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Puerarin mitigates traumatic brain injury via ferroptosis-related targets and immune modulation: An integrated bioinformatics and experimental analysis

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Abstract: **Background**: Ferroptosis is closely involved in the pathological process of traumatic brain injury (TBI), and Puerarin has potential neuroprotective activity. **Objectives**: This study aims to investigate the main targets and underlying mechanisms of Puerarin against traumatic brain injury (TBI) from the perspective of ferroptosis. **Methods**: The GSE104687 dataset (194 TBI and 182 control samples) was obtained from Gene Expression Omnibus (GEO) and differentially expressed genes (DEGs) were identified using the R package limma. The ferroptosis-related genes (FRGs) were compiled from GeneCards and MSigDB and Puerarin-related targets were retrieved from PubChem, Similarity Ensemble Approach (SEA) and SwissTargetPrediction. Four key ferroptosis-related DEGs (FDTRDEGs) were obtained by intersecting DEGs with FRGs and Puerarin targets: NQO1, VCP, PML and RELA. Finally, a mouse TBI model was established to evaluate Puerarin's effects on brain injury, neurological deficits, brain water content and molecular changes across sham, TBI and TBI + Puerarin groups. **Results**: In the TBI model, Puerarin treatment reduced brain injury, inflammation, neurological deficits, and brain water content in TBI mice and reversed the TBI-induced expression changes of the four key genes at both mRNA and protein levels. **Conclusions**: Puerarin may protect against TBI by targeting ferroptosis-related pathways, with core genes NQO1, VCP, PML and RELA playing key roles. It may also modulate immune cell infiltration through these targets, contributing to its therapeutic effects.

Keywords: Ferroptosis; Immune infiltration; Network pharmacology; Traumatic brain injury (TBI); *Puerarin*

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INTRODUCTION

The high incidence and disability rate of traumatic brain injury (TBI) make it a significant global public health challenge. The age-standardized prevalence of TBI increased by 8.4% from 1990 to 2016 ("Global, regional and national burden of traumatic brain injury and spinal cord injury, 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016," 2019). In China, the number of TBI patients exceeds that of most other countries. The estimated TBI-related mortality rate in China is approximately 13 per 100,000 people, which is comparable to rates reported in other countries (Tang *et al.*, 2021). TBI directly threatens physical health and may cause long-term neurological dysfunction, including sensorimotor, psychological and cognitive impairments, severely reducing patients' quality of life (Dams-O'Connor *et al.*, 2023). Additionally, in the United States, the annual medical cost of non-fatal TBI exceeds $40.6 billion. For patients requiring hospitalization, the average cost per patient is $51,241, imposing a heavy financial burden on families (Crozes *et al.*, 2024).

In TBI, ferroptosis may exacerbate brain tissue damage by intensifying oxidative stress, disrupting cellular membrane integrity and inducing inflammatory responses. Ferroptosis-related genes (FRGs) may influence neuronal damage and repair in TBI through the regulation of apoptosis and oxidative stress pathways (Ren *et al.*, 2025). Ferroptosis was prioritized over apoptosis or necroptosis because it uniquely drives TBI pathology through iron-dependent lipid peroxidation, which is reversible and pharmacologically targetable (Jiang *et al.*, 2021). Unlike apoptosis, ferroptosis involves glutathione depletion and Glutathione Peroxidase 4 (GPX4) inactivation, making it a key contributor to oxidative damage in TBI (Tang *et al.*, 2021). For instance, it is demonstrated that mechanical trauma directly activates ferroptosis and inflammatory pathways via cytoskeletal disruption (Zhan *et al.*, 2025). Modulating FRGs may help alleviate oxidative stress and apoptosis caused by TBI, thereby improving patient prognosis (Song *et al.*, 2021). Therefore, investigating the differential expression of FRGs in TBI is of great significance for elucidating its molecular mechanisms and may contribute to TBI diagnosis and therapy.

The treatment goals for TBI are to minimize the impact of primary injury, prevent and manage secondary injury and promote neurological recovery. Although various treatment strategies are currently employed for TBI, including surgical intervention, pharmacological therapy, rehabilitation and cell therapy, there are still many limitations. For instance, there is a lack of drugs targeting the core pathological mechanisms of TBI and due to individual differences, the severity of TBI and patients' responses to drugs vary widely, making precise treatment and optimal recovery difficult to achieve (Tani *et al.*, 2022).

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Hence, finding more effective therapeutic strategies remains a key task in TBI research.

In recent years, some studies have suggested that *Puerarin* may have therapeutic potential for brain or neuronal injuries. *Puerarin*, an isoflavone compound extracted from *Pueraria lobata* (kudzu root), possesses multiple pharmacological effects, such as antioxidant, anti-inflammatory and neuroprotective properties. For example,it is found that *Puerarin* could reduce cerebral ischemia-reperfusion injury by inhibiting oxidative stress (Xu G *et al.*, 2025; Chauhan *et al.,* 2024) demonstrated that *Puerarin* exerted neuroprotective effects by reducing neuronal apoptosis. Cheng *et al. (*2024*)* showed that natural ferroptosis inhibitors (e.g., from medicinal plants) attenuate neuronal injury. Structurally, *Puerarin* shares antioxidant properties with flavonoids from Ampelopsis grossedentata, as highlighted previously, underscoring its role in redox and immune modulation (Wu RR *et al.* 2023). These findings suggest that *Puerarin* may have potential in treating TBI.

However, the specific mechanisms by which *Puerarin* treats TBI remain unclear. In this study, we will investigate the mechanism of action of *Puerarin* on TBI through network pharmacology analysis, thereby laying the foundation for its clinical application.

MATERIALS AND METHODS

Data source

The TBI dataset GSE104687 (Homo sapiens) was downloaded from the Gene Expression Omnibus (GEO) database, including 194 TBI samples and 182 normal control samples (Miller *et al.*, 2017). Ferroptosis-related genes (FRGs) were collected using the keyword "Ferroptosis" from the GeneCards database and the MSigDB database, with 64 and 339 FRGs obtained respectively. After removing duplicate genes, 375 FRGs remained.

Drug target screening

The targets of *Puerarin* were retrieved from three databases: PubChem, Similarity Ensemble Approach (SEA) and SwissTargetPrediction. A total of 197 drug target-related genes (FDTRGs) were obtained.

TBI-related differentially expressed genes

DEGs were identified using the R package limma (v3.58.1) with thresholds set at $|logFC| > 0.5$ and adjusted p-value (FDR) < 0.05. Batch effects in GSE104687 were corrected using the ComBat algorithm (sva package v3.48.0). Database versions: GeneCards (v4.16), MSigDB (2023.1), PubChem (2024). A heatmap was created using the R package *pheatmap* (Version 1.0.12) to show the expression of these key genes. The process is illustrated in the workflow (Fig. 1). Adjusted *p-values* (Benjamini-Hochberg FDR) were applied to control false discovery rate. GSE104687 batch effects were corrected using ComBat with empirical Bayes estimation.

Functional enrichment

R package *clusterProfiler* (Version 4.10.0) was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the key genes. GO/KEGG enrichment analysis was performed using clusterProfiler (v4.10.0), *p-values* were corrected by Benjamini-Hochberg, FDR < 0.05 was considered significant.

Protein-protein interaction (PPI)

The STRING database is used to identify known and predicted protein interactions. The STRING database was used to set the species to human and to select confidence scores≥ 0.150 to build the PPI network.

