

# Cigarette smoking is associated with abnormal ferroptosis in human sperm

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## Research

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# Abstract

**Purpose** The effects of cigarette smoking on male semen quality are controversial, and the molecular mechanisms underlying how cigarette smoking affects semen quality are not clear yet. **Methods** In this study, semen samples from 70 heavy smokers and 75 non-smokers receiving infertility treatment were included. Basic semen parameters in non-smokers and heavy smokers were evaluated. Levels of ferroptosis glutathione (GSH), lipid reactive oxygen species (ROS), iron and GSH-dependent peroxidase 4 (GPX4) protein level) were observed in human semen and in GC-2 Spd cells exposed to cigarette smoke condensate (CSC). **Results** Heavy smokers had significantly higher abnormalities (sperm viability and sperm progressive motility) than non-smoking counterparts. Comparing non-smokers group, GSH level was reduced in the group of heavy smokers ( $P<0.05$ ). However, the level of lipid ROS and iron were significantly increased ( $P<0.05$ ). Besides, GSH level was reduced following treatment with CSC for 24 h, while lipid ROS and iron levels were increased ( $P<0.05$ ). However, the levels were reduced after being co-cultured with Ferrostatin-1 (Fer-1) ( $P<0.05$ ). The level of GPX4 protein was reduced after being treated with CSC in 24 h, and increased after being co-cultured with Fer-1 ( $P<0.05$ ). **Conclusion** Cigarette smoking is associated with abnormalities of ferroptosis in human sperm.

## Introduction

Infertility affects 10–15% of couples worldwide and has become a public health problem in recent years [1]. Male infertility accounts for approximately half of these problems, and decreased semen quality has been widely reported in recent years [2, 3]. Cigarette smoking is considered to be a risk to male infertility as it could be directly and indirectly toxic to spermatogenesis [4–6]. Even though the reason for the decline of semen quality remains unknown, the correlation between smoking and semen quality has been reported in many studies [7–11]. However, the effect of cigarette smoking on sperm quality reported by some studies was shown to be limited [12, 13]. Therefore, the effect of cigarette smoking on semen quality and its mechanism needs to be clarified.

Cigarette smoke contains high concentrations of nitric oxide, peroxynitrite, and free radicals which can potentially induce the production of cellular reactive oxygen species (ROS) in the human body [12, 13]. Thus, this might be the main reason for the decreased semen quality caused by cigarette smoking, as some studies have also reported [4, 16]. Moreover, it has also been reported that the basic semen parameters could be influenced by cigarette smoking [17–19]. Based on these findings, we believe that cigarette smoking could impair the sperm quality by the generation of ROS, but what is its potential mechanism?

Ferroptosis is an identified and non-apoptotic regulated cell death with dependency of iron and lipotoxicity, which is firstly observed in cancer cells with oncogenic Ras mutation and could be induced by erastin [20]. Ferroptosis occurs depending on the process of lipid peroxidation activated by non-enzymatic (Fenton reactions) and enzymatic mechanism (lipoxygenases) [21]. It is characterized by a production of reactive oxygen species from accumulated iron and lipid peroxidation [22]. Ferroptosis can

be induced in the classic way of the inactivation of GPX4, which is the major protector of cellular peroxidation damage [23]. One of the main mechanisms of inactivation of GPX4 is the deprivation of glutathione (GSH) [23]. It can be said that the likelihood of ferroptosis is determined by the balance between ROS production and peroxidation-antioxidant system [24]. This imbalance can result in a number of diseases, including hemochromatosis, neurotoxicity, cancer, liver injury and so on [22, 25–27]. Furthermore, Li et al showed ferroptosis was a pervasive and dynamic type of cell death induced by oxygen-glucose deprivation and reoxygenation injury in Sertoli cells [22, 25–27], Bromfield also showed oxidative stress induces ferroptosis in spermatids [29]. In summary, we hypothesize that cigarette smoke might reduce the semen quality via the inducing lipid peroxidation and the further occurrence of ferroptosis.

