Identification and functional analysis of differentially expressed genes associated with cerebral ischemia/reperfusion injury through bioinformatics methods

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Abstract. Cerebral ischemia/reperfusion (I/R) injury results in detrimental complications. However, little is known about the underlying molecular mechanisms involved in the reperfusion stage. The aim of the present study was to identify a gene expression profile associated with cerebral ischemia/reperfusion injury. The GSE23160 dataset, which comprised data from sham control samples and post-I/R injury brain tissues that were obtained using a middle cerebral artery occlusion (MCAO) model at 2, 8 and 24 h post-reperfusion, was downloaded from the Gene Expression Omnibus database. The differentially expressed genes (DEGs) in the MCAO samples compared with controls were screened using the GEO2R web tool. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for DEGs was performed using the online tool DAVID. Furthermore, a protein-protein interaction (PPI) network was constructed using the STRING database and Cytoscape software. In total, 32 DEGs at 2 h post-reperfusion, 39 DEGs at 8 h post-reperfusion and 91 DEGs at 24 h post-reperfusion were identified, while 15 DEGs were common among all three groups. GO analysis revealed that the DEGs at all three time-points were enriched in ‘chemotaxis’ and ‘inflammatory response’ terms, while KEGG pathway analysis demonstrated that DEGs were significantly enriched in the ‘chemokine signaling pathway’. Furthermore, following PPI network construction, Cxcl1 was identified as the only hub gene that was common among all three time-points. In conclusion, the present study has demonstrated a global view of the potential molecular differences following cerebral I/R injury and may contribute to an improved understanding of the reperfusion stage, which may ultimately aid in the development of future clinical strategies.

Introduction

Globally, stroke is the second most frequent cause of mortality and the primary cause of serious long-term disability worldwide (1). Of all strokes, 87% are ischemic (2). Various mechanisms underlying ischemic stroke are driven by cell-cell interactions within brain, including excitotoxicity, calcium dysregulation, oxidative and nitrosative Stress, cortical spreading depolarizations, inflammation, necrosis, necroptosis and autophagy (3). In addition to a narrow therapeutic time window (4), ischemic stroke remains difficult to manage.

Although reperfusion has been proven to be beneficial for ischemic stroke (5), reperfusion may result in detrimental secondary damage, which is termed ischemia/reperfusion (I/R) injury. Early reperfusion of ischemic brain tissue has been associated with various negative consequences, including blood-brain barrier breakdown, which may result in cerebral edema and/or brain hemorrhage, neurovascular damage and neuronal death (6). Angiogenesis and vasculogenesis have also been detected following reperfusion (7). In addition, inflammation is induced by reperfusion injury and contributes negatively to long-term disease prognosis (8). The inflammatory response may result in subsequent oxidative injury, excitotoxicity and neuronal cell death (9). Chemokines, produced by resident microglial cells and other immune cells in the brain, contribute to the recruitment of circulating leukocytes and exaggerate the inflammatory response. Chemokines have been demonstrated to have both deleterious and beneficial roles in ischemia/reperfusion injury (10).

Microarray analysis has been previously employed to identify molecular variations in cerebral I/R injury (11,12). However, gene expression profiles at different reperfusion periods have not been investigated extensively. Therefore, the present study employed a microarray dataset from the Gene Expression Omnibus (GEO) database and screened for differentially expressed genes (DEGs) between control samples and...
cerebral I/R samples at 2, 8 and 24 h post-reperfusion, and subsequently analyzed the functions and interactions of these DEGs. The results of the current study may aid in improving the understanding of the molecular mechanisms underlying cerebral I/R injury.

Materials and methods

Microarray data. Microarray gene expression profiles from GSE23160 (12) were obtained from the GEO database (http://www.ncbi.nlm.nih.gov/geo/), which is based on the platform of GPL6885 using Illumina MousRef-8 v2.0 Expression BeadChip (Illumina, Inc., San Diego, CA, USA). All of the samples were taken from male C57BL/6J mice (8-10 weeks). Following 2 h suture-induced middle cerebral artery occlusion (MCAO), the animals underwent reperfusion for 2, 8 or 24 h. Tissue extractions at 2, 8 and 24 h post-reperfusion and sham controls (n=4 per group) were included in this dataset.

