Cover Letter

Dear editor,

Sincerest thanks for your response and reviewers' comments on our manuscript entitled "Role of ferroptosis in the process of diabetes-induced endothelial dysfunction"(ID: 57891), which will help us improve it to a better scientific level. We sincerely apologize for the great time it has taken us to respond to these comments, and hope that a revised version of the manuscript will be considered by World Journal of Diabetes. We have modified the paper in response to the insightful reviewers' comments. The main corrections in the paper and the responds to the reviewers' comments are as flowing:

Responds to the reviewers' comments:

Reviewer #1:

1. Response to comment: (This submission demonstrated the role of ferroptosis in diabetes-induced endothelial dysfunction. It has novelty and the following concerns need to conduct. Mediation of ferroptosis in endothelial dysfunction in peripheral diseases needs to introduce.)

Response: Sincerest thanks to you for your good comments. It is really true as Reviewer suggested that mediation of ferroptosis in endothelial dysfunction in peripheral diseases should be introduced. And we have added it to the introduction section according to your comments.

Bai et al. indicated that ferroptosis might occur during the initiation and development of atherosclerosis (AS). And inhibition of ferroptosis could protect against the aggravation of AS in thoracic aorta through attenuating lipid peroxidation and endothelial dysfunction[1].

[1] Bai T, Li M, Liu Y, Qiao Z, Wang Z. Inhibition of ferroptosis alleviates atherosclerosis through attenuating lipid peroxidation and endothelial dysfunction in mouse aortic endothelial cell. Free Radic Biol Med. 2020 Nov 20;160:92-102.

(lines 22 to 25, page 5)

2. Response to comment: (In the introduction, "an in-depth analysis of this topic" seems not suitable in scientific publication.)

Response: Sincerest thanks to you for your good comments. We have rewrite this sentence according to your suggestion.

Therefore, this study aimed to investigate the role and regulatory mechanism of ferroptosis in diabetes-induced endothelial dysfunction.

(lines 3 to 4, page 6)

3. Response to comment: (Promocell needs the source in detail because it supplied primary HUVECs.)

Response: Sincerest thanks to you for your good comments. We have added the details of Promocell according to your suggestion.

Primary HUVECs were purchased from Promocell (C-12200, Heidelberg, Germany).

(lines 23 to 24, page 6)

4. Response to comment: (Identification of ferroptosis was not showed in the methods.)

Response: Sincerest thanks to you for your good comments. Ferroptosis is a form of regulated cell death and is biochemically characterized by the accumulation of lipid peroxides and reactive oxygen species (ROS) [1]. Ferroptosis-inducing factors can directly or indirectly affect glutathione peroxidase through different pathways, resulting in a decrease in antioxidant capacity and accumulation of lipid ROS in cells, ultimately leading to oxidative cell death[2]. GPX4, a glutathione peroxidase, utilizes reduced glutathione to convert lipid hydroperoxides to lipid alcohols, thereby mitigating lipid peroxidation and inhibiting ferroptosis[3]. The levels of GPX4 in ferroptosis cells are down-regulated, while the levels of COX2 and FTH1 are up-regulated. The other ferroptosis-related assays and tools mainly include BODIPY 581/591 C11 (sensor for lipid ROS levels) and GSH and GSSG Assay Kit (quantifies glutathione levels). To identify ferroptosis, cell viability was assessed by CCK-8, the protein levels of GPX4, FTH1, and COX-2 were determined using Western blot assays, the generation of ROS was determined by the BODIPY™ 581/591 C11 and the intracellular glutathione/oxidized glutathione (GSH/GSSG) ratio was measured with the GSH and GSSG Assay Kit in the current study.

[1] Stockwell BR, Friedmann Angeli JP, Bayir H, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. Cell. 2017 Oct 5;171(2):273-285.

[2] Li J, Cao F, Yin HL, et al. Ferroptosis: past, present and future. Cell Death Dis. 2020 Feb 3;11(2):88.

[3] Seibt TM, Proneth B, Conrad M. Role of GPX4 in ferroptosis and its pharmacological implication. Free Radic Biol Med. 2019 Mar;133:144-152.

5. Response to comment: (HG (30 mM) used in current study needs the reference(s) to support, particularly the osmotic factor must rule out.)

Response: Sincerest thanks to you for your good comments. HG (30 mM) used in the current study mainly refers to the literature of Li et al., Long et al., and Zhu et al.[1,2,3]. In the study of Li et al., the rat aortic endothelial cells (RAECs) were incubated with HG (30 mM) to mimic the diabetes environment [1]. And in the study of Long et al., the human retinal endothelial cells (HRECs) were cultured in HG (30 mM) to mimic the diabetes environment [2]. Similarly, in the study of Zhu et al., the human umbilical vein endothelial cells (HUVECs) were treated with HG (30 mM) to mimic the diabetes environment [3].

And it is really true as Reviewer suggested that the osmotic factor must rule out. To maintain constant isotonicity or osmolality, normal glucose (NG, 5 mM) media was supplemented with D-mannitol (25 mM, final concentration) in the current study. We are very sorry for our forget writing it and we have added it to the manuscript according to your comments.

[1] Li XX, Ling SK, Hu MY, Ma Y, Li Y, Huang PL. Protective effects of acarbose against vascular endothelial dysfunction through inhibiting Nox4/NLRP3 inflammasome pathway in diabetic rats. Free Radic Biol Med. 2019 Dec;145:175-186.

[2] Long L, Li Y, Yu S, Li X, Hu Y, Long T, Wang L, Li W, Ye X, Ke Z, Xiao H. Scutellarin Prevents Angiogenesis in Diabetic Retinopathy by Downregulating VEGF/ERK/FAK/Src Pathway Signaling. J Diabetes Res. 2019 Dec 28;2019:4875421.

[3] Zhu M, Chen J, Tan Z, Wang J. Propofol protects against high glucose-induced endothelial dysfunction in human umbilical vein endothelial cells. Anesth Analg. 2012 Feb;114(2):303-9.

(Figure legend 1)

6. Response to comment: (Images for ferroptosis, either in Figure 1 or Figure 2, failed to show in clear.)

Response: Sincerest thanks to you for your good comments. We have changes Figure 1 and Figure 2 according to your suggestion. The previous Figures were failed to show in clear during the conversion in PDF format. The adjusted picture is shown in Figure 1 and Figure 2.

