

Methotrexate combined with methylprednisolone for the recovery of motor function and differential gene expression in rats with spinal cord injury

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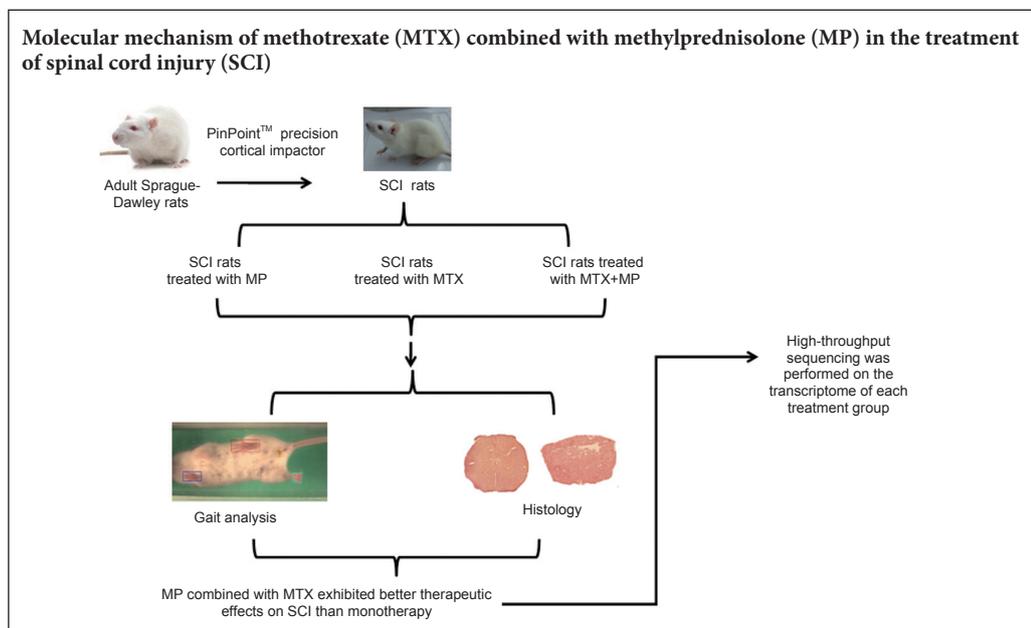
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Graphical Abstract



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Abstract

Methylprednisolone is a commonly used drug for the treatment of spinal cord injury, but high doses of methylprednisolone can increase the incidence of infectious diseases. Methotrexate has anti-inflammatory activity and immunosuppressive effects, and can reduce inflammation after spinal cord injury. To analyze gene expression changes and the molecular mechanism of methotrexate combined with methylprednisolone in the treatment of spinal cord injury, a rat model of spinal cord contusion was prepared using the PinPoint™ precision cortical impactor technique. Rats were injected with methylprednisolone 30 mg/kg 30 minutes after injury, and then subcutaneously injected with 0.3 mg/kg methotrexate 1 day after injury, once a day, for 2 weeks. TreadScan gait analysis found that at 4 and 8 weeks after injury, methotrexate combined with methylprednisolone significantly improved hind limb swing time, stride time, minimum longitudinal deviation, instant speed, footprint area and regularity index. Solexa high-throughput sequencing was used to analyze differential gene expression. Compared with methylprednisolone alone, differential expression of 316 genes was detected in injured spinal cord treated with methotrexate and methylprednisolone. The 275 up-regulated genes were mainly related to nerve recovery, anti-oxidative, anti-inflammatory and anti-apoptotic functions, while 41 down-regulated genes were mainly related to proinflammatory and pro-apoptotic functions. These results indicate that methotrexate combined with methylprednisolone exhibited better effects on inhibiting the activity of inflammatory cytokines and enhancing antioxidant and anti-apoptotic effects and thereby produced stronger neuroprotective effects than methotrexate alone. The 316 differentially expressed genes play an important role in the above processes.

Key Words: nerve regeneration; spinal cord injury; methotrexate; methylprednisolone; gait; gene expression profile; inflammation; oxidative stress; apoptosis; nerve repair; Solexa gene sequencing; secondary lesion; neural regeneration

Introduction

The incidence of spinal cord injury (SCI) caused by many events, including traffic accidents, mining and construction accidents, seismic and natural disasters, has tended to rise year on year. The pathology of SCI usually includes primary injury and secondary injury (Tator, 1991). Primary injury often occurs in the spine, leading to SCI (Ray et al., 2016; Yu et al., 2016). The secondary damage includes inflammation, oxidative stress, neuronal apoptosis, intracellular and extracellular ion imbalance and a series of pathological reactions (Luo et al., 2013). Therefore, to avoid second injury and to promote the survival of axons and neurons, therapies aim to reduce or eliminate the destructive pathological response and to promote the regeneration, repair and functional reconstruction of nerve tissue in the chronic phase.

