Di-Dang-Tang ameliorates cognitive dysfunction in vascular dementia rats via inhibition of ferroptosis through PGK1/NRF2/GPX4 Signaling Pathway

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Research Article

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Abstract

Background

VaD is a prevalent neurodegenerative disorder characterized by cognitive dysfunction and neuronal degeneration. This study investigates the potential therapeutic effects of Di-Dang-Tang (DDT) in a rat model of VaD and elucidates the underlying molecular mechanisms.

Methods

VaD rats were treated with DDT, and their cognitive function was assessed using behavioral tests. Neuronal apoptosis and degeneration in the hippocampal CA1 region were evaluated. The impact of DDT on ferroptosis, a regulated cell death process, was also examined. Furthermore, the protein expression of the PGK1/NRF2/GPX4 pathway, involved in cellular homeostasis and oxidative stress response, was assessed. The influence of PGK1 overexpression on the neuroprotective effects of DDT and its effects on ferroptosis were investigated.

Results

DDT administration significantly alleviated cognitive dysfunction in VaD rats and attenuated neuronal apoptosis and degeneration in the hippocampal CA1 region. DDT treatment inhibited ferroptosis in this brain region. Moreover, DDT regulated the protein expression of the PGK1/NRF2/GPX4 pathway. Interestingly, overexpression of PGK1 abolished the protective effects of DDT on cognitive dysfunction in VaD, and it also eliminated the DDT-induced reduction of ferroptosis.

Conclusion

Our findings demonstrate the neuroprotective effects of DDT in VaD, improving cognitive function and mitigating neuronal apoptosis and degeneration. DDT treatment inhibits ferroptosis and modulates the PGK1/NRF2/GPX4 pathway. The impact of PGK1 overexpression suggests its involvement in mediating DDT's therapeutic actions. Understanding these mechanisms contributes to the development of targeted therapies for cognitive decline in VaD.

1. Introduction

Vascular dementia (VaD) stands as an aging-associated debilitating syndrome characterized by severe cognitive impairment, primarily stemming from ischemic stroke, hemorrhagic stroke, and cerebrovascular diseases, which result in reduced blood flow to the brain. It holds the second highest incidence rate, following only Alzheimer's disease, making it the second most common cause of dementia [1, 2]. While preventive measures, such as the control of vascular risk factors and the use of antiplatelet and
anticoagulation therapies, have been established for the underlying cerebrovascular diseases, there is currently no symptomatic treatment available for VaD [3]. The pathogenesis of VaD is very complicated, encompassing various processes such as the disruption of the central cholinergic system, oxidative stress-induced injury, inflammatory reactions, pyroptotic cell death, excitatory amino acid toxicity, autophagy, and intracellular calcium overload [4–9]. Notably, recent research has revealed abnormal iron deposition in extensive cortical regions of subcortical ischemic VaD patients, which correlates with the severity of cognitive dysfunction and neuronal damage [10]. Studies employing cerebral ischemia-reperfusion injury models have further demonstrated that iron deposition plays a crucial role in stroke-induced neuronal death [11].

Ferroptosis is a distinctive form of cell death that diverges from conventional cell death pathways, such as apoptosis, proptosis, necrosis, and autophagy, with regards to morphological, biochemical, and genetic characteristics [12]. The key hallmarks of ferroptosis encompass iron accumulation induced lipid peroxidation [12]. The regulatory mechanisms underlying ferroptosis involve glutathione metabolism, lipid peroxidation reactions, and iron homeostasis, all of which are closely associated with the pathogenesis of aging, neurodegenerative diseases, ischemia-reperfusion injury, as well as cardiovascular and cerebrovascular disorders [13]. Ferroptosis inducers can be inhibited by ferroptosis inducers. Neurons treated with ferroptosis inducers exhibited significant alterations in iron metabolism, glutathione depletion, excessive reactive oxygen species (ROS) production, and pronounced lipid peroxidation [14]. Hence, lipid peroxidation and oxidative stress represent significant factors contributing to neuronal damage in VaD patients.

Di-Dang-Tang (DDT) is formulated from a combination of multiple medicinal herbs, including *Rheum officinale baill* (Dahuang), *Tabanus bivittatus Mats* (Mengchong), *seeds of Prunus persica* (L.) *Batsch* (Taoren), and *Hirudo nipponica Whitman* (Shuizhi). Some publications have highlighted the therapeutic potential of DDT in specific diseases, such as cardiovascular disorders, liver injury, neurodegenerative diseases, and cancer [15, 16]. Moreover, pharmacological activities of DDT have been demonstrated to be diverse, including anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective effects [15]. Mechanistically, the beneficial effects of DDT have been attributed to its bioactive constituents, such as salvianolic acids, paeoniflorin, and astragalosides, which interact with multiple signaling pathways. Notably, DDT has been shown to regulate the activation of SIRT1-mediated AKT/NRF2/HO-1 pathway, ASK1/MKK7/JNK signaling pathway, and the GRP78-IRE1/PERK Pathways, and so on [15–17]. These molecular interactions contribute to the modulation of various cellular processes, including inflammation, oxidative stress, immune response, and neuronal protection [18]. The evidence suggests that DDT ameliorats pathological changes by regulating key molecular targets, and restoring cellular homeostasis. The elucidation of its mechanisms of action provides valuable insights into its clinical applications and offers opportunities for the development of novel therapeutic strategies.

