Lentinan alleviates arsenic-induced ferroptosis-related hepatic lipid accumulation, insulin resistance via AMPK/ORP8/ULK1-mediated lipophagy

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Date: 2026-02-28T21:57:58+00:00

Abstract

Arsenic-induced mitochondrial dysfunction leads to lipid metabolism disorder, release of mitochondrial ferritin (FTMT), activating ferritinophagy and subsequent ferroptosis in cells. Ferroptosis is characterized by elevated ferrous ion (Fe2+) levels and decreased glutathione peroxidase 4 (GPX4) expression, which increases the risk of insulin resistance (IR). This study investigated the regulatory role of Lentinan (LNT) or AMP-activated protein kinase (AMPK) signaling in sodium arsenite (SA)-induced ferroptosis using male mice and hepatocyte models. In vivo experiments showed that LNT antagonized SA-induced ferroptosis indices, attenuated IR and hepatic lipid accumulation in mice. Detailedly, LNT intervention reduced the levels of hepatic lipid peroxidation derivant oxysterols, Fe2+, and autophagy-related MAP1LC3B, and downregulated IR index, while increased the mRNA levels of AMPK, uncoordinated 51-like kinase 1 (ULK1). Further, the intervention with LNT or AMPK agonist metformin (Met) in vitro experiments revealed their antagonisms against SA-induced ferroptosis, lipid accumulation and IR in AML12 hepatocytes, characterized by the downregulated levels of long-chain acyl-CoA synthetase 4 (ACSL4), Glucose (Glu), and the elevated levels of GPX4, FTMT, and glucose transporter 4 (GLUT-4) in hepatocytes. Also, immunoblotting analysis showed that LNT or Met intervention increased the ratio of LC3-II to LC3-I, upregulated lipophagy receptor-like oxysterol-binding protein-related protein 8 (ORP8), and the phosphorylated AMPK or ULK1 levels, indicating the activation of lipophagy pathway. Further, co-immunoprecipitation experiments demonstrated the enhanced ORP8 & ULK1 co-expression in AML12 hepatocytes. In conclusion, LNT ameliorates SA-induced ferroptosis-related hepatic lipid accumulation and insulin resistance, potentially through activation of AMPK/ORP8/ULK1-mediated lipophagy pathway.

Full Text

Preamble

Lentinan alleviates arsenic-induced ferroptosis-related hepatic lipid accumulation and insulin resistance via AMPK/ORP8/ULK1-mediated lipophagy

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Abstract

Arsenic-induced mitochondrial dysfunction leads to lipid metabolism disorder and release of mitochondrial ferritin (FTMT), activating ferritinophagy and subsequent ferroptosis in cells. Ferroptosis is characterized by elevated ferrous ion (Fe^{2+}) levels and decreased glutathione peroxidase 4 (GPX4) expression, which increases the risk of insulin resistance (IR). This study investigated the regulatory role of Lentinan (LNT) and AMP-activated protein kinase (AMPK) signaling in sodium arsenite (SA)-induced ferroptosis using male mice and hepatocyte models.

In vivo experiments showed that LNT antagonized SA-induced ferroptosis indices, attenuated IR and hepatic lipid accumulation in mice. Specifically, LNT intervention reduced hepatic levels of lipid peroxidation derivatives (oxysterols, Fe^{2+}) and autophagy-related MAP1LC3B, downregulated IR indices, and increased mRNA levels of AMPK and uncoordinated 51-like kinase 1 (ULK1).

In vitro experiments with LNT or the AMPK agonist metformin (Met) revealed their antagonism against SA-induced ferroptosis, lipid accumulation, and IR in AML12 hepatocytes, characterized by downregulated levels of long-chain acyl-CoA synthetase 4 (ACSL4), glucose (Glu), and elevated levels of GPX4, FTMT, and glucose transporter 4 (GLUT-4). Immunoblotting analysis showed that LNT or Met intervention increased the ratio of LC3-II to LC3-I, upregulated the lipophagy receptor oxysterol-binding protein-related protein 8 (ORP8), and activated the phosphorylated AMPK/ULK1 lipophagy pathway. Co-immunoprecipitation experiments demonstrated enhanced ORP8-ULK1 co-expression in AML12 hepatocytes.

