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Glycine alleviates ovarian granulosa cell ferroptosis induced by ER α -mediated internalization of polystyrene microplastics



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Polystyrene microplastics (PSs), pervasive environmental contaminants found in food and human tissues, pose an emerging threat to reproductive health. Elucidating the mechanisms underlying PS-induced toxicity and identifying effective interventions to mitigate adverse effects are therefore critically important. Here, our findings demonstrated that PS exposure in 5-week-old female SPF Kunming mice leads to decreased serum hormone levels and reduced transzonal projections. Furthermore, this study revealed that PS-induced ferroptosis in ovarian granulosa cells. Mechanistically, ER α -mediated PS internalization led to activation of the YAP1-ACSL4 signalling and subsequent lipid peroxidation. Moreover, we demonstrated that glycine effectively alleviate PS-induced ferroptosis by modulating lysosome-dependent ferritin degradation in a PAT1-dependent manner, thereby restoring iron homeostasis. Taken together, these findings revealed that PS exposure triggers ACSL4 overexpression and iron overload in ovarian granulosa cells, whereas glycine restored iron homeostasis via lysosome-mediated ferritinophagy. This study provides critical insights into the reproductive health risks of PS exposure and offers a potential intervention strategy.

The increase in global contamination by microplastics (MPs) has emerged as a critical environmental challenge, posing a top risk to ecological, economic, and public health^{1,2}. Despite the implementation of regulatory measures aimed at mitigating plastic pollution, conventional plastics such as polyvinyl chloride, polystyrene, and polyethylene remain extensively used³. Accumulating evidence has demonstrated that adults may ingest up to ~5 g of MPs per week through sources such as tap water, bottled beverages, food, and food packaging, whereas infants exhibit even higher exposure levels, with as many as 11.2 million particles of MPs per week, primarily from feeding bottles, which indicates that human exposure to MPs is pervasive⁴. These MPs have been detected in various tissues, including blood, follicular fluid, endometrial tissue, testis, and placenta⁵. Research has revealed that MP exposure can induce physical damage and trigger inflammatory responses in organs such as the lungs, intestines, and liver^{6–8}. Notably, recent epidemiological studies have detected MPs in the follicular fluid of 69% of women and in semen samples of 55% of men, highlighting urgent concerns

regarding their impact on reproductive health. However, their toxicological effects on the female reproductive system, particularly the ovaries, remain poorly understood. A better understanding of the effects of MPs on the ovary is thus crucial for assessing their long-term health consequences.

Damage induced by environmental pollutants is closely associated with the disruption of iron homeostasis. Previous studies have demonstrated that various environmental toxins, including lead, cadmium, and bisphenol compounds, can induce iron homeostasis imbalance and trigger ferroptosis^{9–11}, which is a form of cell death characterized by iron overload, ROS accumulation, and lipid peroxidation¹². Mechanistically, ferroptosis is driven by two interdependent processes: lipid peroxidation and iron overload. On the one hand, polyunsaturated fatty acids (PUFAs) recognized by ACSL4 are incorporated into phospholipid membranes through acyl-CoA synthetase activity to form phospholipid-PUFAs, which are highly susceptible to peroxidation under conditions of ROS accumulation, leading to oxidative damage to cellular membranes and excess lipid peroxidation¹³.

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On the other hand, iron overload exacerbates free radical generation through the Fenton reaction¹⁴. When the intracellular Fe^{2+} concentration exceeds the physiological threshold, it reacts with H_2O_2 to produce hydroxyl radicals that accelerate lipid peroxidation. The synergistic interplay between dysregulated iron metabolism and excessive ROS production forms the core molecular mechanism underlying ferroptosis. In particular, recent studies have suggested the role of PS in ferroptosis, which influences the function of the testis, lung, and heart¹⁵. However, the detailed *in vivo* transfer process, potential toxicity, and mechanism of PS in the ovary remain unclear.

Given the established multiorgan toxicity of MP exposure, identifying a feasible and safe preventive strategy is imperative. Glycine (Gly), the smallest neutral amino acid, serves as a rate-limiting substrate for glutathione (GSH) synthesis, thereby increasing intracellular GSH levels and reducing ROS generation. Prior studies have demonstrated the capacity of Gly, including its ability to regulate lipid peroxidation during porcine oocyte maturation and early embryonic development¹⁶, mitigate intestinal and hepatic injury through iron homeostasis regulation^{17,18}, and confer ferroptosis resistance when it is combined with high-intensity interval training¹⁹. These findings indicate the potential of Gly in the inhibition of ferroptosis, yet its efficacy in counteracting PS-induced toxicity remains unclear.

In this study, we aimed to investigate whether PS exposure induces ferroptosis to cause ovarian injury. To this end, we constructed a mouse model of subchronic exposure to 500 nm PS to quantify *in vivo* accumulation levels. Transcriptome and molecular analyses further elucidated PS-induced female reproductive dysfunction. To investigate the mechanism underlying microplastic-induced ferroptosis, we assessed the ferrous ion content, lipid peroxidation markers, and key ferroptosis-related proteins. Genetic knockdown experiments revealed that the YAP-ACSL4 pathway is critical for PS-triggered ferroptosis in ovarian granulosa cells. Notably, we revealed that Gly improved PS-induced ovarian injury. Our findings advance the understanding of PS toxicity and suggest that Gly supplementation is a new strategy for preventing the environmental risk of MPs.

Results

PS accumulates in the ovary to induce ferroptosis

To investigate the association between PS and ovarian dysfunction, the stability of commercially obtained PS was verified (Supplementary Fig. 1), and PS was administered orally ($60 \text{ mg}\cdot\text{kg}^{-1}$) for 28 days to establish a mouse model of ovarian dysfunction. In this study, we found that PS-treated mice presented no significant reduction in body weight (Fig. 1a). However, PS entered the ovary via the esophagus (Fig. 1b), causing a significant decline in the ovary index (Fig. 1c). Notably, H&E staining indicated that PS treatment induced loosening of the cumulus cell layer, suggesting a potential mechanism for ovarian dysfunction (Fig. 1d). Furthermore, decreased concentrations of serum estradiol, progesterone and AMH indicated impaired hormone secretion by ovarian granulosa cells (Fig. 1e–g). Transzonal projections (TZPs) are extensions of the cumulus cell that penetrate the zona pellucida and represent the role of granulosa cells in material transport to oocytes. Therefore, we next detected the effect of PS on TZPs, and the data demonstrated that there was a significant reduction in the number of TZPs (Fig. 1h–i). Collectively, these findings demonstrate that mice exposed to PS exhibit severe ovarian dysfunction.

To explore the underlying mechanisms of PS-induced ovarian damage, we subsequently performed ovarian transcriptome profiling. The analysis revealed 1530 differentially expressed genes (DEGs) in the ovaries of PS-exposed mice compared with those of control mice (647 upregulated and 883 downregulated, Supplementary Fig. 2a), identified via a threshold of $|\text{fold change}| \geq 2$ and P value < 0.05 . KEGG pathway and GO enrichment analyses highlighted pathways related to ferroptosis (including oxidative phosphorylation, ferroptosis, iron ion homeostasis, and iron ion binding) as the pathways most notably enriched in the PS-exposed mouse ovaries (Supplementary Fig. 2b–c). Although PS exposure did not significantly affect the total Fe content (Fig. 1j), the transcriptome findings were empirically validated by subsequent results, including increased ovarian malondialdehyde (MDA) levels (Fig. 1k), decreased glutathione (GSH) levels

(Fig. 1l), and abnormal expression of key ferroptosis regulators ACSL4, ALOX15, SLC7A11, and GPX4 (Fig. 1m). Furthermore, immunofluorescence analysis of mouse ovaries was conducted to detect the expression of ACSL4, and the data revealed its marked upregulation (Fig. 1n), suggesting that ACSL4 could be an important protein in PS-induced ovarian ferroptosis. Taken together, these findings demonstrate that PS accumulates in the ovary, induces ferroptosis, and ultimately causes ovarian dysfunction.

PS activates ferroptosis in ovarian granulosa cells

Following the results mentioned above, the mouse granulosa cell line kk-1 was selected to establish *in vitro* models for further investigation into the mechanism by which PS triggers the ferroptosis. The results of the CCK8 assay indicated that treatment with $100 \mu\text{g}/\text{mL}$ PS for 12 h significantly reduced the viability of kk-1 cells (Fig. 2a), similar to the results of co-staining with calcein AM/PI (Fig. 2b). Furthermore, fluorescence labeling (Supplementary Fig. 3a) and transmission electron microscopy (TEM) (Supplementary Fig. 3b) revealed that PS was internalized by kk-1 cells, indicating substantial cellular uptake. Furthermore, we explored the mechanism by which PS enters kk-1 cells. Molecular docking analysis revealed a stable binding energy of $-5.5 \text{ kcal}/\text{mol}$ between styrene (representing PS) and ER α , indicating a strong interaction (Supplementary Fig. 3c). Additionally, we observed increased ER α protein expression and co-localization between PS and ER α (Supplementary Fig. 3d), suggesting its potential role in mediating PS endocytosis. Thus, we demonstrated that PS enters granulosa cells via ER α -mediated uptake.

