly important for arsenic-exposed women. In fact, the methylation of inorganic arsenic to DMA increases during the course of pregnancy (Concha *et al*,2003;Hopenhayn *et al*,1998; Vahter 2007).

1.6 Impaired Fetal Growth

There are certain indications of impaired fetal growth in mothers exposed to arsenic via drinking water during pregnancy (Hopenhayn *et al*,2003;Yang *et al*,2003).Two ecological cross-sectional studies from north eastern Taiwan (up to 3600 µg/L; 85% above 50 µg/L in the drinking water) and northern Chile (on average 40 µg/L in the water) showed non significant decreases in birth weight of 30 and 57 grams, respectively (from 3133 and 3398g, respectively). Our recent population-based cohort study involved 1578 mother-infant pairs in rural Bangladesh, with measurements of

arsenic concentrations in maternal urine samples collected in early and late gestation (median 80 µg/L, 90th percentile 400 µg/L). Arsenic exposure showed a significant negative association with size at birth at urine arsenic concentrations below 100 µg/L, but no further effect at higher exposure levels. The reason for this dose-response pattern is not known. The total As-associated decrease in birth weight was 170 g (average birth weight 2681 g), and the head and chest circumferences were reduced by 5 mm and 14 mm, respectively.

1.7 Mechanisms of Fetal Toxicity

Considering the high placental transfer of arsenic and its documented toxicity, the reported effects on fetal development seem to be less strong than could be expected, especially in populations with prevalent poor nutrition. Possibly, the increased methylation efficiency during pregnancy results in less arsenic being transferred to the fetus (Hood *et al*,1988), either because of the increased rate of excretion in maternal urine with advancing gestation (Concha *et al*,1998) or variation in the rate of transfer among the arsenic metabolites, as discussed above. Also, the methylated arsenic metabolites, at least in their pentavalent form, are less fetotoxic (Hood 1998; Irvine , Boyer and DeSesso 2006). As there is a wide inter individual variation in arsenic methylation, it is important to evaluate whether women with low methylation capacity, either due to genetic polymorphisms or otherwise impaired one carbon metabolism, are more susceptible to arsenic-induced adverse pregnancy outcomes.

1.8 Arsenic contamination

Arsenic is a ubiquitous metalloid element in the bedrock, sediments, and soils. From these minerals and deposits, it is easily dissolved to surrounding aquifers. Therefore, drinking water derived from groundwater is a common source of human exposure to inorganic arsenic (Intl. Agency Res. Cancer. 2004;Natl. Res. Counc. 2001). In addition, people may be exposed via ambient air in areas with industrial emissions or coal burning, particularly when arsenic-rich coal is used for indoor stoves in poorly vented homes (Wang *et al*,2009).

An increasingly important source of exposure to inorganic arsenic is food, such as cereals and vegetables, especially in areas where arsenic-rich groundwater is used for irrigation (e.g.Adomako *et al*,2009). Highly elevated arsenic concentrations in seafood consist mainly of arsenobetaine, arsenocholine, and arsenosugars, which are of less toxicological significance but need to be considered when arsenic in urine is used as an indicator of exposure to inorganic arsenic.

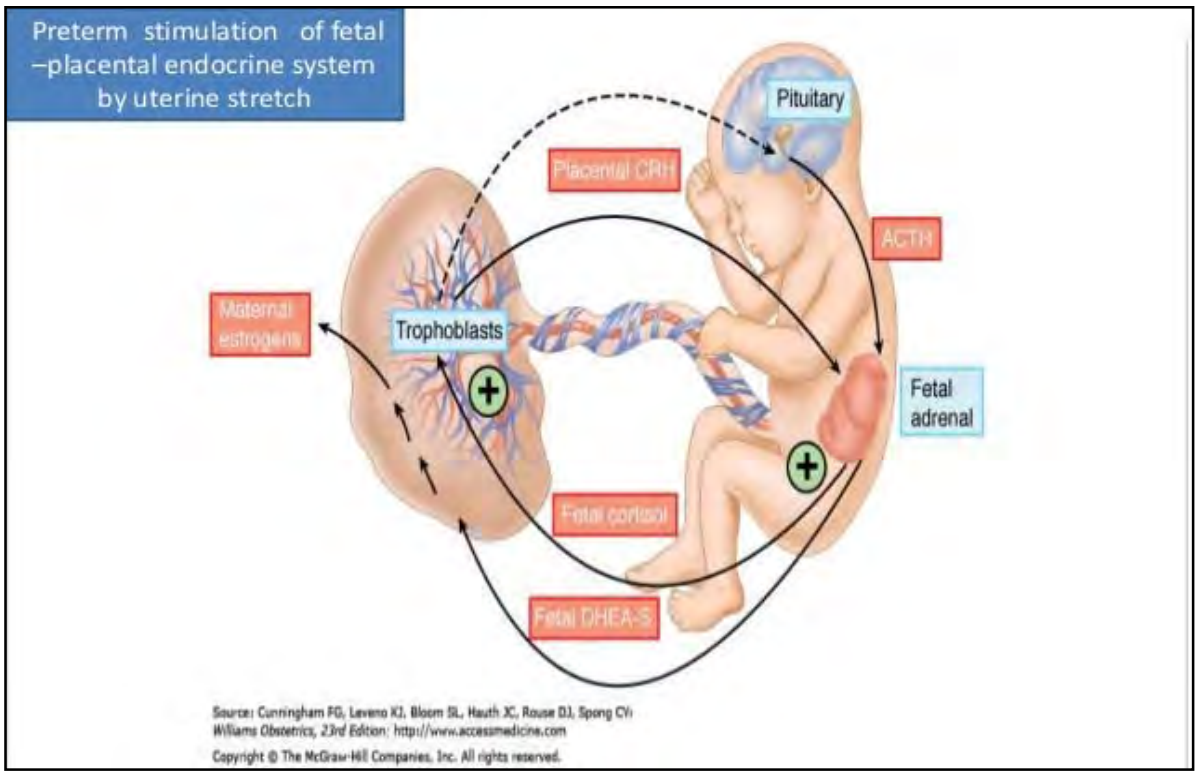

Figure 1.1: preterm stimulation of fetal- placental endocrine system by uterine stretch (Vahter, 2009)

Arsenic is a highly potent toxicant and carcinogen (International Agency for Research on Cancer 2004; National Research Council (NRC) 2001). Worldwide, millions of individuals are drinking well water with arsenic levels above the World Health Organization (WHO) guideline value of 10 µg/L (Kinniburg, 2001; Nordstrom, 2002; WHO, 2004). Arsenic concentrations are high in certain geologic formations, and

arsenic can dissolve easily to groundwater and contaminate local tube wells and other public water supplies. This has been a problem in many parts of the world, including Argentina, Bangladesh, Chile, China, Hungary, India, Taiwan, and parts of the United States (Chowdhury *et al*, 2000; Nordstrom, 2002; Smith, 2000).

Millions of people worldwide are potentially exposed predominantly to inorganic arsenic from drinking water contaminated by naturally occurring sources(Tchounwou *et al*, 1999).

1.9 Arsenic toxicity with glucose intolerance:

Arsenic-induced diabetes may occur through induction of insulin resistance and beta-cell dysfunction by arsenic (or its methylated metabolites) via induction of oxidative stress or interferences in signal transduction or gene expression (Tseng, 2004). Individual factors (e.g., nutritional status, genes) may also influence arsenic toxicity (Vahter, 2007).

Few studies have explored the effects of arsenic on human pregnancy outcomes (Huyck *et al*, 2007; Rahman *et al*, 2007; Vahter *et al*, 2006) and none have investigated risk of diabetes in pregnant women, even though diabetes is a major potential complication of pregnancy with adverse effects for both mothers and infants.

Gestational diabetes (GD) occurs when resistance to circulating insulin leads to hyperglycemia, and this impaired glucose metabolism is first detected during pregnancy. GD has an estimated prevalence of 1–14% of all pregnancies depending on race/ethnicity and diagnostic criteria used (ADA, 2004; Ferrara, 2007).

GD is associated with 30–60% increased risk of developing diabetes in later life in the mother and also poses intergenerational risks to the fetus (Metzger, 2007). Diabetes in pregnancy is associated with increased risk of major congenital malformations, macrosomia (birth weight > 4,000 g or > 90th percentile for gestational age), and

complications during delivery and in the perinatal period including stillbirth (Fetita *et al*, 2006).

1.10 Arsenic toxicity with birth defects

Arsenic crosses the placenta in both animals(Hanlon and Ferm,1987) and humans,(Concha *et al*,1998) and experimental studies support a role for arsenicas a developmental toxicant. Reproductive studies in anarsenic-contaminated community surrounding a Swedish copper smelter found that women working in the smelter or living nearby gave birth to infants of lower birth weight, and had a higher incidence of spontaneous abortions and congenital malformations (Nordstrom ,Beckman and Nordenson,1978; Nordstrom ,Beckman and Nordenson,1979). Although arsenic levels were high, confounding from other chemicals or lifestyle factors could not be excluded. Studies around a copper smelter in Bulgaria found a higher incidence of toxemia in pregnant women (Tabacova *et al*,1994)and lower birth weight in newborns,(Tabacova *et al*,1994) but again, the results were limited by methodologic concerns.

Investigations focused on arsenic exposure from drinking water have reported increased rates of miscarriage (Aschengrau, Zierler and Cohen,1989;Borzsonyi *et al*,1992)stillbirths (Borzsonyi *et al*,1992)and congenital heart disease(Engel and Smith,1994;Zierler*et al*,1988).In the county of Antofagasta, Chile, rates of stillbirths and infant mortality increased over a time period coinciding with a substantial increase in the arsenic concentration (from 90 ug/L to 800ug/L) in the local public drinking water supply (Hopenhayn-Rich *et al*, 2001).A retrospective survey of mothers in Bangladesh compared self reported pregnancy outcomes in two villages with contrasting arsenic levels in drinking water (mean 240 ug/L vs. 20ug/L), and found increases in miscarriages, stillbirths and preterm births in the more highly exposed village(Ahmad *et al*, 2001).

Exposure to elevated levels of inorganic arsenic (iAs) in drinking water is a major public health concern because iAs has been linked with numerous adverse health outcomes (Kapaj *et al*, 2006; Rahman *et al*, 2009). The detrimental effects from long-

term exposure include skin lesions, cardiovascular disease, peripheral vascular disease, diabetes mellitus, and cancers of the urinary bladder, skin, lung, liver, and prostate (Del Razo *et al*, 2011; International Agency for Research on Cancer, 2004; Maull *et al*, 2012; Rahman *et al*, 2009; Tseng, 2007). It is also evident that certain populations, including pregnant women and their unborn children, are particularly susceptible to the adverse effects of iAs exposure(Vahter, 2009). For instance, prenatal iAs exposure has been associated with a greater risk of adverse pregnancy outcomes such as preterm birth, low birth weight, and or fetal loss (Vahter, 2009).

1.11 Apgar score:

Virginia Apgar (1909-1974) was an American obstetrical anesthesiologist. She was a leader in the fields of anesthesiology and teratology,and introduced obstetrical considerations to the established field of neonatology. To the public, however, she is best known as the inventor of the Apgar score, a way to quickly assess the health of newborn children immediately after birth.

Apgar score is used for rapid assessment of newborns.Low five-minute Apgar score has been associated with increased risk of severe neurologic outcome, but data on milder outcomes, particularly in the long term, are limited. We aimed to examine the association of five minute.Apgar score with prevalence of neurologic disability and with cognitive function in early adulthood.