In this study, we first collected the semen samples from heavy smokers and non-smokers and evaluated the semen quality via comparing the parameters. Moreover, the occurrence of ferroptosis was also detected in semen and GC-2spd cell line after the treatment of cigarette smoke condensate, while also detecting the level of GPX4 in the GC-2spd cell, which is the key regulator of ferroptosis. We aim to find out the potential mechanism of the sperm impairment caused by cigarette smoking.

## **Materials And Methods**

### **Study subjects**

This study was declared exempt by the ethics committee of the Guangzhou Women and Children's Medical Center. All studies involving human subjects were conducted in accordance with guidelines laid down in the Declaration of Helsinki. Written informed consents were obtained by the participants, and the subjects' data on medical history, lifestyle, and smoking status with a structured questionnaire were collected. Individuals who had never smoked were defined as non-smokers. Current smokers with smoking dose more than 1 pack per day over 10 years or smoking dose more than 2 packs per day over 5 years were defined as heavy smokers. They were men who visited the reproductive medicine center in the Guangzhou Women and Children's Medical Center for infertility treatment during March 2017 to December 2017. Non-smokers were randomly selected from patients who were age-matched to heavy smokers. All subjects underwent physical examinations and at least two semen analyses. Men who were unhealthy or had a known cause of defective spermatogenesis, such as varicocele, infection, obstruction of the vas deferens, chromosomal abnormalities, or microdeletions of the azoospermia factor region on the Y chromosome were excluded. Patients who were diagnosed with azoospermia, severe oligozoospermia (sperm concentration  $< 5 \times 10^6$  cells/mL), hemospermia, leukospermia, and necrozoospermia were also excluded. Finally, 70 heavy smokers and 75 non-smokers were recruited.

### **Semen Collection And Analysis**

Semen samples were collected in sterile containers from patients by masturbation after 2–7 days of sexual abstinence. Samples were allowed to liquefy for at least 30 min at room temperature. Analysis of semen volume, pH, sperm concentration, motility, vitality, sperm morphology, and computer-assisted semen analysis were carried out according to WHO guidelines [30]. Samples were centrifuged at 1000 ×g for 10 min, and seminal plasma and cell pellets were separated and

stored at -80 °C until analysis.

## **Cell Culture And Cigarette Smoke Condensate Treatment**

GC-2spd cell lines were purchased from the American Tissue Culture Collection (ATCC) and maintained in Dulbecco modified Eagles media (DMEM, Gibco, USA) supplemented with heat-inactivated 10% fetal bovine serum (Biological Industries, Israel) in a 95% air-humidified incubator with 5% CO<sub>2</sub> at 37 °C as our present study [31].

Cigarette smoke condensate (CSC) was prepared according to the study [32] and resuspended at a concentration of 50 mg/mL in dimethyl sulfoxide (DMSO) as stock solution. For smoke-exposure experiments, cells were cultured in medium with 400 µg/mL of CSC. After exposure to CSC for 24 hours, cells were used for further study.

## **Cell Viability Assay**

Cell viability was determined with a Cell Counting Kit-8 (CCK-8) assay. Briefly, cells were plated at a density of  $5 \times 10^4$  cells/well in a 96-well plate with 100 µl of medium. Following treatment, cell viability was evaluated with CCK-8 according to manufacturer's instructions, and absorbance was measured at 450 nm with a microplate reader. Cell viability was expressed as the percentage of live cells vs. controls (set at 100%).

## **Iron Assay**

The relative iron concentration in cell lysates was assessed using the Iron Assay Kit (no. ab83366; Abcam) according to the manufacturer's instructions and our present study [33].

## **Lipid Peroxidation Assay**

The relative malondialdehyde (MDA) concentration in cell lysates was assessed using a Lipid Peroxidation (MDA) Assay Kit (no. ab118970; Abcam) according to the manufacturer's instructions.