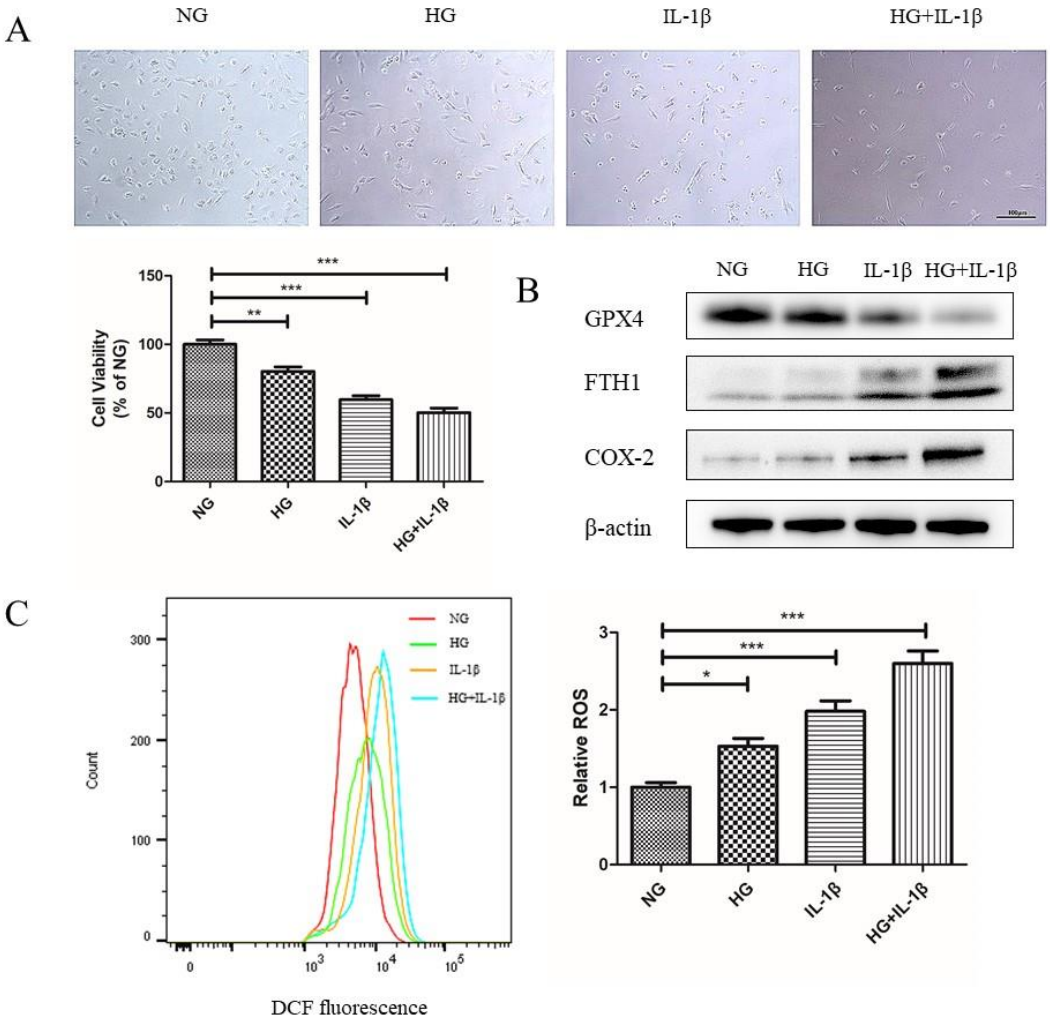

Figure 1 Effects of high glucose (HG) and interleukin-1β (IL-1β) on ferroptosis in human umbilical vein endothelial cells (HUVECs).

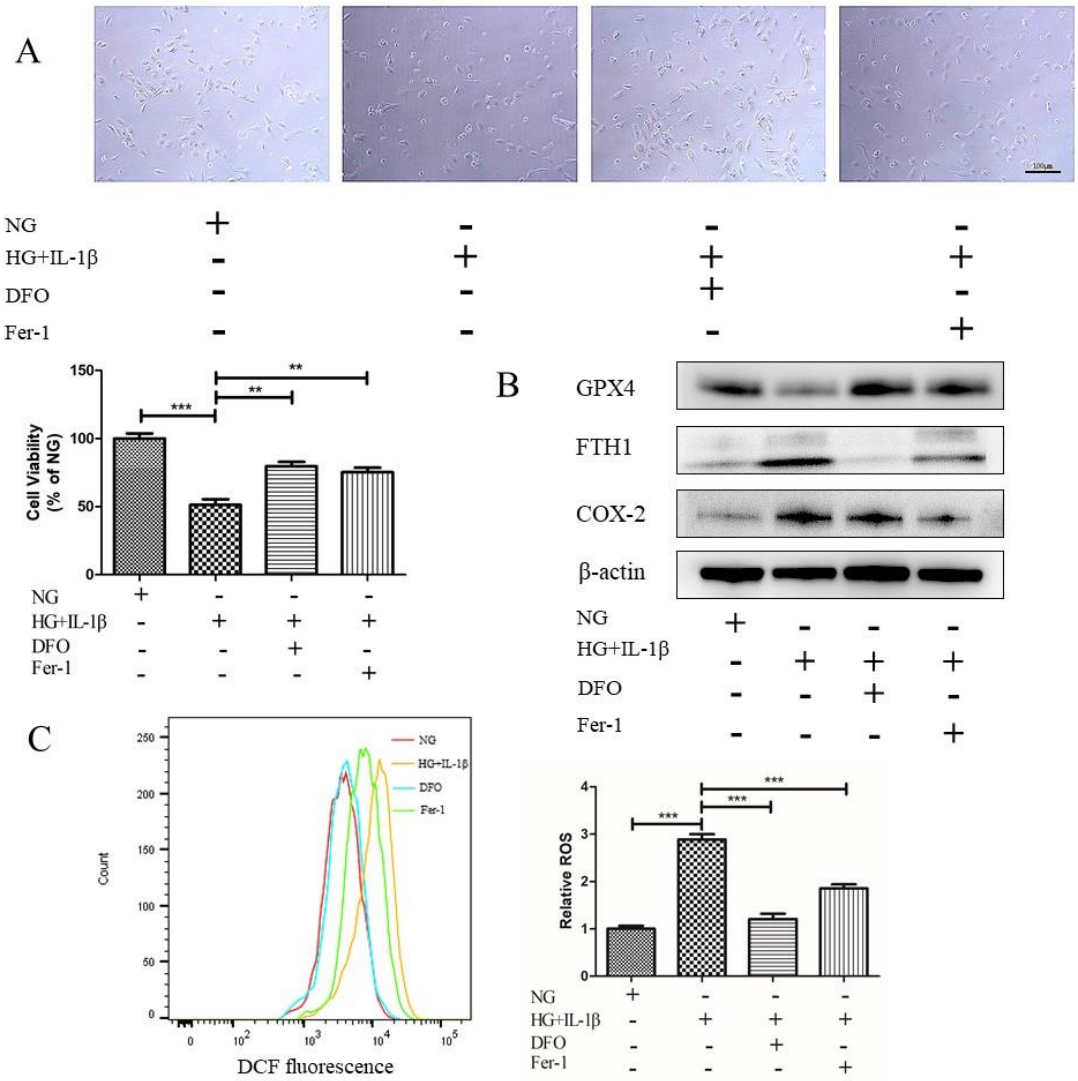

Figure 2 Ferroptosis inhibitor attenuates the ferroptosis of HUVECs induced by HG and IL-1β.

7. Response to comment: (In Figure 5, legends remain unknown. Please revise it in detail.)

Response: Sincerest thanks to you for your good comments. We have revised it in detail according to your suggestion.