Methylprednisolone (MP) acts through a variety of mechanisms to prevent the occurrence of secondary SCI and is currently the only food and drug administration-approved drug for the treatment of acute SCI (Bracken, 2012). However, high doses of MP lead to many side effects, including glucocorticoid-induced infection, diabetic complications, and impaired wound healing, and can endanger the lives of patients. Therefore, many experts suggest that high-dose MP shock therapy should not be used as a conventional treatment, although it can be used as a selective treatment strategy (Zhang et al., 2013).

Methotrexate (MTX) is a common anti-rheumatic drug that can improve the disease state and has anti-inflammatory and immunosuppressive effects (Keith et al., 2012). MTX is generally used to compensate for the poor efficacy of glucocorticoid or other anti-rheumatic drugs. MTX can also be combined with hormones in early rheumatoid arthritis, so as to reduce hormone doses, thereby alleviating hormone side effects (Pincus et al., 2015). MTX can be taken long-term because of low cost, various routes of administration, steady long-term efficacy and high safety; namely, it can be taken as an anchor drug (Saeki et al., 2012). Low doses of MTX can be used in the treatment of SCI to ameliorate inflammation (Sanli et al., 2012), oxidative stress and apoptosis (Bakar et al., 2013; Kertmen et al., 2013), thereby preventing the occurrence and development of secondary injury.

Therefore, large-dose MP pulse therapy has been implemented in the early stage of SCI, followed by low-dose MTX maintenance treatment to compensate for the toxic effects of MP. Under this regimen, MTX can protect the remaining synaptic and neuronal functions. Most studies have explored the therapeutic effect on SCI of MTX or MP alone. This study, however, explored the molecular mechanisms of combined MP and MTX administration for treating SCI by high-throughput sequencing.

Materials and Methods

Animals

Fifty male Sprague-Dawley rats weighing 245 ± 20 g were provided by Hunan Slack Kingma Company, China (license No. 43004700001369). All rats were housed at room temperature ($25 \pm 2^\circ\text{C}$) and 65% humidity with a 12 hour light/dark cycle. Bedding was changed every two days. The rats were allowed free access to food and water.

To allow rats to adapt to the treadmill environment, fifty rats were adaptively trained on a ZH-PT computer controlled ani-

mal treadmill (Anhui Zhenghua, Huaibei, China) for 5 days before the operation. All rats were randomly assigned to the sham group (sham operation), SCI group (model establishment), MP group (SCI + MP alone), MTX group (SCI + MTX alone) and MP + MTX group (SCI + MP combined with MTX). Ten rats from each group were used for gait analysis, six for histology and the remaining four for Solexa gene sequencing.

All procedures were approved by the Experimental Animal Ethics Committee of Jiangxi University of Traditional Chinese Medicine (approval No. A20150107). The experiment followed the United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No.85-23, revised 1986).

Surgical procedure for SCI

SCI models were prepared using a modified method reported by Gu et al. (2011). Rats were anesthetized with 5% chloral hydrate and fixed on a stereotaxic apparatus in the prone position. After shaving the thoracolumbar back region, the skin was disinfected with iodophor. Taking the T₁₀ spinous process as the center, a 4.0 cm-long median longitudinal incision was made to separate the muscle and fascia from both sides of the spine. The spinous process and lamina were gently removed, and the spinal dura mater was exposed. The PinPoint™ Precision Cortex Striker (diameter 3.0 mm, Hatteras Instrument Cary, NC, USA) was moved to the top of the T₁₀ dural surface and centered on the median vessel. When the contact head, which has a high-precision contact sensor, touched the surface of the dura mater, an audible warning was generated. Subsequently, the three-dimensional coordinates were determined. T₁₀ injury was produced with an impact depth of 1.0 mm; an impact velocity of 2.0 m/s; and a residence time of 200 ms. Immediately after rat tail spasm and retraction flutter of lower extremities, cold gauze was used to stop bleeding. Muscles and skin were sutured layer by layer.

The sham group was operated as above, but no impact was performed. The preparation and postoperative nursing of SCI models were in accordance with procedures in a previous study (Gu et al., 2011). After surgery, rats were injected with 5 mL Ringer's lactate solution to compensate for blood loss. The rectal temperature was maintained at $37 \pm 1^\circ\text{C}$ with a thermostatically controlled heating pad. Penicillin and gentamicin sulfate (2.5 mL/kg) were intramuscularly injected twice a day for 7 days. If urinary tract infection occurred, the antibiotics were administered for 10 days. At a fixed time, urination was induced 2 to 3 times every day, until spontaneous urination.

Therapeutic schedule

MP (Methylprednisolone Sodium Succinate for Injection; batch number: Z07305) was provided by Pfizer Pharmaceutical Belgian Company (Belgian, Ixelles). MTX (0.05 g/1 mL, batch number: 271021-2) was purchased from the Lingnan Pharmaceutical Co., Ltd. (Guangzhou, China).

