Identification of Cuproptosis-Related Genes in Nonalcoholic Fatty Liver Disease

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Received 28 August 2022; Revised 30 January 2023; Accepted 10 February 2023; Published 21 February 2023

Academic Editor: Massimo Lucarini

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Nonalcoholic fatty liver disease (NAFLD) is the most prevalent hepatic pathology worldwide. However, the precise molecular mechanisms for NAFLD are still not sufficiently explained. Recently, a new mode of cell death (cuproptosis) is found. However, the relationship between NAFLD and cuproptosis remains unclear. We analyzed three public datasets (GSE89632, GSE130970, and GSE135251) to identify cuproptosis-related genes stably expressed in NAFLD. Then, we performed a series of bioinformatics analyses to explore the relationship between NAFLD and cuproptosis-related genes. Finally, 6 high-fat diet (HFD-) induced NAFLD C57BL/6J mouse models were established to carry out transcriptome analysis. The results of gene set variation analysis (GSVA) revealed that the cuproptosis pathway was abnormally activated to a certain degree. Among three datasets, two cuproptosis-related genes (DLD and PDHB) were correlated with stromal score and NAFLD activity score, and immune score (DLD, R = 0.26, p < 0.001; PDHB, R = 0.27, p < 0.001) and principal component analysis (PCA) of the cuproptosis-related genes showed that the NAFLD group separated from the control group, with the first two principal components accounting for 58.63%-74.88% of the variation. Among three datasets, two cuproptosis-related genes (DLD and PDHB, p < 0.01 or 0.001) were stably upregulated in NAFLD. Additionally, both DLD (AUC = 0.786 – 0.856) and PDHB (AUC = 0.771 – 0.836) had favorable diagnostic properties, and the multivariate logistics regression model further improved the diagnostic properties (AUC = 0.839 – 0.889). NADH, flavin adenine dinucleotide, and glycine targeted DLD, and pyruvic acid and NADH targeted PDHB in the DrugBank database. The DLD and PDHB were also associated with clinical pathology, especially with steatosis (DLD, p = 0.0013 – 0.025; PDHB, p = 0.002 – 0.0026) and NAFLD activity score (DLD, p = 0.004 – 0.02; PDHB, p = 0.003 – 0.031). What is more, DLD and PDHB were correlated with stromal score (DLD, R = 0.38, p < 0.001; PDHB, R = 0.31, p < 0.001) and immune score (DLD, R = 0.26, p < 0.001; PDHB, R = 0.27, p < 0.001) in NAFLD. Furthermore, Dld and Pdhb were also significantly upregulated in the NAFLD mouse model. In conclusion, cuproptosis pathways, especially DLD and PDHB, could be potential candidate genes for NAFLD diagnostic and therapeutic options.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), as a metabolic disease, is the most prevalent hepatic pathology worldwide with a prevalence of approximately 25% [1]. It ranges from simple steatosis (SS) to advanced stage of nonalcoholic steatohepatitis (NASH), the latter rapid progression toward liver cirrhosis, and hepatocellular carcinoma (HCC) [2, 3]. Currently, sensitive biomarkers for the diagnosis of NAFLD are still lacking, and liver biopsies, as an invasive method, are still considered the gold standard for diagnosis and prognosis [4]. Although numerous studies have attempted to study the pathogenesis and progression of NAFLD, there are still no effective drugs for NAFLD other than lifestyle changes [5]. Moreover, the development of NAFLD is a complex process and is still not sufficiently explained [6].
Consequently, it is crucial to explore the mechanisms involved in the pathogenesis of NAFLD to identify new potential targets for diagnosis and therapy.

Recently, Tsvetkov et al. [7] have shown a new mode of cell death, copper-dependent cell death, which is called “cuproptosis.” It can be simply summarized that copper directly binds to lipoylated components of the tricarboxylic acid (TCA) cycle and subsequent Fe-S cluster protein loss causes proteotoxic stress that triggers cell death [8]. The expression changes of ten genes (known as cuproptosis-related genes) involve in cuproptosis, among which seven genes (ferredoxin 1 (FDX1), lipoyl synthase (LIAS), lipoyl-transfers 1 (LIPT1), dihydrolipoamide dehydrogenase (DLD), dihydrolipoamide S-acetyltransferase (DLAT), pyruvate dehydrogenase E1 subunit alpha 1 (PDHA1), and pyruvate dehydrogenase E1 subunit beta (PDHB)) were upregulated, and three genes (metal-regulatory transcription factor 1 (MTFI), glutaminase (GLS), and cyclin-dependent kinase inhibitor 2A (CDKN2A)) were downregulated [7].