Functional similarity analysis

The GO semantic similarity provides a way to measure how similar genes are in terms of their biological function. The R package GOSemSim was used to calculate GO semantic similarity for the key genes. Then, the geometric mean of similarity scores for Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) categories were also calculated. Finally, the results were visualized using the ggplot2 package.

Immune infiltration analysis

The Immune infiltration analysis (CIBERSORT) with a reference gene signature matrix was used and filtered out immune cell scores less than or equal to zero. The immune cell infiltration matrix for dataset GSE104687 was obtained and shown as a bar plot. CIBERSORT was run with the LM22 signature matrix (v1.04) using 1000 permutations. Immune cell scores were filtered to exclude samples with CIBERSORT p-value > 0.05.

Data on animals

15 healthy adult male C57BL/6 mice (4–6 months, 20–25 g) were housed under controlled conditions (20–25°C, humidity: 60%, 12 h light/dark cycle) and acclimated for 7 days prior to the experiment. 15 mice were randomly assigned to three groups (n=5 per group) using computer-generated randomization sequences. Data collectors were blinded to group allocation during behavioral testing and molecular analyses. All experiments included three biological replicates, each with two technical replicates. Briefly, mice were anesthetized with 5% chloral hydrate and the scalp was shaved, disinfected and incised longitudinally along the midline between the ears. The skin was retracted to expose the skull and a mark was made with gentian violet 4 mm lateral to the sagittal suture and 4 mm posterior to the coronal suture on the right side. At the marked site, a craniotomy (diameter: 8 mm) was performed using a dental drill and the bone window was gently rinsed with normal saline using a 10 mL syringe to remove bone

debris, ensuring the integrity of the dura mater. The impact probe was carefully placed within the bone window and stabilized using dental impression material to seal the surrounding gaps. The impact force was set to 0.05 MPa, inducing TBI through pressure-driven hydraulic impact on the brain tissue.

The mice were then randomly divided into three groups: Sham, TBI and TBI + *Puerarin*, with 5 mice in each group. At 30 minutes post-injury, mice in the TBI + *Puerarin* group received *Puerarin* treatment (100 mg/kg/d, intraperitoneal injection, frequency). The TBI group was intraperitoneally injected with the same volume of normal saline. Neurological deficit scores were assessed before treatment and on days 3, 14 and 28 post-treatment. The evaluation included the following tasks: circling, hemiplegia, straight-line walking, startle reflex, exploratory behavior, square beam balance (5 mm), round beam balance (5 mm), square beam walking (3 cm, 2 cm and 1 cm widths). A score of 0 was assigned for successful completion and 1 for failure, with higher scores indicating more severe neurological impairment (Cox *et al.*, 2023). Brain water content was measured as a percentage (%) of wet-to-dry weight ratio. Finally, quantitative PCR (qPCR) and Western blot (WB) were used to evaluate mRNA and protein expression levels of NQO1, RELA, VCP and PML in the brain tissue homogenates.

Experimental quality was controlled by blinding during data collection and analysis. All procedures followed Animal Research: Reporting of *in-vivo* experiments (ARRIVE) guidelines, with sample size justified by power analysis (n=5 per group provided 80% power to detect effect sizes >1.5 at α=0.05).

Statistical analysis

All data processing and analysis in this study were done using R software (Version 4.2.2). To compare three or more groups, the Kruskal-Wallis test was used. Correlations between molecules were calculated using Spearman's rank correlation.

RESULTS

Drug target screening

All experimental results met pre-set quality thresholds, with no outliers excluded. To identify the TBI-related targets of *Puerarin*, the PubChem, SEA and SwissTargetPrediction databases were first used. Bioinformatics methods were mainly used to explore the biological characteristics of TBI (Fig. 1). Data cleaning operations, such as annotation probes, were performed on dataset GSE104687 (Fig. 2). Then, a drug-TBI target interaction network was constructed based on the targets obtained from these databases (Fig. 3).

Ferroptosis- and drug target–related differentially expressed genes in TBI

To obtain ferroptosis- and drug target–related genes (FDTRGs), ferroptosis-related genes (FRGs) and drug target–related genes (DTRGs) were intersected and a Venn diagram was drawn (Fig. 4A). A total of 29 FDTRGs were identified (Fig. 4B). To identify ferroptosis- and drug target–related differentially expressed genes (FDTRDEGs), the 1,513 DEGs were intersected with the 29 FDTRGs and a Venn diagram was created (Fig. 4C). Four FDTRDEGs were obtained: NQO1, VCP, PML and RELA, which were selected as key genes (KeyGenes) for further analysis (Fig. 4D).

Functional enrichment analysis

These analyses included BP, CC, MF and biological pathways. The four KeyGenes were used for the enrichment analysis. The results showed that the four KeyGenes were mainly enriched in BP such as cellular response to oxidative stress, NADH metabolic process, cellular response to chemical stress, regulation of protein catabolic process and response to oxidative stress (Fig. 5).

PPI Network

PPI analysis of the four key genes (KeyGenes) was conducted using STRING with a low confidence score (0.150) and a PPI network was built (Fig. 6A). Similar genes were predicted via GeneMANIA and their interaction network showing physical interactions and shared domains was visualized (Fig. 6B).

Differential expression analysis, correlation analysis and ROC curve analysis

The results showed that the expression of RELA, PML, VCP and NQO1 was highly statistically significant ($p < 0.05$) (Fig. 7A). The ROC curve (Fig. 7B-E) showed that the expression values in RELA, PML, VCP and NQO1 had a low accuracy in the diagnosis of the Control group and the TBI group ($0.5 < AUC < 0.7$). The results showed that RELA was positively correlated with PML and PML was positively correlated with NQO1. The Key Genes PML and VCP, RELA and VCP were negatively correlated. We calculated GO terms, sets of GO terms through R package GOSemSim, semantic similarity between gene products and gene clusters (Fig. 7F). Then, the results of the functional similarity analysis between the Key Genes are visualized by boxplot (Fig. 7G).

Immune infiltration analysis

A bar chart showed their proportions in TBI samples (Fig. 8A). A correlation heatmap (Fig. 8B) revealed strong correlations among most immune cells, with NK cells, both resting and activated, showing the strongest negative correlation ($r = -0.563$, $p < 0.05$). A correlation bubble plot (Fig. 8C) showed that RELA had the strongest negative correlation with T cells gamma delta ($r = -0.42$, $p < 0.05$) among key genes and immune cells.