At present, there is no corresponding research on the molecular mechanism behind DDT treatment of VaD. Therefore, we hypothesized that DDT treats VaD by regulating Ferroptosis. To confirm our hypothesis, we made series experiments and use Donepezil (DPZ) which was a well-known VaD
treatment drug as a positive control of DDT treatment. What’s more, we overexpressed PGK1 to explore the hypothesis that the treatment of DDT to VaD have something to do with PGK1. It is hoped that our research can provide a potential research direction for understanding the therapeutic effect of DDT on VaD and the future treatment of VaD.

2. Materials and methods

2.1. Drugs and reagents

The constitution of DDT comprised an intricate amalgamation, consisting of 48g Dahuang, 30 dry Mengchong, 20 Taoren, and 30 dry Shuizhi. Following the procurement of these botanical components, a meticulous preparatory protocol unfolded: a preliminary phase involving their immersion in water, succeeded by a measured application of heat. The resultant product was then subject to a rigorous cycle of decompression and concentration, meticulously calibrated to yield a concentration of 1 gram per milliliter. The final distillate was judiciously preserved within a controlled refrigerative environment, maintained at 4°C, awaiting subsequent utilization in our investigative journey. Loflomine (400mg/kg) and DPZ 10mg/kg were prepared for the following experiment. The ingredients of DDT was identified using Mass Spectrometry (Figure S1).

2.2. Animal Groups and treatment

Ethical approval: All animal procedures were accordance with Declaration of Helsinki, and were contingent on the Ethics Committee of Nanjing University of Chinese Medicine (granted number was 2208A129) and were executed in line with the rules of the Care and Use of Laboratory Animals. This article does not incorporate any studies with human participants.

Male rats weighing between 250 and 300g were used to establish the 2VO model. Anesthesia was induced by intraperitoneal injection of isoflurane (at a dosage of 350-400 mg/kg body weight) or sodium pentobarbital (at a dosage of 50-60 mg/kg body weight). After anesthetizing rats and sterilizing the surgical area, a midline incision was made in the neck, and blunt dissection was performed to isolate and permanently ligate the bilateral carotid arteries with a size 1 suture. Suture the incision, and perform local disinfection before returning the rats to their cages (with one rat per cage) once fully awake. Before closing the local wound, 3-5 drops of penicillin were applied to prevent infection. Following the surgery, the rats were housed under normal conditions for 3 weeks, and from the 4th week onwards, they were subjected to group-specific drug treatment. The Morris water maze analysis system was used for behavioral testing, including the spatial navigation experiment and the spatial exploration test.

In certain experimental paradigms, vehicle AAV-OE-PGK1 (Shanghai Jikai Biotechnology Co., Ltd.) was injected into the one-side cerebral ventricle of the mice (5ul of 1.0×1012 TU/ml vehicle per injection), and 2VO was immediately carried out.

2.3. Experimental Design
A cohort of 80 male rats (male, 250~300g), eight of the 80 rats were used as a sham-operated group, and the rest of the rats were used for the establishment of the 2VO model.

In one series, rats were randomly divided into six groups: the sham-operated (sham) group; the 2VO group; the low DDT (L-DDT) group (comprising 1.62g/kg raw medicinal content, with an extract ratio of 0.178g/kg); the medium DDT (M-DDT) group (comprising 3.24g/kg raw medicinal content, with an extract ratio of 0.356g/kg); the high DDT (H-DDT) group (comprising 6.48g/kg raw medicinal content, with an extract ratio of 0.712g/kg); and the DPZ group (10mg/kg). In another series, the rats was randomly assigned to four groups: the sham-operated (sham) group; the high DDT (H-DDT) group (comprising 6.48g/kg raw medicinal content, with an extract ratio of 0.712g/kg); the overexpression-PGK1 (OE-PGK1) group; and the overexpression-PGK1+ high-DDT (OE-PGK1+ H-DDT) group. After completing the entire experiment, we conducted HE staining to investigate potential pathological changes in slices of the five main organs (Lung, Liver, Spleen, Stomach, Heart) of rats after the treatment of DDT (Figure S2). No obvious pathological change can be detected after DDT treatment.

2.4. Water Maze Swimming Test

After 3-week administration period and 4-week administration period, Morris water maze test (MWM) was taken to evaluate the spatial learning and memory capabilities of the rats in each experimental group. In this test, the pool is divided into four quadrants (NE, SE, SW, NW) in four directions and a transparent plastic platform was placed below the water surface within the center of first quadrant of the maze to serve as a hidden escape platform. An escape latency threshold of 120s was set for the rats. If a rat successfully located the platform within 120s and remained on it for at least 10s, the exact latency time was recorded. Conversely, if a rat failed to find the platform within 120s, an latency time of 120s was recorded. Each rats is trained 4 times a day, with an interval of 30 minutes between the two training and 5 consecutive days. On the 6th day, remove the original platform and put any one water entry point of the rats into the water. All rats must be the same water entry point to record the number of times the rats crossed the original platform within 2 minutes [19].

