Arsenic induces pancreatic dysfunction and ferroptosis via mitochondrial ROS-autophagy-lysosomal pathway

Sen Wei, Tianming Qiu, Xiaofeng Yao, Ningning Wang, Liping Jiang, Xue Jia, Ye Tao, Zhidong Wang, Pei Pei, Jingyuan Zhang, Yuhan Zhu, Guang Yang, Xiaofang Liu, Shuang Liu, Xiance Sun

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ABSTRACT

Chronic arsenic exposure is a significantly risk factor for pancreatic dysfunction and type 2 diabetes (T2D). Ferroptosis is a newly identified iron-dependent form of oxidative cell death that relies on lipid peroxidation. Previous data have indicated that ferroptosis is involved in various diseases, including cancers, neurodegenerative diseases, and T2D. However, the concrete effect and mechanism of ferroptosis on pancreatic dysfunction triggered by arsenic remains unknown. In this study, we verified that ferroptosis occurred in animal models of arsenic-induced pancreatic dysfunction through assessing proferroptotic markers and morphological changes in mitochondria. In vitro, arsenic caused execution of ferroptosis in a dose-dependent manner, which could be significantly reduced by ferrostatin-1. Additionally, arsenic damaged mitochondria manifested as diminishing of mitochondrial membrane potential, reduced cytochrome c level and production of mitochondrial reactive oxygen species (MtROS) in MIN6 cells. Using the Mito-TEMPO, we found the autophagy level and subsequent ferroptotic cell death induced by arsenic were both alleviated. With autophagy inhibitor chloroquine, we further revealed that ferritin regulated ferroptosis through the MtROS-autophagy pathway. Collectively, NaAsO2-induced ferroptotic cell death is relied on the MtROS-dependent autophagy by regulating the iron homeostasis. Ferroptosis is involved in pancreatic dysfunction triggered by arsenic, and arsenic-induced ferroptosis involves MtROS, autophagy, ferritin.

1. Introduction

Arsenic contamination is a common environmental pollution and a threat to global public health. Around 200 million people are exposed to World Health Organization (WHO)’s permissible limit of 10 μg/L of arsenic in drinking water, including China, Bangladesh, and Latin America (Medina-Pizzali et al., 2018; Sorg et al., 2014; Jiang et al., 2012). Long-term arsenic exposure is closely related to many diseases, including cardiovascular disease, hepatosis and cancer. Additionally, growing evidences have revealed that arsenic exposure is associated with a high risk of type 2 diabetes (T2D) (Grau-Perez et al., 2017; Beck et al., 2017) and the prevalence of T2D is 9% in arsenic exposure region (Islam et al., 2012).

The molecular mechanisms of T2D include the failure of quick response to insulin in cells (insulin resistance), and the reduction of insulin secretion caused by the dysfunction of pancreatic β cells (Liu et al., 2012). This study verified that ferroptosis occurred in animal models of arsenic-induced pancreatic dysfunction through assessing proferroptotic markers and morphological changes in mitochondria. In vitro, arsenic caused execution of ferroptosis in a dose-dependent manner, which could be significantly reduced by ferrostatin-1. Additionally, arsenic damaged mitochondria manifested as diminishing of mitochondrial membrane potential, reduced cytochrome c level and production of mitochondrial reactive oxygen species (MtROS) in MIN6 cells. Using the Mito-TEMPO, we found the autophagy level and subsequent ferroptotic cell death induced by arsenic were both alleviated. With autophagy inhibitor chloroquine, we further revealed that ferritin regulated ferroptosis through the MtROS-autophagy pathway. Collectively, NaAsO2-induced ferroptotic cell death is relied on the MtROS-dependent autophagy by regulating the iron homeostasis. Ferroptosis is involved in pancreatic dysfunction triggered by arsenic, and arsenic-induced ferroptosis involves MtROS, autophagy, ferritin.
et al., 2014; Wei et al., 2018). Previous data have suggested that chronic arsenic exposure can affect insulin secretion in pancreatic β cells (Carmean et al., 2019). In-depth, deficiency of L-Nfe2l1 and dysfunction of mitochondrial metabolism may be involved in the molecular mechanism of pancreatic β cells damage induced by arsenic (Cui et al., 2017; Dover et al., 2018). However, it is not clear whether ferroptosis is involved arsenic-induced pancreatic β cells damage.