In conclusion, LNT ameliorates SA-induced ferroptosis-related hepatic lipid accumulation and insulin resistance potentially through activation of the AMPK/ORP8/ULK1-mediated lipophagy pathway.

Keywords: Lentinan; Sodium arsenite; Ferroptosis; Hepatic lipid accumulation; Insulin resistance; Lipophagy

1. Introduction

Sodium arsenite (SA), a prevalent species of inorganic arsenic (iAs), contaminates groundwater and food worldwide. When iAs enters the body through drinking water or food, it is primarily metabolized by the liver, causing hepatic toxic damage [1]. Epidemiological studies have shown that environmental arsenic exposure significantly increases the risk of non-alcoholic fatty liver disease (NAFLD) [2] and type 2 diabetes mellitus (T2DM) [3] in humans. Animal experiments have confirmed that arsenic exposure induces non-alcoholic steatohepatitis (NASH) lesions in rats [4].

In vitro studies have further revealed that mitochondria are the primary target of SA-induced hepatotoxicity. SA exposure can induce mitochondrial swelling, mitochondrial membrane potential ($\Delta\Psi$m) depolarization, mitochondrial oxidative stress, and dysfunction of aerobic respiration in hepatocytes [5,6]. Consequently, mitochondrial damage triggers ferritinophagy and its dependent cellular ferroptosis by releasing mitochondrial reactive oxygen species (mtROS) and mitochondrial ferritin (FTMT) [7]. Previous studies have found that chronic SA exposure promotes ferritinophagy-mediated ferroptosis in chickens, which is associated with activation of the adenosine 5' -monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR)/uncoordinated 51-like kinase 1 (ULK1) autophagy signaling pathway [8].

Oxidative stress is considered an important characteristic of arsenic exposure-induced cytotoxicity [9]. Within cells, excessive reactive oxygen species (ROS) react with polyunsaturated fatty acids in lipid membranes, inducing lipid peroxidation and leading to cellular ferroptosis [10]. To prevent irreversible oxidative damage, cells produce adaptive responses to restore redox homeostasis. Glutathione (GSH) is an important scavenger of ROS. When GSH is oxidized to glutathione disulfide (GSSG) by glutathione peroxidase 4 (GPX4), free radicals are reduced, mitigating ferroptosis. Conversely, decreased GPX4 activity or direct degradation of GPX4 in cells can lead to increased iron-dependent ROS, thereby inducing cellular ferroptosis [11].

Lentinan (LNT) is a bioactive polysaccharide extracted from shiitake mushrooms. The main chain structure of LNT molecules consists of β-(1$→$3)-D-glucopyranosyl units, with side chains formed by glucose polymers connected via β-(1$→$6) and β-(1$→$3) bonds. Recent in vivo studies have demonstrated that LNT promotes autophagic flux through the AMPK/mTOR signaling pathway, accelerating the clearance of myelin debris by Schwann cells and thereby facilitating recovery [12]. Additionally, an in vitro study showed that LNT inhibits lipopolysaccharide (LPS)-induced oxidative stress-associated inflammation and apoptosis in bovine mammary epithelial cells (BMECs) [13]. Our recent research revealed that LNT protects against SA-induced liver pathological damage and inflammation [14]. Furthermore, Yang X' s study demonstrated that

LNT supplementation alleviates high-fat diet (HFD)-induced hepatic steatosis in mice [15].