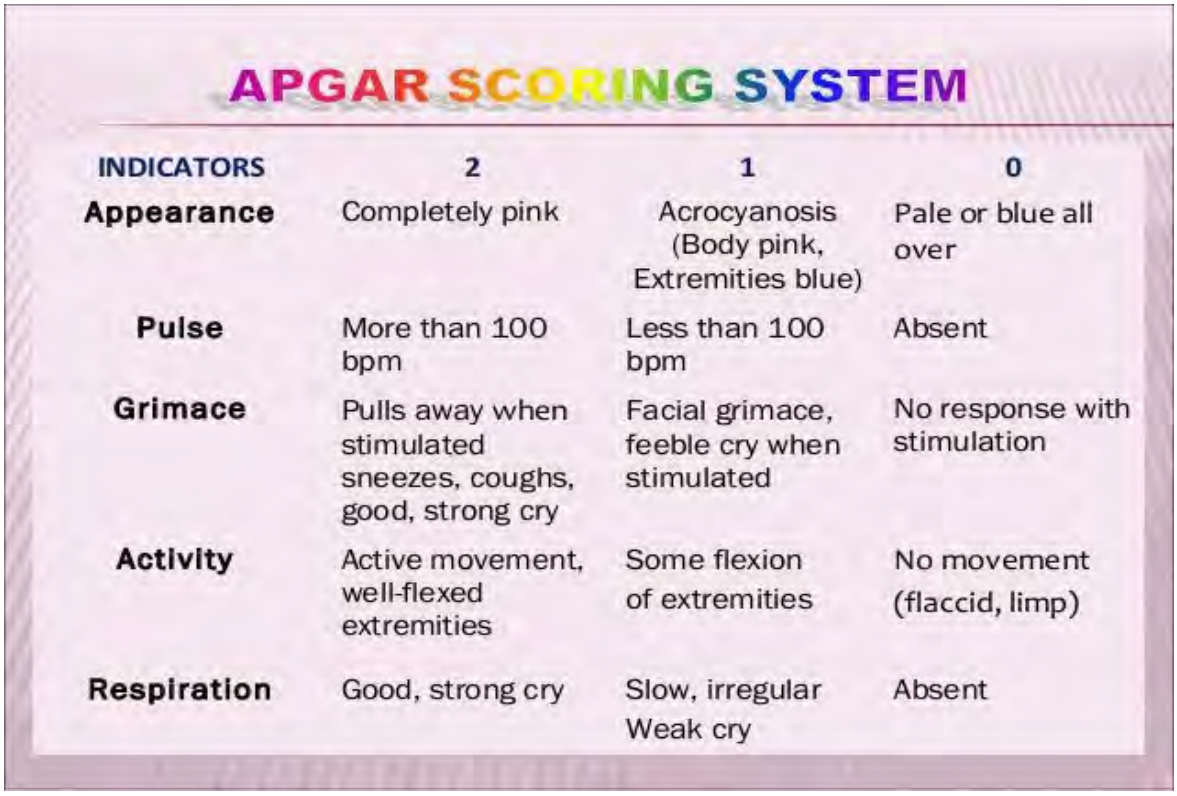

Figure 1.2: Apgar scoring system (source: Google)

The test is generally done at one and five minutes after birth,and may be repeated later if the score is and remains low. Scores 7 and above are generally normal, 4 to 6 fairly low, and 3 and below are generally regarded as critically low.

A low score on the one-minute test may show that the neonate requires medical attention (Casey,McIntire and Leveno,2001), but does not necessarily indicate a long-term problem, particularly if the score improves at the five-minute test. An Apgar score that remains below 3 at later times such as 10, 15, or 30 minutes may indicate longer-term neurological damage, including a small but significant increase in the risk of cerebral palsy. However, the Apgar test's purpose is to determine quickly whether a newborn needs immediate medical care. It is not designed to predict long term health issues (Curr. Res. Anesth. Analg, 1953)

A score of 10 is uncommon, due to the prevalence of transient cyanosis, and does not substantially differ from a score of 9. Transient cyanosis is common, particularly in babies born at high altitude. A study that compared babies born in Peru near sea level with babies born at very high altitude (4340 m) found a significant average difference in the first Apgar score, but not the second. (Gonzales and Salirrosas ,2005)

In recent years, doubts have been cast on the value of the Apgar score. Studies found that the Apgar score failed to predict specific neurologic outcomes of the term infants, a use for which it was never intended(Nelson and Ellenberg ,1981). What's more, it was once inappropriately adopted alone to diagnose asphyxia (AAP Committee on Fetus and Newborn and ACOG Committee on Obstetric Practice ,2006).

In order to place the Apgar score in its proper perspective, the Neonatal Resuscitation Program Guidelines state that ''Apgar scores should not be used to dictate appropriate resuscitation actions, nor should interventions for depressed infants be delayed until the 1 minute assessment'' (Vahter 2009).Furthermore, the Apgar score also has its own limitations. A number of factors may influence an Apgar score such as drugs, trauma, congenital anomalies, infections, hypoxia, hypovolemia,and preterm birth. Up to date, there are few consistent data on the significance of the Apgar score in preterm infants. Because elements of the score such as tone, color and reflex irritability partially depend on the physiologic maturity of the infants, this situation may lead to a healthy preterm infant with no evidence of asphyxia receiving a lower score only because of immaturity (AAP Committee on Fetus and Newborn and ACOG Committee on Obstetric Practice ,2006).

Association of Apgar score at five minutes with long-term neurologic disability and cognitive function.Most newborns with Apgar scores below 7 grow up healthy, but risks of neuro developmental disability among them are greater than among those with higher Apgar scores, particularly in the short term(Thorngren-Jerneck and Herbst,2001;Nelson and Ellenberg,1984; Moster *et al*,2001; Sun *et al*,2006).

Increased risks have been reported for neonatal seizures (Cebrian *et al*,1983); neonatal intracranial hemorrhage (Thorngren-Jerneck and Herbst,2001); cerebral palsy (Thorngren-Jerneck and Herbst,2001;Nelson and Ellenberg,1984;Moster *et al*,2001); mental retardation (Thorngren-Jerneck and Herbst,2001); and epilepsy (Sun *et al*,2006; Ehrenstein *et al*, 2006). There are also reports of association between five-minute Apgar scores below 7 and risk of motor and developmental impairments at school age, including symptoms of attention deficit (Moster , Lie and Markestad, 2002) and speech and language problems (Krebs , Langhoff-Roos and Thorngren-Jerneck, 2001). Less is known about long-term or mild neurodevelopment disability among newborns with low Apgar scores.

Moreover, no published study is available on pregnancy outcomes in relation to arsenic exposure through drinking water in Bangladesh. Therefore, we explore this study was to identify pertinent information regarding pregnancy outcomes of the women who were chronically exposed to arsenic through drinking water, and also to estimate the Apgar score and association between adverse pregnancy outcomes in exposed and non-exposed groups.

1.12 Objectives

1.12.1 General Objectives: The general objective was undertaken to explore the association of chronic arsenic exposure with gestational diabetes mellitus (GDM) and neonatal outcome in a Bangladeshi population.

1.12.2 Specific Objectives:

The specific objectives of the study were:

- To investigate the arsenic exposure during pregnancy in arsenic affected area.
- To assessed the maternal and neonatal outcome in arsenic exposure mother.
- To explore the association between arsenic affected mother with maternal and neonatal outcome.

2. METHODOLOGY

2.1 Study design:

It was an observational cross-sectional study. The study comprised face to face interview and urine, blood and using water sample were analyzed.

2.2 Study area:

This study was conducted in three rural districts of the People's Republic of Bangladesh, Chandpur, Faridpur and Madaripur. These districts were selected based on evidence of presence of arsenic (As) -contaminated tube wells. Databases from the local public health offices recorded 73%, 26% and 79% of tube wells with As levels higher than 50 g/L in the Sadar (district center) of Chandpur, Faridpur and Madaripur respectively. These records were provided to the government offices through nation-wide surveys conducted by local health workers and non-governmental organizations in 2003.

2.3 Study period:

Total study period started from 4th January to 6th December, 2014. The study period expanded with literature review and protocol preparation,data analysis and final thesis submission. Data were collected March to August 2014 Study period.

2.4 Study participants:

We recruited 317 pregnant women and used datasets from 263 (age in yrs, M±SD, 21±3.7) pregnant women residing in an arsenic affected area of Bangladesh and 54 mothers exclude the final analysis.

The participants for this study were pregnant women and their newborns with no known medical conditions. The recruitment of participants started from March to August 2014. It was carried out at one hospital and clinic at each district. Most of our participants were in their later half of the second or third trimester when enrolled. Pregnant women

who were in the early second trimester were invited to return for recruitment when they reached the later stage of pregnancy.

Gynecologists of each centre conducted health checks for pregnancy complications such as preeclampsia, intrauterine growth restriction, placenta previa, and placental abruption; medical history such as diabetes mellitus and renal disease; and neonatal outcome was assessed using Apgar score measured by a Specialist Obstetrician.Pregnant women who had tobacco and alcohol consumption habits and non-singleton birth history were excluded in this study.

2.5 Inclusion criteria

- Screening of women with at 27-38 weeks of gestation.
- Previous history of GDM or glucose intolerance.
- History of GDM-associated adverse pregnancy outcomes and
- Maternal age ≥25 yrs.
- No pre-existing diabetes mellitus.

2.6 Exclusion criteria

- Multi fetal pregnancy.
- History of non-singleton birth.
- Any disease that affected pregnancy e.g. thyroid disorder, heart disease, hypertension etc.
- Mental illness

2.7 Data collection procedure:

Local health workers were recruited to administer informed consent was taken from each subject (fulfilling the inclusion criteria) in an appropriate form (Appendix-I). The exact nature and purpose of the study was explained to gestational diabetes in short and were asked for the willingness to participate and then motivated them to visit clinical laboratory for the blood investigation.The study participants to collect the following information: age, education, occupation, time living at residence, smoking status and

alcoholic beverage consumption during pregnancy (both defined as yes or no and frequency), daily prenatal supplement intake (yes or no), residence location (urban or rural), source and daily consumption of drinking and cooking water, and source of bathing water. In addition, information on previous pregnancy outcomes including number of pregnancies and number of previous pregnancy losses was gathered from questionnaires. Information on birth outcomes/measures including newborn birth weight, newborn length, gestational age, head circumference, placental weight, and 5-min Appearance, Pulse, Grimace, Activity, Respiration (APGAR) score was gathered at time of delivery by the physician.

Social demographic data including: age, education, occupation, and socioeconomic status (SES) were recorded. To analyze SES, information on possession of assets (table, chair or bench, wrist watch or clock, bed, radio, colored TV, refrigerator, bicycle and mobile phone), and household status (availability of electricity, number of rooms, presence of flushed toilets, and building materials for roof, walls and floorings) was collected. Participants were also asked their last menstrual periods and parity. Gestational week at the time of maternal sampling was calculated from the dates of sample collection and last menstrual period.

At delivery, recruited local nurses recorded the birth date and mode of delivery. The length of gestation was calculated from the dates of delivery and last menstrual period. Information collected for the newborn included sex, Apgar score, recumbent length and birthweight.

Data on adverse outcomes were collected, including preterm birth (gestational age < 37 weeks), low birth weight (LBW; < 2,500 g), small for gestational age (SGA; birth weight < 10th percentile), and large for gestational age (LGA; birth weight > 90th percentile). SGA and LGA categories were based on newborn data collected from northern regions of Mexico (Montes-Nunez et al, 2011; Rios et al, 2008). During hospitalization, a protocol clinical examinationof the newborns was performed.
Anthropometric data were measured once, before breast feeding started.

Infants were weighed without diapers and using anelectronic digital infant scale. Length was measured in the supine position, using a stadiometer composed of a stationary head-board and a movable footboard. Knees and hipswere extended using gentle force and the footboard pressedagainst the balls of the feet (BMC Public Health 2013).

The Apgar score was measuredon a scale from 1 to 10, at 1 and 5 minutes after delivery.Infants were evaluated on a scale of 0 to 2 according to five categories (skin color, muscle tone, reflexes, respiratory effortand heart rate), and the points from each category added together to determine the total score.

Apgar scale (evaluate @ 1 and 2 minutes postpartum)				
	Sign	**2**	**1**	**0**
A	Activity (muscle tone)	Active	Arms and legs flexed	absent
P	Pulse	>100 bpm	<100 bpm	absent
G	Grimace (reflex irritability)	Sneezes, coughs, pulls away	grimaces	No response
A	Appearance(skin color)	Normal over entire body	Normal except extremities	Cyanotic or pale all over
R	Respirations	Good, crying	Slow, irregular	absent

2.8 The Apgar score was measured by following signs used are as follows:

2.8.1 **Heart Rate**: This was found to be the most important diagnostic and prognostic of the five signs. A heart rate of 100-140 was considered good and given a score of two, a rate of under 100 received a score of one, and if no heart beat could be seen, felt or heard the score was zero.

2.8.2 **Respiratory Effort**: An infant who was apneic at 60 seconds afterbirth received a score of zero, while one who breathed and cried lustilyreceived a two rating. All other types of respiratory effort, such as irregular, shallow ventilation were scored one. An

infant who had gasped once at thirty or forty-five seconds after birth, and who then became apneic, received a zero score, since he was apneic at the time decided upon for evaluation.

2.8.3 **Reflex Irritability**: This term refers to response to some form of stimulation. The usual testing method was suctioning the oropharynx and nares with a soft rubber catheter which called forth a response of facial grimaces, sneezing or coughing. Although spontaneous micturition and defecation are not a response to an applied stimulus, they were considered to be favorable signs if they occurred.