Ferroptosis is a novel form of cell death triggered by lipid peroxidation in an iron-dependent way (Dixon et al., 2012; Gao and Jiang, 2018; Angeli et al., 2017). Recently, the Cell Death Naming Committee (NCDD) has classified ferroptosis as one of the regulated cell death (RCD) (Galluzzi et al., 2018). The morphological, biochemical and genetic features of ferroptosis are obviously different from other forms of programmed cell death (Dixon and Stockwell, 2017; Dover et al., 2018). However, it is not clear whether ferroptosis is involved arsenic-induced pancreatic β cells damage.

Ferroptosis is a novel form of cell death triggered by lipid peroxidation in an iron-dependent way (Dixon et al., 2012; Gao and Jiang, 2018; Angeli et al., 2017). Recently, the Cell Death Naming Committee (NCDD) has classified ferroptosis as one of the regulated cell death (RCD) (Galluzzi et al., 2018). The morphological, biochemical and genetic features of ferroptosis are obviously different from other forms of programmed cell death (Dixon and Stockwell, 2014; Yang et al., 2016). In brief, ferroptosis is defined by three indispensable hallmarks, including the impaired lipid peroxide repair capacity caused by the loss of glutathione peroxidase 4 (GPX4) activity, the availability of reducto-active iron, and oxidation of polyunsaturated fatty acid (PUFA)-containing phospholipids (Dixon and Stockwell, 2019). Currently, three biomarkers are available for identifying the occurrence of ferroptosis: protein marker (GPX4 and COX-2), lipid peroxidation, and lipid reactive oxygen species (ROS) (Li et al., 2017; Wang et al., 2017). Ferroptosis is supposed to involve in the evolution of many diseases, including ischemia-reperfusion injury (Li et al., 2019), intracerebral hemorrhage (Li et al., 2017), and Parkinson’s disease (Guiney et al., 2017). Additionally, ferroptosis can be suppressed by the specific inhibitor, ferrostatin-1 (Fer-1) (Yang and Stockwell, 2016) and desferrioxamine (DFO) (Ma et al., 2016). Bruni et al. indicated that Fer-1 or DFO could improve human islet viability and function impaired by small molecules, erastin or RSL3 (Bruni et al., 2018). However, the molecular mechanism of ferroptosis in diabetes is still unexplored and remains mysterious.

It is well known that the evolution of ferroptosis lies at the intersection of amino acid, lipid, and iron metabolism (Stockwell et al., 2017). Iron is highly reactive and can produce excessive ROS by Fenton reactions (Nishizaki and Iwahashi, 2015), resulting in cellular oxidative...
damage. Recently, it has been reported that abnormal iron metabolism upregulates the ferroptosis level in Rhabdomyosarcoma (Liu et al., 2017). Hence, the fine-tuning of iron level is closely related to cellular ferroptosis.

It is well accepted that mitochondria play a crucial role in cellular metabolism. In cysteine loss-triggered ferroptosis, mitochondrial tri-carboxylic acid (TCA) cycle and electron transport chain (ETC) serves as the main source for cellular lipid peroxide production (Gao et al., 2019). Additionally, our previous research have found that arsenic can induce mitochondrial dysfunction in INS-1 cells (Zhang et al., 2019). Mitochondrial ROS (MtROS) is one of the main indicators of mitochondrial damage, and lipid ROS plays a core role in the execution of ferroptosis (Feng and Stockwell, 2018). Hence, whether MtROS can affect ferroptosis remains to be elucidated.

Autophagy is a fundamental metabolic process in mammalian cells, transmitting cellular substance to the lysosome for degradation (Jiang and Thompson, 2015). Previous data have demonstrated that autophagy regulates ferroptosis through degrading ferritin (Gao et al., 2015; Hou et al., 2016). Ferritinophagy, a kind of selective autophagy of ferritin, has been reported to promote ferroptosis (Mancias et al., 2014). Our previous study have found that chronic arsenic exposure leads to pancreatic dysfunction via islet autophagy (Wu et al., 2018a). However, the underlying relationship between mitochondrial dysfunction, autophagy and ferroptosis caused by arsenic remains unknown.

The current study aimed to determine whether ferroptosis was involved in the islet’s dysfunction or progression of T2D caused by arsenic, and to reveal the underlying mechanisms. We assumed that arsenic induced MtROS-dependent autophagy by regulating the iron homeostasis, in turn led to ferroptotic cell death and islet dysfunction.

