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Effects of Inorganic Arsenic on Type 2 Diabetes Mellitus In Vivo: the Roles and Mechanisms of miRNAs

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Abstract

Accumulating studies have shown that chronic exposure to iAs correlates with an increased incidence of diabetes. In recent years, miRNA dysfunction has emerged both as a response to iAs exposure and independently as candidate drivers of metabolic phenotypes such as T2DM. However, few miRNAs have been profiled during the progression of diabetes after iAs exposure in vivo. In the present study, high iAs (10 mg/L NaAsO$_2$) exposure mice models of C57BKS/Lepr^{db} (*db/db*) and C57BLKS/J (WT) were established through the drinking water, the exposure duration was 14 weeks. The results showed that high iAs exposure induced no significant changes in FBG levels in either *db/db* or WT mice. FBI levels, C-peptide content, and HOMA-IR levels were significantly increased, and glycogen levels in the livers were significantly lower in arsenic-exposed *db/db* mice. HOMA-β% was decreased significantly in WT mice exposed to high iAs. In addition, more different metabolites were found in the arsenic-exposed group than the control group in *db/db* mice, mainly involved in the lipid metabolism pathway. Highly expressed glucose, insulin, and lipid metabolism-related miRNAs were selected, including miR-29a-3p, miR-143-3p, miR-181a-3p, miR-122-3p, miR-22-3p, and miR-16-3p. And a series of target genes were chosen for analysis, such as *ptp1b, irs1, irs2, sirt1, g6pase, pepck and glut4*. The results showed that, the axles of miR-181a-3p-*irs2*, miR-181a-3p-*sirt1*, miR-22-3p-*sirt1*, and miR-122-3p-*ptp1b* in *db/db* mice, and miR-22-3p-*sirt1*, miR-16-3p-*glut4* in WT mice could be considered promising targets to explore the mechanisms and therapeutic aspects of T2DM after exposure to high iAs.

Keywords Arsenic · T2DM (type 2 diabetes mellitus) · microRNAs · Insulin resistance · Glucose

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Introduction

Diabetes is an increasing health concern worldwide. According to the international diabetes federation, diabetes will affect 693 million individuals globally by 2045, and type 2 diabetes mellitus (T2DM) accounts for more than 90% of all diabetes cases [1]. T2DM is a metabolic disease characterized by impairments in the secretion or action of the glucose-lowering hormone insulin and is associated with genetic, environmental, metabolic, and behavioral risk factors [2–5]. Lifestyle elements such as exercise and diet are important contributing factors, and obesity often precedes some forms of T2DM [6]. Recent evidence suggests that various toxicants, such as arsenic, play an important role in the induction of insulin resistance in T2DM [7–9].

Arsenic is a ubiquitous toxic metalloid that is extensively present in the environment. A recent report by the United Nations International Children's Emergency Fund (UNICEF) mentions that approximately 140 million people

from 70 different countries are exposed to inorganic arsenic (iAs) in their drinking water [10]. Chronic exposure to iAs is associated with numerous adverse health effects, including the typical skin triad, cancer, cardiovascular diseases, neurological disorders, and diabetes [11–15]. Accumulating epidemiological studies have shown that chronic exposure to iAs correlates with an increased incidence of diabetes by interfering with glucose homeostasis [16]. However, the molecular mechanisms underlying these defects remain poorly characterized and merit further investigation. In recent years, miRNA dysfunction has emerged both as a response to iAs exposure and independently as candidate drivers of metabolic phenotypes such as T2DM [17, 18].

miRNAs are non-coding RNAs approximately 18–22 nucleotides in length and are characterized as key regulators of genes at the post-transcriptional level [19]. miRNAs bind to the 3′ untranslated region of mRNAs, thus regulating the expression of the target genes [18]. Studies on miRNAs have offered leads to the diagnosis of certain metabolic diseases such as T2DM [20, 21]. A series of miRNAs has been recognized as biomarkers for controlling glucose homeostasis and T2DM [22, 23]. At present, miRNAs have gained extensive attention as biomarkers for rapid diagnosis and as targets for treatment of diabetes, due to their regulatory functions in the initiation and progression of diabetes [24–26]. In addition, several diabetes-related miRNAs have been identified; for example, decreased expression levels of plasma miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486, and increased expression levels of miR-28-3p and miR-143 are associated with diabetes [27, 28].

In summary, miRNAs have been profiled under various factors; however, few miRNAs have been profiled during the progression of diabetes after iAs exposure in vivo. Therefore, in this study, we used a typical T2DM mouse model (C57BKS/Lepr[db], *db/db* mice) and C57BLKS/J (WT) mice to explore the roles and underlying mechanisms of iAs exposure-related miRNAs in the development of T2DM.

Materials and Methods

Animal Treatment

Twenty C57BKS/Lepr[db] (*db/db*) and twenty-eight C57BLKS/J (WT) five-week-old male mice (as found in previous studies that male rodent have more severe glucose intolerance patterns with respect to females [29]) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were housed in stainless steel cages under controlled conditions of 25 ± 3 °C, 50 ± 5 % humidity, fed with SPF-grade rat and mice reproduction fodder purchased from Beijing Keao Xieli Feed Co.,Ltd, and

a 12 h light/dark cycle with water ad libitum. Following acclimation for four weeks, random fasting blood glucose (FBG) was determined for all *db/db* mice. Those mice with FBG above 11.1 mmol/L for three times in succession were assigned to two groups according to FBG levels and body weight. C57BLKS/J (WT) mice were also divided into two groups based on FBG levels and body weight. For both types of mice, the control groups were provided with deionized water, and the arsenic exposure groups were provided deionized water with 10 mg/L sodium arsenite water solution. The exposure duration was 14 weeks, and the sodium arsenite solution was prepared weekly. Water consumption was measured in all the groups. Body weight and FBG levels were measured weekly. After 14 weeks of exposure, all mice were deprived of food for 6 h and then euthanized to collect the pancreatic, liver, and gastrocnemius of the right hind leg (Skeletal muscles are responsible for about 80 % of glucose disposal under insulin-stimulated conditions and play a key role on the development of insulin resistance [30]). Plasma was acquired for analysis. We followed animal use and experimental procedures approved by the Animal Care and Use of Committee of Harbin Medical University.