2.8.4 **Muscle Tone**: This was an easy sign to judge, for a completely flaccid infant received a zero score, and one with good tone, and spontaneously flexed arms and legs which resisted extension were rated two points. We are unable to agree with Flagg's description of spasticity (Flagg ,1944) as a sign of asphyxiation of the infant. The use of analeptics in the baby did not influence this score because of the standardized early time of observation and rating.

2.8.5 **Color**: This is by far the most unsatisfactory sign and caused themost discussion among the observers. All infants are obviously cyanotic at birth because of their high capacity for carrying oxygen and theirrelatively low oxygen content and saturation (Eastman,1930).The disappearance ofcyanosis depends directly on two signs previously considered respiratory effort and heart rate.

Comparatively few infants were given a full score of two for this sign, and many received zero in spite of their excellent score for other signs. The foreign material so often covering the skin of the infant at birth interfered with interpreting this sign, as did the inherited pigmentation of the skin of colored children, and an occasional congenital defect. Many children for reasons still mysterious to us, persist in having cyanotic hands and feet for several minutes in spite of excellent ventilation, and added oxygen. A score of two was given only when the entire child was pink. Several hundred children were

rated at three or five minutes as well as at sixty seconds and in almost all cases a score of two could be given for color at these later times.

2.9 Data collection technique

Face to face interview in between fasting and 2 hr (OGTT) blood collection, participants were asked with questionnaires by the data collector. Each participant took approximately 20 minutes to complete the questionnaire.

2.10 Anthropometric Measurement

Weight

Body weight was measured on a lever balance (Detecto-Medic, Detecto Scales, Inc, USA). The balance was calibrated every day before use. The body weight was measured bare footed to the nearest 0.1 kg with clothes on. The average weight (0.5 kg) of the clothes was later substracted from the measured weight. The measurement of weight done after the bladder has been emptied and before a meal.

Height

Heights of the subjects were measured barefooted in the standing position with a stander scale to the nearest 0.1 cm (Detecto-Medic, Detecto Scale Inc., USA).

During measuring height some precautions were taken. When measuring height, the subjects stands straight with the head positioned such that the Frankfurt plane is horizontal, feet together, knees straight, and heels, buttocks and shoulder blades in contact with the vertical surface of the stadiometer.

Method of calculation of Body Mass Index (BMI)

Body mass index was calculated from the body weight and height of the subjects using the following formula weight in kg divided by height in meter Square.

$$BMI = \frac{\text{Weight in kg}}{(\text{Height in meter})^2}$$

2.11 Measurement of blood pressure as per ACOG (Fernando, 1993):

Blood pressure measured in lying position keeping the sphygmomanometer at the level of heart. When systolic B.P more than 140 mm of Hg or diastolic BP more than 90 mm Hg it confirmed on two different occasions at least 6 hours apart. Hypertension is defined following the criteria of American College of obstetrics and Gynaecology (ACOG-Fernando, 1992) According to ACOG hypertension is defined as BP equal to as or more than 140/ 90 mm Hg; rise of systolic BP 30 mm Hg or rise of diastolic BP 15 mm Hg or more.

2.11.1 Pregnancy Induced Hypertension (PIH): Gestational hypertension (GH) and Preeclampsia (PE) are together considered as Pregnancy induced hypertension or PIH.

2.12 Procedure of the Collection sample (Blood and Urine):

Urine and blood samples were collected by recruited local phlebotomists.

All participants were requested to fast over night (8-10hour) and not to take any kind of medicine or not to smoke on the previous day. They were requested to attend the sample collection room of the hospital on the next morning. 5 cc of fasting blood and 2cc two hour after 75 gm glucose were collected following all aseptic precaution from the ante-cubital vein using disposable plastic syringe. Collected blood samples were kept in capped and airtight glass test tubes. Prior to sample collection the containers and test tubes were deionized as follows:

- The container and test tubes were washed with detergent.
- Those then were rinsed with double distilled deionzed water several times and then dried in an oven at 60^0C.

After collection of samples the test tubes were sealed with parafilm. Serum was separated by centrifugation (10 minutes at a rate of 3000 rpm) at 20^0 C immediately after the blood was taken. Urine sample also collected same time with selected urine container.

2.12.1 Gestational Diabetes Mellitus (GDM):

GDM diagnosis by WHO criteria (1999): 2hr post load glucose>=200mg/dl (11.1mmol/l) during an OGTT. The test should be performed as described by WHO using a glucose load containing the equivalent of 75-g anhydrous glucose dissolved in water.

2.12.2 Diagnostic criteria for impaired glucose homeostasis

FPG >=110 to <126mg/dl (6.1 to 7.0mmol/l)

2hr post load glucose 140 to <200mg/dl (7.75 to 11.1mmol/l)

2.12.3 Normal

FPG <110mg/dl (6.1mmol/l)

2 hr post load glucose <140mg/dl (7.75mmol/l)

Separated serum was allocated in different eppendrop and preserved immediately at -27^{0}C for the subsequent analysis. Before analysis sample was allowed to thaw and then analyzed for fasting glucose, 2 hours after glucose, triglyceride, total cholesterol.

2.13 Measurement Arsenic exposure:

2.13.1 Arsenic exposure by usable water: Degree of chronic arsenic exposure was assessed by the level of As in the usable (drinking, cooking, washing and bathing) water at the respective households.

2.13.2 As exposure by Urine sample:

Urine samples were diluted with 1.5% nitric acid and 2% 1-butanol, and filtered through 0.45 µm pore membranes. Maternal urinary-As (U-As) concentrations were measured using an octopole collision/reaction cell inductively coupled-plasma mass spectrometry (Agilent 7500 ce - Agilent Technology, Hachioji, Japan). Certified Reference Material No.18 “Human Urine” (National Institute for Environmental Studies, Tsukuba, Japan) was used for quality control. The mean detection limit was 0.97 µg/L.

2.14 Statistical analysis

The data obtained by questionnaire were first checked for the completeness by the investigator. After checking the completeness, coding was done. Data entry, cleaning, editing and final analysis was done in SPSS 16. Chi square test, frequency distribution used to analyze the association between the variables. P value of <0.05 considered sufficient for rejecting the null hypothesis of no difference among groups.

2.15 Ethical considerations

All necessary ethical and administrative approvals were obtained from the appropriate authorities before the commencement of the study. The protocol was approved by the ethical review committee of Bangladesh University of Health Sciences before the start of study. Because of this study involved blood and Urine sample collection it needed it an invasive procedure and may rise strong ethical issues. Detailed information regarding the study objectives, procedure and risk and benefits involved were provided during first contact with participants. They had opportunity to discuss if they required further information and clarification. Informed written consent was obtained before the interview. All literate individual participants read the consent paper by themselves and signed. For the illiterate participants, the data collector read, translated and explained the consent paper to them and if they agreed, their thumb impression or sign was taken. Pregnant women were assured that all the information would be kept confidential and would only be used for research purpose. Social and cultural values of the pregnant mothers were respected and considered as required. Allocated interviewers were female. Subjects found to have adverse effect due to glucose intake were handled by the health professionals immediately. Scientific objectivity was maintained and personal harm to the subjects and respondents was guarded. Respondents or subjects were informed about the nature of the research methods that were used and they were free to withdraw from the study at any time.

2.16 Laboratory analysis:

2.16.1 The glycemic status of the subjects was assessed by:

- Fasting Blood Glucose (FBG)

- 2 hour after 75 gm glucose (OGTT)

Glucose level of the study subjects was measured by using Glucose Oxidase method (Randox, UK) (AppendixIII).

2.16.2 Lipidemic status was assessed by:

- Serum total cholesterol by enzymatic endpoint method (cholesterol Oxidase/ Peroxidase) (Randox Laboratories, UK) (Appendix IV)
- Serum triglyceride by enzymatic-colorimetric (GPO-PAP) method (Randox laboratories, UK) (Appendix V)
- Estimation serum High Density Lipo protein by enzymatic colorimetric (cholesterol CHOD-PAP) methodin auto analyzer (Analyzer Medical System, Rome, Italy) (Friedewald WT, 1972)
- LDL cholesterol level in serum was calculated by using by Friedewald formula (Friedewald WT. 1972)

2.16.3 As level in water (WAs) was measured by ultraviolet/visible spectrophotometry. (Appendix-VIII)

2.16.4 Urinary-As was measured by inductively coupled plasma-mass spectrometry and adjusted with specific gravity ($U\text{-}As_{SG}$). (Agilent 7500cc-Agilent Technology, Hachioji, Japan).

3. RESULTS

3.1 Proportion of GDM and non-GDM participants

GDM was diagnosed according to American Diabetic Association Guideline, 2013. The cut off point was fixed based on fasting glucose value of >5.1 mmol/L and 2hr after glucose value of >7.00 mmol/L.

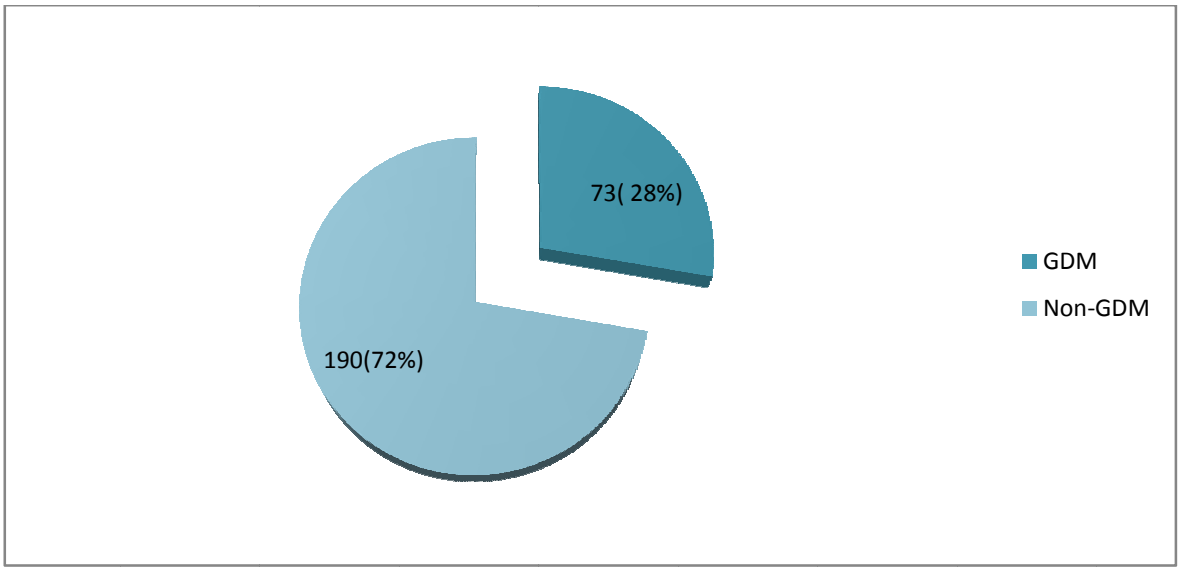

Figure 3.1: The proportion of GDM and non-GDM in the study subjects.

This pie chart shows the proportion of GDM and Non-GDM subjects. Total of 263 participants were included in the study. Among them 73(28%) were GDM and 190(72%) were non-GDM.

3.2 The socio-demographic status of study participants

Table 3.1 presents the sociodemographic status of all participants. The maximum numbers of the pregnant women were completed secondary or higher secondary school, 162 (61.59%). Among them most of the participants are housewives, 253(96.2%). About 127(48.28%), study subjects were from Madaripur districts and 145(55.14%), were living in rural area. About 121(41.8%) tube-well water were used for cooking and 193(73.38%) tube-well water were used for drinking.

Table 3.1: The socio-demographic status of all pregnant women (n=263):

Characteristic	**n(%)**
Education	
-Illiterate	13 (4.9%)
-Completed primary school	78(29.68%)
-Completed secondary or higher school	162(61.59%)
-Completed college or above	10(3.83%)
Occupation	
Housewife	253(96.2%)
Service	10(3.8%)
Region	
-Chandpur	53 (20.17%)
-Faridpur	83 (31.55%)
-Madaripur	127 (48.28%)
Area	
-Urban	64(24.33)
-Rural	145(55.14)
-Semi urban	54(20.53)
Source of cooking water	
- Tube well	121 (41.8%)
- Pond/river	110 (46.0%)
- Tap (deep tube well or filtered pond water)	32 (12.2%)
Source of drinking water	
- Tube well	193 (73.38%)
- Pond/river	2 (0.77%)
- Tap (deep tube well or filtered pond water)	68 (25.85%)

Results are expressed as Mean± SD. n= number of subjects. Percentages were analyzed by cross tab analysis.