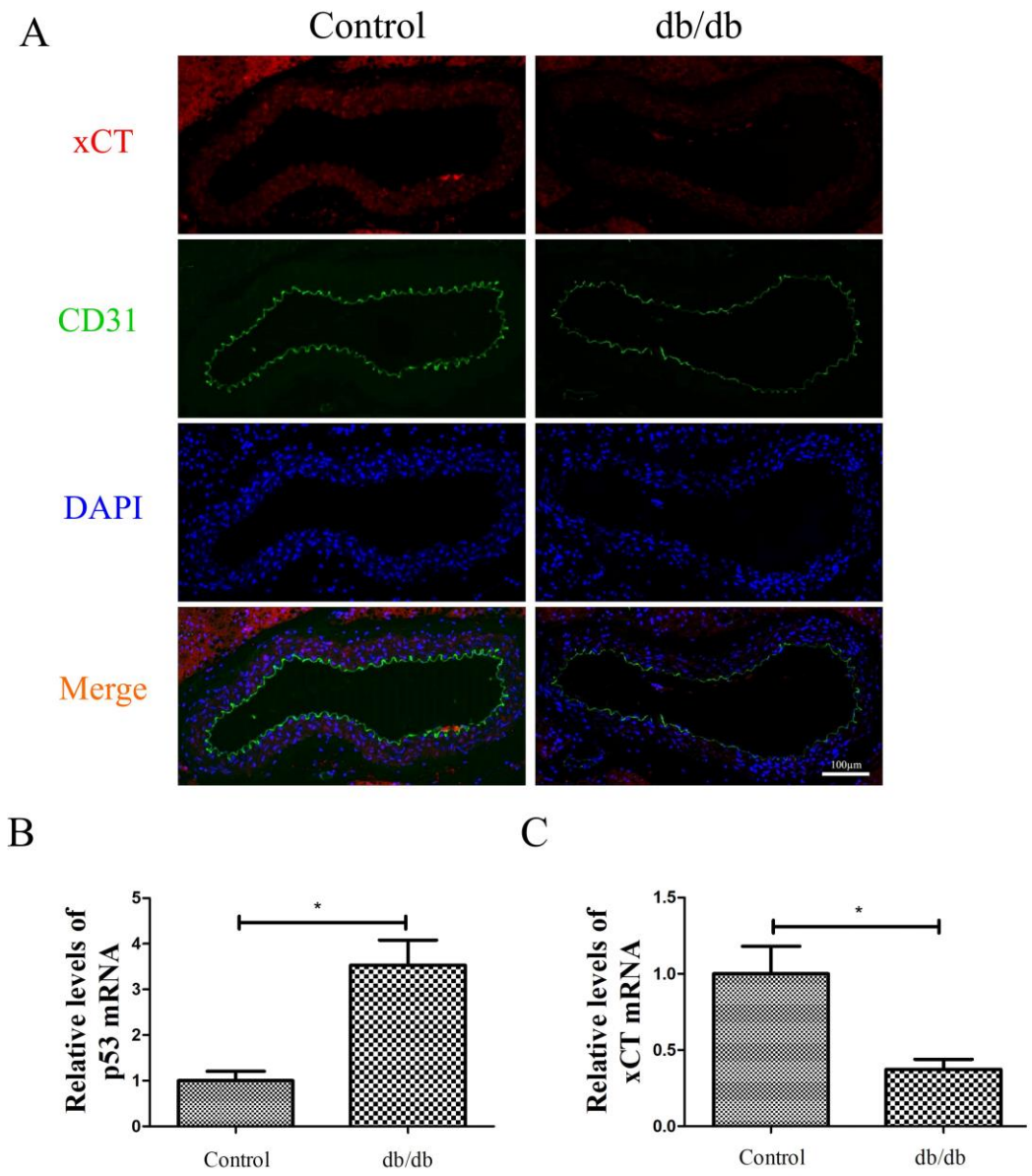

Figure 5 Detection of xCT and p53 expression in the aorta of db/db mice.

8. Response to comment: (In Figure 6, hyperglycemia seems better than DM and proposal model for working model in the wording. Additionally, action site of cytokine did not indicate. Why?)

Response: Sincerest thanks to you for your good comments. Although the molecular mechanisms of endothelial dysfunction are not completely understood, hyperglycemia and inflammation are considered the key causes of diabetes-induced endothelial dysfunction [1-3]. It is really true as Reviewer suggested that hyperglycemia plays a critical role in diabetes-induced endothelial dysfunction. For instance, hyperglycemia in diabetes decreases vasodilation through the decreased bioavailability of nitric oxide (NO) and prostacyclin (PGI2) [4]. Additionally, increased inflammation is a major contributor to diabetes-induced endothelial dysfunction

[5]. Therefore, in the current study, HUVECs were treated with HG (30 mM) and IL-1β (10 ng/mL) to mimic the diabetes environment.

The results of this study indicated that HG and IL-1β can activate the p53-xCT-GSH axis and cause increased endothelial cell ferroptosis and endothelial dysfunction. Given the above, we added hyperglycemia and inflammation in the working model according to your comments.

[1] Xiang L, Mittwede PN, Clemmer JS: Glucose Homeostasis and Cardiovascular Alterations in Diabetes. COMPR PHYSIOL 2015, 5(4):1815-1839.

[2] Vanhoutte PM, Shimokawa H, Feletou M, Tang EH: Endothelial dysfunction and vascular disease - a 30th anniversary update. Acta Physiol (Oxf) 2017, 219(1):22-96.

[3] Shenouda SM, Widlansky ME, Chen K, Xu G, Holbrook M, Tabit CE, Hamburg NM, Frame AA, Caiano TL, Kluge MA et al: Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. CIRCULATION 2011, 124(4):444-453.

[4] Muzaffar S, Jeremy JY, Angelini GD, Shukla N: NADPH oxidase 4 mediates upregulation of type 4 phosphodiesterases in human endothelial cells. J CELL PHYSIOL 2012, 227(5):1941-1950.

[5] Sharma A, Rizky L, Stefanovic N, Tate M, Ritchie RH, Ward KW, de Haan JB. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator dh404 protects against diabetes-induced endothelial dysfunction. Cardiovasc Diabetol. 2017 Mar 3;16(1):33.