Glucose and Lipid Measurements

Total of 50 µL plasma were used to detect glucose and lipid content using an automatic biochemical analyzer (Hitachi 3100, Hitachi Limited). Hexokinase method was applied to detect the content of glucose, Enzyme Colorimetric assay for triglyceride and total cholesterol, Selective Clearance assay for LDLc, and Selective Inhibition assay for HDLc according to the manufacturer' s instructions (China Medical System Limited).

Fasting Blood Insulin and C-Peptide Determination

Fasting blood insulin (FBI) and C-peptide were determined using a commercial kit, Mouse Metabolic Magnetic Bead Panel 96 Well Plate Assay (Cat. # MMHMAG-44K, Merck Life Sciences). Briefly, the plasma samples were centrifuged at $3000 \times g$ for 5 min. Thereafter, 10µL of the supernatant was prepared for analysis. According to the manufacturers' instructions, 200 µL of assay buffer was added to each well, and 10 µL of the appropriate matrix solution was added to the background, standards, control, and sample wells, followed by incubation with 25 µL beads overnight at 4 °C with shaking. After detection, streptavidin phycoerythrin was successively added to each well, and the plate was read on a Luminex® 200™ instrument. The mean fluorescence intensity data were analyzed using a curve-fitting method for calculating the concentrations of FBI and C-peptide.

Metabolomic Analysis

High-purity nitrogen, columns, centrifuge tubes, methanol, acetonitrile, acetonitrile–water mixture solution, and sample bottles were prepared. Plasma (100 µL) was added to 200 µL methanol and vortexed for 2 min. The samples were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was transferred to another centrifuge tube and blow-dried with liquid nitrogen. For reconstitution, 100 µL acetonitrile–water mixture solution was added, vortexed for 2 min, and centrifuged. The liquid was transferred to a sample bottle. A Waters UPLC HSS T3 column was used to separate the samples, with an injection volume of 2 µL. Data analyses of spectra were performed using MetaboAnalyst 2.0 (http://www.metaboanalyst.ca/MetaboAnalyst), which also provides PLS-DA and KEGG pathway analysis outputs. After importing the detected data into Progenesis QI software (Waters, USA) for peak identification, peak extraction and normalization, the data were then analyzed using the SIMCAP 13.0 software (Umetrics, USA) to screen the different metablites.

Hematoxylin and Eosin Staining and PAS Staining

Parts of the liver were extracted and fixed in 4% paraformaldehyde solution for at least one week. Thereafter, the samples were dehydrated in a graded alcohol series and embedded in paraffin wax. Sections of 4 µm thickness were stained with hematoxylin and eosin and photographed using an Olympus CX43 microscope for pathological analysis. Glycogen levels were evaluated using the periodic acid-Schiff (PAS) staining method. Three sections per slice were imaged using an Olympus CX43 microscope and analyzed using ImagePro Plus 6.0.

In Silico Analysis

The selection of miRNAs was based on the previous studies providing information about the represented miRNAs involved in Type 2 Diabetes process [31]. In Silico analysis was performed using an online bioinformatics program, mirecords/miRDB, which utilizes miRanda, Target scan, Pic Tar, etc., to predict potential target genes.

RNA Isolation and Quantitative Real-time PCR (RT-qPCR)

Total RNA was extracted from the liver and muscles using a Qiagen miRNeasy Mini Kit (Qiagen Bio, Germany). cDNAs was prepared by reverse transcription using the Takara Mir-X™ miRNA First-Strand Synthesis Kit and PrimeScript™ RT reagent Kit with gDNA Eraser for miRNA and mRNA, respectively (Takara Bio. Japan). All qPCR analyses were performed on a QuantStudio™ 5 Real-Time PCR system using Takara TB GREEN Premix Ex Taq (Takara Bio, Japan). For miRNA quantification, a total of 6 (Table S1) miRNAs related to insulin resistance, glucose transport, insulin sensitivity, and adipocyte differentiation were chosen. miRNA levels were quantified by normalization to U6. For mRNA quantification, seven differentially miRNA-regulated genes (Table S2) were selected and the mRNA levels were normalized to β-actin. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical evaluations were carried out using Graphpad Prism 8. All experimental values were expressed as the Means ± SD. Differences among groups were calculated using Student's t-test. A P-value less than 0.05 was considered statistically significant.

Results

Effects of Arsenic Exposure on the Body Mass and Ratios of Liver to Body Weight and Pancreas to Body Weight

Throughout the exposure period, the total arsenic intake was significantly higher in *db/db* mice than in WT mice exposed to 10 mg/L sodium arsenite (17.39 mg versus 5.25 mg) (Fig. 1A). In both *db/db* and WT mice, the arsenic content in urine considerably increased in the iAs-exposed group compared to that in the control group (Fig. 1B).

At the end of the exposure period, 10 mg/L iAs had no significant effect on the terminal body weight and liver/body weight ratios ($P > 0.05$) (Fig. 1C and Fig. 1E), whereas it was evident that arsenic exposure significantly reduced the pancreas/body weight ratio ($P < 0.05$) (Fig. 1D). Additional details are provided in Fig. 1.