3.3 Proportion of using cooking water sources of the participants

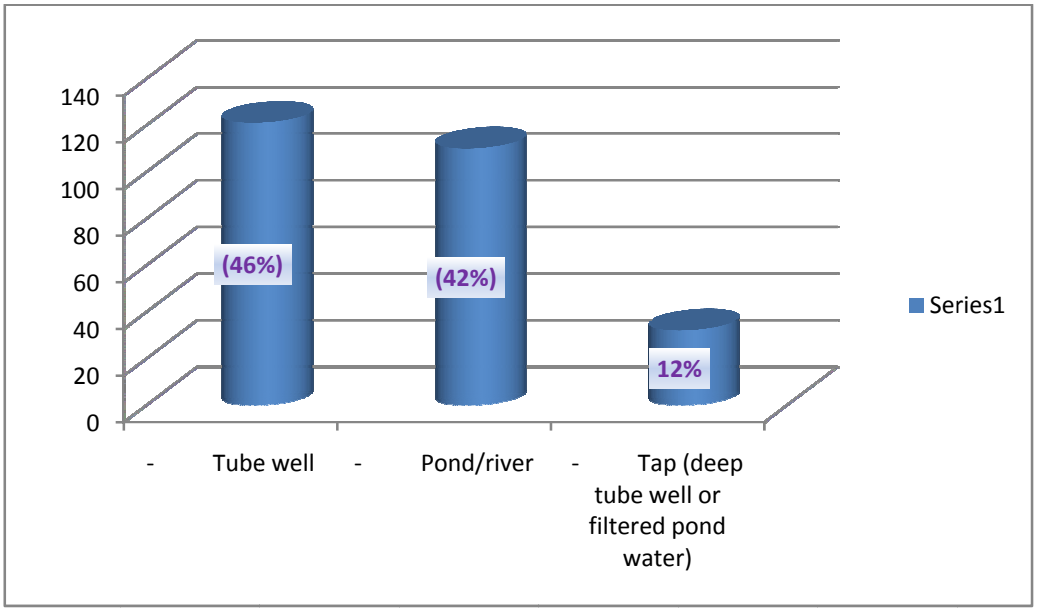

Figure 3.3: The proportion study subjects using different sources of water for cooking

This bar chart shows the proportion of cooking water source used by the participants. About 46% of tube well water,42% of pond or river water and 12% tap (deep tubewell or filtered pond water) were used by the participants.Maximum participant (121, 41.8%)were used tube well water for cooking.

3.4 Maternal status of study subjects

Table 3.2 shows the maternal status of the study Non-GDM and GDM subjects. Maternal status was measured by parity and types of delivery of the study subjects.

The parity showed only 23.3% were primi para and 76.7% were multi para having GDM. The difference between primi para and multi para were not significant.

The types of delivery also showed no significant difference between normal (43.8%) and caesarian section (56.2%) in GDM subjects.

Table 3.2: The Maternal status of study Non-GDM and GDM subjects

Characteristic	Non-GDM n(%)	GDM n(%)	P value
Parity			
- Primi para	69(36.3)	17(23.3)	0.159
- Multi para	121(63.7)	56(76.7)	
Types of delivery			
- Normal	99(52.1)	32(43.8)	0.308
- C-section	91(47.9)	41(56.2)	

Results are expressed as number and (percentages, %) n= number of subjects. The significance difference was analyzed by cross tab chi squire test. Significance level were calculated $p<0.05$.

3.5 Clinical characteristics of the study participants

The clinical characteristics of Non-GDM and GDM subjects are shown in Table 3.3. The Age parameter (years,M±SD) showed no significant difference between GDM and Non-GDM subjects. The Body mass index (kg/m^2,M±SD) showed significantly higher in GDM subjects (25.5±3.7) compare to the Non-GDM subjects (23.31±3.1), ($p<0.001$).The Systolic blood pressure (mm of Hz, M±SD)) showed significantly higher in GDM subjects (113.9±11.2) compared to the Non-GDM subjects (80±4.5),($p<0.023$).The Diastolic blood pressure (mm of Hz, M±SD) showed significantly higher in GDM subjects (95±3.1) compared to the Non-GDM subjects (75.2±7.1),($p<0.013$).The Mean blood pressure (mm of Hz, M±SD) also showed significantly higher in GDM subjects (95.2±7.8) compared to the Non-GDM subjects (88.4±1.2),($p<0.041$).

Table 3.3: The clinical characteristics of Non-GDM and GDM subjects (n=263).

Characteristic	Non-GDM	GDM	P value
Age (yrs)	21.53±3.76	21.57±3.2	0.939
BMI (kg/m^2)	23.31±3.1	25.5±3.7	<0.001
SBP (mm of Hz)	80±4.5	113.9±11.2	0.023
DBP(mm of Hz)	75.2±7.1	95±3.1	0.013
MBP	88.4±1.2	95.2±7.8	0.041

Results are expressed as mean± *SD and* median (range), n= number of subjects. HTN=Hypertension, BMI=Body Mass Index, WHR=Waist hip ratio, SBP=Systolic Blood pressure, DBP=Diastolic Blood Pressure MBP=Mean Blood pressure

3.6 Glycemic and Lipidemic status of study participants

Table 3.4 represents the biochemical characteristics of Non-GDM and GDM subjects. The S Fasting glucose value (mmol/l, M±SD) showed significantly higher in GDM subjects (7.1±1.9) compare to the Non-GDM subjects (4.3±0.53), ($p<0.001$). The S 2 hr after glucose value (mmol/l, M±SD) showed significantly higher in GDM subjects (6.3±0.56) compare to the Non-GDM subjects (11.43±2.4), ($p<0.001$).The S TG (mg/dl ,M±SD) showed significantly higher in GDM subjects (187.2±72.7) compare to the Non-GDM subjects (243.4±7.7), ($p<0.012$).The S Chol (mg/dl, M±SD) showed significantly higher in GDM subjects (118±4.3) compare to the Non-GDM subjects (208±1.2), ($p<0.045$). The S HDL-Cholesterol (mg/dl ,M±SD) and LDL-Cholesterol (mg/dl ,M±SD) showed no significant difference between GDM and Non-GDM subjects. The S Creatinine (mg/dl, M±SD) showed significantly higher in GDM subjects (0.69±0.28) compare to the Non-GDM subjects (0.79±0.13),($p<0.001$).The S Albumine (mg/dl, M±SD) showed significantly higher in GDM subjects (27.6±4.5) compare to the Non-GDM subjects (27.1±2.3), ($p<0.001$).

Table 3.4: The Glycemic and Lipidemic status of Non-GDM and GDM subjects

Characteristic	Non-GDM	GDM	P value
S Fasting glucose value (mmol/l)	4.3±0.53	7.1±1.9	0.001
S 2 hr after glucose value (mmol/l)	6.3±0.56	11.43±2.4	0.001
S TG (mg/dl)	187.2±72.7	243.4±7.7	0.012
S Chol (mg/dl)	118±4.3	208±1.2	0.045
S HDL-Cholesterol (mg/dl)	43.3±10.9	33.3±5.9	0.451
LDL-Cholesterol(mg/dl)	126.9±32.2	211.9±2.2	0.065
S Creatinine (mg/dl)	0.69±0.28	0.79±0.13	0.001
S Albumine (mg/dl)	27.6±4.5	27.1±2.3	0.001

Results are expressed as Mean± SD. n= number of subjects. S TG=Serum Tri-Glyceride, T Chol=Total Cholesterol, HDL=High Density Lipoprotein, LDL=Low Density Lipoprotein.

3.7 Arsenic status in study participants

Table 3.5 showed arsenic level in water in non-GDM and GDM subjects. Water arsenic level was significantly higher in the GDM as compared to the Non-GDM group [WAs, µg/l, median (range), 62(34-354) versus 3.6 (1.02-99), $p<0.001$].Urine arsenic level was also significantly higher in the GDM as compared to the Non-GDM group [U-As, µg/L, median (range), 60 (1.9–248) versus 2.21(1.31-89), $p<0.001$].

Table 3.5: Arsenic status in Non-GDM and GDM subjects (n=263)

Characteristic	Non-GDM	GDM	P value
Water Arsenic level (µg/L)	2.32±1.98 3.6 (1.02-99)	14.8±1.98 62 (34-354)	<0.001
U-As (µg/L)	2.21 (1.31-89)	60 (1.9–248)	<0.001

The results express mean±SD and median (range) where as applicable. Test of the significance level p<0.5 and analyzed by independents 't' test. W-As Water arsenic level; U-As=Urinary arsenic level.

3.8 Odds ratio of arsenic exposure in the study participants

Table 3.6 showed odds ratio of water arsenic exposure in non-GDM and GDM subjects. The GDM subjects showed that 100 % subjects were high in Arsenic exposure and non-GDM showed 45% High exposure. The Odds ratio with CI also support that 1.7 (1.502-1.925) almost 2 times chance of developing gestational diabetes.

Table 3.6: The odds ratio of arsenic exposure in GDM and Non-GDM subjects

Characteristic	Non-GDM	GDM	P value	Odds ratio 95% CI
Low Arsenic exposure	104(54.7%)	0(0%)	<0.001	1.7 (1.502-1.925)
High Arsenic exposure	86(45.3%)	73(100%)		

The result were express as number and percentages, n(%) and odds ratio, the significance level were analyzed by *chi* X^2 test. Odds ratio represent the chance of happening disease. Arsenic exposure categorize by cut-off value, >10ug/L.

3.9 Anthropometric measurement of Neonatal of the study subjects: Table-7

Table 3.7 represents the anthropometric measurement of Neonatal of the study subjects. The fetal weight (Kg,M±SD) were significantly higher in GDM subjects (3.8±1.02) compared to the Non-GDM subjects (3.03±0.27), (p=0.001). APGARScore of the neonates from GDM mothers was significantly lower compared to the neonates from Non-GDM mothers (APGER Score, M±SD, 4.7±0.8 vs 6.4±0.67, p<0.001).

Table 3.7: The Anthropometric measurement of Neonatal of the study subjects

Characteristic	Non-GDM	GDM	P value
Fetal Wt (gm)	2048 (729-3000)	2470 (850-3200)	0.620
Fetal Wt (Kg)	3.03±0.27	3.8±1.02	0.001
Apgar Score (in 5 minutes)	6.4±0.67 7(6-8)	4.7±0.8 5(4-8)	<0.001

The results express mean±SD and median (range) where as applicable. Test of the significance level p<0.5 and analyzed by independent‘t’ test.

3.10 Odds ratio of APGAR score in neonates of the study participants

Table 3.8 showed odds ratio of APGAR score in non-GDM and GDM subjects. The GDM subjects showed 31.5% Apgar score and non-GDM showed 46.3% Apgar score. The Odds ratio with CI also support 5.033 (0.301-5.033) that almost 5 times chance of happening adverse effect of neonate of a GDM mother on later life.

Table 3.8: The odds ratio of APGAR score in neonates of the study subjects

Characteristic	Non-GDM	GDM	P value	95% CI odds ratio
Low APGER score <7	102(53.7)	50(68.5)	0.029	5.033 (0.301-5.033)
High APGER score >7	88(46.3)	23(31.5)		

The result were express as number and percentages, n(%) and odds ratio, the significance level were analyzed by *chi* X^2 test. Odds ratio represent the chance of happening disease. Low APGAR score were categorize by cut-off value, >7 in 5 minits.