Identification of DEGs. GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/), an R-based web application (13), was employed to analyze DEGs between MCAO samples and sham samples. P<0.05 and |logFC|≥1.2 were set as the threshold criteria to identify genes that were differentially expressed in MCAO models. Subsequently, the DEGs at 2, 8 and 24 h post-reperfusion were screened for subsequent analyses. A Venn diagram was produced to indicate the intersection among DEGs in the various MCAO groups using FunRich software (version 2.1.1; www.funrich.org) (14).

Functional enrichment analysis of DEGs. To identify the biological processes, cellular components, molecular functions and biological pathways that the DEGs were significantly enriched in, Gene Ontology (GO) enrichment (15) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (16) were performed using the online tool Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/). P<0.05 was considered to indicate a significantly enriched term or pathway, which was calculated using a hypergeometric test. Heat map illustration of DEGs was performed with heat map illustrator software (version 1.0.3.7; http://hemi.biocuckoo.org) (17).

Construction of the PPI network. To further investigate the underlying molecular mechanisms of cerebral I/R injury, protein-protein interaction (PPI) networks for the DEGs were constructed using the Search Tool for the Retrieval of Interacting Genes (STRING) database (http://www.string-db.org/) (18). A combined score of >0.4 was selected to construct the PPI networks. The obtained PPI networks at 24 h post-reperfusion were subsequently visualized using Cytoscape software (version 3.5.1) (19). Finally, the topological properties of the networks at 2, 8 and 24 h post-reperfusion were analyzed and the degree of each node was calculated; genes with a degree >10 were defined as hub genes.

Results

Identification of DEGs. As demonstrated in Fig. 1, 32 DEGs at 2 h post-reperfusion, 39 DEGs at 8 h post-reperfusion and 91 DEGs at 24 h post-reperfusion were identified in the MCAO samples compared with the controls. Among them, 15 DEGs were common to all three injury samples, including C-C motif chemokine ligand (CCL)7, suppressor of cytokine signaling 3, CCL4, activating transcription factor 3, lipocalin 2, hemoglobin α adult chain 1, gap junction protein β2, CD14 antigen, CCL3, heat shock protein 1A, S100 calcium-binding protein A8 (calgranulin A), C-X-C motif chemokine ligand 1 (CXCL1), epithelial membrane protein 1, tissue inhibitor of metalloproteinase 1 and zinc finger protein 36, all of which were upregulated in the MCAO samples (Table I). Heat map and PPI network analysis at 2 and 8 h post-reperfusion are not presented due to the small number of identified DEGs.

Bioinformatics analyses of DEGs. To further the understanding of the screened DEGs and determine their potential roles following I/R injury, GO functional and KEGG pathway enrichment analyses were performed. A total of 51 GO enriched terms for biological processes at 2, 8 and 24 h post-reperfusion were obtained. The 10 most enriched GO terms according to the P-value for 2, 8 and 24 h post-reperfusion groups are presented in Table II. Furthermore, ‘chemotaxis’ (GO:0006935), ‘inflammatory response’ (GO:0006954), ‘immune response’ (GO:0006955), ‘G-protein coupled receptor signaling pathway’ (GO:0007186), ‘response to toxic substance’ (GO:0009636), ‘neutrophil chemotaxis’ (GO:0030593), ‘positive regulation of tumor necrosis factor production’ (GO:0032760), ‘positive regulation of GTPase activity’ (GO:0043547), ‘lymphocyte chemotaxis’ (GO:0048247), ‘positive regulation of inflammatory response’ (GO:0050729), ‘cell chemotaxis’ (GO:0060326), ‘chemokine-mediated signaling pathway’ (GO:0070098), ‘positive regulation of ERK1 and ERK2 cascade’ (GO:0070374), ‘cellular response to interferon-gamma’ (GO:0071346), ‘cellular response to interleukin-1’ (GO:0071347), ‘monocyte chemotaxis’ (GO:0002547) and ‘cellular response to tumor necrosis factor’ (GO:0071356) were significantly enriched at all three post-reperfusion time-points (2, 8 and 24 h).

