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Exposure to Alcohol Causes Ferroptosis Activation Associated with Suppression of Heat Shock Responses-mediated iron metabolism disorder in *Caenorhabditis elegans*

Yanan Zhao^{1,2}, Bin Li¹, Ran Cao¹, Leyao Chen¹, Binghua Dong¹, Siting Ju¹, Ziyang Huang¹, Hui Teng^{1,*}, Lei Chen^{1,2,*}

¹. College of Food Science and Technology, Guangdong Ocean University, Zhanjiang, Guangdong 524000, People's Republic of China; Guangdong Provincial Key Laboratory of Aquatic Product Processing and Safety, Zhanjiang, Guangdong 524000, People's Republic of China; Guangdong Province Engineering Laboratory for Marine Biological Products, Zhanjiang, Guangdong 524000, People's Republic of China; Guangdong Provincial Engineering Technology Research Center of Seafood, Zhanjiang, Guangdong 524000, People's Republic of China; Key Laboratory of Advanced Processing of Aquatic Product of Guangdong Higher Education Institution, Zhanjiang, Guangdong 524000, China;

². Shenzhen Institute of Guangdong Ocean University, Shenzhen 518108, China

ABSTRACT: Exposure to alcohol caused toxicity in *Caenorhabditis elegans* (*C. elegans*). However, the underlying mechanisms for this induced toxicity by alcohol remain largely unclear. We examined possible association of ferroptosis activation with toxicity of alcohol. This study revealed that alcohol (400 mM-700 mM)-related iron toxicity suppressed head thrash, pharyngeal pumping of *C. elegans*, generated reproductive toxicity and oxidative stress, while rescued by ferroptosis inhibitor Fer-1 (100 μ mol/L) and iron chelating agent DFO (50 μ mol/L). Meanwhile, alcohol resulted in increased Fe²⁺ levels due to iron metabolism dysfunction, promoting iron uptake by increasing gene level of *Smf-3*, suppressing iron storage and efflux by decreasing gene level of *Ftn-1* and *Fpn-1.1*. Besides, alcohol caused increased MDA content, decreased GSH content, as well as alterations in the expression *Gpx-1*, *Ftn-1*, and *Acs-17*, facilitating ferroptosis. Moreover, alcohol-caused shorten lifespan, abnormal physiological state, reproductive toxicity as well as disturbed iron homeostasis and ferroptosis were all exacerbated in *hsf-1* and *skn-1* mutation *C. elegans*. The study offers novel insights into the mechanisms of alcohol-induced iron metabolism dysfunction and ferroptosis in *C. elegans*.

Keywords: Ferroptosis, Iron toxicology, Heat Shock Responses, *Caenorhabditis elegans*

1. Introduction

Alcohol abuse represents a significant public health burden in Western societies^[1]. Chronic excessive alcohol consumption contributes to progressive liver damage, including hepatic steatosis, fibrosis, and alcoholic hepatitis. In a substantial proportion of patients, persistent alcohol use leads to cirrhosis, markedly elevating the risk of hepatic decompensation, hepatocellular carcinoma (HCC), and overall mortality^[2-4]. Although the precise mechanisms underlying alcoholic liver disease (ALD) remain incompletely elucidated, current evidence implicates oxidative stress, lipid peroxidation, cytokine dysregulation, and iron overload as

*Corresponding author:
chenlei841114@hotmail.com (L. Chen).

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key pathogenic drivers^[5,6]. Currently, the treatment of alcoholic liver injury is unsatisfied and there is no effective drug for its treatment in clinic yet. With both prevalence and mortality rates rising annually, ALD has emerged as a major public health concern and strategies to mitigate ALD are urgently required.

Ferroptosis represents a distinct iron-dependent cell death modality characterized by iron-catalyzed lipid peroxidation and plasma membrane damage^[7]. To be specific, iron accumulation in the form of Fe^{2+} can be extremely toxic. The redox-active Fe^{2+} species forms labile iron pools that catalyze Fenton reactions, generating oxidative stress capable of inducing caspase-independent programmed cell death. This process typically manifests with concurrent glutathione (GSH) depletion and progressive accumulation of lipid peroxidation products^[8]. Emerging evidence positions ferroptosis as a key mechanistic pathway in foodborne contaminants, such as 1,3-dichloro-2-propanol^[9] and high fat diet^[10]. The *Caenorhabditis elegans* (*C. elegans*) has emerged as a robust model organism for ecotoxicological studies due to its sensitivity to environmental stressors and suitability for toxicity assessment at ecologically relevant concentrations^[11–13]. Our previous studies and other independent research groups have confirmed that Alcohol exposure could cause significantly iron overload, which could be serve as a reliable and well-recognized foodborne inducer for constructing validated iron toxicity model^[14,15]. Nevertheless, its underlying mechanisms for iron toxicity still needs to be further determined in nematodes.

Heat shock factor 1 (HSF-1), a master regulator of stress responses, was initially identified for its essential role in transcriptional activation of heat shock proteins (HSPs) during thermal stress^[16,17]. Recent advances reveal HSF1's broader regulatory functions in cellular stress adaptation through non-HSP-related target genes, such as the ubiquitin-proteasome system, autophagy, and immune response. Of note, HSF1 also exerts a critical role in ferroptosis by regulating heat shock protein beta-1(HSPB1)-mediated Kelch-like ECH-associated protein 1/nuclear factor-like 2 (KEAP/NRF2) redox-sensitive signaling pathway, a pivotal role in protecting cells against iron toxicology and oxidative injury through regulating the transcription of antioxidant and detoxification genes^[18].

In this study, *C. elegans* was used to systematically explore the potential toxic impacts and the underlying mechanisms of alcohol caused iron toxicity and ferroptosis. Through integrated analyses of lifespan parameters, physiological biomarkers, oxidative stress markers, and transcriptional profiling of iron metabolism and ferroptosis-related genes within the HSF1/Nrf2 signaling context, we strive to assess the pathological consequences of alcohol on iron toxicity in nematodes through the ferroptosis pathway. Our findings demonstrate that alcohol exposure disrupts HSF1/Nrf2-regulated iron homeostasis through coordinated upregulation of iron uptake pathways coupled with suppression of iron storage and export mechanisms, ultimately driving ferroptotic in *C. elegans*.

2. Materials and methods

2.1 *C. elegans* strains

The following *C. elegans* strains were used in the current study: N2 (wild-type strain); EU1, *skn-1*(zu67), PS3551, *hsf-1*(sy441), LD1, *ldls7[skn-1: GFP+rol-6(su1006)]*, CF1824, *muEx265[hsf-1p::hsf-1(cDNA)+myo-3::GFP]*, all provided by CGC (Caenorhabditis Genetic Center).

2.2 *C. elegans* culture and infection model

All nematodes were cultured on a 9 cm nematode growth medium (NGM) fed with *Escherichia coli* (*E. coli*) OP50 in a temperature-controlled incubator at 20°C. Bleaching solution (2% HOCl, 0.45 M NaOH) was used to synchronize pregnant hermaphrodite to release eggs. L4 stage nematodes were collected on NGM and exposed to 400 mM, 500 mM, 600 mM and 700 mM alcohol for 24 h, and then the nematodes were cleaned and transferred to new NGM plates^[19,20]. Treated nematodes were collected for the following assays, and the nematodes were fed with *E. coli* OP50 as the control group.

2.3 Lifespan Assay

At least 150 worms were transferred randomly to the NGM plates, and each group consisted of three separate plates. The dead and surviving worms were counted daily until all of the nematodes died^[21,22]. Nematodes that exhibited embryos hatching within adults before the eggs were laid (internal hatch) or that escaped from the NGM were excluded from the statistical data.

2.4 Body Size Assay

At least 30 N2 nematodes exposed to alcohol were gathered and transferred to new NGM plates per group; then, the images of the nematodes under the stereomicroscope (LIOO, China) were obtained, and the body length and width were quantified with ImageJ 2.3.0.

2.5 Motility Assay

The treated N2 nematodes were made morphological observations after alcohol exposure for 24h. The categorization of movement class was established as follows: Motion A, autonomous and fluid movements; Motion C, movements limited to the head and tail when probed with a platinum wire; and Motion B, an intermediary between Motion A and C. The nematode count in each treated group exceeded 80.

2.6 Fertility Assay

10-15 N2 nematodes were transferred to the normal food-free NGM plates and added to the lysate (0.5 mL of 0.5 M NaOH, 0.5 mL of 4.0% NaClO, and 3 mL of H₂O). After the worm body was lysed, the eggs in the uterus were exposed for counting. 10-15 nematodes were selected stochastically and cultured until the end of the spawning period to count all offspring. And no FUDR was added to the NGM in this assay.

2.7 ROS and Lipofuscin Accumulation Assay

The gathered N2 worms were rinsed three times with buffer solution and then transferred to a black 96-well plate, with 50 worms allocated to each well. The 50 μ mol/L DCFH-DA was added and incubated for 30 min at 37 °C. Then, the ROS level was measured using a microplate reader (Thermo, China) (Ex: 485 nm; Em: 535 nm). The same treatment with 50 N2 nematodes per well, and lipofuscin accumulation level were measured using the microplate reader (Ex: 350 nm; Em: 460 nm).

2.8 Antioxidant Enzyme Activity and Metabolic Product Assay

At least 500 treated N2 worms were acquired and subjected to three rounds of washing of buffer and homogenized with a tissue grinder (Coyote, China). The suspension was centrifuged, and the supernatant was collected for subsequent assays. The activities of superoxide dismutase (SOD, A001-1-1), catalase (CAT, A007-2-1) and glutathione peroxidase (GSH, A006-2-1), as well as the level of malondialdehyde (MDA, A003-1-1) and iron (Fe^{2+}), were assessed by commercial kits according to the instructions of the manufacturer. The data were standardized to protein content using a BCA kit. All kits were purchased from Beyotime Biotech (Beijing, China).

2.9 Caclein-AM staining

Collect the treated nematodes and add 5 μm of Caclein-AM fluorescent staining solution for 1 h. Then wash them 2-3 times with M9 buffer solution. Determine the fluorescence intensity of the nematodes under a fluorescence microscope and measure the fluorescence intensity of 10 nematodes in each group.

2.10 Quantitative Real-Time PCR

Total RNA was extracted from treated N2 worms, and cDNA was synthesized with the instructions of the FastKing RT kit (with gDNA). The qRT-PCR was performed on the QuantStudio Real-Time PCR System (Thermo) according to the instructions in the SuperReal PreMix Plus (SYBR Green) kit. The qRT-PCR results were analyzed by using the $2^{-\Delta\Delta\text{Ct}}$ method, with β -action as an internal reference gene. The kits were acquired from TIANGEN Biotech Co., Ltd. (Beijing, China), and all primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primer information is displayed in Table S1.

2.11 Fluorescence Visualization

The translocation of GFP+rol-6 and the fluorescence intensity of *hsf-1p::hsf-1(cDNA)+myo-3::GFP* were assessed by observing the entire bodies of DL1 and CF1824. The nematodes were fixed onto 2% agarose slides and anesthetized with 5 mM levamisole. Images of GFP were captured by a fluorescence-inverted microscope (Leica, Germany) and analyzed by ImageJ 2.3.0. At least 30 worms were randomly chosen per group randomly.

2.12 Gas chromatography analysis of tissue alcohol concentration in vivo

According to the methods of Alaimo et al^[23]. and Wu et al^[24]., after treatments, the worms were washed with M9 buffer (3 g/L KH_2PO_4 , 6 g/L Na_2HPO_4 , 5 g/L NaCl, 1 ml 1 M MgSO_4) three times. Subsequently, the worms were re-suspended in 500 μl of M9 solution to facilitate cell fragmentation while placed in an ice bath. Once the worms were fully disintegrated, the mixture was centrifuged at 8,000 r/min for 5 min at 4°C. To the supernatant, N-butanol was introduced at a final concentration of 20 ppm, serving as the internal standard. The mixture was filtered through 0.23- μm PTFE filters for gas chromatography to determine tissue alcohol concentrations. To generate an alcohol standard curve, the internal standard n-butanol at a final concentration of 20 ppm and an alcohol standard at 5, 10, 20, 30, and 40 ppm final concentrations were used.