There has been recognition that copper metabolism disorder can lead to a variety of chronic liver diseases, such as Wilson disease (WD), NAFLD, and liver cirrhosis [9]. Zhang et al. indicated that serum copper levels were positively correlated with body mass index (BMI), leptin, and insulin resistance, which are all risk factors for NAFLD [2]. Dev et al. found a dramatic increase in hepatic copper levels, resulting in obesity and hepatic steatosis in the hepatocyte-specific knockout of Atp7b WD mouse model [10].

In this research, we seek to comprehensively investigate the molecular alterations and clinical relevance of the cuproptosis-related genes in NAFLD. This study highlights the importance of cuproptosis-related genes in NAFLD and lays a foundation for future studies of cuproptosis in NAFLD.

2. Materials and Methods

2.1. Data Retrieving and Processing from GEO. Data from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) fulfilled the inclusion criteria below: (1) publication date from 2010 to 2022; (2) containing NAFLD and normal tissue samples; (3) providing detailed clinicopathological information. The exclusion criteria were as follows: (1) duplicated research; (2) patients who underwent bariatric surgery or with severe diseases; (3) data that could not be analyzed; (4) animal or cell experiments. Subsequently, the gender expression profiles of GSE89632 [11], GSE130970 [12], and GSE135251 [13] were downloaded from GEO. Eventually, 24 control patients, 19 SS patients, and 19 NASH patients in GSE89632, 6 control patients and 72 NAFLD patients in GSE130970, 10 control patients, and 216 NAFLD patients were included in this study (Table 1). Since the datasets were from a public database, patient consent and ethics committee approval were not required. Then, the gene array data (GSE89632) was converted into the quantile normalized values, and the read count data (GSE130970 and GSE135251) was standardized by the transcripts per million (TPM). The overall research design is shown in Figure 1.

2.2. Gene Set Variation Analysis (GSVA) and Single-Sample Gene Set Enrichment Analysis (ssGSEA). GSVA, a pathway enrichment method that estimated variation of pathway activity, was performed to evaluate the role of the cuproptosis pathway in NAFLD using R package “GSVA” [14]. In addition, the stromal score, immune score, and immune cells’ marker enrichment were ssGSEA and they were calculated by “estimate” (http://bioinformatics.mdanderson.org/estimate/), and “GSVA” R packages. The immune gene sets were downloaded from Charoentong et al. [15]. Then, the spearman correlation analysis between DLD/PDHB expression and immune cells was performed. The results were visualized using the “ggplot2” R package.

2.3. Correlation Analysis of Cuproptosis-Related Genes, Protein-Protein Interaction (PPI) Network Construction, and the Prediction of Potential Drugs. The “ggcorrplot” R package was used to recognize the correlation between cuproptosis-related genes by the Spearman correlation analysis. The STRING database (https://string-db.org/) was utilized to construct a PPI network with an interaction score > 0.4. The prediction of potential drugs was performed in the DrugBank database (https://go.drugbank.com).

2.4. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analyses. To explore the potential pathways of DLD and PDHB, the GO and KEGG analyses were performed using “clusterProfiler” [16] and “org.H.s.e.bg.db” R package. The median of the risk model scores of DLD and PDHB was selected as the cut-off value.

2.5. Animal Model and Experiment Design. A total of 6 male C57BL/6j mice (weight, 23.47 ± 1.18 g; age, 6 weeks) were purchased from the medical laboratory animal center of Guangdong (Guangzhou, China). All the mice were acclimatized under a temperature of 24 ± 2 °C, a relative humidity of 55 ± 10%, and a 12 h light/dark cycle for 10 days before the commencement of the animal experiment. All animal experiments were approved by the experimental animal ethics committee of Jinan University and were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jinan University (IACUC-20190916-09).

After acclimatization, the 6 mice were randomly divided into the normal control group (NC, n = 3) and the NAFLD group (n = 3). The NC group mice were fed with standard mouse diet, and the NAFLD group mice were induced by a high-fat diet (HFD) (containing 34% fat, 2% cholesterol, 26% carbohydrate, 26% protein, and 12% basic feed (w/w)) for 8 weeks before sacrificing.