Previous studies have found that ferroptosis not only accelerates liver cell death but also promotes the accumulation of lipid peroxidation products, which inhibit key enzymes involved in fatty acid oxidation. This inhibition prevents free fatty acids (FFAs) from entering mitochondria for β-oxidation metabolism, leading instead to their esterification into triglycerides (TG) in hepatocytes—a process associated with the progression of alcoholic liver injury and NAFLD [16,17]. It has been demonstrated that metformin (Met)-mediated activation of the AMPK signaling pathway is accompanied by induction of lipophagy in hepatocytes to alleviate hepatic steatosis [18]. Currently, the potential mechanism by which LNT counteracts SA-induced hepatic ferroptosis remains unclear. The present study explores the protective role of LNT in regulating SA-induced hepatotoxicity from the perspective of AMPK signaling and lipophagy.

2. Materials and Methods

2.1 Main reagents

Biochemical or Enzyme-linked immunosorbent assay (ELISA) reagent kits for Insulin (INS, DM-X6649), Malondialdehyde (MDA, DM-X6743), Superoxide Dismutase (SOD, DM-X6899), Catalase (CAT, DM-X6641), TG (DM-X6707), Total cholesterol (TC, DM-X6798), Oxysterols (DM-K202), Microtubule associated protein 1 light chain 3B (MAP1LC3B, DM-X6702), and FFAs (DM-X6708) were purchased from Shanghai Duma Biotechnology Co., Ltd. Ferrous ion (Fe^{2+}, BC5415) and Glucose (Glu, BC2505) assay kits were from Beijing Solarbio Biotechnology Co., Ltd. Glucose transporter 4 (GLUT-4, NBP2-82175) was obtained from Novus Biologicals.

2.2 Animal experiments

2.2.1 Animal experiment protocol

Mice were purchased from Hunan Slack Company (SCXK (Xiang) 2021-0002), with a body weight of 26.5 ± 1.0 g. The mice were 9–10 weeks old and housed in an environment maintained at 20–24 °C and 40–60% humidity, with a 12 h light/dark cycle. The experimental protocol was approved by the Animal Ethics Committee of Guilin Medical University (GLMC20230712).

Sodium arsenite ($NaAsO_2$, SA, CAS 7784-46-5, Sigma-Aldrich) was used as the toxic exposure agent, and Lentinan (LNT, CAS 37339-90-5, Shanghai Duma Biotechnology Co., Ltd) was used as the intervention agent. The experiment was divided into four groups ($n = 6$ per group): Control (mice fed under conventional conditions with free access to drinking water); SA exposure (SA administered by gavage at a dose of 5.0 mg/kg body weight (bw) every other day for 6 weeks (w)); LNT control (LNT administered by gavage at a dose of 50.0 mg/kg bw every other day for 6 w); and LNT + SA (LNT administered by gavage at 50.0 mg/kg bw, followed 12 h later by SA gavage at 5.0 mg/kg bw, every other day for 6 w). At the end of the experiment, mice were fasted for 24

h, then anesthetized and euthanized. Serum and liver tissues were collected for further analysis.

2.2.2 Liver tissue Oil Red O staining

Frozen liver tissues were prepared using a Cryotime FSE frozen slicer and placed into a cryostat. OCT embedding medium was added to prepare 5 μm tissue sections, which were then stained with Oil Red O. Under an optical microscope (DMB5-2231P1, Xiamen Motic Company), the distribution of lipids in the hepatic tissue was observed. Orange-red particles or clumps represent fat deposits, while blue indicates nuclear staining.

2.2.3 Hepatic or serum ELISA experiment

Mouse liver tissue was excised, and residual blood was washed away with PBS buffer solution. Liver tissue homogenate was prepared by mixing the tissue with pre-cooled physiological saline at a ratio of 1 g to 9 mL (10% w/v) using a Dounce homogenizer in an ice bath. The homogenate was centrifuged at 8,000 × g and 4 °C for 10 minutes (min), and the supernatant was collected and kept on ice for analysis. A full wavelength scanning multifunctional microplate reader (Thermo Fisher Scientific, USA) was used to detect levels of lipid peroxidation markers (MDA, SOD, CAT) by the ELISA method.