## **Glutathione Assay**

The relative glutathione (GSH) concentration in cell lysates was assessed using the Glutathione Assay Kit (no. CS0260; Sigma) according to the manufacturer's instructions.

## Western Blot Analysis

Following treatment, cells were washed twice with cold PBS and extracted with RIPA lysis buffer on ice. Extracted cells were then sonicated and centrifuged at 15,000 g for five minutes. Protein extraction was carried out in accordance with existing protocols (Beyotime, China). Protein content was determined using a BCA protein assay kit, according to manufacturer's instructions. Equivalent amounts of protein were separated on 10–15% SDS-polyacrylamide gels and blotted onto a nitrocellulose membrane. After blocking at room temperature for two hours with 5% non-fat milk in TBS with 0.1% Tween-20, membranes were probed with GPX4 (1:5,000 dilution), GAPDH (1:5,000 dilution) and HRP conjugated IgG antibodies (1:10,000 dilution); and visualized by exposure to Gel Doc XR (BioRad, USA). Relative band intensity was then determined by standard scanning densitometry normalized with GAPDH.

## Statistical analysis

The chi-square test was used to compare groups of categorical variables, and an independent sample t-test was used to analyze numerical data in two groups. The nonparametric Mann-Whitney test was used to analyze differences between nonhomogeneous variances. One-way ANOVA with Tukey or Dunnett posttest was used to analyze data in multiple groups (SPSS version 17.0 for Windows). A two-sided P value of 0.05 was considered statistically significant.

## Results

### Semen parameters of study subjects

The mean age and semen parameters of 75 non-smokers and 70 heavy smokers are given in Table 1. The two groups had similar average age (34.4 years vs. 35.8 years). The sperm vitality (65.4% vs. 59.4%), and sperm progressive motility (45.5% vs. 36.9%) between the two groups were significantly different, but other basic semen parameters, including sperm concentration ( $64.6 \times 10^6$  /mL vs.  $56.5 \times 10^6$  /mL), semen volume (3.2 mL vs. 3.2 mL), abnormal sperm morphology (95.1% vs. 96.1%), and sperm counts ( $216.5 \times 10^6$  cells vs.  $189.6 \times 10^6$  cells) were not significantly different (Table 1).

### Seminal GSH, lipid ROS and iron level in non-smokers and heavy smokers

To investigate the ferroptosis level between non-smokers and heavy smokers, GSH, lipid ROS and iron level were detected in the semen. Comparing non-smokers group, GSH level was reduced in the group of

heavy smokers ( $P < 0.05$ ). However, the level of lipid ROS and iron were increased ( $P < 0.05$ ) (Fig. 1A, B, C). Collectively, these indicated that ferroptosis level was increased in heavy smokers.

## Cell death level in GC-2spd cells after CSC treatment

The effect of CSC on the viability of GC-2spd cells was investigated using the CCK-8 assay. Cell viability was reduced in a dose-dependent manner following treatment with CSC in 24 hours (Fig. 2A). To determine whether CSC-induced toxicity was caused by apoptosis independent, Z-VAD-fmk (a broad caspase spectrum inhibitor) were co-culture with CSC. And the level of CSC-induced toxicity was reduced, but still not totally inhibited by the co-treatment of Z-VAD-fmk ( $P < 0.05$ ). Next, Ferrostatin-1 (Fer-1, an ferroptosis inhibitor) and Z-VAD-fmk were co-culture with CSC. The level of CSC-induced toxicity was reduced dramatically ( $P < 0.01$ ) (Fig. 2B).

