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Research article

Astragalin alleviates oligoasthenospermia via promoting nuclear translocation of Nrf2 and reducing ferroptosis of testis

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ABSTRACT

Oligoasthenospermia (OAS) is a global human developmental disease and the most common type of male infertility. There are currently no sufficiently effective therapeutic strategies for OAS. *Wuziyanzong* Pill (WZYZP) is a traditional Chinese prescription for the clinical treatment of male infertility, and its efficacy is well known in China. Therefore, due to the complexity of traditional Chinese medicine, the specific mechanism of action of WZYZP on OAS has not been elucidated. Astragalin (AG), one of the main active substances in WZYZP, has good antioxidant effect. The aim of this research is to investigate whether AG, the active substance in WZYZP, can treat OAS by promoting Nrf2 nuclear translocation and inhibiting ferroptosis. The OAS model was established by intraperitoneal injection of cyclophosphamide, and the therapeutic effects of AG and WZYZP on OAS were evaluated by detecting sperm quality, sex hormone levels and testicular pathological changes after intragastric administration of AG and WZYZP. Western blot was used to measure the expression levels of TFR1, SLC7A11, GPX4 and FTH1. The nuclear translocation of Nrf2 was detected by immunofluorescence staining and nuclear/intracellular expression of Nrf2. The results showed that AG could improve sperm quality and serum sex hormone levels in OAS rats, reduce the expression of testicular Fe^{2+} and TFR1, up-regulate testicular SLC7A11, GPX4 and FTH1, and inhibit testicular ferroptosis. At the same time, AG can promote the expression and nuclear translocation of Nrf2 in the testis of OAS rats. AG can alleviate OAS via promoting nuclear translocation of Nrf2 and inhibiting ferroptosis of testis.

1. Introduction

According to epidemiological statistics, infertility continues to affect 15 % of couples around the world by the end of the 20th

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Abbreviations:

AG	astragalin
CP	cyclophosphamide,
ELISA	enzyme-linked immunosorbent assay
GSH	glutathione
GSH-px	glutathione peroxidase
H&E	Hematoxylin-Eosin
LIN	linearity
MDA	malondialdehyde
Nrf2	nuclear factor erythroid 2-related factor 2
OAS	oligoasthenospermia
ROS	reactive oxygen species
VAP	velocity average path
VCL	velocity curved line,
VSL	velocity straight line,
SOD	superoxide dismutase
STR	straightness
WB	western bolt
WZYZP	Wuziyanzong Pill

century [1], which is still a sociological and medical problem related to global human development. The results show that "male factors" account for about 40 %, including oligoasthenospermia and oligozoospermia, which are mainly related to the amount, concentration, motion morphology of sperm [2]. Oligoasthenospermia (OAS), the most common types of male infertility, is defined as less than 42 % sperm motility or less than 30 % forward movement of sperm in semen parameters. Metabolic syndrome [3], drug [4], oxidative stress [5] and other factors are the main pathogenesis of OAS. However, the mechanism of OAS is complex and not well defined.

Under the physiological conditions, the oxidation and antioxidant system in the body are in a balanced state, and metabolism or drugs and other factors will destroy this balance, resulting in a sharp up-regulation in the content of reactive oxygen species (ROS) in the body [6]. Because sperm is impressionable to attack of ROS and lipid peroxidation, oxidative stress is often the most important reason for OAS [7]. Studies showed that superfluous ROS could disrupt the cellular balance of Fe^{2+} and induce non-programmed cell death [8].

Previous study confirmed that sperm of OAS patients can undergo ferroptosis due to lipid peroxidation, and the degree of ferroptosis is negatively correlated with GPX4 and SCL7A11 [9]. Inhibition of lipid peroxidation related gene SLC7A11 could significantly reduce ferroptosis in testicular cells of oligozoospermia mice [10]. Nuclear factor erythroid 2-related factor 2 (Nrf2) plays an indispensable and important role in the body's antioxidant system. It can be dissociated from keap1 and enter the nucleus as a transcription factor to up-regulate the body's antioxidant system [11]. Nrf2 is also an important protective mechanism for the body to defend against lipid peroxidation and ferroptosis [12,13]. Studies have reported that Nrf2 can be used as a target to regulate spermatogenic cell apoptosis in OAS rats and treat OAS [14]. In related studies, sulforaphane can prevent ferroptosis of myocardial cells in diabetic mice by up-regulating Nrf2 [15]. Therefore, regulating ferroptosis via targeting Nrf2 may be a viable therapeutic strategy.

Wuziyanzong Pill (WZYZP) is a famous traditional Chinese medicine, which is often used to ameliorate m ale reproductive dysfunction in clinical practice [16]. Astragalin (AG), one of the main components of WZYZP [17], has been reported to reduce LPS-induced lung injury in rats by enhancing Nrf2 activity [18]. Previous studies have shown that AG can significantly improve spermatogenesis in CP-induced OAS mice [19]. At the same time, a study has demonstrated that AG can improve spermatogenesis in streptozotocin-induced diabetic mice by inhibiting oxidative stress [20]. Therefore, we hypothesized that AG may improve OAS by promoting Nrf2 nuclear translocation and inhibiting ferroptosis of spermatogenic cells.

2. Materials and methods

2.1. Materials

Astragalin (480-10-4) was provided by Yuanzhi Biotechnology Co., LTD. (Nanjing, Jiangsu, China). *Wuziyanzong* Pill (Z11020173) was purchased from Tongrentang Co., LTD. (Beijing, China). Blood glucose test strips and blood glucose meters were from Tianjin Jiuan Medical Co., LTD. Tissue ROS test kit (DHE) (HR8821) was from Biolaibo Biotech Co., LTD (Beijing, China). GSH and GSSG Assay Kit (S0053), MDA test kit (S0131S), Cellular Glutathione Peroxidase (GSH-px) Assay Kit with NADPH (S0056), and Total Superoxide Dismutase (SOD) Assay Kit with NBT (S0109) was purchased from Biyuntian Biotechnology Co., LTD. (Shanghai, China). Rat LH (Luteinizing Hormone) ELISA Kit (E-EL-R0026c), Ferrous Iron Colorimetric (Fe^{2+}) Assay Kit (E-BC-K773-M) and QuicKey Pro Rat The ELISA Kit for T (Testosterone) ELISA kit (E-OSEL-R0003) was from Elabscience Co., LTD. (Wuhan, Hubei, China). Rabbit anti-mouse antibodies for SLC7A11 (DF12509), GPX4 (DF6701), TFR1 (AF5343), FTH1 (DF6278), Nrf2 (AF0639) and Histone H3 (AF0863) were

from Affinity Co., LTD (Shanghai, China). Rabbit anti-mouse antibody (AF5003) for β-Actin and HRP-labeled Goat Anti-Rabbit IgG (H + L) (A0208) were from Biyantian Co., LTD. (Shanghai, China). One-Step PAGE Gel Fast Preparation Kit (10 %) (E303-01) was purchased from Vazyme (Nanjing, Jiangsu, China). Nuclear and Cytoplasmic Protein Extraction Kit (P0027), 5X SDS-PAGE Sample Loading Buffer (P0015L) and Ultra Sensitive ELISA Assay Kit with Fluorescent HRP Substrate (P0207L) was from Biyantian Co., LTD. (Shanghai, China).