Mouse serum samples were collected and centrifuged at 4,000 rpm and 4 °C for 10 min. The resulting serum supernatant was used to determine levels of TG, TC, oxysterols, glucose, insulin, and Fe^{2+} by ELISA. Optical density (OD) values were measured according to the manufacturer' s instructions using an ELISA reader. The insulin resistance (IR) index was calculated as serum insulin (mIU/L) multiplied by serum glucose (mmol/L) divided by 22.5 [19].

2.2.4 Determination of mRNA levels in liver

Total RNA from liver samples was extracted using the TRIzol method. The concentration and purity of the RNA were determined using a NanoDrop 2000 spectrophotometer, with concentrations ranging from 200 to 2000 ng/μL, and an OD_{260}/OD_{230} ratio between 1.8 and 2.2. Reverse transcription was performed by adding the appropriate reagents according to the protocol to convert mRNA into cDNA. Subsequently, SYBR Green Mix, primers, and cDNA template were combined in appropriate proportions for PCR amplification. β-actin was used as an internal reference. After real-time quantitative polymerase chain reaction (RT-qPCR) was performed using an Applied Biosystems instrument, the relative quantification of the target gene was analyzed using the $2^{-\Delta\Delta CT}$ method [20].

The sequences of the forward and reverse primer pairs for each gene are as follows: - ACSL4: 5' -ATTGGTCAGGGATATGGGCT-3' and 3' -AGAGGAGCTCCAACTCTTCCA-5'- AMPK: 5'-GTGACCAATGGTGTTCGTGC-3'and 3'-CACGCCCTTTCTCATCCACT-5'- ULK1: 5'-AGAGGGCTGTGTACCGAGAT-3' and 3' -TCCTAGAGAGAACAGGGGGC-5'

2.3 AML12 hepatocytes experiment

2.3.1 CCK-8 assay and experimental strategy

Frozen hepatocytes were recovered in a cell culture incubator (ESCO, Singapore). AML12 hepatocytes were seeded into cell culture flasks with Dulbecco' s Modified Eagle Medium (DMEM) and cultured at 37°C in 5% CO_2. To determine the optimal concentration of LNT intervention, LNT was added to the culture medium of 4.0 μmol/L SA-exposed AML12 hepatocytes at concentrations of 0.0, 5.0, 15.0, 25.0, or 35.0 μmol/L. Cell viability was assessed using the CCK-8 assay. Specifically, 10 μL of CCK-8 solution was added to each well and incubated at 37°C for 4 h. Absorbance was measured at 450 nm using a multifunctional microplate reader. Cell viability was calculated as: [(experimental well absorbance − blank well absorbance) / (control well absorbance − blank well absorbance)] × 100%. Each LNT intervention concentration was tested in triplicate.

After the optimal LNT dose was determined, the following four groups were established to explore the intervention effects of LNT or AMPK signaling: Control (hepatocytes cultured routinely in DMEM medium for 48 h); SA group (hepatocytes exposed to 4.0 μmol/L SA for 48 h); LNT + SA group (hepatocytes exposed to 4.0 μmol/L SA for 6 h, subsequently adding 25.0 μmol/L LNT for co-treatment for 42 h); and Met + SA group (hepatocytes exposed to 4.0 μmol/L SA for 6 h, then adding 2.0 mmol/L Met for co-treatment for 42 h, based on Park J et al.' s study [18]).

2.3.2 Lipid droplet fluorescence staining and lipid content

100.0 μL cell suspension was inoculated into each well of a 6-well plate. SA exposure or LNT intervention was performed when the cells reached 70% to 80% confluence. After the experiment, 1.0 mL of cell fixative was added to each well and incubated for 20–30 min. The fixative was then removed, and the wells were washed 2–3 times with deionized water. Next, 1.0 mL of 60% BODIPY staining solution (Nanjing Beyotime Biotechnology Co., Ltd, C2053M) was added to each well and incubated in the dark at room temperature for 20–30 seconds. A microplate reader was used to excite green fluorescence at approximately 493/503 nm, and the green fluorescence characteristics were evaluated using an inverted fluorescence microscope (Leica GmbH, Germany). For the assay of lipid content, the adherent cell suspension was collected and mixed with ELISA reaction reagents according to the manual instructions to determine the levels of TG and FFAs in AML12 hepatocytes.