Next, we investigated whether PS induces ferroptosis in kk-1 cells. The results showed that the PS-treated kk-1 cells presented elevated intracellular ROS and lipid peroxidation (Fig. 2c–e), along with increased levels of the end product of lipid peroxidation MDA and a reduced content of GSH (Fig. 2g–h). In addition, there was a marked increase in the levels of ferrous ions and total Fe in the kk-1 cells treated with PS (Fig. 2f, i). Given that mitochondrial injury is a hallmark of ferroptosis, we assessed mitochondrial parameters. These cells showed elevated mitochondrial ferrous ion levels and decreased mitochondrial membrane potential (Fig. 2j–k). Moreover, TEM revealed mitochondrial ultrastructural damage, including membrane disruption and reduced cristae density in PS-exposed kk-1 cells (Fig. 2l). Consistent with the *in vivo* findings, treatment of $100 \mu\text{g}/\text{mL}$ PS for 12 h markedly upregulated ACSL4 and ALOX15 (Fig. 2m). Collectively, these findings demonstrated that PS activates ferroptosis in ovarian granulosa cells.

Fer-1 inhibits PS-induced ferroptosis in ovarian granulosa cells

To further investigate the role of ferroptosis in PS-induced kk-1 cell damage, we co-treated kk-1 cells with the ferroptosis inhibitor Ferrostatin-1 (Fer-1) and PS. CCK8 assay revealed that Fer-1 treatment significantly attenuated PS-triggered cell death (Fig. 3a), which was corroborated by co-staining with calcein AM/PI (Fig. 3b). Assessment of the levels of cellular ROS and lipid ROS indicated that both cellular and lipid ROS were significantly lower in the Fer-1 and PS co-treatment group than in the PS group (Fig. 3c–d). Correspondingly, Fer-1 and PS co-treatment reversed the PS-induced alterations in the MDA and GSH levels (Fig. 3f–g). Additionally, the content of total Fe and ferrous ions also decreased in response to co-treatment with Fer-1 and PS (Fig. 3e, h). Co-exposure to PS and Fer-1 also mitigated mitochondrial damage, as evidenced by reduced mitochondrial ferrous ion accumulation (Fig. 3i), restored membrane potential (Fig. 3j), and improved mitochondrial ultrastructure (Fig. 3k). Collectively, these findings showed that Fer-1 alleviates the ferroptosis induced by PS in granulosa cells.

The YAP-ACSL4 pathway contributes to PS-induced ferroptosis in granulosa cells

Based on the significant ACSL4 upregulation in granulosa cells following PS exposure (Fig. 2m), we speculated that the ACSL4-dependent pathway mediated PS-induced ferroptosis. To test this hypothesis, we employed siRNA-mediated gene silencing to target ACSL4 expression in kk-1 cells,

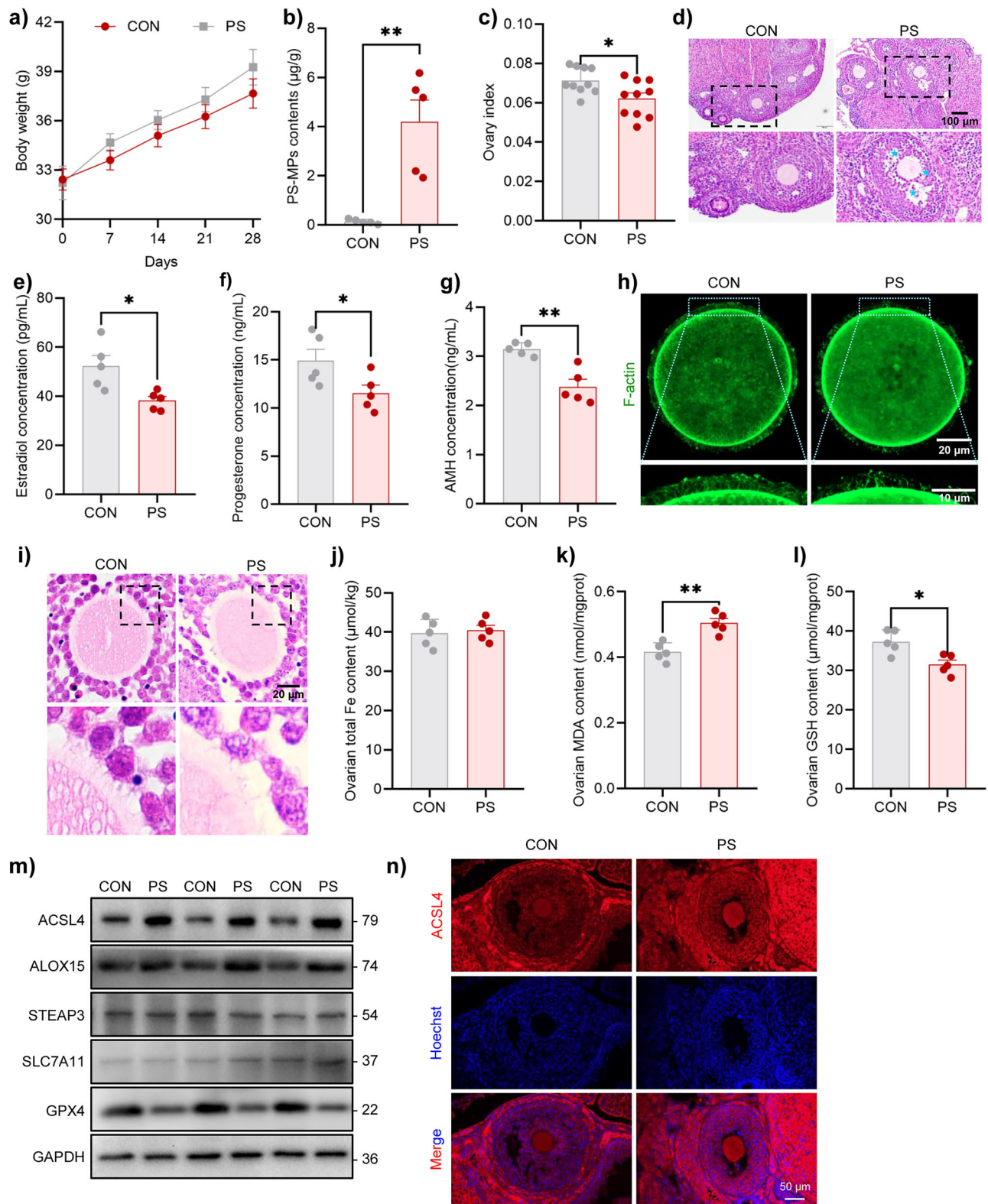


Fig. 1 | Ovarian toxicity induced by PS exposure in female mice. a Body weights of the mice after PS exposure ($n = 10$). **b** PS contents in mouse ovaries following PS treatment ($n = 5$). **c** Ovary indices of ovaries after PS exposure ($n = 10$). **d** H&E-stained ovaries. **e–g** Concentrations of estradiol, progesterone and AMH in the serum ($n = 5$). **h** TZPs stained with F-actin in oocytes. The bottom panel shows a higher magnification of the area outlined in the upper panel. **i** TZPs in ovaries

stained with H&E. **j–l** The levels of ovarian total Fe, MDA and GSH ($n = 5$). **m** Western blot analysis of ferroptosis-related proteins in PS-treated mice. **n** Representative immunofluorescence images of ACSL4 in ovaries. * $P < 0.05$, ** $P < 0.01$, by two-tailed unpaired t test. Data are presented as the mean $\pm$ SEM. The loading control (GAPDH) was used for normalization.

