Iron stress affects the survival of Toxoplasma gondii

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Research Article

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Abstract

Background

Iron possesses redox abilities and plays a crucial role in biosynthesis, energy metabolism, and other biological processes. It represents an indispensable nutrient for the survival of Toxoplasma gondii. In response to Toxoplasma infection, host cells employ a defensive strategy referred to as "nutritional immunity" to restrict the availability of iron, thus impeding T. gondii from obtaining sufficient amounts of this vital element. The present research aims to examine the impact of iron stress on T. gondii, including iron deficiency and iron overload, and to explore the feasibility of disturbing the iron homeostasis as a potential treatment for toxoplasmosis.

Methods

An iron-deficient environment was induced by supplementing the culture medium with the permeable iron chelator, deferoxamine mesylate (DFO), while ammonium iron(II) sulfate was utilized as an iron supplement to establish an iron overload environment. Experiments were conducted to assess the impact of different iron levels on T. gondii's proliferation ability, invasion ability, escape ability, and plaque formation. Additionally, the redox ability of T. gondii under varying iron stress conditions was examined. Transcriptome analysis was employed to investigate the differential gene expression of T. gondii under iron deficiency and iron overload. Lastly, iron chelation therapy and iron supplementation therapy were administered to mice infected with T. gondii to assess the potential of targeting iron homeostasis disruption for the treatment of toxoplasmosis.

Results

Iron supplementation and the iron chelating agent significantly influence the growth of T. gondii. Low iron stress inhibits the proliferation of T. gondii and greatly reduces plaque formation, whereas high iron stress increases the invasion ability while significantly reducing proliferation. Altered iron levels perturb the redox capacity of T. gondii, resulting in a substantial increase in oxidation products (GSSG and MDA), reactive oxygen species (ROS), and superoxide anions under high iron stress. Under iron deficiency, specific genes pertaining to bradyzoites are up-regulated, thereby facilitating the conversion of tachyzoites to bradyzoites in the Pru strain. Conversely, under iron overload, a significant up-regulation of surface protein genes in T. gondii occurs, leading to an enhanced adhesion ability. Notably, the administration of iron supplements and iron chelating agents has no discernible effect on the mortality rates of Toxoplasma-infected mice. Nevertheless, mice infected with T. gondii exhibit significant weight loss and aggravated symptoms following iron supplementation therapy.

Conclusions
This study unequivocally confirms the essentiality of iron as a nutrient for \textit{T. gondii} survival. Iron stress, including iron deficiency and iron overload, affects the growth of \textit{T. gondii}.

\section{Background}

\textit{Toxoplasma gondii} is an obligate intracellular parasite capable of infecting most warm-blooded species and almost all of the nucleated cells [1, 2]. Upon invading host cells, the parasite relies on a substantial uptake of nutrients from hosts to satisfy its nutritional requirements for rapid proliferation, including lipids, proteins, and various metal ions [3, 4]. Among these metal ions, iron holds particular significance as the most abundant biological metal ion, assuming a critical role as an essential cofactor for numerous enzymes and regulatory proteins [5, 6]. However, excessive iron concentrations in the cytosol or other sites can be potentially toxic [7, 8]. Consequently, organisms, including bacteria and parasites, have evolved diverse strategies to acquire essential iron and detoxify iron overload[9]. Recent investigations on \textit{Plasmodium spp.}, belonging to the same genus as \textit{T. gondii}, have demonstrated that iron deficiency anemia protects against malaria, while iron supplementation increases susceptibility and morbidity [10–12]. Unlike \textit{Plasmodium spp.}, which primarily parasitize erythrocytes containing high iron levels in the form of hemoglobin during the asexual lifecycle [13], \textit{T. gondii} encounters more iron stresses as it parasitizes various cells throughout its lifecycle. Consequently, perturbing iron homeostasis is considered an attractive strategy for controlling \textit{T. gondii} infection, although its impact on the parasite remains unclear.

Iron constitutes the third most abundant element in \textit{T. gondii}, after zinc and copper, accounting for 5% of the total elemental content of the parasite [14]. Iron plays a crucial role in several essential cellular processes during \textit{T. gondii} infection, including iron-sulfur cluster biogenesis [15–17], heme biosynthesis [18, 19], iron-dependent redox regulation [20–22], ATP production and energy transfer [22]. Furthermore, iron helps \textit{T. gondii} resist growth inhibition caused by interferon [23]. Although iron is vital for the intracellular parasitic process, it is challenging for \textit{T. gondii} which heavily relies on host-derived nutrients [24]. Consequently, \textit{T. gondii} has developed various mechanisms to tightly regulate iron homeostasis, including rhoptry proteins ROP4 and ROP2 binding human lactoferrin [25, 26] and the vacuolar iron transporter (VIT) mediating iron detoxification [27].