2.13 Statistical Analyses

All of the experiments are conducted in triplicate in this study. The data were analyzed GraphPad Prism 8.0. The log-rank (Mantel-Cox) test was applied to examine the survival curve. All results are presented as the

mean $\pm$ standard deviation (SD). In all statistical analyses, values are mean $\pm$ standard error of three independent experiments, $P < 0.05$ was accepted as statistically significant.

3. Results

3.1 The effect of alcohol on *C. elegans* lifespan

Tissue xenobiotic concentration serves as a critical biomarker for evaluating its accumulation^[24]. Quantitative analysis revealed dose-dependent alcohol bioaccumulation in *C. elegans* following 24h exposure to 400-700 mM concentrations, with tissue levels ranging from 22.46 to 33.79 ng/ μ g (Figure 1A), confirming the retention of alcohol in *C. elegans* body. Given the established correlation between lifespan reduction and organismal health deterioration in *C. elegans*, we conducted longitudinal survival analyses to evaluate alcohol 's toxicity. The selected concentration range (400-700 mM) was determined through preliminary dose-response studies and literature validation^{[13,25][26]}. As demonstrated in Figure 1B and Table 1, alcohol exposure significantly shortened the average, median, and maximum lifespan of nematodes. By the 10th day of cultivation under normal conditions without alcohol, 50 % of the worms remained alive, whereas alcohol-treated cohorts exhibited complete mortality by day 16 (400 mM), day 14 (500 mM), and day 11 (600 and 700 mM). Moreover, the average lifespans upon 400 mM and 500 mM decreased by 30%, and the median lifespans were 6 and 7 days, respectively. The average lifespan upon 600 mM and 700 mM decreased by 50% and 60% respectively, and the median lifespan of both was 5 days, which was significantly lower compared with the control group. These findings underscore the potent toxic effect of alcohol in *C. elegans*.

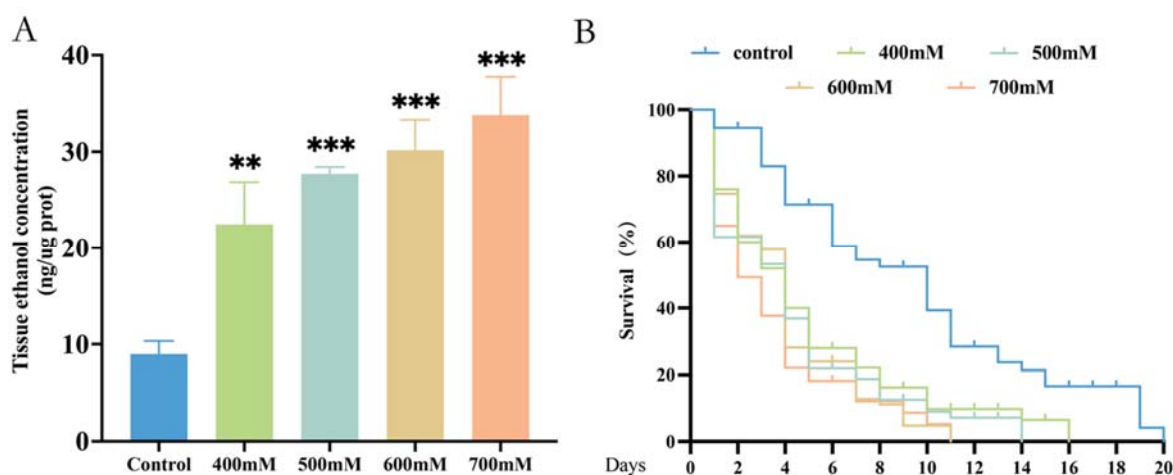


Figure 1. The effete of alcohol on *C. elegans* lifespan. (A) Tissue alcohol concentration in *C. elegans*; (B) Effect of alcohol on the lifespan of *C. elegans*. Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control.

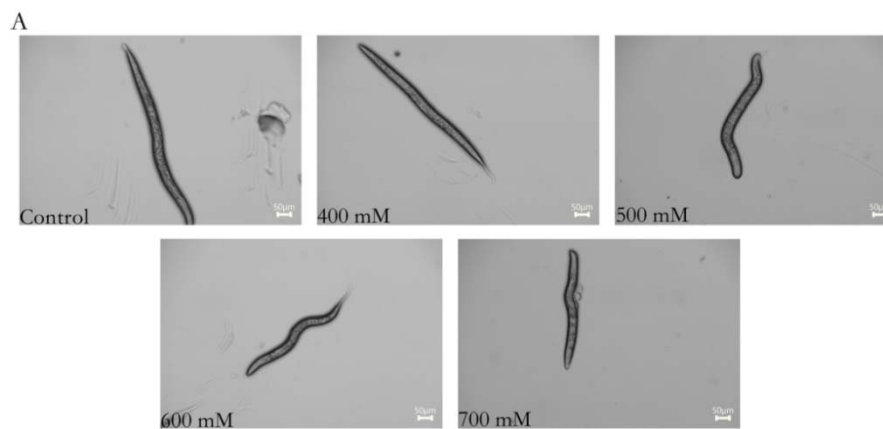
Table 1. Lifespan of N2 with Different Concentration Alcohol Treatments

Treatment	Median survival (days)	Mean lifespan (days)	Maximum lifespan (days)	Effect (%)
Control	10	10 $\pm$ 0.3	20	
400 mM	4	5 $\pm$ 0.43**	16	-47.3%
500 mM	4	5**	14	-51.5%
600 mM	4	4 $\pm$ 0.4**	11	-51.7%
700 mM	2	4 $\pm$ 0.4**	11	-57.3%

Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control.

3.2 The effect of alcohol on phenotype and movement of *C. elegans*

Body size parameters serve as sensitive morphological indicators in *C. elegans*. Quantitative morphometric analysis demonstrated significant alcohol-induced reductions in both body width and length across treatment groups (Figure A-C). While 400 mM exposure showed non-significant decreases compared to controls, dose-dependent morphological alterations became statistically significant at higher concentrations. Locomotor activity is an important behavioral measure of aging, as it decreases with the aging of worms, which is a result similar to muscle degeneration in human muscle loss syndrome. Results presented in Figure 2 D-E revealed that alcohol exposure significantly reduced the frequency of both body bends and pump pharynx in a dose-dependent way across all parameters. Detailed movement classification revealed progressive alcohol-induced motor dysfunction (Figure 2 F). Most nematodes in the control group could move spontaneously and were mainly in Motion A. However, after exposure to alcohol, the worms exhibited Motion A significantly reduced, with increased proportion of Motion B and Motion C. Over 23% of the worms exhibited Motion B with a small number of Motion C, and the 600 mM group showed the most Motion C. Trajectory analysis further corroborated these findings (Figure 2 G). Control worms maintained characteristic sinusoidal locomotion with directional persistence, while alcohol-exposed specimens exhibited disorganized movement patterns characterized by erratic directional changes and reduced path linearity, indicating that body activity in alcohol group was limited.



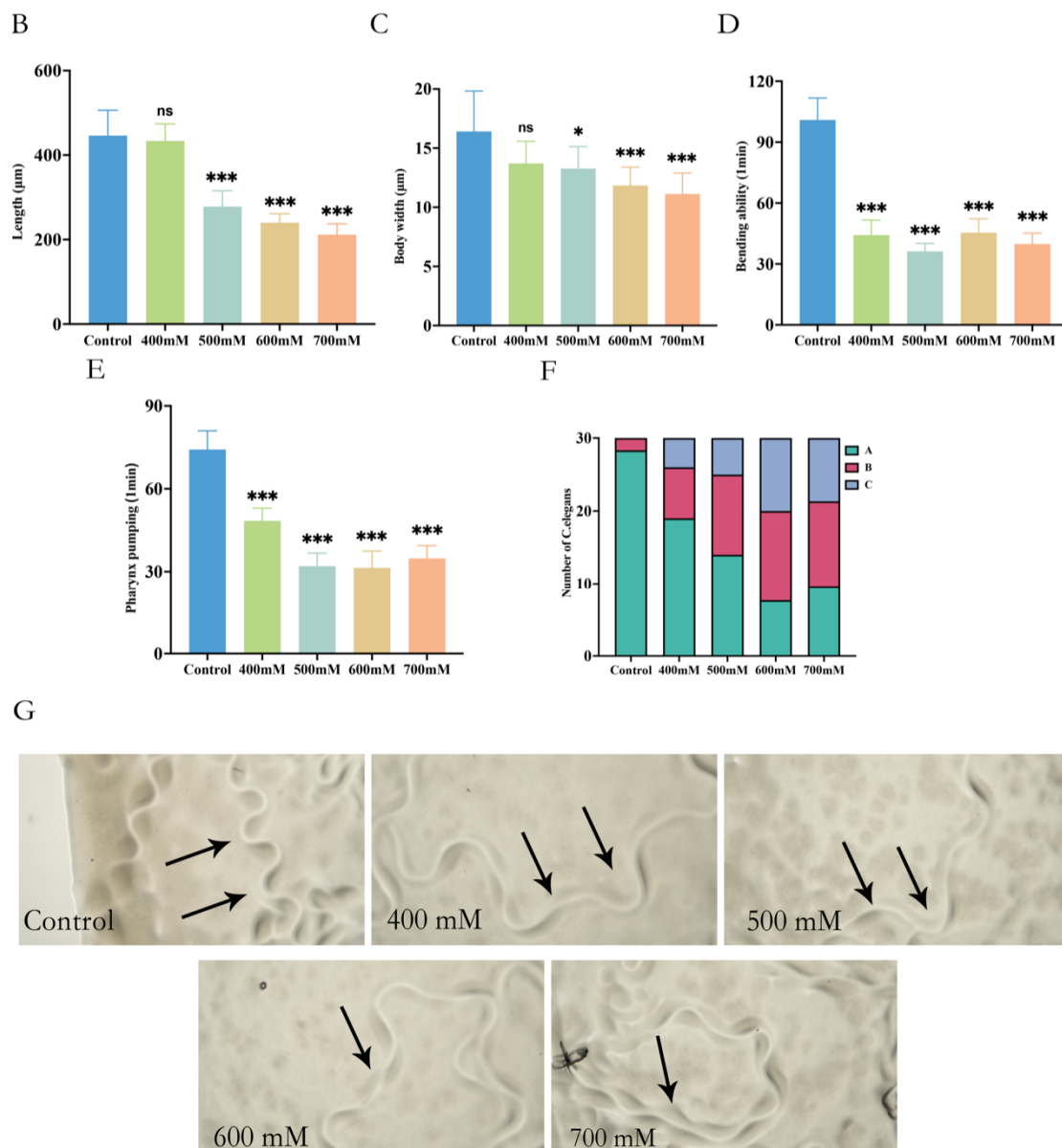


Figure 2. The effete of alcohol on *C. elegans* phenotype and movement. (A) Effect of alcohol on body size of *C. elegans*; (B) Effect of alcohol on body length of *C. elegans*; (C) Effect of alcohol on body width of *C. elegans*; (D) Effect of alcohol on bending ability of *C. elegans*; (E) Effect of alcohol on pharynx pumping of head bending of *C. elegans*; (F); Effect of alcohol on movement classification of *C. elegans*; (G) Effects of alcohol on locomotion trajectory of *C. elegans*. Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control.

3.3 Effects of alcohol exposure on reproductive toxicity of *C. elegans*

Given the established reproductive toxicity profile of alcohol, we conducted longitudinal fecundity assessments over a 5-day exposure period. As shown in Figure 3A, the average brood amount of the control group nematodes was 35 ± 0.5 , 38 ± 0.33 , 23 ± 0.67 , 25 ± 0.67 and 20 within 5 days, alcohol reduced it to 14 ± 0.17 , 27 ± 0.83 , 21 ± 0.5 and 21 ± 0.5 in days 1, and 9 ± 0.5 , 8 ± 0.67 , 13 ± 0.33 and 7 ± 0.67 in days 2. Higher concentrations (500–700 mM) caused progressive fecundity decline culminating in complete reproductive cessation from day 3 onward. Total egg production analysis revealed significant concentration-response relationships (Figure 3B). Control worms produced 143.0 ± 0.17 offspring over the observation period, but alcohol reduced it to 42 ± 0.83 , 38 ± 0.16 , 42 ± 0.5 and 31 ± 0.33 at concentration 400 mM, 500 mM, 600 mM and 700 mM, respectively.