2.6. Histological Analysis. At the end of the study, the mice were sacrificed to measure liver mass and liver mass index by using the following formula: live mass index = liver mass/body mass × 100%. Afterward, the liver samples were immersed in 10% formalin neutral buffer solution for 48 h, then processed routinely, embedded in paraffin, sectioned to 5 μm thickness and stained with hematoxylin and eosin (H&E). Lipid accumulation in liver was analyzed by Oil red O (ORO) staining (Sigma). Slides were observed with a light microscope (Leica DMi 8).
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2.7. Transcriptome Analysis of Mice Liver Tissues. The quality and integrity of total RNA were supervised on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The RNA sequencing library was generated from 250 ng total RNA using Ribo-o ff rRNA Depletion Kit (Vazyme BioTech, Nanjing, China) for rRNA depletion followed by VAHTS ™ Universal V6 RNA-seq Library Prep Kit for Illumina (Vazyme BioTech, Nanjing, China) according to the manufacturer’s protocols. The libraries were sequenced on Illumina NovaSeq 6000 (Illumina, California, USA). Sequences were aligned to the mm10 mouse reference genome using STAR Aligner. Then, reads aligning to genes were counted using htseq-count, and analysis of differentially expressed genes (DEGs) was performed using the “limma” R package [17] and later visualized by volcano plot using “ggplot2” R package (https://ggplot2.tidyverse.org/). The threshold for the DEGs was set as $p$ value $< 0.05$ and $|\log_2 \text{fold change (FC)}| \geq 0.5$. The raw data was stored in the ArrayExpress database (accession: E-MTAB-11980, https://www.ebi.ac.uk/arrayexpress/).

2.8. Statistical Analysis. Statistical analysis was performed using R software (Version 4.1.3, http://www.r-project.org). Statistical comparisons between two groups of continuous data were performed using the $t$-test or Mann–Whitney $U$ -test according to the test condition and the Kruskal-Wallis test for multiple comparisons among the groups. Principal
Figure 2: Continued.
component analysis (PCA) was applied to display the overall differences of cuproptosis-related genes and visualized by the "ggplot2" R package. Venn diagram was performed using the "ggvenn" R package. The multivariate logistics regression risk model was constructed in DLD and PDHB. A receiver-operating characteristic (ROC) curve was conducted to assess the diagnostic value of the genes and the model by "pROC" R package and visualized by the "ggplot2" R package. A difference with $p < 0.05$ was considered significant.

3. Results

3.1. Cuproptosis Pathway Plays a Role in NALFD. To determine whether the cuproptosis pathway played a role in NALFD, GSVA and PCA were performed. The results showed that the enrichment score of the cuproptosis pathways in GSE89632 and GSE130970 was significantly increased ($p < 0.05$) in the NALFD group compared with the control group, but not in GSE135251 ($p = 0.22$, Figures 2(a)–2(c)). Next, the principal component analysis (PCA) of the cuproptosis-related genes showed that the NALFD group separated from the control group, with the first two principal components accounting for 58.63%–74.88% of the variation (Figures 2(d)–2(f)). These results suggest that the cuproptosis pathway is activated to some degree in NALFD.

3.2. Differential Expression of Cuproptosis-Related Genes. Subsequently, the expression levels of the cuproptosis-related genes were explored in the three datasets, respectively. The cuproptosis-related genes were changed to varying degrees in the three datasets, in which DLD ($p < 0.001$ in GSE89632, $p = 0.004$ in GSE130970, $p < 0.001$ in GSE135251) and PDHB ($p = 0.001$ in GSE89632, $p = 0.007$ in GSE130970, $p = 0.001$ in GSE135251) were stably and significantly upregulated in the three datasets (Figures 3(a)–3(d)). The outcomes illustrate that DLD and PDHB play a part in the pathogenesis and progression of NALFD.