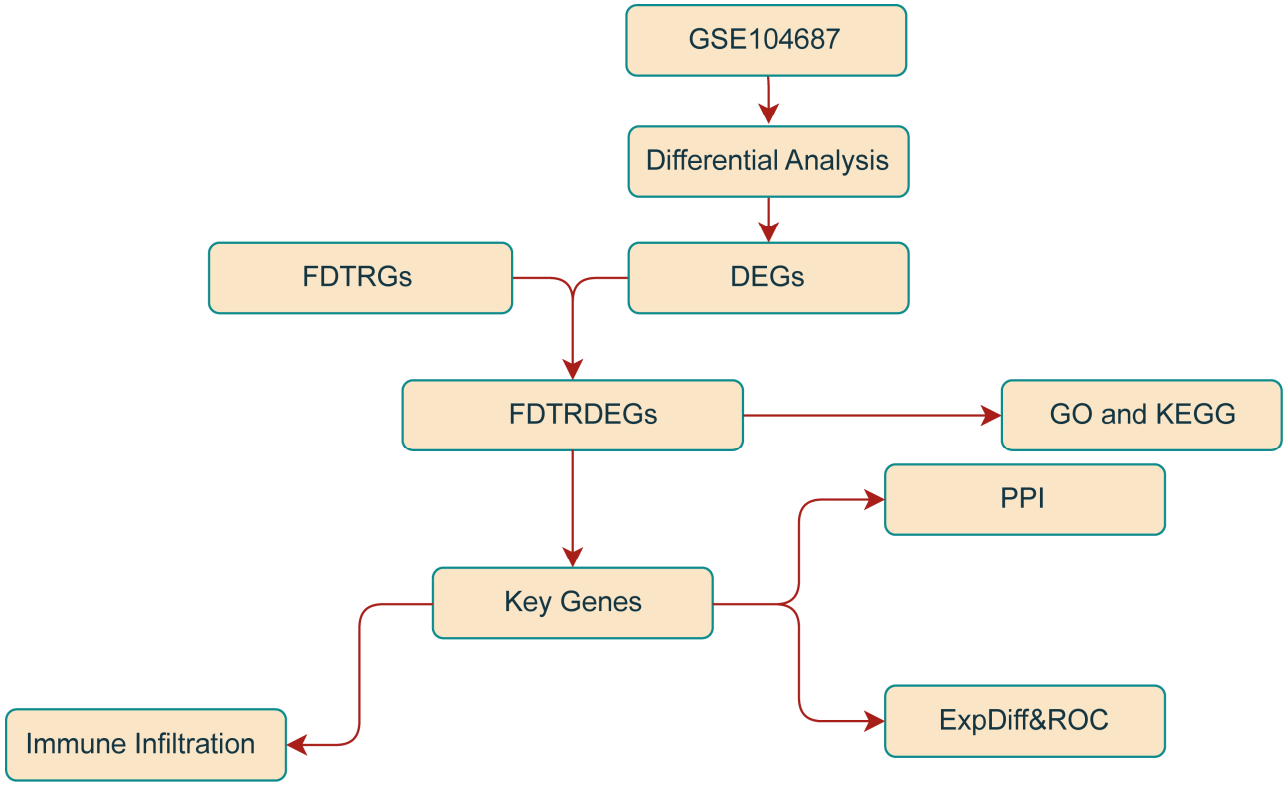

Fig. 1: Technology roadmap of the study.

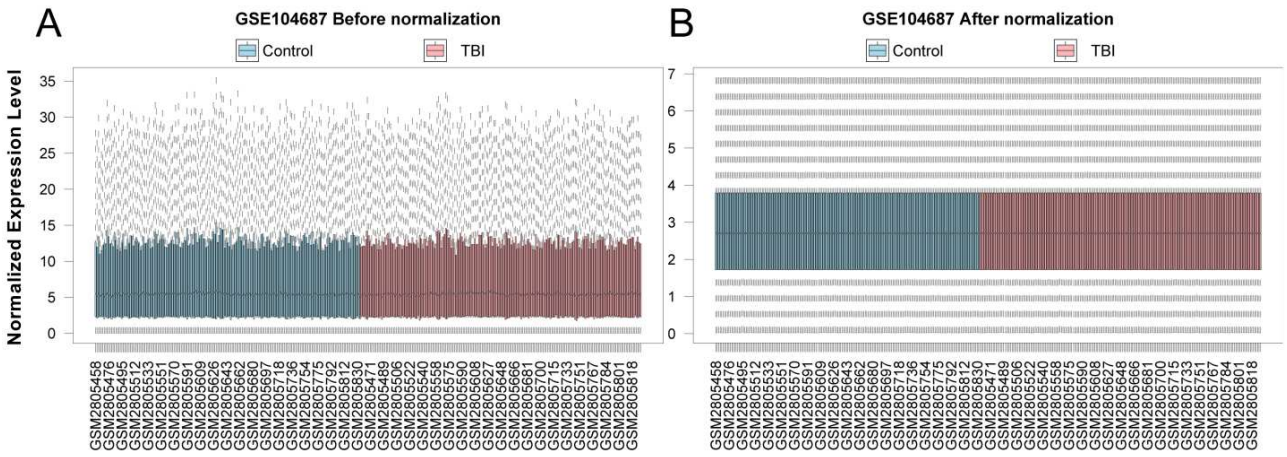

Fig. 2: Batch effects removal of dataset.
(A) Box plot of gene expression distribution between GSE104687 samples before correction. (B) Box plot of gene expression distribution among corrected GSE104687 samples. Blue represents the Control group in the dataset and pink represents the TBI group in the dataset.

Puerarin improves neurological and molecular changes in TBI model

The TBI mouse model was successfully established. Macroscopic observation of brain tissue revealed that, compared with the TBI group, the TBI + *Puerarin* group exhibited a smaller area of brain injury and a milder local inflammatory response (Fig. 9A), indicating a partial amelioration of tissue damage. Following treatment, the TBI + *Puerarin* group showed reduced neurological deficit score (Fig. 9B) and decreased brain water content (Fig. 9C). mRNA expression levels were quantified as fold changes relative to sham group. NQO1 and VCP were downregulated, while RELA and PML were upregulated in the TBI group. These molecular changes were reversed by *Puerarin* treatment (Figs. 9D-F).