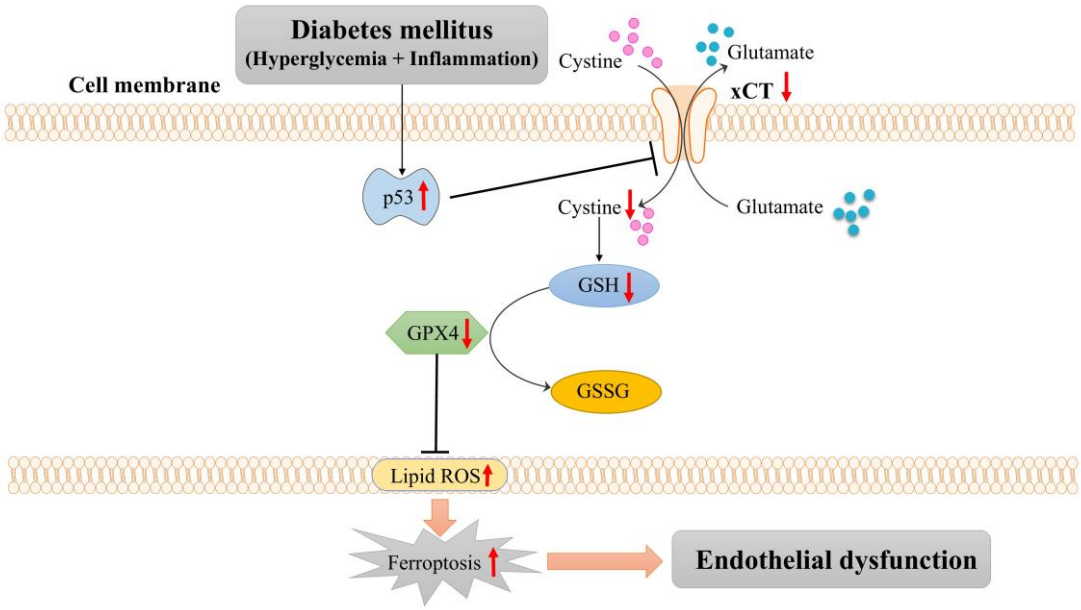

Figure 6 Working model: the activation of the p53-xCT-GSH axis and ferroptosis plays a vital role in diabetes-induced endothelial dysfunction.

9. Response to comment: (Data of PCR did not show. Why?)

Response: Sincerest thanks to you for your good comments. Data of PCR was showed in Figure 3A. And we further detected the levels of p53 mRNA and xCT

mRNA in the aorta of db/db mice. The data of PCR was shown in Figure 5(B and C).

Special thanks to you for your good comments.

Reviewer #2:

1. Response to comment: (It is said that coronary artery atherosclerosis resulted from inflammation of local lesion, however, in this study, human umbilical vein endothelial cell was cultured in vitro, how to mimic the inflammatory environment which played an important role in the formation of atherosclerosis.)

Response: Sincerest thanks to you for your good comments. Although the molecular mechanisms of endothelial dysfunction are not completely understood, hyperglycemia and inflammation are considered the key causes of diabetes-induced endothelial dysfunction [1-3]. It is really true as Reviewer suggested that coronary artery atherosclerosis resulted from inflammation of local lesions. And increased inflammation is a major contributor to diabetes-induced endothelial dysfunction [4]. Previous studies have shown that serum Il-1β levels in diabetic patients are significantly increased[5]. HUVECs were treated with IL-1β (10 ng/mL) to mimic the inflammatory environment in the current study. Additionally, hyperglycemia plays a critical role in diabetes-induced endothelial dysfunction. For instance, hyperglycemia in diabetes decreases vasodilation through the decreased bioavailability of nitric oxide (NO) and prostacyclin (PGI2) [6]. Therefore, in the current study, HUVECs were treated with HG (30 mM) and IL-1β (10 ng/mL) to mimic the diabetes environment.

[1] Xiang L, Mittwede PN, Clemmer JS: Glucose Homeostasis and Cardiovascular Alterations in Diabetes. COMPR PHYSIOL 2015, 5(4):1815-1839.

[2] Vanhoutte PM, Shimokawa H, Feletou M, Tang EH: Endothelial dysfunction and vascular disease - a 30th anniversary update. Acta Physiol (Oxf) 2017, 219(1):22-96.

[3] Shenouda SM, Widlansky ME, Chen K, Xu G, Holbrook M, Tabit CE, Hamburg NM, Frame AA, Caiano TL, Kluge MA et al: Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. CIRCULATION 2011, 124(4):444-453.

[4] Sharma A, Rizky L, Stefanovic N, Tate M, Ritchie RH, Ward KW, de Haan JB. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator dh404 protects against diabetes-induced endothelial dysfunction. Cardiovasc Diabetol. 2017 Mar 3;16(1):33.

[5] von Scholten BJ, Reinhard H, Hansen TW, et al. Markers of inflammation and endothelial dysfunction are associated with incident cardiovascular disease, all-cause mortality, and progression of coronary calcification in type 2 diabetic patients with microalbuminuria. J Diabetes Complications. 2016 Mar;30(2):248-55.

[6] Muzaffar S, Jeremy JY, Angelini GD, Shukla N: NADPH oxidase 4 mediates

upregulation of type 4 phosphodiesterases in human endothelial cells. J CELL PHYSIOL 2012, 227(5):1941-1950.

2. Response to comment: (In previous studies, the injured endothelial cell caused by inflammation or other reasons might undergo necrosis or apoptosis; in this study, the author found that ferroptosis might involve in this process, so the author should which one play a more important role.)

Response: Sincerest thanks to you for your good comments. It is really true as Reviewer suggested that the injured endothelial cell caused by inflammation or other reasons might undergo necrosis or apoptosis. We added experiments based on your suggestion.

HUVECs were treated with NG, HG (30 mM) and IL-1β (10 ng/mL), the ferroptosis inhibitor, desferrioxamine (DFO, 100 μM) or Ferrostatin-1 (Fer-1, 10 μM), the necroptosis inhibitor necrostatin-1s (Nec-1s, 2 μM), and the apoptosis inhibitor Z-VAD-FMK (Z-VAD, 5 μM). After treatment for 48 h, cell viability was determined using CCK-8. As shown in Figure 2(A), DFO, Fer-1, Nec-1s, and Z-VAD partially rescue HG and IL-1β mediated cell death. It demonstrated that in addition to necrosis and apoptosis, ferroptosis may play an important role in cell death induced by hyperglycemia and inflammation. Therefore, this study aimed to investigate the role and regulatory mechanism of ferroptosis in diabetes-induced endothelial dysfunction.