Additionally, DEGs were enriched in various GO cellular component terms; at 2 and 8 h post-reperfusion, DEGs were enriched in ‘extracellular region’ (GO:0005576), while...
Table I. DEGs between cerebral I/R and sham control samples that were common among 2, 8 and 24 h post-reperfusion time-points.

| Gene symbol | Gene name                  | Post-reperfusion time-point | 2 h     | 8 h     | 24 h    |
|-------------|----------------------------|----------------------------|---------|---------|---------|
|             |                            | P-value | Log FC  | P-value | Log FC  | P-value | Log FC  |
|             |                            |         |         |         |         |         |         |
| ATF3        | Activating transcription factor 3 | 5.01x10^-6 | 2.062964 | 3.56x10^-5 | 1.692171 | 7.49x10^-3 | 1.591308 |
| CCL3        | C-C motif chemokine ligand 3 | 5.13x10^-10 | 3.259239 | 7.05x10^-6 | 3.291652 | 4.81x10^-4 | 2.300334 |
| CCL4        | C-C motif chemokine ligand 4 | 1.32x10^-10 | 4.429954 | 1.29x10^-4 | 4.74245 | 5.83x10^-3 | 3.679271 |
| CCL7        | C-C motif chemokine ligand 7 | 2.18x10^-3 | 1.220192 | 7.63x10^-3 | 1.426973 | 4.00x10^-3 | 1.707457 |
| CD14        | CD14 antigen               | 1.38x10^-7 | 1.757282 | 5.92x10^-7 | 2.578573 | 2.56x10^-3 | 2.353608 |
| CXCL1       | C-X-C motif chemokine ligand 1 | 3.29x10^-6 | 1.806643 | 4.81x10^-6 | 2.878375 | 1.43x10^-3 | 2.596958 |
| EMP1        | Epithelial membrane protein 1 | 1.68x10^-6 | 1.688657 | 6.78x10^-7 | 1.386369 | 1.89x10^-3 | 2.161470 |
| GJB2        | Gap junction protein β2    | 3.16x10^-8 | 1.999569 | 1.15x10^-7 | 1.717101 | 9.13x10^-5 | 1.822626 |
| HBA-A1      | Hemoglobin α, adult chain 1 | 5.38x10^-7 | 2.436023 | 2.15x10^-4 | 3.757753 | 4.84x10^-4 | 3.807134 |
| HSPA1A      | Heat shock protein 1A      | 1.69x10^-6 | 3.054788 | 3.80x10^-5 | 2.545466 | 1.59x10^-2 | 1.299110 |
| LCN2        | Lipocalin 2                | 4.06x10^-9 | 1.312245 | 4.47x10^-15 | 3.250233 | 1.72x10^-3 | 3.409733 |
| S100A8      | S100 calcium-binding protein A8 (calgranulin A) | 2.29x10^-6 | 1.240885 | 1.66x10^-6 | 1.655962 | 1.90x10^-3 | 3.780381 |
| SOCS3       | Suppressor of cytokine signaling 3 | 3.54x10^-8 | 2.009771 | 1.74x10^-8 | 2.004998 | 5.48x10^-4 | 2.361302 |
| TIMP1       | Tissue inhibitor of metalloproteinase 1 | 1.02x10^-4 | 2.376190 | 3.61x10^-18 | 3.403190 | 1.52x10^-5 | 3.874533 |
| ZFP36       | Zinc finger protein 36     | 2.01x10^-3 | 1.595428 | 6.26x10^-7 | 1.406482 | 1.17x10^-2 | 1.330419 |

Table II. Top 10 enriched GO biological process terms for DEGs between cerebral ischemia/reperfusion and sham control samples.

A, Top 10 enriched GO biological process terms for DEGs at 2 h post-reperfusion

| GO ID   | GO term                                | Count | %     | P-value | Genes                                  |
|---------|----------------------------------------|-------|-------|---------|----------------------------------------|
| GO:0030593 | Neutrophil chemotaxis                  | 5     | 15.63 | 5.65x10^-6 | CXCL1, CCL3, S100A8, CCL4, CCL7        |
| GO:0032570 | Response to progesterone               | 4     | 12.50 | 1.80x10^-5 | FOS, OXT, FOSB, GJB2                   |
| GO:0006954 | Inflammatory response                  | 7     | 21.88 | 2.24x10^-5 | CXCL1, CCL3, S100A8, CCL4, CCL7, CD14, IL1A |
| GO:0045944 | Positive regulation of transcription from RNA polymerase II promoter | 10    | 31.25 | 2.99x10^-5 | FOS, CCL3, EGR2, ATF3, EGR4, FOSB, NPSA4, JUNB, IL1A, CYR61 |
| GO:0071356 | Cellular response to tumor necrosis factor | 5     | 15.63 | 3.59x10^-3 | LCN2, ZFP36, CCL3, CCL4, CCL7         |
| GO:2000503 | Positive regulation of natural killer cell chemotaxis | 3     | 9.38  | 4.25x10^-3 | CCL3, CCL4, CCL7                      |
| GO:0006935 | Chemotaxis                             | 5     | 15.63 | 4.73x10^-5 | CCL3, S100A8, CCL4, CCL7, CYR61       |
| GO:0051951 | Response to cAMP                       | 4     | 12.50 | 8.99x10^-5 | FOS, OXT, FOSB, JUNB                  |
| GO:0070098 | Chemokine-mediated signaling pathway   | 4     | 12.50 | 1.13x10^-4 | CXCL1, CCL3, CCL4, CCL7               |
| GO:0050729 | Positive regulation of inflammatory response | 4     | 12.50 | 1.69x10^-4 | CCL3, S100A8, CCL4, CCL7              |