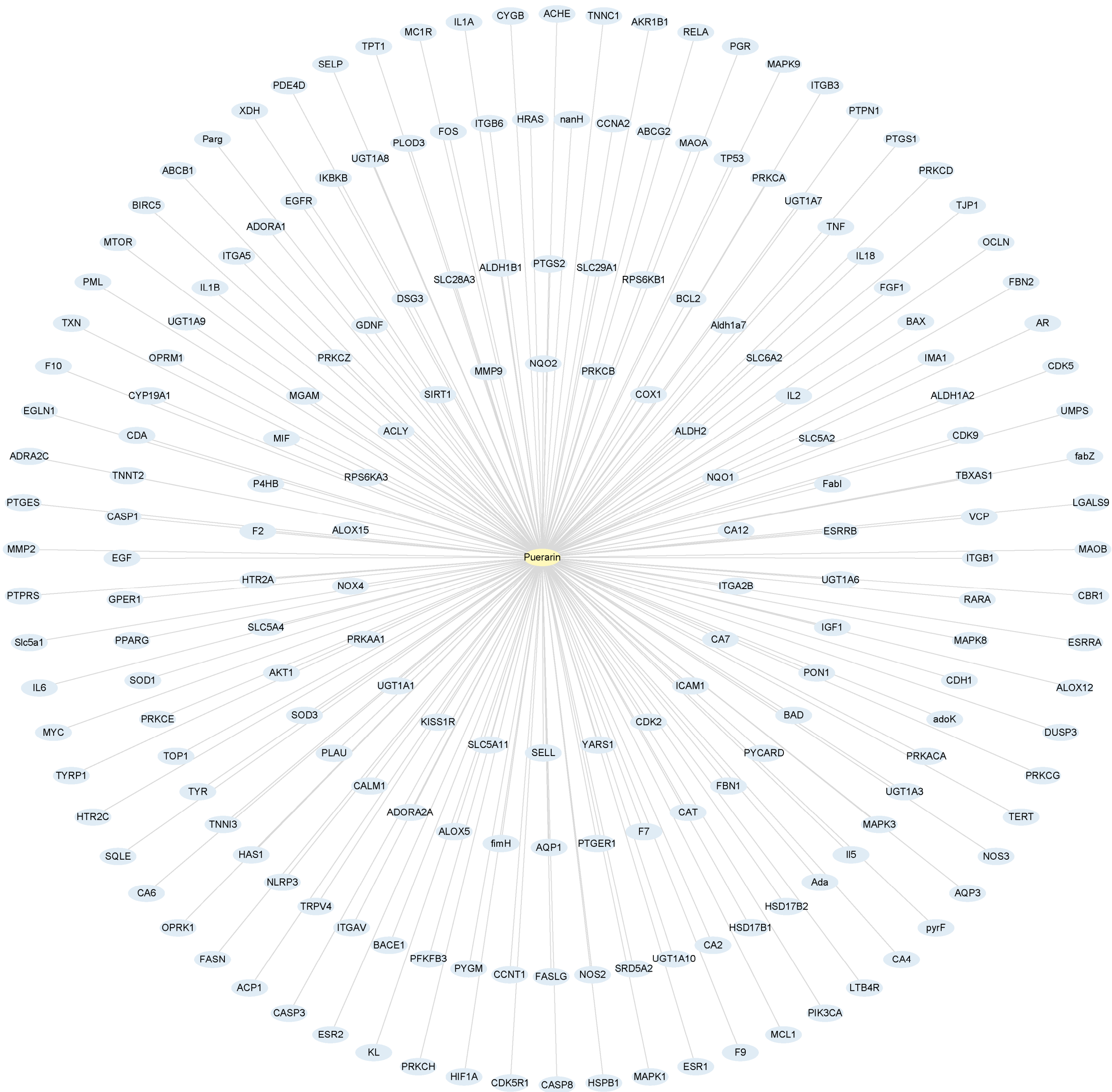

Fig. 3: Drug–TBI target interaction network.
Yellow nodes represent drug names. Blue nodes indicate target genes related to the medicinal herbs. Nodes with more connections have higher degree values, suggesting greater significance in the network. Top-ranked bioactive compounds may be key nodes in the network and play important roles in treating traumatic brain injury.

DISCUSSION

With the accelerated modernization of traditional Chinese medicine, screening pharmacologically active, clinically valuable components from traditional Chinese medicinal herbs has become a research hotspot. Puerarin demonstrates neuroprotective effects in preclinical studies, suggesting clinical potential for TBI treatment (Huang *et al.*, 2022; Liu *et al.*, 2023). Elucidating *Puerarin*'s molecular mechanisms in ameliorating TBI is crucial for clinical translation. The molecular mechanisms of *Puerarin* in treating TBI from the perspective of ferroptosis were investigated. Utilizing bioinformatics analysis, potential molecular targets were efficiently identified and an in-depth functional enrichment analysis was conducted.

Our screening criteria for ferroptosis related genes referred to the identification process of lipid oxidation related genes established by Xing *et al. (*2024*).* In this study, a total of 1,513 differentially expressed genes (713 upregulated and 800 downregulated) were identified from the GSE104687 dataset.

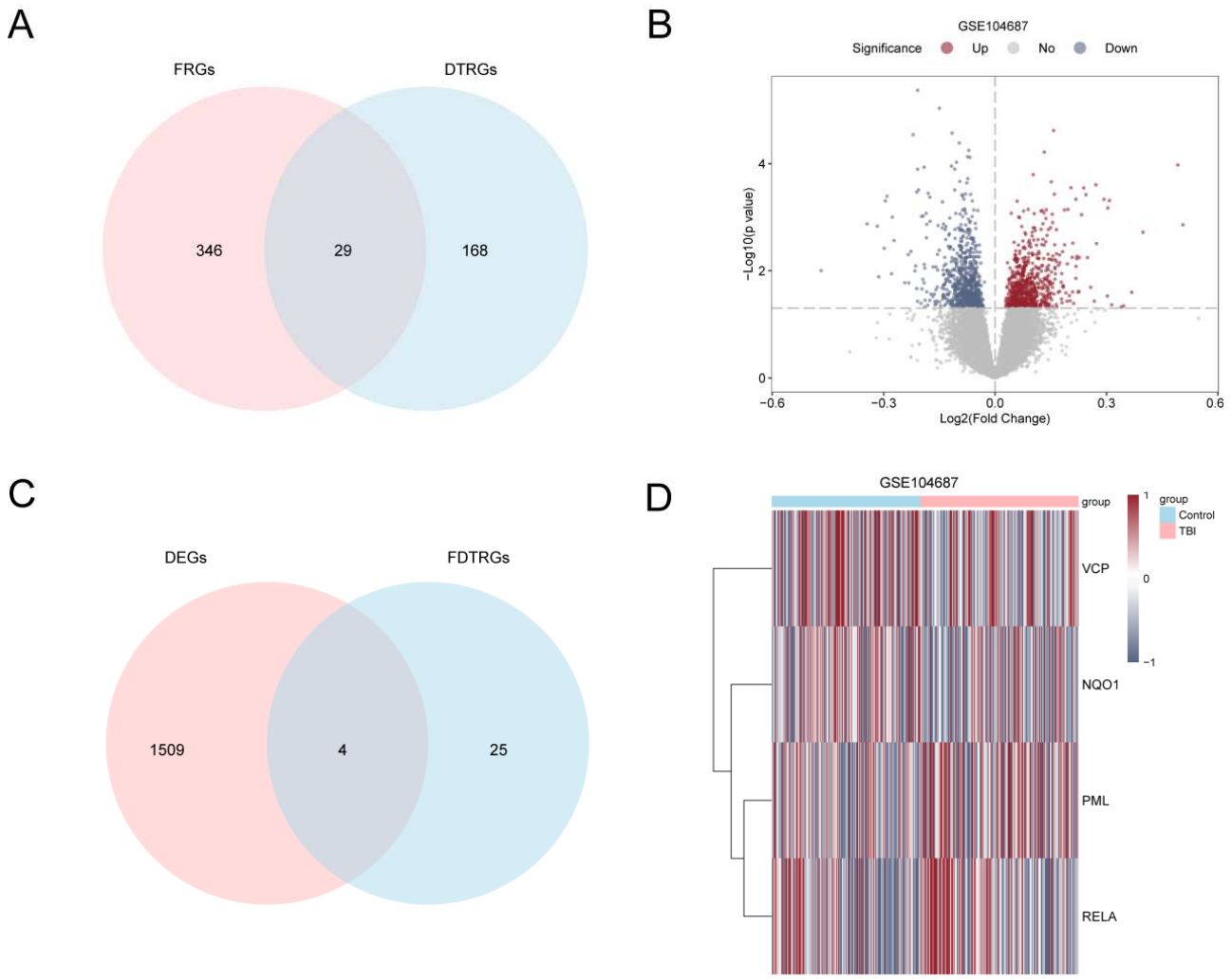

Fig. 4: Differential gene expression analysis.
(A) Venn diagram of ferroptosis-related genes (FRGs) and drug target–related genes (DTRGs); (B) Volcano plot of differentially expressed genes between the Traumatic Brain Injury (TBI) and Control groups in the combined GEO datasets; (C) Venn diagram of differentially expressed genes (DEGs) and ferroptosis- and drug target–related genes (FDTRGs) in the combined GEO datasets; (D) Heatmap of key genes (KeyGenes) in the combined GEO datasets. Pink represents TBI group and blue represents the Control group.