3.3. Correlation Analysis of Cuproptosis-Related Genes and PPI Network Construction. The Spearman correlation analysis showed that there were potential interactions between the cuproptosis-related genes (Figures 3(e) and 3(f)). Moreover, DLD and PDHB both had strong correlations in the three datasets ($R = 0.51$, $p < 0.0001$ in GSE89632; $R = 0.53$, $p < 0.0001$ in GSE130970; $R = 0.77$, $p < 0.0001$ in GSE135251). Afterward, the PPI analysis further suggested the potential interactions among them. DLD and PDHB had more PPI edges than other genes, suggesting that DLD and PDHB were hub genes (Figure 3(h)).

3.4. DLD and PDHB Are Associated with Clinical Characteristics of NALFD and the Prediction of Potential Drugs. Next, we further explored the relationship between DLD/PDHB and clinical characteristics, respectively. First, the diagnostic values of DLD and PDHB were evaluated through ROC analysis. By observing the area under the curve (AUC), both DLD and PDHB had favorable diagnostic properties (AUC = 0.786/0.771 in GSE89632, AUC = 0.856/
Figure 3: Continued.
Log2 normalized values

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Figure 3: Continued.
Figure 3: Continued.
0.836 in GSE130970, and AUC = 0.837/0.802 in GSE135251 (Figures 4(a)–4(c)). Then, GSE130970 was used for the training set and GSE135251 for the testing set. The multivariate logistics regression model further improved the diagnostic properties (AUC = 0.889 in GSE130970 and AUC = 0.839 in GSE135251), and the risk model was summarized using the following equation: \( \text{Response} = -1.63 + 0.24\log_2(\text{DLD}[\text{TPM}]+1) + 0.20\log_2(\text{PDHB}[\text{TPM}]+1) \) (Figures 4(b) and 4(c)). These results indicate that DLD and PDHB may be potential biomarkers in the diagnosis of NAFLD.

After that, the association between DLD/PDHB and clinical pathology was analyzed separately. In GSE89632, DLD was significantly positively associated with steatosis \( (p = 0.0013) \), ballooning \( (p = 0.028) \), lobular inflammation \( (p = 0.048) \), and NAS \( (p = 0.02) \) (Figure 4(d)); PDHB was significantly positively relative to steatosis \( (p = 0.002) \), ballooning \( (p = 0.027) \), NAS \( (p = 0.031) \), and fibrosis \( (p = 0.034) \) (Figure 4(e)). In GSE130970, DLD was significantly positively associated with steatosis \( (p = 0.025) \), lobular inflammation \( (p = 0.007) \), and fibrosis \( (p = 0.0064) \) (Figure 4(f)); PDHB was only significantly positively relative to steatosis \( (p = 0.0026) \) (Figure 4(g)). In GSE135251, both DLD \( (p = 0.004) \) and PDHB \( (p = 0.003) \) were positively associated with NAS, but not fibrosis (Figure 4(h)). We then predicted the potential drugs for DLD and PDHB in the DrugBank database and showed that nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), glycine targeted DLD, and pyruvic acid and NADH targeted PDHB (Figure 4(i)). All in all, DLD and PDHB are associated with the clinical pathology of NAFLD.

3.5. DLD and PDHB Are Associated with Metabolic Pathways and Copper Toxicity. Subsequently, GO and KEGG analyses were implemented for pathway analysis by comparing the low- and high-risk scores of DLD and PDHB in GSE130970. The GO results showed that the high-risk score of DLD and PDHB was associated with the acetyl-CoA metabolic process and fatty acid metabolic process, and detoxification of copper ion pathway was enriched in the low-risk score group (Figure 5(a)). Besides, the KEGG results illustrated that the high-risk score of DLD and PDHB was associated with fatty acid metabolism, glycolysis/gluconeogenesis, and pyruvate metabolism (Figure 5(b)). These results indicate that high-expressed DLD and PDHB appear to increase copper toxicity and are related to the acetyl-CoA metabolic process and pyruvate metabolism, both important substrate sources for the TCA cycle.