B, Top 10 enriched GO biological process terms for DEGs at 8 h post-reperfusion

| GO ID   | GO term                                | Count | %     | P-value | Genes                                  |
|---------|----------------------------------------|-------|-------|---------|----------------------------------------|
| GO:0030593 | Neutrophil chemotaxis                  | 10    | 25.64 | 8.69x10^-15 | CXCL1, CCL12, CCL3, S100A8, LGALS3, CCL9, CCL4, CCL7, FCGR3, CCL17 |
‘membrane’ (GO:0016020) was significantly enriched at both 8 and 24 h post-reperfusion (Table III). Furthermore, Table IV indicates that DEGs were significantly enriched in ‘cytokine activity’ (GO:0005125) and ‘chemokine activity’ (GO:0008009) GO molecular function terms at 2, 8 and 24 h post-reperfusion.

Table V presents the KEGG pathways that were significantly enriched in DEGs. KEGG analysis indicated that 'chemokine path
signaling pathway’, ‘cytokine-cytokine receptor interaction’ and ‘toll-like receptor signaling pathway’ were significantly enriched in DEGs at 2, 8 and 24 h post-reperfusion. Furthermore, as demonstrated in Fig. 2, a total of 11 chemokine signaling pathway-associated genes were overexpressed in 24 h post-reperfusion injury samples compared with the sham control samples.

**PPI network analysis.** Genes with an interaction degree >10 in the PPI network analysis of DEGs at 2, 8 and 24 h post-reperfusion were defined as hub genes, which are listed in Table VI. CXCL1 was the only gene that was considered to be a hub gene at 2, 8 and 24 h post-reperfusion (Table VI). The constructed PPI network of 24 h post-reperfusion samples is presented in

### Table III. GO cellular component terms for DEGs between cerebral ischemia/reperfusion and sham control samples.

#### A. Enriched GO cellular component terms for DEGs at 2 h post-reperfusion

| GO ID     | GO term                                      | Count | %     | P-value       | Genes                                                                 |
|-----------|----------------------------------------------|-------|--------|---------------|-----------------------------------------------------------------------|
| GO:0005576| Extracellular region                         | 14    | 43.75  | 9.56x10^-7   | CXCL1, CCL3, AVP, S100A8, PMCH, OXT, CCL4, CCL7, TIMP1, LCN2, NPTX2, IL1A, CD14, CYR61 |
| GO:0005615| Extracellular space                          | 12    | 37.50  | 1.02x10^-5   | LCN2, CXCL1, AVP, CCL3, S100A8, PMCH, OXT, CCL4, CCL7, CD14, IL1A, TIMP1 |