2.2. Animals and groups

Thirty 8 weeks old SPF male Sprague-Dawley (SD) rats were fed in the animal room of the Central Laboratory of Jiangsu Health Vocational College. The rats were fed with water and food freely under 12 h light/12 h dark cycle. The rearing temperature was 21 ± 2 °C and the relative humidity was 60 ± 10 %. Animal qualification certificate number was (SCXKSU 2021-0021). Cyclophosphamide (CP, 35 mg/kg/d, 5 days) was injected intraperitoneally to induce asthenozoospermia in 4 groups, and 0.9 % normal saline was injected in another group as control. Blood samples were collected from the inner canthus one week later to determine the serum testosterone level, that was to measured whether the model was valid. AG and WZYZP were dissolved in 0.5 mg/mL sodium carboxymethyl cellulose solution for intragastric administration. The grouping and administration results were as follows:

Con:2 mL/d, 0.5 mg/mL CMCC-Na.
CP:2 mL/d, 0.5 mg/mL CMCC-Na.
AGL:20 mg/kg/d AG.
AGH:40 mg/kg/d AG.
WZYZP:2 g/kg/d WZYZP.

During the experiment, all rats were given water and food AD libitum, and drugs were administered by gavage at a fixed time each day (Fig. 1). After 21 days of CP intervention, blood from the abdominal aorta, bilateral testis and epididymis were collected. The left testis was fixed in 4 % paraformaldehyde, and the right testis was frozen and stored in a refrigerator at −80 °C. The bilateral epididymis were placed in Hams' F10 medium preheated in a water bath heating box and incubated at 37 °C for 30 min until complete dissociation of epididymal sperm was achieved. Sperm concentration and motility were detected by CFT-9203 sperm quality detection and analysis system (Ruiqi Co., LTD, Jiangsu, China).

2.3. Hematoxylin-Eosin (H&E) staining

The right testis of every rat were fixed in 4 % paraformaldehyde fixative for 72 h, and made into 5 μm sections after embedding in paraffin. The sections were stained with H&E staining, and the pathological changes of the testis tissue and the amount of Leydig cells and spermatogenic cells were observed under an orthotopic optical microscope.

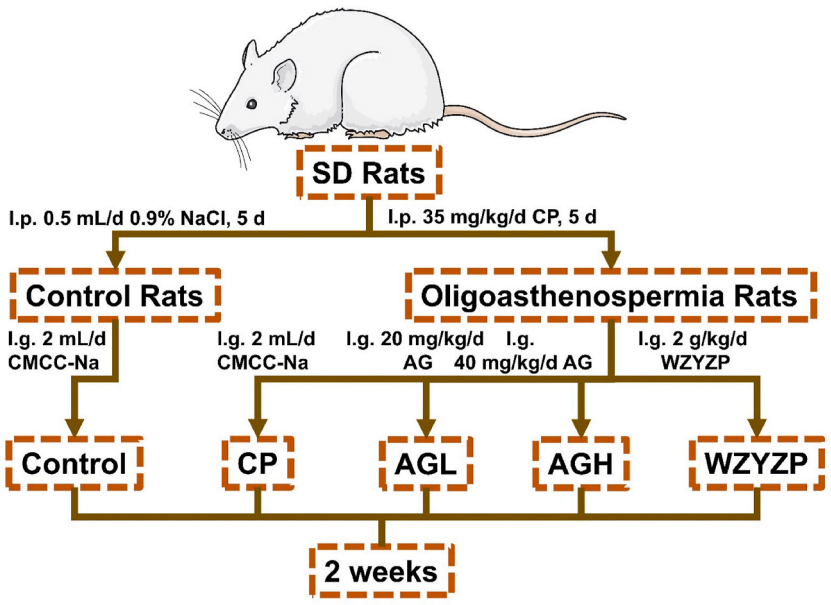

Fig. 1. Drug administration. (SD rats which intraperitoneally injected CP to induce OAS model were orally administered with different doses of AG and WZYZP.)

2.4. Immunofluorescence

Testicular tissues in 4 % paraformaldehyde fixative were fixed for 72 h, embedded in paraffin, and sectioned at 5 μm. After antigen repair and DNA denaturation, 3 % BSA solution was used as blocking solution at 26 °C for 30 min. Samples were incubated with configured antibodies to rabbit derived Nrf2 mixture in a wet box at 4 °C overnight. The mixed secondary antibody was prepared with FITC-labeled donkey anti-rabbit IgG. The sections after incubating with the primary antibody were placed in a wet box and incubated with the mixed secondary antibody in the section ring for 1 h at room temperature. After sealing with glycerol, the staining was observed under Leica DM IL LED fluorescence inverted microscope and sent to a laser confocal microscope for scanning.