Indeed, hosts also employ elaborate strategies to withhold iron and prevent \textit{T. gondii} from acquiring sufficient iron. Transferrin and lactoferrin bind nearly all free iron in serum, bodily fluids, and tissues, while ferritin chelates intracellular free iron ions. This mechanism, known as nutritional immunity, reduces the concentration of available iron to $10^{-24}\text{M}$, thereby impeding the infection of pathogens [28, 29]. Consequently, when \textit{T. gondii} invades host cells, the intracellular iron levels fail to meet the parasite's nutritional demands for rapid proliferation, creating an iron-deficient environment for \textit{T. gondii}. In reality, the body's iron levels are dynamically regulated, fluctuating between deficiency and overload under different physiological processes. Given \textit{T. gondii}'s sophisticated lifestyle [30], enabling replication in diverse cells, it is adept at adapting to varying iron stresses to ensure an adequate iron supply. Consequently, our study aims to investigate the impact of iron stresses on \textit{T. gondii}, including iron...
deficiency and iron overload, as well as the parasite's response to these stresses. We discovered that *T. gondii* requires an appropriate iron content for its vital activities. Notably, we observed that iron deficiency affects multiple metabolic processes in *T. gondii*, triggering the transformation of tachyzoites into bradyzoites, whereas iron overload influences redox ability but enhances adhesion ability. Moreover, we noted worsened clinical symptoms in mice infected with *T. gondii* following iron supplementation therapy. Thus, careful consideration should be given to iron chelation therapy and iron supplementation therapy during *T. gondii* infection.

**Methods**

**Host cells and parasite culture**

In this study, we utilized the RHΔku80, RH-Luc, and PRU strains. Tachyzoites of *Toxoplasma gondii* were cultured in human foreskin fibroblast (HFF, maintained in our laboratory) cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS) at 37°C with 5% of CO₂.

**In vitro inhibition assay**

1 × 10³ RH-Luc tachyzoites were inoculated into HFFs grown in 96-well plates using DMEM containing gradient diluted iron chelating agents DFO (Topscience, China) or iron supplements ammonium iron(II) sulfate [66]. Dimethyl sulfoxide (DMSO) was used as a control. After 72 hours of culture, relative luminescence units (RLU) were measured using a fluorescence microplate reader (Tecan, Spark 10M, Switzerland) and the Firefly Luciferase Reporter Gene Assay Kit (Beyotime Biotech, China). The inhibition rate was calculated using the formula: inhibition rate = [(RLU_{DMSO} - RLU_{experimental group})/RLU_{DMSO}] × 100%. Nonlinear regression curve fitting was employed to determine median lethal concentration (IC50) and 95% confidence interval (CI). Tree independent experiments were performed.

**Immunofluorescence Assays**

Immunofluorescence assays (IFAs) were carried out as previously described [68]. Briefly, HFFs infected with tachyzoites were fixed with 4% formaldehyde, permeabilized with 0.25% Triton X-100 in phosphate-buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA) in PBS. The cells were then incubated with primary antibody diluted in 3% BSA/PBS for 1 hour at 37 °C. After washing with PBS, the cells were incubated with secondary antibodies (FITC-conjugated goat anti-mouse IgG (H + L), 1:100, or Cy3-conjugated goat anti-rabbit IgG (H + L), 1:100) for 1 hour at 37 °C. Finally, the cells were sealed with an antifade mounting medium containing DAPI, and images were captured using a fluorescence microscope.

**Invasion assay**

1 × 10⁶ tachyzoites were incubated with DFO (50µM) or ammonium iron(II) sulfate (200µM) for 1 hour and then inoculated into HFFs [69]. After 1 hour of invasion, the cells were washed with PBS and fixed
with 4% formaldehyde. Non-invaded (attached) tachyzoites were stained with rabbit anti-SAG1 before permeabilization, while invaded tachyzoites were stained with mouse anti-IMC1 after permeabilization with 0.25% Triton X-100. Red-labeled Cy3-conjugated goat anti-mouse IgG (H + L) was used to label all tachyzoites, while green-labeled FITC-conjugated goat anti-rabbit IgG (H + L) was used to label attached tachyzoites. The invasion efficiency was determined by counting the ratio of red-labeled or green-labeled tachyzoites to host cells in several random fields under a fluorescence microscope. Three independent experiments were performed.