The necks of rats in sham and SCI groups were subcutaneously injected with 0.5 mL normal saline 30 minutes after injury. The rats in the MP group were injected with MP (30 mg/kg, 187 μL ; Pfizer, Belgian, Ixelles) *via* the tail vein. The rats in the MTX group were subcutaneously injected with MTX (0.3 mg/kg, 1.5 μL ; Lingnan Pharmaceutical Co., Ltd.), once every other day, for 2 weeks. The rats in the MP + MTX group were injected with MP (30 mg/kg, 187 μL) *via* the tail vein 30 minutes before

injury, and were then subcutaneously injected with MTX (0.3 mg/kg, 1.5 μ L) at 1 day after injury, once a day, for 2 weeks.

Gait analysis

The hind limb gait, including hind limb swing time, stride time, minimum longitudinal deviation, instant speed, footprint area and regularity index, was recorded using the small animal gait analyzer (Clever Sys. Inc., Reston, VA, USA). The video files were analyzed using Treadscan 4.0 system software (Clever Sys. Inc.) (Zhang et al., 2012). Gaits were tested at 2, 4, and 8 weeks after injury. Before the test, each rat ran freely for 30–45 seconds and was then placed on the walking table of the gait analyzer. The speed of the walking table increased from 7 cm/s to 15 cm/s over 20 seconds. Each rat was tested three times.

Histology

Sections containing injured spinal cord tissue were generated as previously described (Pinzon et al., 2008). Briefly, at 8 weeks, all animals were anesthetized and perfused intracardially with normal saline solution followed by 4% paraformaldehyde solution. Cord segments of about 1 cm surrounding the injured site were dissected, embedded in paraffin, and cut vertically into serial sections. One section at the center of impact (+0 mm) and sections at around 3 and 5 mm from the impact site were selected and stained with Harris' hematoxylin and eosin for routine pathological examination.

Total RNA extraction and Solexa gene sequencing

Rats were decapitated 8 weeks after surgery. Four rats were selected from each group for RNA extraction. An approximately 10 mm spinal cord section surrounding the damage center was isolated. Total RNA extraction and cDNA synthesis were performed as previously described (Liu et al., 2016). Briefly, total RNA from each sample was isolated using an RNeasy Protect Mini Kit (Qiagen, Dusseldorf, Germany). RNA purity and quantity were assessed with a SmartSpec Plus (Bio-Rad, Hercules, CA, USA). cDNA synthesis was performed using an iScript cDNA Synthesis Kit (Bio-Rad). The amplification procedure was: pre-denaturation for 30 seconds at 98°C; 18 cycles of denaturation for 10 seconds at 98°C, annealing for 30 seconds at 65°C, extension for 15 seconds at 72°C; extension for 7 minutes at 72°C; holding at 8°C. The amplification products were sequenced after gel purification by HiSeq2000 high-throughput sequencing (Illumina, San Diego, CA, USA). The obtained image files were converted into digital signals. The results were analyzed and processed after removing impurities and linker sequences.

Quality evaluation and RNA-Seq bioinformatic analysis

Original HiSeq2000 Single-end sequencing images were transformed into raw data. Adapters and low quality, contaminated data were removed from the raw data and the length distribution was analyzed. Sequences were then matched against the rat genome. The ratios of sequence to the genome and unique gene were calculated using SOAP software (BGI, Shenzhen, China). To annotate and classify gene functions for each sequence, all assembled unique sequences were searched against GenBank's non-redundant database, the Swiss-Prot protein database (<http://www.expasy.ch/sprot>), the KEGG pathways database (Kanehisa et al., 2008), and the COG database ([\[ncbi.nlm.nih.gov/COG/\]\(http://ncbi.nlm.nih.gov/COG/\)\) using BLASTx and against nucleotide \(nt\) using BLASTn with a threshold of \$E < 1.0E-5\$. According to protein \(nr\) annotation information, we used Blast2GO software \(Conesa et al., 2005\) to obtain GO comment information from Unigene. AGRIGO and WEGO software were used to conduct GO functional classification \(Ye et al., 2006\). R program was used to identify differentially expressed genes from different samples. Differential expression was analyzed between two groups \(SCI and sham groups, MP and SCI groups, and MTX + MP and MP groups\) using DEGseq software \(Wang et al., 2010\). The abundance of gene expression was measured using per million mapped fragments \(Mortazavi et al., 2008\). The differentially expressed genes in different treatment groups were screened using a digital gene expression profile difference gene detection method \(Audic and Claverie, 1997\). The \$P\$ -value of each differentially expressed gene was computed. The Benjamini–Hochberg false discovery rate \(Pang et al., 2013\) was applied to correct \$P\$ -values. The genes suppressed at an estimated \$|\log_2\(-\text{Fold change}\)| \geq 1\$ and with a false discovery rate adjusted \$P\$ -value \$\leq 0.05\$ were considered to be differentially expressed \(Wu et al., 2010\). All differentially expressed genes were mapped to each term of the Gene Ontology database \(<http://www.geneontology.org/>, release date: Aug 1st, 2012\) and the gene numbers were calculated from each GO term. The rigorous Bonferroni correction method was used for \$P\$ value correction \(Chen et al., 2014\). When the corrected GO term had a cutoff value of no more than 0.05, it was defined as a significant enrichment.](http://www.</p>
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Quantitative polymerase chain reaction (qPCR)