2.5. Nissl staining and HE staining

After the fresh brain tissue is fixed in 10% neutral formarline solution for 48 hours, it is buried in conventional dehydration. Then dewax these brain slices and wash it with distilled water Put slices into Tolidine blue Stain, place the dyeing tank in an incubator at 50-60°C and infect 25-50 min. The brain slices are successively dealt with distilled water, 70% alcohol, 95% alcohol, anhydrous ethanol, and xylene. For HE staining, slices were initially dewaxed by sequential immersion in xylene and different alcohol concentrations. Hematoxylin and eosin were employed to respectively stain the nucleus and cytoplasm. Subsequently, the slices were dehydrated using alcohol, anhydrous ethanol, and xylene. After removing them from xylene and allowing slight drying, they were sealed with neutral gum.

2.6. Fluoro-Jade B
Fluorescent dye Fluoro-Jade B (FJB) is an anionic fluorescein derivatives that can be used for histological staining of degeneration neurons. Fluoro-Jade B can be compatible with several other labeling programs, including immunofluorescence and Nissl staining. Sections were reacted with 0.0002% FJB in 0.1% acetic acid while using FJB staining to detect degenerated neurons [20].

2.7. Determination of ROS

ROS were detected using an ROS (S0033M, Beyotime, Jiangsu, China) assay kit. Detection was performed using a NovoCyte flowcytometer with NovoExpress analysis software (ACEA Biosciences, San Diego, CA, USA).

Dihydroethidium (DHE; Sigma-Aldich) was used to detect intracellular ROS in the hippocampal CA1 region of VaD rats. For DHE staining, brain tissues homogenized with hippocampal CA1 region and centrifuged. Then, the supernatant was taken out and exposed to 10 uM DHE for 30 min at 37 °C or ROS detection in cultured cells. Fluorescence microscopy, laser confocal microscope, fluorescence spectrophotometer, luminescence enzyme marker, flow cytometer, etc. are used to detect fluorescence, so as to determine the level of ROS in the hippocampal CA1 region of VaD rats.

2.8. GSH and MDA Detection

Malondialdehyde (MDA) and Glutathone (GSH) were measured using an MDA (Solarbio, Beijing, China) or GSH (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) assay kit following the standard protocol. Briefly, the double-antibody sandwich method was used to measure the optical density of each well at a wavelength, and the contents of MDA and GSH were calculated.

2.9. Detection of Cellular Fe²⁺ Ions Generation

The concentration of Fe²⁺ in hippocampal tissues was determined using the Fe²⁺ detection kit (A039-2-1, Nanjing Jicheng Bioengineering Institute, Nanjing, China) following the recommended protocols provided by the manufacturer. Free Fe²⁺ reacts with Iron Probe to produce a stable complex with absorbance at 593nm. Then analyze it with microplate reader.

2.10. Immunofluorescence assay

The brain sections (4mm) were subjected to antigen retrieval. Then, primary antibodies against NRF2 (Art No.12721S, Dilution ratio: 1:400, Cell Signaling Technology Co, Ltd.) and corresponding secondary antibody Anti-rabbit IgG (H+L) (Art No.14708, Dilution ratio: 1:5000, Cell Signaling Technology Co, Ltd.) were added. After incubating, the slices were stained. Finally, the content was determined by flow cytometry.

2.11. Immunohistochemistry assay
The brain sections were subjected to antigen retrieval, and then incubated with primary antibodies Glutathione peroxidase 4 (GPX4, Art No.ab125066, Dilution ratio: 1:200, Abcam Limited Co, Ltd.) and NEUM (Art No.ab 177487, Dilution ratio: 1:500, Abcam Limited Co, Ltd.) at 4 °C. The slides were incubated with corresponding secondary antibodies Goat anti-Rabbit IgG H&L (HRP, Art No.ab205718, Dilution ratio: 1:1000; Abcam Limited Co, Ltd.). After incubating and staining, a microscope system was used to capture fluorescence. The immunohistochemistry images was analyzed by the ImageJ software.