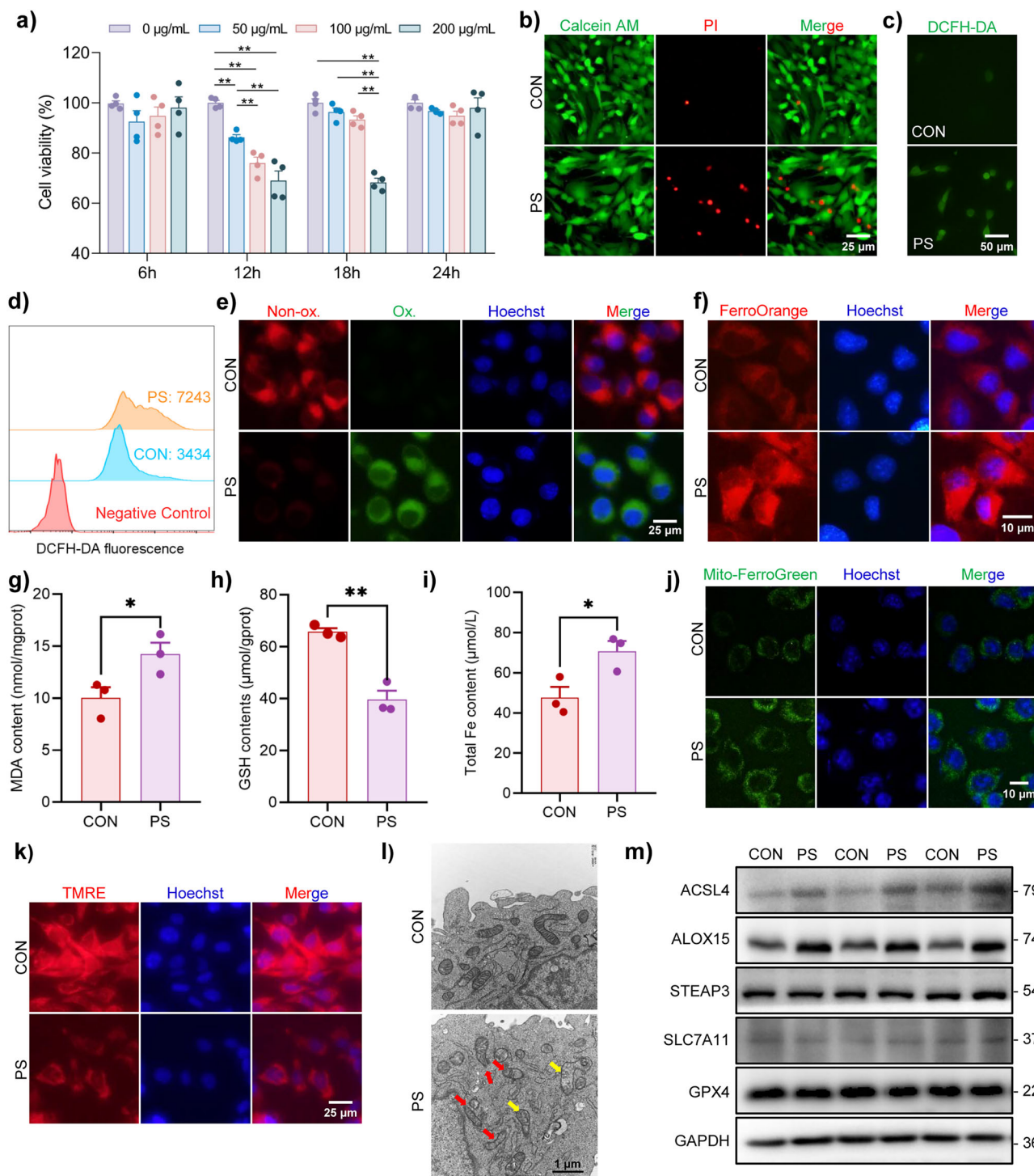


Fig. 2 | PS triggered ferroptosis via interaction with ERα in ovarian granulosa cells. a Viability of kk-1 cells treated with various doses of PS (50, 100 or 200 µg/mL) for 6, 12, 18, or 24 h. **b** Representative images of the survival rate of kk-1 cells co-stained with calcein AM and PI after 100 µg/mL PS treatment for 12 h. **c, d** Representative images of ROS levels in kk-1 cells following PS treatment via fluorescent microscopy and flow cytometry. **e** Representative fluorescent images of lipid ROS in PS-exposed kk-1 cells. **f** Representative fluorescent images of ferrous ions in kk-1 cells after PS treatment. **g–i** The levels of MDA, GSH and total Fe in kk-1 cells after PS treatment. **j** Representative fluorescent images of mitochondrial ferrous

ions in PS-exposed kk-1 cells. **k** Representative images of the mitochondrial membrane potential in PS-exposed kk-1 cells stained with TMRE. **l** TEM images of mitochondria in kk-1 cells. The red arrow indicates mitochondrial membrane rupture, and the yellow arrow indicates mitochondrial crista reduction. **m** Western blot analysis of ferroptosis-related proteins in PS-treated kk-1 cells. * $P < 0.05$, ** $P < 0.01$, by two-tailed unpaired t test and one-way ANOVA with the LSD test. Data are presented as the mean $\pm$ SEM of three independent experiments. The loading control (GAPDH) was used for normalization.

and the efficient knockdown of *Acs14* was confirmed by western blot analysis (Supplementary Fig. 4). The results of the CCK8 assay indicated that *Acs14* knockdown significantly reversed the reduction in viability of PS-treated kk-1 cells (Fig. 4a). Importantly, the knockdown of *Acs14* in kk-1 cells effectively

relieved key ferroptosis-related hallmarks, including excessive lipid peroxidation (Fig. 4b), intracellular ferrous ion accumulation (Fig. 4c) and ROS overproduction (Fig. 4d). Biochemical analysis further revealed that *Acs14* knockdown reversed the PS-induced alterations in the MDA, GSH and total

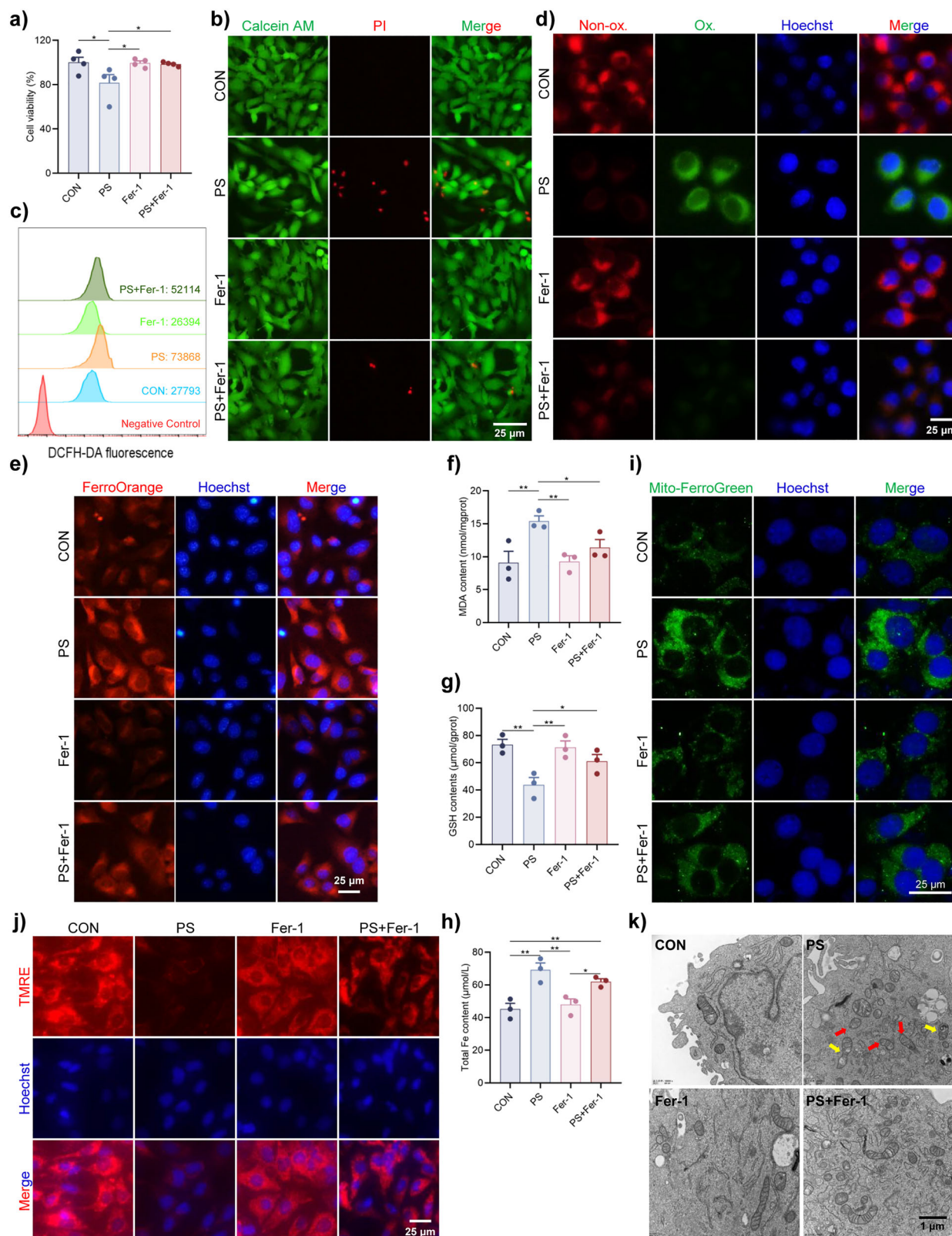


Fig. 3 | Fer-1 attenuated PS-induced ferroptosis in ovarian granulosa cells. **a** Cell viability of kk-1 cells treated with PS and Fer-1 for 12 h. **b** Representative images of kk-1 cells co-stained with calcein AM and PI in response to PS and Fer-1 treatment. **c** Flow cytometry results of lipid ROS in kk-1 cells. **d** Representative fluorescent images of lipid ROS in kk-1 cells after co-exposure to PS and Fer-1. **e** Representative fluorescent images of ferrous ions in kk-1 cells after PS and Fer-1 treatment. **f–h** The levels of MDA, GSH, and total Fe in kk-1 cells after PS and Fer-1 co-treatment.

i Representative fluorescent images of mitochondrial ferrous ions in kk-1 cells after co-exposure to PS and Fer-1. **j** Representative images of the mitochondrial membrane potential in kk-1 cells stimulated with PS and Fer-1 stained with TMRE. **k** TEM images of mitochondria in kk-1 cells. The red arrow indicates mitochondrial membrane rupture, and the yellow arrow indicates mitochondrial crista reduction. * $P < 0.05$, ** $P < 0.01$, one-way ANOVA with the LSD test. Data are presented as the mean $\pm$ SEM of three independent experiments.