Effects of iAs on Glucose, Insulin Metabolism, and Lipid Profiling

The results showed that exposure of 10 mg/L iAs induced no significant changes in FBG levels in either *db/db* or WT mice ($P > 0.05$) (Fig. 2A). FBI levels were not significantly altered in WT mice exposed to 10 mg/L iAs, whereas it was highly increased in the mice of the arsenic-exposed group compared with those in the control group of *db/db* mice ($P < 0.05$) (Fig. 2B). Furthermore, we determined the C-peptide content to reflect the real scenario of insulin secretion, owing to its longer half-life than insulin. Similar changes to that of FBI were found in the C-peptide content, which was significantly increased in the arsenic exposure group in *db/db* mice; however,
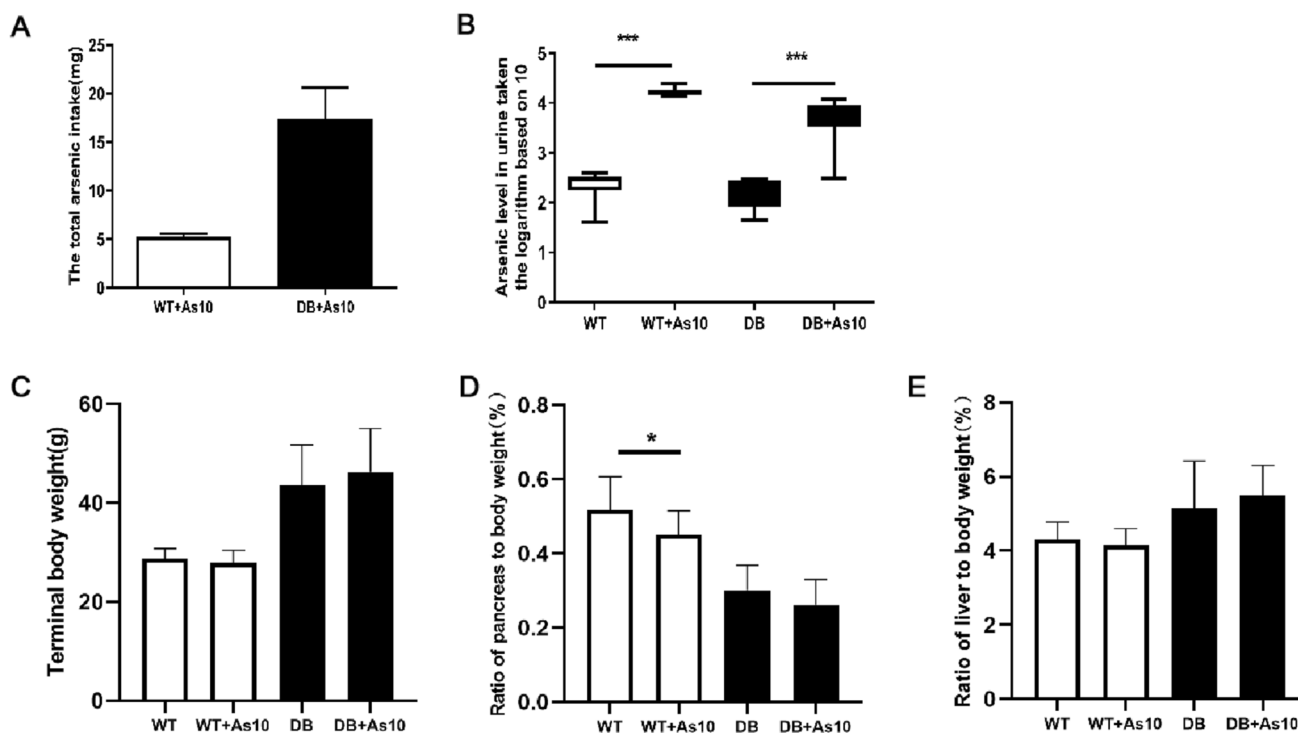
Fig. 1 Influences on the body mass and liver, pancreas to body weight ratio induced by arsenic exposure DB means diabetic control mice; DB+As10 means diabetic mice exposed to 10 mg/L inorganic arsenic; WT means wild type control mice; WT+As10 means wild type mice exposed to 10 mg/L inorganic arsenic. **A** The total arse-nic intake throughout the exposure period; **B** arsenic contents in urine taken the logarithm based on 10; **C** the terminal body weight; **D** ratio of pancreas to body weight; **E** ratio of liver to body weight. N=14(WT mice), and N=10 (DB mice). *P < 0.05, ***P < 0.001

no obvious change was observed in the arsenic-exposed WT mice (Fig. 2C). HOMA-IR and HOMA-β% were calculated based on a previous study [32]. The arsenic exposure dose of 10 mg/L significantly increased HOMA-IR levels in *db/db* mice, compared with that of the corresponding control group mice, whereas no significant change was found in WT mice (Fig. 2D). In addition, HOMA-β% was decreased significantly in arsenic-exposed WT mice (P < 0.05) but increased non-significantly in *db/db* mice exposed to arsenite (P = 0.061) (Fig. 2E).

Furthermore, the lipid concentrations were determined to evaluate the influence of iAs. The results revealed that iAs exposure had no significant effect on the levels of triglyc-erides, TCH, HDL-c, or LDL-c. However, it is noteworthy that exposure of 10 mg/L arsenic increased the concentration of TCH (P = 0.080) and LDL-c (P = 0.072) in *db/db* mice. Further details are provided in Fig. 2F.

Pathological Changes in Liver Induced by iAs Exposure

The liver is a crucial metabolic organ that acts as a hub for the metabolism of various tissues. As shown in Fig. 3, iAs exposure affected the liver. Compared with the control group mice, arsenic-exposed WT mice exhibited more obvious edema, and the cytoplasm became looser in WT mice. In *db/db* mice, cavitation denaturation was apparent in both groups, and iAs exposure increased the degree of hepatic edema. Moreover, the central vein was also congested.