To detect the expression level of selected unigenes, quantitative real-time PCR was performed as described previously (Chen et al., 2014). A 5 μ L aliquot of cDNA was blended with Super Mix (Invitrogen) and the PCR primers in a 20 μ L reaction volume. The iQ5 instrument (Bio-Rad, Hercules, CA, USA) was used to perform quantitative PCR under the following conditions: 95°C for 30 seconds, followed by 40 cycles of 95°C for 15 seconds and 60°C for 35 seconds. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method using β -actin as an internal control (Chen et al., 2011). Gene-specific primers used are listed in **Table 1**. The RNA library used was generated from a separate sample from that used for RNA-seq. Three replicates were performed for each sample.

Statistical analysis

Data analysis was performed using SPSS 16.0.2 statistical software (SPSS, Chicago, IL, USA). All data are presented as the mean \pm SD. Comparisons among groups were performed by one-way analysis of variance followed by Dunnett's test. Gene matching randomness was analyzed using the Kolmogorov-Smirnov test (Lee et al., 2017). A value of $P < 0.05$ was considered statistically significant.

Results

Effects of MP and MTX on motor function in rats with SCI

Swing time

As shown in **Figure 1A**, the swing time in the MP group was significantly shorter than that of the SCI group ($P < 0.05$) from 2 to 8 weeks post-injury. At 8 weeks post-injury, improvement was significantly enhanced by combined MP and MTX compared with the use of MP alone ($P < 0.05$).

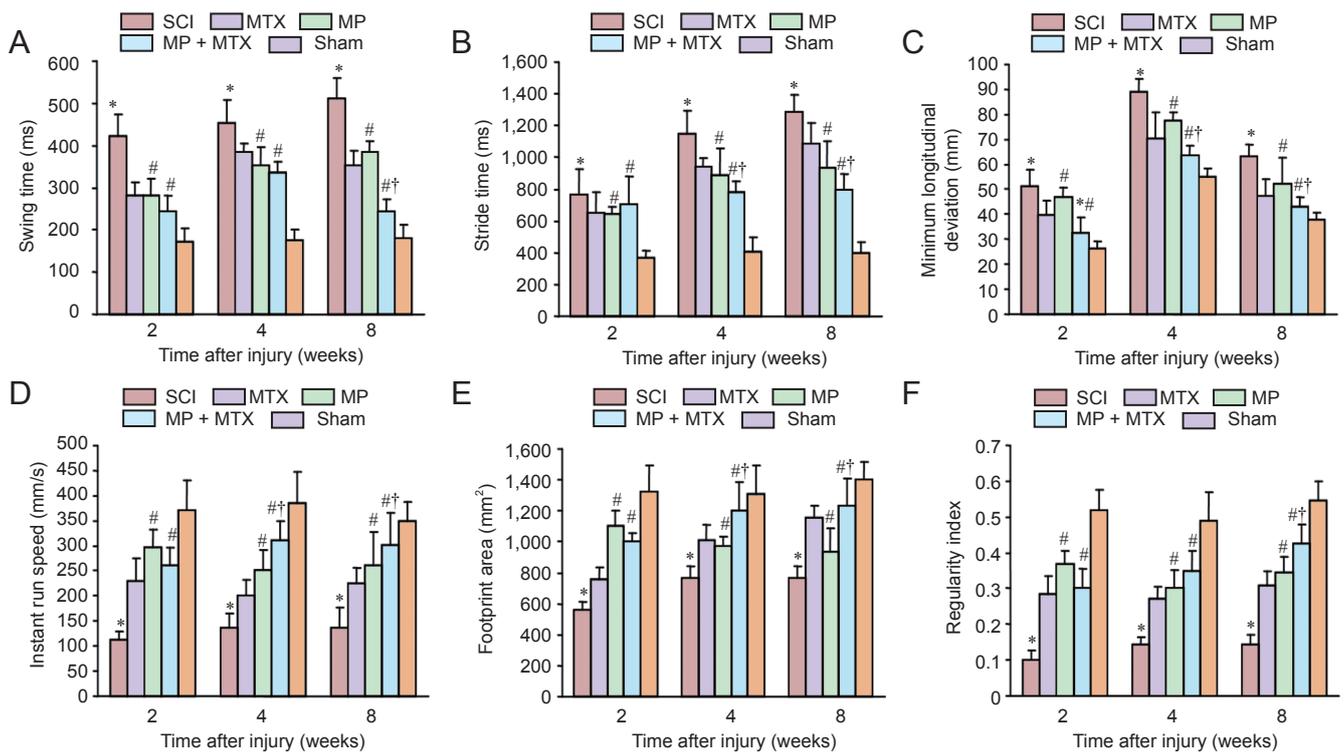


Figure 1 Comparison of gait parameters among groups at 2, 4 and 8 weeks after SCI.