#### B. Enriched GO cellular component terms for DEGs at 8 h post-reperfusion

| GO ID     | GO term                                      | Count | %     | P-value       | Genes                                                                 |
|-----------|----------------------------------------------|-------|--------|---------------|-----------------------------------------------------------------------|
| GO:0005615| Extracellular space                          | 16    | 41.03  | 4.81x10^-8   | CXCL1, CCL3, FLT1, S100A8, LGALS3, PMCH, CCL9, CCL4, CCL7, TIMP1, CCL17, LCN2, CCL12, SERPINA3N, DMKN, CD14 |
| GO:0005576| Extracellular region                         | 14    | 35.90  | 1.39x10^-5   | CXCL1, CCL3, S100A8, LGALS3, PMCH, CCL9, CCL4, CCL7, TIMP1, CCL12, SERPINA3N, DMKN, CD14 |
| GO:0009897| External side of plasma membrane             | 5     | 12.82  | 3.17x10^-3   | LGALS3, OSMR, TLR2, CD14, FCRG3                                      |
| GO:0016020| Membrane                                     | 20    | 51.28  | 4.79x10^-2   | GPR84, FLT1, S100A8, LGALS3, OSMR, FKBP5, MS4A6D, TLR2, SLC10A6, GJB2, FCGR3, HBA-A1, CH25H, PLIN4, HMOX1, ITGAD, SLC15A3, GBP2, EMP1, CD14 |

#### C. Enriched GO cellular component terms for DEGs at 24 h post-reperfusion

| GO ID     | GO term                                      | Count | %     | P-value       | Genes                                                                 |
|-----------|----------------------------------------------|-------|--------|---------------|-----------------------------------------------------------------------|
| GO:0009897| External side of plasma membrane             | 8     | 8.70   | 6.31x10^-4   | FCGR2B, LGALS3, PDPN, CCL21C, FCRG4, TLR2, CD14, CXCL10              |
| GO:0048237| Rough endoplasmic reticulum lumen            | 3     | 3.26   | 4.29x10^-4   | LYZ2, LYZ1, CHIL3                                                    |
| GO:0009986| Cell surface                                 | 10    | 10.87  | 2.33x10^-3   | SLC11A1, THBD, FCRG2B, LGALS3, TNFRSF12A, IFITM3, FCRG4, TLR2, CD14, ANXA2 |
| GO:0005886| Plasma membrane                              | 34    | 36.96  | 8.00x10^-3   | GPR182, GPR84, S100A8, IFITM2, TNFRSF12A, IFITM3, VIM, S100A9, TLR2, CD52, FPR2, SLC11A1, P2RY6, DAB2, PLIN2, HMOX1, TGM2, STRA6, CLEC4D, ANGPT2, ACTB, PDPN, LILRB4A, GJB2, ANXA2, CYBA, THBD, FCRG2B, HSPB1, SCN4B, RGS9, EMP3, CD14, EMP1 |
| GO:0016020| Membrane                                     | 44    | 47.83  | 1.14x10^-2   | GPR182, GPR84, GFA, TSPO, S100A8, IFITM2, TNFRSF12A, IFITM3, S100A9, TLR2, CD52, FPR2, FXR5, GLIPR2, SLC11A1, P2RY6, DAB2, PLIN2, HMOX1, CH25H, TGM1, TGM2, STRA6, CLEC4D, ACTB, LGALS3, PDPN, MS4A6D, LILRB4A, GJB2, ANXA2, HBA-A1, CYBA, RAB32, THBD, FCRG2B, SCN4B, RGS9, EMP3, SLC15A3, GBP2, CD14, EMP1, MVP |

GO, Gene Ontology; DEGs, differentially expressed genes.
Fig. 3, which contains 67 nodes and 281 edges. Each node represents a DEG and each edge represents a PPI between two DEGs. At 24 h post-reperfusion, 23 genes served as hub genes, and of these hub genes, CCL2 exhibited the highest degree (Fig. 3).

Discussion

In the present study, 32 DEGs at 2 h, 39 DEGs at 8 h and 91 DEGs at 24 h post-reperfusion injury were identified between cerebral I/R and sham control samples. Previous studies have performed bioinformatics analysis to identify DEGs between MCAO models and controls (11,20-23). However, to the best of our knowledge, the present study is the first to perform global gene expression profiling at three time-points following reperfusion, and the findings may lead to improvements in the understanding of the pathophysiological process of cerebral I/R injury. DEGs associated with inflammation have previously been associated with cerebral I/R injury (20,22), and the results of the present study were consistent with these previous reports, indicating a persistent inflammatory response in cerebral I/R injury.