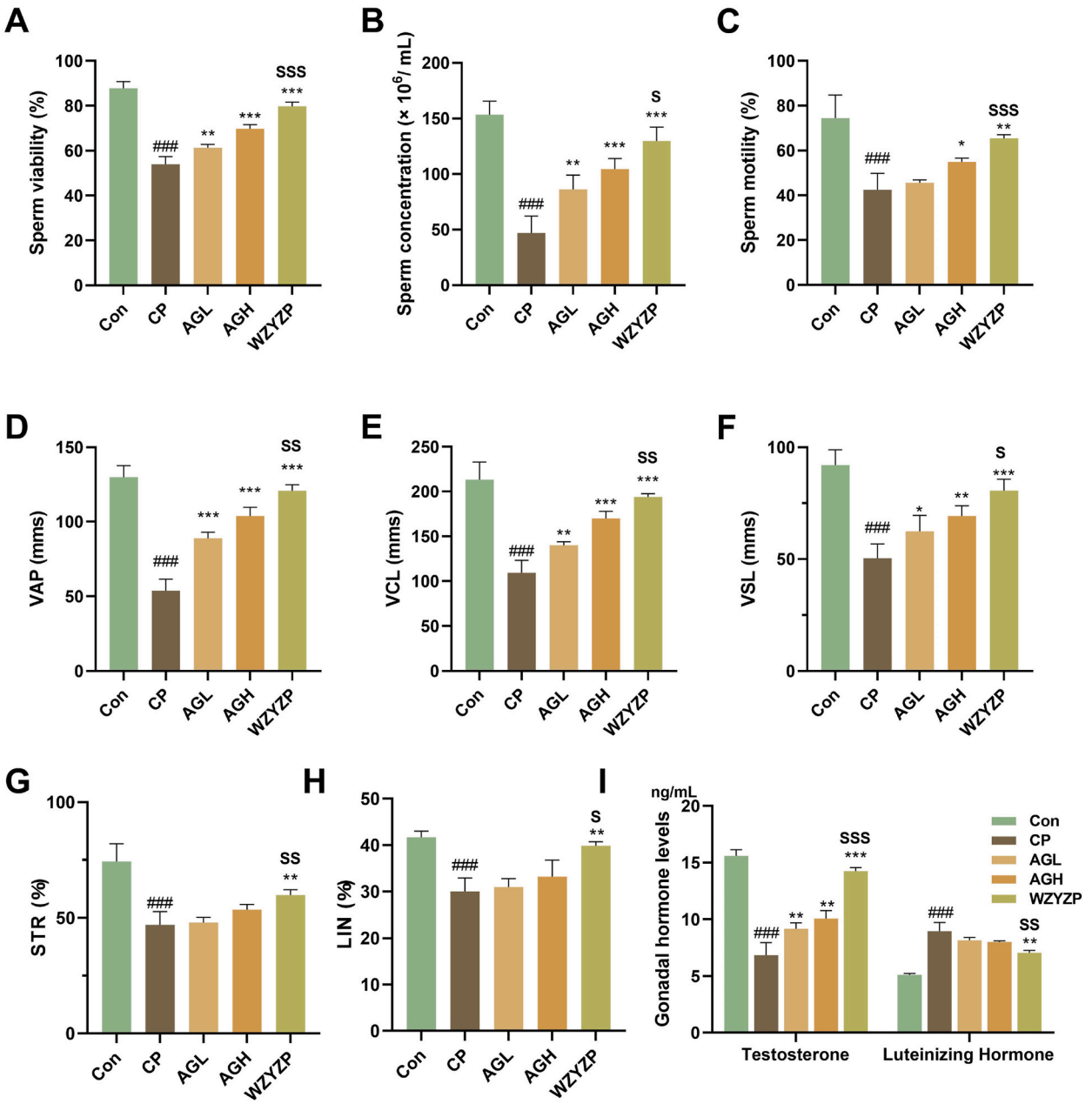

Fig. 2. Effects of AG on sperm quality and sex hormone levels in OAS rats. Sperm (A) viability, (B) concentration, (C) motility, (D) VAP, (E) VCL, (F) VSL, (G) STR, (H) LIN, (I) Expression levels of Testosterone and Luteinizing hormone in Con, CP, AGL, AGH and WZYZP group. Values are expressed as mean ± SEM (n = 6). (*p < 0.05, **p < 0.01, ***p < 0.001 vs CP; $^{###}$p < 0.001 vs Con; ^{S}p < 0.05, ^{SS}p < 0.01, ^{SSS}p < 0.001 vs AGH).

2.5. Enzyme-Linked Immunosorbent assay

Rat blood serum was used as samples for testosterone and luteinizing hormone determination. Testicular tissues were ground with a tissue grinder for ROS, MDA, SOD, GSH, GSH-px, and Fe^{2+} measurements. All assays were performed according to the instructions of commercially available kits and tested in a multifunctional microplate reader.

2.6. Western Bolt

The testicular tissue was washed with PBS three times, and lysed by adding RIPA lysate containing 1 mmol/L LPMSF (100 μL of RIPA lysate per 10 mg of tissue). After grinding for 15 min, the samples in ice box were lysed for 30 min. After quantification with BCA, adding sample loading buffer and denaturing in a metal bath at 100 °C for 10 min, the protein expression was detected by Western screening. Then 30 μg of protein was added in gel, and the target protein was cut and transferred to PVDF membrane after constant pressure electrophoresis of 80 V-30 min and 120 V-55 min. After blocking PVDF membranes by incubating them with BSA-TBST for 2 h, they were incubated with target antibody (1 : 950) and HRP - antibody (1 : 1200), successively. After washing, ECL was used to image the gel imaging system.