2. Materials and methods

2.1. Animal

Groups of 18 specific pathogen free (SPF) Adult male Sprague-Dawley rats (300 g – 350 g) obtained from Institute of Genome Engineered Animal Models for Human Disease of Dalian Medical University (China). The rats were divided randomly into 3 groups, control, low-dose of NaAsO2 (2.5 mg/kg) and high-dose of NaAsO2 (5 mg/kg), 6 animals in each group. NaAsO2 (CAS No. 7784-46-5) was gained from Sigma Aldrich. Rats were housed and handled according to protocols approved by Dalian Medical University Animal Care and Use Committee. The rats were subjected to NaAsO2 at a dose of 0, 2.5 and 5 mg/kg by gavage for 5 months. Control group was given distilled water using the above method.

2.2. Cell culture

The MIN6 cell was originated from a mouse insulinoma cell line (Miyazaki et al., 1990) and cultivated in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal bovine serum (Hyclone, USA) and antibiotics (HYC-SV30010, Hyclone, USA) and 50 μM β-mercaptoethanol under 5% CO2 at 37 ℃. 1.299 mg of NaAsO2 was dissolved in 1 ml phosphate buffered saline (PBS) to composition stock solution of 10 mM. The MIN6 cells were treated with 0 or 4 μM NaAsO2 for 24 h.

Fig. 3. NaAsO2 induced ferroptosis in MIN6 cells. The MIN6 cells were treated with NaAsO2 and erastin. (A) The toxicity of NaAsO2 on cell viability (n = 6). (B) The protein levels of GPX4, COX-2 after treatment with NaAsO2 and erastin (10 μM) (n = 3). (C) The relative GSH, T-SOD, and MDA content were measured in cells by corresponding kits (n = 3). (D) Detection of cell lipid ROS by BODIPY 581/591 C11 staining (scale bar = 50 μm). (E) The relative content of insulin released was measured using an ELISA kit (n = 3). All results are expressed as the mean ± SD. *P < 0.05, **P < 0.001 vs. control group.

2.3. Pretreatment of cells

Before treatment with NaAsO2, cells were pre-treated with 10 μM ferroptosis specific inhibitor Fer-1 (SML0583, Sigma-Aldrich, USA), 70 μM MtROS scavenger Mito-TEMPO (C4234, ApexBio, USA), 10 μM lysosomal inhibitor chloroquine (CQ) (C6628, Sigma-Aldrich, USA) and 10 μM DFO (ab120727, Abcom, USA) for 6 h. Additionally, 10 μM
Ferroptosis inducer erastin (E7781, Sigma-Aldrich, USA) was treated for 24 h.

2.4. Cell viability assay

The cytotoxicity for the MIN6 cell caused by NaAsO$_2$ was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Firstly, MIN6 cells were plated in 96-well plates and treated with various concentrations of NaAsO$_2$ (0, 1, 2, 4, 8, 16, 32 $\mu$M) for 24 h. Then, MTT (0.5 mg/ml) was added and hatched for 4 h at 37 $^\circ$C. Finally, the absorbance at 570 nm wavelength was counted by the absorbance at 570 nm wavelength.

2.5. Serum fasted insulin content assay

The content of serum fasted insulin was measured according to the manufacturer’s instructions (ER1113, Finetest, China).

2.6. Iron assay

The serum iron and non-heme iron content in tissue were calculated according to the manufacturer’s instructions (A039 and A039-2, Nanjing Jincheng Bioengineering Institute, China). The relative iron content in cell lysates was measured by Intracellular Iron Colorimetric Assay Kit (E1042, Beijing Applygen Technologies Inc., China).

2.7. GSH, MDA, and T-SOD content assay

The relative content of GSH, MDA, and T-SOD was detected according to the manufacturer’s instructions (A006-2, A003-1, and A001-3, Nanjing Jincheng Bioengineering Institute, China).

2.8. Lipid ROS assay

Lipid ROS was measured by the Molecular Probes BODIPY 581/591 C11 (D3861, Invitrogen, USA). The MIN6 cells were seeded in 24-well plates and pretreated with inhibitors and NaAsO$_2$ as previously described, and incubated with the kit reagent at a working concentration of 5 $\mu$M for 30 min in the dark. Then cells were washed three times with phosphate buffered saline (PBS). Images were acquired under a fluorescence microscope (Olympus, Tokyo, Japan).