PAS staining showed that the glycogen levels in the livers of the arsenic-exposed group were significantly lower (P < 0.01) than those in the control group of *db/db* mice (Fig. 3E-I). However, no significant alteration in glycogen levels was observed in WT mice exposed to 10 mg/L arsenic (P > 0.05).

Profiling of Metabolites in the Plasma After iAs Exposure

The influence of iAs exposure on metabolite concentra-tion in the plasma was determined. In negative ion mode, 9350 metabolic substances were found in WT group, 9695 in WT+As10 group, 10,122 in DB group, and 9892 in DB+As10 group, respectively. In positive ion mode, 21,353 metabolites were found in WT group, 22,442 in WT+As10 group, 21,796 in DB group and 21,900 in DB+As10 group, respectively. PLS-DA plot showed better separation of the plasma metabolites in the *db/db* mice exposed to 10 mg/L iAs than in the WT mice under either positive or negative ion mode (Fig. 4). More metabolites were found
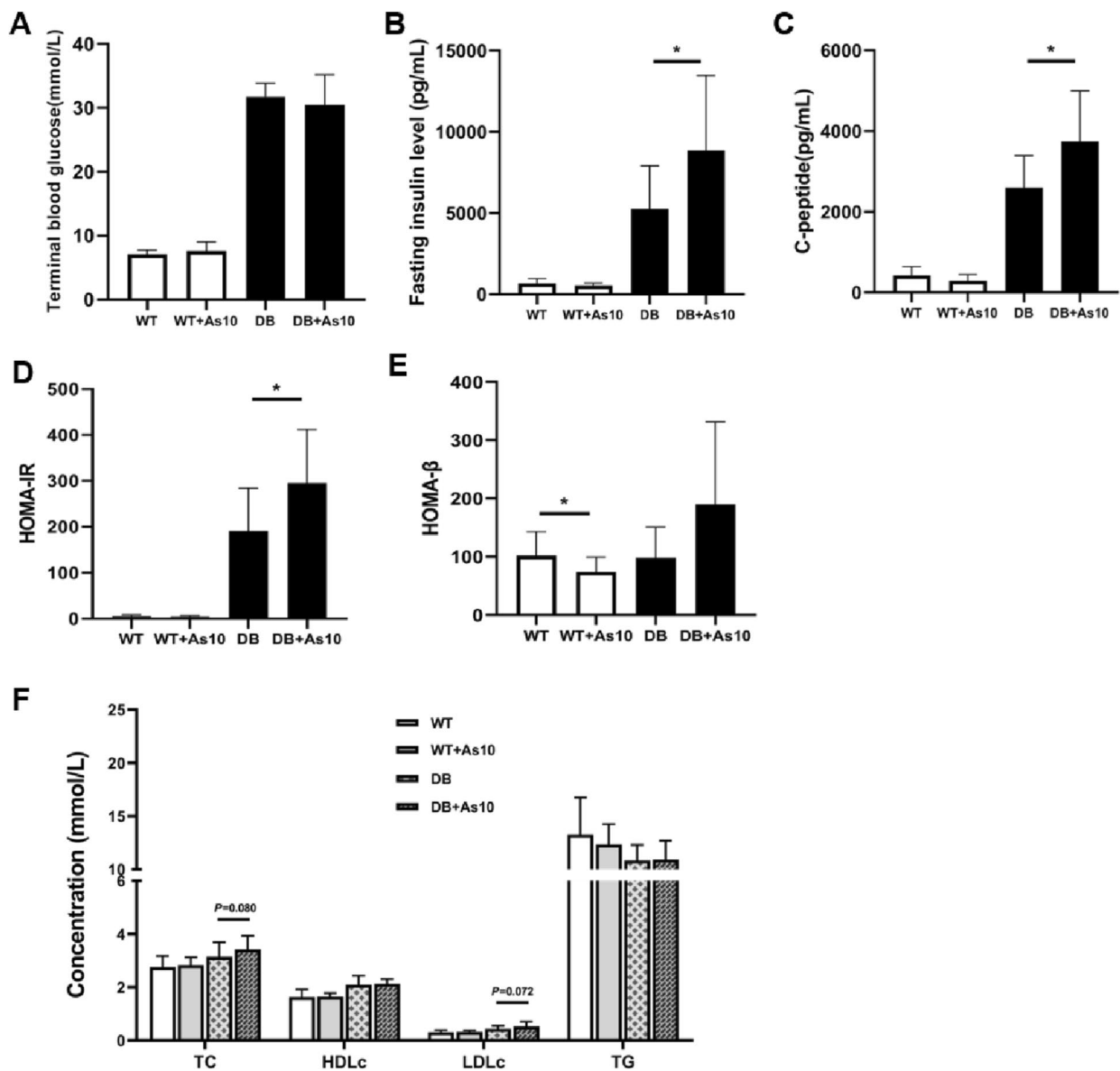
Fig. 2 Effects of iAs on glucose, insulin metabolism and lipid profiling DB means diabetic control mice; DB+As10 means diabetic mice exposed to 10 mg/L inorganic arsenic; WT means wild type control mice; WT+As10 means wild type mice exposed to 10 mg/L inorganic arsenic. **A** the terminal blood glucose; **B** fasting blood insulin level; **C** fasting blood C-peptide content; **D** HOMA-IR; **E** HOMA-β%; **F** lipid profiling. N=14 (WT mice), and N=10 (DB mice). *$P < 0.05$

in the arsenic-exposed group than in the corresponding control group in *db/db* mice (6 and 3 metabolites, respectively). In *db/db* mice, N-acetylmuramoyl-alanine amidase, (4E)-1-(3,4-dihydroxyphenyl)tetradec-4-en-3-one, and LysoPI(20:4(5Z,8Z,11Z,14Z)/0:0) were involved in the lipid metabolism pathway. In addition, D-tryptophan and L-Aspartyl-4-phosphate were involved in amino acid metabolism. In WT mice, two metabolites were correlated with the lipid metabolism pathway: LysoPC(20:1(11Z)/0:0) and 16(17)-EpDPE. Moreover, D-tryptophan was also found different metabolite, similar to that in *db/db* mice (Table 1).