2.12. Western Blotting

Hippocampal tissue, stored at −80°C, was thawed and dissolved in RIPA lysis buffer supplemented with a mixture of protease inhibitors and phosphatase inhibitors. The resulting solution was centrifuged at 12,000 g for 10 minutes, and the supernatant was collected for further analysis. The protein concentration in the supernatant was determined using the BCA Protein analysis kit (Thermo Fisher Scientific, Inc.). Subsequently, 25 μg of the isolated proteins were separated by electrophoresis on 10% or 12% SDS polyacrylamide gels and then transferred onto PVDF membranes. Following blocking with 5% skim milk for 1 hour, the membranes were incubated overnight at 4°C with primary antibodies. Dilute the antibody, and immerse the PVDF membrane with protein into square preservation box, and oscillate for 1-2 hours. After the incubation is completed, absorb the second antibody. Finally, use a pipette to absorb the appropriate amount of light-emitting liquid to cover the PVDF film, and the ELC light-emitting instrument is exposed and the image is collected. The primary antibodies used were as follows: Glutathione peroxidase 4 (GPX4, Art No.ab125066, Dilution ratio: 1:200), Goat anti-Rabbit IgG H&L (HRP, Art No.ab205718, Dilution ratio: 1:1000), and SLC7A11 (Art No.ab275411, Dilution ratio: 1:1000) was purchased from Abcam Limited Co, Ltd.. NRF2 (Art No.12721S Dilution ratio: 1:400), NFR2 (Art No.12721 Dilution ratio: 1:1000), Phosphoglycerate Kinase 1 (PGK1, Art No.63536, Dilution ratio: 1:1000), GAPDH (Art No.5174, Dilution ratio: 1:5000), LaminB1 (Art No.17416, Dilution ratio: 1:1000) and Anti-rabbit IgG (H+L) (Art No.14708, Dilution ratio: 1:5000) were purchased from Cell Signaling Technology(CST) Co, Ltd.. The western blot can be measured by the ImageJ software.

2.13. Statistical analysis

Statistical analysis and graphical representation of the data were performed using GraphPad Prism 8.0 software. Statistical significance was denoted by p< 0.05. All statistical variables are presented as mean ± standard deviation (SD) based on a minimum of three independent experiments. The two-tailed Student's t-test was employed to assess differences between two groups, while one-way analysis of variance (ANOVA) was used for comparisons among multiple groups.

3. Results

3.1. DDT alleviates cognitive dysfunction in VaD rats

The timeline shows the progress of the whole experiment (Fig.1A). 2VO is a widely used as the VaD model. It takes 21 days to build up. The DDT treatment started on the 21th day and lasted for 4 weeks.
To evaluate cognitive dysfunction in VaD rats, we performed Morris Water Maze tests, which assess animal analysis, judgment ability, and problem-solving skills through search strategy and touchability indicators. Trajectory analysis revealed that the search strategy employed by the DDT and DPZ groups closely resembled that of normal rats, indicative of improved cognitive function (Fig.1B). Statistical analysis unveiled a significant increase in latency time for the 2VO group compared to the Sham group (Fig.1C, \( p < 0.01 \)). Moreover, the latency time of the L-DDT group, M-DDT group, and H-DDT group was significantly shorter than that of the 2VO group (Fig.1C, \( p < 0.01 \)). Additionally, relative to the Sham group, the platform crossing frequency and time spent with objects were markedly reduced in the 2VO group (Fig.1D, \( p < 0.01 \); Fig.1E, \( p < 0.01 \)). Conversely, the L-DDT group, M-DDT group, H-DDT group, and DPZ group exhibited an increased platform crossing frequency and more time spent with objects compared to the 2VO group (Fig.1D, \( p < 0.05 \); Fig.1E, \( p < 0.05 \)). Furthermore, the resistance index (RI) of the 2VO group was significantly lower than that of the Sham group (Fig.1F, \( p < 0.01 \)). However, the RI of the L-DDT group (Fig.1F, \( p < 0.05 \)), M-DDT group (Fig.1F, \( p < 0.05 \)), H-DDT group (Fig.1F, \( p < 0.05 \)), and DPZ group (Fig.1F, \( p < 0.05 \)) exceeded that of the 2VO group. In summary, our findings demonstrate that DDT treatment effectively ameliorates cognitive impairment in VaD rats, offering potential therapeutic strategies for addressing cognitive dysfunction in VaD.

3.2. DDT attenuates neuronal apoptosis and degeneration in the hippocampal CA1 region of VaD rats

Treat the hippocampal CA1 region of VaD rats with Nissl staining to detect neuronal apoptosis and degeneration in the hippocampal CA1 region of VaD rats. In the Sham group, neural cells in the CA1 area of the hippocampus had complete morphology and structure and abundant Nissl particles in the cytoplasm. In the 2VO group, the number of nerve cells in the CA1 area of the hippocampus was significantly reduced, and the Nissl granules were not obvious (Fig.2A). From the experimental data analysis, the number of cells in the CA1 region of the hippocampus in the rat group 2VO was significantly less than that in the Sham group (Fig.2B, \( p < 0.01 \)). Compared with the 2VO group, the number of neurons increased in the L-DDT group (Fig.2B, \( p < 0.05 \)), and remarkably increased in the M-DDT group (Fig.2B, \( p < 0.01 \)) and in the H-DDT group (Fig.2B, \( p < 0.01 \)). The Fluoro-Jade B staining was used to observe the number of degenerative neurons in different groups (Fig.2C), and by data analysis, the degree of neurodegeneration was significantly higher in the 2VO group than in the Sham group (Fig.2D, \( p < 0.01 \)). Meanwhile, compared with the 2VO group, the degree of neurodegeneration was decreased in the L-DDT group (Fig.2D, \( p < 0.05 \)), observably decreased in the M-DDT group (Fig.2D, \( p < 0.01 \)) and in the H-DDT (Fig.2D, \( p < 0.01 \)). These results revealed that DDT actually attenuates neuronal apoptosis and degeneration in the hippocampal CA1 region of VaD rats.