2.9. Observation of mitochondria

The mitochondria of the pancreas from rats were observed with transmission electron microscopy as described previously. The pancreas was fixed with 2.5% glutaraldehyde for 1 day, and post-fixed in 1% osmium tetroxide for 1 h. Dehydration of the sample was done in a concentration gradient of ethanol alcohols. Finally, the pellet was embedded in Epon resin and then was stained with lead citrate and uranyl acetate. Electron micrographs were analyzed with a Tecnai Spirit electron microscope.
2.10. Measurement of MtROS

MIN6 cells were incubated with 100 nM Mito-Tracker Green (C1048, Beyotime, China) in low-serum media at 37 °C for 30 min, and incubated in 5 μM MitoSox Red (M36008, Thermo, USA) in phosphate buffered saline (PBS) at 37 °C for 30 min subsequently, protected from light. Finally, cells were washed three times gently with warm PBS buffer and immediately observed under confocal microscopy (Olympus, Tokyo, Japan).

2.11. Measurement of mitochondrial membrane potential (MMP)

MMP was measured by tetraethylbenzimidazoly-lcarbocyanine iodide (JC-1) staining. The cells were treated with NaAsO2 and stained with 5 μM JC-1 (C2005, Beyotime, China) for 20 min at 37 °C, protected from light, washed twice with PBS and observed by the fluorescence microscope. MMP was analyzed by the ratio of red/green fluorescence intensity using Image - Pro Plus 6.0 software.

2.12. Western blot analysis

The total proteins from cell samples or pancreas were lysed with cell lysis buffer (92590, Merck Millipore, USA) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (KGP610, KeyGEN, China), 1 mM protease inhibitor (KGP603, KeyGEN, China) and 1 mM phosphatase inhibitors (KGP602, KeyGEN, China). Mitochondrial and cytoplasmic proteins were extracted according to the manufacturer’s instructions (C3601, Beyotime, China). The concentration of protein isolated was quantified with the BCA Protein Assay Kit (MK164230, Thermo, USA). The proteins were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride (PVDF) membranes (ISEQ00010, Merck Millipore, USA) by a wet electrophoretic transfer method. The Membranes were blocked with 10% nonfat milk for 1 h at 37 °C, then incubated it overnight at 4 °C with primary antibodies against LC3B (ab192890, 1:1000, Abcam, USA), p62 (18420-1-AP, 1:1000, Proteintach, China), GPX4 (ab125066, 1:1000, Abcam, USA), COX-2 (WL01750, 1:500, Wanleibio, China), FTH1 (ab75972, 1:1000, Abcam, USA), NCOA4 (DF4255, 1:1000, Affinity, China), Cyt c (10993-1-AP, 1:500, Proteintach, China) and GAPDH (AF7021, 1:1000, Affinity, China). Finally, the Membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (ZB-2301, ZSGB-BIO, China) for 2 h at room temperature. Protein expression level was detected using an ECL kit (P0018, Beyotime, China) and Bio-Rad ChemiDoc MP imaging system, and was analyzed by ImageJ software.

2.13. Quantitative real-time PCR

Total RNA was isolated from rat Pancreas using RNAiso Plus (Takara), then cDNA synthesis was executed by using the PrimeScript ™ RT reagent Kit (AG11705, ACCURATE BIOTECHNOLOGY, HUNAN). The relative expression levels of the gene were analyzed by the ΔΔCt method. The sequences of primers were as follows: Ptgs2: forward 5′-cttcgggagcacaacagagt-3′ and reverse 5′-aagttaaacgctcaggtg-3′; GAPDH: forward 5′-gaagctggtcatcaacggga-3′ and reverse 5′-gaaggggaggagatgac-3′.

Fig. 5. MtROS was involved in ferroptosis and dysfunction induced by NaAsO2. (A) The expression levels of Cyt c was measured by western blotting, NaAsO2 (4 μM). (B) MMP and (C) MtROS were measured by JC-1 and MitoSOX probes. (D) The efficiency of TEMPO (70 μM), and its effect on GPX4 and COX-2 in NaAsO2-treated MIN6 cells. (E) Effects of NaAsO2 and TEMPO on relative GSH, T-SOD, and MDA content in MIN6 cells. (F) Effects of TEMPO on the NaAsO2-induced accumulation of lipid ROS by BODIPY 581/591 C11 staining (scale bar = 50 μm). (G) Effects of TEMPO on the NaAsO2-induced insulin released using an ELISA kit. (H) The expression of FTH1 and NCOA4 by western blotting. (I) Effects of TEMPO on the NaAsO2-induced accumulation of cellular iron content was assayed by kits. All results are expressed as the mean ± SD, n = 3. *P < 0.05, **P < 0.001 vs. control group, #P < 0.05, ##P < 0.001 vs. NaAsO2 group.