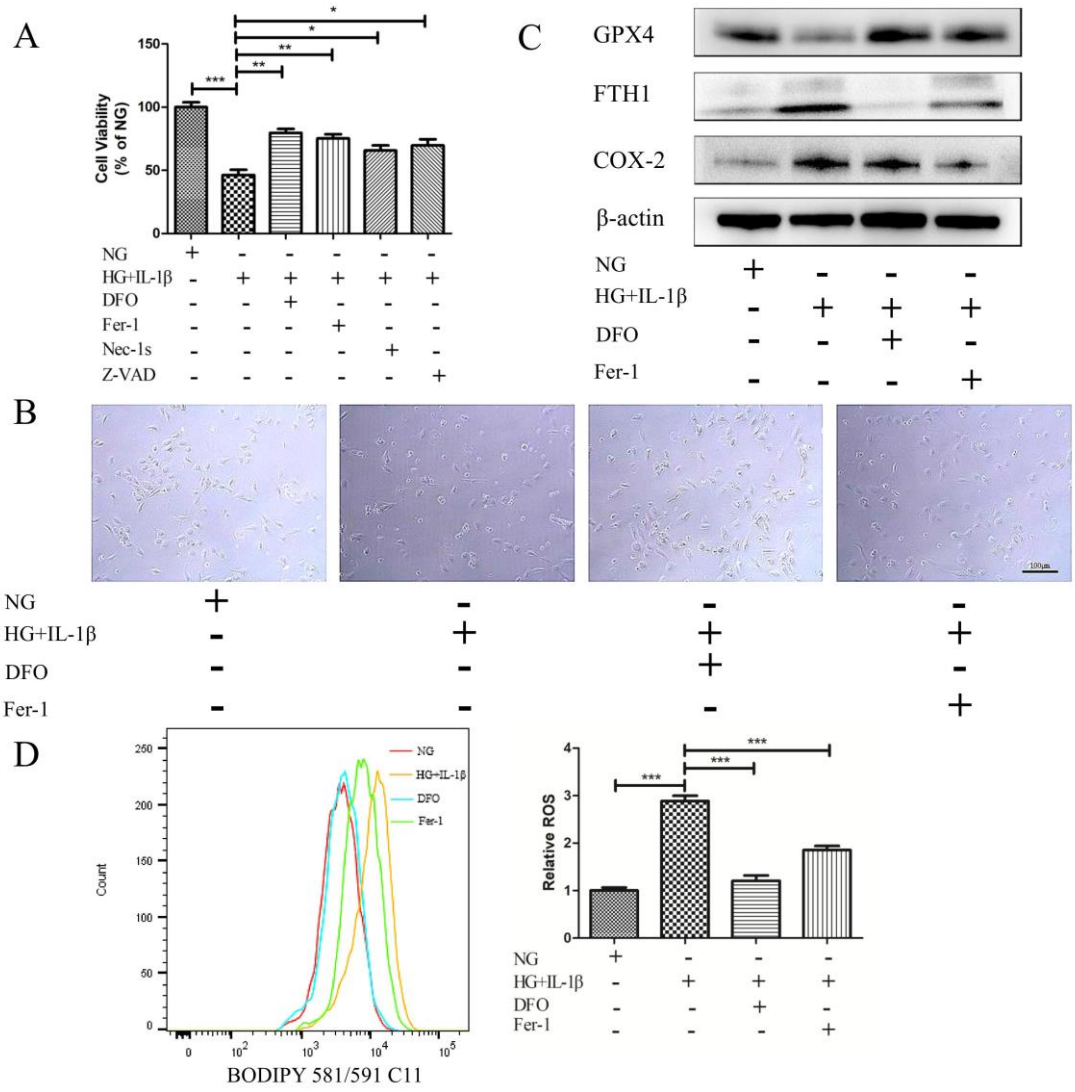

Figure 2 Ferroptosis inhibitor attenuates the ferroptosis of HUVECs induced by HG and IL-1β.

3. Response to comment: (Cox-2 is pathological factor which is mainly secreted by inflammatory cells, does vein endothelial cell secret this inflammatory cytokines. In figure 1 and figure 2, the author detected the expression of Cox-2, FTH1 and GPX4, is there any connection between them?)

Response: Sincerest thanks to you for your good comments. It is really true as Reviewer suggested that Cox-2 is pathological factor which is mainly secreted by inflammatory cells. Besides, COX-2 is expressed in vein endothelial cell[1-3].

Ferroptosis-inducing factors can directly or indirectly affect glutathione peroxidase through different pathways, resulting in a decrease in antioxidant capacity and accumulation of lipid ROS in cells, ultimately leading to oxidative cell death[4]. GPX4, a glutathione peroxidase, utilizes reduced glutathione to convert lipid hydroperoxides

to lipid alcohols, thereby mitigating lipid peroxidation and inhibiting ferroptosis[5]. COX2 and FTH1 were also considered as markers of ferroptosis[6,7]. The levels of GPX4 in ferroptosis cells are down-regulated, while the levels of COX2 and FTH1 are up-regulated[8]. The other ferroptosis-related assays and tools mainly include BODIPY 581/591 C11 (sensor for lipid ROS levels) and GSH and GSSG Assay Kit (quantifies glutathione levels). To identify ferroptosis, cell viability was assessed by CCK-8, the protein levels of GPX4, FTH1, and COX-2 were determined using Western blot assays, the generation of ROS was determined by the BODIPY™ 581/591 C11 and the intracellular glutathione/oxidized glutathione (GSH/GSSG) ratio was measured with the GSH and GSSG Assay Kit in the current study.

[1] Akarasereenont P, Techatraisak K, Chotewuttakorn S, Thaworn A. The expression of cyclooxygenase-2 in human umbilical vein endothelial cell culture from preeclampsia. J Med Assoc Thai. 1999 Feb;82(2):167-72.

[2] Abbasi N, Akhavan MM, Rahbar-Roshandel N, Shafiei M. The effects of low and high concentrations of luteolin on cultured human endothelial cells under normal and glucotoxic conditions: involvement of integrin-linked kinase and cyclooxygenase-2. Phytother Res. 2014 Sep;28(9):1301-7.

[3] Takata Y, Nomura K, Ishibashi K, Kido K, Sasamori Y, Hiraike H, Ayabe T, Atsumi GI. Elevated Expression of Vascular Adhesion Molecule-1, Plasminogen Activator Inhibitor-1, Cyclooxygenase-2, and Thrombomodulin in Human Umbilical Vein Endothelial Cells from Hospitalized Gestational Diabetes Mellitus Patients. Biol Pharm Bull. 2019;42(5):807-813.

[4] Li J, Cao F, Yin HL, et al. Ferroptosis: past, present and future. Cell Death Dis. 2020 Feb 3;11(2):88.

[5] Seibt TM, Proneth B, Conrad M. Role of GPX4 in ferroptosis and its pharmacological implication. Free Radic Biol Med. 2019 Mar;133:144-152.

[6] Chen B, Chen Z, Liu M, Gao X, Cheng Y, Wei Y, Wu Z, Cui D, Shang H. Inhibition of neuronal ferroptosis in the acute phase of intracerebral hemorrhage shows long-term cerebroprotective effects. Brain Res Bull. 2019 Nov;153:122-132.

[7] Li Z, Jiang L, Chew SH, Hirayama T, Sekido Y, Toyokuni S. Carbonic anhydrase 9 confers resistance to ferroptosis/apoptosis in malignant mesothelioma under hypoxia. Redox Biol. 2019 Sep;26:101297.

[8] Ingold I, Berndt C, Schmitt S, et al. Selenium Utilization by GPX4 Is Required to Prevent Hydroperoxide-Induced Ferroptosis. Cell. 2018 Jan 25;172(3):409-422.e21. doi: 10.1016/j.cell.2017.11.048. Epub 2017 Dec 28. PMID: 29290465.

4. Response to comment: (In figure 2b, DFO could is a kind chelating agent of iron ion, how could it modulate the expression of GXP4?)

Response: Sincerest thanks to you for your good comments. Deferoxamine (DFO) is an iron chelator that binds free iron in a stable complex, preventing it from engaging

in chemical reactions. And DFO is a ferroptosis inhibitors which can which can significantly rescue cell death though deplete the iron. Recent studies have shown that DFO can significantly reduce the level of reactive oxygen species (ROS) in cells and increase the level of GPX4[1,2,3]. In our view, DFO may modulate the expression of GXP4 indirectly, it may be related to the decrease of ROS. And this is a very valuable question, we will further study it in the future.