(A–F) Comparison of swing time, stride time, minimum longitudinal deviation, instant run speed, footprint area and regularity index. Data are expressed as the mean \pm SD ($n = 10$ per group, one-way analysis of variance followed by Dunnett's test). * $P < 0.05$, vs. SCI group; # $P < 0.05$, vs. MP group; † $P < 0.05$, vs. sham group. SCI: Spinal cord injury; MP: methylprednisolone; MTX: methotrexate.

Stride time

As shown in **Figure 1B**, the stride time was significantly longer between SCI and sham groups after injury ($P < 0.05$). The MP + MTX group achieved a significant better therapeutic effect between 4 and 8 weeks post-injury compared with the MP group ($P < 0.05$).

Minimum longitudinal deviation

As shown in **Figure 1C**, the minimum longitudinal deviation in the MP group was significantly decreased compared with the SCI group ($P < 0.05$). The restorative effects in the MP + MTX group were significantly better at each time point compared with the MP group ($P < 0.05$).

Instant run speed

As shown in **Figure 1D**, the instant run speed was significantly decreased in the SCI group at each time point after injury compared with the sham group ($P < 0.05$). The instant run speed in the MP group was significantly improved compared with the SCI group ($P < 0.05$). The instant run speed in the MP + MTX group at 4 and 8 weeks post-injury was significantly increased compared with the MP group ($P < 0.05$).

Footprint area

As shown in **Figure 1E**, at 2–8 weeks after injury, the footprint area in the SCI group was significantly decreased compared with the sham group ($P < 0.05$). The footprint area in the MP group was significantly increased compared with the SCI group at each time point ($P < 0.05$). The improvements in the MP + MTX group were significantly more at 4 and 8 weeks post-injury

compared with the MP group ($P < 0.05$).

Regularity index

As shown in **Figure 1F**, 2–8 weeks after injury, the regularity index in the SCI group was significantly lower than that of the sham group ($P < 0.05$). The regularity index was significantly improved in the MP group compared with the SCI group 2 weeks post-injury ($P < 0.05$). The MP + MTX group and the MP group showed similar restorative effects at 2 and 4 weeks, but the MP + MTX group was significantly more improved at 8 weeks compared with MP group ($P < 0.05$).

Effects of MP and MTX on histology in SCI rats

The results of hematoxylin-eosin staining are shown in **Figure 2**. Eight weeks after injury, white matter and gray matter structures were complete in the sham group. The number of neurons was decreased and the vacuole was very severe in the SCI group. In the MP + MTX and MP groups, there were various degrees of tissue loss and cyst formation, and all the damage had a tendency to spread to the adjacent area. Myelin sheath swelling and the loose structure of the white matter and the white matter bundle were improved in the MP + MTX group. Total glial cell proliferation, tissue voids, and the size of cysts at the injury center and in adjacent areas were significantly smaller in the MP + MTX group compared with those in the MP group.

Gene sequencing

Evaluation of sequencing results

Quality evaluation and RNA-Seq bioinformatic analysis showed that linker sequences were removed from the original data.

Table 1 Primer sequences used in quantitative polymerase chain reaction

Gene	Primer	Sequence	Product size (bp)
<i>β-Actin</i>	Actin-1	5'-GGC ACC ACA CCT TCT ACA AT-3'	961
	Actin-2	5'-TAG CCT TGG CGA GCA ACG GT-3'	
<i>Ftl1</i>	Ftl-1	5'-GCC GCC TTA CAA GTC T -3'	
	Ftl-2	5'-GTG TTC TTT TAG TTC GGA GT-3'	1086
<i>Omg</i>	Omg-1	5'-TGG ATG TTT CTA AGA ATA TG-3'	
	Omg-2	5'-ACT GGT GTC GTA ACT CGT AA-3'	926
<i>Mif</i>	Mif-1	5'-CGC TTT GTA CCG TCC TCC G -3'	
	Mif-2	5'-TTC CAC CCG TAG CGA TGG CCA-3'	826
<i>Uqcrh</i>	Uqcrh-1	5'-ATA TCC GTC GAG TGA CCC TTA-3'	
	Uqcrh-2	5'-ACG TCT AGT CAA ATA ATT CTA-3'	530
<i>Ncdn</i>	Ncdn-1	5'-GAG GAC ACA GAA CCA GAA GCC-3'	
	Ncdn-2	5'-GAA ACG AAC CCC GGT TAG AA-3'	979
<i>Syp</i>	Syp-1	5'-GGC TTC GTG AAG GTG CTG CAG-3'	
	Syp-2	5'-AGA GAA CTC GAG AAC GGG T-3'	908
<i>Gstp1</i>	Gstp1-1	5'-GAC CCT CAA GCT CAC GTA CT-3'	
	Gstp1-2	5'-CGT ATC GAA AGG AAC AGA GG-3'	446
<i>Tgfb3</i>	Tgfb3-1	5'-GGG CAA GGC AAA GAG CTT-3'	
	Tgfb3-2	5'-GGC ACG TAG AGA AGG TCG-3'	735
<i>Thbs1</i>	Thbs1-1	5'-TGG CCA GCG TTG CCA-3'	
	Thbs1-2	5'-GGT ACC TCT GGT CAC TGA-3'	746
<i>Cdk5r2</i>	Cdk5r2-1	5'-GTC GCT ATT ACC GCT GCT CTA T-3'	
	Cdk5r2-2	5'-GTG TCT CTT CTG TGG TGG C-3'	798