2.14. Glucose-stimulated insulin secretion assay

MIN6 cells were seeded in 24-well plates and pretreated with inhibitors and NaAsO2 as previously described. For Glucose-stimulated insulin secretion experiment, MIN6 cells were incubated in glucose-free Krebs-Ringer Bicarbonate solution (KRB) at 37 °C for 30 min. Then cells were incubated in KRB buffer supplemented with 2.8 mM or 17.6 mM concentrations of glucose at 37 °C for 2 h. Finally, the supernatant was collected and analyzed with the mouse INS (Insulin) ELISA Kit (EM0260, Finetest, China).

2.15. Pathological analysis

Parts of pancreas tissue from rats were fixed in 4% paraformaldehyde for 24 h, dehydrated in a concentration gradient of ethanol alcohols, embedded in paraffin, and then stained with hematoxylin-eosin (H&E) for microscopic analyses.

2.16. Statistical analysis

Data were expressed as means ± standard deviation (SD) from at least three independent experiments performed in triplicate and analyzed using SPSS 20.0. Comparisons were analyzed using one-way ANOVA or t-test, and P < 0.05 was considered statistically significant.

3. Results

3.1. NaAsO2 induced pancreatic dysfunction in rats

The effect of NaAsO2 on rat pancreas was investigated by H&E staining and serum fasted insulin levels (Fig. 1A, B). As showed in Fig. 1A, in the control group, the islets cells were round and oval, with uniform distribution and complete islet structure. Compared with the control group, islet cell hypertrophy was observed in NaAsO2-exposed rats (2.5 mg/kg and 5 mg/kg), with irregular islet margin and uneven distribution of islet cells. Moreover, NaAsO2 exposure (2.5 mg/kg and 5 mg/kg) significantly decreased serum fasted insulin levels (Fig. 1B).

As manifested in Fig. S1A, the body mass was obviously reduced by NaAsO2 (5 mg/kg). Meanwhile, NaAsO2-exposed rats (2.5 mg/kg and 5 mg/kg) showed significantly impaired insulin sensitivity and glucose tolerance compared with the control group (Fig. S1B, C). These data indicated that NaAsO2 induced pancreatic dysfunction in rats.

3.2. NaAsO2 induced ferroptosis in vivo and in vitro

To explore whether NaAsO2 induced ferroptosis in vivo, we first...
measured the protein expression of GPX4 and COX-2 in pancreatic tissues (Fig. 2A). Compared with the control group, GPX4 expression was obviously down-regulated in the 5 mg/kg group. Whereas, COX-2 expression was significantly upregulated in NaAsO2-exposed rats (2.5 mg/kg and 5 mg/kg). Moreover, the content of iron in serum and pancreas were both apparently increased in the 5 mg/kg group (Fig. 2B, C). Further, levels of GSH, T-SOD, and MDA in the pancreatic tissues were assessed: GSH and T-SOD contents were significantly lower in the NaAsO2-exposed group (5 mg/kg) than that in the control group. Otherwise, the content of MDA conspicuously rose in the NaAsO2-exposed groups (2.5 mg/kg and 5 mg/kg) (Fig. 2D). Importantly, the Ptg2 mRNA levels were elevated dramatically in the NaAsO2-exposed groups (2.5 mg/kg and 5 mg/kg) (Fig. 2E). As shown in the mitochondrial ultrastructural micrographs, membrane rupture and reduction or disappearance of cristae were observed in the NaAsO2-exposed group (Fig. 2F). These data indicated that NaAsO2 induced ferroptosis in vivo.

Then, we applied ferroptosis inducer Erastin to verify whether NaAsO2 could induce ferroptosis in vitro. As showed in Fig. 3A, the toxicological effect of NaAsO2 on MIN6 cells performed a significant dose-dependent manner. Moreover, the protein levels of GPX4 and COX-2 (Fig. 3B), the contents of GSH, T-SOD, and MDA (Fig. 3C), and lipid ROS levels (Fig. 3D) showed a distinct dose-dependent change after NaAsO2 or Erastin treatment compared with the control group, respectively. Meanwhile, the glucose-stimulated insulin secretion of MIN6 cells was impaired in the 4 μM NaAsO2 and Erastin group (Fig. 3E). Thus, NaAsO2 could trigger ferroptosis in vivo and vitro.