Effects of iAs on miRNAs

Highly expressed glucose, insulin, and lipid metabolism-related miRNAs were selected based on previous study results, including miR-29a-3p, miR-143-3p, miR-181a-3p, miR-122-3p, and miR-22-3p in the liver, and miR-16-3p in muscles (Fig. 5). In *db/db* mice, an iAs exposure dose of 10 mg/L exerted a remarkable influence on the levels of miR-22-3p, miR-122-3p, and miR-181a-3p, whereas no significant alterations were found in the miR-29a-3p and miR-143-3p levels in the liver. In muscles of these mice, miR-16-3p levels
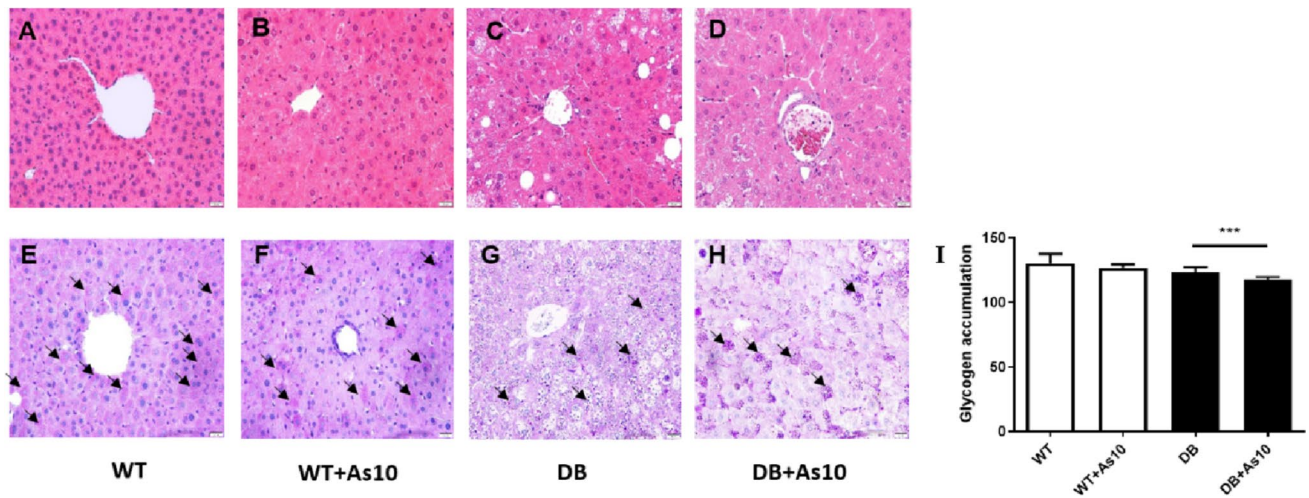
Fig. 3 H&E staining and PAS results of liver (×400) DB means diabetic control mice; DB+As10 means diabetic mice exposed to 10 mg/L inorganic arsenic; WT means wild type control mice; WT+As10 means wild type mice exposed to 10 mg/L inorganic arse- nic. **A**–**D** Were the respective H&E staining pictures; **E**–**H** were the respective pictures of PAS staining; and **I** was statistical result of liver glycogen levels, black arrows indicate glycogen. $N=5$. ***$P < 0.001$

were not significantly decreased. In WT mice, an iAs exposure dose of 10 mg/L highly increased miR-22-3p and miR-122-3p levels but significantly decreased levels of miR-143-3p. However, the miR-29a-3p and miR-181a-3p levels did not change significantly. In the muscles of WT mice, miR-16-3p levels significantly decreased.

Effects of iAs on Chosen Diabetes-Related Genes

To explore the underlying mechanism of the selected miR-NAs on the progression of T2DM induced by high iAs exposure, a series of target genes those play important roles in insulin transmission, glucose uptake, and lipid metabolism were selected for analysis. The selected genes were as follows: *ptp1b*, regulated by miR-122-3p, which is involved in lipid metabolism and insulin resistance; *irs1*, regulated by