3.3. DDT inhibits ferroptosis in the hippocampal CA1 region of VaD rats

Ferroptosis, an iron-dependent form of programmed cell death, differs from apoptosis, necrosis, and autophagy. We examined the impact of DDT on ferroptosis by measuring Fe\(^{2+}\), GSH, and MDA levels in rat brains using ELISA. ROS levels significantly increased in the 2VO group compared to the Sham group (Fig.3A; Fig.3B, \( p < 0.01 \)), but decreased in the L-DDT group (Fig.3A; Fig.3B, \( p < 0.05 \)), M-DDT group (Fig.3A; Fig.3B, \( p < 0.05 \)), and H-DDT group (Fig.3A; Fig.3B, \( p < 0.05 \)).
Fig.3B, \( p<0.01 \), and H-DDT group (Fig.3A; Fig.3B, \( p<0.01 \)) compared to the 2VO group. MDA levels increased in the 2VO group compared to the Sham group (Fig.3C, \( p<0.01 \)), while DDT treatment led to decreased MDA levels in the L-DDT group, M-DDT group, and H-DDT group compared to the 2VO group (Fig.3C, \( p<0.01 \)). The ferrous iron content exhibited an increase in the 2VO group relative to the Sham group (Fig.3D, \( p<0.01 \)), whereas it demonstrated a decrease in the L-DDT group, M-DDT group, and H-DDT group compared to the 2VO group (Fig.3D, \( p<0.01 \)). Conversely, GSH levels were lower in the 2VO group when compared to the Sham group (Fig.3E, \( p<0.01 \)), whereas DDT treatment resulted in elevated GSH levels in the L-DDT group, M-DDT group, and H-DDT group relative to the 2VO group (Fig.3E, \( p<0.01 \)). Overall, our findings from ELISA analysis demonstrate that DDT administration effectively inhibits ferroptosis in the hippocampal CA1 region of VaD rats.

3.4. DDT regulates the protein expression of PGK1/NRF2/GPX4 pathway of VaD rats

Accumulating evidence suggests that Nrf 2 can inhibit SLC7A11 regulation in tissue injury after cerebral ischemia [21, 22]. SLC7A11 is the upstream mediator of GPX4 and is critical to regulating ferroptosis. Therefore, we used western blot and Immunofluorescence and immunohistochemistry assay to investigate the expression levels of SLC7A11, GPX4, Nrf2, and PGK1 after DDT treatment. According to the experimental data analysis, the expression of GPX 4 in the 2VO group was significantly lower than that in Sham group (Fig.4A; Fig.4B, \( p<0.01 \); Figu.4G, \( p<0.05 \)). Compared with the 2VO group, the expression of GPX4 in the L-DDT, M-DDT and H-DDT group significantly increased (Fig.4A; Fig.4B, \( p<0.01 \); Fig.4G, \( p<0.01 \)). The experimental data demonstrated higher expression of Nuclear-Nrf2 in the 2VO group relative to the Sham group (Fig.4C; Fig.4D, \( p<0.01 \)). Conversely, compared to the 2VO group, the L-DDT group showed increased expression of Nuclear-Nrf2 (Fig.4C; Fig.4D, \( p<0.05 \)), while the M-DDT group and H-DDT group exhibited significantly higher expression of Nuclear-Nrf2 (Fig.4C; Fig.4D, \( p<0.01 \)). Data analysis revealed that SLC7A11 expression in the 2VO group was significantly lower than that in the Sham group (Fig.4E; Fig.4F, \( p<0.01 \)). However, SLC7A11 expression significantly increased in the L-DDT group (Fig.4E; Fig.4F, \( p<0.05 \)), M-DDT group (Fig.4C; Fig.4E, \( p<0.01 \)), and H-DDT group (Fig.4E; Fig.4F, \( p<0.01 \)) compared to the 2VO group. These findings indicate that DDT treatment elevated the expression of the PGK1/NRF2/GPX4 pathway. The expression of PGK1 in the 2VO rats was significantly higher than that in the Sham group (Fig.4E; Fig.4H, \( p<0.01 \)). In comparison to the 2VO group, the expression of PGK1 decreased in the L-DDT group (Fig.4E; Fig.4H, \( p<0.05 \)), M-DDT group (Fig.4E; Fig.4H, \( p<0.01 \)), and H-DDT group (Fig.4E; Fig.4H, \( p<0.01 \)).