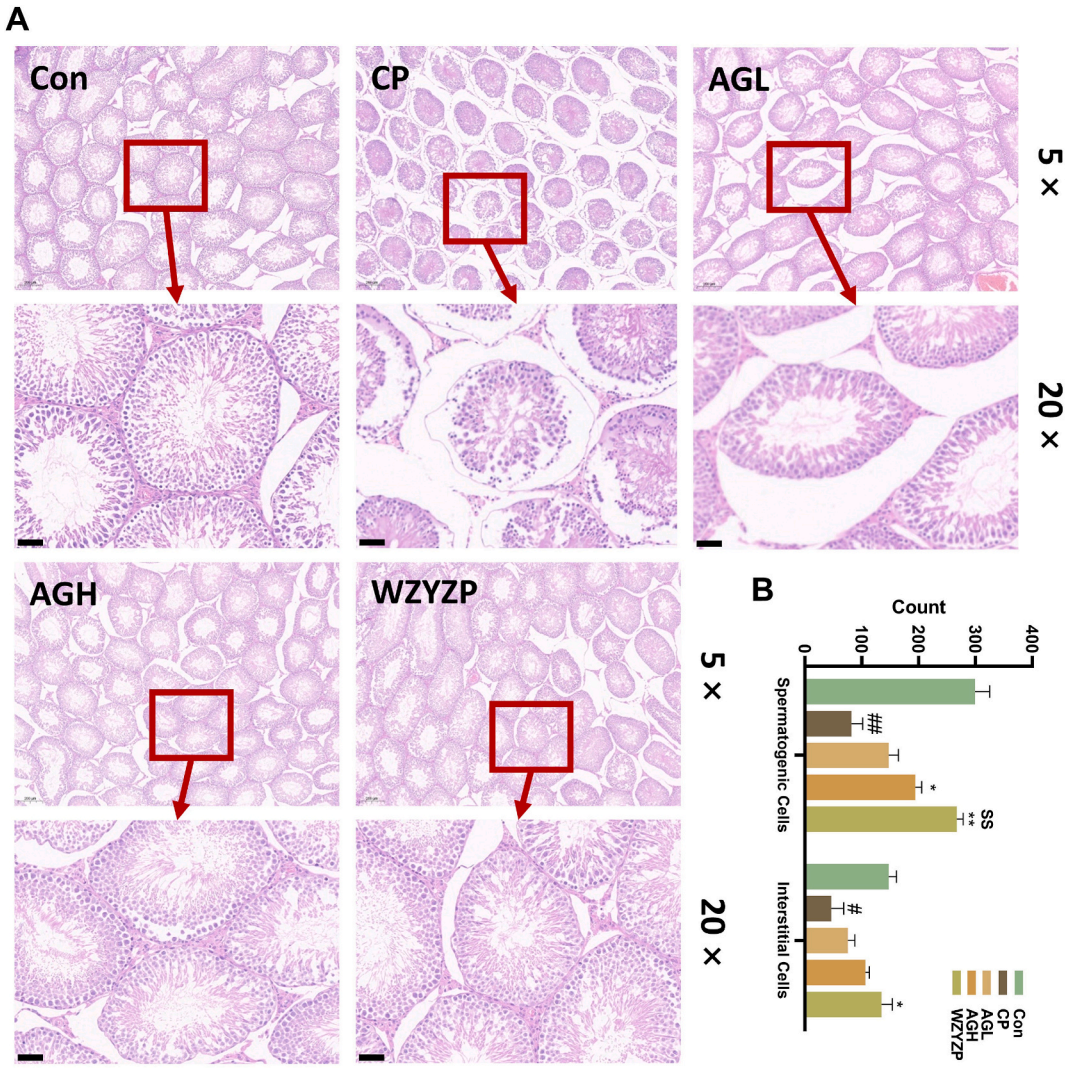

Fig. 3. Effects of AG on morphology of testis in OAS rats. (A) H&E staining of testis in OAS rat. (Original magnifcation × 50 & 200; scale bar represents 50 μm). (B) Count of spermatogenic cells and count of interstitial cells in the field of view at a magnification of 200×. Values are expressed as mean ± SEM (n = 3). (*$p < 0.05$ vs CP; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Con; $^{SS}p < 0.01$ vs AGH).

2.7. Statistics analysis

Graphpad prism 8.3.0 was used for statistical and visualization processing. One-way ANOVA and *t*-test were used for difference analysis between two groups and multiple groups, and the results were presented as mean ± SEM. An asterisk indicates a statistically significant difference (*P < 0.05, **P < 0.01, ***P < 0.001).

3. Results

3.1. AG can alleviate CP-induced OAS in rats

The effect of AG on the sperm amount and quality of OAS rats was evaluated by measuring sperm motility parameters, sperm viability, motility and sperm count. As shown in Fig. 2, the sperm motility ($p < 0.001$), sperm viability ($p < 0.001$) and sperm number ($p < 0.001$) of the CP group were significantly decreased compared with the Con group, while those of the AG group were significantly improved in a dose-dependent manner (Fig. 2A–C). The same was true for the examination of sperm motility rates, including velocity average path (VAP), straightness (STR), velocity curved line (VCL), linearity (LIN) and velocity straight line (VSL) (Fig. 2D–H). These results indicate that AG can improve sperm quality in OAS rats ($p < 0.05$). The result showed that sperm quality of WZYZP group was better than that of the AG group, indicating that AG may not be the only active substance in WZYZP that can treat OAS in rats. The results of sex hormone assays showed that CP significantly declined testosterone ($p < 0.001$) and luteinizing hormone ($p < 0.001$) in rats, which were reversed by WZYZP (Fig. 2 I). AG significantly increased testosterone ($p < 0.001$) in OAS rats, but had no significant effect on luteinizing hormone (Fig. 2 I).

To further explore the therapeutic effect of AG on OAS in rats, we examined the morphological changes of rat testicular tissue by H&E staining. The results showed that the CP group had significantly atrophied seminiferous tubules, enlarged tubule Spaces, and significantly reduced numbers of spermatogenic cells ($p < 0.001$) and Leydig cells ($p < 0.05$), compared with the Con group. After the intervention with AG, the above conditions were relieved. The gap between the seminiferous tubules became smaller, and the number of spermatogenic cells recovered and arranged more closely (Fig. 3A and B). These results indicate that oral administration of AG