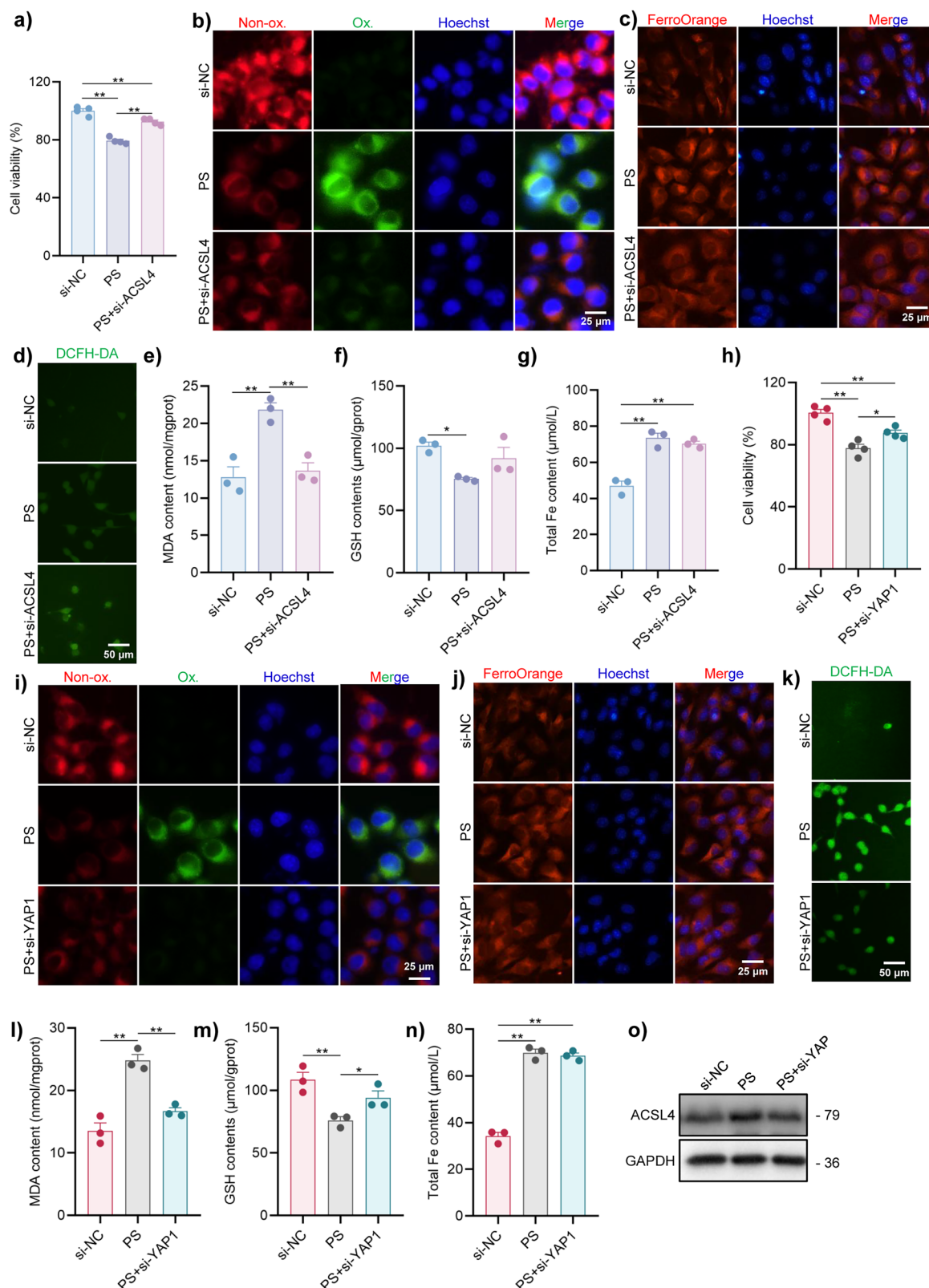


Fig. 4 | PS induced ferroptosis via the YAP1-ACSL4 pathway in ovarian granulosa cells. a Viability of *kk-1* cells after *Acsl4* knockdown. **b** Representative fluorescent images of lipid ROS in *Acsl4*-knockdown *kk-1* cells. **c** Representative images of ferrous ions in *Acsl4*-knockdown *kk-1* cells stimulated with PS. **d** Representative images of ROS levels in *Acsl4*-knockdown *kk-1* cells following PS treatment via fluorescent microscopy. **e–g** The levels of MDA, GSH and total Fe in *Acsl4*-knockdown *kk-1* cells after PS treatment. **h** Viability of *kk-1* cells after *Yap1* knockdown. **i** Representative fluorescent images of lipid ROS in *Yap1*-knockdown

kk-1 cells. **j** Representative images of ferrous ions in *Yap1*-knockdown *kk-1* cells stimulated with PS. **k** Representative images of ROS levels in *Yap1*-knockdown *kk-1* cells following PS treatment via fluorescent microscopy. **l–n** The levels of MDA, GSH, and total Fe in *Yap1*-knockdown *kk-1* cells after PS treatment. **o** Effects of *Yap1* knockdown on the PS-increased ACSL4 protein in *kk-1* cells. * $P < 0.05$, ** $P < 0.01$, one-way ANOVA with the LSD test. Data are presented as the mean $\pm$ SEM of three independent experiments. The loading control (GAPDH) was used for normalization.

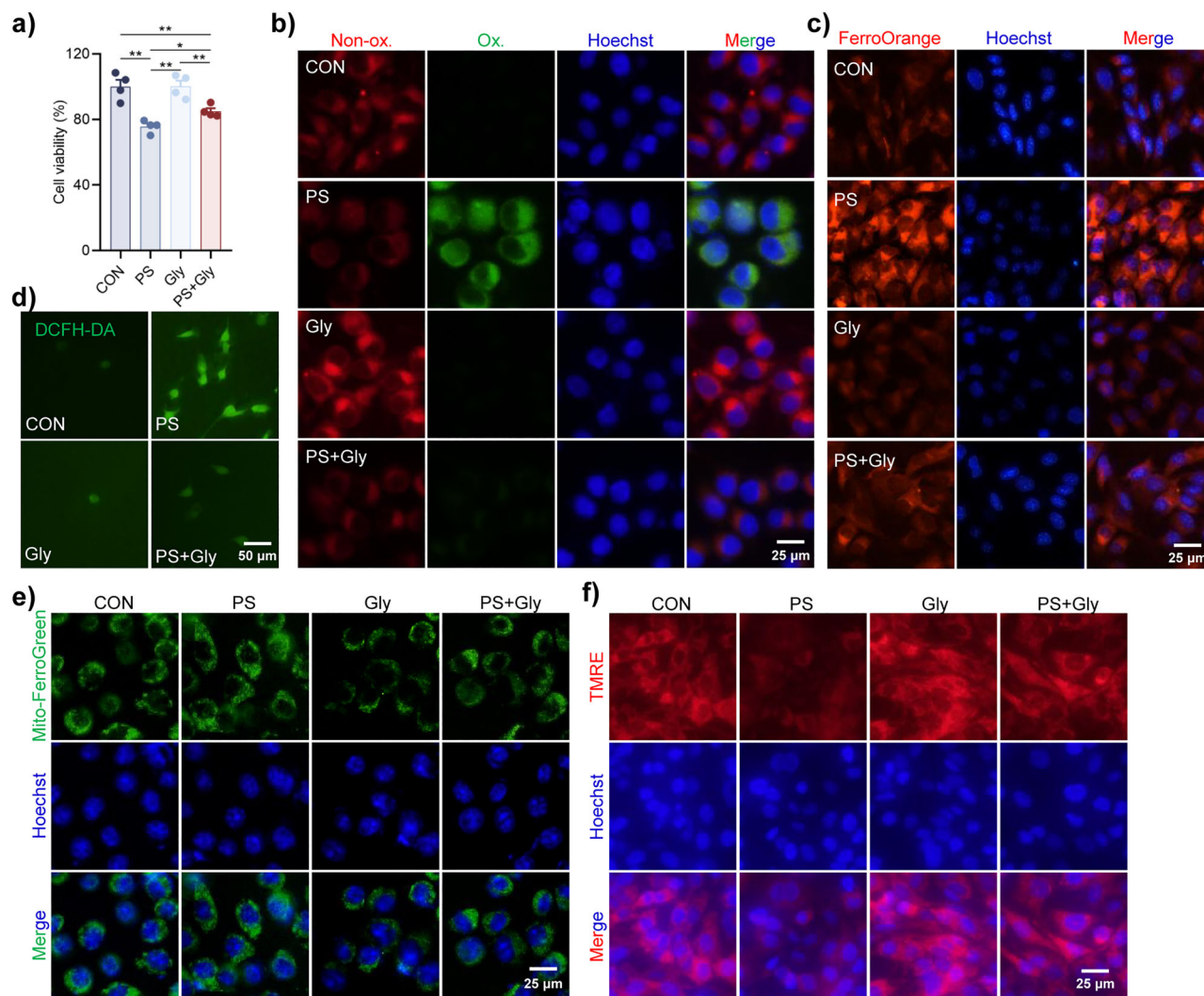


Fig. 5 | Gly reversed PS-induced ferroptosis in ovarian granulosa cells. a Viability of kk-1 cells treated with PS and Gly for 12 h. **b** Representative fluorescent images of lipid ROS in kk-1 cells after co-exposure to PS and Gly. **c** Representative fluorescent images of ferrous ions in kk-1 cells after PS and Gly treatment. **d** Representative

fluorescent images of the cellular ROS of kk-1 cells. **e** Representative fluorescent images of mitochondrial ferrous ion in kk-1 cells after co-exposure to PS and Gly. **f** Representative images of the mitochondrial membrane potential in kk-1 cells stimulated with PS and Gly stained with TMRE.