3.6. DLD and PDHB Are Positively Correlated with Stromal Score and Immune Score. Considering that GSE135251 had the largest sample size, we chose it to explore the relationship between the DLD/PDHB and the immune microenvironment. DLD was positively correlated with stromal score \( (R = 0.38, p = 7.18 \times 10^{-9}) \) and immune score \( (R = 0.26, p = 9.15 \times 10^{-5}) \) (Figure 6)). Meanwhile, DLD was
Figure 4: Continued.
14.0 Kruskal–Wallis, \( p = 0.0013 \)
13.5
13.0
DLD expression
12.5
12.0
0 12 3 <3 3–4 >4 0 1–2 3–4
Steatosis Lobular inflammation NAS Fibrosis
Ballooning
(d)

14.0
13.5
13.0
DLD expression
12.5
12.0
0 12 3 0 1 2 <3 3–4 >4 0 1–2 3–4
Steatosis Lobular inflammation NAS Fibrosis
Ballooning
(e)

14.0
13.5
13.0
DLD expression
12.5
12.0
0 12 3 0 1 2 <3 3–4 >4 0 1–2 3–4
Steatosis Lobular inflammation NAS Fibrosis
Ballooning
(f)

14.0
13.5
13.0
DLD expression
12.5
12.0
0 12 3 0 1 2 <3 3–4 >4 0 1–2 3–4
Steatosis Lobular inflammation NAS Fibrosis
Ballooning
(g)

14.0
13.5
13.0
DLD expression
12.5
12.0
0 12 3 0 1 2 <3 3–4 >4 0 1–2 3–4
Steatosis Lobular inflammation NAS Fibrosis
Ballooning
(h)

Figure 4: Continued.
positively associated with multiple immune cells, especially with gamma delta T cell, CD4+ T cell, and immature dendritic cell \((R > 0.3, p < 0.05, \text{Figure } 6(c))\). On the other hand, \(PDHB\) was also positively correlated with stromal score \((R = 0.31, p = 4.89e^{-06}, \text{Figure } 6(d))\) and immune score \((R = 0.27, p = 7.34e^{-05}, \text{Figure } 6(e))\). \(PDHB\) was also positively associated with multiple immune cells, especially with memory CD4 T cell, gamma delta T cell, and immature dendritic cell \((R > 0.3, p < 0.05, \text{Figure } 6(f))\). These results indicate that \(DLD\) and \(PDHB\) have an impact on the immune microenvironment.

3.7. Further Validation of \(DLD\) and \(PDHB\) in the NAFLD Mouse Model. Mice fed HFD develop hepatic steatosis, mimicking the NAFLD of humans \([18]\). We conducted an animal experiment, and after HFD was fed to NAFLD mouse group for 8 weeks, a significant increase in the body mass, liver mass, and liver mass index was observed in the NAFLD
Figure 6: Continued.
group compared with the NC group (Table 2). Besides, the results of liver pathology showed that liver sections from the NALFD group had hepatocyte swelling, ballooning degeneration, and different sizes of lipid droplets (Figures 7(a) and 7(b)). Oil Red O staining revealed the accumulation of neutral lipids in NAFLD (Figures 7(c) and 7(d)). These results demonstrate that the NAFLD mouse model was successfully established.

Subsequently, transcriptome analysis of mice liver tissues indicated that $Dld$ and $Pdhb$ were also significantly upregulated in the NAFLD group compared with the NC group (Table 2). Besides, the results of liver pathology showed that liver sections from the NALFD group had hepatocyte swelling, ballooning degeneration, and different sizes of lipid droplets (Figures 7(a) and 7(b)). Oil Red O staining revealed the accumulation of neutral lipids in NAFLD (Figures 7(c) and 7(d)). These results demonstrate that the NAFLD mouse model was successfully established.

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4. Discussion

In this study, we first explored the relationship between 10 cuproptosis-related genes and NAFLD. Then, $DLD$ and $PDHB$ were stably upregulated in NAFLD among the three datasets. Besides, the $DLD$ and $PDHB$ were also associated with the clinical characteristics (especially steatosis and NAS) and the immune microenvironment in NAFLD. NADH, FAD, and glycine targeted $DLD$, and pyruvic acid and NADH targeted $PDHB$. In addition, the high-expressed $DLD$ and $PDHB$ appeared to increase copper toxicity and had impacts on the TCA cycle by affecting the acetyl-CoA and pyruvate, further suggesting that the cuproptosis might affect the development of NAFLD. Furthermore, the NAFLD mouse model was established to determine the expression signature of $Dld$ and $Pdhb$. These outcomes suggest that $DLD$ and $PDHB$ promoted hepatic steatosis and trigger liver inflammation through the cuproptosis.