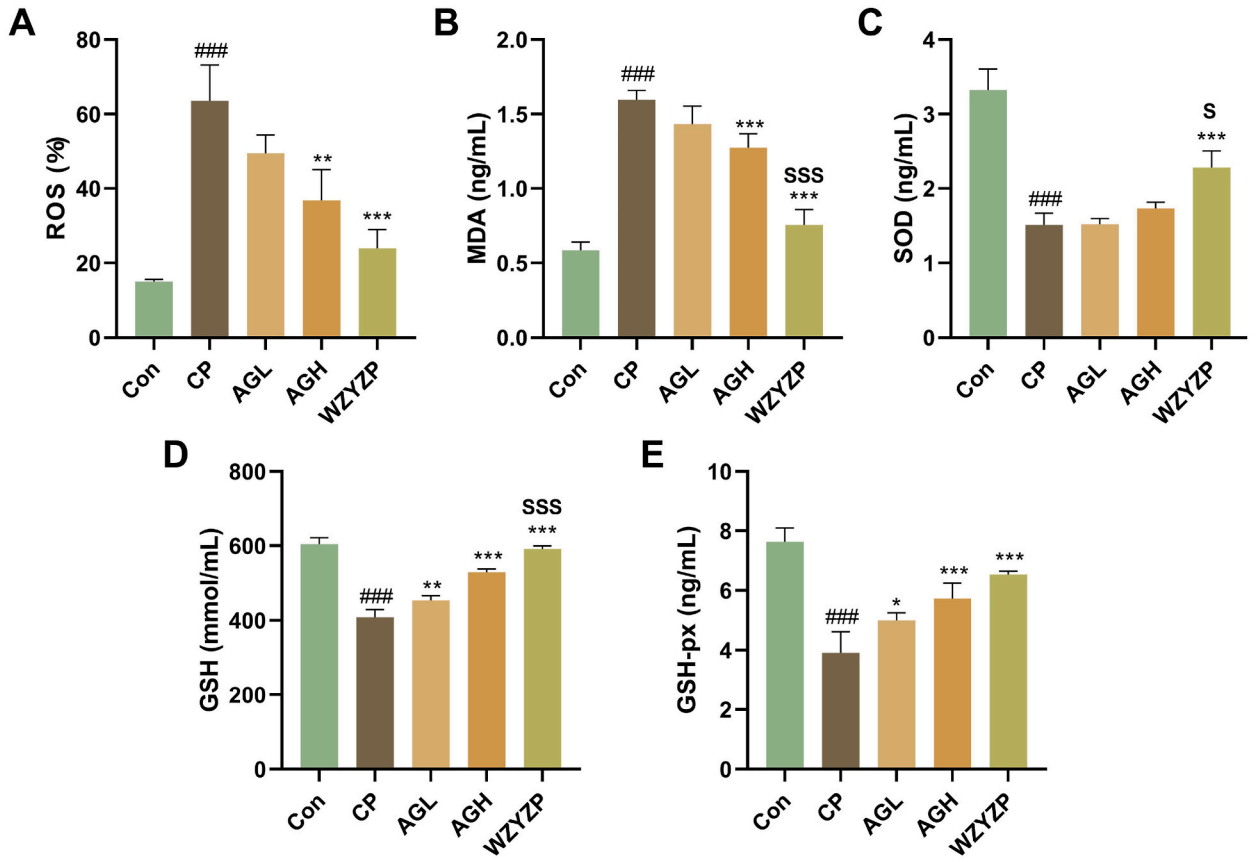

Fig. 4. Effects of AG on lipid peroxidation of testis in OAS rats. Content of ROS (A), MDA (B) and GSH (D) in testis after treating with AG and WZYZP. Activity of SOD (C) and GSH-px (E) were detected via ELISA. Values are expressed as mean ± SEM (n = 6). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs CP; $^{\#\#\#}p < 0.001$ vs Con; $^{S}p < 0.05$, $^{SS}p < 0.01$, $^{SSS}p < 0.001$ vs AGH).

ameliorates OAS in rats.

3.2. AG can inhibit lipid peroxidation in OAS rats

Lipid peroxidation of sperm cells and spermatogenic cells in the testis is an important mechanism for the pathogenesis of OAS [21]. We evaluated the effect of AG on the level of testicular lipid peroxidation in OAS rats by measuring the concentration of ROS, MDA, SOD, GSH and GSH-px in testicular tissue. The results showed that the ROS level ($p < 0.001$) and MDA level ($p < 0.001$) in the testis tissue of the rats were significantly up-regulate after CP intervention, and the SOD level ($p < 0.001$), GSH level ($p < 0.001$) and GSH-px level ($p < 0.001$) were significantly decreased, indicating that the level of lipid peroxidation in the testis of the rats with OAS induced by CP was significantly increased. After AGH intervention, the ROS level ($p < 0.01$) and MDA level ($p < 0.001$) in testicular tissue were significantly decreased, and the GSH level ($p < 0.001$) and GSH-px level ($p < 0.001$) were significantly up-regulated (Fig. 4A–E), indicating that AG can inhibit lipid peroxidation in the testis of OAS rats. However, AG alone had a significantly weaker inhibitory effect on testicular lipid peroxidation than WZYZP (Fig. 4B–D).