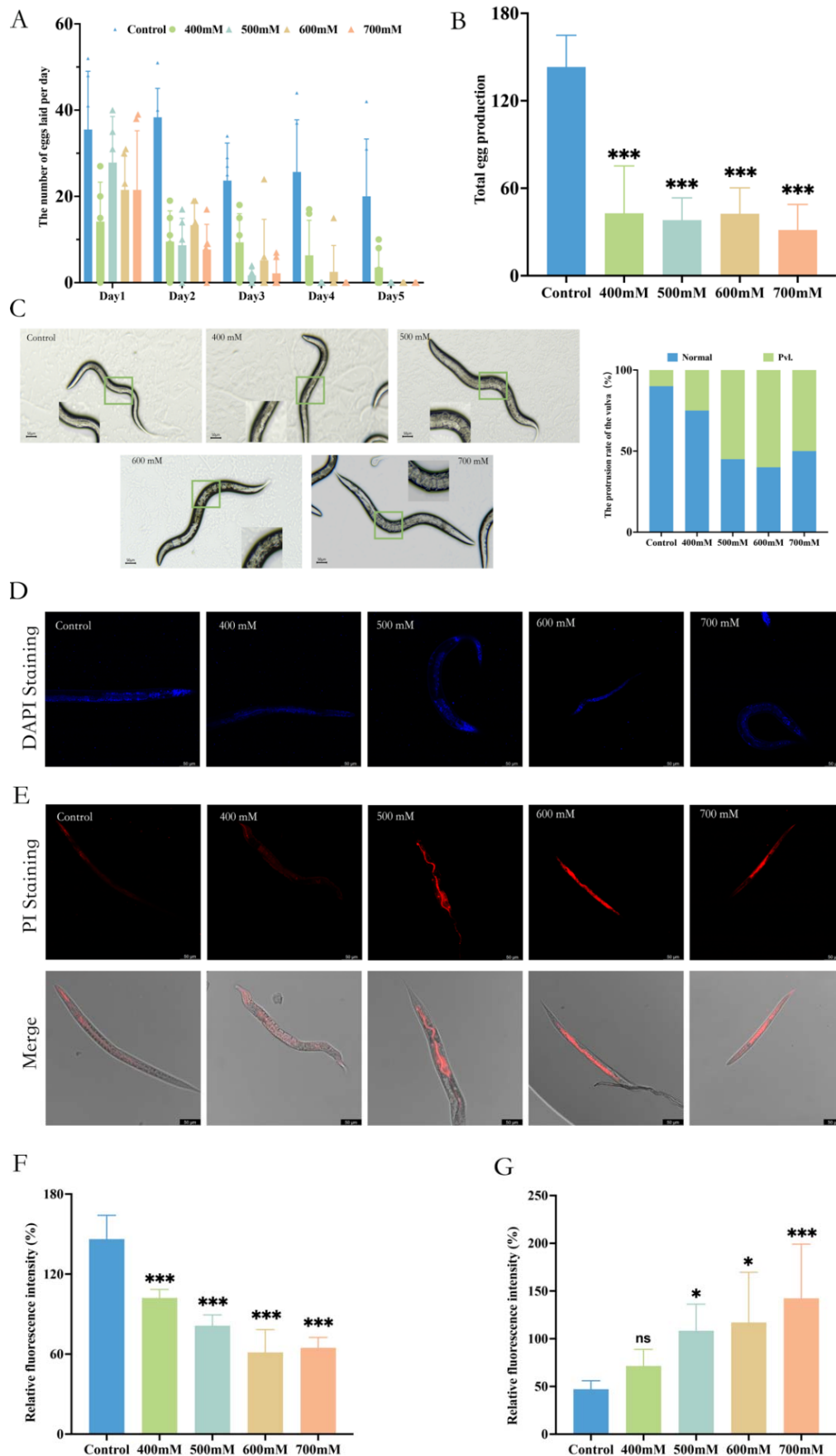
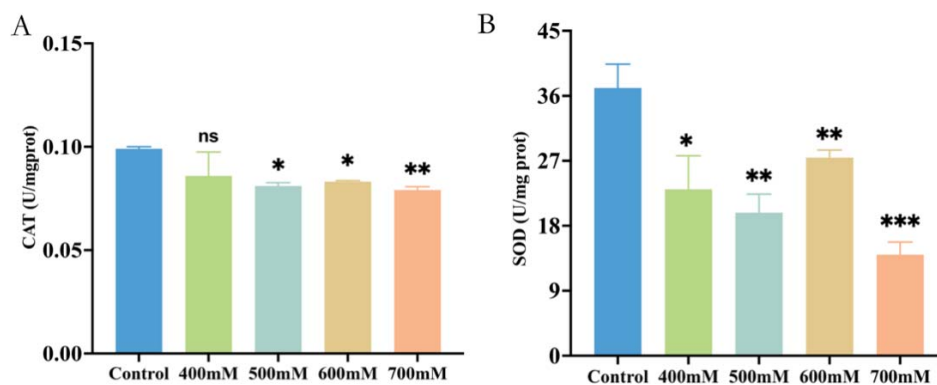


Figure 3. Effects of alcohol exposure on reproductive toxicity of *C. elegans*. (A) The effect of alcohol on the number of eggs laid per day; (B) The effect of alcohol on total eggs production; (C) The effect of alcohol on vulval protrusion in *C. elegans*; (D) Representative images of DAPI staining in *C. elegans*; (E) Representative images of PI staining in *C. elegans*; (F) Relative fluorescence intensity of DAPI staining; (G) Relative fluorescence intensity of PI staining. Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control.

Vulval lesions disrupt oocyte deposition and embryonic development, compromising reproductive system functionality. Our analysis revealed significant alcohol-induced vulval morphogenic defects, particularly protruding-vulva (Pvl) phenotypes, and the influence of alcohol concentration above 500 mM is particularly obvious, and the proportion of Pvl is as high as 45%. Germ cell integrity was further assessed through DAPI-based cytological profiling (Figure 3D, F). Fertile nematodes have gonads and eggs visible in their bodies, while non-fertile nematodes have neither gonads nor eggs developed^[27]. In the control group, the nuclear size of the mitotic region of the nematodes was normal and the intercellular arrangement spaces were tight, and the fluorescence intensity was high. However, in the nematodes exposed to different concentrations of alcohol, some of the nuclei in the mitotic region decrease, and the intercellular arrangement spaces increased, with a significant decrease in fluorescence intensity. Moreover, maintaining membrane integrity is crucial for cell survival and significant defects in membrane integrity can lead to cell death. As shown in Figure 3E and G, exposure to alcohol alters membrane permeability, as indicated by PI staining. The above results indicate that alcohol can significantly exert toxicity on the reproductive system of nematodes, thereby affecting their growth and development.

3.4 Effects of alcohol exposure on oxidative stress of *C. elegans*

Alcohol disturbs normal metabolism and related enzyme activities in nematodes. The effects of alcohol on antioxidant capacity in worms were investigated by measuring the activities of SOD and CAT (Figure 4A-B). Compared with the control group, SOD and CAT activities were significantly decreased in the different dose of alcohol group, except the level of CAT in 400 mM alcohol administration. Accumulation of ROS and lipofuscin are important markers of oxidation damage in *C. elegans*. The accumulation of lipofuscin in nematodes is closely related to the oxidative damage, and its accumulation always be with the increase of continuous attack by ROS produced from the outside, especially in intestinal cells. Thus, we further explored whether alcohol exacerbates the generation of lipofuscin and ROS in nematodes. As shown in Figure 4C-F, there was no significant change in lipofuscin and ROS accumulation in nematodes exposed to alcohol with 400 mM, while lipofuscin and ROS accumulation significantly increased in nematodes exposed to alcohol with a concentration of more than 500 mM, indicating alcohol could stimulate oxidative stress in *C. elegans*.



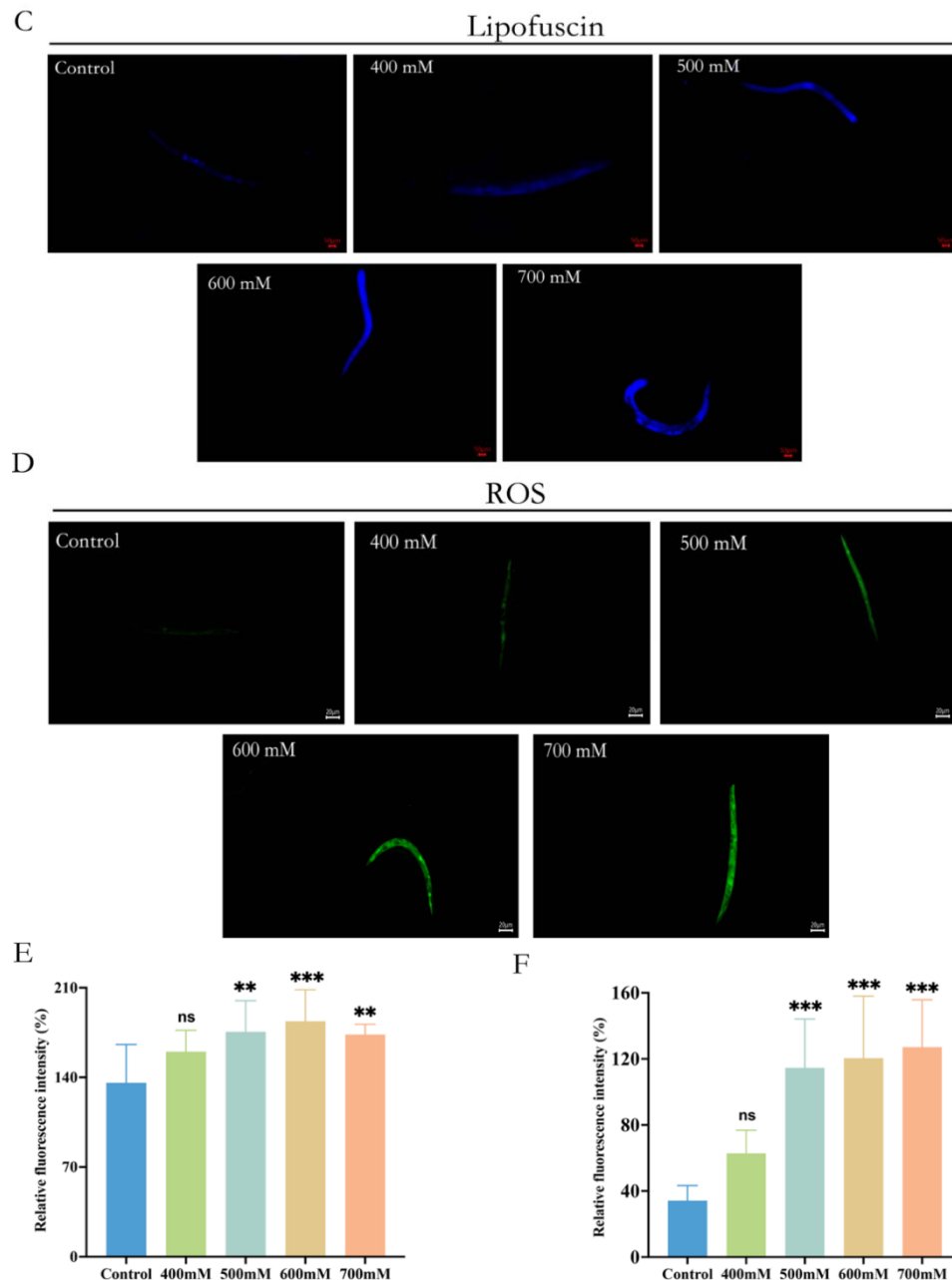


Figure 4. Effects of alcohol exposure on oxidative stress of *C. elegans*. (A) The effect of alcohol on the content of CAT; (B) The effect of alcohol on the content of SOD; (C) Representative images of lipofuscin fluorescence in *C. elegans*; (D) Representative images of ROS staining in *C. elegans*; (E) Relative fluorescence intensity of lipofuscin staining; (F) Relative fluorescence intensity of ROS staining. Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control.