[1] Bruni A, Pepper AR, Pawlick RL, Gala-Lopez B, Gamble AF, Kin T, Seeberger K, Korbutt GS, Bornstein SR, Linkermann A, Shapiro AMJ. Ferroptosis-inducing agents compromise in vitro human islet viability and function. Cell Death Dis. 2018 May 22;9(6):595. doi: 10.1038/s41419-018-0506-0. PMID: 29789532; PMCID: PMC5964226.

[2] Kose T, Vera-Aviles M, Sharp PA, Latunde-Dada GO. Curcumin and (-)-Epigallocatechin-3-Gallate Protect Murine MIN6 Pancreatic Beta-Cells Against Iron Toxicity and Erastin-Induced Ferroptosis. Pharmaceuticals (Basel). 2019 Feb 6;12(1):26.

[3] Li Y, Yang H, Ni W, Gu Y. Effects of deferoxamine on blood-brain barrier disruption after subarachnoid hemorrhage. PLoS One. 2017 Mar 1;12(3):e0172784. doi: 10.1371/journal.pone.0172784.

5. Response to comment: (In figure 3a, the mean of this set of experiments is difficult to understand, why the author set this experiment.)

Response: Sincerest thanks to you for your good comments. GPX4 uses GSH to protect cells from ferroptosis by eliminating phospholipid peroxides[1]. GSH is one of the major cellular non-protein antioxidants, which is a tripeptide anti-oxidant consisting of glutamate, glycine and cysteine[2]. Jiang et al. found that ferroptosis as a p53-mediated activity during tumor suppression and p53 sensitizes cells to ferroptosis by negative regulation of xCT (SLC7A11), a key component of the cystine/glutamate antiporter, and inhibiting cystine uptake[3]. The ability of p53 to transcriptionally repress xCT was shown to lead to decreased cystine import, which would lead to reduced glutathione production and increased ROS, an important component of ferroptosis [3]. And previous research has established that hyperglycemia promotes the activation of p53[4,5]. Therefore, we set this experiment to further determine whether the p53-xCT-GSH axis is involved in HG and IL-1β induced ferroptosis.

[1] Yang WS, SriRamaratnam R, Welsch ME, Shimada K, Skouta R, Viswanathan VS, et al. Regulation of ferroptotic cancer cell death by GPX4. Cell 2014;156:317-31.

[2] Meister A. Selective modification of glutathione metabolism. Science 1983;220:472-7.

[3] Jiang L, Kon N, Li T, Wang SJ, Su T, Hibshoosh H, et al. Ferroptosis as a p53-mediated activity during tumour suppression. Nature 2015;520:57-62.

[4] Wu Y, Lee S, Bobadilla S, Duan SZ, Liu X. High glucose-induced p53

phosphorylation contributes to impairment of endothelial antioxidant system. Biochimica et biophysica acta Molecular basis of disease 2017;1863:2355-62.

[5] Chen YW, Chenier I, Chang SY, Tran S, Ingelfinger JR, Zhang SL. High glucose promotes nascent nephron apoptosis via NF-kappaB and p53 pathways. American journal of physiology Renal physiology 2011;300:F147-56.

6. Response to comment: (In previous researched, p53 was regarded as a protect factor for cell to defense oxidative stress, because p53 could work as a transcription factor to regulate the expression of some proteins of antioxidant in stress. However, in this paper, knockdown of p53 lead to the upregulation of Xct which played an antioxidant role, the conclusion is contradicted with most other conclusions, how to explain it.)

Response: Sincerest thanks to you for your good comments. It is really true as Reviewer suggested that p53 was regarded as a protect factor for cell to defense oxidative stress. p53 regulates the expression of an assortment of genes involved in maintaining homeostasis, including those involved in cell cycle regulation, redox homeostasis (i.e. antioxidant enzyme production), DNA replication and repair, apoptosis, and autophagy[1-5]. Recent studies suggest that p53 plays a significant role in the development of metabolic diseases, including diabetes. It has been demonstrated that endothelial p53 is activated in diabetes and that it negatively regulates glucose metabolism by modulating mitochondrial biogenesis and glucose uptake into skeletal muscle [6]. Previous ex vivo studies have demonstrated that adenoviral-mediated overexpression of p53 leads to impairment of endothelium-dependent vasodilation [7,8]. In agreement with these reports, mice with heterozygous systemic p53 knockout exposed to a high-fat diet show improvement of endothelial function and lower circulating cholesterol levels [9]. It was recently reported that transplantation of human $CD34^+$ cells with silencing of p53 improved recovery of blood flow after ischemia in diabetic mice [10]. Taking these findings into account, pathophysiological stress such as hyperglycemia or ischemia may initiate p53 activation in endothelial cells, leading to vascular dysfunction. And the results of our study indicated that the p53-xCT-GSH axis induced by HG and IL-1β can repress the expression of xCT and inhibit cystine uptake, ultimately causing increased endothelial cells ferroptosis and endothelial dysfunction. From the perspective of ferroptosis and GSH synthesis disorders, we describe a new mechanism of endothelial vascular injury in diabetes.

[1] Holley AK, Clair DK S. Watching the watcher: regulation of p53 by mitochondria. Future Oncol. 2009;5(1):117–130.

[2] Levine AJ, Feng Z, Mak TW, et al. Coordination and communication between the p53 and IGF-1-AKT-mTOR signal transduction pathways. Genes Dev. 2006;20:267–75.

[3] Saleem A, Adhihetty PJ, Hood DA. Role of p53 in mitochondrial biogenesis and

apoptosis in skeletal muscle. Physiol Genomics.
2009;37(1):58–66.
[4] Saleem A, Carter HN, Hood DA. P53 is necessary for the adaptive changes in cellular milieu subsequent to an acute bout of endurance exercise. Am J Physiol Cell Physiol. 2014;306(3): C241–C249.
[5] Smeenk L, van Heeringen SJ, Koeppel M, et al. Characterization of genome-wide p53 binding sites upon stress response. Nucleic Acids Res. 2008;36(11):3639–3654.
[6] M. Yokoyama, S. Okada, A. Nakagomi, J. et al. Inhibition of endothelial p53 improves metabolic abnormalities related to dietary obesity, Cell Rep. 7 (5) (2014) 1691–1703.
[7] C.S. Kim, S.B. Jung, A. Naqvi, et al. p53 impairs endothelium-dependent vasomotor function through transcriptional upregulation of p66shc, Circ. Res. 103 (12) (2008) 1441–1450.
[8] A. Kumar, C.S. Kim, T.A. Hoffman, et al. p53 impairs endothelial function by transcriptionally repressing Kruppellike factor 2, Arterioscler. Thromb. Vasc. Biol. 31 (1) (2011) 133–141.
[9] F. Leblond, S. Poirier, C. Yu, et al. The anti-hypercholesterolemic effect of low p53 expression protects vascular endothelial function in mice, PLoS ONE 9 (3) (2014) e92394.
[10] N. Kundu, C.C. Domingues, C. Chou, et al. Use of p53-silenced endothelial progenitor cells to treat ischemia in diabetic peripheral vascular disease, J. Am. Heart Assoc. 6 (4) (2017).