Through multiple bioinformatics analyses, four ferroptosis-related differentially expressed genes (FDTRDEGs) were finally identified: NQO1, VCP, PML and RELA. Our PPI network and enrichment analysis revealed that these four key targets constitute a core module that regulates neuronal ferroptosis. NQO1 is involved in various important intracellular biochemical reactions, including the cyclic metabolism of vitamin K, antioxidant responses and resistance to oxidative stress. Among these, its antioxidant function is one of the most critical (Preethi *et al.*, 2022). VCP is crucial for maintaining cellular and organ homeostasis, particularly in the nervous system (Ahlstedt *et al.*, 2022). VCP cooperates with cofactors to regulate proteostasis and clear protein aggregates and damaged organelles (Chu *et al.*, 2023). Mutations in VCP are associated with neurodegeneration and other diseases, highlighting its importance for brain function (Alshaikh *et al.*, 2024). PML, as a unique subnuclear structure, can recruit a variety of proteins and participate in numerous cellular functions through mechanisms such as transcriptional regulation and post-translational modification (Vogiatzoglou *et al.*, 2022). These functions include the regulation of apoptosis, angiogenesis, cell migration, adhesion and proliferation, DNA damage repair and the differentiation of hematopoietic cells. RELA is a key component of the NF-κB signaling pathway, which is activated in response to a variety of cellular stressors (e.g., oxidative stress, mechanical injury, etc.).

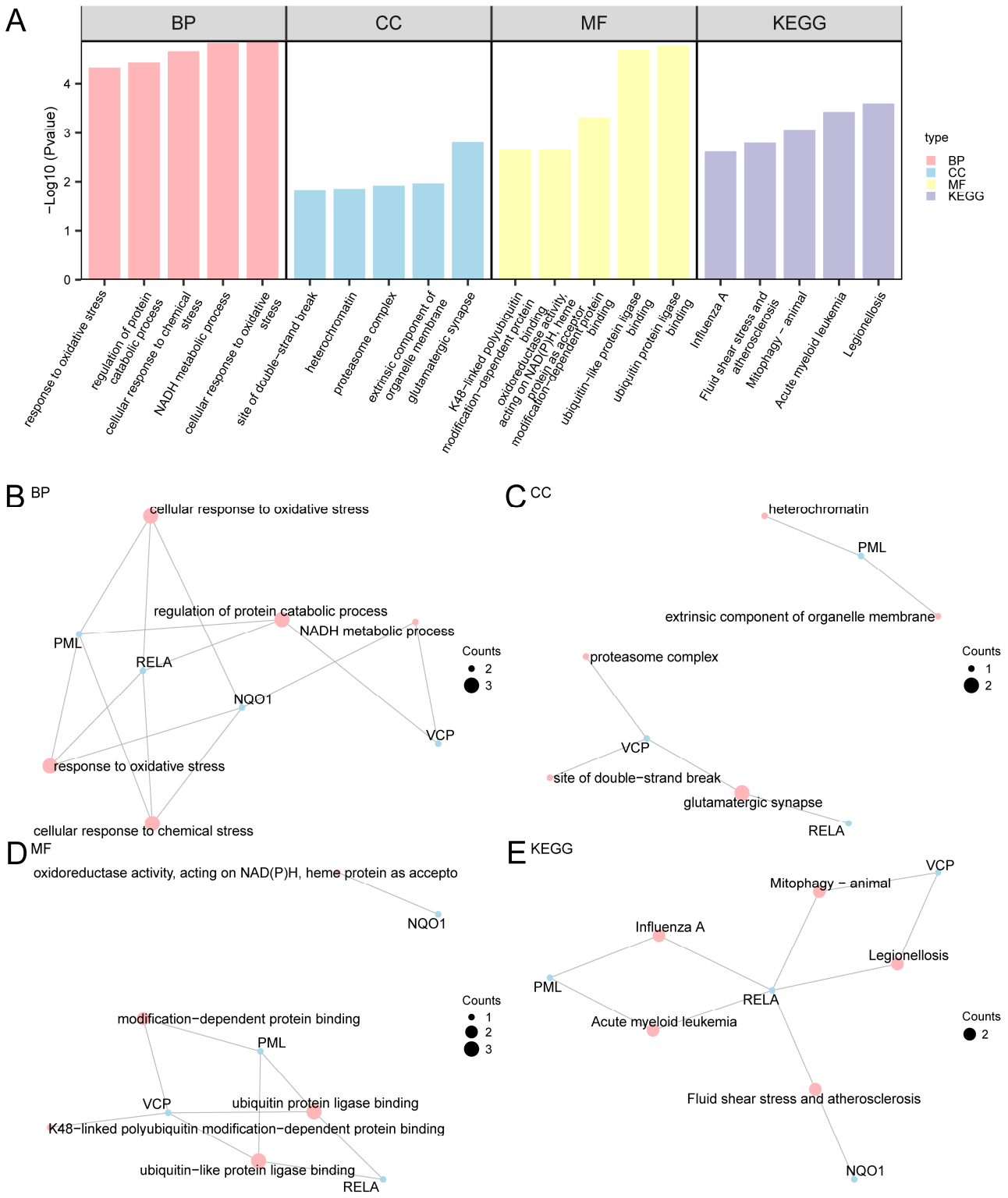

Fig. 5: GO and KEGG enrichment analysis.
(A) Bar chart showing the results of Gene Ontology (GO) and KEGG pathway enrichment analysis of the key genes (KeyGenes), including Biological Process (BP), Cellular Component (CC), Molecular Function (MF) and KEGG pathways. The x-axis represents GO terms and KEGG terms (B–E) Network diagrams showing the GO and KEGG enrichment results of the key genes (KeyGenes): BP; (B) CC; (C) MF; (D) and KEGG; (E) Pink nodes represent terms, blue nodes represent genes and edges indicate the relationships between terms and genes.