**Replication assay**

1 × 10^5 freshly released tachyzoites were inoculated in HFFs grown on coverslips [68]. After 1 hour of invasion, HFFs were washed with PBS, and the culture medium was replaced with DMEM containing DFO or iron supplements ammonium iron(II) sulfate. After 24 hours of incubation at 37°C with 5% of CO₂, the cells were washed with PBS, fixed with 4% formaldehyde and stained with an anti-GAP45 antibody. Tachyzoites in 100 parasitophorous vacuoles (PVs) were counted in several random fields using a fluorescence microscope.

**Egress assay**

1 × 10^5 tachyzoites were inoculated into HFFs and cultured for 28 hours [67]. The parasites were stimulated with 5% ethanol in DMEM to induce egress. Once the parasites started to release from the PVs, the coverslips were fixed with 4% paraformaldehyde. IFA were performed using an anti-GAP45 antibody (1:300) to label the tachyzoite membrane. The egress efficiency was determined by counting the number of released and unreleased PVs in multiple random fields under a fluorescence microscope. Three independent experiments were conducted.

**Plaque assay**

100 freshly released tachyzoites were inoculated into HFFs grown in 12-well plates [69]. After 1 hour of invasion, HFFs were washed with PBS, and the culture medium was replaced with medium containing DFO or ammonium iron(II) sulfate. Following 7 days of culture, HFFs were washed with PBS, fixed with 4% formaldehyde and stained with 2% crystal violet.

**Detection of iron, reactive oxygen species, superoxide anion, and mitochondrial membrane potential**

The concentration of iron(II) ions was measured using the fluorescence probe FerroOrange (Dojindo, Japan). The level of reactive oxygen species (ROS) in *T. gondii* was assessed using the fluorescence probe DCFH-DA (Solarbio, China). The concentration of superoxide anion was determined using the fluorescence probe dihydroethidium (DHE, Beyotime Biotech, China). Changes in mitochondrial membrane potential were evaluated using the fluorescence probe JC-1 (Beyotime Biotech, China). Freshly released tachyzoites were incubated in a culture medium containing DFO or ammonium iron(II) sulfate for 1 hour. After washing with PBS, the parasites were treated with FerroOrange, DCFH-DA, DHE, or JC-1,
and then resuspended in 500 µL of PBS following two additional washes. Finally, the stained tachyzoites was analyzed using flow cytometry or a fluorescence microplate reader [67].

**GSSG and MDA Determination**

A total of $1 \times 10^7$ tachyzoites were incubated in a culture medium containing DFO or ammonium iron(II) sulfate for 1 hour. After washing with PBS, the parasites were lysed by freezing in liquid nitrogen and thawing at 37°C in three cycles. The supernatant of each sample was collected for measurement of GSSG or MDA using a GSH and GSSG Assay Kit or Lipid Peroxidation MDA Assay Kit, following the manufacturer's instructions (Beyotime, China).

**Transcriptomics analysis**

HFF cells infected with tachyzoites were cultured in the medium supplemented with DFO (50µM) or ammonium iron(II) sulfate (200µM). After 24 hours of incubation, the cells were lysed, and the parasites were collected. The collected parasites were washed twice with precooled PBS and resuspended in 1 mL TRIzol (Sangon Biotech, Shanghai). The samples were then flash-frozen in liquid nitrogen and sent to Shanghai Applied Protein Technology Co., Ltd. for transcriptome data analysis. Advanced Heatmap Plots was performed using the OmicStudio tools at https://www.omicstudio.cn.

**Mouse infectivity studies**

Female BALB/c mice (Vitalriver, Beijing) were intraperitoneally injected with iron chelating agents DFO (300 mg/Kg) or with iron supplements ammonium iron(II) sulfate (100 mg/Kg) or with phosphate-buffered saline (PBS) as a vehicle control [70]. The injections were administered one day prior to intraperitoneal injection of 100 RH tachyzoites, and treatment was continued for seven days. A control group comprised of non-infected and untreated mice was included. The mice were anesthetized (i.p.) and euthanized by cervical dislocation on day 8 post-infection. Blood samples were collected by puncturing the retroorbital plexus, and tissue samples (heart, liver, spleen, lungs, kidneys, brain, and lymph nodes) were collected, fixed in 10% buffered formalin, and processed for paraffin embedding and sectioning.

**Statistical Analysis**

Graphs were created and statistical analyses were performed using GraphPad Prism software v8.0. All data were analyzed using unpaired two-tailed Student’s t tests or two-way ANOVA tests.