## Ferroptosis level in GC-2spd cells after CSC treatment

To investigate the ferroptosis level induced by CSC in the GC-2spd cells, GSH, lipid ROS and iron level were measured. GSH level was reduced following treatment with CSC in 24 h, and lipid ROS and iron level were increased ( $P < 0.05$ ). However, the level of them were reduced after co-cultured with Fer-1 ( $P < 0.05$ ) (Fig. 3A, B, C). Besides, the level of GPX4 protein was reduced after treating with CSC for 24 h, and restored after the cells were treated with CSC and Fer-1 ( $P < 0.05$ ) (Fig. 3D, E). According to these results, CSC-induced toxicity in the GC-2spd cells was also contributed by ferroptosis.

## Discussion

Although cigarette smoking is considered as a major risk factor for several human diseases, such as lung cancer and chronic obstructive pulmonary disease (COPD) [34, 35], the effects of smoking on male infertility are controversial. Some studies have reported that cigarette smoking could significantly decrease basic semen parameters [17–19], whereas other studies had shown that cigarette smoking did not affect semen quality [12, 13]. In the present work, a case–control study was conducted between heavy smokers and non-smokers, the results showed that sperm vitality, and sperm progressive motility between the two groups were significantly different. The sperm quality was lower in the group of heavy smokers.

The molecular mechanisms' underlying smoking-affect semen quality are not clear yet. Smoking has been demonstrated to increase DNA fragmentation and damage in sperm mainly by inducing apoptosis [36, 37]. Caspase-independent apoptosis resulted from oxidative stress mediates this process. However, this mechanism is not solely responsible for affecting semen quality in heavy smokers.

Ferroptosis, a new regulated cell death identified by Brent R. Stockwell's lab in 2012, is mediated by an iron dependent accumulation of lipid reactive oxygen species (ROS). Study by Li et al showed that

ferroptosis is a pervasive and dynamic type of cell death induced by Oxygen-glucose deprivation and reoxygenation injury in Sertoli cells [28], Elizabeth G explored the contribution of ferroptosis to the demise of germline cells during periods of acute stress in vivo [29]. Previous studies have verified that the lethal lipid peroxidation reaction is accelerated by intracellular iron in the germ cells, whereas other divalent cationic metals have no effect [38, 39].

Based on these discoveries, we present the first findings of ferroptosis in the semen from heavy smokers. In this study, we found that GSH level was reduced in the group of heavy smokers. However, the level of lipid ROS and iron were increased. These results indicate that the ferroptosis level was increased in heavy smokers. For the further proof that the effect on sperm quality of heavy smokers by ferroptosis, GC-2spd cell line was adopted in this study. The cell was treated with CSC, then we found that lipid ROS and iron level increased and level of GSH dropped. Fer-1, which acts as a lipid ROS scavenger and suppresses ferroptosis but not apoptosis, necrosis or autophagy [22, 40], was also used as a protector of resisting ferroptosis. According to the results, Fer1 significantly depressed CSC-induced cell death and lipid ROS generation, this indicated that lipid peroxidation might be associated with CSC-induced cell death in GC-2spd cells. Besides, the level of GPX4 protein was found to be downregulated in GC-2spd cell after the cells were treated with CSC, however, GPX4 restored by treating the cells with CSC and Fer-1. Based on these findings, CSC-induced toxicity in the GC-2spd cells was also contributed by ferroptosis. This conclusion indicates that smoking is associated with abnormalities of human sperm because the ferroptosis induced by lipid peroxidation. The major factors regulating ferroptosis are lipid peroxidation and iron metabolism signaling [21, 22]. Two mechanisms maybe responsible for inducing ferroptosis: (1) the indirect way of inhibiting GSH via the deprivation of the precursor Cys (cystine), as a result of the subsequent influence on the system  $xc^-$ , and (2) inactivating GPX4 directly by compounds such as RSL3[21, 23]. A variety of inhibitors and inducers of ferroptosis have been reported to regulate the accumulation of lethal lipid reactive oxygen species (ROS) derived from iron metabolism turbulence [22, 41]. In this study, the level of GPX4 protein and GSH level were reduced and the level of MDA and iron were increased. Reduced GSH serves a role in antioxidant defense, and the GSH-GPX4 interaction is critical for the regulation of redox [21]. Depletion of GSH, a basal cofactor of GPX, impairs GPX4 function which can lead to cellular redox state changes and ferroptosis [21]. But the mechanism of ferroptosis induction and the associated signaling pathway responsible for regulating cell death still needs to be further proven.