3.5 Effect of alcohol exposure on iron metabolism of *C. elegans*

Our previous studies have confirmed that alcohol-caused damage significantly associated with iron metabolism dysfunction *in vivo* and *in vitro*^[15,28], thus we further explore whether alcohol could disturb iron homeostasis in *C. elegans*. Organisms iron levels are controlled by several iron regulatory proteins. DMT1 (encoded by *smf-3*), ferritin (encoded by *ftn-1*) and ferroportin (encoded by *Fpn.1-1*), responsible for cytosolic ferrous iron uptake, ferrous iron storage in the form of ferric iron and ferrous iron exports out of the cytosolic environment, respectively. As shown in Figure 5A-C, exposure to alcohol significantly decreased the expression of *ftn-1* and *Fpn.1-1*. Interestingly, alcohol induced a U-phase climate of the gene expression of *smf-3* and reach the highest level at 500 mM of alcohol. These results indicated that alcohol stimulated iron

homeostasis dysfunction by increasing iron uptake as well as suppressing iron storage and efflux.

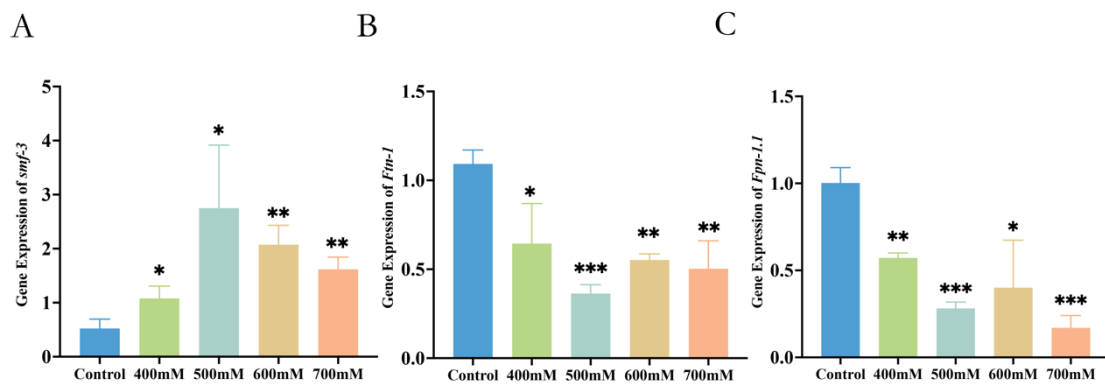
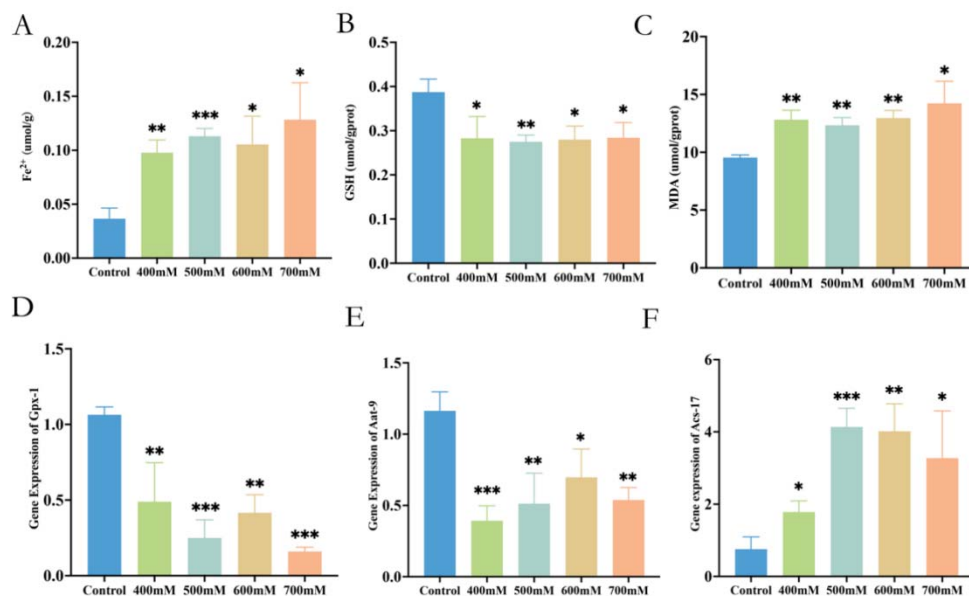


Figure 5. Effect of alcohol exposure on iron metabolism. (A) mRNA transcription levels of *smf-3*; (B) mRNA transcription levels of *Ftn-1*; (C) mRNA transcription levels of *Fpn-1.1*. Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control.

3.6 Effect of alcohol exposure on ferroptosis

Iron accumulation is one of the most important biomarkers of ferroptosis which always exerts its effects by activating iron toxicology-mediated lipid peroxidation. MDA, which is produced as a result of lipid peroxidation, is frequently utilized as a biomarker to indicate the occurrence of this process. The GSH can limit ferroptosis by inhibiting lipid peroxidation. As shown in Figure 6A and G, significant iron accumulation was observed in varieties alcohol exposed nematodes based on the detected level of Fe^{2+} as well as the Caclein-AM staining. Meanwhile, the MDA content was also significantly increased by alcohol (Figure 6C). In contrast, relative GSH content was significantly decreased in alcohol exposed nematodes (Figure 6B). At the same time, the ferroptosis-related gene expressions of *gpx-1* and *aat-9* were down-regulated, while *acs-17* was up-regulated (Figure D-F). The above results indicated that alcohol exposure could induce ferroptosis in *C elegans*.



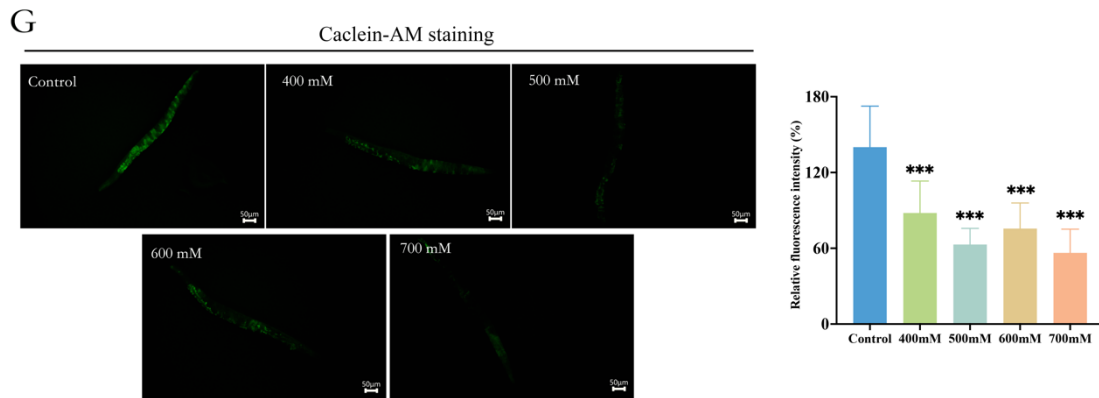
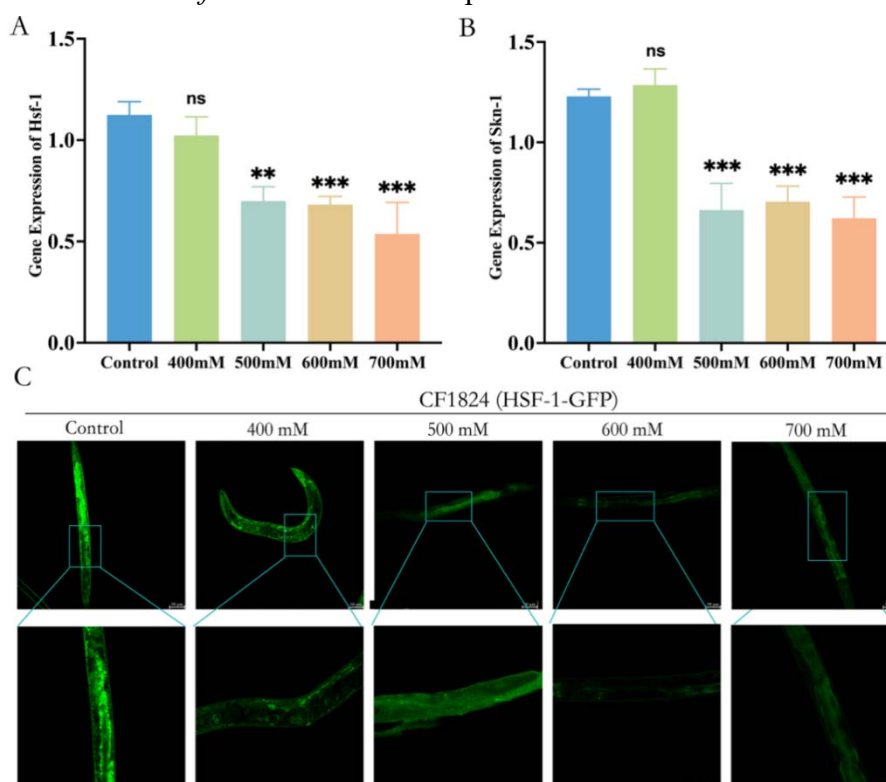


Figure 6. Effect of alcohol exposure on ferroptosis related indicators. (A) The effect of alcohol on the content of Fe^{2+} in *C. elegans*; (B) The effect of alcohol on the content of GSH in *C. elegans*; (C) The effect of alcohol on the content of MDA in *C. elegans*; (D) mRNA transcription levels of *Gpx-1*; (E) mRNA transcription levels of *Aat-9*; (F) mRNA transcription levels of *Acs-17*; (G) Representative images of Caclein-AM staining in *C. elegans*. Values are presented as mean $\pm$ SD. * $P < 0.05$ vs Control, ** $P < 0.01$ vs Control.

3.7 Hsf-1/skn-1 pathway involved in alcohol-induced ferroptosis in *C. elegans*

To gain insights into the molecular mechanisms underlying alcohol-mediated ferroptosis, we first assessed the expression of HSF-1 (encoded by *hsf-1*) and NRF2 (encoded by *skn-1*), the vital protein associated ferroptosis. As present in Figure 7A-B, exposure to alcohol at various concentration significantly decreased the expression of *hsf-1* and *skn-1*, except for 400 mM. We also focused on two *C. elegans* strain CF1824 (HSF-1: GFP) and LD1 (SKN-1:GFP) to detect the expression and translocation to the nucleus of *hsf-1* and *skn-1* which response for iron metabolism and oxidation stress. As shown in Figure 7C-F, alcohol exposure significantly reduces the nuclear translocation of the *hsf-1* and *skn-1* protein. Notably, 400 mM alcohol administration did not significantly change the nuclear translocation of the *hsf-1* or *skn-1* protein, while the suppression effect of *skn-1* reached greatest at 500 mM alcohol administration. The results above highlighting the essential role of *hsf-1* and *skn-1* in this process.