**Results**

1. **Toxoplasma infection enhances cellular iron levels in HFF cells**

Pathogens face challenges in acquiring sufficient iron to meet their metabolic needs due to the host's regulated iron metabolism, which aims to reduce the concentrations of intracellular and extracellular free iron ions. As a result, the low iron levels in host cells are not conducive to the rapid proliferation of *Toxoplasma gondii* after invading the cells. However, our study revealed an upregulation of transferrin receptor 1 (TfR1) expression in HFF cells over time following *Toxoplasma* infection (Fig. 1A). Most cells
acquire iron via TfR1-mediated endocytosis, while the iron-bound transferrin binds to TfR1 and the complex is internalized into endosomes [31, 32]. The increase in TfR1 expression indicates an increased demand for iron in T. gondii-infected cells. To further investigate the changes in cellular iron content upon T. gondii infection, we quantified the total iron content in HFF cells. The total iron content in cells infected with T. gondii for 24 hours was significantly higher compared to uninfected cells (Fig. 1B). Furthermore, using a probe specifically designed to detect divalent iron ions, we observed a much higher fluorescence intensity in T. gondii-infected cells compared to uninfected cells (Fig. 1C and D). These findings suggest that intracellular iron levels continue to rise during the rapid proliferation of T. gondii in host cells.

2. Iron stress affects the growth of Toxoplasma gondii

To understand the role of iron ions in the interaction between T. gondii and host cells, it is essential to investigate the impact of different iron ion environments on the biological activities of T. gondii, including iron deficiency and iron overload. We constructed an iron-deficient environment by adding the permeable iron chelator deferoxamine mesylate (DFO) to the culture medium [33], while ammonium iron(II) sulfate was used as an iron supplement to create an iron overload environment [34]. We attempted to use the impermeable iron chelator bathophenanthroline disulfonic acid (BPDS) and iron(III) chloride but found that only high concentrations of these additives could damage T. gondii (Fig. S1 C-D).

The inhibitory effect of DFO on the growth of T. gondii was determined by using RH strain with fluorescein in an iron-deficient environment, and the median lethal concentration of DFO was found to be 13.56 µM (Fig. 2A, Fig. S1 A). In the DFO-induced iron-deficient environment, the proliferation and invasion ability of T. gondii were both significantly inhibited (Fig. 2B, C and D), leading to a reduced area of plaque formation caused by Toxoplasma infection (Fig. 2I).

In the iron overload environment supplemented with divalent iron, the proliferation of T. gondii was inhibited (Fig. 2E, F and H, Fig. S1 B), and the ability to form plaque decreased (Fig. 2J). Interestingly, as the concentration of exogenous divalent iron in the culture medium increased, the ability of T. gondii to invade host cells also increased (Fig. 2G).

These findings suggest that the process of rapid proliferation of tachyzoites in cells requires appropriate iron levels, and excessively high or low iron concentrations can affect the growth of T. gondii.

3. Iron availability affects the oxidation-reduction ability of Toxoplasma gondii

Considering iron's ability to shuttle between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states [7], it plays a significant role in the redox process of organisms. We further investigated whether the effect of iron stress on the growth of T. gondii is caused by an imbalance in iron-dependent redox capacity. By using a fluorescent probe for divalent iron ions, we observed changes in the iron level of the parasite when T. gondii was incubated with the iron chelator DFO or the iron supplement ammonium iron(II) sulfate (Fig. 3A). Moreover, mitochondrial membrane potential, as indicated by JC-1, a cation probe reflecting mitochondrial integrity [35], remained unaffected by iron deficiency or iron overload in T. gondii (Fig. 3D).
However, under iron overload, but not iron deficiency, *T. gondii* produced significant oxidation products such as oxidized glutathione (GSSG) and malondialdehyde (MDA), a product of lipid peroxidation (Fig. 3B and C). Additionally, iron overload induced *T. gondii* to generate more reactive oxygen species (ROS) and superoxide anions (Fig. 3E and F). These findings support the result that iron overload diminishes *T. gondii*’s proliferation.