3.3. Fer-1 inhibited cellular ferroptosis and dysfunction induced by NaAsO2

To further investigate the role of ferroptosis in NaAsO2-induced β cells dysfunction, ferroptosis specific inhibitor Fer-1 was utilized. As shown in Fig. 4A, compared with NaAsO2-exposed group, Fer-1 increased the protein level of GPX4 and reduced COX-2 protein expression effectively. Additionally, the levels of GSH and T-SOD were obviously increased and MDA content was reduced by pretreatment with Fer-1 (Fig. 4B). Fer-1 also obviously reduced the intensity of green fluorescence showed by BODIPY 581/591 C11 staining under NaAsO2 stress (Fig. 4C). Meanwhile, the glucose-stimulated insulin secretion response of MIN6 cells was recovered by Fer-1 (Fig. 4D). Taken together, these data suggested that Fer-1 inhibited the NaAsO2-induced ferroptosis and dysfunction in MIN6 cells.
3.4. MtROS was involved in ferroptosis and dysfunction induced by NaAsO2

As manifested in Fig. 5A, Cyt c was released from the mitochondria to the cytoplasm after NaAsO2 treatment. The intensity of green fluorescence was significantly increased after NaAsO2 treatment, indicating that the MMP of cells was conspicuously reduced (Fig. 5B). Furthermore, compared with the control group, NaAsO2 obviously augmented the levels of MtROS showed by microscopy (Fig. 5C). In general, these data uncovered that NaAsO2 induced mitochondrial dysfunction in MIN6 cells.

To confirm the correlation between MtROS and ferroptosis induced by NaAsO2, we pretreated MIN6 cells with Mito-TEMPO, the MtROS scavenger. As depicted in Fig. 5D, the increased COX-2 and reduction of GPX4 expression induced by NaAsO2 were regained by Mito-TEMPO. In addition, Mito-TEMPO increased the levels of GSH and T-SOD, and reduced MDA content (Fig. 5E). The over-production of lipid ROS induced by NaAsO2 was also decreased after treatment with Mito-TEMPO (Fig. 5F). Particularly, Mito-TEMPO obviously increased cellular insulin secretion in the presence of NaAsO2 (Fig. 5G). Moreover, the decreased expression of FTH1 and NCOA4 (autophagy cargo receptor), and increased cellular free iron content were all reversed by Mito-TEMPO (Fig. 5H, I). Together, these data demonstrated that the MtROS-dependent autophagy was an active regulator for NaAsO2-induced ferroptosis.

3.5. Ferroptosis was triggered by MtROS-dependent autophagy

The relationship between MtROS and autophagy has been well confirmed, however, whether MtROS caused by NaAsO2 is associated with autophagy is unclear. As shown in Fig. 6A, the increased autophagy biomarker LC3-II levels and decreased autophagy substrate p62 levels caused by NaAsO2 were reversed by Mito-TEMPO. It indicated that NaAsO2-induced upregulation of autophagy was relied on MtROS. To further confirm the change of autophagic flux, we used the autophagy inhibitor chloroquine (CQ) in MIN6 cells. Upregulation of LC3 by NaAsO2 was reinforced by CQ, and the downregulation of p62 was reversed by CQ since CQ restrained the fusion of autophagosome and autolysosome (Fig. 6B).

To explore the link between autophagy and ferroptosis, we used pharmacological inhibitor of autophagy. Indeed, lysosomal inhibitor CQ can significantly block NaAsO2-induced ferroptosis in MIN6 cells. NaAsO2-induced downregulated expression of GPX4 and upregulated expression of COX-2 was overturned by CQ (Fig. 6C). After pretreatment with CQ, impaired GSH and T-SOD activity and overloaded MDA under NaAsO2 exposure were alleviated (Fig. 6D). Additionally, the NaAsO2-induced lipid ROS accumulation was apparently inhibited by CQ (Fig. 6E). Simultaneously, CQ facilitated the insulin secretion effectively that restrained by NaAsO2 in MIN6 cells (Fig. 6F). Together, these data demonstrated that the MtROS-dependent autophagy was an active regulator for NaAsO2-induced ferroptosis.