In conclusion, this study indicates that cigarette smoking induced abnormal ferroptosis in human semen and eventually impairs sperm quality. Accordingly, this finding may be used as a clinical basis for the development of new efficacious treatments for smoking-induced cell death and male infertility.

## Abbreviations

GSH: glutathione; ROS: lipid reactive oxygen species; GPX4: GSH-dependent peroxidase 4; CSC: cigarette smoke condensate; ATCC: American Tissue Culture Collection; DMEM: Dulbecco modified Eagles

media;CCK-8: Cell Counting Kit-8; MDA: malondialdehyde; Fer-1: Ferrostatin-1; COPD: chronic obstructive pulmonary disease

## Declarations

### Acknowledgements

Not applicable

### Authors' contribution

ZHO and LS developed the concept of the study; All authors contributed to data accumulation; ZHO, YD, ZHC, YY and QRW contributed to data analysis; ZHO and QRW performed the experiment; ZHO wrote the manuscript. All authors contributed to revisions of the manuscript, and approved of the final submission. ZHO takes responsibility for the accuracy of the data analysis.

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**Availability of data and materials** Data were obtained from the referenced publications. Further information is available by contacting Dr. Ou at zhanhui-ou@hotmail.com.

### Ethics approval and consent to participate

The study has been approved by the Ethics Committee of our Institution (2017021201). Oral and written consent was obtained for all willing participants prior to registering for this study. Patient Informed consent to participate in this study. The authors have consented for publication.

### Competing interests

The authors declare that they have no competing interests.

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## Table 1

Table 1. The semen parameters of 75 non-smokers and 70 heavy smokers.

Parameter	Non-smokers	Heavy smokers	P
N	75	70	
Age (y)	34.4±5.7	35.8±4.5	0.101
Sperm volume (mL)	3.2±0.12	3.2±0.14	0.836
Sperm concentration (X10 <sup>6</sup> )	64.6±1.80	56.5±4.23	0.081
Sperm count (X10 <sup>6</sup> cells/mL)	216.5±15.50	189.6±18.23	0.262
Sperm progressive motility (%)	45.5±1.41	36.9±1.88	0.000
Abnormal sperm morphology (%)	95.1±0.37	96.1±0.22	0.230
Sperm vitality (%)	65.4±1.71	59.4±2.14	0.030

Note: Data are presented as mean ± SE.