3.5. Overexpression of PGK1 abolish the protect effect of DDT on VaD caused cognitive dysfunction

To investigate the effects of PGK1 overexpression to the protect effect of DDT on VaD caused cognitive dysfunction, Western blot, MWM and Nissl staining were taken. The timeline shows the progress of the whole experiment (Fig.5A). 2VO model, a widely used as the VaD model took 21days to built up. The DDT treatment started on the 21th day and lasted for 4 weeks, and injected the brain localization unilaterally with AAV- OE-PGK1 at the same time.
The result of MWM revealed that the 2VO group had longer latency time compared with the H-DDT group (Fig.5B; Fig.5C, $p<0.01$), while the OE-PGK1+ H-DDT group also had longer latency time compared with the H-DDT group(Fig.5C, $p<0.05$). Meanwhile, the Platform crossing frequency of the 2VO group was lower compared with the H-DDT group (Fig.5D, $p<0.05$), while the Platform crossing frequency of the OE-PGK1+H-DDT group also lower compared with the H-DDT group(Fig.5D, $p<0.05$). These findings that the spatial learning and memory capabilities of the 2VO group and the OE-PGK1+H-DDT group were both worse than the H-DDT group indicated that the overexpression of PGK1 inhibits DDT's treatment of VaD.

Western blot was used to confirm the overexpression of PGK1. Our results showed that the PGK1 was actually overexpressed in the OE-PGK1 group (Fig.5E; Fig.5F, $p<0.01$). What’s more, the result of Nissl staining revealed that the H-DDT group had more normal neuron than the 2VO group (Fig.5G; Fig.5H, $p<0.01$) and the OE-PGK1+H-DDT group (Fig.5G; Fig.5H, $p<0.01$), which further confirmed the construction above. All these results indicate that the overexpression of PGK1 will inhibit the therapeutic effect of DDT on VaD caused cognitive dysfunction.

3.6. Overexpression of PGK1 eliminates the DDT-caused reduction of ferroptosis in VaD rats

To investigate the effect of PGK1 overexpression to the DDT-caused reduction of ferroptosis, we measured the levels of Fe$^{2+}$, ROS and MDA in the brains of rats across different groups using ELISA. The level of ROS in the H-DDT group was lower than that of the 2VO group (Fig.6A; Fig.6B, $p<0.01$) and the OE-PGK1+H-DDT group (Fig.6A; Fig.6B, $p<0.01$). The H-DDT group had lower level of MDA than the 2VO group (Fig.6C, $p<0.01$) and the OE-PGK1+H-DDT group (Fig.6C, $p<0.01$). In addition, the H-DDT group had lower level of Fe$^{2+}$ than the 2VO group (Fig.6D, $p<0.01$) and the OE-PGK1+H-DDT group (Fig.6D, $p<0.01$). These results confirm that PGK1 overexpression eliminates the reduction DDT caused of ferroptosis in VaD rats.

3.7. Overexpression of PGK1 decreased the DDT treatment induced elevation of key factors in the PGK1/NRF2/GPX4 pathway

To gain insight into the effect of PGK1 overexpression on PGK1/NRF2/GPX4 pathway, we identified the expression of SLC7A11, GPX4 and Nrf2. Immunofluorescence staining of NRF2 (Fig.7A) revealed that NRF2 expression in the nucleus was downregulated after overexpression of PGK1. The western blot revealed that the level of SLC7A11 expression in the H-DDT group was higher than that in the 2VO group (Fig.7C, Fig.7D, $p<0.01$) and the OE-PGK1+H-DDT group (Fig.7C, Fig.7D, $p<0.01$). Meanwhile, the level of GPX4 expression in the H-DDT group was higher than that in the 2VO group (Fig.7C, Fig.7E, $p<0.01$) and the OE-PGK1+H-DDT group (Fig.7C, Fig.7E, $p<0.01$). What’s more, the NRF2 expression in the nucleus was markedly decreased after PGK1 overexpression in the OE-PGK1+H-DDT group compared with the H-DDT group (Fig.7B, $p<0.01$; Fig.7F; Fig.7G, $p<0.01$), while the NRF2 expression in the nucleus was also markedly decreased in the 2VO group compared with the H-DDT group (Fig.7B, $p<0.01$; Fig.7F; Fig.7G, $p<0.01$). These findings indicated that PGK1/NRF2/GPX4 pathway elevated by DDT could be decreased by overexpression of PGK1, and that will decreased the efficacy of DDT.
4. Discussion

VaD is an age-related neurodegenerative disease that affects cognitive abilities, and was often thought to have a relation with stroke, hypertension, diabetes, and atherosclerosis [23]. Our experimental data suggest that DDT can improve the spatial learning and memory capabilities in VaD rats. Some previous studies revealed that ferrostatin-1 also significantly repaired cognitive dysfunction in VaD rats [24]. Therefore, we made a hypothesis that DDT can treat cognitive dysfunction by inhibiting ferroptosis.

Ferroptosis is a recently identified type of cell death that relies on the overproduction of iron and ROS, resulting in marked lipid peroxides. GSH serves as a reducing agent for GPX4, which was the key enzyme of the process, and can transform toxic lipid peroxides into non-toxic lipid alcohols, preventing the aggregation of ROS and subsequent lipid peroxidation, ultimately leading to ferroptosis [25]. Notably, some former studies investigated the manifestation of ferroptosis in hippocampal tissue of VaD rats. Morphological alterations such as mitochondrial shrinkage and crest reduction or disappearance were observed, alongside decreased expression of GPX4 and xCT, which are negative regulators of ferroptosis [26]. Also, our experimental data found a decrease in GSH and GPX4 synthesis, and an accumulation of Fe$^{2+}$, ROS, and MDA in the 2VO group. A key focus of ferroptosis research is investigating whether specific inhibitors of ferroptosis could enhance nerve repair. One such inhibitor is Ferrostatin-1, a first-generation synthetic compound that has demonstrated effective inhibition of ferroptosis in vitro [27]. However, its limited in vivo efficacy due to plasma and metabolic instability remains a challenge.