miR-22-3p; *irs2* regulated by miR-181a-3p, both with a function in insulin signal transmission; *sirt1*, regulated by miR-22-3p and miR-181a-3p, which are involved in insulin resistance; and g*6pase,* regulated by miR-143-3p, which plays a role in *AKT* activation, glucose phosphorylation, and insulin resistance. In addition, *pepck* plays an important role in gluconeogenesis. For the muscles, *glut4* regulated by miR-16-3p implicated in the regulation of glucose uptake was selected for analysis. The results showed that iAs exposure induced significant changes in the expression of diabetes-related genes chosen for assessment: *ptp1b*, *irs2*, *g6pase*, *sirt1* for the liver, and *glut4* for muscles. However, no evidence of high regulation of *irs1* or *pepck* under 10 mg/L arsenic exposure in *db/db* mice could be obtained (Fig. 6). In WT mice, an iAs exposure dose of 10 mg/L highly upregulated *ptp1b*, *irs1*, and *glut 4* and decreased the levels of *sirt1*, whereas *g6pase*, *pepck*, and *irs2*
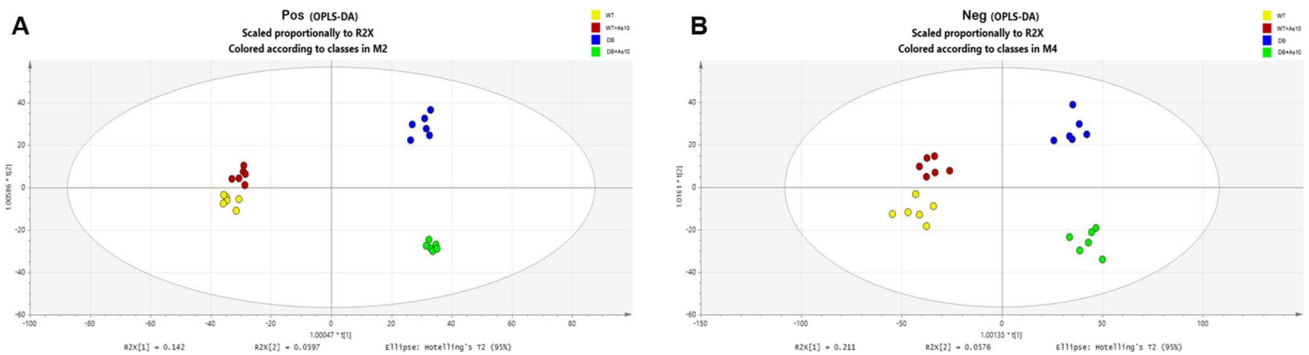

Fig. 4 The metabolites profiling of plasma induced by iAs exposure DB means diabetic control mice; DB+As10 means diabetic mice exposed to 10 mg/L inorganic arsenic; WT means wild type control mice; WT+As10 means wild type mice exposed to 10 mg/L inorganic arsenic. **A** Positive ion mode; **B** Negative ion mode. $N=6$

Table 1 Altered metabolites and biological pathways induced by iAs exposure

Significant metabolic variations compared with the corresponding control group	Positive ion mode	Pathway	Negative ion mode	Pathway
10 mg/L (WT mice)	LysoPC(20:1(11Z)/0:0)	Glycerophospholipid Metabolism Lipid metabolism pathway	D-Tryptophan	Amino acid metabolism
			16(17)-EpDPE	Lipid metabolism pathway
10 mg/L (*db/db* mice)	D-Tryptophan	Amino acid metabolism	L-Aspartyl-4-phosphate	Amino acid metabolism
	N- Acetylmuramoyl-O- Alanine amidase	Lipid metabolism pathway	3,5-Di-tert-butyl-4-hydroxyben-zaldehyde	Oxidative stress
			LysoPI(20:4(5Z,8Z,11Z, 14Z)/0:0)	Glycerophospholipid metabo-lism Lipid metabolism pathway
			(4E)-1-(3,4-dihydroxyphenyl) tetradec-4-en-3-one	Lipid metabolism pathway

levels were not significantly changed. In summary, the axles of miR-181a-3p-*irs2*, miR-181a-3p-*sirt1*, miR-22-3p-*sirt1*, and miR-122-3p-*ptp1b* in the liver may play a role in insulin resistance in high-arsenic-exposed *db/db* mice. In WT mice, axles of miR-22-3p-*sirt1* and miR-16-3p-*glut4* participated in the reduced insulin sensitivity induced by iAs-exposure.

Discussion

The effects of arsenic exposure on diabetes have gained more recognition recently, and many studies have attempted to understand the exact mechanism through which arsenic-induced diabetes development occurs. In this study, we assessed the effects of iAs exposure on miRNA profiles in the progression of T2DM in vivo and the possible underlying mechanism.

A typical T2DM mouse model (*db/db* mice) was used in this study. In *db/db* mice, leptin receptor gene knockout leads to a disorder in the leptin signaling pathway, which induces obesity-type insulin resistance. As age increases, fasting blood glucose levels in these organisms increase significantly from 8 weeks of age. Hence *db/db* is currently an ideal animal model for T2DM. Various inorganic arsenic concentrations spanning from 0.1 mg/L to more than 50 mg/L in drinking water have been studied before in vivo studies [33, 34]. However, a specific concentration range for susceptibility to T2DM has not yet been defined. According to the exposure time, iAs that is too low could not be enough to elicit an expected response. However, a very high concentration could produce non-desired new factors such as cancer, as shown in previous studies [35]. Therefore, arsenite concentrations of 10 mg/L and 50 mg/L were sufficient to produce the expected
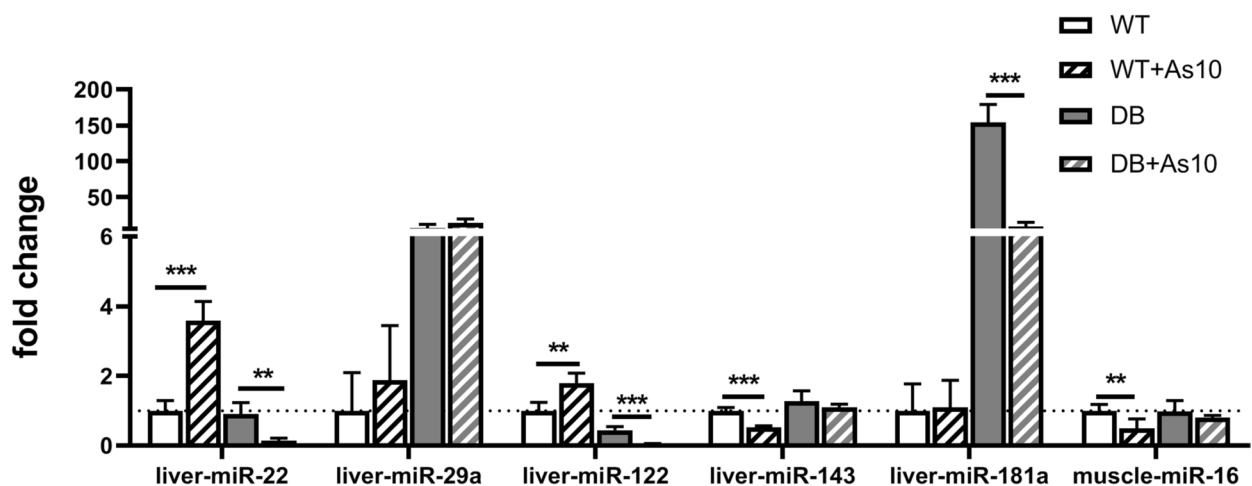