Ftl1: Ferritin light chain 1; Cdk5r2: cyclin-dependent kinase 5 activator 2; Omg: oligodendrocyte-myelin glycoprotein; Mif: macrophage migration inhibitory factor; Uqcrh: ubiquinol-cytochrome c reductase hinge protein; Ncdn: neurochondrin; Syp: synaptophysin; Gstp1: glutathione S-transferase P; Tgfb3: transforming growth factor beta-3; Thbs1: thrombospondin-1.

Reads with a length of < 20 bp were filtered out. Sequences containing an unknown base ratio of > 4% were removed. Retention of valid data was > 70%, which was in line with requirements for subsequent analyses. The gene matching ratio was up to > 50% (Table 2). Matching randomness analysis of all reads showed no significant differences in length distribution and uniform distribution; the randomness and uniformity were in line with the expected requirements.

Differential gene expression after SCI injury

The difference in gene expression between two groups (SCI and sham, MP and SCI, MTX+MP and MP) showed that overall

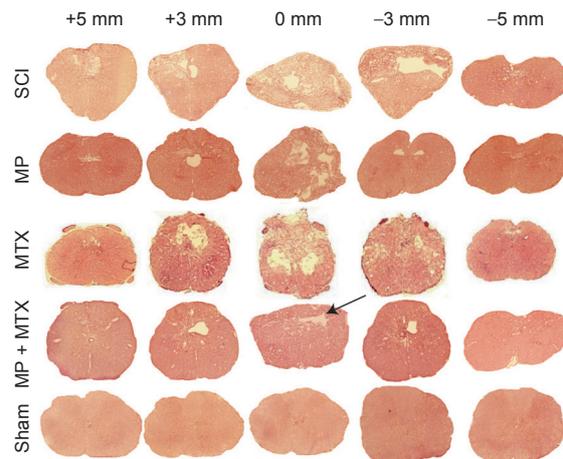


Figure 2 Histopathological changes of the spinal cord at 8 weeks post-trauma (hematoxylin-eosin staining, × 20).

Vertical cross-sections were taken from the injured site (+0 mm), and 3 and 5 mm rostral (+) and caudal (–) to the epicenter. Eight weeks after injury, the SCI, MP + MTX and MP groups had different degrees of tissue loss and cyst occurrence, and there was a tendency of damage to spread to the adjacent area. After MP + MTX combination therapy, fibrous scar formation was present at the lesion; total glial cell proliferation, tissue voids, and cyst area were significantly less compared with the use of MP or MTX alone (as indicated by the arrow). MP: Methylprednisolone; MTX: methotrexate; SCI: spinal cord injury.

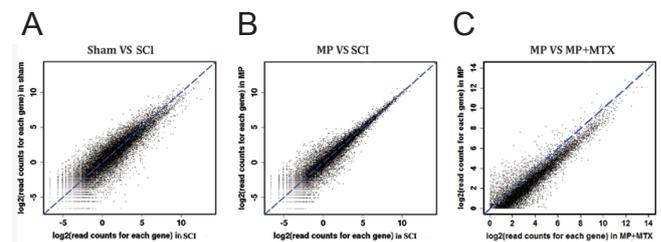


Figure 3 Scatter plots of gene expression differences between two groups after normalization.

Differential expression was analyzed between two groups (SCI and sham groups, MP and SCI groups, MTX + MP and MP groups) using DESeq software (Wang et al., 2010). (A) Comparison of the sham group and SCI group. (B) Comparison of the MP group and SCI group. (C) Comparison of MP group (30 mg/kg MP) and MP + MTX group (30 mg/kg MP + 0.3 mg/kg MTX). Sham group and SCI groups were subcutaneously injected with 0.5 mL saline. MP: Methylprednisolone; MTX: methotrexate; SCI: spinal cord injury.

gene expression was roughly similar between groups (Figure 3). Thus, the scatters overlapped and showed a diagonal line.

According to GO classification, differentially expressed genes were split into three categories: biological processes, cellular components and molecular functions; each category contains a large number of subgroups. All gene GO classifications included reproduction, transcription factor activity, cell killing, immune process, catalytic activity, transport activity, metabolism, and anti-oxidation. Fifty-four regulatory gene subclasses were related to life activities.