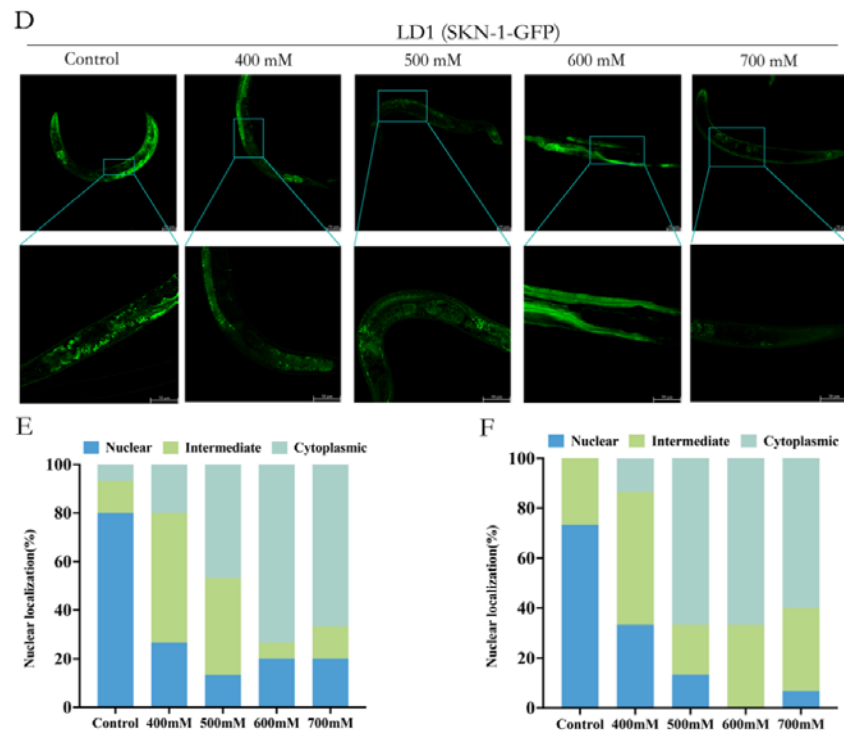


Figure 7. *Hsf-1/skn-1* pathway involved in alcohol-induced ferroptosis in *C. elegans*. (A) mRNA transcription levels of *Hsf-1*; (B) mRNA transcription levels of *Skn-1*; (C) Fluorescence image of CF1824 (HSF-1-GFP) expression upon different concentration of alcohol treatment; (D) Fluorescence image of LD1 (SKN-1-GFP) expression upon different concentration of alcohol treatment; (E) Relative fluorescence intensity of HSF-1-GFP expression; (F) Relative fluorescence intensity of SKN-1-GFP expression. Values are presented as mean $\pm$ SD. * $P < 0.05$ vs Control, ** $P < 0.01$ vs Control.

3.8 The effect of *Fer-1* and *DFO* on phenotype and movement in alcohol-exposed *C. elegans*

Fer-1 is the potent ferroptosis inhibitor and *DFO* is the potent iron chelating. The nematodes were treated with *Fer-1* (100 μ mol/L) or *DFO* (50 μ mol/L) and/or 500 mM alcohol. The results shown that *Fer-1* and *DFO* could partially save the shorten lifespan induced by alcohol by 21% and 24.4%, respectively (Figure 8A, Table 2). Meanwhile, the effete of *Fer-1* or *DFO* on *C. elegans* phenotype and movement were also investigated. As shown in Figure 8B-D, *Fer-1* or *DFO* abolish the toxic impacts of alcohol exposure on the body bends, head thrashes and pump pharynx of the N2 nematodes. Besides, *Fer-1* and *DFO* significantly reverse alcohol-induced decrease of the body width and body length of nematodes (Figure 8E, G, H). Moreover, nematode motion trajectories in the different treatment groups were assessed, and the results shown that the alcohol-induced nonuniform and non-coordinated trajectory was also improved by *Fer-1* and *DFO* (Figure 8F, I). These results suggested that the shortened lifespan, physiological phenotype and movement disorders of nematodes induced by alcohol are related to ferroptosis, and inhibiting ferroptosis may significantly relieve alcohol-induced injury in nematodes.

Table 2. Lifespan of N2 with Different Treatments

Treatment	Median survival	Mean lifespan	Maximum lifespan	Effect (%)
Control	11	8 $\pm$ 0.9	18	
500 mM	4	5 $\pm$ 0.7**	11	-41.7%
<i>Fer-1</i>	6	7 $\pm$ 0.03**	15	-21%
<i>DFO</i>	8	6 $\pm$ 0.73**	16	-24.4%

Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control. *** $P < 0.01$ vs Alcohol.

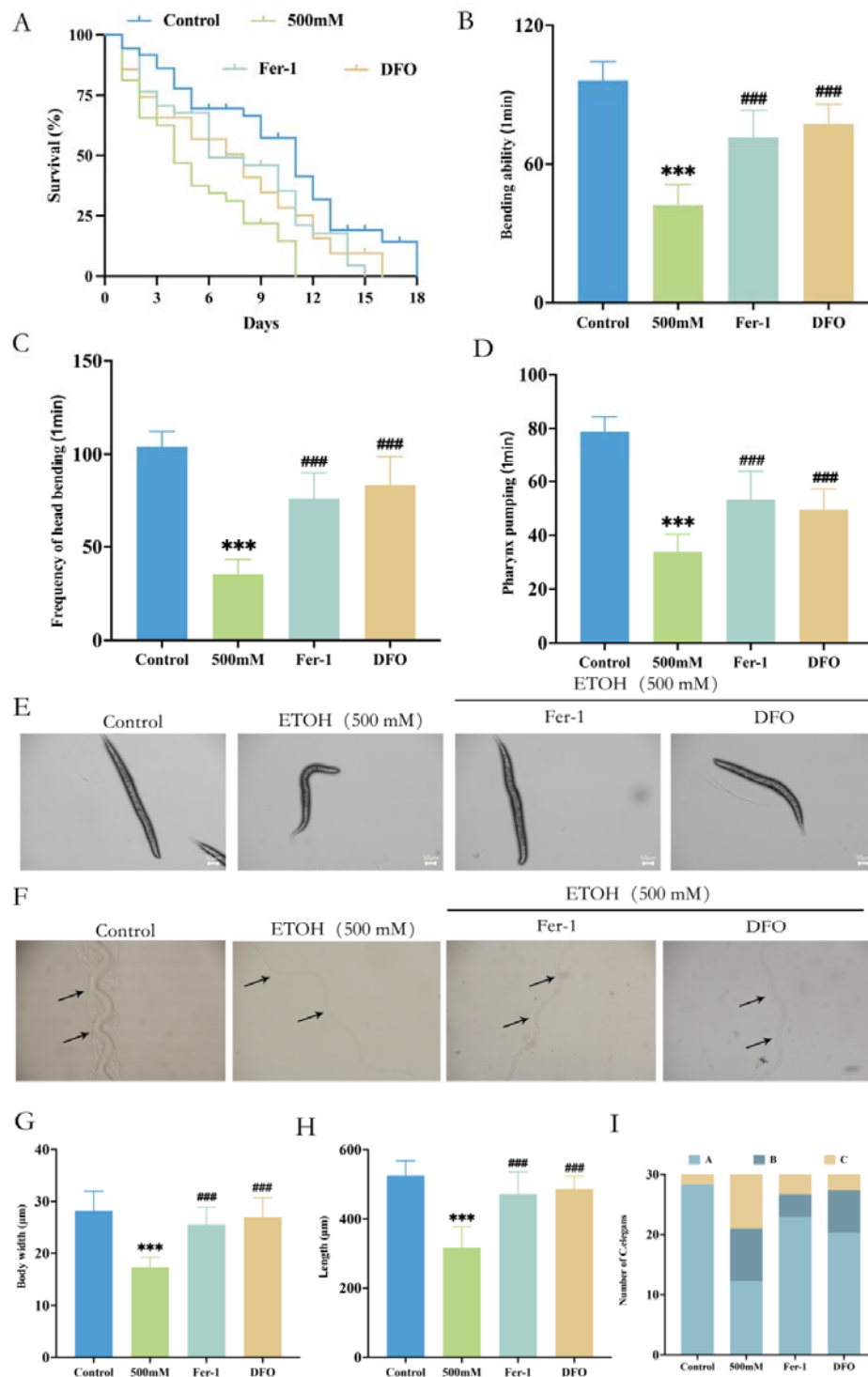
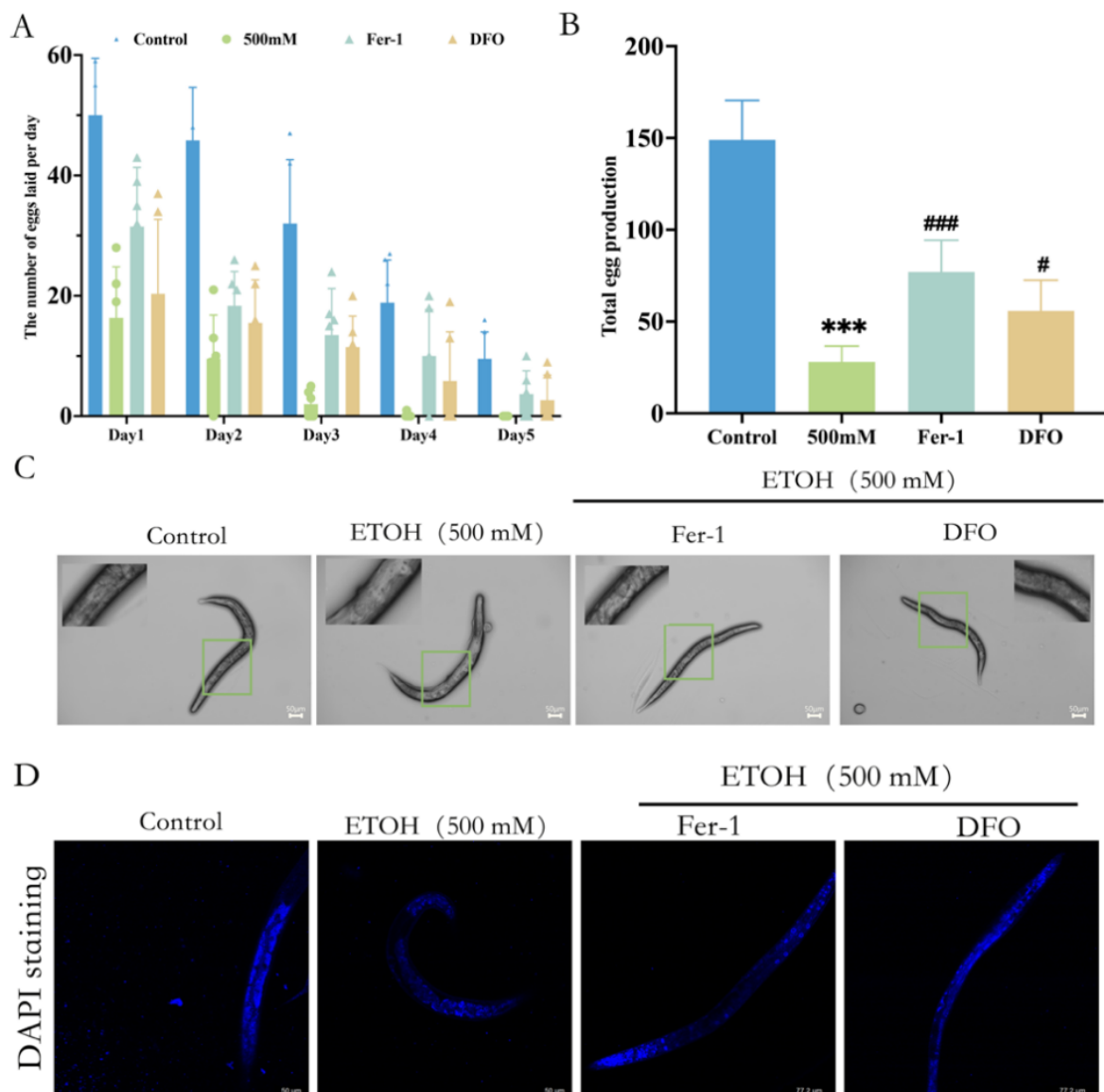


Figure 8. The effect of Fer-1 and DFO on phenotype and movement in alcohol-exposed *C. elegans*. (A) Effect of Fer-1 and DFO on the lifespan of *C. elegans*; (B) Effect of Fer-1 and DFO on frequency of head bending of *C. elegans*; (C) Effect of Fer-1 and DFO on pharynx pumping of head bending of *C. elegans*; (D) Effect of Fer-1 and DFO on bending ability of *C. elegans*; (E) Effect of alcohol on body size of *C. elegans*; (F) Effects of Fer-1 and DFO on locomotion trajectory of *C. elegans*. (G) Effect of Fer-1 and DFO on body length of *C. elegans*; (H) Effect of Fer-1 and DFO on body width of *C. elegans*; (I) Effects of Fer-1 and DFO on locomotion trajectory of *C. elegans*. Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control. ### $P < 0.01$ vs Alcohol.