3.11 Correlation between Water arsenic Level and 2 hr after blood glucose value in GDM subjects.

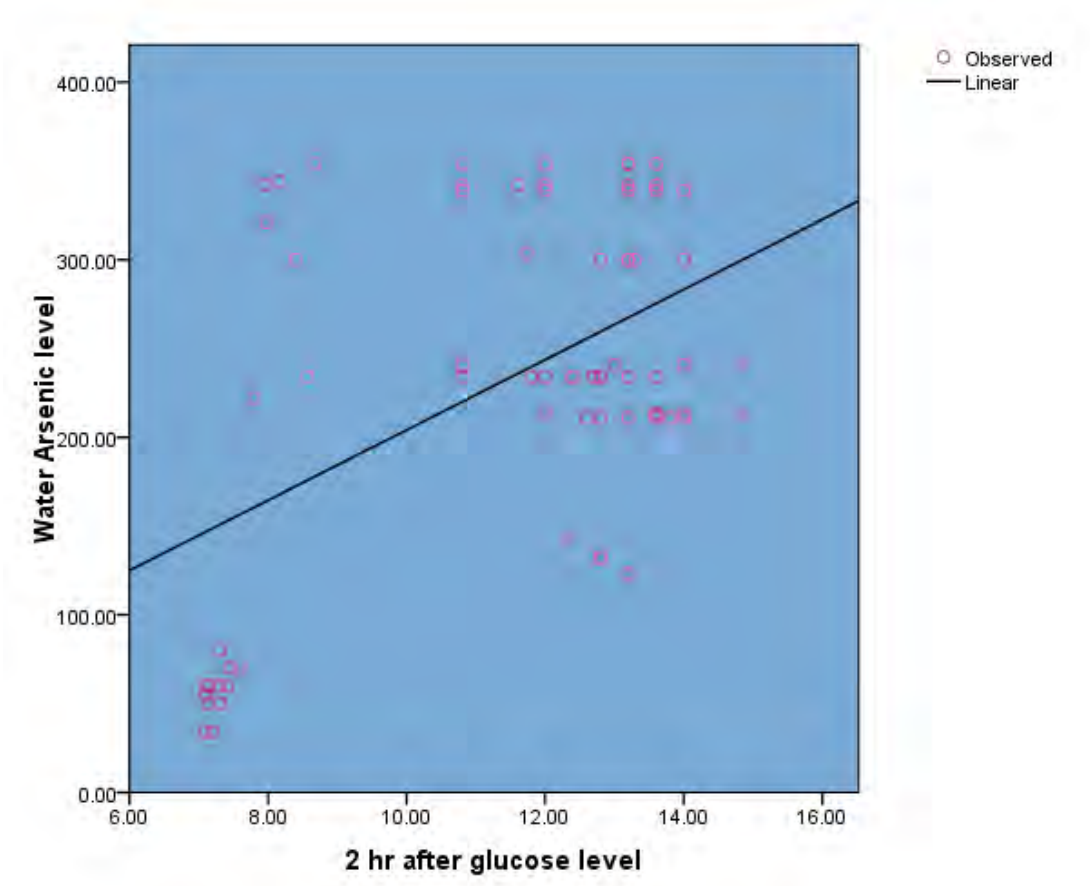

Figure 3.3: The Correlation between Water arsenic Level and 2 hr after blood glucose value in GDM subjects.

Figure 3.3 represents that correlation between water arsenic level and 2 hr after blood glucose value inGDM subjects.This diagram showed that water level and blood glucose level are significant positively co-related with each other (r=0.429;p=<0.001).

3.12 Correlation of Water arsenic Level and 2 hr after blood glucose value in Non-GDM subjects

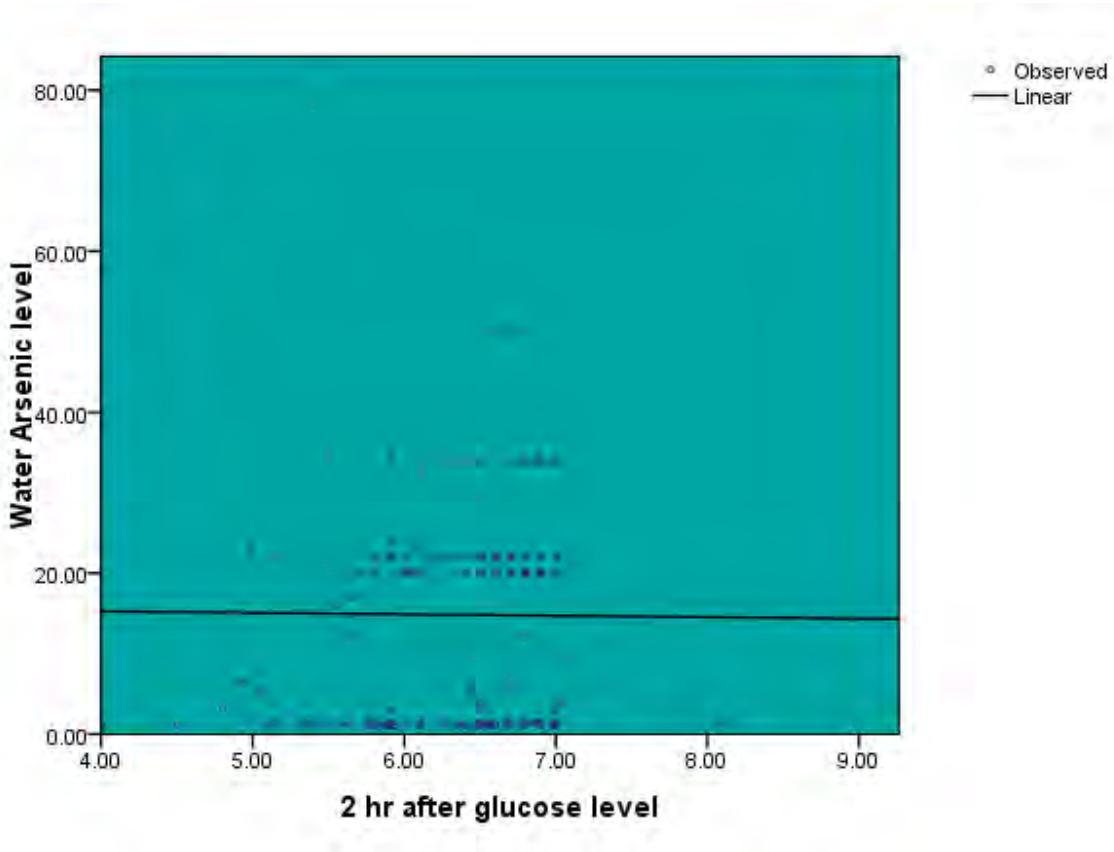

Figure 3.4: The Correlation of Water arsenic Level and 2 hr after blood glucose value in Non-GDM subjects.

This curve showed no correlation between water arsenic level and blood glucose level (r=0., p=0.) in Non GDM subjects.

3.13 Correlation between Water arsenic Level and Urinary total arsenic level in GDM subjects

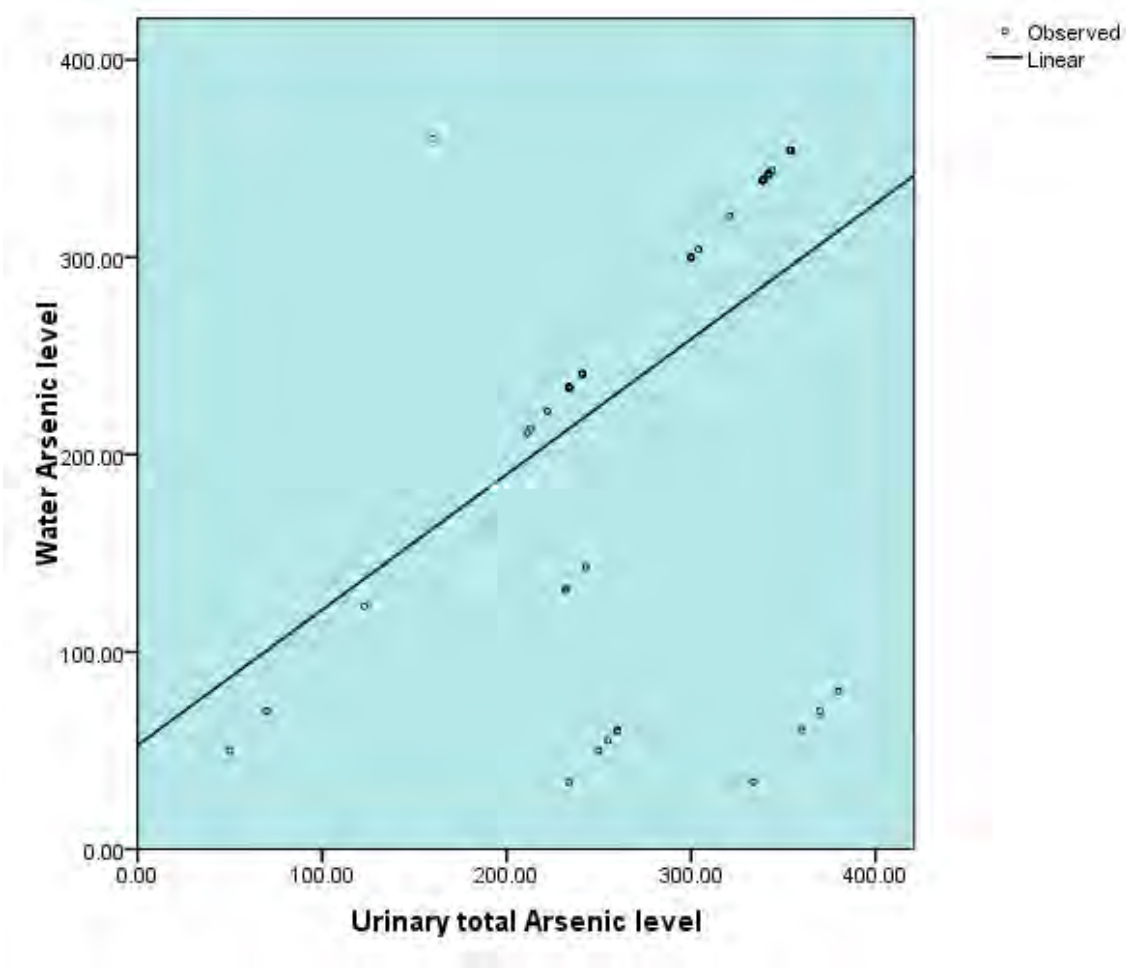

Figure 3.5: The Correlation between Water arsenic Level and Urinary total arsenic level in GDM subjects.

Figure 3.5 represents that correlation between water arsenic level and Urinary total arsenic level in GDM subjects. This diagram showed that water level and blood glucose level are significant positively co-related with each other (r=0.234;p=<0.001).

3.14 Correlation of Water arsenic Level and Low Apgar score in GDM subjects

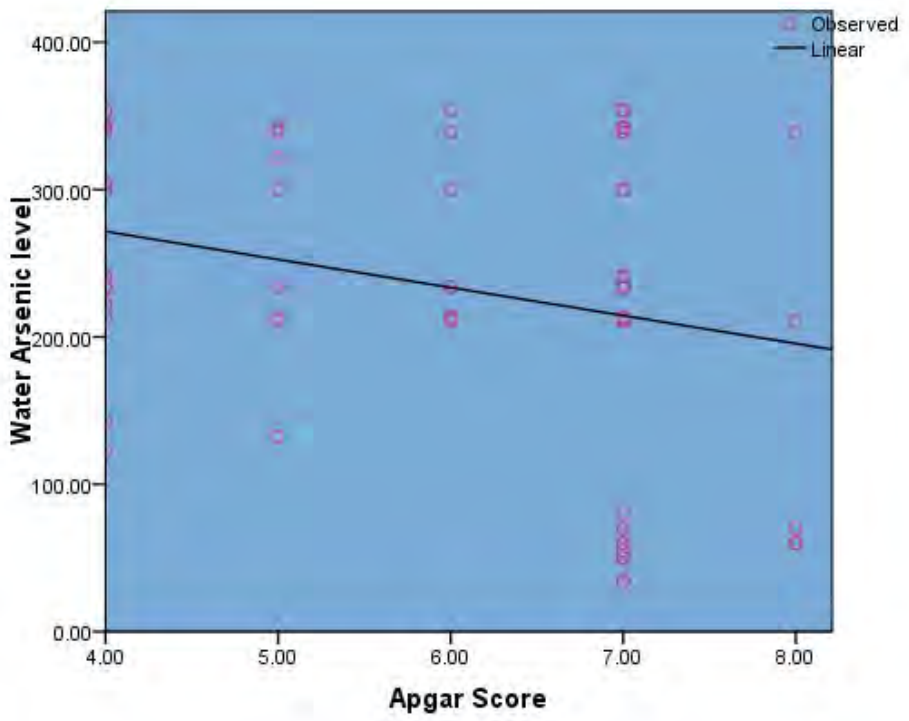

Figure 3.6: The Correlation of Water arsenic Level and Low Apgar score in GDM subjects.

Figure 3.6 represents that correlation between water arsenic level and low ApgarScore. This curve estimated that, the water arsenic level and Apgar score are negatively correlated with each other, when arsenic level is increased then Apgar score is decreased ($r=0.233$; $p<0.041$).