Table IV. Enriched GO molecular function terms for DEGs between cerebral ischemia/reperfusion and sham control samples.

| GO ID   | GO term                        | Count | %    | P-value      | Genes                                                                 |
|---------|--------------------------------|-------|------|--------------|----------------------------------------------------------------------|
| GO:0005125 | Cytokine activity               | 6     | 18.75| 2.48x10^{-5} | CXCL1, FOS, CCL3, CCL4, CD14, IL1A, FOS, CCL3, CCL4, CD14             |
| GO:0008009 | Chemokine activity               | 4     | 12.50| 6.79x10^{-5} | FOS, CCL3, CCL4, CD14                                               |
| GO:000978  | RNA polymerase II core promoter proximal region sequence-specific DNA binding | 6     | 18.75| 2.84x10^{-4} | FOS, EGR2, ATF3, FOSB, NPAS4, JUNB                                 |
| GO:001077  | Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding | 5     | 15.63| 9.82x10^{-4} | FOS, EGR2, FOSB, NPAS4, JUNB                                      |
| GO:003690  | Double-stranded DNA binding      | 3     | 9.38 | 2.13x10^{-2} | FOS, FOSB, JUNB                                                       |
| GO:003677  | DNA binding                      | 8     | 25.00| 2.85x10^{-2} | ZFP36, FOS, EGR2, ATF3, EGR4, FOSB, NPAS4, JUNB                     |

| GO ID   | GO term                        | Count | %    | P-value      | Genes                                                                 |
|---------|--------------------------------|-------|------|--------------|----------------------------------------------------------------------|
| GO:0005125 | Cytokine activity               | 8     | 20.51| 1.54x10^{-7} | CXCL1, CCL12, CCL3, CCL9, CCL4, CCL7, CCL17                         |
| GO:0008009 | Chemokine activity               | 7     | 17.95| 3.98x10^{-10}| CXCL1, CCL12, CCL3, CCL9, CCL4, CCL7, CCL17, TIMP1, CXCL10, IL11   |
| GO:0005125 | Cytokine activity               | 8     | 20.51| 1.54x10^{-7} | CXCL1, CCL12, CCL3, CCL9, CCL4, CCL7, CCL17                         |
| GO:0008009 | Chemokine activity               | 7     | 17.95| 3.98x10^{-10}| CXCL1, CCL12, CCL3, CCL9, CCL4, CCL7, CCL17, TIMP1, CXCL10, IL11   |

| GO ID   | GO term                        | Count | %    | P-value      | Genes                                                                 |
|---------|--------------------------------|-------|------|--------------|----------------------------------------------------------------------|
| GO:0005125 | Cytokine activity               | 11    | 11.96| 1.69x10^{-14}| CXCL1, CCL12, CCL3, CCL2, CCL21C, CCL9, CCL21A, PF4, CCL4, CCL7, CXCL10 |
| GO:0008009 | Chemokine activity               | 12    | 13.04| 4.78x10^{-9} | CXCL1, CCL12, CCL3, CCL2, CCL9, PF4, CCL4, CCL7, SPP1, TIMP1, CXCL10, IL11 |
| GO:0005125 | Cytokine activity               | 5     | 5.44 | 8.52x10^{-6} | CCL3, CCL21C, CCL9, CCL21A, CCL4                                   |
| GO:0048020 | CCR chemokine receptor binding  | 4     | 4.35 | 3.43x10^{-2} | CCL2, PF4, CCL7, CXCL10                                           |
| GO:0031727 | CCR2 chemokine receptor binding | 3     | 3.26 | 1.30x10^{-4} | CCL12, CCL2, CCL7                                               |
| GO:0008201 | Heparin binding                 | 4     | 4.35 | 4.94x10^{-2} | HBA-A1, CYBA, HMOX1, IDO1                                         |

GO, Gene Ontology; DEGs, differentially expressed genes.
Chemokines have been reported to have complex and essential roles in I/R injury, which involves extensive leukocyte and neutrophil infiltration, subsequently exaggerating the ischemic area (24). Following ischemic stroke, chemokines are primarily produced by resident microglial cells in the brain and infiltrating immune cells, which leads to further leukocyte recruitment and activation (25). In the present study, CCL2 exhibited the highest degree in the PPI network at 24 h post-reperfusion, which is consistent with previous research.