4. **Low iron stress induces the conversion of tachyzoites to bradyzoites in Toxoplasma gondii**

As a Eukaryote, *T. gondii* possesses complex and diverse proteins involved in various lifecycle stages. To gain a deeper understanding of the impact of iron deficiency on *T. gondii*, we performed RNA sequencing (RNA-seq) to investigate the transcriptomic changes following 24 hours of iron deficiency. Among 8,477 genes, 409 were identified as differentially expressed genes (log2 fold ≥ 1 or ≤ -1, P  0.05), with 238 significantly up-regulated and 171 down-regulated genes in the iron-deficient condition (50 µM DFO added to the media) compared to normal media (Fig. 4A). Notably, several genes specifically expressed during bradyzoite stage were found among the up-regulated genes, including LDH2 [36–39], BRP1 [40], H2A1 [41], and MIC13 [42]. Comparing the 50 genes with the highest differential expression levels among the up-regulated genes and the 30 genes with the highest differential expression levels among the down-regulated genes with the transcriptomics of *T. gondii* at different lifecycle stages in the database (including tissue cysts, chronic infection, and merozoites) [43–45], we found similarities between the transcriptomics of *T. gondii* under iron deficiency and the transcriptomics observed during tissue cysts and chronic infection (Fig. 4B). We performed KEGG enrichment analysis to gain insights into the biological roles of the significantly up-regulated genes. The most enriched KEGG terms included valine, leucine, and isoleucine degradation, glycolysis/gluconeogenesis, and pyruvate metabolism (Fig. S2 A). These results suggest that iron deficiency affects synthesis and metabolism, potentially compensating for its normal biological function.

PRU strain was cultured in culture medium with DFO for 72 hours. Cyst-wall staining using *Dolichos biflorus lectin* (DBL) showed the presence of positive bradyzoite cysts, although some vacuoles stained faintly with DBL (Fig. 4C and E). BAG1 antibody staining showed that nearly 40% of vacuoles converted into bradyzoites (Fig. 4D and F). This suggests that the tachyzoites of *T. gondii* have a propensity to differentiate into bradyzoites under iron deficiency, and iron deficiency is a stressor driving differentiation.

5. **The adhesion ability of Toxoplasma gondii is enhanced in high iron environment**

Similarly, we employed RNA-seq to investigate the transcriptomic changes following 24 hours of iron overload to further understand the effect of iron overload on *T. gondii*. The volcano plot demonstrated significant up-regulation of 674 genes and down-regulation of 122 genes under iron overload conditions (Fig. 5A, Fig. S2 B). Notably, several SAG1-related sequences (SRS) superfamily proteins were among the upregulated genes, which are believed to mediate attachment to host cells [46–48]. *T. gondii* can successfully invade host cells through the following stages: 1) gliding along a host cell surface, with parasite surface proteins interacting with cell surface or substrate receptors; 2) active invasion and forming the “moving junction” (MJ) at the close contact area between the parasite’s apex and the host
cell; 3) resulting in the formation of parasitophorous vacuoles [49–51]. To determine the number of *T. gondii* that adhered during the invasion process, we found that the adhesion ability of *T. gondii* was significantly enhanced by the addition of divalent iron ions to the culture medium or by pre-incubating with divalent iron ions prior to invading the host cells (Fig. 5B).

### 6. Iron supplementation therapy enhances the virulence of *Toxoplasma gondii* in mice

To evaluate the potential of iron deficiency and iron overload as treatments for toxoplasmosis, we conducted iron chelation therapy and iron supplementation therapy to mice infected with *Toxoplasma gondii*. Neither the mice receiving iron chelation therapy nor those receiving iron supplementation therapy showed any change in the mortality rate caused by acute toxoplasmosis resulting from RH tachyzoite infection, although intraperitoneal injection of DFO delayed the time of death (Fig. 6A, Fig. 3A-C). Notably, iron supplementation in *Toxoplasma*-infected mice led to significant weight loss (Fig. 6B). Although iron chelation treatment reduced the number of *T. gondii* in the peritoneal fluid and lungs of mice, it promoted dissemination and increased parasite burden in other tissues, including the kidneys, spleen, and liver (Fig. 6C). Considering the relationship between the availability of iron and host immunity, we further evaluated the effects of iron chelation therapy and iron supplementation therapy on the expression of cytokines. As expected, the concentration of IFN-γ and IL-10 in serum was significantly increased due to *T. gondii* infection. Interestingly, both iron deficiency and iron overload decreased the level of IFN-γ and IL-10 in *Toxoplasma*-infected mice. However, in uninfected mice, iron overload increased the levels of IFN-γ and IL-10 (Fig. 6D and F). Examination of liver pathological sections showed an increased area of inflammatory necrosis caused by *T. gondii* infection in mice treated with iron supplementation (Fig. 6F).