There were 443 differentially expressed genes between the SCI and sham groups (269 genes were up-regulated and 174 genes were down-regulated). GO enrichment analysis showed that the upregulated genes in the SCI group were mainly involved in wound repair, ionic equilibrium, stress response, organ development, biosynthesis, signal transduction, inflammation

Table 2 The matching of each sample sequence with the gene and the unique matching statistics

Sample name	Clean reads	Gene mapped reads	Gene unique reads	Gene unique ratio (%)
Sham(1)	10222537	6859558	6067189	88.45
Sham(2)	11809428	8005393	7056818	88.15
Sham(3)	11757893	7936577	7001648	88.22
Sham(4)	10894532	7038957	6077436	86.34
Model(1)	11045928	5889613	4997939	84.86
Model(2)	9088736	5511297	4765099	86.46
Model(3)	10534624	5885694	5046982	85.75
Model(4)	11354692	7782506	6857165	88.11
MP(1)	12164382	6020647	5148380	85.51
MP(2)	10439937	5518130	4754520	86.16
MP(3)	9870349	5922209	5158244	87.10
MP(4)	11235209	6913024	6012257	86.97
MTX(1)	8505469	4529368	3900117	86.11
MTX(2)	10757998	6500278	5658204	87.05
MTX(3)	9592743	6020545	5248020	87.17
MTX(4)	9682878	5234607	4528227	86.51
MTX+MP(1)	11075612	7546652	6602707	87.49
MTX+MP(2)	9059197	6262652	5363719	85.65
MTX+MP(3)	12280483	8894591	7726607	86.87
MTX+MP(4)	10162864	7256664	6357966	87.62

(1), (2), (3), and (4) refer to four parallel samples. The data from the table indicate that the randomness analysis of the four samples we obtained from the sequencing showed no significant difference in the distribution of the read length and the uniform distribution uniformity is good, in line with the requirements of the analysis.

tion, oxidation and cell apoptosis, suggesting that pathological manifestations after injury might be related to the regulation and control of these genes. Among them, *Dcn* [Q01129, \log_2 (-Fold change) = 2.44, $P = 0.0074$] can regulate and control neurons and glial cells in the central nervous system to produce decorin (Jungmann et al., 2012). Decorin has anti-fibrosis and anti-inflammation effects (Esmaili et al., 2014), which are involved in the wound repair process. The upregulation of *Dcn* expression might be associated with activation of the wound repair mechanism. The *Ftl1* [P29391, \log_2 (Fold change) = 2.944, $P = 0.00151$], *Ftl2* [P49945, \log_2 (Fold change) = 2.678, $P = 1.091E-81$] and *Tcirg1* genes [Q13488, \log_2 (Fold change) = 2.278, $P = 0.00481$] are responsible for regulating the intracellular and extracellular ion balance (Capecci and Forgac, 2013; Tao et al., 2014). The upregulation of this kind of gene might reflect the activation of the ion balance regulatory pathway in response to ion imbalance.

Compared with the sham group, genes related to regulation of neuronal regeneration were down-regulated in the SCI group (Table 3), which corresponded to neurophysiological dysfunction after SCI, and might be the most direct reason for sense and motor function-related disorders. Genes with greater differential expression included *Hsp90aa1*, encoding heat shock protein 90, [P82995, \log_2 (Fold change) = -4.21, $P = 2.21E-05$], which corrects the misfolding of synuclein and regulates the neuroprotective effect in neurodegenerative diseases (Sundaramurthi et al., 2012). The gene encoding cyclin-dependent kinase, *Cdk5r2*, [Q13319, \log_2 (Fold change) = -3.31, $P = 2.55E-09$] plays a crucial role in the oligodendrocyte differentiation

Table 3 Examples of down-regulated GO items of differentially expressed genes associated with regeneration of neurons in spinal cord injury and sham groups

Classification number	<i>P</i>	Term
GO:0001764	4.01E-08	Neuron migration
GO:0006836	1.31E-06	Neurotransmitter transport
GO:0007409	1.66E-04	Axonogenesis
GO:0031133	2.14E-04	Regulation of axon diameter
GO:0007268	2.33E-04	Synaptic transmission
GO:0007413	2.83E-04	Axonal fasciculation
GO:0008088	3.68E-04	Axon cargo transport
GO:0048168	9.17E-04	Regulation of neuronal synaptic plasticity
GO:0050772	9.17E-04	Positive regulation of axonogenesis
GO:0042552	1.53E-03	Myelination
GO:0002175	2.92E-03	Protein localization to paranode region of axon
GO:0022010	3.40E-03	Central nervous system myelination
GO:0050905	5.06E-03	Neuromuscular process
GO:0050885	5.50E-03	Neuromuscular process controlling balance
GO:0048812	8.39E-03	Neuron projection morphogenesis

P-value of the difference test was corrected by Bonferroni correction method.

and myelination repair process (Bankston et al., 2013). The gene encoding contactin-2, *Cntn2*, [P22063, \log_2 (Fold change) = -3.38, $P = 0.0116$] is a member of the neural recognition family. It participates in the formation and maintenance of the nervous system (Mohebiany et al., 2014). The *Omg* gene [Q63912, \log_2 (-Fold change) = -2.85, $P = 2.89E-12$] can regulate neurons in the central nervous system and oligodendrocytes to express the myelin protein. Myelin is strongly associated with development and maturation of the nervous system (Vourch and Andres, 2004). In addition, genes down-regulated in the SCI group compared with the sham group involved biological oxidation, substance and energy metabolism, organ development, ion transportation, and signal transduction, indicating that the down-regulation of these genes might be related to the pathological process after injury.