## Figures

Figure 1

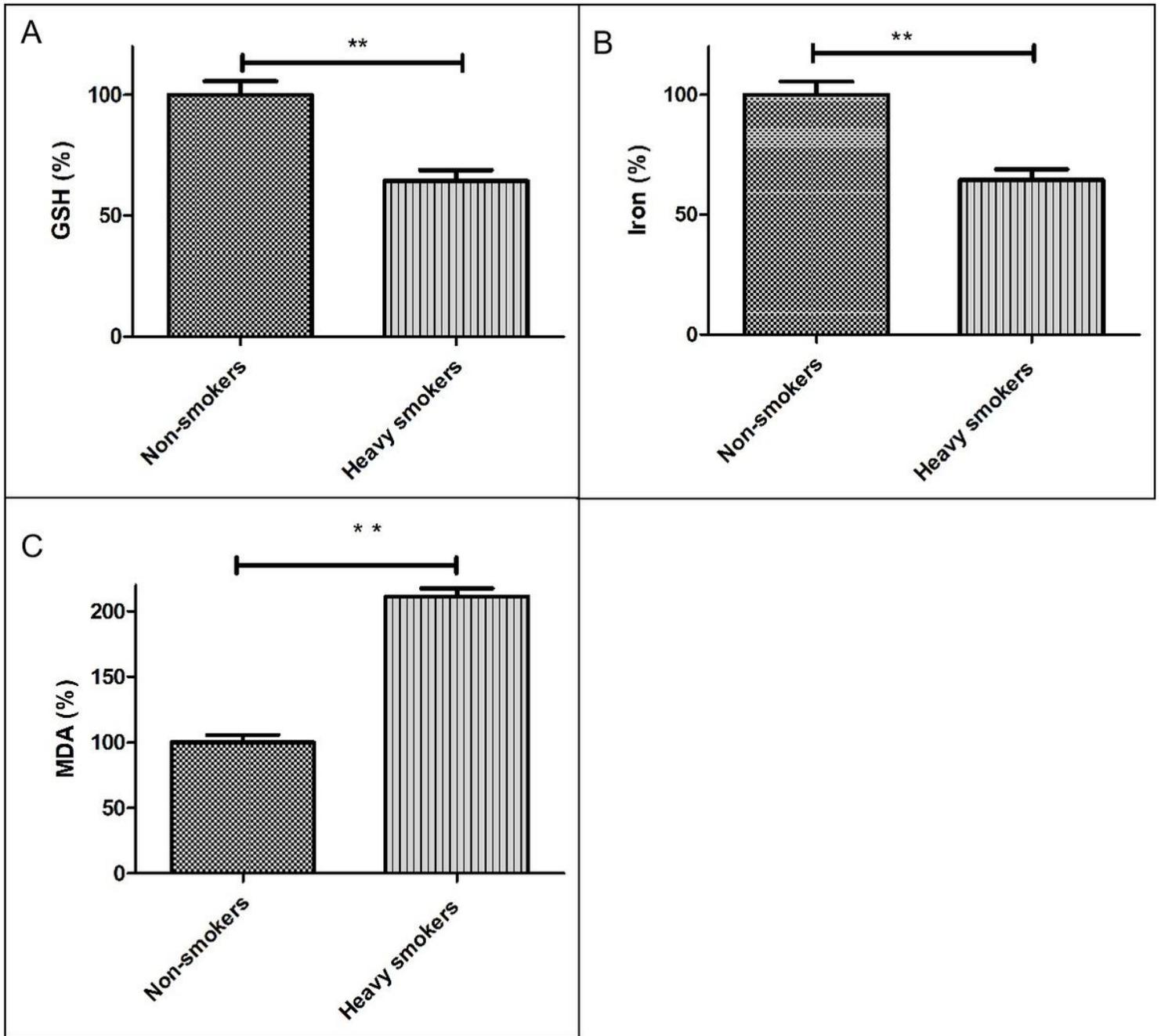


Figure 1

Seminal GSH, lipid ROS and iron level in non-smokers and heavy smokers. A. GSH level in the semen of heavy smokers and non-smokers. B. Iron level in the semen of heavy smokers and non-smokers. C. Lipid ROS level in the semen of heavy smokers and non-smokers. \* $P < 0.05$ , \*\* $P < 0.01$  represents the statistical difference compared to the respective group (n = 3).