3.9 Effects of Fer-1 and DFO on reproductive toxicity in alcohol-exposed *C. elegans*

Next, the effect of Fer-1 and DFO on reproductive toxicity in nematodes exposed to alcohol was evaluated. As shown in Figure 9A, alcohol-decreased eggs laid were all reversed by Fer-1 and DFO from days

1 to days 5. Especially from the days3, the alcohol exposure group almost stopped egg-laying, while the egg-laying quantity of the nematodes was significantly improved upon the intervention of Fer-1 and DFO and could continue to egg-lay for 5 days. Similarly, Fer-1 and DFO also abolished the decreased total egg production induced by alcohol (Figure 9B). Meanwhile, alcohol-stimulated Pvl was reversed by Fer-1 and DFO, from 75% to 40% and 35%, respectively (Figure 9 C,G). We further adopted the visualization treatment of the germ cells of nematodes via DAPI staining and the results shown that the decreased nuclei in the mitotic region intercellular arrangement spaces were improved by Fer-1 and DFO, manifested as significantly increased fluorescence intensity (Figure 9D, F). Moreover, alcohol-stimulated high membrane permeability was counteracted by Fer-1 and DFO supplement in *C. elegans* (Figure 9E). These results above suggested that alcohol-induced reproductive toxicity is closely related to ferroptosis.



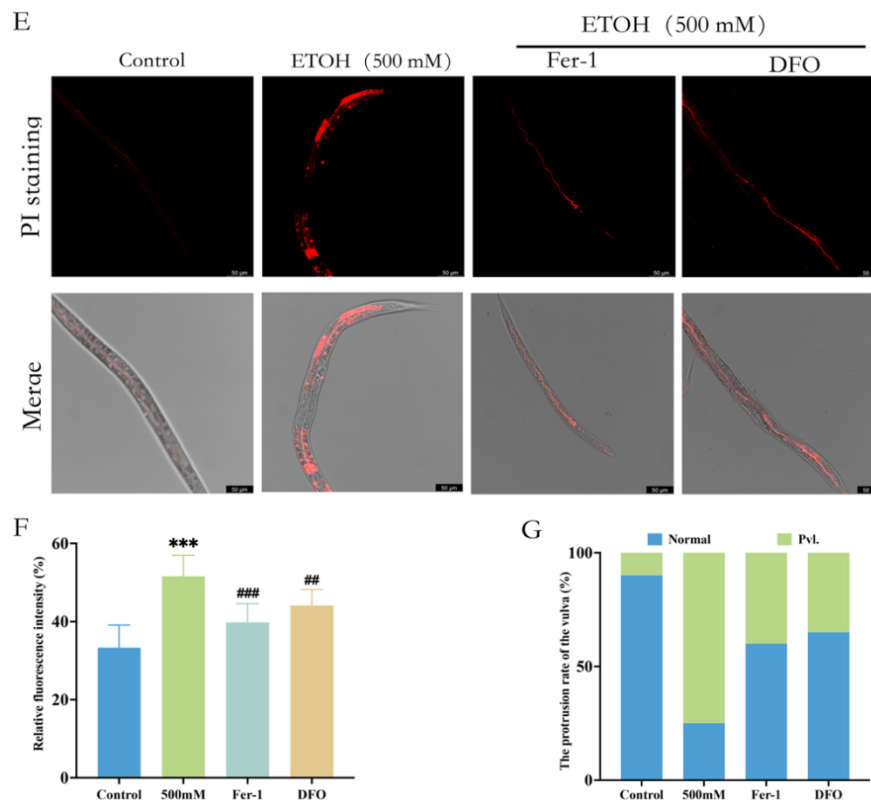


Figure.9 Effects of Fer-1 and DFO on reproductive toxicity in alcohol-exposed *C. elegans*. (A) The effect of Fer-1 and DFO on the number of eggs laid per day; (B) The effect of Fer-1 and DFO on total eggs production; (C) The effect of Fer-1 and DFO on vulval protrusion in *C. elegans*; (D) Representative images of DAPI staining in *C. elegans*; (E) Representative images of PI staining in *C. elegans*; (F) Relative fluorescence intensity of DAPI staining; (G) Relative fluorescence intensity of PI staining. Values are presented as mean $\pm$ SD. ** $P < 0.01$ vs Control, # $P < 0.05$ vs Alcohol, ### $P < 0.01$ vs Alcohol.

3.10 Effects of Fer-1 and DFO on oxidative stress in alcohol-exposed *C. elegans*

We detected the level of antioxidant enzymes SOD and CAT upon Fer-1 and DFO supplement, as well as the production of lipofuscin and ROS (Figure 10A-F). Compared with the control group, the antioxidant enzymes SOD and CAT in the alcohol group were significantly decreased, while supplement with Fer-1 and DFO significantly reversed the occurrence of this situation. Then, the generation of lipofuscin in nematodes was observed. Compared with the control group, the accumulation of lipofuscin in the alcohol-treated nematodes increased significantly, and the accumulation of lipofuscin decreased after the intervention of Fer-1 and DFO. Moreover, the production of ROS in nematodes was observed under a fluorescence microscope. Consistent with expectation, the increased ROS accumulation in the alcohol group was reduced after Fer-1 or DFO intervention. The above results indicate that ferroptosis inhibitors can alleviate oxidative stress in nematodes exposed to alcohol, thereby reducing the occurrence of ferroptosis.

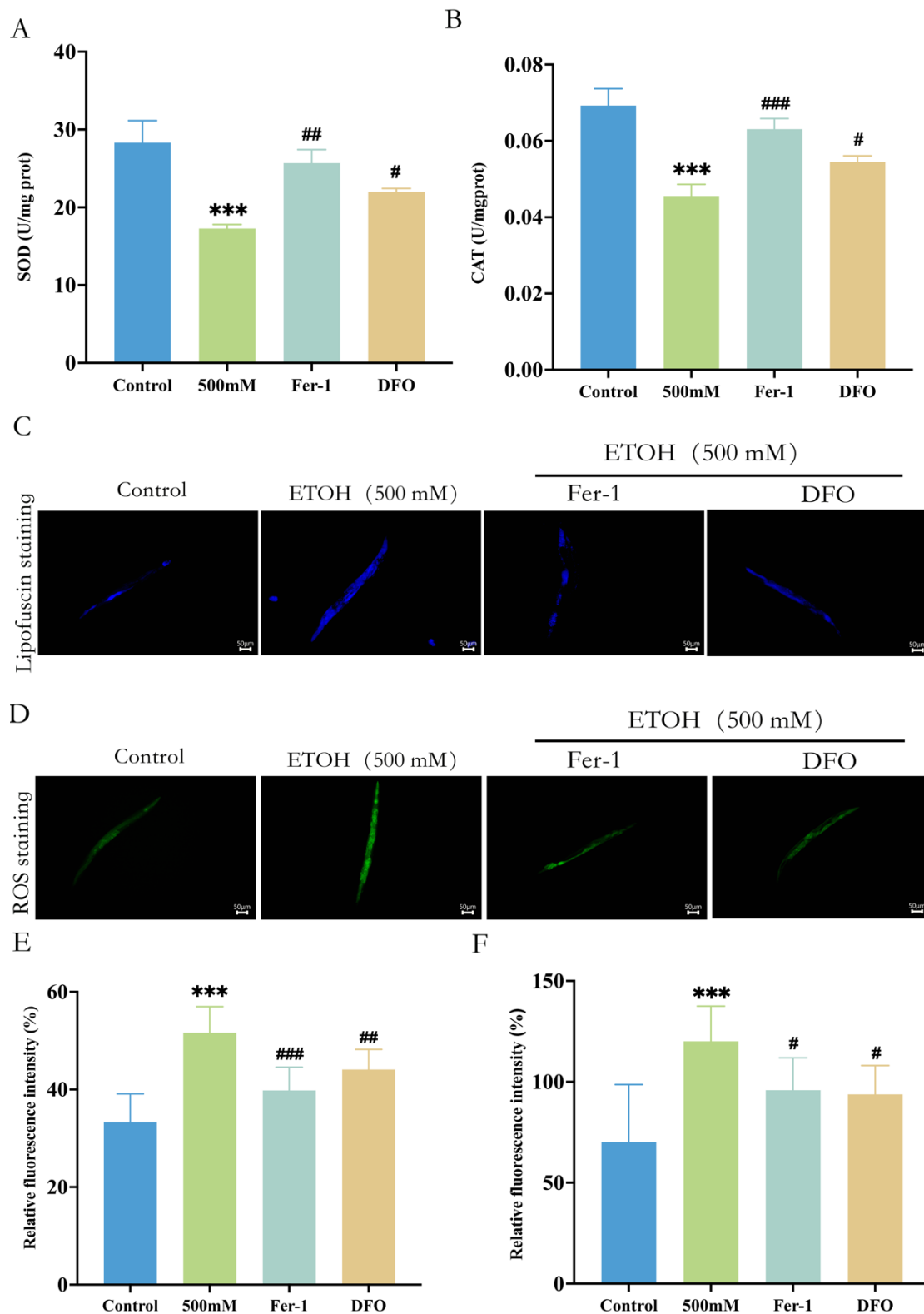


Figure 10. Effects of Fer-1 and DFO exposure on oxidative stress in alcohol-exposed *C. elegans*. (A) The effect of Fer-1 and DFO on the content of SOD; (B) The effect of Fer-1 and DFO on the content of CAT; (C) Representative images of lipofuscin fluorescence in *C. elegans*; (D) Representative images of ROS staining in *C. elegans*; (E) Relative fluorescence intensity of lipofuscin staining; (F) Relative fluorescence intensity of ROS staining. Values are presented as mean $\pm$ SD. ** P < 0.01 vs Control, # P < 0.05 vs Alcohol, ### P < 0.01 vs Alcohol.

3.11 The effect of Fer-1 and DFO on iron metabolism and ferroptosis in alcohol-exposed *C. elegans*

Fer-1 and DFO were used to detect iron metabolism-related indicators in alcohol-exposed nematodes. The decreased expression of *Ftn-1*, *Fpn.1-1* as well as increased expression of *Smf-3* caused by alcohol were

all reversed by Fer-1 and DFO (Figure 11A-C). We further focus on alcohol-induced ferroptosis. As expected, the significantly increased contents of Fe^{2+} , MDA and decreased content of GSH in the alcohol group were all reversed upon Fer-1 and DFO administration (Figure D-F). The Caclein-AM staining was also used to detect the Fe^{2+} level by the fluorescence intensity, which consist with the result obtained above (Figure J). Meanwhile, ferroptosis vital biomarkers *Gpx-1*, *Aat-9* and *Acs-17* were also be detected and found that Fer-1 and DFO improved the abnormal changes of these three genes induced by alcohol (Figure G-I). The above results indicate that alcohol-caused iron metabolism and ferroptosis could be restored by Fer-1 and DFO in *C. elegans*.