3.15 Correlation of Water arsenic Level and Low Apgar score in Non-GDM subjects

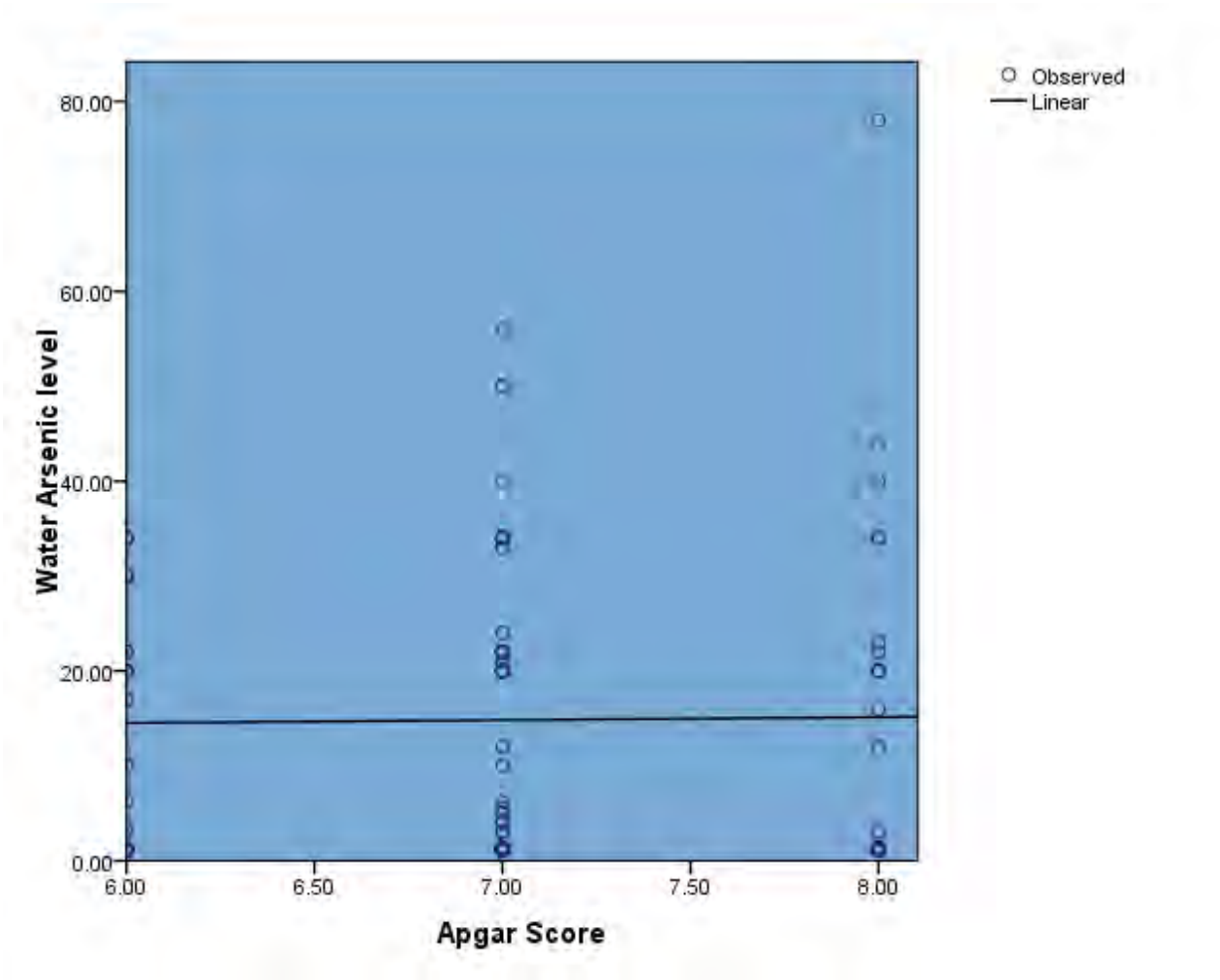

Figure 3.7: The Correlation of Water arsenic Level and Low Apgar score in Non-GDM subjects.

Figure 3.7 showed that correlation between water arsenic level and low Apgar score in Non-GDM subjects.This diagram showed no significant correlation between water arsenic level and Apgar score.

4. DISCUSSION

Arsenic exposure is a well-recognized public health problem; millions of people world-wide are potentially exposed inorganic arsenic from drinking water contaminated by naturally occurring sources (Tchounwou *et al*, 1999). Chronic exposure to arsenic is associated with a number of adverse health effects (Yoshida *et al*, 2004).

Suggestive evidence links chronic exposure to high arsenic levels in drinking water with increased diabetes risk in Taiwan and Bangladesh. Methodologic problems, however, limit the causal interpretation of this association. The use of average drinking water and the lack of individual measures of arsenic possibly lead to underestimate the exposure (Navas acien, 2008).

Although the present cross-sectional study was designed with exploration of arsenic contamination and associated health effects (maternal and neonatal) as its primary objectives, it gives an idea about the proportion of pregnant women converting to GDM in a tertiary hospital setup. Out of 263 pregnant women followed up from third trimester up to delivery 73 (28%) developed GDM. Prevalence of GDM up to 17% has been reported in a population based study in India (Seshiah *et al*, 2008); thus the present prevalence rate is much higher than that in the general population. The subjects in the present study, however, were collected from the Department of Gynecology and Obstetrics in few tertiary hospitals where a higher prevalence of GDM is expected. Large scale population based studies are required to find out a more accurate account of the conversion to GDM in general population.

Environmental exposure to arsenic may result in abnormal glucose metabolism which, in turn, increases the risk of developing diabetes, through several plausible mechanisms (Longnecker and Daniels, 2001). Arsenic is a known endocrine disruptor (Tseng, 2004) and may disrupt the glucocorticoid receptor (Kaltreider *et al*, 2001), which regulates a wide range of biological processes in humans including insulin sensitivity. It is also possible that presence of diabetes or prediabetic conditions alter arsenic metabolism in such a way as to cause higher levels in the body. However, experimental evidence

suggest that oxidative stress and insulin resistance can be induced by arsenic, suggesting a biological plausibility to arsenic-induced diabetes (Tseng *et al,* 2004).

The present data shows an urinary total arsenic concentration of 60 (1.9–248) µg /l [(95% CI, 1.7 (1.502-1.925)]. NHANES (National Health and Nutrition Examination Survey) data suggest almost similar findings; as per its report the average urinary total arsenic concentration is 8.30 µg/L (95% CI, 7.19–9.57) (Caldwell *et al*, 2008). Using data from the same nationally representative cross-sectional survey of the U.S. population ,Navas-Acien and colleagues (2008) found that participants with type 2 diabetes had a 26% higher level of total arsenic (95% CI, 2.0–56.0) and the OR for type 2 diabetes comparing participants at the 80th versus the 20th percentiles was 3.58 for the level of urinary total arsenic (95% CI, 1.18–10.83). Saldana (2007) has also shown an association of environmental exposure (agricultural pesticides) and increased risk of GDM among wives of licensed pesticide applicators. Another study, used sensitive laboratory biomarkers of both the exposure (arsenic) and outcome (plasma glucose). Levels of total arsenic exposure (measured in maternal blood and hair) in their study were higher than reported background levels of arsenic in unexposed individuals (ATSDR 2007). Their results taken together suggest that, a significant portion of US women of childbearing age may be at risk for GDM due to environmental exposures such as arsenic.

In this cross-sectional study, daily arsenic contaminated GDM women were compared with Non-GDM women. Women diagnosed with diabetes in third trimester of pregnancy, differed already during these pregnancies in several parameters (Serum Tri-Glyceride, Total Cholesterol, High Density Lipoprotein, Low Density Lipoprotein, creatinine, albumin etc) during their pregnancies. The mothers who were exposed with arsenic during pregnancy, had more complications, such as development of diabetes later. They also had caesarean delivery more often; (31.3%) delivered by caesarean section compared to (68.7%) women who did not develop diabetes.

Among this population of pregnant women with relatively high exposure, arsenic concentration was associated with impaired glucose tolerance during pregnancy and therefore may be associated with increased risk of GDM. Women in the highest quartile of urine arsenic exposure had almost two times 1.7(1.502-1.925) higher odds of impaired GTT than women in the lowest quartile of exposure, and there was a statistically significant ($P<0.001$) trend in risk of impaired GTT by increasing quartile of exposure. On the other hand, a study from Korea did find an association between arsenic exposure and diabetes in people exposed to normal, background exposure levels of arsenic, especially women (Kim and Lee, 2011).In a recent study it was shown that people from rural communities in the US with high rates of diabetes, arsenic was associated with poorer diabetes control (a higher HbA1c) in people with diabetes (Gribble *et al*, 2012). Vahter et al (2006), claimed that higher arsenic concentration is associated with gestational diabetes mellitus and pregnancy complications.

The Apgar score system used to estimate the probability of survival of the infant (Metzger *et al*, 2002; HAPO study, 2009) and to appraise the need for resuscitation (Aberg *et al*, 2002). An additional score obtained at five minutes of age gained universal acceptance after the report from the Collaborative Perinatal Project which showed a stronger relation between the five-minute score and neonatal mortality than the one-minute score (Crowther *et al*, 2005). However, it has been suggested that the Apgar score is antiquated and that its predictive value has been considerably weakened by the institution of prompt and effective neonatal care.

The relationship between five-minute Apgar scores and infant survival analysis indicates that the Apgar score is not only useful for neonatal period and term infants as it was 50 years ago, but also meaningful for post-neonatal period and preterm infants. Hence, Apgar score could still be a good and convenient predictor of infant death. Apgar scores are used as an indicator of the well being of the newborn.

The Apgar scores of the infants were lower among the women with GDM or later Type 2 diabetes compared to women without diabetes. In the multivariate analysis calculating

risk for low Apgar scores in the whole cohort, pre pregnancy weight of the mother, GDM or later Type 2 diabetes, low birth weight and short gestational length were significant risk factors at 5 min but for low Apgar at 10 min only low birth weight and short gestational length were significant. This indicates that prematurity for any reason in general is the dominating condition behind sustained low Apgar score.

There was a significantly ($P<0.001$) higher value of drinking water arsenic level (14.8±1.98) in GDM compared to Non-GDM (2.32±4.34) subjects in the third trimesters. It seems that the involvement is indirect as there was significant correlation of fasting or 2-hr blood glucose level (11.43±2.4 vs 6.3±0.56; $p<0.001$) with higher urine arsenic level in GDM. Their newborns were heavier despite a shorter gestational length and had significantly lower Apgar scores (5.7±0.8), which was indicating poor neonatal outcome. The cut-off value is categorize 75^{th} percentile <7 and odds ratio of APGAR score is (CI, 5.033 (0.301-5.033) which shows that chance of risk is almost 5 times higher.

In a recent study on Bengali population with much higher levels of exposure, blood arsenic levels were highly correlated with creatinine-adjusted urinary concentrations (r = 0.85) and with drinking-water arsenic concentration (r = 0.75) (Hall *et al*. 2006). In our findings, on Pearson's correlation analysis in GDM subjects, WAs levels were found to have a significant positive correlation with both fasting and postprandial serum glucose ($r=0.638;p=<0.05$) and the urinary arsenic level ($r=0.234;p<0.001$) and also the low APGER Score of the neonates were found to have a significant negative correlation with serum glucose ($r=0.233$; $p=0.041$).

The limitation of the present study was that water and urine sample collected at the time of third trimester or before delivery as a biomarker of arsenic exposure, which was measured at least 24 to 28 weeks of pregnancy. That may have a strong association with outcome. Another limitation is that arsenic exposure measuring sample were blood and hair and nail were not analyzed. Because arsenic binds to the sulfhydryl groups in keratin, the primary component in hair and nails, this is where the highest levels of

arsenic tend to be found (Liu *et al*, 2008). Therefore, studies of chronic arsenic exposure often use hair or nails as biomarkers of cumulative exposure. Although nails or hair may best represent cumulative exposure, keratin-bound arsenic is isolated from the body's further metabolic processes and may be less biologically active than blood arsenic. Because of these differences in biomarkers of arsenic exposure, hair was used in a subset of women with samples available, to provide estimation of long-term exposures, with every 1 cm of hair representing each month of prior exposure (Kurttio *et al*, 1998). It was believed that due to chronic exposures, arsenic level in circulation reaches a steady-state level and it may better reflect an individual's total intake of internal arsenic (Hall *et al*, 2006).

In summary, GDM is a major potential complication of pregnancy associated with negative health effects for both the mother and infant. Understanding the effects of environmental exposure on impaired glucose tolerance during pregnancy may have substantial public health importance beyond the direct effects on GDM. Studies are needed to investigate environmental, behavioral and biological factors that may contribute to a risk of development and progression of diabetes and its related maternal and neonatal complications. Such studies should incorporate culturally specific lifestyle factors into treatment and prevention strategies to reduce risk across racial, ethnic, and socioeconomic groups. Future research in this area will be important for understanding whether different pathophysiologic mechanisms or risk factors are responsible for increased diabetes risk and especially in children. Genetic background may also play a role in the susceptibility of individuals to the effects of arsenic. For example, a study from parts of Mexico with historically high levels of arsenic exposure found that people who had certain genes are more likely to develop diabetes when exposed to arsenic (Drobna *et al*, 2012). In addition, better understanding of modifiable risk factors for GDM such as diet (antioxidant rich food) and activity patterns (such as breast feeding practices) related to environmental exposures may lead to efforts at primary prevention.