TCM offers several advantages, including abundant resources, low toxicity and side effects, and the ability to target multiple channels and mechanisms [28]. Numerous TCM formulations have shown successful outcomes in the treatment of nervous system diseases. DDT, a valuable traditional Chinese medicine formula recorded in the Treatise on Typhoid Fever, has long been recognized for its therapeutic properties, including reducing the extent of neurological deficits and promoting absorption of hematomas [16]. It also has the ability to target multiple channels. Moreover, TCM, with its rich resources, minimal toxic effects, and ability to target multiple channels and mechanisms, offers a promising avenue for drug development. While the neuroprotective role of DDT has been observed in rat models of Intracerebral hemorrhage [16], its effects in VaD have yet to be investigated. Thus, the present study utilized DDT as a therapeutic intervention to evaluate its neuroprotective effects in VaD rats and elucidate the underlying mechanisms. Our findings revealed a significant improvement in MWM scores of rats in the DDT+2VO group compared to the VaD group, although the scores remained lower than those of the sham group overall. These results suggest that DDT promotes cognitive function recovery in VaD rats. Moreover, the expressions of GSH and GPX4 were upregulated in the hippocampal tissue of the DDT+2VO treatment group. Collectively, these findings indicate that DDT enhances cognitive function recovery and attenuates ferroptosis in VaD rats.

PGK1 is an essential enzyme that involved in the first ATP-generating step of the glycolytic pathway [29]. It catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP in the...
aerobic glycolysis pathway [30]. The expression of PKG1 will significantly affect the formation of ATP in rats. This will further affect the expression of other DNAs in rats. PGK1 is one of key metabolic enzymes in the glycolytic, and can regulate ferroptosis by some way [31]. Our study showed that the overexpression of PGK1 will inhibit the therapeutic effect of DDT on VaD, so we can infer that DDT can regulate ferroptosis by influencing the expression of PKG1.

GPX4 is an antioxidant which can be observed in cell membrane and cytoplasm [32]. Inhibition of GPX4 activity leads to the accumulation of lipid peroxides, resulting in ferroptosis [33]. In rats, inhibition of GPX4 activity will cause neurodegenerative changes and neuronal damage, which finally leads to cognitive dysfunction [34]. Our results showed that inhibition of GPX4 activity was serious in the hippocampus of VaD rats. However, the activity of GPX4 was increased in the hippocampus of VaD rats after treated by DDT. What's more, ferroptosis in the hippocampus and the damage of hippocampal neurons both decreased as a result. Those findings showed that DDT inhibits the occurrence of ferroptosis in VaD hippocampal neurons by regulating the activity of GPX4. NRF2 plays the key regulatory factor in maintaining an oxidative steady state in cells [33, 35]. NRF2 and GPX4 are key factors of ferroptosis, and down-regulation of NRF2 and GPX4 can lead to deviant changes in antioxidant systems and intracellular oxidation, which further result in accumulating of ROS and cell death [36].

Our experiments above demonstrated that DDT regulates PGK1/NRF2/GPX4 Signaling Pathway to influence ferroptosis, and then treats VaD rats, so further experiment could be done to explore the molecular mechanism behind. This study also had some limitations. First, the experiment only took place on hippocampal neuronal cells, hence, other could be selected to be subjects for further studies. What's more, immune inflammatory response also played an important role in the complex pathological mechanism of VaD. Therefore, further study is needed.

5. Conclusions

In conclusion, this study provides compelling evidence for the beneficial effects of DDT in ameliorating cognitive dysfunction in VaD rats. DDT administration demonstrated significant neuroprotective properties, as evidenced by the attenuation of neuronal apoptosis and degeneration specifically in the hippocampal CA1 region, a critical area affected in VaD. Additionally, DDT exhibited the ability to inhibit ferroptosis, a form of cell death associated with oxidative stress and dysregulated iron metabolism, in the same hippocampal region. The study also highlighted the involvement of the PGK1/NRF2/GPX4 pathway in mediating the protective effects of DDT. Notably, the study revealed that overexpression of PGK1 counteracted the beneficial effects of DDT, suggesting its role in the underlying mechanism. These findings emphasize the potential therapeutic value of DDT in the management of cognitive dysfunction related to VaD and underscore the importance of further investigating the intricate interplay between DDT, the PGK1/NRF2/GPX4 pathway, and ferroptosis in neurodegenerative disorders.