3.6. Autophagy triggered ferroptosis by regulating iron homeostasis

To investigate how autophagy regulated ferroptosis under NaAsO2 stress, we pretreated cells with CQ to explore the role of autophagy in NaAsO2-induced augmented expression of cellular iron content. The increased level of iron content induced by NaAsO2 was obviously decreased by CQ (Fig. 7A). Cellular free iron concentration was mainly controlled by ferritin. Therefore, we measured the expression of ferritin light chain FTH1 and NCOA4. As depicted in Fig. 7B, the inhibition of protein FTH1 and NCOA4 by NaAsO2 was both reversed by CQ. The above results indicated that the iron homeostasis was dominated by autophagy in NaAsO2-exposed MIN6 cells.

Iron has been proved to be required for ferroptosis (Dixon et al., 2012; Doll et al., 2017). Here, we found the concentration of iron was increased in rat pancreas after NaAsO2 exposure (Fig. 2C). In vitro, we pretreated cells with DFO, to detect the role of iron in NaAsO2-induced ferroptosis. The decreased GPX4 and increased COX-2 expression induced by NaAsO2 were both reversed by DFO (Fig. 7C). As observed in Fig. 7D, DFO restored the levels of GSH and T-SOD effectively and...
reduced MDA content in NaAsO2-exposed cells. Additionally, DFO significantly reduced lipid ROS level provoked by NaAsO2, as observed by microscopy (Fig. 7E). The insulin secretion in MIN6 cells was also improved by DFO (Fig. 7F). Collectively, autophagy regulated NaAsO2-induced ferroptosis via iron homeostasis.

4. Discussion

The present study demonstrated that NaAsO2 triggered mitochondrial injury, and upregulated autophagy, ultimately led to ferroptotic cell death and islet ß cell dysfunction. Mechanically, we revealed that NaAsO2-induced ferroptotic cell death depended upon the MtROS-dependent autophagy by regulating the iron homeostasis. Additionally, insulin release of islet ß cell was improved via inhibiting the MtROS-autophagy-ferritin pathway.

Dixon et al. firstly found the novel kind of RCD-ferroptosis: a non-apoptotic cell death depended on iron (Dixon et al., 2012; Latunde-Dada, 2017; Masaldan et al., 2018; Toyokuni et al., 2017). It has been reported that ferroptosis is involved in the process of numerous disease models, including hemochromatosis (Wang et al., 2017), intestinal ischemia/reperfusion (Li et al., 2019) and hepatocellular carcinoma (Sun et al., 2016). So far, few studies have linked ferroptosis to the process of arsenic-induced pancreatic dysfunction. To our knowledge, only Tang et al. have indicated that 50 mg/L of arsenite can trigger ferroptosis-related neurodegenerative diseases (Tang et al., 2018a). Hence, we hypothesized that ferroptosis might play a crucial role in pancreatic dysfunction caused by arsenic contamination. Here, the animal model of pancreatic dysfunction was established after NaAsO2 gavage for five months, evidenced by aberrant morphological changes and decreased insulin secretion of islet. To verify the presence of ferroptosis in NaAsO2-induced pancreatic dysfunction, we measured the expression of ferroptosis related proteins, contents of GSH, T-SOD, MDA, and mitochondrial morphology in pancreatic tissues. Moreover, the Ptg2 mRNA as a biomarker for the induction of ferroptosis in vivo (Li et al., 2017; Ye et al., 2019) was assessed. In general, we found the proferroptotic factors were all significantly upregulated, and the dose of arsenic applied in our study (5 mg/kg) produced a similar effect to the 50 mg/L of arsenite used by Tang et al. (Tang et al., 2018a). Thus, ferroptosis was involved in rat pancreatic dysfunction induced by NaAsO2.