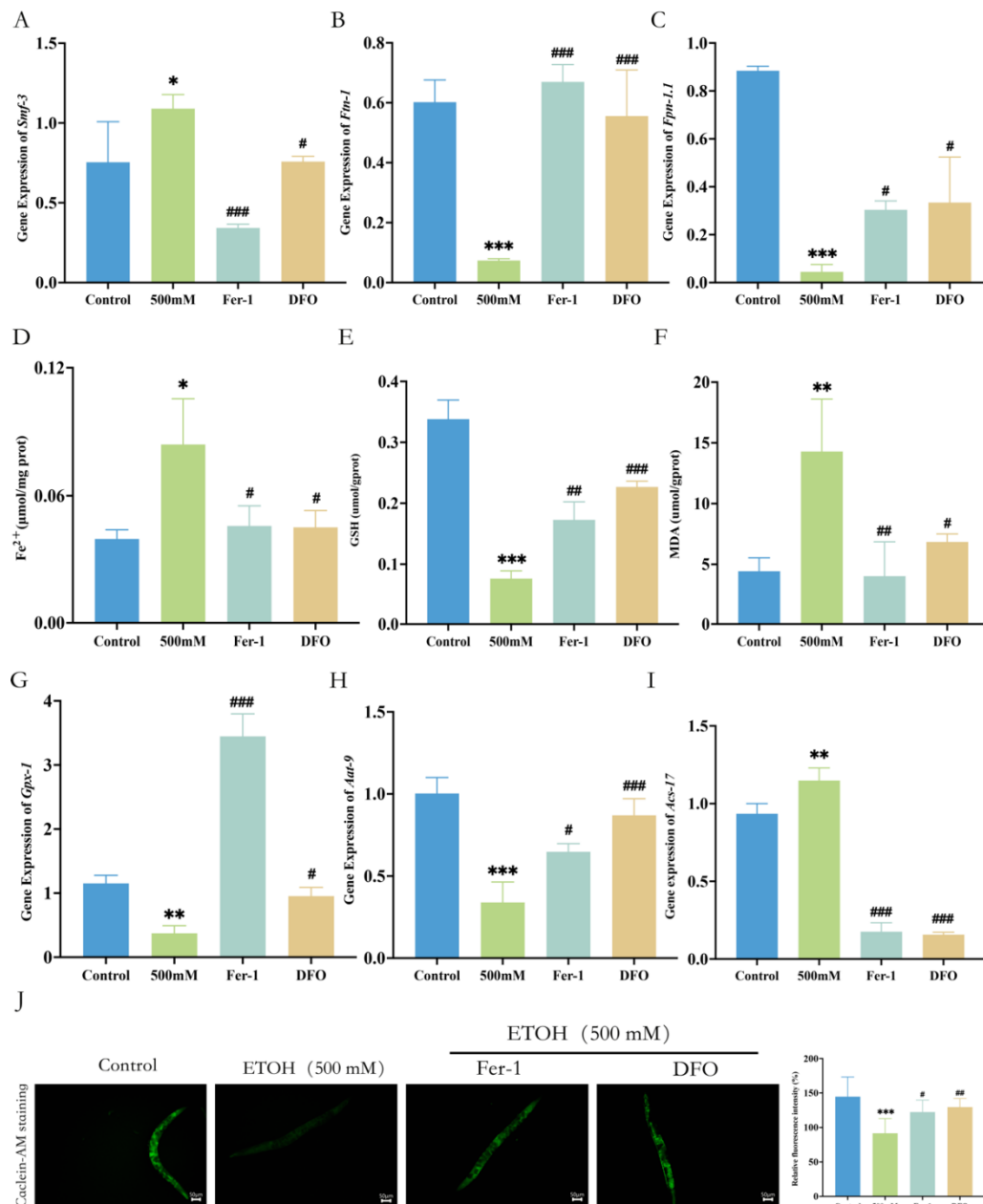
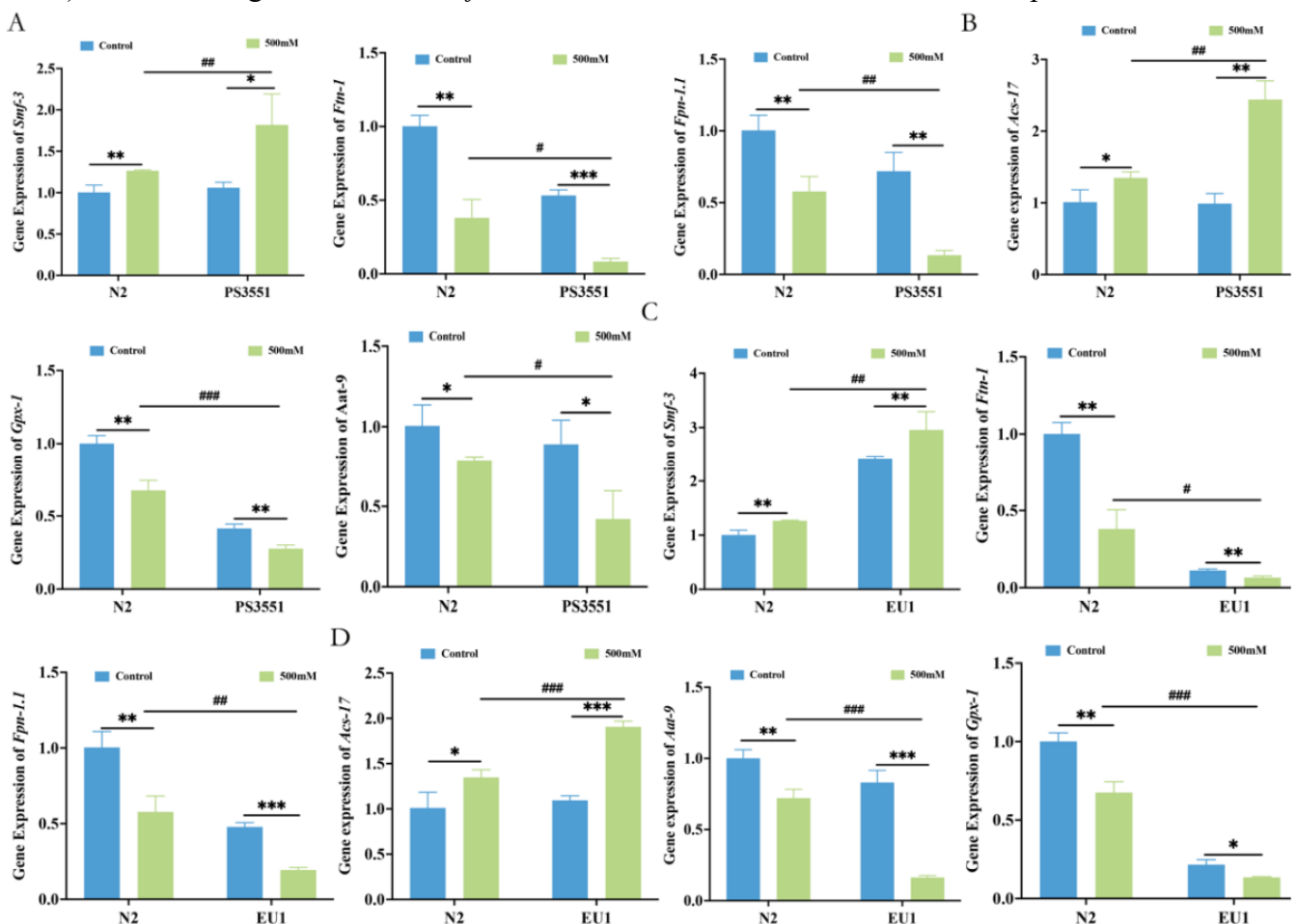


Figure 11. The effect of Fer-1 and DFO on iron metabolism and ferroptosis in alcohol-exposed *C. elegans*. (A) mRNA transcription levels of *Smf-3*; (B) mRNA transcription levels of *Ftn-1*; (C) mRNA transcription levels of *Fpn-1.1*; (D) The effect of alcohol on the content of Fe^{2+} in *C. elegans*; (E) The effect of Fer-1 and DFO on the content of GSH in *C. elegans*; (F) The effect of Fer-1 and DFO on the content of MDA in *C. elegans*; (G) mRNA transcription levels of *Gpx-1*; (H) mRNA transcription levels of *Aat-9*; (I) mRNA transcription levels of *Acs-17*; (J) Representative images of Caclein-AM staining in *C. elegans*. Values are presented as mean $\pm$ SD. * P < 0.05 vs Control, ** P < 0.01 vs Control, # P < 0.05 vs Alcohol, ## P < 0.01 vs Alcohol.

3.12 Alcohol induced ferroptosis by suppressing *hsf-1/skn-1* pathway in *C. elegans*

To investigate the molecular pathways involved in alcohol-induced ferroptosis, we conducted epistasis experiments to evaluate the contributions of *hsf-1* and *skn-1*. We focused on the strain PS3551 and EU1, which carrying a missense mutation that produces a truncated, nonfunctional HSF-1 protein and NRF2, respectively. As shown in Figure 12 A-C, alcohol-induced iron metabolism dysfunction, manifested as increased gene expression of *Smf-3* as well as decreased gene expression of *Fpn-1.1* and *Ftn-1*, were all enhanced in these two mutants. Meanwhile, genes expression of ferroptosis markers *Gpx-1*, *Aat-9* and *Acs-17* were also detected. Compared with the wild-type nematodes, alcohol-exposure nematodes of PS3551 and EU1 significantly reduced the level of *Gpx-1* and *Aat-9*, while promote the level of *Acs-17* (Figure 12 D-F). Besides, the level of iron as well as ROS, two vital markers of ferroptosis, were also investigated. As expected, alcohol-induced iron overload was further intensified in strain PS3551 and EU1, manifested as lower fluorescence intensity of Caclein-AM staining (Figure 12 M-O). Meanwhile, ROS accumulation caused by alcohol also be enhanced in two mutant strain, as shown by higher fluorescence intensity (Figure 12 P-R). Furthermore, the alcohol-surged content of MDA as well as decreased content of GSH were also aggravated in two mutant strain (Figure 12 T-V). These findings indicate that *hsf1/skn-1* is essential for alcohol-induced ferroptosis.



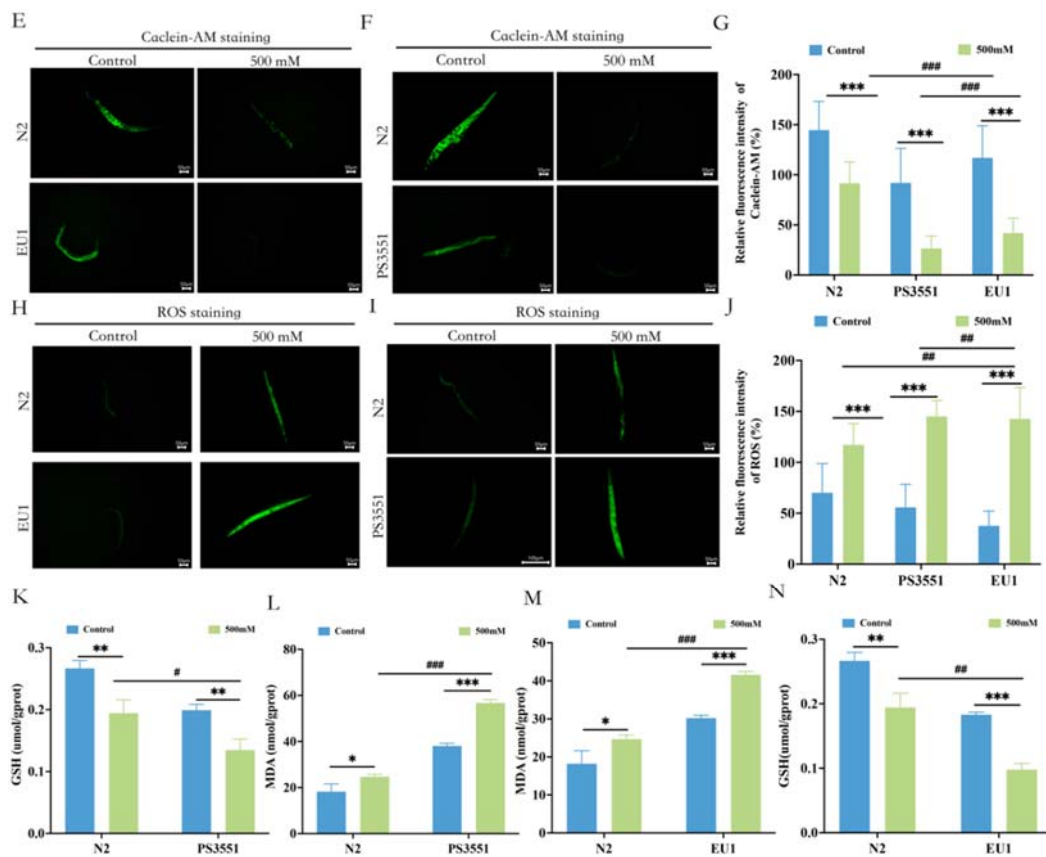


Figure 12. Alcohol induced ferroptosis by suppressing *hsf-1/skn-1* pathway in *C. elegans*. (A) mRNA transcription levels of iron metabolism-related genes in *C. elegans* of N2 and PS3551; (B) mRNA transcription levels of ferroptosis-related genes in *C. elegans* of N2 and PS3551; (C) mRNA transcription levels of iron metabolism-related genes in *C. elegans* of N2 and EU1; (D) mRNA transcription levels of ferroptosis-related genes in *C. elegans* of N2 and EU1; (E) C. elegans fluorescence staining in *C. elegans* of N2 and EU1; (F) C. elegans fluorescence staining in *C. elegans* of N2 and PS3551; (G) Relative fluorescence intensive of C. elegans staining; (H) ROS fluorescence staining in *C. elegans* of N2 and PS3551; (I) ROS fluorescence staining in *C. elegans* of N2 and EU1; (J) Relative fluorescence intensive of ROS staining; (K) The content of GSH in *C. elegans* of N2 and PS3551; (L) The content of MDA in *C. elegans* of N2 and PS3551; (M) The content of GSH in *C. elegans* of N2 and EU1; (N) The content of MDA in *C. elegans* of N2 and EU1.