7. Response to comment: (In figure 3c, the expression of CD31, a marker of endothelial, was changed in different group, why?)

Response: Sincerest thanks to you for your good comments. We have readjusted the figure according to your comments. The adjusted picture is shown in Figure 3C.

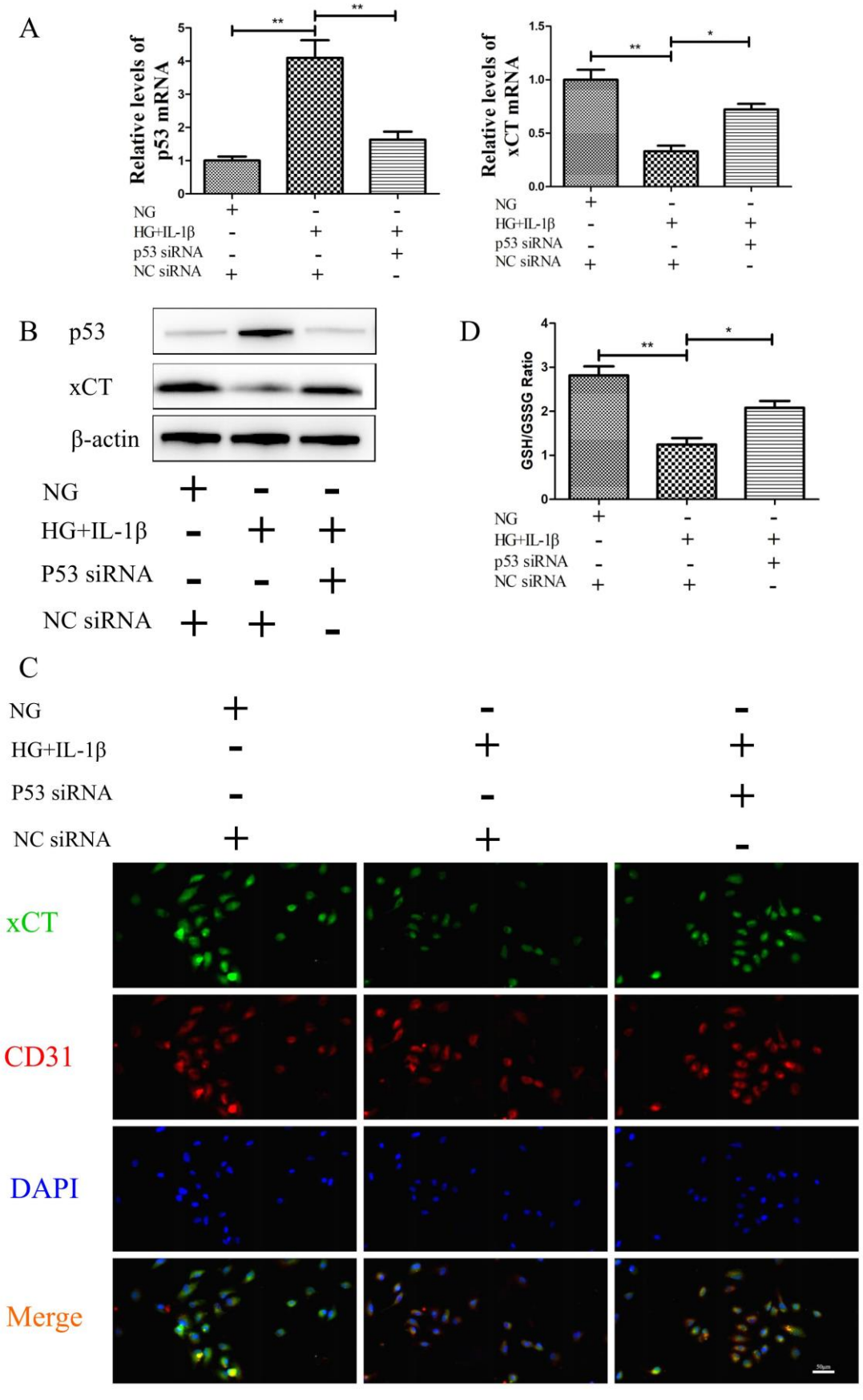

Figure 3 HG and IL-1β activate the p53-xCT-GSH axis in HUVECs.

8. Response to comment: (In the study, GXP4 was detected in many experiments, however the author just want to certify that p53-xCT-GSH axis played a role in ferroptosis of HUVECs, so why GXP4 was detected?)

Response: Sincerest thanks to you for your good comments. Glutathione (GSH) is synthesized from glycine, glutamate, and cysteine, among which cysteine is the rate-limiting precursor. Most cells obtain cysteine through the import of extracellular cystine - an oxidized dimeric form of cysteine - via the amino acid transporter xCT [1,2]. GPX4, a glutathione peroxidase, utilizes reduced glutathione to convert lipid hydroperoxides to lipid alcohols, thereby mitigating lipid peroxidation and inhibiting ferroptosis[3,4,5]. Inhibition of xCT could suppress the production of GSH, leading to a downregulation of GPX4[6]. And Genetic studies performed in cells and mice established GPX4 as the key regulator and marker of ferroptosis[7,8]. To identify ferroptosis, the protein levels of GPX4 was determined using Western blot assays, cell viability was assessed by CCK-8, the generation of ROS was determined by the BODIPY™ 581/591 C11 and the intracellular glutathione/oxidized glutathione (GSH/GSSG) ratio was measured with the GSH and GSSG Assay Kit in the current study.

[1] Stockwell BR, Friedmann Angeli JP, Bayir H, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. Cell. 2017 Oct 5;171(2):273-285.

[2] Conrad M, Sato H. The oxidative stress-inducible cystine/glutamate antiporter, system x (c) (-) : cystine supplier and beyond. Amino Acids. 2012 Jan;42(1):231-46.

[3] Seibt TM, Proneth B, Conrad M. Role of GPX4 in ferroptosis and its pharmacological implication. Free Radic Biol Med. 2019 Mar;133:144-152.

[4] Friedmann Angeli JP, Schneider M, Proneth B, et al. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. Nat Cell Biol. 2014 Dec;16(12):1180-91. doi: 10.1038/ncb3064. Epub 2014 Nov 17.

[5] Yang WS, SriRamaratnam R, Welsch ME, et al. Regulation of ferroptotic cancer cell death by GPX4. Cell. 2014 Jan 16;156(1-2):317-331.

[6] Konstorum A, Tesfay L, Paul BT, et al. Systems biology of ferroptosis: A modeling approach. J Theor Biol. 2020 May 21;493:110222.

[7] Sakai O, Yasuzawa T, Sumikawa Y, et al. Role of GPx4 in human vascular endothelial cells, and the compensatory activity of brown rice on GPx4 ablation condition. Pathophysiology. 2017 Mar;24(1):9-15.

[8] Yu H, Guo P, Xie X, Wang Y, Chen G. Ferroptosis, a new form of cell death, and its relationships with tumourous diseases. J Cell Mol Med. 2017 Apr;21(4):648-657.

9. Response to comment: (In figure 5a, Prussian Blue stain could show the iron ions

in blue color, however, the lesion the author showed is brown, why? In addition, as the author said, the lesion is endothelial, however, the brown lesions are located at the tunica media. Another, the brown lesion looked like a nonspecific coloring; the author should supply more convincing data.)