Table V. Enriched KEGG pathways for DEGs between cerebral ischemia/reperfusion and sham control samples.

| KEGG entry | Pathway name                               | Count | %     | P-value     | Genes                                      |
|------------|--------------------------------------------|-------|-------|-------------|--------------------------------------------|
| mmu05132   | Salmonella infection                       | 6     | 18.75 | 1.27x10^-6 | CXCL1, FOS, CCL3, CCL4, CD14, IL1A         |
| mmu04380   | Osteoclast differentiation                 | 5     | 15.63 | 2.68x10^-4 | FOS, SOCS3, FOSB, JUNB, IL1A               |
| mmu04620   | Toll-like receptor signaling pathway       | 4     | 12.50 | 2.11x10^-1 | FOS, CCL3, CCL4, CD14                     |
| mmu04062   | Chemokine signaling pathway                | 4     | 12.50 | 1.34x10^-2 | CXCL1, CCL3, CCL4, CCL7                   |
| mmu04060   | Cytokine-cytokine receptor interaction     | 4     | 12.50 | 2.41x10^-2 | CCL3, CCL4, CCL7, IL1A                    |
| mmu05166   | HTLV-I infection                           | 4     | 12.50 | 3.35x10^-2 | ZFP36, FOS, EGR2, ATF3                    |

| KEGG entry | Pathway name                               | Count | %     | P-value     | Genes                                      |
|------------|--------------------------------------------|-------|-------|-------------|--------------------------------------------|
| mmu04062   | Chemokine signaling pathway                | 7     | 17.95 | 2.28x10^-5 | CXCL1, CCL12, CCL3, CCL9, CCL4, CCL7, CCL17|
| mmu04060   | Cytokine-cytokine receptor interaction     | 6     | 15.38 | 8.01x10^-4 | CCL12, CCL3, FLT1, OSMR, CCL4, CCL7        |
| mmu05132   | Salmonella infection                       | 4     | 10.26 | 1.72x10^-3 | CXCL1, CCL3, CCL4, CD14                   |
| mmu04620   | Toll-like receptor signaling pathway       | 4     | 10.26 | 3.60x10^-3 | CCL3, TLR2, CCL4, CD14                    |
| mmu04145   | Phagosome                                  | 4     | 10.26 | 1.61x10^-2 | TLR2, TUBB6, CD14, FCGR3                   |
| mmu05142   | Chagas disease (American trypanosomiasis)  | 3     | 7.69  | 4.02x10^-2 | CCL12, CCL3, TLR2                         |

| KEGG entry | Pathway name                               | Count | %     | P-value     | Genes                                      |
|------------|--------------------------------------------|-------|-------|-------------|--------------------------------------------|
| mmu04062   | Chemokine signaling pathway                | 11    | 11.96 | 2.50x10^-7 | CXCL1, CCL12, CCL3, CCL2, CCL21C, CCL9, CCL21A, PF4, CCL4, CCL7, CCL10 |
| mmu04060   | Cytokine-cytokine receptor interaction     | 11    | 11.96 | 1.95x10^-6 | CCL12, CCL3, CCL2, TNRFSF12A, CCL21C, CCL21A, PF4, CCL4, CCL7, CCL10, IL11 |
| mmu05323   | Rheumatoid arthritis                       | 6     | 6.52  | 1.43x10^-4 | CCL12, CCL3, CCL2, TLR2, MMP3, IL11        |
| mmu04620   | Toll-like receptor signaling pathway       | 6     | 6.52  | 3.80x10^-4 | CCL3, TLR2, CCL4, CD14, SPP1, CCL10        |
| mmu04668   | TNF signaling pathway                      | 6     | 6.52  | 5.41x10^-4 | CXCL1, CCL12, CCL2, SOCS3, MMP3, CCL10    |
| mmu04145   | Phagosome                                  | 7     | 7.61  | 6.73x10^-4 | ACTB, CYBA, FCGR2B, FCGR4, TLR2, TUBB6, CD14|
| mmu05144   | Malaria                                    | 4     | 4.35  | 3.20x10^-3 | HBA-A1, CCL12, CCL2, TLR2                |
| mmu05164   | Influenza A                                | 6     | 6.52  | 3.99x10^-3 | ACTB, CCL12, CCL2, SOCS3, HSPA1A, CCL10   |
| mmu05142   | Chagas disease (American trypanosomiasis)  | 4     | 4.35  | 2.58x10^-2 | CCL12, CCL3, CCL2, TLR2                   |

KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes.
Figure 2. Heat map illustration of chemokine signaling pathway-associated genes in 24 h post-reperfusion and sham samples. A total of 11 chemokine signaling pathway-associated genes were included. The color code depicts the value of each gene following median normalization, with blue indicating the lowest and red as the highest value.