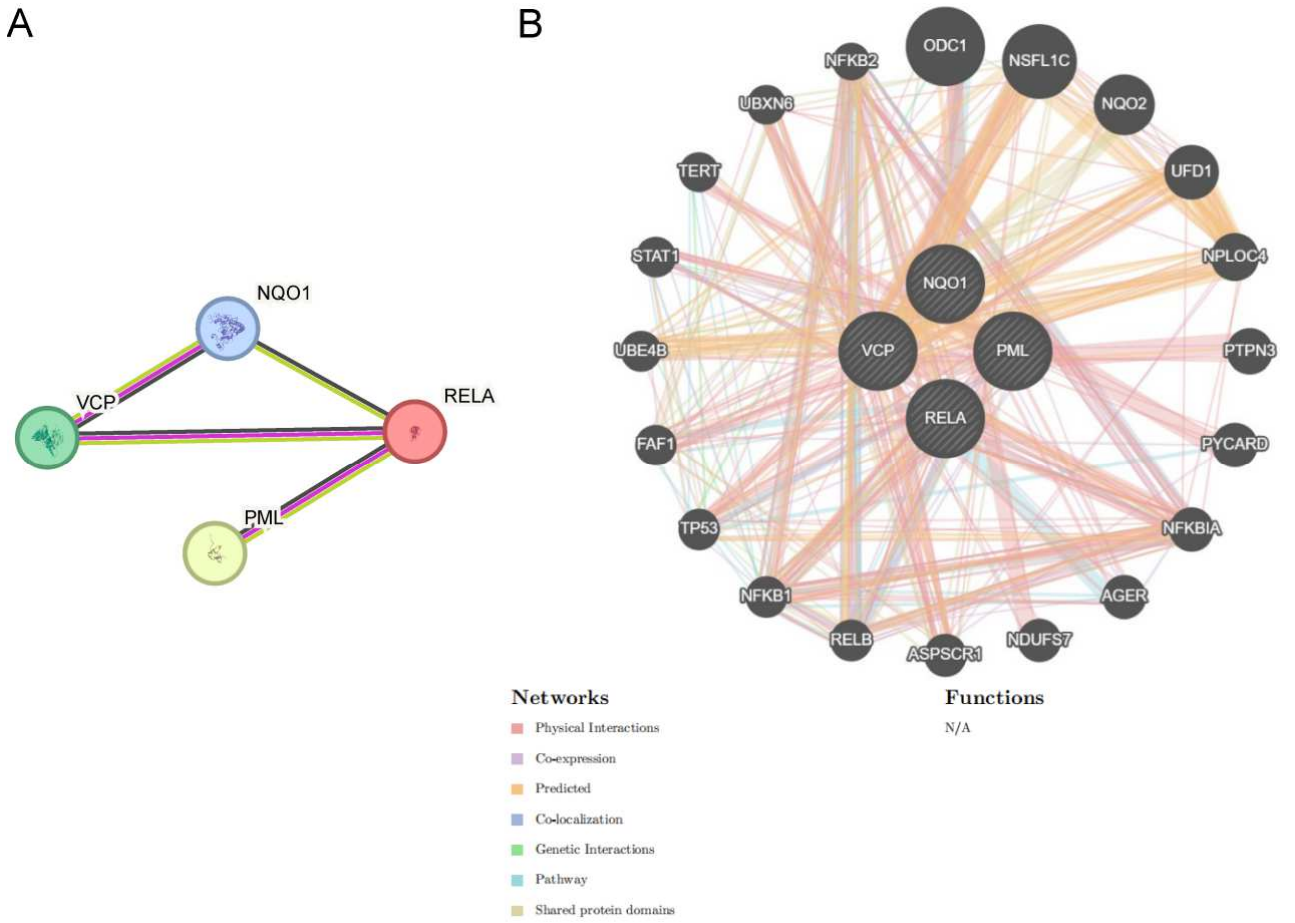

Fig. 6: Protein-protein interaction network.
(A) PPI network of the KeyGenes; (B) An interaction network of functionally similar genes was predicted based on the KeyGenes. Circular nodes represent genes, and their sizes reflect the attributes and characteristics of the genes. Lines represent relationships between genes, such as interactions or functional associations, with line thickness indicating the strength of the association or the importance of the interaction.

These four key ferroptosis-related genes are closely associated with TBI progression and represent major targets of *Puerarin* in mitigating TBI (Mo *et al.*, 2022; Wang *et al.*, 2022; Deng *et al.*, 2023). This multi-target synergistic effect is consistent with the mechanism by which flavonoids regulate oxidative damage through the p53 pathway (Wu *et al*., 2024). We hypothesized that *Puerarin* may exert its protective effects on TBI by modulating multiple ferroptosis-related targets. Furthermore, KEGG analysis indicated that the top five enriched pathways were Legionellosis, Acute myeloid leukemia, Mitophagy—animal, Fluid shear stress and atherosclerosis and Influenza A. Taken together, the potential neuroprotective mechanisms of *Puerarin* against TBI are closely linked to these ferroptosis-related targets and signaling pathways. Liang *et al.* found that RELA plays a key role in the reconstruction of neural connections between the prefronto-amygdala after trauma (Liang *et al.*, 2024), which provides a new explanation for *Puerarin* to improve cognitive impairment after TBI.

The multi-omics study further confirmed that reprogramming of lipid metabolism is a core feature of ferroptosis (Jiang *et al.*, 2025), which was confirmed by our KEGG enrichment results (atherosclerosis, influenza and other lipid-related pathways). After TBI, immune cell infiltration is a key pathological process (Al-Khateeb *et al.*, 2024). Microglia and monocytes respond rapidly, with monocytes differentiating into macrophages that participate in the inflammatory response (Cáceres *et al.*, 2024). For example, T cells, including CD4+ and CD8+ subsets, infiltrate the injured tissue and exert either pro-inflammatory or anti-inflammatory effects (Xu *et al.*, 2021). The temporal dynamics of immune cell infiltration are distinct; for example, T cell numbers initially increase within a few days after injury and then decline (Wu J. *et al.*, 2024). Collectively, these immune cells shape the post-injury inflammatory response and tissue repair. In this study, analysis of the GSE104687 dataset revealed that 22 types of immune cells were enriched in TBI, consistent with previous findings (Wang *et al.*, 2014).