Response: Sincerest thanks to you for your good comments. In the current study, the presence of tissue-iron was detected by the Prussian Blue-DAB staining instead of Prussian Blue staining and the iron ions were shown in brown color. However, It is really true as Reviewer pointed out that the brown lesions are located at the tunica media instead of the intima. Therefore, we deleted this part of the experiment. And we further detected the levels of p53 mRNA and xCT mRNA in the aorta of db/db mice. The data of PCR was shown in Figure 5(B and C).

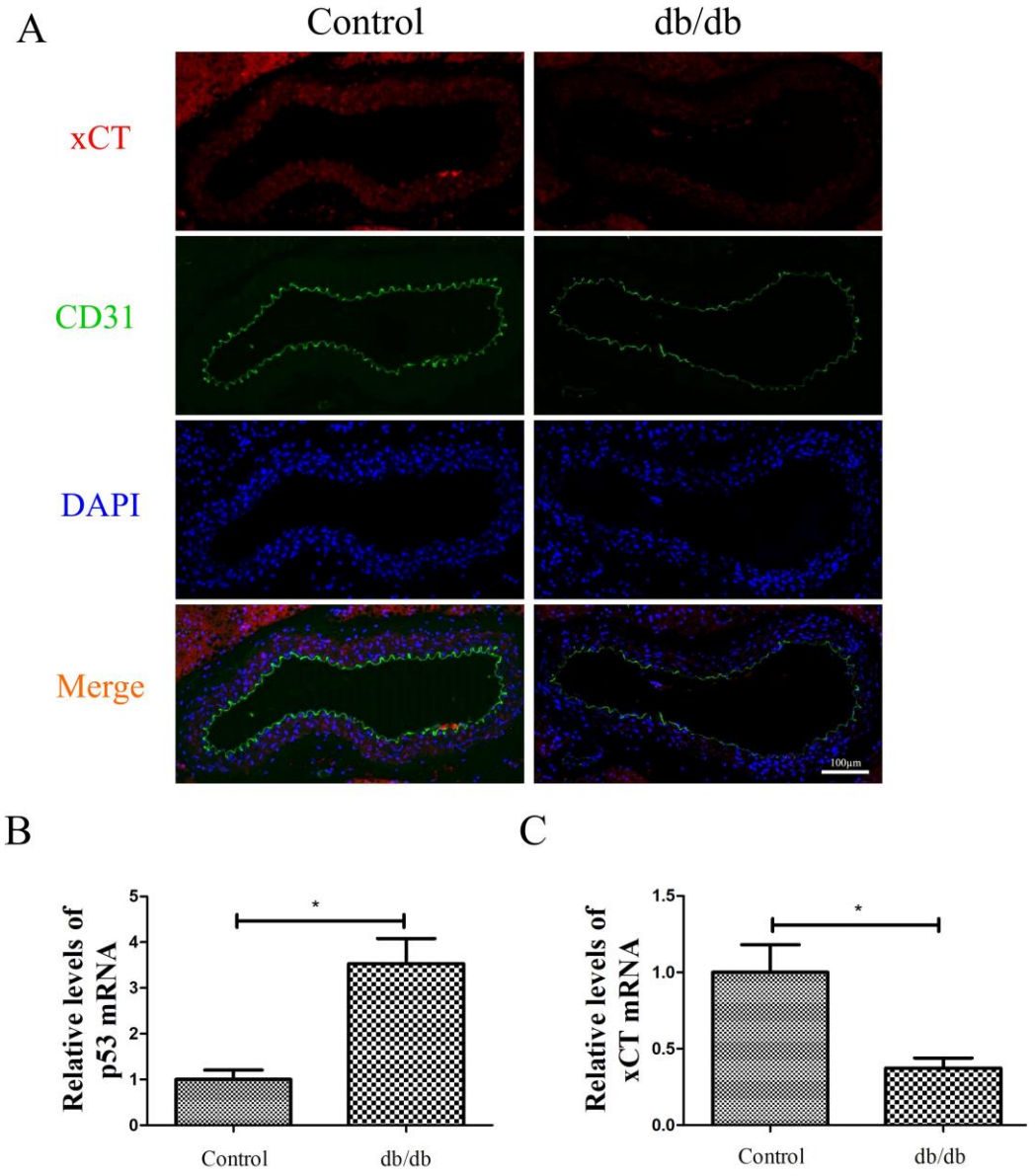

Figure 5 Detection of xCT and p53 expression in the aorta of db/db mice.

10. Response to comment: (There are many grammatical errors, the language needs further polishing. For examples: (1) And then tested the cell viability (Lack of subject) ; (2) ferroptosis related marker should be ferroptosis-related marker.)

Response: Sincerest thanks to you for your good comments. And grammatical error of the manuscript has been modified according to your suggestion.

Special thanks to you for your good comments.

Responds to Editor's comments:

1. Response to comment: (The language classification is Grade C. Please visit the following website for the professional English language editing companies we recommend: https://www.wjgnet.com/bpg/gerinfo/240*)*

Response: Sincerest thanks to you for your good comments. And grammatical error of the manuscript has been modified according to your suggestion.

2. Response to comment: (The "Author Contributions" section is missing. Please provide the author contributions)

Response: Sincerest thanks to you for your good comments. We have provided the author contributions according to your suggestion. In addition, we added YAO YY to the author in our paper. In fact, she made a significant contribution to the manuscript revision. All authors listed in the previous version of our paper agree to the change and order of the author list in this version. If any questions, please don't hesitate to let me know.

Author Contributions

LUO EF, LI HX, WANG D, and TANG CC conceived and designed the experiments. LUO EF, LI HX, QIN YH, and LI LQ performed the experiments and data analyses and wrote the manuscript. YAN GL, QIAO Y, and HOU JT contributed to the quality control of data and algorithms. YAO YY helped perform the data analysis and manuscript revision with constructive discussions. All authors read and approved the final manuscript.

(lines 20 to 25, page 1)

3. Response to comment: (The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor)

Response: Sincerest thanks to you for your good comments. We have provided the original figure documents (PowerPoint) according to your suggestion.

4. Response to comment: (PMID and DOI numbers are missing in the reference list. Please provide the PubMed numbers and DOI citation numbers to the reference list and list all authors of the references. Please revise throughout)

Response: Sincerest thanks to you for your good comments. We have provided the PubMed numbers and DOI citation numbers to the reference list and listed all authors of the references according to your suggestion.

Special thanks to you for your good comments.

Responds to comments of Editorial office director:

1. Response to comment: (The study was supported by the National Natural Science Foundation of China; and Fundamental Research Funds for the Central Universities. Please upload the approved grant application form(s) or funding agency copy of any approval document(s).)

Response: Sincerest thanks to you for your good comments. We have upload the approved grant application form(s) or funding agency copy of any approval document(s) according to your suggestion.

Special thanks to you for your good comments.

We appreciate for Editors/Reviewers' warm work earnestly, and tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We sincerely hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions.

Sincerely yours,
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