2.3.3 Evaluation of insulin mediated signaling in hepatocytes

For the evaluation of insulin-mediated Glu and GLUT-4 signaling, AML12 hepatocytes were exposed to 4.0 μmol/L SA for 6 h, with or without addition of 100 nM insulin (I6634, Sigma-Aldrich) for the remaining 42 h of SA exposure. Following the method described in Section 2.3.1, intervention with 25.0 μmol/L LNT or 2 mM Met was performed in SA-exposed hepatocytes. At the end of experiments, cells were centrifuged at 800 × g for 5 min at 4 °C. The collected

supernatant was used to measure the levels of Glu and GLUT-4 in hepatocytes using the ELISA procedure described in Section 2.2.3.

2.3.4 Western-Blotting (WB) experiment

Hepatocytes were collected, and RIPA lysis buffer containing 1% protease inhibitor was added to lyse the cells. Total protein was then extracted from the hepatocytes, and protein concentration was quantitatively measured using a BCA assay kit. The cell lysate was placed in a water bath at 95 °C for 10 min. Immunoblotting was performed to determine the relative levels of the autophagosome marker LC3, GPX4, long-chain acyl-CoA synthetase 4 (ACSL4), p-AMPK, p-ULK1, FTMT, and oxysterol-binding protein-related protein 8 (ORP8).

The WB procedure was performed as follows: preparation of SDS-PAGE gel, electrophoresis, membrane transfer, and blocking. Primary antibodies were used as follows: anti-LC3 I/II (Invitrogen, PA1-16931), anti-GPX4 (Abcam, ab231174), anti-ACSL4 (Invitrogen, PA5-27137), anti-phospho-AMPK$ $1/2 (Abcam, ab133448), anti-AMPK (Abcam, ab80039), anti-FTMT (Invitrogen, MA568234), anti-phospho-ULK1 (Invitrogen, PA5-104556), and anti-ORP8 (Invitrogen, PA5-110065), which were added separately and incubated overnight at 4 °C. Subsequently, enzyme-labeled secondary antibodies were added and incubated at room temperature for 2 h. Finally, an enhanced chemiluminescence (ECL) kit was used for detection and imaging. WB bands were evaluated by an infrared laser scanning imaging system (USA LI-COR Corporation, Odyssey 9120), which were analyzed by relative quantification of band integral absorbance (IA), and β-actin was used as the internal reference (IA_0).

2.3.5 Co-immunoprecipitation (Co-IP) experiment

The experimental procedures for lysis, protein extraction, and protein concentration determination of hepatocytes were performed following the same protocols used for WB. Subsequently, a portion of the cell lysate was taken to prepare the input sample, while the remaining lysate was incubated with primary antibodies (anti-ORP8 and anti-ULK1 (Abcam, ab167139)) for Co-IP reactions, alongside a parallel negative control incubated with isotype IgG. After antibody incubation, magnetic beads were added to the supernatant for bead precipitation and washing. Loading buffer was then added to the samples from the experimental group, negative control, and input, respectively, followed by WB analysis. Relative quantification was performed by analyzing the levels of IA, with β-actin serving as the IA_0.

2.4 Statistical Analysis

SPSS version 28.0 and GraphPad Prism version 8.0 were used for data analysis and statistical plotting. Experimental data are presented as mean ($\bar{x}$) $\pm$ standard deviation (SD), and statistical differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. A p-value of 0.05 was used as the standard of statistical significance.

3. Results

3.1 Mouse body weight and liver coefficient

As shown in , at the end of the experiment, the overall weight of mice in each group exhibited an increasing trend. There were no significant differences in body weight among the groups except for the most significant weight gain in the LNT control group. Liver weight in the SA exposure group showed a significant increase compared with the control, while liver weight in the LNT+SA group and LNT control group were lower than that in the SA exposure group ($P < 0.05$). Similarly, the liver coefficient (liver weight/body weight) in the SA exposure group was significantly higher than that in the control group, while the liver coefficient in the LNT control and LNT + SA groups was lower than that in the SA exposure group ($P < 0.05$).