4. Discussion

Ferroptosis is recognized as a distinct form of programmed cell death that relies on iron, setting it apart from other forms of cellular demise^[29,30]. This process is marked by oxidative damage that is mediated by iron, leading to the formation of free radicals and the subsequent build-up of lipid peroxides within the cell. At the heart of the ferroptosis mechanism lies the role of ferrous iron, which plays a crucial role in catalyzing the peroxidation of polyunsaturated fatty acids (PUFAs) found in cell membranes^[31]. The present study investigated the potential toxic impacts, and the underlying mechanisms of alcohol-caused ferroptosis by hindering iron metabolism function via HSF1/NRF2 pathway in *C. elegans*.

Exposure to environmental contaminants may modify numerous physiological functions in organisms. Exposure to alcohol significantly shortened the lifespan of *C. elegans*, corroborating previous findings from Wu et al^[24] who found that the median lifespan of 3% of alcohol-treated nematodes was 10 days, and all alcohol-stimulated nematodes were dead by day 12. *C. elegans* exhibit a range of behaviors such as head thrash, body bending and pharyngeal pumping. In our study, we investigated the effects of alcohol on these behaviors in *C. elegans* and noted significant behavioral inhibitions, including decreased head thrash, body bending, and

pharyngeal pumping. To confirm the role of ferroptosis in alcohol-caused damage, two small molecular ferroptosis inhibitors were used. Specifically, the antioxidant mechanism of Fer-1 is to directly eliminate lipid free radicals and block the spread of lipid peroxidation, thereby protecting the integrity of the cell membrane^[32]. The antioxidant mechanism of DFO is through chelating iron ions to form a stable hexacoordination complex, reducing the occurrence of Fenton reactions catalyzed by Fe^{2+} within cells, thereby reducing the production of ROS^[33]. Interestingly, these behavioral inhibitions were restored by the Fer-1 and DFO, indicating the effect of iron overload and ferroptosis in alcohol-induced shorten lifespan and abnormal locomotion behavior.

Research indicated that ferroptosis is also linked to the induction of reproductive toxicity in organisms exposed to various pollutants^[34–36], and a rise in sterility was often observed alongside the activation of ferroptosis in *C. elegans*. The spawning period of nematodes is a crucial stage in their reproductive cycle, involving oocyte maturation, fertilization, eggshell formation and the spawning process. Egg-laying mainly occurs during the adult stage, with the peak egg-laying period being 2 to 3 days (approximately 48 to 72 h) before the adult stage and continuously lay eggs for approximately 4 to 5 days. It is reported that alcohol at low concentration (0.5%-1%) had a relatively small impact on the number of eggs laid. In this study, nematodes exposed to alcohol at 400 mM-700 mM significantly affect the reproductive system of nematodes.

To determine whether the death of individual cells in the *C. elegans* model occurs before the death of the organism and the relay process of cell death within the organism, we used PI staining to observe the dead cells in the organism during the alcohol exposure. PI is a fluorescent staining agent that can bind to DNA but cannot pass through the membranes of living cells, thus enabling the identification of the nuclei of recently dead or dying cells^[37]. We found that alcohol exposure significantly induced cell death of *C. elegans*, especially in intestinal cell. In order to further verify the effect of alcohol on inducing cell death in *C. elegans*, nematodes were stained by DAPI to visualize germ cells in nematodes' gonad. Fertile nematodes have gonads and eggs visible in their bodies, while non-fertile nematodes have neither gonads nor eggs developed. Consistent with the result of PI staining, alcohol also causes nematodes sterility in a dose-dependent. As expected, Fer-1 and DFO significantly improve alcohol-stimulated reproductive toxicity.

The “free radical theory” posits that the primary underlying factor behind numerous diseases is the detrimental impact of ROS on cellular constituents, and ROS accumulation has been proved to be responsible for the iron mediated toxicity^[38]. Lipofuscin is an endogenous intestinal autofluorescent substance that accumulates over time in cells due to oxidative damage and accumulation of metabolic waste^[39]. Lipofuscin content is closely related to ROS production^[40]. Damaged mitochondria impair the ability to degrade lipofuscin of cells. The accumulation of lipofuscin ultimately makes organic more susceptible to oxidative stress to promote the level of ROS. Currently, lipofuscin and ROS can be slowed by a variety of drugs of antioxidants or free radical scavengers but are not able to be removed completely from the cell. Alcohol led to excessive accumulation of ROS and lipofuscin in *C. elegans*, potentially accelerating damage through pro-oxidative effects. However, Fer-1 or DFO alleviated the overproduction of ROS and lipofuscin induced by

alcohol. SOD and CAT are two key antioxidant enzymes that jointly participate in the elimination of ROS and maintain the redox balance of cells. We evaluated the activities of two important antioxidant enzymes in nematodes after the action of alcohol at different concentrations. Similar to the results of ROS and lipofuscin, alcohol reduced the level SOD and CAT, which reversed by Fer-1 and DFO, respectively.

Ferroptosis is a novel form of iron-dependent programmed cell death^[29,30]. The accumulation of Fe^{2+} is one of the hallmarks for ferroptosis^[35]. We observed significantly iron accumulation after alcohol exposure by Caclein-AM staining and iron commercial kit. Iron metabolism depends on a series of iron regulatory proteins which responsible for iron uptake, iron storage and iron efflux. In ferritin pathway, ferritin acting as an iron storage protein inhibits Fenton reaction by converting Fe^{2+} into Fe^{3+} . It is reported that exposure to 6-PPDQ caused toxicity and ferroptosis in *C. elegans* by suppressing *ftn-1* gene level^[36]. Similarly, the observed decrease in *ftn-1* expression in alcohol exposed nematodes. *Smf-3*, a homologue to the human divalent metal transporter (DMT-1), as a functional null mutation partially inhibits the cell death by regulating ferrous iron uptake, and numerous studies have identified the role of *Smf-3* in iron metabolism and ferroptosis. Peng et al^[41] found that DMT1 knockout abolishes neuronal $\text{A}\beta$ -associated pathologies by reducing neuronal iron uptake and ferroptosis; praeruptorin A has been proved to suppress doxorubicin-induced cardiomyopathy by regulating DMT1-mediated ferroptosis^[42]. Our results indicated that alcohol caused significantly increase of gene level of *Smf-3*. In addition, we also detected the gene level of *fpn-1.1* which responsible for iron efflux, and the results presented a significantly decrease upon alcohol exposure, which consistent with the results obtained from our previous experiments in cells and animals (not published yet). Based on the findings of this study, it was determined that alcohol stimulates iron homeostasis imbalance in *C. elegans*.

GPX4 is an essential enzyme that plays a crucial role in countering oxidative stress by converting liposomal peroxides, particularly lipid hydro-peroxides, into lipid alcohols inside cells. This transformation is vital as it helps avert the formation of harmful ROS triggered by elevated Fe^{2+} levels. GSH, the predominant antioxidant found in cells, aids in neutralizing ROS through the catalytic action of GPX4. Additionally, the vulnerability of cells to ferroptosis is simultaneously influenced by both the activity of GPX4 and the concentration of GSH^[43]. SLC7A11, a vital transporter, contribute to transportation of cysteine into cells, and lacking SLC7A11 reduce the raw materials for the synthesis of GSH, lowering the synthesis of GPX4, and ultimately inducing ferroptosis. Consequently, a decrease in GPX4 results in the buildup of lipid peroxides, increasing the vulnerability of cells to ferroptosis^[44]. *Gpx-1* and *Aat-9* is the homologs of GPX4 and SLC7A11 in *C. elegans*. In the study, expression of *gpx1* and *aat-9* as well as GSH content were decreased after alcohol exposure, implying that the activities of GPX4 and GSH were important mechanisms of alcohol-induced ferroptosis in *C. elegans*. ACSL4 has been recognized as an essential isoenzyme in the metabolism of PUFAs, playing a pivotal role in facilitating ferroptosis. This is achieved through its involvement in the biosynthesis of arachidonoyl-CoA and the initiation of phospholipid peroxidation. The activation of ACSL4 leads to an increase in lipid peroxidation linked to ferroptosis^[45]. *Acs-17* is a homolog of ACSL4 in *C. elegans*, and we observed a significant upregulation of *Acs-17* expression in nematodes exposed to alcohol. Simultaneously, a

notable increase of MDA content was detected after alcohol exposure, indicating the severe lipid peroxidation.

HSF-1 safeguards the proteome by regulating the production of heat shock proteins (HSPs), which act as molecular chaperones^[46]. Consistent with this role, reducing the levels of HSF-1 results in heightened protein aggregation, impaired tissue function, and reduced survival rates. In contrast, enhancing HSF-1 expression supports the integrity of the proteome, fosters tissue health, and contributes to a longer lifespan. Research indicates that HSF-1 facilitates longevity through the remodeling of the mitochondrial network in a ubiquitin-1-dependent manner^[47]. Moreover, increased activity of HSF-1 is associated with enhanced longevity in fasted *C. elegans*^[48]. *Skn-1* transcription factor plays a crucial role in the cellular response to oxidative stress, and disrupting the *skn-1*-mediated stress response mechanism increases a series of damage, including ferroptosis, which reduces lifespan. Qian et al^[49] investigated the effect of isobavachalcone on *Cryptococcus neoformans*-induced ferroptosis in *C. elegans* and found that targeting inhibiting *skn-1* may negate the positive effect of isobavachalcone, indicating the vital role of *skn-1* mediated GSH-GPX-1 axis upon ferroptosis. In this study, alcohol down-regulated the expression of *hsf-1* and *skn-1*, indicating a suppression of HSF1 and NRF2 synthesis in *C. elegans* due to alcohol exposure. Moreover, alcohol-caused decrease of gene level of *hsf-1* and *skn-1* were exacerbated in mutation strain *C. elegans*, revealing the association between alcohol-caused ferroptosis activation and HSF1/NRF2 pathway.

This study brings groundbreaking viewpoints to damage mechanisms of food-borne iron toxicity in *C. elegans*. Alcohol-related iron toxicity suppressed head thrash, pharyngeal pumping in *C. elegans*, generated reproductive toxicity and oxidative stress, while rescued by Fer-1 and DFO. Meanwhile, alcohol resulted in increased Fe²⁺ levels due to iron metabolism dysfunction, inducing promoting iron uptake by increasing gene level of *Smf-3*, suppressing iron storage and efflux by decreasing gene level of *Ftn-1* and *Fpn-1.1*. Besides, alcohol caused increased MDA content, decreased GSH content, as well as alterations in the expression *Gpx-1*, *Ftn-1*, and *Acs-17*, facilitating ferroptosis. Moreover, alcohol-caused shorten lifespan, abnormal physiological state, reproductive toxicity as well as disturbed iron homeostasis and ferroptosis were all exacerbated in *hsf-1* and *skn-1* mutation *C. elegans*. The study offers novel insights into the mechanisms of alcohol-induced ferroptosis in *C. elegans*.

Supporting information statement

Table S1 The primer information

Declaration of competing interest

The authors declare that there are no conflicts of interest to declare.

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