The above findings imply that arsenic contamination may play a role in glucose intolerance and may be associated with an increased risk of GDM; it may contribute to

lower apger score in babies. Our results probably represent more than a potential causal association between arsenic and GDM; it may also provide clues to long-observed health disparities that might have prevented public health interventions.

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APPENDICES

APPENDIX- I

Data Collection Sheet

ID: **Date**

Institution:

Particulars of the patients:-

Name:

Age:

Address

a) **Present :**

Telephone No:

b) **Permanent:**

Contact person address:

Telephone no:

Education: 1□Masters 2□Graduate 3□ HSC 4□SSC 5□Primary 6 □Illiterate

Occupation: 1□Housewife 2□Service 3□Students 4□Others

Location: 1□Urban 2□Semi urban 3□Rural

Family Members:

Monthly family income:

Menstrual history: MP/MC

L.M.P

E.D.D

Obstetrical history: Para

Gravida

ALC

Risk factors:-

Obesity	Yes[1]/No[0]
Positive history of diabetes (sibs/parents)	Yes[1]/No[0]
H/O delivery of large infant (>400 gm)	Yes[1]/No[0]
H/O still birth	Yes[1]/No[0]
/O prematurity	Yes[1]/No[0]
H/O unexplained neonatal death	Yes[1]/No[0]
H/O pre eclampsia	Yes[1]/No[0]
H/O traumatic delivery e associated neurological disorder in the infant	Yes[1]/No[0]
Poor reproductive history (>3 spontaneous abortions in the 1st or 2nd trimester)	Yes[1]/No[0]
Chronic hypertension	Yes[1]/No[0]

Physical / Clinical Examination (Mother):

Observations	**1st visit Date……**	**2nd visit Date…….**
Height		
Weight		
BMI		
BP		
Height of Uterus		

Biochemical Investigation :

Observations	1st visit Date…….	2nd visit Date……..
Fasting blood glucose (mmol/l)		
Blood glucose 2 hr after 75 gm of glucose (mmol/l)		
Serum TG (mg/dl)		
Serum T-chol (mg/dl)		
Serum HDL (mg/dl)		
Serum LDL (mg/dl)		

Physical / Clinical Examination (Neonates):

Observations	1st visit Date…….
Length	
Weight	
APGAR score at 5 minits	
Activity (muscle tone)	
Active 2	
Arms and legs flexed 1	
Absent 0	
Pulse	
>100 bpm 2	
<100 bpm 1	
Absent 0	
Grimace (reflex irritability)	
Sneezes, coughs, pulls away 2	
Grimaces 1	
No response 0	
Appearance (skin color)	

Normal over entire body 2	
Normal except extremities 1	
Cyanotic or pale all over 0	
Respirations	
Good, crying 2	
Slow, irregular 1	
Absent 0	

Interviewer signature

APPENDIX- II

Estimation of plasma blood glucose (Randox laboratories, UK).

Plasma glucose was estimated by enzymatic colorimetric (GOD-PAP) method

Principle (Barham, Trinder, 1972)

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red violet quinoneimine dye as indicator.

$$\text{Glucose} + H_2O \xrightarrow[Oxidase]{Glucose} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{Peroxidase} \text{Quinoneimine} + 4H_2O$$

Reagents

Contents	*Initial concentration of solution*
Buffer	
Phosphate Buffer	0.1 mol/l, pH 7.0
Phenol	11 mol/1
GOD-PAP Reagent	
4-aminophenazone	0.77 mmol/1
Glucose oxidase	≥1.5 kU/1
Peroxidase	≥1.5 kU/1
Standard	
Glucose	5.55 mmol/1 (100 mg/dl)

Additional Reagent

Uranyl Acetate 0.16% cat NO DP 647 2x 500 ml

Materials required

Microcentrifuge tube

Micropipettes and pipettes with disposable tips

AUTOLAB (Analyzer Medical system, Rome, Italy)

Procedure

Procedure for glucose GOD-PAP assay without deproteinisation. The instrument was calibrated before estimation.

Plasma and reagent were taken in specific cup. They were arranged serially into the Auto lab Analyzer (Analyzer Medical system, Rome, Italy). The Auto lab was programmed for the estimation of glucose and allowed to run with following procedure:

5 µl sample and 500 µl reagent were mixed and incubated at 37^0 C for 10 minutes. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Optical densities or absorbances were fed into a computer and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using following formula.

$$\text{Glucose concentration (mmol/l)} = \frac{A_{Sample}}{A_{S\,\tan dard}} \times 5.5$$

APPENDIX- III

Estimation of serum total cholesterol (Randox laboratories, UK)

Total cholesterol was measured by enzymatic endpoint method (cholesterol Oxidase/Peroxidase) method in auto analyzer (Analyzer Medical System, Rome, Italy) using reagent of Randox laboratories, UK.

Principle (Trinder, 1988)

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantiphyrine in the presence of phenol and peroxidase.

$$\text{Cholesterolester} + H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholestene-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{phenol} + \text{4-aminoantiphyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + H_2O$$

Reagent composition

Contents	Initial Concentration of Solution
Reagent	
4-Aminoantipyrine	0.30 mmol/l
Phenol	6 mmol/l
Peroxidase	$\geq$ 0.5 U/ml
Cholesterol esterase	$\geq$ 0.15 U/ml
Cholesterol oxides	$\geq$ 0.1 U/ml
Pipes Buffer	80 mmol/l; pH 6.8
Standard	5.17 mmol/l (200 mg/dl)

Materials

Microcentrifuge tube

Micropipettes and pipettes

Disposable tips

AUTOLAB (Analyzer Medical system, Rome, Italy)

Procedure

Plasma and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the AUTOLAB. 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the Auto lab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration of cholesterol in sample was calculated by using software program with the following formula.

$$\text{Cholesterol concentration (mg/dl)} = \frac{A_{Sample}}{A_{S\tan dard}} \times \text{concentration of standard.}$$

APPENDIX- IV

Estimation of plasma triglycerides

Plasma triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in auto analyzer (Analyzer Medical System, Rome, Italy) using reagent of Randox laboratories, UK.

Principle

The triglyceride is determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen- peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase (Trinder 1969).

$$\text{Triglyceride} + H_2O \xrightarrow{Lipase} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Kinase]{Glycerol} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow[Oxidase]{Glycerol\ Phosphate} \text{dihydroxy acetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4- chlorophenol} \xrightarrow{Peroxidase} \text{quinoneimine} + HCl + 4\,H_2O$$

Reagents

Contents	*Concentrations in the Test*
Buffer	
Pipes Buffer	40 mmol/l, pH 7.6
4-choloro-phenol	5.5 mmol/l
Magnesium-ions	17.5 mmol/l
2. Enzyme Reagent	
4-aminophenazone	mmol/l
ATP	1.0 mmol/l
Lipases	>150 U/ml
Glycerol-3-phosphate oxidase	1.5 U/ml
Peroxidase	0.5 U/ml

3. Standard 2.29 mmol/l (200 mg/dl)

Materials

Micropipettes and pipettes

Disposable tips

AUTOLAB (Analyzer medical system, Rome, Italy)

Procedure

Plasma and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the AUTOLAB. 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the AUTOLAB.

The reaction occurred in reaction cell. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Triglyceride concentration was calculated by using software program in AUTOLAB with the following formula.

Triglyceride concentration (mg/dl) = $\frac{A_{Sample}}{A_{S\,\tan\,dard}} \times$ Concentration of the standard.

Appendix- V

Estimation of serum high density lipoprotein (HDL)

Plasma high-density lipoprotein (HDL) was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method in auto analyzer (Analyzer Medical System, Rome, Italy) (Friedewald WT, 1972)

Principle

HDL (High Density Lipoproteins) are separated from chylomicrons, VLDL (very low density lipoproteins) and LDL (Low density lipoproteins) by the addition of a precipitating reagent (phosphotungstic acid-magnesium chloride) to serum or plasma. After centrifugation, the cholesterol contents of HDL fraction which remains in the supernatant are determined by the enzymatic colorimetric method using CHOD- PAP.

Reagent composition

Contents

Buffer

Enzymes

Standard 50 mg/dl (1.29 mmol/l)

Materials

Microcentrifuge tube, Micropipettes and pipettes

Disposable tips

AUTOLAB

Procedure

Samples (200 ml) and precipitating reagents (500 µl) were taken in a microcentrifuge tube. Then it was mixed and allowed to sit for 10 minutes at room temperature. Then it was centrifuged at 4000 rpm for 10 minutes.

The supernatant was used as sample for determination of cholesterol content by the CHOD-PAP method. The sample and reagents were taken in specific cup or cell. They were arranged serially then ID number for test was entered in the AUTOLAB.

Then 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the AUTOLAB. The reaction occurred in reaction cell. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration was calculated by using software program.

Appendix- VI

Estimation of LDL cholesterol level in serum

LDL cholesterol level in serum was calculated by using by Friedewald formula (Friedewald WT. 1972)

Formula

$$\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL Cholesterol} + \frac{1}{5} x \text{ Triglyceride})$$

Appendix- VII

ARSENIC ESTIMATION SPECTROPHOTOMETER

Experimental Procedure

Apparatus

A Secomam Anthelie NUA 002 UV-VIS spectro- photometer with 1 cm quartz cell was used for the absorbance measurements and a WTW pH 330, pH meter was used for pH measurements.

Reagents

All chemicals used were of analytical grade and distilled water was used for dilution of reagents and samples. Standard arsenic (III) stock solution (1000 µg mL-1) was prepared by dissolving 0.1732 g of NaAsO2 in 100 mL of water. Working standard solution was prepared by dilution of stock solution. Hydrochloric acid, 0.4 M, potassium iodate, 2%, sodium acetate, 2 M were used. A 0.05% solution of variamine blue was prepared by dissolving 0.05 g of variamine blue in 25 mL of ethanol and making up to 100 mL with distilled water. The solution was stored in an amber bottle.

Method

Preparation of calibration curve

An aliquot of a sample solution containing 0.2-14 µg mL-1 of arsenic(III) was transferred into a series of 10 mL calibrated flasks. Then, potassium iodate (2%, 1 mL) and hydrochloric acid (0.4 M, 1 mL) were added and mixture was gently shaken. This was followed by addition of variamine blue (0.05%, 1 mL) and 2 mL of 2 M sodium acetate solution. The solution was kept for 5 min and made up to the mark with distilled water. The absorbance of the coloured species was measured at 556 nm against the corresponding reagent blank.

Determination of arsenic in urine and serum

Arsenic is reported to be present in trace amounts in normal urine and serum 27(Patty 1963). If a person is affected by arsenic poisoning the amount of

arsenic in urine and serum increases. To check the validity of the method synthetic samples were prepared by adding known amounts of arsenic to serum and urine samples. The samples were deprotonised with trichloroacetic acid and filtered. Aliquots were then analyzed for arsenic by the proposed and reported methods 29(Pillai,Sunita & Gupta;2000).

Determination of arsenic in hair and nails

People drinking arsenic contaminated water have been reported to have high arsenic in their hair and nails. About 0.5 to 1.0 g of hair and nail samples were placed in a tube containing 10 mL of nitric acid. The tube was closed and heated on a hot plate at 100°C for 5 min. After 24 h the lid was opened and 1 mL of concentrated nitric acid was added and evaporated at 100°C until 1 mL of solution remained. Few drops of 10% KI were added to convert As(V) to As(III). The presence of any excess of iodine, indicated by light brown colour was destroyed by adding few drops of ascorbic acid 28(Sandell 1959). The sample was cooled, diluted to 5 mL and analyzed by the proposed and also by reported method29(Pillai,Sunita & Gupta;2000).

Determination of arsenic in plant material

A sample of plant material (5 g) was digested with 10 mL of HNO3 for about 20 min. After cooling, 1 mL of perchloric acid was added and heating was continued for about another 10 min. As(V) if any, is reduced to As(III) by the process described above. The solution was transferred to a 25 mL volumetric flask and diluted to volume with water. Aliquots of the sample were analysed by the recommended procedure and also by the reported method 29(Pillai,Sunita & Gupta;2000).

Determination of arsenic in soil

A known weight (1 g) of a soil sludge sample was placed in a 50 mL beaker and extracted 4 times with a 5 mL portion of concentrated HCl. The extract was boiled for about 30 min. As(V) if any, is reduced to As(III). The solution was cooled and diluted to 25 mL with distilled water. Aliquots of the sample

were analysed by the recommended method and also by the reported method 29(Pillai,Sunita & Gupta;2000)..

Determination of arsenic in natural and polluted water

Water samples from a river receiving effluent of steel plant and fertilizer factory were collected in polyethylene bottles and filtered through Whatman 41 filter paper. As(V) if any, is reduced to As(III) by the process described. Arsenic content was determined directly according to the recommended method and also by the reported method 29(Pillai,Sunita & Gupta;2000).