### Discussion

In our study, we conducted an extensive investigation into the impact of iron on the survival of *Toxoplasma gondii*. We discovered that iron plays a crucial role in addition to its effect on proliferation. Specifically, iron deficiency promotes the transformation of *Toxoplasma* tachyzoites into bradyzoites, while high iron levels enhance the adhesion ability of *T. gondii*. Furthermore, we successfully obtained transcriptome data of *T. gondii* under both iron deficiency and iron overload conditions, providing valuable insights into the iron metabolism process of the parasite. This data will greatly contribute to our further understanding of how *T. gondii* interacts with iron.

Iron is an essential micronutrient for all living organisms, serving as a component of iron-containing organic compounds and playing a vital role in various iron- and heme-containing proteins involved in energy metabolism and oxygen transport [52, 53]. However, excessive iron accumulation leads to oxidative stress and tissue injury. Mammals have developed intricate iron homeostatic mechanisms to meet metabolic needs and minimize the risks associated with iron overload toxicity [7, 8]. The hemoglobin of erythrocytes contains the most of body iron (∼70%), and the rest portion is distributed in muscle myoglobin or stored within ferritin in the liver. Only a small part of iron (<1%) binds to plasma iron carrier transferrin and circulates in the blood. Most cells acquire iron from transferrin through receptor-
mediated endocytosis, while excess intracellular iron is stored in cytosolic ferritin [6]. Acquiring iron from the host is a fundamental factor in the infection and development of pathogens, particularly intracellular pathogens, and the battle between host and pathogen for iron acquisition is complex and persistent [54]. Although the exact mechanisms by which *T. gondii* overcomes the barriers of "nutritional immunity" and successfully obtains iron are not fully understood [55, 58], the importance of iron in the lifecycle of *T. gondii* has been extensively demonstrated. As an intracellular parasite, *T. gondii* must acquire sufficient iron from host cells for rapid proliferation [71]. Strategies aimed at preventing *T. gondii* from obtaining sufficient iron or disrupting iron homeostasis hold promise for the treatment of toxoplasmosis. In the case of *Plasmodium spp.*, withholding iron from vital parasite metabolic pathways has been proposed as a potential antimalarial chemotherapeutic strategy [10–12]. *T. gondii* has the ability to infect a wide range of nucleated cells, indicating its capability to deal with iron stress challenges. Indeed, we observed an increase in intracellular iron ion levels and the expression of transferrin receptor after *T. gondii* invasion. However, the up-regulation of the transferrin receptor in response is not directly mediated by iron deficiency in host cells caused by excessive iron uptake, but rather by an uncharacterized secreted protein [56]. During the intracellular growth of the parasite, *T. gondii* may secrete specific proteins to modulate the iron metabolism pathway of host cells, ensuring its survival.

Considering the presence of host factors, directly reducing the level of iron ions in the intracellular parasite environment is challenging. Addition of iron chelating agents can reduce free iron ions in the solution, but the difference in inhibiting the growth of *T. gondii* between impermeable BPDS and permeable DFO suggests that the parasite's growth relies on the host's iron source. As expected, both iron deficiency and iron overload inhibit proliferation of *T. gondii*.

Iron deficiency affects various aspects of *T. gondii*, primarily by inhibiting specific metabolic pathways, which explains the conversion of tachyzoites to bradyzoites under iron deficiency. However, the extent of this transformation may depend on the iron state of the host cell. Parasitophorous vacuoles treated with 50µM DFO are significantly smaller than those treated with 10µM DFO, suggesting that iron is not the sole determinant of the transition from tachyzoites to bradyzoites.

The unexpected enhancement of adhesion ability in iron overload environment can be attributed to the up-regulation of SAS proteins. All SRS domains segregated into eight distinct domain subfamilies, and most SRS gene contain two SRS domains [46]. The up-regulation of SAS proteins mainly consists of fam7 and fam8, known to be associated with adhesion [46]. Further research is required to investigate the contribution of these up-regulated SRS proteins to iron sensing and adhesion. The inhibitory effect of excessive iron on *T. gondii* proliferation can be explained by its impact on redox capacity. However, based on the 50% lethal concentration of iron supplements for both *T. gondii* and host cells, *T. gondii* appears more susceptible to the toxic effects of excessive iron. Unlike eukaryotes, *T. gondii* lacks ferritin for storing ingested iron and preventing iron toxicity [9]. Instead, iron is stored in a vacuole via the vacuolar iron transporter [27, 57]. Combining an inhibitor of iron detoxification protein with iron supplementation may be a potential treatment for toxoplasmosis.
The results indicating the inhibitory effects of iron deficiency and iron overload on the growth of *T. gondii* in vitro are promising, underscoring the potential of iron therapy for toxoplasmosis. However, the results of iron chelation therapy or iron supplementation therapy for acute *Toxoplasma*-infection in mice were disappointing. Despite altering the iron level in the body (Fig. S3 A-C), infected mice were unable to escape death, and iron supplementation even increased susceptibility to clinically significant toxoplasmosis. The intraperitoneal injection of the drug significantly reduced the parasite burden in the ascites. Both iron chelation therapy and iron supplementation therapy are limited to specific tissues and only prioritize altering the iron level at the injection site. It is understandable that transmission ability is enhanced under iron deficiency. To ensure better survival, *T. gondii* must migrate to an iron-rich environment, although proving this through experiments is challenging due to the complex environment within the body.