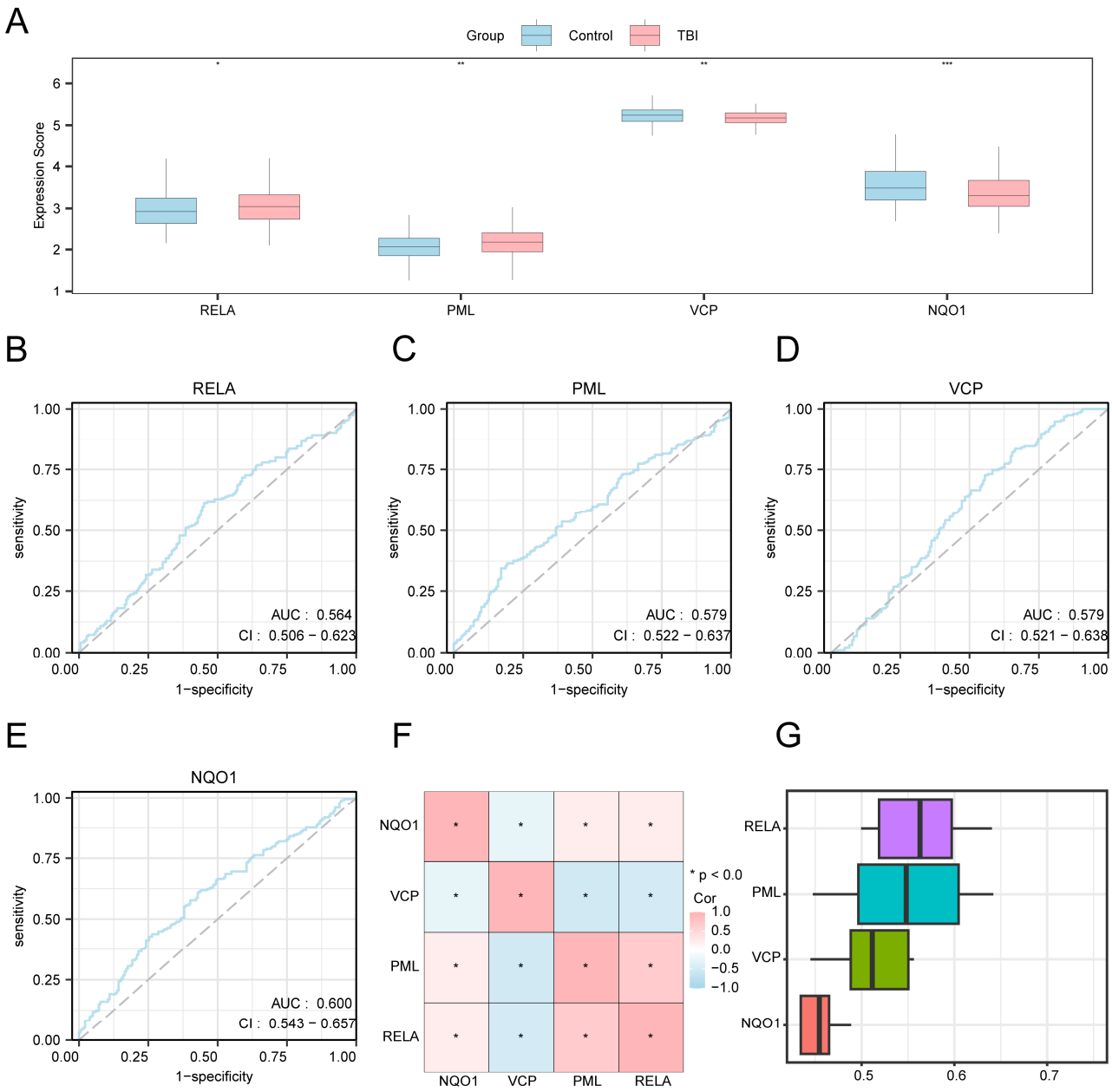

Fig. 7: Differential expression and ROC curve.
(A) Group comparison diagram of Key Genes in the traumatic brain injury (TBI) group and the Control group of dataset GSE104687. B-e. ROC curves of Key Genes RELA; (B) PML; (C) VCP; (D) and NQO1; (E) in dataset GSE104687; (F) Correlation analysis among Key Genes; (G) Functional similarity analysis of Key Genes. The symbol * is equivalent to $P < 0.05$ and has a certain statistical significance. ** represents a p-value < 0.01 and is highly statistically significant; *** represents p value < 0.001, highly statistically significant.

Moreover, we found that the four key ferroptosis-related genes exhibited strong correlations with most immune cell types. Notably, resting NK cells and activated NK cells showed the most significant and strongest negative correlations, whereas RELA was the opposite for γδT cells. The immunological consequences of ferroptosis in TBI are underscored by its role in releasing damage-associated molecular patterns (DAMPs), which activate microglia and recruit T cells (Li *et al.*, 2024). Our data align with studies showing that ferroptosis inhibitors suppress pro-inflammatory cytokine release, highlighting crosstalk between ferroptotic death and immune dysregulation.

Finally, we preliminarily validated the protective effects of *Puerarin* in a mouse TBI model. Dose selection was based on the PK-PD model proposed, which confirmed that *Puerarin* exposure in brain tissue was dose-dependent with NQO1/RELA regulation (Sun J. *et al.*, 2025).

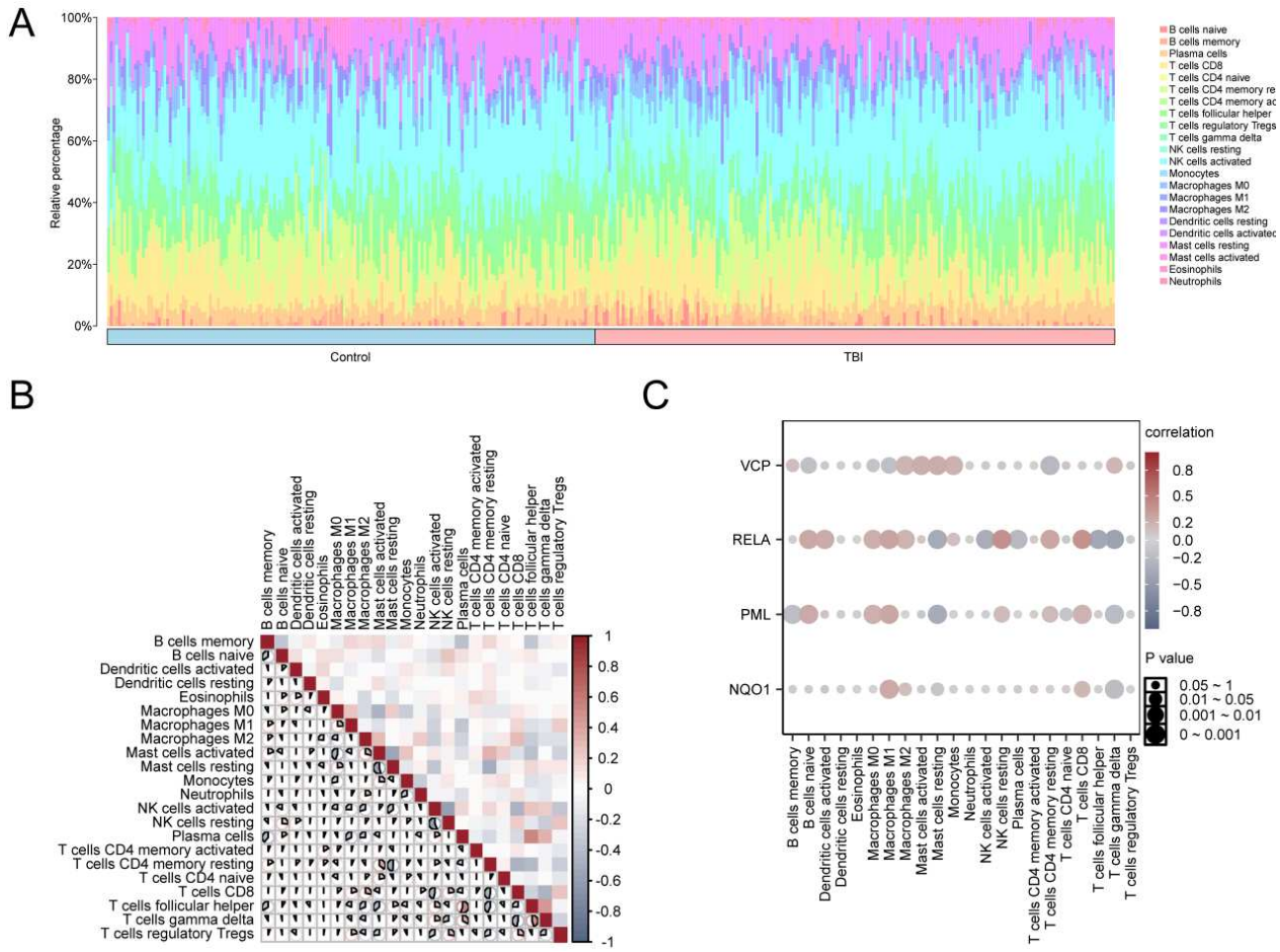

Fig. 8: Combined datasets immune infiltration analysis by CIBERSORT algorithm.
(A) Bar chart showing the proportions of immune cells in dataset GSE104687. (B) Correlation heatmap of immune cells in dataset GSE104687. (C) Bubble chart showing the correlation between immune cell infiltration levels and KeyGenes in dataset GSE104687. r value: <0.3, weak or no correlation; 0.3-0.5, a weak correlation; 0.5-0.8, a moderate correlation; ≥ 0.8, a strong correlation. Blue represents the control group and pink represents the TBI group. Red indicates a positive correlation, green indicates a negative correlation and the color intensity reflects the strength of the correlation.