Figure 2

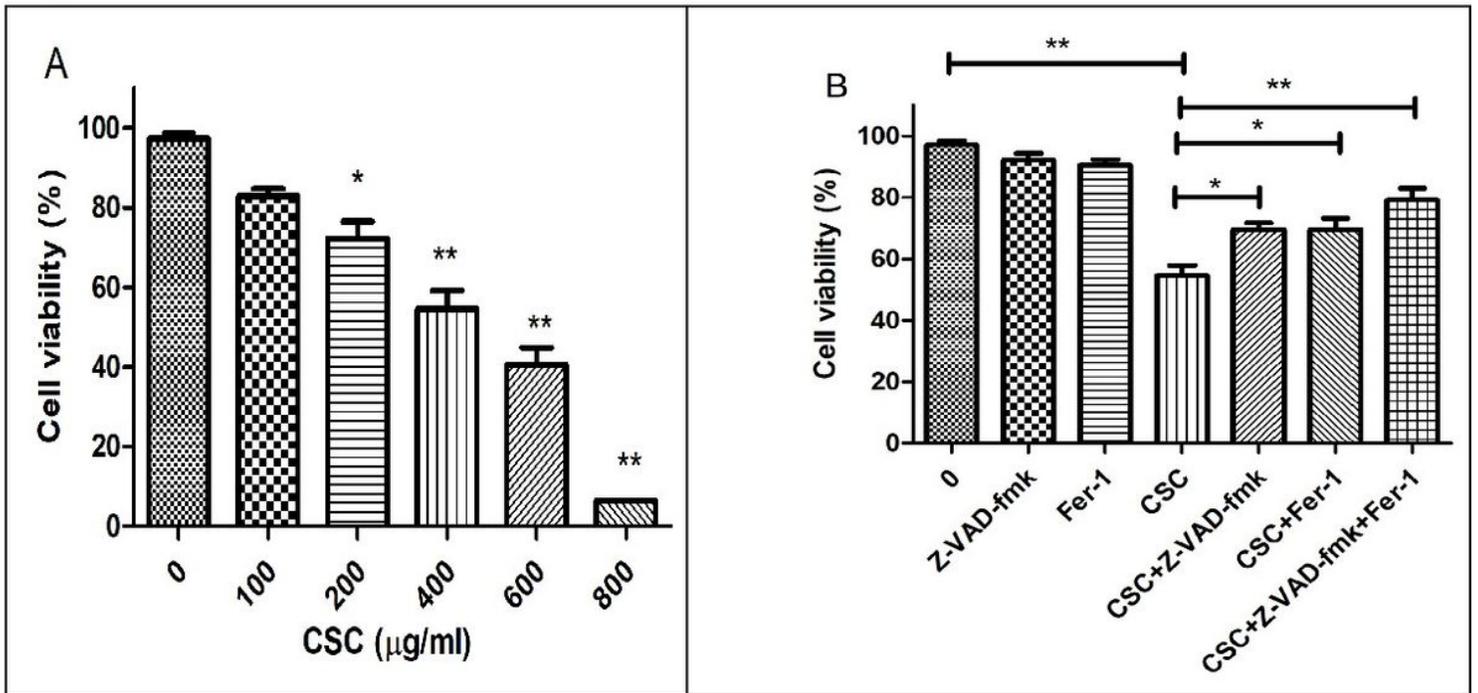


Figure 2

Exposure to CSC-triggered cell death in GC-2spd cells. A. GC-2spd cells were treated with 0–800 μg/mL of CSC for 24 h. Cell viability was measured by CCK-8 assay (n = 3). B. GC-2spd cells were detected after treatment with/without 400 μg/mL of CSC for 24 h in the presence or 10 μmol/L of Fer-1 and Z-VAD-fmk respectively, and treated with 400 μg/mL of CSC for 24 h following pre-incubation with 10 μmol/L of Fer-1 and Z-VAD-fmk or Fer-1 combined with Z-VAD-fmk for 24 h. \*P<0.05, \*\*P<0.01 represents the statistical difference compared to the respective group (n = 3).