When evaluating the potential of iron therapy, it is essential to consider its impact on the host, particularly the close connection between the host's iron status and immunity [58–60]. Interferon gamma (IFN-γ) is the primary cytokine responsible for activating cell-autonomous immune responses and inhibiting the growth of intracellular parasites [61–63]. *Toxoplasma*-infection increases the concentration of IFN-γ in the serum, as does intraperitoneal injection of iron supplements in uninfected mice. However, it is surprising that the level of IFN-γ is reduced in infected mice following intraperitoneal injection of iron supplements. Additionally, iron chelation therapy also lowers the level of IFN-γ in infected mice. IL-10 plays a role in controlling inflammatory responses during acute *Toxoplasma* infection. Similar patterns were observed in the levels of IL-10 in the serum of mice under different treatments [64, 65], including an increase caused by injection of iron supplements alone and a decrease caused by iron chelation therapy or iron supplementation therapy in infected mice. It appears that the effect of iron supplementation on immunity in uninfected animals is comparable to that of *Toxoplasma* infection. Iron homeostasis influences immune system of host, including innate and adaptive immunity [60].

Although our studies have revealed important discoveries, it is crucial to acknowledge the limitations of our research. Firstly, we cannot definitively determine whether the effects of iron chelating agents and iron supplementation on *Toxoplasma gondii* are direct or if they influence the parasite indirectly through their impact on host cells. Secondly, due to the diverse biological effects of iron, our attempts to determine the impact of iron deficiency on *Toxoplasma* metabolism have been limited. Thirdly, given the complex environment within the host organism, further research is required to understand the effects of iron chelation therapy and iron supplementation therapy on the host's immune system. These limitations underscore the need for future investigations that delve deeper into these aspects to achieve a more comprehensive understanding of the interactions between iron and *Toxoplasma gondii*, as well as its implications for the host.

**Conclusions**

In conclusion, this study demonstrates that iron is a vital nutrient for the survival of *T. gondii*. Iron deficiency affects various metabolic processes in *T. gondii*, inducing the transformation of tachyzoites
into bradyzoites, while iron overload affects redox ability but enhances adhesion ability. Iron chelation therapy and iron supplementation therapy for *T. gondii* infection should be approached with caution, as clinical symptoms in mice infected with *T. gondii* were exacerbated after iron supplementation therapy.

**Declarations**

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**Availability of data and materials**

We agree that the data supporting our findings in this study can be found.

**Authors’ contributions**

ZY and MY carried out the study. ZY and ZZ analyzed experimental data. ZS and YP assisted in conducting animal experiments. JL helped to draft the manuscript. QL conceived of the study, and participated in its design and coordination. All authors have read and agreed to the final version of the manuscript.

**Ethics approval and consent to participate**

The study was conducted following the guidelines of the Care and Use of Laboratory Animals, Ministry of Science and Technology of China. This work was reviewed and approved by the Laboratory Animal Welfare and Animal Experimental Ethical Committee of China Agricultural University (Certificate No.: CAU-AW31901202-2-1).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Figures**
Toxoplasma-infection induces an increase in intracellular iron levels. A, western blot analysis identified an upregulation of TfR1 expression in HFF cells with increasing duration of Toxoplasma infection. B, total intracellular iron ion content in HFF cells changes over the course of T. gondii infection, with higher levels observed at 24 hours post-infection compared to uninfected cells. C, fluorescence intensity of HFF cells stained with divalent iron ion probe FerroOrange, demonstrating stronger fluorescence in cells infected with T. gondii for 24 or 48 hours compared to uninfected cells. D, fluorescence assays showing changes