The results indicated that *Puerarin*-treated TBI mice exhibited improvements in brain tissue appearance, brain water content and neurological deficit scores, suggesting amelioration of injury.

These findings are consistent with previous studies (Wang it is, 2014; Wang it is, 2022), further supporting the therapeutic potential of *Puerarin* in the treatment of TBI. *Puerarin*'s bioavailability and brain penetration support its therapeutic relevance. Studies indicate that *Puerarin* crosses the blood-brain barrier via glucose transporters, achieving peak brain concentrations within 1–2 hours post-administration, with a half-life sufficient to sustain neuroprotective effects (Long *et al.*, 2024). Through multiple bioinformatics analyses, four FDTRDEGs were identified: NQO1, VCP, PML and RELA. In the TBI group, NQO1 and VCP were down-regulated, while RELA and PML were up-regulated compared with the sham group. After treatment with *Puerarin*, the abnormal expression of these four genes was reversed.

Taken together, we suggest that the neuroprotective effect of puerperin may be achieved through multiple mechanisms: Direct ferroptosis inhibition: stabilization of REDOX balance via the NQO1-VCP axis; (2) NF-κB-ferroptosis interaction: RELA promoted inflammation and amplified ferroptosis and puerarin might break this positive feedback; (3) Indirect immune regulation: it can reduce TNFα/IL-6 and other pro-inflammatory factors and indirectly alleviate ferroptosis stress.

This multi-target property is consistent with the action characteristics of natural products, but the contribution of each mechanism needs to be verified through conditional gene-knockout experiments. However, this study has many limitations.

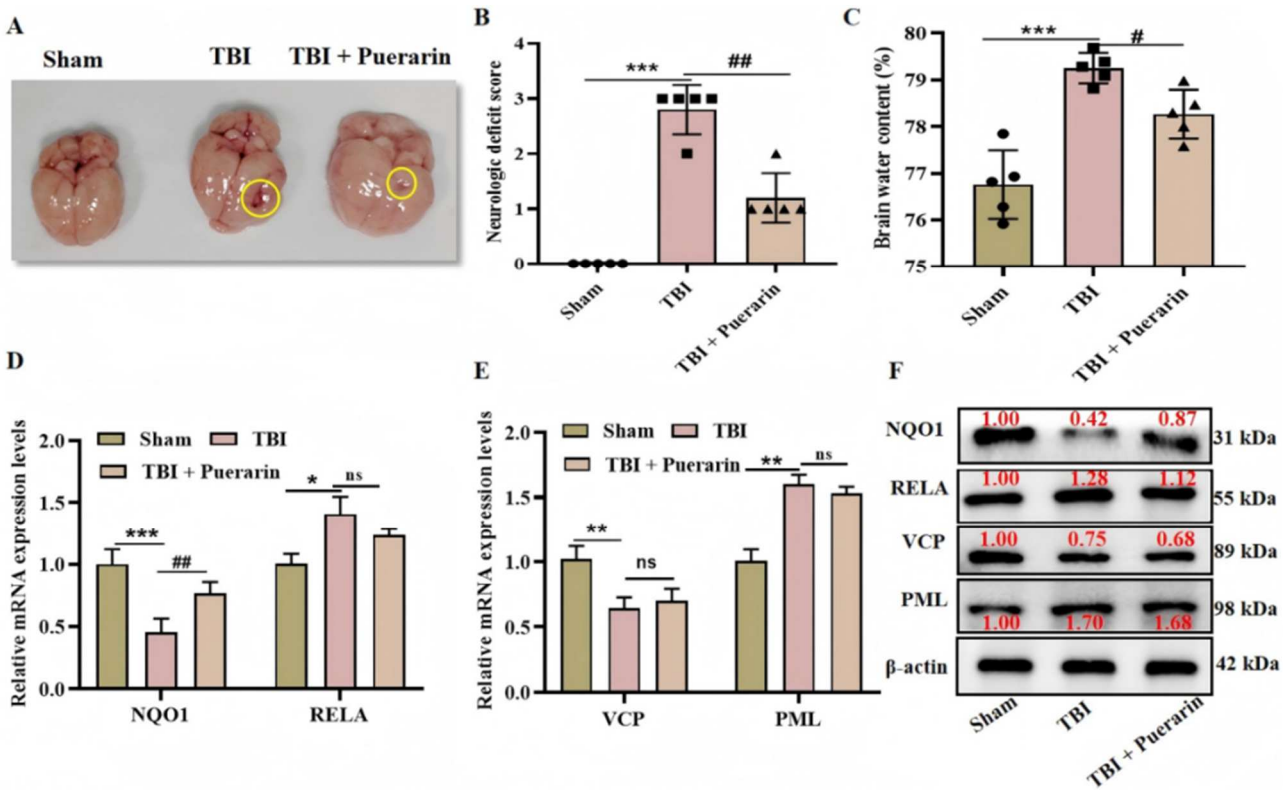

Fig. 9: Effect of *Puerarin* on brain injury and molecular changes in TBI model.
(A) Macroscopic appearance of brain tissue; (B) Neurologic deficit score; (C) Brain water content; (D) Relative mRNA expression levels of NQO1 and RELA; (E) Relative mRNA expression levels of VCP and PML; (F) Protein expression levels of NQO1, RELA, VCP and PML.

First, this study did not directly examine core ferroptosis markers such as GPX4 and ACSL4, so it is not possible to fully differentiate whether *Puerarin* acts as a specific inhibitor of ferroptosis or a generalized antioxidant. Future experiments need to simultaneously detect the GSH/GSSG ratio, MDA content and mitochondrial morphology. Second, the changes in the proportions of immune cells analyzed by CIBERSORT have not been verified experimentally using methods such as flow cytometry, which limits the reliability of the conclusions. Future studies should combine immunohistochemistry and cell-counting technologies for cross-platform validation. Third, our animal experiments were relatively simple and preliminary; further in-depth studies are required to elucidate the pharmacological mechanisms of *Puerarin*. Furthermore, our bioinformatics analysis relied on public datasets (e.g., GSE104687) that may underrepresent non-Asian populations and the animal model validation was conducted in a single species and sex. Although no Chinese-language RCTs were included in this study (as it is not a meta-analysis), future work should incorporate multi-ethnic clinical cohorts to minimize potential publication bias and enhance generalizability.

CONCLUSION

Through the combination of bioinformatics and experimental verification, this study showed that *Puerarin* may inhibit ferroptosis by regulating NQO1, VCP, PML, RELA and other targets, accompanied by changes in immune cell infiltration patterns. However, ferroptosis inhibition may only be part of its neuroprotective mechanism. Future studies should integrate PK-PD modeling, neuroimaging biomarkers and conditional gene knockout models to accurately analyze the contribution of each mechanism.

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Authors' contributions
Tiezhu Guo: Designed the research study; Zhiqiang Zhao: Performed the experiments, organized the database and drafted the manuscript; Fan Li and Zhenfen Cui: Provided help and advice on data collection and statistical analysis. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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Data availability statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval
All animal experiments were conducted in strict accordance with the guidelines for the care and use of laboratory animals. The experimental protocol was reviewed and approved by the Ethics Committee of Heji Hospital (DW2022055), affiliated with Changzhi Medical College. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest
The author(s) declare(s) no conflict of interest.

Supplementary data
https://www.pjps.pk/uploads/2026/05/SUP1779367326.pdf

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