APPENDICES

APPENDIX- I

Data Collection Sheet

ID: **Date**

Institution:

Particulars of the patients:-

Name:

Age:

Address

a) **Present :**

Telephone No:

b) **Permanent:**

Contact person address:

Telephone no:

Education: 1□Masters 2□Graduate 3□ HSC 4□SSC 5□Primary 6 □Illiterate

Occupation: 1□Housewife 2□Service 3□Students 4□Others

Location: 1□Urban 2□Semi urban 3□Rural

Family Members:

Monthly family income:

Menstrual history: MP/MC

L.M.P

E.D.D

Obstetrical history: Para

Gravida

ALC

Risk factors:-

Obesity	Yes[1]/No[0]
Positive history of diabetes (sibs/parents)	Yes[1]/No[0]
H/O delivery of large infant (>400 gm)	Yes[1]/No[0]
H/O still birth	Yes[1]/No[0]
/O prematurity	Yes[1]/No[0]
H/O unexplained neonatal death	Yes[1]/No[0]
H/O pre eclampsia	Yes[1]/No[0]
H/O traumatic delivery e associated neurological disorder in the infant	Yes[1]/No[0]
Poor reproductive history (>3 spontaneous abortions in the 1st or 2nd trimester)	Yes[1]/No[0]
Chronic hypertension	Yes[1]/No[0]

Physical / Clinical Examination (Mother):

Observations	**1st visit Date……**	**2nd visit Date…….**
Height		
Weight		
BMI		
BP		
Height of Uterus		

Biochemical Investigation :

Observations	1st visit Date…….	2nd visit Date……..
Fasting blood glucose (mmol/l)		
Blood glucose 2 hr after 75 gm of glucose (mmol/l)		
Serum TG (mg/dl)		
Serum T-chol (mg/dl)		
Serum HDL (mg/dl)		
Serum LDL (mg/dl)		

Physical / Clinical Examination (Neonates):

Observations	1st visit Date…….
Length	
Weight	
APGAR score at 5 minits	
Activity (muscle tone)	
Active 2	
Arms and legs flexed 1	
Absent 0	
Pulse	
>100 bpm 2	
<100 bpm 1	
Absent 0	
Grimace (reflex irritability)	
Sneezes, coughs, pulls away 2	
Grimaces 1	
No response 0	
Appearance (skin color)	

Normal over entire body 2	
Normal except extremities 1	
Cyanotic or pale all over 0	
Respirations	
Good, crying 2	
Slow, irregular 1	
Absent 0	

Interviewer signature

APPENDIX- II

Estimation of plasma blood glucose (Randox laboratories, UK).

Plasma glucose was estimated by enzymatic colorimetric (GOD-PAP) method

Principle (Barham, Trinder, 1972)

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red violet quinoneimine dye as indicator.

$$\text{Glucose} + H_2O \xrightarrow[Oxidase]{Glucose} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{Peroxidase} \text{Quinoneimine} + 4H_2O$$

Reagents

Contents	*Initial concentration of solution*
Buffer	
Phosphate Buffer	0.1 mol/l, pH 7.0
Phenol	11 mol/1
GOD-PAP Reagent	
4-aminophenazone	0.77 mmol/1
Glucose oxidase	≥1.5 kU/1
Peroxidase	≥1.5 kU/1
Standard	
Glucose	5.55 mmol/1 (100 mg/dl)

Additional Reagent

Uranyl Acetate 0.16% cat NO DP 647 2x 500 ml

Materials required

Microcentrifuge tube

Micropipettes and pipettes with disposable tips

AUTOLAB (Analyzer Medical system, Rome, Italy)

Procedure

Procedure for glucose GOD-PAP assay without deproteinisation. The instrument was calibrated before estimation.

Plasma and reagent were taken in specific cup. They were arranged serially into the Auto lab Analyzer (Analyzer Medical system, Rome, Italy). The Auto lab was programmed for the estimation of glucose and allowed to run with following procedure:

5 µl sample and 500 µl reagent were mixed and incubated at 37^0 C for 10 minutes. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Optical densities or absorbances were fed into a computer and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using following formula.

$$\text{Glucose concentration (mmol/l)} = \frac{A_{Sample}}{A_{S\,\tan dard}} \times 5.5$$

APPENDIX- III

Estimation of serum total cholesterol (Randox laboratories, UK)

Total cholesterol was measured by enzymatic endpoint method (cholesterol Oxidase/Peroxidase) method in auto analyzer (Analyzer Medical System, Rome, Italy) using reagent of Randox laboratories, UK.

Principle (Trinder, 1988)

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantiphyrine in the presence of phenol and peroxidase.

$$\text{Cholesterolester} + H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholestene-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{phenol} + \text{4-aminoantiphyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + H_2O$$

Reagent composition

Contents	Initial Concentration of Solution
Reagent	
4-Aminoantipyrine	0.30 mmol/l
Phenol	6 mmol/l
Peroxidase	$\geq$ 0.5 U/ml
Cholesterol esterase	$\geq$ 0.15 U/ml
Cholesterol oxides	$\geq$ 0.1 U/ml
Pipes Buffer	80 mmol/l; pH 6.8
Standard	5.17 mmol/l (200 mg/dl)

Materials

Microcentrifuge tube

Micropipettes and pipettes

Disposable tips

AUTOLAB (Analyzer Medical system, Rome, Italy)

Procedure

Plasma and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the AUTOLAB. 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the Auto lab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration of cholesterol in sample was calculated by using software program with the following formula.

$$\text{Cholesterol concentration (mg/dl)} = \frac{A_{Sample}}{A_{S\tan dard}} \times \text{concentration of standard.}$$

APPENDIX- IV

Estimation of plasma triglycerides

Plasma triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in auto analyzer (Analyzer Medical System, Rome, Italy) using reagent of Randox laboratories, UK.

Principle

The triglyceride is determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen- peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase (Trinder 1969).

$$\text{Triglyceride} + H_2O \xrightarrow{Lipase} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Kinase]{Glycerol} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow[Oxidase]{Glycerol\ Phosphate} \text{dihydroxy acetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4- chlorophenol} \xrightarrow{Peroxidase} \text{quinoneimine} + HCl + 4\,H_2O$$

Reagents

Contents	*Concentrations in the Test*
Buffer	
Pipes Buffer	40 mmol/l, pH 7.6
4-choloro-phenol	5.5 mmol/l
Magnesium-ions	17.5 mmol/l
2. Enzyme Reagent	
4-aminophenazone	mmol/l
ATP	1.0 mmol/l
Lipases	>150 U/ml
Glycerol-3-phosphate oxidase	1.5 U/ml
Peroxidase	0.5 U/ml

3. Standard 2.29 mmol/l (200 mg/dl)

Materials

Micropipettes and pipettes

Disposable tips

AUTOLAB (Analyzer medical system, Rome, Italy)

Procedure

Plasma and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the AUTOLAB. 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the AUTOLAB.

The reaction occurred in reaction cell. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Triglyceride concentration was calculated by using software program in AUTOLAB with the following formula.

Triglyceride concentration (mg/dl) = $\frac{A_{Sample}}{A_{S\,\tan\,dard}} \times$ Concentration of the standard.

Appendix- V

Estimation of serum high density lipoprotein (HDL)

Plasma high-density lipoprotein (HDL) was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method in auto analyzer (Analyzer Medical System, Rome, Italy) (Friedewald WT, 1972)

Principle

HDL (High Density Lipoproteins) are separated from chylomicrons, VLDL (very low density lipoproteins) and LDL (Low density lipoproteins) by the addition of a precipitating reagent (phosphotungstic acid-magnesium chloride) to serum or plasma. After centrifugation, the cholesterol contents of HDL fraction which remains in the supernatant are determined by the enzymatic colorimetric method using CHOD- PAP.

Reagent composition

Contents

Buffer

Enzymes

Standard 50 mg/dl (1.29 mmol/l)

Materials

Microcentrifuge tube, Micropipettes and pipettes

Disposable tips

AUTOLAB

Procedure

Samples (200 ml) and precipitating reagents (500 µl) were taken in a microcentrifuge tube. Then it was mixed and allowed to sit for 10 minutes at room temperature. Then it was centrifuged at 4000 rpm for 10 minutes.

The supernatant was used as sample for determination of cholesterol content by the CHOD-PAP method. The sample and reagents were taken in specific cup or cell. They were arranged serially then ID number for test was entered in the AUTOLAB.

Then 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the AUTOLAB. The reaction occurred in reaction cell. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration was calculated by using software program.

Appendix- VI

Estimation of LDL cholesterol level in serum

LDL cholesterol level in serum was calculated by using by Friedewald formula (Friedewald WT. 1972)

Formula

$$\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL Cholesterol} + \frac{1}{5} x \text{ Triglyceride})$$

Appendix- VII

ARSENIC ESTIMATION SPECTROPHOTOMETER

Experimental Procedure

Apparatus

A Secomam Anthelie NUA 002 UV-VIS spectro- photometer with 1 cm quartz cell was used for the absorbance measurements and a WTW pH 330, pH meter was used for pH measurements.

Reagents

All chemicals used were of analytical grade and distilled water was used for dilution of reagents and samples. Standard arsenic (III) stock solution (1000 µg mL-1) was prepared by dissolving 0.1732 g of NaAsO2 in 100 mL of water. Working standard solution was prepared by dilution of stock solution. Hydrochloric acid, 0.4 M, potassium iodate, 2%, sodium acetate, 2 M were used. A 0.05% solution of variamine blue was prepared by dissolving 0.05 g of variamine blue in 25 mL of ethanol and making up to 100 mL with distilled water. The solution was stored in an amber bottle.

Method

Preparation of calibration curve

An aliquot of a sample solution containing 0.2-14 µg mL-1 of arsenic(III) was transferred into a series of 10 mL calibrated flasks. Then, potassium iodate (2%, 1 mL) and hydrochloric acid (0.4 M, 1 mL) were added and mixture was gently shaken. This was followed by addition of variamine blue (0.05%, 1 mL) and 2 mL of 2 M sodium acetate solution. The solution was kept for 5 min and made up to the mark with distilled water. The absorbance of the coloured species was measured at 556 nm against the corresponding reagent blank.

Determination of arsenic in urine and serum

Arsenic is reported to be present in trace amounts in normal urine and serum 27(Patty 1963). If a person is affected by arsenic poisoning the amount of

arsenic in urine and serum increases. To check the validity of the method synthetic samples were prepared by adding known amounts of arsenic to serum and urine samples. The samples were deprotonised with trichloroacetic acid and filtered. Aliquots were then analyzed for arsenic by the proposed and reported methods 29(Pillai,Sunita & Gupta;2000).

Determination of arsenic in hair and nails

People drinking arsenic contaminated water have been reported to have high arsenic in their hair and nails. About 0.5 to 1.0 g of hair and nail samples were placed in a tube containing 10 mL of nitric acid. The tube was closed and heated on a hot plate at 100°C for 5 min. After 24 h the lid was opened and 1 mL of concentrated nitric acid was added and evaporated at 100°C until 1 mL of solution remained. Few drops of 10% KI were added to convert As(V) to As(III). The presence of any excess of iodine, indicated by light brown colour was destroyed by adding few drops of ascorbic acid 28(Sandell 1959). The sample was cooled, diluted to 5 mL and analyzed by the proposed and also by reported method29(Pillai,Sunita & Gupta;2000).

Determination of arsenic in plant material

A sample of plant material (5 g) was digested with 10 mL of HNO3 for about 20 min. After cooling, 1 mL of perchloric acid was added and heating was continued for about another 10 min. As(V) if any, is reduced to As(III) by the process described above. The solution was transferred to a 25 mL volumetric flask and diluted to volume with water. Aliquots of the sample were analysed by the recommended procedure and also by the reported method 29(Pillai,Sunita & Gupta;2000).

Determination of arsenic in soil

A known weight (1 g) of a soil sludge sample was placed in a 50 mL beaker and extracted 4 times with a 5 mL portion of concentrated HCl. The extract was boiled for about 30 min. As(V) if any, is reduced to As(III). The solution was cooled and diluted to 25 mL with distilled water. Aliquots of the sample

were analysed by the recommended method and also by the reported method 29(Pillai,Sunita & Gupta;2000)..

Determination of arsenic in natural and polluted water

Water samples from a river receiving effluent of steel plant and fertilizer factory were collected in polyethylene bottles and filtered through Whatman 41 filter paper. As(V) if any, is reduced to As(III) by the process described. Arsenic content was determined directly according to the recommended method and also by the reported method 29(Pillai,Sunita & Gupta;2000).