Figure 1
in intracellular iron ion levels using FerroOrange (red) and green fluorescent protein-labeled Toxoplasma gondii (green) in infected (red arrow) and uninfected (blue arrow) HFF cells, with higher fluorescence intensity observed in infected cells. ** $p < 0.01$; *** $p < 0.001$

**Figure 2**

Iron deficiency and iron overload inhibits *Toxoplasma* growth. A and E, half lethal concentration curve depicting the effects of different concentrations of iron chelating agents and iron supplements. RH-Luc
tachyzoites were inoculated in 96-well plates with gradient-diluted DFO or Fe$^{2+}$ in DMEM for 72 hours, and relative light units (RLUs) were measured. B and F, replication ability of RHΔku80 strains treated with different concentrations of iron chelating agents (10 μM and 50μM) and iron supplements (200 μM and 1000μM). Iron deficiency and iron overload hinder Toxoplasma replication. C and G, invasive ability of RHΔku80 strains under different concentrations of iron chelating agents and iron supplements. Invasive ability decreases under iron deficiency conditions but increases under iron overload conditions. D and H, escape ability of RHΔku80 strains exposed to different concentrations of iron chelating agents and iron supplements. Escape ability is enhanced under iron overload conditions. L and J, plaque formation area decreases in RHΔku80 strains treated with different concentrations of iron chelating agents and iron supplements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Figure 3

Iron status induces imbalance of Toxoplasma redox capacity. A, fluorescence intensity of the fluorescent probe FerroOrange reflects changes in iron levels of Toxoplasma gondii under conditions of iron deficiency and iron overload. Iron chelating agents reduce parasite iron levels, while iron supplements increase them. B and C, detection of oxidation products GSSG and MDA in Toxoplasma gondii under...
conditions of iron deficiency and iron overload. Under iron overload, the content of parasite oxidation products increases. D-F, analysis of changes in mitochondrial membrane potential (JC-1), ROS level (DCFH-DA), and superoxide anion concentration (DHA) in *T. gondii* under iron deficiency and iron overload. High iron levels increase ROS and superoxide anions but do not alter mitochondrial membrane potential.

** p < 0.01; *** p < 0.001; ns, not significant.
Iron deficiency promotes the conversion of tachyzoites to bradyzoites. A, volcano scatter plot illustrating gene differential expression under iron deficiency stress through transcriptome analysis. B, heat map comparing *Toxoplasma* transcription under iron deficiency to different life stages, including tissue cysts, chronic infection, and merozoites. Similarities between the transcriptome of *T. gondii* under iron deficiency and transcriptome during tissue cyst and chronic infection are observed. C and E, immunofluorescence detection of the proportion of DBA-positive parasitophorous vacuoles under iron deficiency using DBA (green) and mouse anti-SAG1 (red). D and F, immunofluorescence detection of the proportion of bradyzoite specifically expressed genes BAG1 in parasitophorous vacuoles under iron deficiency using rabbit anti-BAG1 (green) and mouse anti-SAG1 (red).

**Figure 5**

Iron overload affects the adhesion ability of *Toxoplasma gondii*. A, volcano scatter plot showing gene differential expression under iron overload stress through transcriptome analysis. B, difference in *Toxoplasma* adhesion ability in different iron level environments, including transfer to cells after incubating with DFO or Fe²⁺ for 1 hour or adding BPDS or Fe²⁺ to the culture medium. Only when *T. gondii* contacts with iron ions does its adhesion ability increase. *** *p* < 0.001.
Figure 6

Iron chelation therapy and iron supplementation therapy reveal complex biological processes. A, Survival curve of BALB/c mice intraperitoneally infected with 100 tachyzoites of RHΔku80, treated with iron chelation therapy and iron supplementation therapy. Neither the mice receiving iron chelation therapy nor those receiving iron supplementation therapy showed any change in the mortality rate. B, Weight change of BALB/c mice infected with RHΔku80, treated with iron chelation therapy and iron supplementation.
therapy. Iron supplementation in *Toxoplasma*-infected mice resulted in significant weight loss. C, parasite burden in different organs of infected mice after iron chelation therapy and iron supplementation therapy. Iron chelation treatment reduced the number of *T. gondii* in the peritoneal fluid and lungs of mice, but promoted dissemination and increased parasite burden in other tissues, including the kidneys, spleen, and liver. D, detecting the concentration of IFN-γ and IL-10 in the serum of infected mice. E, Examination of liver pathological sections showed an increased area of inflammatory necrosis caused by *T. gondii* infection in mice treated with iron supplementation. * p < 0.05; ** p < 0.01; *** p < 0.001; ns, not significant.

**Supplementary Files**

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