Table 1. Mice weight, liver weight, and liver coefficients in each group

Group	Weight (g)	Liver weight (g)	Liver coefficients (%)
Control	24.28$±0.50\|1.03±0.14\|4.22±0.49\|\|$SAexposure$\|24.27±0.82\|1.39±0.12*\|5.71±0.37 *\|\|$LNTcontrol$\|25.42±0.70*\|1.16±0.05 *#\|4.57±0.20#\|\|$LNT+SA$|24.22±0.71|1.16±0.09#|4.78±$0.28#		
F value (P)	4.22 (0.02)	11.87 (< 0.001)	19.40 (< 0.001)

Data were expressed as $\bar{x}$±SD, $n = 6$; SA exposure group, SA 5.0 mg/kg·bw; LNT intervention, 50 mg/kg·bw. *Compared with Control, $P < 0.05$; #Compared with SA exposure group, $P < 0.05$.

3.2 LNT alleviated arsenic-induced lipid accumulation in liver

As shown in [FIGURE:1], in the control group, the cellular structure of liver tissue was clearer, with fewer fat particles and no obvious red lipid droplets, indicating low fat content. In the SA exposure group, the liver cells exhibited enhanced coloration, and clustered red lipid droplets appeared around the cells, indicating lipid deposition in hepatic tissue. After LNT intervention (LNT + SA), the deposition of red lipid droplets and the degree of fat accumulation in liver tissue were reduced compared to the SA group, indicating that LNT effectively improved SA-induced lipid metabolism disorders in mice.

Figure 1 [FIGURE:1]. Characteristics of hepatic lipid distribution in each group (Oil Red O staining, $×$40). I, Control; II, SA exposure (5.0 mg/kg bw); III, LNT control (50 mg/kg bw); IV, LNT + SA.

Figure 4

Figure 1: Figure 4

3.3 Characteristics of oxidative stress, lipid content, ferritinophagy, and insulin resistance

As shown in [FIGURE:2] (a,b,c), compared with the control group, the SA exposure group showed increased levels of hepatic lipid peroxidation product MDA, ferritinophagy markers Fe^{2+} and MAP1LC3B, as well as elevated serum lipid content (TG, TC) and oxidized product of cholesterol (oxysterols), while hepatic levels of antioxidant enzymes SOD and CAT were decreased ($P < 0.05$). Compared with the SA exposure group, LNT intervention (LNT + SA) resulted in decreased levels of MDA, Fe^{2+}, TG, TC, and oxysterols, along with elevated levels of SOD, CAT, and MAP1LC3B ($P < 0.05$).

As shown in [FIGURE:2] (d), compared with the control group, the SA exposed group exhibited increased levels of Glu, INS, and IR index ($P < 0.05$). Compared with the SA exposure group, the intervention with LNT (LNT + SA) resulted in decreased levels of serum Glu and IR index ($P < 0.05$), while the difference in INS content was not significant ($P > 0.05$). These results suggest that LNT intervention counteracts SA-induced hepatic oxidative stress, lipid accumulation, ferritinophagy, and IR (detailed data shown in Table S1).

Figure 2 [FIGURE:2]. Characteristics of oxidative stress, lipid content, ferritinophagy, and insulin resistance (IR). (a) Characteristics of hepatic oxidative stress indices SOD, CAT, and MDA; (b) Characteristics of TG, TC, and oxysterols in serum; (c) Characteristics of hepatic MAP1LC3B and serum proportion of Fe^{2+}; (d) Characteristics of IR following SA exposure and LNT intervention. Data were expressed as $\bar{x}\pm$SD, $n = 6$; *Compared with Control, $P < 0.05$; #Compared with SA exposure group, $P < 0.05$.

3.4 Characteristics of ferroptosis and autophagy-related AMPK/ULK1 signaling

As shown in [FIGURE:3] (a),

Figures

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