Figure 3. Protein-protein interaction network for DEGs between cerebral ischemia/reperfusion and sham control samples at 24 h post-reperfusion. Circles represent nodes and lines between nodes represent edges, which indicate DEGs and interactions between two DEGs, respectively. C-C motif chemokine ligand 2 was identified as the hub gene with the highest interaction degree and is indicated in yellow. DEGs, differentially expressed genes.
studies (26,27). Accordingly, CCL2 mRNA expression was initially increased at 6 h post-reperfusion, peaking 2 days later. Additionally, CCL3 was previously described to be upregulated post-I/R injury via the induction of monocyte accumulation in the ischemic brain (28,29), and the expression of CCL3 post-reperfusion has been reported to be time-dependent (30). CCL7, as a mast cell-derived product, has been reported to be involved in the recruitment of inflammatory cells into the ischemic sites (31), subsequently contributing to stroke pathology (32). CXCL1, identified as a hub gene in the PPI networks at 2, 8 and 24 h post-reperfusion in the present study, was reported to be increased in the cerebrospinal fluid of patients that have suffered from a stroke (33). However, both neurotoxic and neuroprotective effects have been demonstrated for chemokines in post-stroke inflammation (28).

It is established that inflammation is a major contributor to stroke pathophysiology, and the immune system has been implicated in all stages of the ischemic cascade, from the acute damaging events to the progression of tissue repair (34). Microglia cells, which are closely associated with inflammation, were reported to become rapidly activated following ischemia (35). Furthermore, various pro-inflammatory factors, including interleukin (IL)-1β, IL-6, tumor necrosis factor-α, reactive oxygen species, nitric oxide and prostaglandin E2, were reported to be produced by activated microglia and contribute to neuronal death in cerebral ischemia (36). In the present study, specific cytokines were not measured. Further studies are required to investigate the association between the chemokine family and pro-inflammatory cytokines, which further elucidate the pathophysiological process following cerebral I/R injury.

The results of the present study revealed that the toll-like receptor signaling pathway was significantly enriched at 2 h post-reperfusion, suggesting early transcriptional activation. TLR2, a vital factor in the inflammatory response and tissue damage, has been reported to be implicated in cerebral ischemic damage (37). Microglia cells produce cytokines and chemokines following the stimulation of TLR2 (38). Furthermore, leukocyte and microglial infiltration, and neuronal death, were reported to be attenuated by TLR2 inhibition (39), indicating a potential novel therapeutic strategy.

In conclusion, the current study identified a set of DEGs that were altered between cerebral I/R injury samples and sham control samples. The findings may provide novel insight into the potential mechanisms underlying the development of cerebral I/R injury. Our future studies will be aimed at unveiling the potential diagnostic and prognostic value of these hub genes, which may ultimately aid the translation of these targets into clinical practice.

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Table VI. Hub genes identified in PPI networks.

A, Hub genes in the PPI network at 2 h post-reperfusion

| Gene | Degree |
|------|--------|
| FOS  | 16     |
| CXCL1| 11     |
| ATF3 | 11     |

B, Hub genes in the PPI network at 8 h post-reperfusion

| Gene | Degree |
|------|--------|
| TLR2 | 14     |
| CXCL1| 13     |
| CD14 | 12     |
| CCL4 | 12     |
| CCL3 | 12     |
| CCL7 | 12     |
| TIMP1| 10     |

C, Hub genes in the PPI network at 24 h post-reperfusion

| Gene | Degree |
|------|--------|
| CCL2 | 37     |
| TLR2 | 28     |
| CCL3 | 19     |
| CD14 | 19     |
| CXCL10|19     |
| CXCL1| 18     |
| CCL7 | 17     |
| CCL4 | 17     |
| SLC11A1|16     |
| TIMP1| 10     |
| MS4A6D|14     |
| LCN2 | 14     |
| LILRB4|14     |
| FPR2 | 14     |
| SOCS3| 13     |
| SLC15A3|12     |
| VIM  | 12     |
| FGFR2B|12     |
| CCL9 | 12     |
| CCL12| 11     |
| PF4  | 11     |
| LGALS3|10     |
| ACTB | 10     |

PPI, protein-protein interaction.
Availability of data and materials

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

Authors' contributions

XS and WB were responsible for study design. XH, HJ and XS were responsible for data acquisition, analysis, and interpretation. WB and ZY drafted the manuscript. ZY interpreted the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The authors declare that they have no competing interests.

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

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