Figure 3

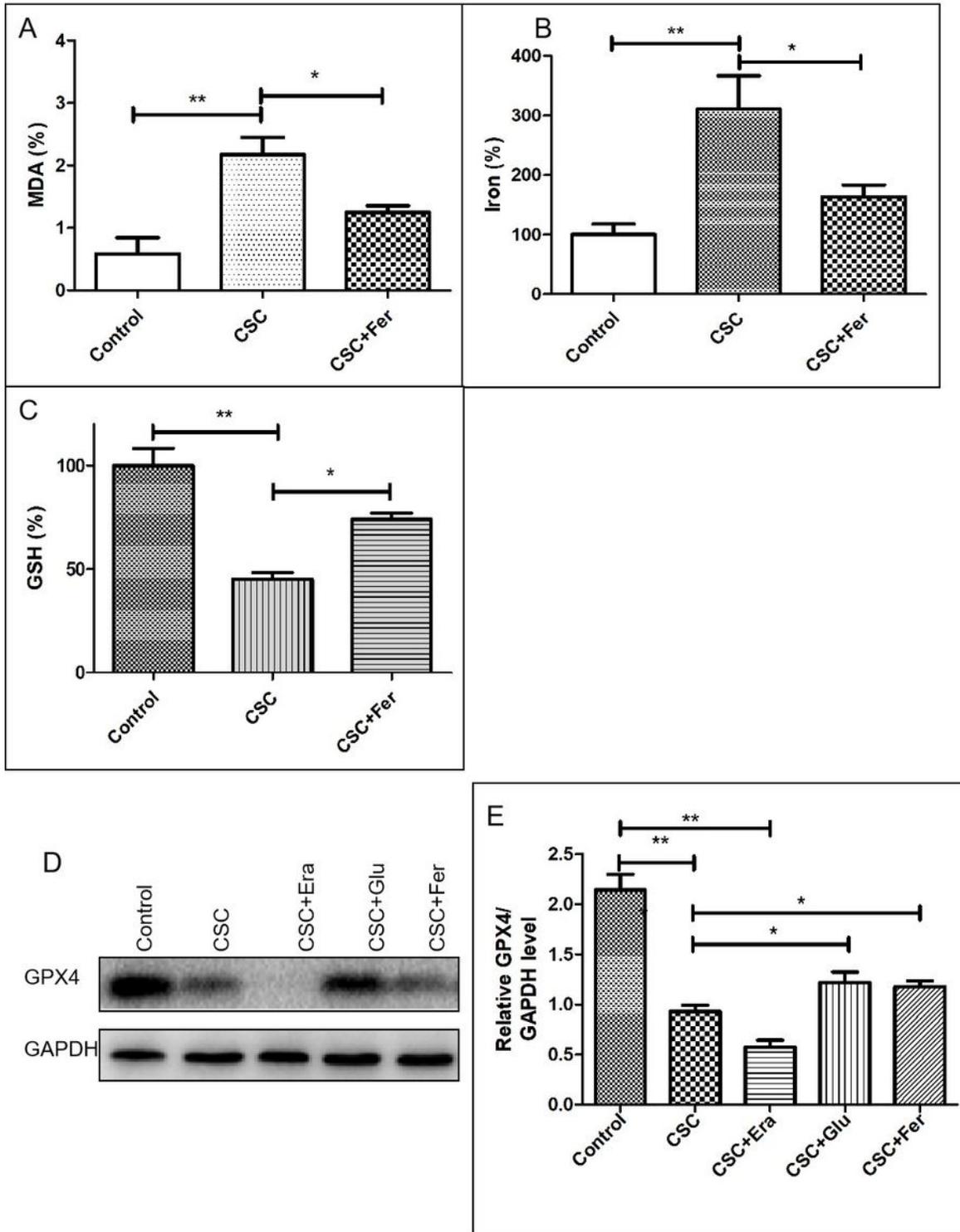


Figure 3

Seminal GSH, lipid ROS and iron level in GC-2spd cells after CSC treatment. A. Lipid ROS level in the GC-2spd cells after CSC treatment and after pre-cultured with Fer-1. B. Iron level in the GC-2spd cells after CSC treatment and after pre-cultured with Fer-1. C. GSH level in the GC-2spd cells after CSC treatment and after pre-cultured with Fer-1. D, E. GPX4 level in the GC-2spd cells after CSC treatment and after pre-

cultured with Fer-1. \*P<0.05 ,\*\*P<0.01 represents the statistical difference compared to the respective group (n = 3).