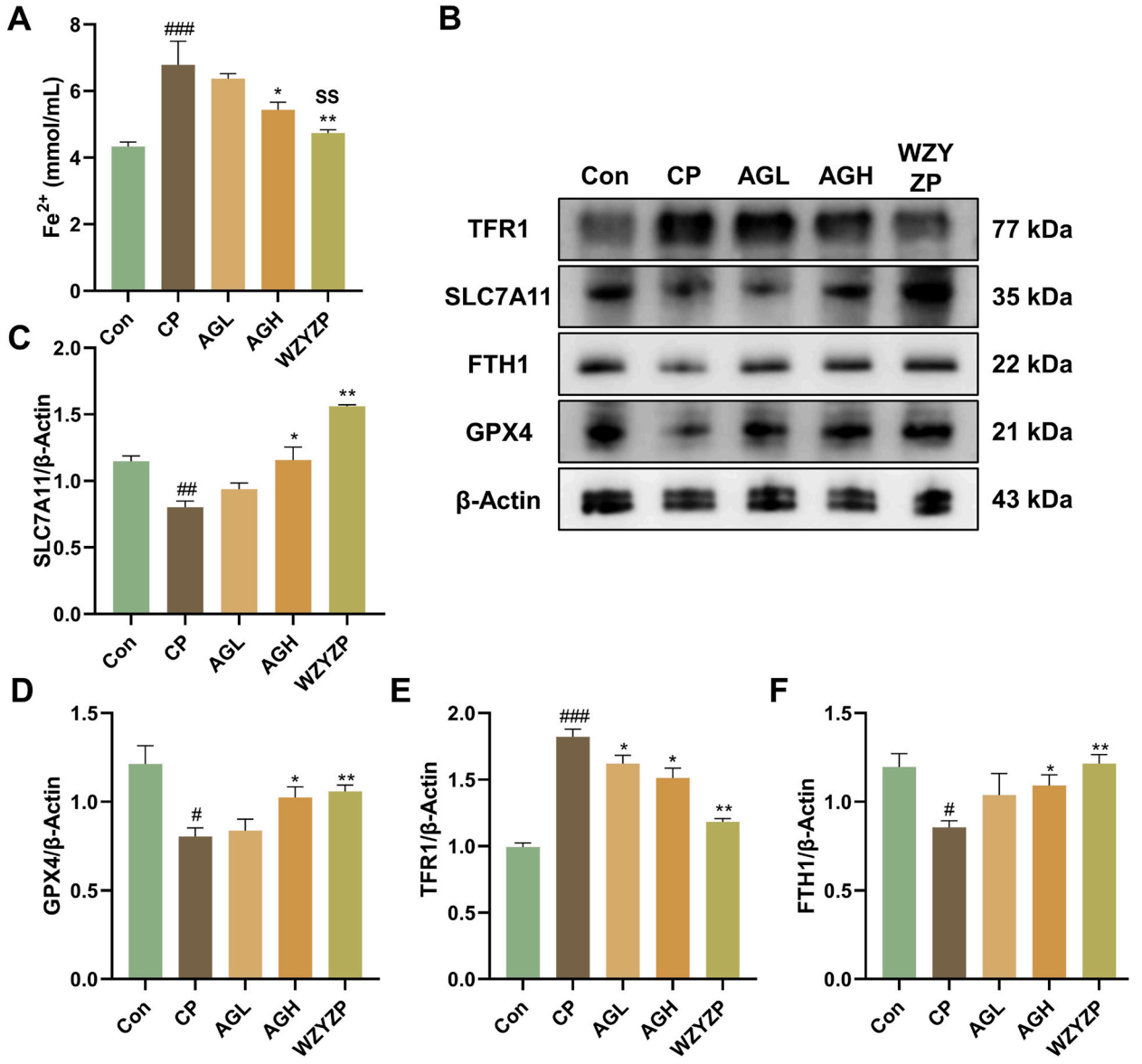

Fig. 5. Effects of AG on ferroptosis of testis in OAS rats. (A) Fe^{2+} content of testis in Con group, CP group, AGL group, AGH group and WZYZP group. Values are expressed as mean ± SEM (n = 6) (*$p < 0.05$ vs. Con; $^{\#}p < 0.05$ vs. CP; $^{S}p < 0.05$ vs. AGH). (B–F) Western blot was used to detect the expressions of SLC7A11 level, GPX4 level, TFR1 level and FTH1 level in the testis of rats. Values are expressed as mean ± SEM (n = 3). (*$p < 0.05$, **$p < 0.01$ vs CP; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs Con; $^{SS}p < 0.01$ vs AGH).

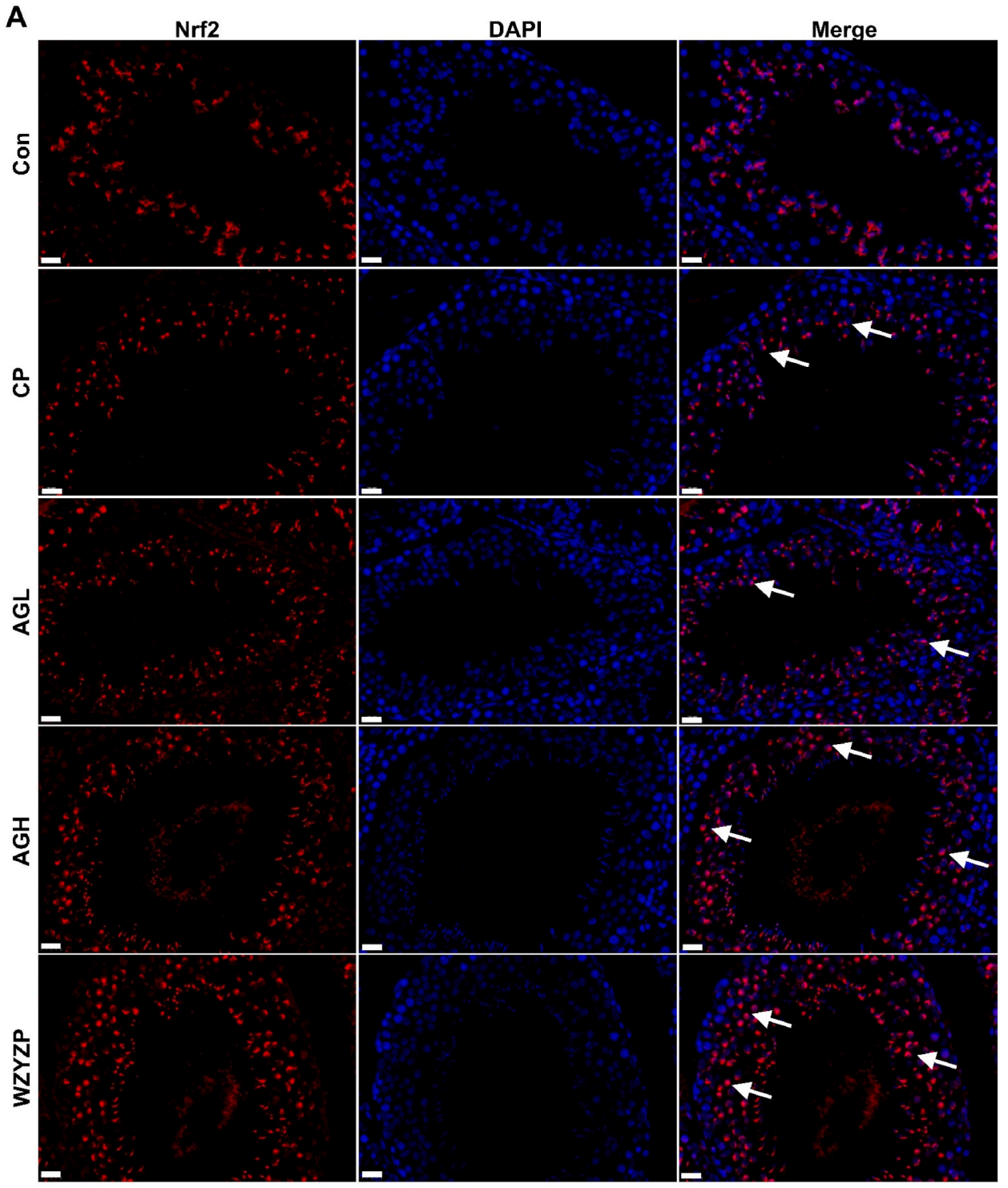

Fig. 6. Effect of AG on Nrf2 nuclear translocation in rat testicular tissue. (A) Nrf2 immunofluorescence staining of rat spermatogenic tubules (Original magnifcation × 400; scale bar represents 10 μm). (B–E) Levels of Nrf2-C (total intracellular Nrf2) and Nrf2-N (Nrf2 in nucleus) expression was detected by western bolt. Values are expressed as mean ± SEM (n = 3). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs CP; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Con).