Fe levels (Fig. 4e–g). Previous studies have suggested that the activation of Hippo pathway and dephosphorylation of YAP1 regulate the expression of ACSL4 in kk-1 cells²⁰. Thus, we determined the regulatory role of YAP1 in PS-induced ferroptosis. Our findings showed that PS exposure not only increased total YAP1 expression but also inhibited its phosphorylation (Supplementary Fig. 5a), thereby facilitating the nuclear translocation of YAP1 (Supplementary Fig. 5b), suggesting that YAP1 plays a crucial role in PS-induced ferroptosis. Following the successful knockdown of *Yap1* (Supplementary Fig. 6), the cell survival rate improved (Fig. 4h), and the level of lipid peroxidation was suppressed (Fig. 4i). Although *Yap1* knockdown failed to reverse the increase in ferrous ion levels (Fig. 4j), it effectively attenuated the excessive accumulation of intercellular ROS (Fig. 4k). Biochemical analysis further revealed that *Yap1* knockdown reversed the PS-induced alterations in MDA, GSH and total Fe levels (Fig. 4l–n). The western blot results confirmed the upstream regulatory function of YAP1 in ACSL4 expression in PS-exposed cells (Fig. 4o). Taken together, these results showed that YAP-ACSL4 pathway contributes to PS-induced ferroptosis in granulosa cells.

Gly alleviates PS-triggered ferroptosis in ovarian granulosa cells

Gly alleviated diquat-induced hepatic injury by inhibiting ferroptosis in weaned piglets¹⁸. Thus, we investigated whether Gly reverses the ferroptosis

induced by PS in kk-1 cells. First, CCK8 assay revealed that Gly treatment significantly relieved PS-triggered ferroptosis (Fig. 5a). Assessment of the level of lipid ROS indicated that lipid ROS was significantly lower in the Gly + PS groups than in the PS group (Fig. 5b). Furthermore, Gly co-treatment reversed the PS-induced alterations in ferrous ion (Fig. 5c) and intercellular ROS levels (Fig. 5d). Co-exposure to PS and Gly also mitigated the mitochondrial damage, as evidenced by reduced mitochondrial ferrous ion accumulation (Fig. 5e) and restored membrane potential (Fig. 5f). Collectively, these findings showed that Gly alleviates the ferroptosis induced by PS in granulosa cells.

Gly relieves the ferroptosis induced by PS via the inhibition of NCOA4-mediated ferritinophagy

The western blot results showed that Gly failed to relieve the expression of ACSL4 upregulated by PS (Fig. 6a), suggesting that there might be another pathway through which Gly improves PS-triggered ferroptosis. Given the established mechanistic hallmarks of ferroptosis, namely lipid peroxidation and dysregulated iron homeostasis, we propose that Gly can alleviate iron overload primarily through NCOA4-mediated ferritinophagy, a selective autophagic process that governs intracellular iron homeostasis. Western blot analysis revealed that PS induced FTH1 downregulation, while upregulating the expression of NCOA4, and Gly co-treatment effectively

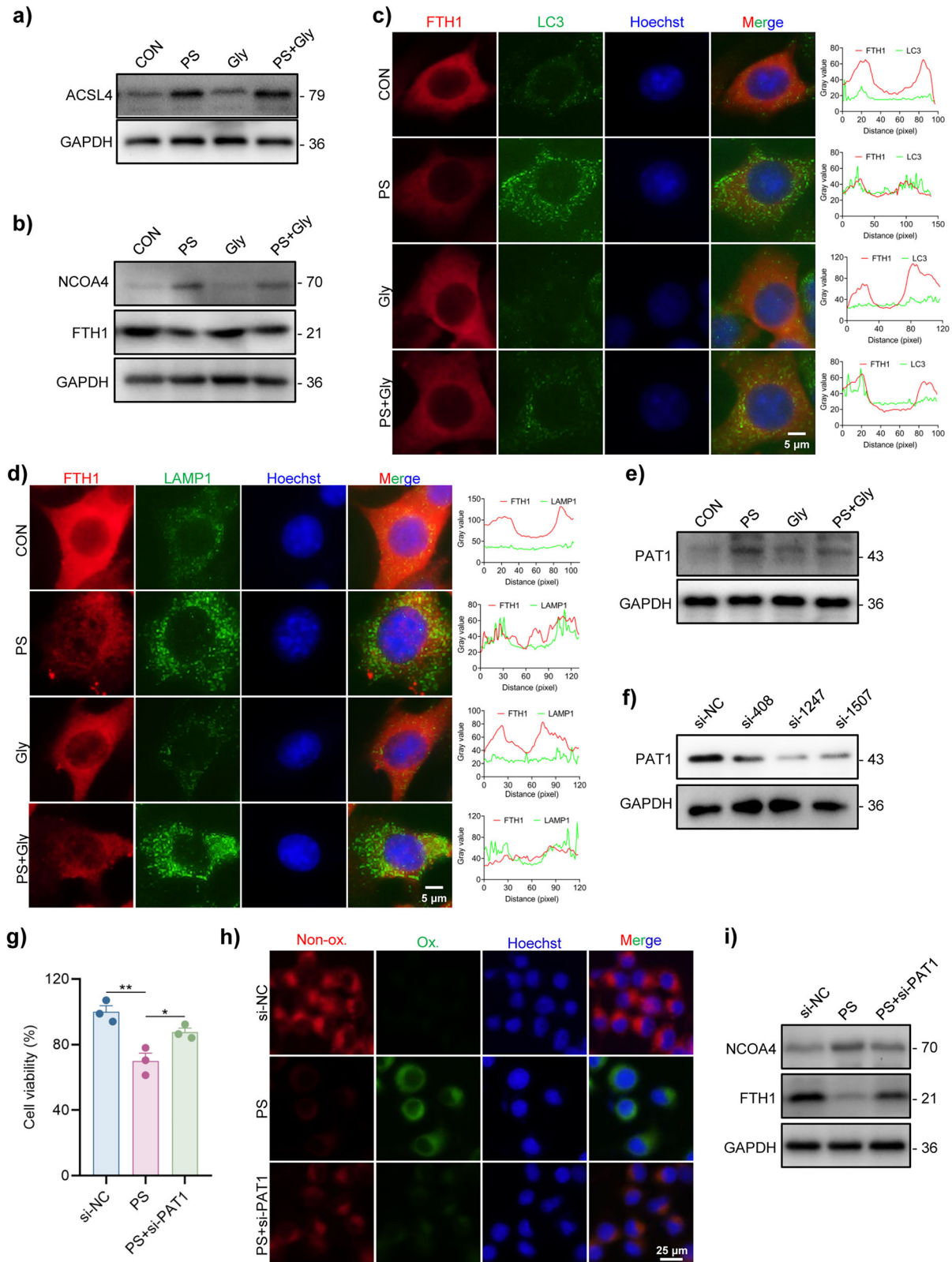


Fig. 6 | Gly interacted with PAT1 to regulate ferritinophagy in ovarian granulosa cells. **a** Protein expression of ACSL4 in kk-1 cells after co-exposure to PS and Gly. **b** Western blot of FTH1 and NCOA4 in kk-1 cells after PS and Gly co-exposure. **c** Representative immunofluorescent images of FTH1 and LC3 and co-localization analysis after co-exposure with PS and Gly. **d** Representative immunofluorescent images staining for FTH1 and LAMP1 and co-localization analysis in kk-1 cells after co-exposure to PS and Gly. **e** Western blot of PAT1 in kk-1 cells after

PS and Gly co-exposure. **f** Western blot analysis of PAT1 protein expression in cells transfected with control siRNA (si-NC) or *Pat1*-targeting siRNAs. **g** Viability of kk-1 cells after *Pat1* knockdown. **h** Effect of *Pat1* knockdown on PS-upregulated lipid ROS in kk-1 cells. **i** Western blot of NCOA4 and FTH1 in *Pat1*-knockdown kk-1 cells after co-exposure to PS and Gly. * $P < 0.05$, ** $P < 0.01$, one-way ANOVA with the LSD test. Data are presented as the mean $\pm$ SEM of three independent experiments. The loading control (GAPDH) was used for normalization.