Abbreviations
VaD  Vascular dementia
DDT  Di-Dang-Tang; DPZ: Donepezil
MWM.  the Morris water maze
the L-DDT group  the low DDT group
the M-DDT group  the medium DDT group
the H-DDT group  the high DDT group
the DPZ group  the DonepezilPZ group
the sham group  the sham-operated group
the OE-PGK1 group  the overexpression-PGK1 (OE-PGK1) group
the OE-PGK1+ H-DDT group  the overexpression-PGK1+ high-DDT group
FJB  Fluorescent dye Fluoro-Jade B
ROS  Reactive Oxygen Species
SD  standard deviation
ANOVA  analysis of variance
MDA  Malondialdehyde
GSH  Glutathione
RI  Resistance Index
DHE  Dihydroethidium
PGK1  Phosphoglycerate kinase 1
NRF2  Nuclear Factor Erythroid 2-related factor
GPX4  Glutathione Peroxidase 4
SLC7A11  Solute Carrier Family 7 Member 11
TCM  Traditional Chinese medicine

Declarations
Authorship contributions

Author contribution Conception and design of the work, Junjie Ma; drafting of manuscript, hiyuan Li; analysis of data, Yongqu Jiang; interpretation, Yongxia Jiang; preparation of figures, Xiaoli Liu. Critical revision, Yue Hu; acquisition of data, Zhou Yang; supervision, Yue Hu; funding acquisition, Zhou Yang. All authors approved the final manuscript and agreed to be personally accountable for the authors’ own contributions.

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Availability of data and materials

The data used in this study to support the findings are available from the corresponding author.

Ethics approval and consent to participate

Consent for publication

No applicable.

Competing interests

This authors declared that they had no competing interests.

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**Figures**
Figure 1

Morris water maze test. A. Diagram showing the design of experiment, including modeling, drug application and subsequent experimental design. B. The trajectory of rats in the MWM. C. The time it takes for animals to successfully find the platform for the first time after entering the water. D. The frequency of the experimental animal crosses the original platform position within 5 days. E. The time the rats stayed on the stage. F. Resistance index in different groups.
Figure 2

DDT attenuates neuronal apoptosis and degeneration in the hippocampal CA1 region of VaD rats. A. Representative images of Nissl staining of the hippocampal CA1 region of VaD rats. Scale bar = 50 μm. B. The number of surviving neurons in the hippocampal CA1 region. C. Representative images of Fluoro-Jade B staining of the hippocampal CA1 region of VaD rats. Scale bar = 50 μm. D. The number of degenerative neurons stained by Fluoro-Jade B.
**Figure 3**

DDT inhibits ferroptosis in the hippocampal CA1 region of VaD rats. A. Representative immunofluorescence images of the hippocampal CA1 region of VaD rats using Dihydroethidium. Scale bar = 50 μm. B. The level of ROS in the hippocampal CA1 region. C. The level of MDA in the hippocampal CA1 region. D. Ferrous Ion Content of the hippocampal CA1 region. E. The number of GSH in the hippocampal CA1 region.

**Figure 4**

DDT regulates the protein expression of PGK1/NRF2/GPX4 pathway of VaD rats. A. Immunohistochemical staining. The nuclei are stained in blue, and the target proteins are stained in brown. B. Quantification of the staining intensity showing the relative expressions of GPX4. C. Representative Western blot images of Nucleus-Nrf2. D. Quantification of the staining intensity showing the expressions of Nucleus-Nrf2. E. Representative Western blot images of PGK1, SLC7A11, GPX4. F, G, and H. Quantification of the staining intensity showing the expressions of PGK1, SLC7A11, GPX4.
Overexpression of PGK1 abolish the protect effect of DDT on VaD caused cognitive dysfunction. A. Diagram showing the design of experiment, including modeling, drug application and subsequent experimental design. B. The trajectory of rats in the MWM. C. The time it takes for animals to successfully find the platform for the first time after entering the water. D. The frequency of the experimental animal crosses the original platform position within 5 days. E. Representative Westerb blot images of PGK1. F.
Quantification of the staining intensity showing the expressions of PGK1. G. Representative images of Nissl staining of the hippocampal CA1 region of VaD rats. Scale bar = 50 μm. H. Quantification of the staining intensity showing the number of surviving neurons.

**Figure 6**

Overexpression of PGK1 eliminates the DDT-caused reduction of ferroptosis in VaD rats. A. Representative immunofluorescence images of the hippocampal CA1 region of VaD rats using Dihydroethidium. Scale bar = 50 μm. B. The level of ROS in the hippocampal CA1 region. C. The level of MDA in the hippocampal CA1 region. D. Ferrous Ion Content of the hippocampal CA1 region.
Figure 7

Overexpression of PGK1 decreased the DDT treatment induced elevation of key factors in the PGK1/NRF2/GPX4 pathway. A. Representative images of Immunohistochemistry assay of NRF2. B. Quantification of Immunohistochemistry staining intensity showing the relative expressions of NRF2 in nucleus. C. Representative Westerb blot images of SLC7A11 and GPX4. D. Quantification of staining intensity showing the expressions of SLC7A11. E. Quantification of staining intensity showing the expressions of GPX4. F. Representative Westerb blot images of NRF2. G. Quantification of staining intensity showing the expressions of NRF2.
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