Copper, a cofactor for various enzymes, is an essential micronutrient required for normal cell function, and the cuproptosis is related to various cancer progressions, such as HCC [19, 20]. Recent studies have determined that serum and liver copper are related to NAFLD, but the specific mechanism is still unclear [21]. Mitochondrial respiration is required for cuproptosis, but upregulation of mitochondrial activity often occurs in an early NAFLD stage, and the expression levels of cuproptosis-related genes ($DLD$ and $PDHB$) are increased in our study, which suggests that cuproptosis may contribute to the progression of NAFL to NASH [7, 22]. However, the mitochondrial function gradually decreases during the progression of NAFLD, suggesting that the effect of cuproptosis is progressively attenuated and...
Figure 7: The hematoxylin and eosin (H&E) staining of NC mouse ((a) ×400) and NAFLD mouse ((b) ×400); the Oil red O (ORO) staining of NC mouse ((c) ×400) and NAFLD mouse ((d) ×400); (e) the volcano plot shown the differential expressed genes in the NAFLD mouse group compare with the NC group.
may lead to HCC eventually [22]. In addition, it is reported that suppression of DLD expression inhibits melanoma growth and tumor proliferation by increasing intracellular reactive oxygen species (ROS) production and thereby inducing autophagy cell death [23]. PDHB catalyzes the conversion of pyruvate to acetyl-CoA, acting as a central node that links glucose metabolism, lipid metabolism, and the TCA [24]. The dysfunction of PDHB can lead to metabolism alteration which is one of the hallmarks of cancer cells [25, 26]. Recent studies show that glycine and hepatic NAD+ decrease in NAFLD individuals, and their supplementation can ameliorate NAFLD, but their mechanisms have not been completely elucidated, and our study may provide new insight for them [27–29]. Besides, the altered pyruvate metabolism, particularly enhanced lactate production, plays an essential role in the NAFLD progression, but further studies are needed to determine whether pyruvic acid supplementation improves NAFLD [30].

FAD, a redox-active coenzyme, is involved in various metabolic pathways, including the beta-oxidation of fatty acid and TCA, and its role in NAFLD is still unknown [31]. Moreover, DLD and PDHB are positively correlated with gamma delta T cell and CD4+ T cell which often accumulate in the liver and subsequent stimulate inflammatory processes in NAFLD [32, 33]. Hence, the cuproptosis, especially DLD and PDHB, may also play a vital role in NAFLD.

The present study had several advantages. Firstly, this was the first study to explore the relationship between NAFLD and cuproptosis-related genes. Secondly, we comprehensively analyzed multiple datasets and screened out stably upregulated cuproptosis-related genes (DLD and PDHB), which were later verified in the NAFLD mouse model, whereas our study also had some limitations. First of all, we did not carry out an in-depth study of cuproptosis on NAFLD. For another, the NAFLD mouse model rather than human tissue was used in this study, which might have influenced the results. But HPD-induced NAFLD mouse model can mimic the NAFLD of humans most accurately [18].

In conclusion, our study presented a systematic analysis of molecular alterations and interactive genes of cuproptosis in NAFLD. Finally, we screened out two cuproptosis-related genes (DLD and PDHB) that were correlated with NAFLD prognosis. Although further research is still needed, we provide useful and novel information to explore the potential candidate genes for NAFLD diagnostic and therapeutic options.

Data Availability

The data can be found in ArrayExpress and GEO databases, accession numbers: E-MTAB-11980, GSE89632, GSE130970, and GSE135251. The coding can be found on GitHub (https://github.com/biomedt/oxidative/tree/main).

Ethical Approval

All animal experiments were approved by the experimental animal ethics committee of Jinan University and was performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jinan University (IACUC-20190916-09).

Conflicts of Interest

The authors declare no conflict of interest.

Authors’ Contributions

Chutian Wu, Xiongxiu Liu, Lixian Zhong, Yun Zhou, Linjing Long, and Tingzhuang Yi contributed equally to this work. Chutian Wu, Xiongxiu Liu, Lixian Zhong, Yun Zhou, Linjing Long, and Tingzhuang Yi conducted the animal experiments and analyzed the study data, helped draft the manuscript, and made critical revisions of the manuscript. Sisi Chen, Yuting Li, Yanfang Chen, Lianli Shen, and Qutong Zeng assisted with data collection and the analysis. Shao-hui Tang supervised the research and edited the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors appreciate the study investigators and staff who participated in this study.

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