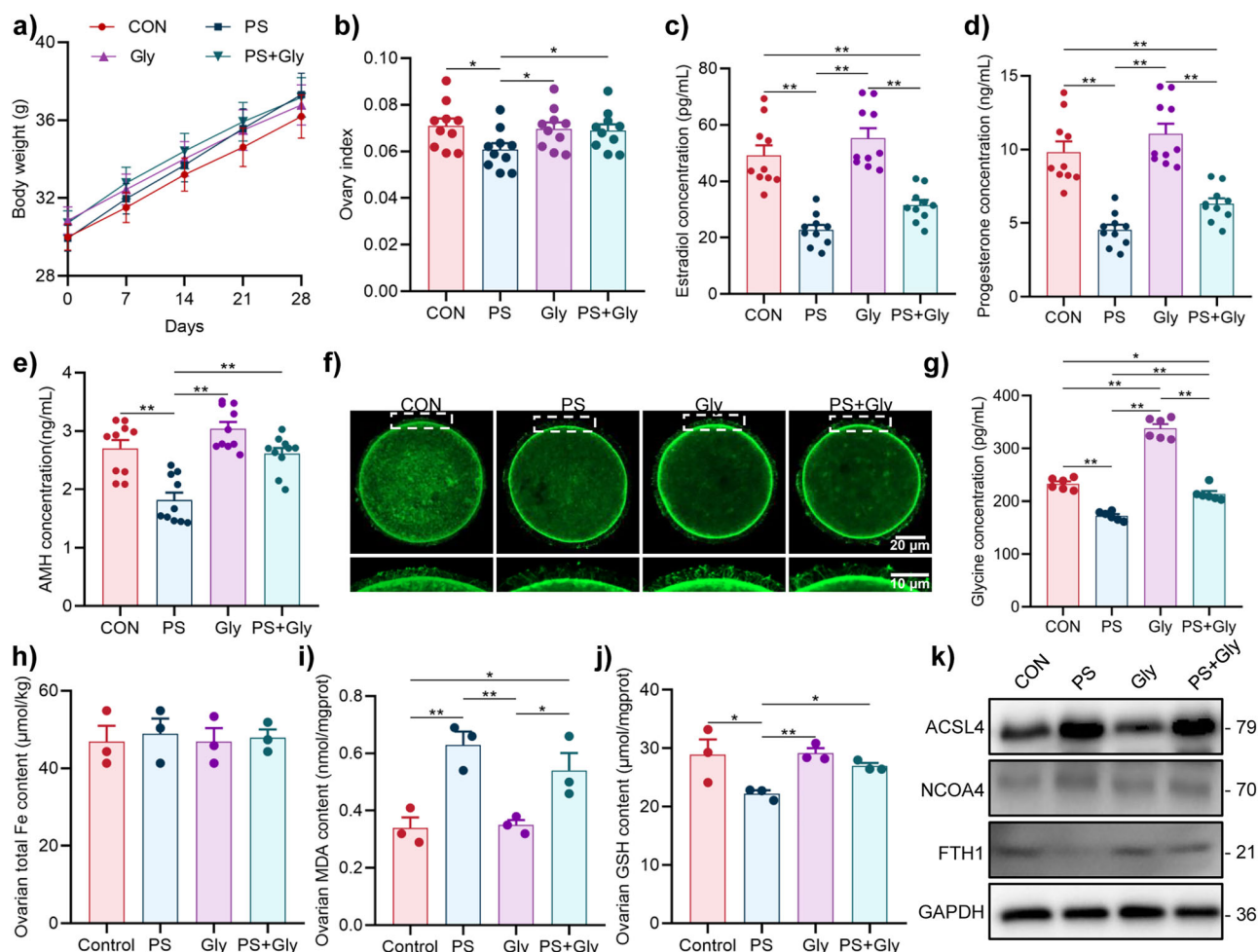


Fig. 7 | Gly mitigated ovarian ferroptosis via PAT1-mediated ferritinophagy in mice. **a** Body weights of the mice after PS, Gly, and PS + Gly exposure ($n = 10$). **b** Ovary indices of ovaries after PS and Gly exposure ($n = 10$). **c–e** Concentrations of estradiol, progesterone and AMH in the serum ($n = 10$). **f** TZPs stained with F-actin in oocytes. The bottom panel shows a higher magnification of the area outlined in the upper panel. **g** Concentration of Gly in mouse ovaries ($n = 6$). **h–j** The levels of

ovarian total Fe, MDA and GSH ($n = 3$). **k** Western blot of ferroptosis-related proteins in mouse ovaries after PS, Gly, and PS + Gly exposure. $*P < 0.05$, $**P < 0.01$, one-way ANOVA with the LSD test. Data are presented as the mean $\pm$ SEM of three independent experiments. The loading control (GAPDH) was used for normalization.

restored these perturbations to normal levels (Fig. 6b). NCOA4-bound FTH1 is encapsulated by autophagosomes and dispatched to lysosomes for degradation, subsequently leading to the release of Fe^{2+21} . The colocalization of FTH1 with LC3 and LAMP1 in PS-treated cells further confirmed ferritinophagy activation (Fig. 6c–d). Proton-coupled amino acid transporter 1 (PAT1) is transcriptionally upregulated under amino acid-deprived conditions to enhance lysosomal degradation efficiency²². We speculate that PAT1 may be involved in the protective effect of Gly against PS-induced ferroptosis. Western blot analysis confirmed that Gly significantly reversed PS-induced PAT1 overexpression (Fig. 6e). To validate the role of PAT1 in ferritinophagy regulation mechanistically, we constructed a siRNA-mediated *Pat1* knockdown model (Fig. 6f). Cell viability and lipid peroxidation assays indicated that *Pat1* silencing significantly reversed the reduction in viability and reduced lipid ROS accumulation (Fig. 6g–h). Furthermore, *Pat1* knockdown ameliorated the PS-triggered abnormal expression of NCOA4 and FTH1 (Fig. 6i). Taken together, these findings demonstrated that Gly relieves PS-induced ferroptosis via the inhibition of NCOA4-mediated ferritinophagy.

Gly improves ovarian functions impaired by PS via the inhibition of ferroptosis

We further evaluated the protective effect of Gly on ovarian damage caused by PS in vivo. As expected, Gly supplementation did not affect body weight

(Fig. 7a) but significantly improved the PS-triggered reduction in the ovary index (Fig. 7b), suggesting reversal of ovarian damage. Serum hormone analysis revealed that Gly restored the PS-induced decreases in the levels of estradiol, progesterone, and AMH (Fig. 7c–e), confirming improved ovarian endocrine function. Phalloidin staining further revealed that Gly restored the density of TZPs (Fig. 7f). Crucially, Gly supplementation prevented PS-induced glycine deficiency, (Fig. 7g) and ferroptosis biomarkers, including elevated MDA and reduced GSH (Fig. 7i, j), while maintaining stable total Fe levels (Fig. 7h). Western blot analysis confirmed that Gly attenuated PS-triggered ferritinophagy activation by restoring expression of NCOA4 and FTH1 without altering ACSL4 expression, which was previously established in our in vitro model (Fig. 7k). Collectively, these results indicated that Gly preserves ovarian function by suppressing PS-triggered ferroptosis.

Discussion

In the present study, we elucidate the molecular mechanism of PS-induced ovarian toxicity and identify Gly as an effective protective agent against PS-induced ferroptosis in the ovary. Although several studies have investigated the effects of PS on male and female reproductive health, most have focused primarily on the phenotypic observations of organ injury and morphological alterations^{23–25}, leaving the underlying molecular mechanisms largely uncharacterized. Our findings revealed that, as an emerging environmental pollutant, PS is internalized into granulosa cells through interaction with

ER α , subsequently activating the YAP1-ACSL4 signalling axis and promoting lipid peroxidation, a hallmark of ferroptosis. Notably, we demonstrated that Gly ameliorates PS-triggered ferroptosis by restoring iron homeostasis via interaction with the amino acid transporter PAT1. These results advance our understanding of MP-induced reproductive toxicity and highlight a potential nutritional intervention strategy to mitigate the adverse health effects of environmental pollutants.

With global annual plastic production nearing 390 million metric tons and a recycling rate of less than 10%, accumulating evidence has demonstrated the presence of MPs in human and animal tissues, including blood, placenta, uterus and follicular fluid^{26–29}, and their strong associations with elevated risks of hematological malignancies, intestinal inflammation, cerebral thrombosis, cardiovascular dysfunction, and neurodegenerative diseases^{30–34}. In our previous work, we demonstrated that NPs can enter mouse testicular tissues, inducing structural damage and impairing sperm quality²⁵. However, the molecular mechanisms underlying PS-induced ovarian injury remain poorly defined. Here, we provide direct evidence that PS exposure induces ferroptosis, an iron-dependent form of programmed cell death, implicated in ovarian senescence, polycystic ovary syndrome, and ovarian insufficiency^{35–37}. The 500 nm PS particles used in this study are environmentally relevant, as they fall within the size range abundantly generated from the fragmentation of larger plastic waste and are present in various consumer products, including cosmetics and industrial abrasives. Of note, Fer-1 effectively attenuated PS-triggered granulosa cell death, strongly supporting the central role of ferroptosis in PS-induced ovarian toxicity. Furthermore, we revealed that PS is internalized into granulosa cells through interaction with ER α , a critical regulator of folliculogenesis and steroidogenesis. Given the high expression of ER α in granulosa cells, the sensitivity of female reproductive tissue to MP exposure is emphasized. Our study revealed that PS is internalized into granulosa cells via interaction with ER α . Moreover, we confirmed that both NPs and MPs can bind to estrogen receptors, triggering downstream cellular dysfunction and tissue damage. These findings provide novel mechanistic insights into the pathophysiological effects of microplastics on ovarian function.

Further investigations revealed that PS activates YAP1, a transcriptional coactivator known to respond to mechanical signals and cytoskeletal dynamics, thereby upregulating ACSL4, which is an essential enzyme that incorporates polyunsaturated fatty acids (PUFAs) into membrane phospholipids prone to peroxidation. In recent years, ferroptosis has been increasingly recognized as an essential mechanism of tissue damage associated with environmental stressors, including heavy metals, airborne particulates, and engineered nanoparticles^{38–40}. While the YAP1-ACSL4 axis has been implicated in ferroptosis-associated pathologies in prior studies^{41–43}, this work presents the first direct evidence that this signalling pathway plays an important role in PS-triggered ovarian granulosa cell toxicity. Our results demonstrated that silencing of either YAP1 or ACSL4 significantly improved PS-induced lipid ROS accumulation and cell death, as confirmed by siRNA-mediated knockdown and rescue experiments. As exogenous microparticles, PS may elicit mechanical stress-mimetic signalling through physical perturbation of the membrane architecture or disruption of cytoskeleton dynamics. Subsequently, YAP1, a key mechanosensitive transcriptional coactivator, is activated and translocated into the nucleus, leading to the activation of the Hippo pathway⁴⁴. On the basis of the above results, we propose a mechanistic model in which PS is internalized into granulosa cells via an ER α -dependent pathway, leading to YAP1 dephosphorylation, its subsequent nuclear translocation, and the transcriptional activation of ACSL4. This cascade ultimately drives iron-dependent lipid peroxidation and causes ferroptosis. This model not only establishes a complete toxicological framework that provides critical mechanistic insights into MP-induced ovarian dysfunction but also extends the pathological implications of ferroptosis in reproductive toxicity induced by environmental contaminants, highlighting potential molecular targets for intervention.