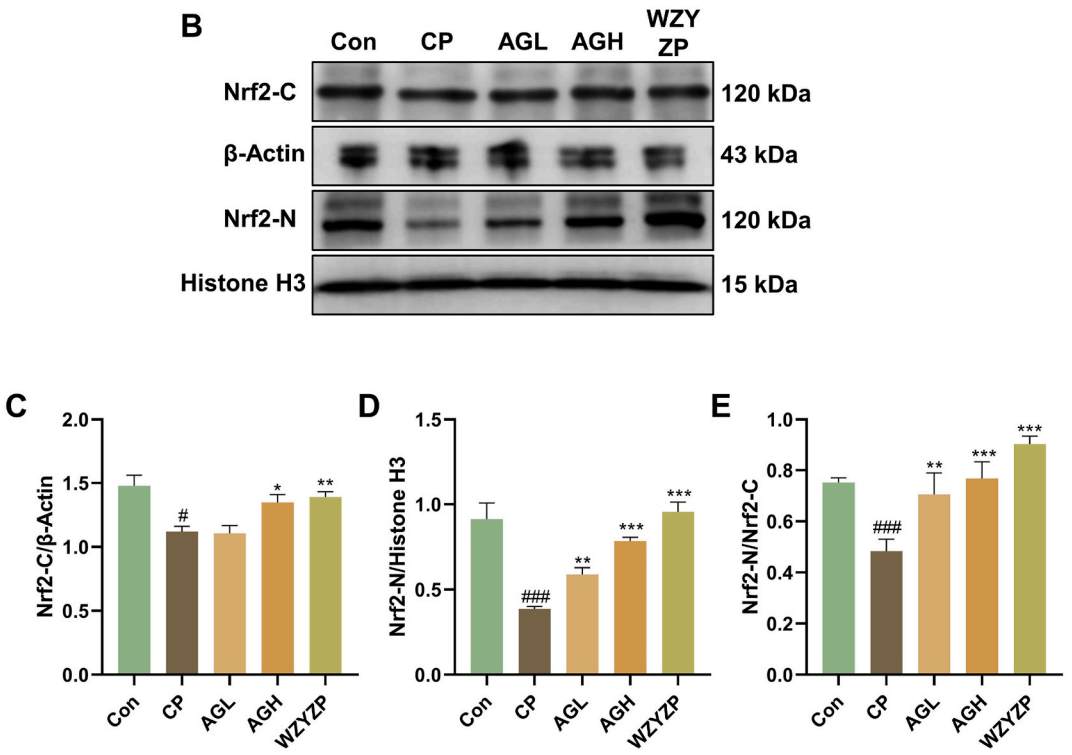

Fig. 6. (*continued*).

3.3. AG can inhibit ferroptosis in the testis of OAS rats

To explore the regulatory effect of AG on ferroptosis of spermatogenic cells in CP-induced OAS rats, we measured the Fe^{2+} content and the expression of ferroptosis-related proteins in the testicular tissue. Our study found a significant increase in Fe^{2+} content ($p < 0.001$) in the testicular tissue of CP-treated rats, which was reversed by administration of either AGH ($p < 0.05$) or WZYZP ($p < 0.01$), and the effect of WZYZP was significantly better than that of AG ($p < 0.01$) (Fig. 5 A). Western Bolt results showed that the expression of TFR1 ($p < 0.001$) was significantly up-regulated and the SLC7A11 level ($p < 0.01$), GPX4 level ($p < 0.05$) and FTH1 level ($p < 0.05$) were significantly decreased in the testicular tissue of OAS rats (Fig. 5B–F). Compared with the CP group, TFR1 was significantly down-regulated in the AGL ($p < 0.05$) and AGH ($p < 0.05$) groups in a dose-dependent manner (Fig. 5B–E). The expression of SLC7A11 level ($p < 0.05$), GPX4 level ($p < 0.05$) and FTH1 level ($p < 0.05$) were also significantly increased in AGH group (Fig. 5B–D,F). The results showed that AG could alleviate OAS in rats by inhibiting ferroptosis of spermatogenic cells.

3.4. AG inhibits ferroptosis in the testis of OAS rats via promoting translocation of Nrf2

To verify that the inhibitory effect of AG on ferroptosis in the testicular tissue of OAS rats was mediated by promoting Nrf2 nuclear translocation, the nuclear translocation of Nrf2 in the testicular tissue was detected by immunofluorescence staining and the ratio of nuclear Nrf2 level to total Nrf2 level. We observed the results of immunofluorescence and found that the coincidence level of Nrf2 and DAPI in the seminiferous tubules in the CP group rats was less than that in the Con group rats (white arrow), and AG could reverse this phenomenon (Fig. 6 A). The Western blot result showed that the total Nrf2 level in the testis tissue of the CP group ($p < 0.05$) decreased significantly, and the expression level of Nrf2 ($p < 0.001$) in the nucleus decreased more significantly. After AGH intervention, the expression levels of total ($p < 0.05$) and nuclear ($p < 0.001$) Nrf2 in testicular tissue were increased, and the level of Nrf2-N/Nrf2-C was significantly higher than that in the CP group ($p < 0.001$) (Fig. 6B–E). These results suggest that the inhibitory effect of AG on testicular ferroptosis in OAS rats may be related to Nrf2 nuclear translocation.

4. Conclusion

AG can improve sperm quality and serum sex hormone levels in CP-induced OAS rats. AG also can alleviate oxidative stress and cell loss in testicular tissue. At the same time, AG reduced the expression of testicular Fe^{2+} and TFR1, up-regulate testicular SLC7A11, GPX4 and FTH1, and inhibit testicular ferroptosis by promoting the expression and nuclear translocation of Nrf2. Therefore, our study showed that AG, the active ingredient of WZYZP, alleviated OAS via promoting nuclear translocation of Nrf2 and reducing ferroptosis of testis (Fig. 7).