Fig. 5 Effects of iAs on miRNAs profiling DB means diabetic control mice; DB+As10 means diabetic mice exposed to 10 mg/L inorganic arsenic; WT means wild type control mice; WT+As10 means wild type mice exposed to 10 mg/L inorganic arsenic. N=5–7. ** $P < 0.01$; *** $P < 0.001$
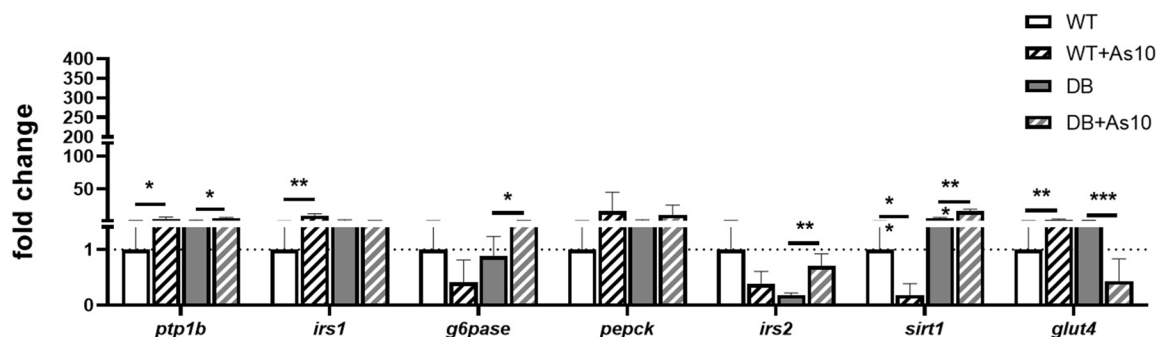
Fig. 6 Effects of iAs on chosen diabetes-related genes DB means diabetic control mice; DB+As10 means diabetic mice exposed to 10 mg/L inorganic arsenic; WT means wild type control mice; WT+As10 means wild type mice exposed to 10 mg/L inorganic arsenic. $N=5$–7. $*P < 0.05$; $**P < 0.01$; $*** P < 0.001$

effects. Our group found that 50 mg/L sodium arsenite significantly affected water intake in mice. Finally, the dose of 10 mg/L was used in this study, which is equivalent to 0.1 mg/L of As^{3+}, nearly 50 times of human beings. The life span of C57BL / 6 mice is about 24 months and the average human life span is 75 years. Based on these data, 14 weeks of arsenic exposure in mice is equivalent to 10 years in humans, which is considered a chronic exposure.

Impairments in insulin secretion, induction of insulin resistance, and glucose homeostasis are key features of T2DM. Several studies have focused on high-fat diet-induced glucose metabolism and arsenic-induced insulin transmission. This study highlighted the effects of arsenic on the diabetic progression of *db/db* mice to restrain the worse outcomes in areas with high arsenic exposure. A previous study explored the effects of arsenite in *db/db* mice; a lower level of arsenic (3 mg/L arsenite) was employed, and the authors found results similar to ours that increased IR by arsenic exposure [36]. However, in contrast to our results, the insulin level significantly decreased, and the glucose level increased. In this study, iAs exposure increased the degree of hepatic edema and significantly decreased glycogen levels in *db/db* mice. Therefore, a high iAs exposure dose of 10 mg/L worsened the progression T2DM by increasing IR. In addition, it was found that the β-cell function is significantly influenced by iAs. Indeed, through the assessment of HOMA-β%, iAs strongly affected the function of β-cells in non-diabetic conditions, inducing a decrease in insulin production, leading to an increase in blood glucose, and possible settlement of diabetes. However, in diabetic conditions, β-cell activity increased under iAs exposure ($P = 0.061$), trying to antagonize the high blood glucose under diabetic conditions in mice.

In recent years, metabolomics technology has developed rapidly and has been applied to analyze the changes in metabolites of patients with pre-diabetes and diabetes [37, 38]. In this study, no significant change was found in the lipid profile; however, 10 mg/L arsenic exposure increased the concentrations of TCH ($P = 0.080$) and LDL-c ($P = 0.072$) in *db/db* mice. To clarify the effects of high arsenic, metabolites in plasma were further analyzed using metabonomics technology, and more number of different metabolites were found in diabetic mice, mainly related to lipid and amino acid metabolism. In WT mice, the different metabolites screened in the iAs-exposed group were mainly related to phospholipid and lipid metabolism. Lysophosphatidylcholine content, which has been reported to be associated with an increased risk of T2DM, significantly decreased in these mice [39]. These results indicate that arsenic exposure affected phospholipid, lipid, and amino acid metabolism and disturbed the metabolic balance, thus aggravating insulin resistance.