It has been reported that mitochondrial dysfunction is involved in the destruction of islet ß cell (Bhattacharjee et al., 2018; Mulder, 2017). Previous studies have demonstrated that mitochondrial dysfunction is an essential process for RCD, including autophagy (Biczo et al., 2018), apoptosis (Zhang et al., 2017) and programmed necrosis (Ou et al., 2017). Recently, Gao et al. found that the role of mitochondria in ferroptosis was reflected in mitochondrial ETC, TCA cycle, and glutaminolysis (Gao et al., 2019). Interestingly, mitochondria act as the center of ATP production, and mitochondria produce lipid ROS due to changes in their metabolic functions, which is a prerequisite for the occurrence of ferroptosis. Furthermore, in the study of Wu et al., tert-butylnaphthoquinone (t-BHQ) induced ferroptotic cell death, accompanied by the production of MtROS, diminishing of MMP, and reduced levels of Cyt c (Wu et al., 2018b). Consistently, our results indicated that NaAsO2 brought about mitochondrial dysfunction, manifested as increased cytoplasmic Cyt c level, loss of MMP, and generation of MtROS. Using Mit-to-TEMPO, we found that the level of ferroptosis was downregulated in MIN6 cells. What’s more, intracellular iron concentration was also reduced by Mit-to-TEMPO. Taken together, our study confirmed MtROS mediated activation of ferroptosis via manipulating the levels of cellular iron.

In our previous research, arsenic induced islet dysfunction via upregulating the autophagy level (Wu et al., 2018c; Bai et al., 2016). However, Zhang et al. found that low concentration of arsenic suppressed activation of autophagy, which is inconsistent with our research (Lau et al., 2013; Dodson et al., 2018a, b). This conclusion may be linked to the diverse dose of arsenic. Many harmful substances have different effects at different dose levels, and arsenic is without exception.

Recently, a growing body of studies have demonstrated that ferroptosis is a mode of cell death that relies on ferritinophagy which is a cargo-specific autophagy (Gao et al., 2016; Tang et al., 2018b). Furthermore, Hou. et al. found that knockout of Atg5 and Atg7 could suppress erastin-induced ferroptosis through reducing cellular iron content and lipid peroxidation in MEFs cells (Hou et al., 2016). In the present study, similar results were obtained. NaAsO2 upregulated the levels of LC3, and reduced the levels of p62 in MIN6 cells, suggesting the autophagy was activated by NaAsO2. More importantly, after the application of the autophagy-specific inhibitor CQ, the levels of ferroptosis and free iron elevated by NaAsO2 were significantly reduced. These results indicated that ferroptosis was a form of autophagic cell death, which was ferritin-dependent. Additionally, the level of autophagy was elevated by MtROS, which was derived from mitochondrial damage induced by NaAsO2. Collectively, we suggested MtROS played an essential role in the executing of ferroptosis, and autophagy manipulated ferroptosis by controlling intracellular free iron levels.

In fact, multiple forms of RCD may take part in islet ß cell function impairment, including pyroptosis (Pei et al., 2019), apoptosis (Pan et al., 2016) and necroptosis (Dutta et al., 2018). Previous researches indicated p53 played an indispensable role in the execution of apoptosis (Dhami et al., 2013; Wang et al., 2015). Moreover, Le. et al. found that p53 regulated ferroptosis through cytokine metabolism in the suppression of tumour cell growth (Jiang et al., 2015). In some recent studies, the molecular crosstalk between ferroptosis and apoptosis via ER stress was further unfolded (Hong et al., 2017; Lee et al., 2018). However, the crosstalk between ferroptosis and other RCD forms in islet ß cells dysfunction induced by NaAsO2 needs to be further clarified.

Cellular metabolism plays an essential role in the induction of ferroptosis. Our study indicates that iron metabolism is autophagy-dependent in ferroptosis induced by NaAsO2. However, other metabolic pathways associated with ferroptosis caused by NaAsO2 are worthy for further investigation.

In summary, this study demonstrates that the ferroptosis is involved in NaAsO2-induced islet ß cells dysfunction, and mitochondrial injury and degradation of ferritin are responsible for the underlying molecular mechanism of ferroptosis. We also indicate that inhibiting ferroptosis improves NaAsO2-induced islet ß cells dysfunction via suppression of MtROS-autophagy-ferritin pathway (Fig. 8). Hence, our study provides a novel target strategy for NaAsO2-induced islet ß cells dysfunction.

5. Author contributions

S.W., T.Q. and X.S. designed the studies. S.W., T.Q., P.P., Y.T., X.J., Z.W. and X.L. assisted with animal experiments. S.W., X.J., T.Q., L.J., J.Z., Y.Z., S.L participated in cell experiments. S.W. wrote the manuscript. X.Y., N.W., T.Q., G.Y. and X.S. revised the manuscript. All authors reviewed the manuscript. X.S. is the guarantor of the article.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest in the present study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.121390.
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