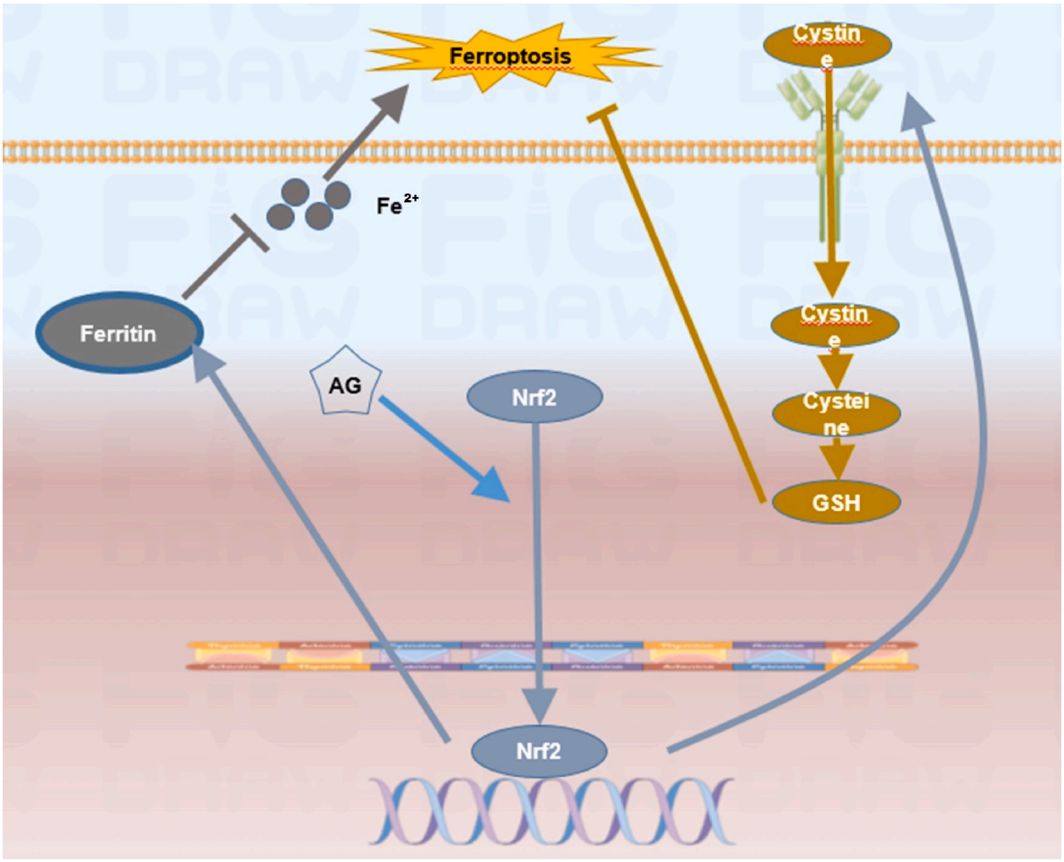

Fig. 7. Astragalin alleviates oligoasthenospermia via promoting nuclear translocation of Nrf2 and reducing ferroptosis.

5. Discussion

According to the criteria of the WHO Human Semen Manual [22], less than 42 % sperm motility or less than 30 % of the sperm moving forward is considered as OAS. OAS has been plaguing the majority of infertile couples in the world, which has caused a significant burden to the society [23]. However, WHO has no adequate and effective treatment options for OAS [24]. WZYZP is a classic Chinese medicinal formulae for the treatment of male infertility, which is included in the Chinese Pharmacopoeia. However, due to the complex composition of traditional Chinese medicine formula, the clearly defined mechanism of action has not been fully elucidated, and WZYZP has not been used internationally. In this study, AG, the main active substance in WZYZP, was found to inhibit lipid peroxidation and ferroptosis in testicular tissue to improve OAS in rats, which contributed to the clarification of the mechanism of action of WZYZP and its clinical application in the treatment of OAS.

Dysregulation of SLC7A11-GPX4 axis and Fe transport are important mechanisms of ferroptosis [25]. The levels of SLC7A11 and GPX4 in semen of asthenozoospermia patients were significantly down-regulated, the mitochondrial membrane potential of sperm cells decreased, and the ferroptosis level increased [9]. Studies have shown that SLC7A11-GPX4 axis can inhibit ferroptosis of vascular smooth muscle cells and promote vascular calcification [26]. In addition, inhibition of SLC7A11 expression reduced the ability of sperm cells to bind to gonadotrophins, suggesting that SLC7A11 may affect sperm quality by regulating extrinsic Fe transport [27]. FTH1 can inhibit ferroptosis by promoting ferritinophagy in Parkinson's disease model [28]. Down-regulation of FTH1 can induce ferroptosis of bladder cancer cells [29]. In our study, we found that AG could up-regulate SLC7A11, GPX4 and FTH1 in the testis of OAS rats, which also supported this view.

Nrf2 is an important antioxidant defense mechanism of the body, and it is also an important mechanism to prevent ferroptosis and lipid peroxidation [12]. Activation of antioxidant pathway mediated by Nrf2 promotes testicular recovery in OAS mice [30]. In patients with refractory seminal vesiculitis, the level of Nrf2 protein and sperm quality were significantly decreased [31]. Activation of Nrf2 can inhibit ferroptosis by up-regulating SLC7A11 [32]. Previous studies have found that AG can alleviate LPS-induced acute lung injury in rats by activating Nrf2 [26]. Kaempferol, the glycoside of AG, has also been reported to activate Nrf2 [33]. Our findings suggest that AG upregulates Nrf2 in spermatogenic cells and spermatid cells and promotes nuclear translocation of Nrf2. A study showed that AG glycoside kaempferol could prevent ferroptosis of hepatocytes by promoting the dissociation of Nrf2 and Keap1 [34], which also proved the correctness of our study. However, the promoting effect of AG on Nrf2 nuclear translocation is greater than the agonizing effect on Nrf2, which is consistent with the results of a 2020 study [35]. However, a study showed that administration of

Nrf2 inhibitor could induce ferroptosis of colorectal cancer cell [36]. Nrf2 knockout also can attenuate oligospermia in male mice by inhibiting ferroptosis [37]. Nrf2 appears to have a dual effect on testicular cell iron death, which may be related to the presence of multiple cells in the testes and the different stages of OAS. This will be explored in our subsequent research.

WZYZP consists of *Schisandrae chinensis fructus*, *Lycii fructus*, *Rubus fructus*, *Cuscutae semen* and P*lantaginis semen*. The therapeutic effect of WZYZP on OAS is well known in China, but its inhibitory effect on ferroptosis has not been studied so far [16]. Our results showed that WZYZP was superior to AG in inhibiting spermatogenic cells and spermatids ferroptosis in OAS rats. This result may be due to the presence of other substances that inhibit ferroptosis. Studies have shown that Schisandrin A is another major substance in WZYZP [17], which can reduce ferroptosis in diabetic nephropathy through AdipoR1 ubiquitination [38]. Quercetin, another substance in WZYZP, has also been reported to inhibit ferroptosis and improve diabetic kidney injury by activating Nrf2/HO-1 signaling pathway [17,39]. This may be the reason why WZYZP inhibited ferroptosis better than AG.

Consent for publications

The authors have read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article.

Data availability statement

The data that has been used is confidential.

Ethics statement

Our study used SPF male Sprague-Dawley rats. All the experimental protocol for the use of animal was approved the Ethics Committee of Jiangsu Health Vocational College (JHVC-IACUC-2023-B019).

Ethics approval and consent to participate

The study complies with all regulations. No human were used in the present research. All animal experiments in this study were conducted in accordance with the regulations of the Ethics Committee of Jiangsu Health Vocational College., JHVC-IACUC-2023-B019.

CRediT authorship contribution statement

Jiayu Cai: Writing – original draft, Validation, Conceptualization. **Lingxiong Song:** Data curation. **Zebo Hu:** Methodology. **Xiaojiao Gao:** Formal analysis. **Yuhan Wang:** Visualization, Software. **Yang Chen:** Visualization, Data curation. **Ke Xi:** Methodology, Investigation, Conceptualization. **Xin Lu:** Writing – review & editing. **Yonghui Shi:** Writing – review & editing.

Declaration of competing interest

There are no conflicts of interest in our work and the manuscript was published with the consent of all authors. On behalf of my co-authors, I would like to state that the work described here is original research that has never been published before and is not under consideration for publication elsewhere.

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