More in vivo studies are needed to evaluate microRNAs as molecular drivers of iAs-associated diabetes [40]. Based on literature review, six miRNAs highly expressed and closely related to glucose and lipid metabolism were assessed, including miR-29a-3p, miR-143-3p, miR-181a-3p, miR-122-3p, and miR-22-3p in the liver and miR-16-3p in the muscles. We found remarkable effects of arsenic on miRNA levels, and the quantification of miRNAs in this study provided more information than a previous study [41]. Among the selected miRNAs, the iAs exposure dose of 10 mg/L highly increased the miR-22-3p and miR-122-3p levels, whereas it significantly decreased the levels of miR-143-3p and miR-16-3p in WT mice. Liver-specific miR-122-3p has been associated with dyslipidemia in both murine and human systems under pathological conditions other than diabetes [42–44]. MiR-22-3p has been shown to repress fatty acid synthesis and elongation [45], while playing an important role in hepatic physiology during insulin resistance and type 2 diabetes [46]. Our results show that iAs exposure increased the levels of miR-122-3p and miR-22-3p in WT mice but not in *db/db* mice. It has been reported that miR-143-3p is involved in the settlement of T2DM [41] by negatively regulating the insulin-AKT signaling pathway [47]. In addition, it was found that miR-143-3p

was obviously decreased in WT mice exposed by 10 mg/L arsenic. The results from another study also validates obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism [28]. Moreover, a previous study explored the expression profile of miRNAs induced by all-trans retinoic acid (ATRA) and arsenic trioxide (As$_2$O$_3$) in acute promyelocytic leukemia (APL) cells during differentiation and found that miR-181a levels were significantly downregulated by arsenic, which was similar to our results [48]. MiR-16-3p in muscles directly regulates glucose in WT mice, and it has been reported that miR-16-3p stimulated glucose uptake in skeletal myoblasts [49]. In addition, miR-29a-3p has been suggested as a master regulator of arsenic-induced post-transcriptional changes in gene expression in a rat insulinoma cell line [50]. Nevertheless, it was not significantly altered by exposure to 10 mg/L arsenic. The above results show that the five selected miRNAs (miR-22-3p, miR-122-3p, miR-143-3p, miR-181a-3p, and miR-16-3p) are involved in the progression of T2DM induced by 10 mg/L arsenic exposure.

In this study, we assessed the effect of iAs on the primary insulin signaling pathway responsible for most of the metabolic actions of insulin, the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway [51]. The decrease or knockout of PTP1B, an enzyme that removes phosphate from tyrosine residues of cellular proteins, impairs insulin sensitivity through the downregulation of PEPCK, the enzyme catalyzing the formation of phosphoenolpyruvate, to alter the process of liver glycogen synthesis and gluconeogenesis [52]. We found that high iAs exposure significantly increased the level of *ptp1b* mRNA in both *db/db* and WT mice. The known negative regulation of *ptp1b* by miR-122-3p indicates that the increase in *ptp1b* expression on iAs exposure was partly due to the decrease in miR-122-3p levels in *db/db* mice. GLUT4 is a primary insulin-regulated glucose transporter on cell membranes, such as skeletal muscle membranes [53]. *Glut4* mRNA expression in muscles decreased significantly under arsenic exposure, indicating decreased glucose intake in muscles. However, miR-16-3p levels were not significantly altered, indicating that other undetectable miRNAs may play a crucial role in the regulation of GLUT4 expression in *db/db* mice. In WT mice, iAs exposure remarkably decreased the levels of miR-16-3p and increased the *glut4* mRNA levels in muscles. The increase in g*6pase* levels confirmed that *db/db* mice livers were highly insulin resistant, as seen in the study by Sun et al. [54]. As miR-143-3p levels did not change remarkably, other important miRNAs of which play an important role need to be further explored. Contrary to our expectations, *irs2* and *sirt1* were highly upregulated after iAs exposure in *db/db* mice; sirt1 is involved in gluconeogenesis and lipid metabolism and downregulates IRS2 [55, 56]. In contrast, *sirt1* mRNA levels were significantly

lower in the WT mice. We speculate that other genes might play important roles in insulin transmission and glucose metabolism in As-induced progression of T2DM in *db/db* mice, in addition to *ptp1b*, *glut4*, and g*6pase*, as mentioned by Renu et al. [57]. Based on the miRNA and their target mRNA profiles, we found that regulation axles, including miR-181a-3p-*irs2*, miR-181a-3p-*sirt1*, miR-22-3p-*sirt1*, and miR-122-3p-*ptp1b* in the liver, may play a role in the insulin resistance of high-arsenic-exposed *db/db* mice. In WT mice, miR-22-3p-*sirt1* and miR-16-3p-*glut4* were involved in the regulation of insulin sensitivity.

We obtained promising findings that have not been obtained in previous studies on miRNAs associated with T2DM progression induced by arsenic exposure using a typical mice model (*db/db* mice) and WT mice. However, some limitations do exist. RNA-Seq was not employed to identify unknown miRNAs associated with iAs-exposure. However, five selected miRNAs were found to be significantly altered. In addition, only one dose of arsenic and exposure period were used; in vivo studies with a defined range of arsenic concentrations and different exposure periods must be conducted in the future.

Conclusions

In summary, our results suggest that high iAs exposure could aggravate the progression of T2DM by altering insulin secretion, insulin resistance, and lipid metabolism and worsen diabetic effects in individuals with diabetes. The results showed that, the axles of miR-181a-3p-*irs2*, miR-181a-3p-*sirt1*, miR-22-3p-*sirt1*, and miR-122-3p-*ptp1b* in *db/db* mice, and miR-22-3p-*sirt1*, miR-16-3p-*glut4* in WT mice could be considered promising targets to explore the mechanisms and therapeutic aspects of T2DM after exposure to high iAs.

Declarations

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