Given the critical role of the YAP1-ACSL4 axis in PS-induced ferroptosis, the development of effective intervention strategies is needed. In this study, we identified Gly as a potent protective agent against PS-induced

ovarian injury that acts through a previously unrecognized mechanism. The concurrent restoration of AMH and E₂ levels, along with the reconstruction of TZPs *in vivo*, indicates that glycine intervention not only rescues granulosa cell viability but also promotes the recovery of key ovarian endocrine and paracrine functions. These findings suggest the high potential of glycine to preserve follicular health and developmental competence following PS insult. Previous studies have shown that Gly modulates lipid peroxidation and ferroptosis across diverse pathological models, including the promotion of porcine oocyte maturation, amelioration of diquat-induced intestinal and hepatic injury in piglets and attenuation of age-related skeletal muscle atrophy in mice^{16–19}, which revealed that the underlying mechanisms have been largely attributed to its antioxidant properties, suppression of iron accumulation and regulation of iron metabolism. Here, we found that Gly preserves FTH1 protein stability by inhibiting lysosome-dependent degradation in a PAT1-dependent manner, thereby reducing the labile iron pool and blocking Fenton action-driven lipid peroxidation. Notably, knockdown of *Pat1* or pharmacological inhibition of its transport activity largely abolished the Gly-mediated restoration of iron homeostasis, suggesting that the cytoprotective function of Gly is mechanistically dependent on PAT1. These findings reveal a critical role for PAT1-mediated amino acid transport in regulating ferroptosis sensitivity in the ovary and highlight a novel molecular axis, Gly-PAT1-FTH1, as a potential therapeutic target for mitigating microplastic-induced ovarian dysfunction.

Although YAP1-ACSL4 has been implicated as a potential target for PS-induced ovarian damage, several limitations of this study must be acknowledged. First, our experimental model was established using a single type and narrowly defined size of MPs; however, environmental MPs exhibit substantial heterogeneity in terms of particle size, shape, surface chemistry, and aging state⁴⁵, all of which can influence their bioavailability, cellular uptake, and toxicological outcomes. We believe that changes in particle size, morphology, and specific surface area may exacerbate tissue damage following the internalization of MPs into biological tissues. Thus, the generalizability of our findings requires further investigation. Second, our mechanistic insights are derived primarily from murine models and *in vitro*-cultured granulosa cells. While these systems provide valuable experimental control, validation of the ER α -YAP1-ACSL4 signalling axis and the protective role of PAT1-mediated Gly transport in human ovarian tissues are essential. Furthermore, the *in vivo* pharmacokinetics of Gly, including its absorption, distribution, and reproductive tissue bioavailability, remain poorly characterized and warrant comprehensive pharmacological evaluation. Finally, although we revealed a previously unrecognized role for PAT1 in regulating iron homeostasis, the underlying molecular mechanisms remain to be fully elucidated. Addressing these limitations will be critical for advancing the field toward mechanistic precision and developing effective interventions against microplastic-induced female reproductive toxicity.

In conclusion, our findings revealed that PS activated the YAP1-ACSL4 signalling to induce ovarian granulosa cell ferroptosis via ER α -mediated internalization. Moreover, Gly effectively mitigates PS-triggered ovarian injury by maintaining iron homeostasis through PAT1-dependent regulation of lysosomal iron metabolism. This work not only fills a critical knowledge gap regarding the impact of microplastics and nanoplastics on female reproductive function but also establishes a theoretical link between plastic and female reproductive dysfunction, which links molecular pathogenesis with potential nutritional intervention strategies and offers new avenues for protecting female reproductive health against plastic.

Methods

Chemicals

Nonfluorescent and fluorescent polystyrene microplastics (PS, Cy5-PS, and FITC-PS, 25 mg/mL in ddH₂O) with a particle size of 500 nm were procured from Zhichuan Technology (Changzhou, China). Before exposure, the PS was sonicated for 30 min (a cycle including sonication for 3 s and an interval for 5 s) via an ultrasonic cell crusher (JY98-IIIN, Ningbo, China) in an ice bath and subsequently diluted to a concentration of 10 mg·mL^{−1} in ddH₂O.

Animal exposure

Sixty 5-week-old female SPF Kunming mice purchased from Dossy Experimental Animals Co. Ltd. (Chengdu, China) were housed in the Laboratory Animal Center of Northwest A&F University under controlled pathogen-free conditions. The environment was maintained at a temperature of $25 \pm 1^\circ\text{C}$, a relative humidity of $55 \pm 10\%$, and a 12 h light/dark cycle. Standard diet and food-free water were provided *ad libitum*.

To assess the toxicity of PS on the female reproductive system, twenty mice were randomly divided into two groups ($n = 10$): the CON group and the PS group. After a 7-day acclimation period, mice received an oral gavage of PS suspension ($60 \text{ mg}\cdot\text{kg}^{-1}$ body weight per day) or an equivalent volume of sterile water daily for 28 consecutive days. Furthermore, forty mice were randomly divided into four groups ($n = 10$) as follows: the CON group, the PS group, the Gly group ($1 \text{ g}\cdot\text{kg}^{-1}$ Gly), and the PS + Gly group ($60 \text{ mg}\cdot\text{kg}^{-1}$ PS + $1 \text{ g}\cdot\text{kg}^{-1}$ Gly) to investigate the role of Gly in PS-exposed mice. After a 7-day acclimation period, the mice were exposed to $200 \mu\text{L}$ of specific solution (ddH_2O and $10 \text{ mg}\cdot\text{mL}^{-1}$ PS, $200 \text{ mg}\cdot\text{mL}^{-1}$ Gly or PS + Gly) by oral gavage for 28 days until the samples were collected.

Blood was collected behind the eyes, placed at 4°C for 2 h and centrifuged at 4000 rpm for 10 min at 4°C to obtain serum. After decapitation, mouse ovaries were collected for subsequent experiments. Environmentally relevant doses are detailed in the Supplementary Information.

Characterization of PS

The morphology of the PS was analyzed via TEM (FEI, Hillsboro, USA), and its hydrodynamic diameter and zeta potential were examined via a Zetasizer (Malvern, Worcestershire, U.K.).

Histological analysis of ovaries

After deparaffinized and rehydrated, 4% PFA-fixed and paraffin-embedded ovaries were sectioned at a thickness of $2 \mu\text{m}$ for hematoxylin and eosin (H&E) staining. All the sections were observed and imaged by using a BX40 microscope (Olympus, Tokyo, Japan).

Py-GC/MS analysis of the PS content

The PS content in the mouse ovary was assessed via Py-GC/MS (Shimadzu, Kyoto, Japan) following the methodology of previous studies⁴⁶. In brief, nitric acid (68%) was used to digest the ovaries and this digestion mixture was concentrated. The sample crucible was subsequently used to analyze the sample. The Py-GC/MS calibration curve for the samples is represented by the equation $y = 107.6x - 1.0549$, with an R^2 value of 0.9931. Finally, the content of PS was determined via a calibration curve.

Collection of oocytes

The isolated ovaries were placed in a modified Krebs-Ringer, M2 medium (Aibei, M1250, Nanjing, China), and the well-developed follicles were punctured with 30-G needles to collect the GV oocytes or COCs. Subsequently, COCs were firstly pipetted into 0.1% hyaluronidase to remove cumulus cells.

Detection of TZPs morphology and distribution

The collected oocytes were fixed with 4% PFA at room temperature for 30 min, incubated with permeabilization buffer (Beyotime, P006) at room temperature for 10 min, and then stained with FITC-labeled phalloidin in the dark for 30 min at 37°C . A confocal microscope LSM980 with Airyscan2 (Zeiss, Oberkochen, Germany) was subsequently utilized to capture images of the TZPs.

RNA-seq analysis

The TRIzol reagent was used to isolate and purify the total RNA according to the manufacturer's procedure. Then, the Illumina NovaSeq™ 6000 (Wekemo Co., Ltd., Shenzhen, China) was used to sequence the strand-specific cDNA library. Differential expression analysis was performed via the DESeq2 package, and the DEGs were selected with $|\text{Log}_2(\text{fold change})| > 1$ and $P < 0.05$. Finally, bioinformatic analysis and visualization

were performed via OmicStudio tools (<https://www.omicstudio.cn/tool>), Bioincloud (<http://www.bioincloud.tech/>), and an online bioinformatics analysis platform (<https://www.bioinformatics.com.cn>).

Cell culture

The contamination-free ovarian granulosa cell kk-1, which was purchased from Bluef (Shanghai) Biotechnology Development Co., Ltd. (RRID: CVCL_6663, Shanghai, China), was cultured in DMEM/F12 (Gibco, Waltham, USA) supplemented with 10% (v/v) FBS (Zeta Life, CA, USA), penicillin (100 U mL^{-1}) and streptomycin ($100 \mu\text{g mL}^{-1}$) (Solarbio, Beijing, China) in a 5% CO_2 incubator at 37°C . Various reagents, including 50, 100, $200 \mu\text{g mL}^{-1}$ PS, $100 \mu\text{g mL}^{-1}$ FITC-PS/Cy5-PS, $4 \mu\text{M}$ Fer-1 (Glpbio, Montclair, USA), and 2 mM Gly (Sigma, St. Louis, MO, USA), were added to the kk-1 cells following the specific experimental protocols.

Cell viability assay

A CCK-8 assay (Glpbio, USA) was used to assess overall cell viability on the basis of mitochondrial dehydrogenase activity, while calcein-AM/PI dual staining (Beyotime, C2015L) was used to distinguish live (calcein-AM positive, green) and dead (PI positive, red) cells simultaneously on the basis of membrane integrity. For the CCK-8 assay, after the cells were stimulated with different intervention factors, the culture medium was removed, and the cells were subsequently incubated with fresh medium supplemented with CCK-8 at 37°C in a 5% CO_2 incubator for 3 h. Finally, the absorbance at 450 nm was quantified and employed to calculate the cell viability. For calcein/PI, after exposure, the cells were fluorescently labeled with a mixture of calcein AM and PI for 30 min and then captured with the BX40 microscope.

Cellular uptake assay

To investigate the distribution of PS in kk-1 cells, a fluorescence microscope was used to detect the ability of kk-1 cells to internalize PS. Briefly, the cells were exposed to $100 \mu\text{g mL}^{-1}$ FITC-PS or Cy5-PS for 0 or 12 h, after which the cells were photographed.

Biochemical and ELISA assays

The contents of total Fe in mouse ovaries and kk-1 cells were detected via a commercial total Fe assay kit (Servicebio, Wuhan, China), and the contents of GSH and MDA in mouse ovaries and kk-1 cells were measured using commercial GSH and MDA assay kits purchased from Solarbio (Beijing, China). The concentrations of estradiol, progesterone, and anti-Müllerian hormone in the serum were measured via commercial ELISA kits (Reed-Biotech, Wuhan, China).

Determination of lipid peroxidation level

Following specific treatment, kk-1 cells were stained with C11 BODIPY 581/591 (Glpbio, Montclair, USA), followed by washing with PBS three times. Finally, a BX40 microscope was used to capture the images.

TEM assay

The subcellular structure of PS-treated kk-1 cells was observed via TEM analysis. In brief, kk-1 cells were fixed in 4% glutaraldehyde at room temperature for 2 h and then dehydrated via a graded series of alcohol and acetone. The samples were subsequently embedded in HistoCore Arcadia (Leica, Wetzlar, Germany) and sectioned via an EMUC7 ultrathin microtome (Leica, Wetzlar, Germany). Finally, the sections were stained with uranyl acetate and lead citrate, and observed via a TECNAI G2 SPIRIT BIO TEM (FEI, Hillsboro, USA).

Detection of cellular and mitochondrial ROS

After specific exposure, kk-1 cells were stained with the fluorescent probes DCFH-DA (Beyotime, S0033) and MitoSOX (GlpBio, GC68230) to detect cellular and mitochondrial ROS, respectively. A BX40 microscope was used to observe the DCFH-DA-labeled cells, and a FACSARIA™ III flow cytometer (BD Biosciences, USA) was used to assess the level of mitochondrial ROS in

MitoSOX-labeled cells. In flow cytometry, the target cell population was circled based on FSC and SSC, and the debris and dead cells were excluded.

Mitochondrial membrane potential ($\Delta\Psi_m$) assay

Following PS treatment, kk-1 cells were fluorescently labeled with TMRE (Beyotime, C2001), and images were captured via a BX40 microscope to detect the mitochondrial membrane potential.

Immunofluorescence assay

Following the treatment described above, the cells were fixed in 4% PFA for 10 min at room temperature and then incubated in commercial blocking buffer (Beyotime, P0102) at room temperature for 20 min followed by incubation with primary antibody against LAMP1 (Santa Cruz, sc-20011, 1:200), FTH1 (Affinity, DF6278, 1:250) and LC3 (Poteintech, 66139-3-Ig, 1:500) at 4 °C overnight. Then, the cells were rinsed with PBST three times for 5 min each, after which they were fluorescently labeled with a goat anti-mouse secondary antibody (Proteintech, RGAM002, 1:500) or a goat anti-rabbit secondary antibody (Proteintech, RGAR004, 1:500).

Western blot

The cells and mouse ovaries were lysed with precooled RIPA buffer (Beyotime, P0013) supplemented with 1% PMSF (Beyotime, ST506) and 2% protease inhibitor (Solarbio, P6730, Beijing, China) after specific treatment. The concentration of total protein was quantified via a BCA assay kit (Beyotime, P0010). 20 μ g of protein was resolved via 12% SDS-PAGE, and then electrophoretically transferred to PVDF membranes (Millipore, ISEQ00010, Billerica, USA) via a Junyi JY-ZY5 blot transfer cell apparatus. The transfer buffer containing 25 mM Tris-base, 192 mM Gly, and 20% methanol was purchased from Epizyme (Shanghai, China). The PVDF membranes were blocked with blocking buffer at room temperature for 30 min followed by incubation with primary antibodies against ACSL4 (Affinity, DF12141, 1:1000), ALOX15 (Affinity, DF13494, 1:1000), SLC7A11 (Affinity, DF12509, 1:1000), GPX4 (Affinity, DF6701, 1:1000), YAP1 (Affinity, DF3182, 1:1000), p-YAP1 (Ser127) (Affinity, AF3328, 1:1000), PAT1 (Proteintech, 24775-1-AP, 1:1000), FTH1 (Affinity, DF6278, 1:1000), NCOA4 (Affinity, DF4255, 1:1000) and GAPDH (Proteintech, 60004-1-Ig, 1:50000) at 4 °C overnight. The next day, after three washes in TBST, the membranes were incubated with secondary antibodies, including an HRP-conjugated anti-rabbit secondary antibody (Proteintech, SA00001-2, 1:5000) and anti-mouse secondary antibody (Proteintech, SA00001-1, 1:5000) at room temperature for 1 h. Chemiluminescence signals were measured with an enhanced chemiluminescence (ECL) star (Sparkjade, ED0025-C), and protein bands were captured via ChemiDoc XRS+ (Bio-Rad). The intensity of each target protein band was normalized to that of its corresponding loading control (GAPDH) from the same sample lane and semiquantitatively analysis was performed via ImageJ software (NIH) and Image Lab Software (Bio-Rad).

Molecular docking

Molecular docking was employed to predict the potential binding of PS and ER α . The structure of ER α was generated by AlphaFold, and styrene was utilized as a representative unit to probe the binding interface between polystyrene and ER α . The protonation state of all the compounds was set at pH = 7.4, and the compounds were expanded to 3D structures via Open Babel⁴⁷. AutoDock Tools (ADT3) were applied to prepare and parametrize the receptor protein and ligands. The docking grid documents were generated by AutoGrid of sitemap, and AutoDock Vina (1.2.0) was used for docking simulation^{48,49}. The optimal pose was selected to analyze the interaction. Finally, the protein-ligand interaction figure was generated via PyMOL.

Cell transfection

Mouse-specific small interfering RNAs, including ACSL4 siRNA (si-ACSL4), YAP1 (si-YAP1), and PAT1 siRNA (si-PAT1), were purchased from GenePharma (Shanghai, China). si-ACSL4, si-YAP1, and si-PAT1

(50 nM) were transfected into kk-1 cells via GP-Transfect-Mate (Gene-Pharma) according to the manufacturer's instructions, and the sequences of these siRNAs are listed in Supplemaentary Table 1.

Detection of cellular and mitochondrial Fe²⁺ levels

After the treatment described above, the cells were fluorescently labeled with FerroOrange (Dojindo, F374, Kumamoto, Japan) and Mito-FerroGreen (Dojindo, M489) at 37 °C for 30 min and imaged via a BX40 microscope.

Statistical and reproducibility

All the experiments in this study were performed at least three times, and the statistical analysis was performed via GraphPad Prism 8.0 software. The data are presented as the means $\pm$ SEM. To assess the significance, a *t*-test was used to compare the means of two different groups, and one-way ANOVA with the least significant difference (LSD) test was used to compare between more than two groups. When *P* was less than 0.05, statistical significance was defined, with *P* < 0.05 and *P* < 0.01 indicating statistical significance.

Ethics approval and consent to participate

This work received approval for research ethics from the Northwest A&F University Animal Care and Use Committee, and a proof/certificate of approval is available upon request (Approval No.: XN2023-0702). We have complied with all relevant ethical regulations for animal use.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Raw RNA-seq data in this study are openly available at [<https://ngdc.cnbc.ac.cn/gsa/browse/CRA039353>]. Source data can be obtained from Supplementary Data. Uncropped and unedited blot images were included in the Supplementary Data. Other data are available from the corresponding author upon reasonable request.

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Author contributions

Guangyu Liu and Jing Lv performed the cell culture experiments, contributed to the initial manuscript draft, and conducted statistical analysis. Jueshun Zhang, Ziqi Wang, Yuanyou Li, Tiantian Lian, Xiaoya Li, and Dmitry Il

provided essential support in data collection, analytical validation, and technical execution. Zhongliang Jiang supervised the research, secured funding, critically revised the manuscript, and approved the final version. All authors reviewed the manuscript and consented to its submission.

Competing interests

The authors declare no competing interests

Additional information

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