Effect of drug therapy on differential gene expression

(1) Differential gene expression between MP and SCI groups
There were 52 differentially expressed genes (33 up-regulated and 18 down-regulated) between MP and SCI groups. GO enrichment analysis showed that the upregulated genes associated with SCI injury and repair mainly reflected nervous system activities, inflammatory processes, biological oxidation, and apoptosis (Table 4).

The up-regulated genes associated with nervous system activities were involved with positive regulation of axonal regeneration, synaptic transportation, and oligodendrocyte generation, which directly reflected the promoting role of MP in nerve regeneration after SCI. Among them, *Gstp1* [P04906, \log_2 (Fold change) = 2.11, $P = 0.00285$], encodes glutathione-S-transferase, an isoenzyme that marks oligodendrocyte maturation in mammals (Tamura et al., 2007). The *Mif* gene [P34884, \log_2 (Fold change) = 2.02, $P = 0.0163$] encodes macrophage migration inhibition factor, a pluripotent cytokine that participates in inflammation and immune responses, and can promote the regeneration of peripheral nerves and inhibit Schwann cell apoptosis (Nishio et al., 2002).

Table 4 Examples of up-regulated GO items of differentially expressed genes associated with secondary damage mechanisms in spinal cord injury and methylprednisolone groups

Classification number	P	Term
Nervous system		
GO:0048680	3.60E-02	Positive regulation of axon regeneration
GO:0048490	1.51E-02	Anterograde synaptic vesicle transport
GO:0014003	3.00E-02	Oligodendrocyte development
Inflammation		
GO:0070664	8.17E-03	Negative regulation of leukocyte proliferation
GO:0071638	9.34E-03	Negative regulation of monocyte chemoattractant protein-1 production
GO:0032691	1.17E-02	Negative regulation of interleukin-1 beta production
GO:0042130	2.43E-02	Negative regulation of T cell proliferation
GO:0032720	3.91E-02	Negative regulation of tumor necrosis factor production
Biological oxidation		
GO:0009231	4.68E-03	Riboflavin biosynthetic process
GO:0009398	4.68E-03	Flavin mononucleotide biosynthetic process
GO:0051771	1.05E-02	Negative regulation of nitric-oxide synthase biosynthetic process generation
GO:0006122	1.74E-02	Mitochondrial electron transport, ubiquinol to cytochrome c
GO:0000302	3.23E-02	Response to reactive oxygen species
GO:0008121	1.07E-02	Ubiquinol-cytochrome-c reductase activity
GO:0043295	1.24E-02	Glutathione binding
Cell apoptosis		
GO:0060547	1.51E-02	Negative regulation of necrotic cell death
GO:0033033	1.63E-02	Negative regulation of myeloid cell apoptotic process
GO:0002906	1.74E-02	Negative regulation of mature B cell apoptotic process
GO:0016505	2.83E-03	Peptidase activator activity involved in apoptotic process

P-value of the difference test was corrected by Bonferroni correction method.

Among the upregulated genes were multiple GO items associated with the inflammatory reaction. Among them, the factors that directly reflect the anti-inflammatory effect of MP were negative regulation of leukocyte proliferation, generation of monocyte chemoattractant protein (MCP-1), interleukin (IL)-1 β , and tumor necrosis factor (TNF), and T cell proliferation. The anti-inflammatory effect might be an important mechanism of MP for promoting nerve function restoration after SCI. The *RT1-Ba* gene [P20037, log₂(Fold change) = 3.24, $P = 0.00186$], encoding class II tissue antigen, participates in the genetic control and regulation of the immune response, and can decrease inflammation after injury by inhibiting the proliferation of T cells (Havik et al., 2007). The *Mif* gene, encoding macrophage migration inhibitory factor, can also inhibit the inflammatory reaction by limiting the excessive phagocytosis of macrophages

Table 5 Examples of down-regulated GO items of differentially expressed genes associated with secondary damage mechanisms in spinal cord injury and methylprednisolone groups

Classification number	P	Term
Neuron regeneration		
GO:0021954	3.28E-02	Neuronal development in central nervous system
GO:0048813	4.20E-02	Dendrite morphogenesis
Inflammation		
GO:0002675	7.55E-04	Positive regulation of acute inflammatory reaction
GO:0046651	1.66E-02	Lymphocyte proliferation
GO:0006954	2.15E-02	Inflammatory reaction
GO:0030595	2.59E-02	Leukocyte chemotaxis
GO:2000343	3.51E-02	Positive regulation of chemokine (C-X-C motif) ligand 2 production
GO:0008009	4.15E-02	Chemokine activity
Biological oxidation		
GO:0070301	4.60E-03	Cellular response to hydrogen peroxide
GO:0022900	1.16E-02	Electron transport chain
GO:0071456	1.34E-02	Cellular response to hypoxia
GO:0070469	1.59E-02	Respiratory chain
GO:0008121	2.27E-02	Ubiquinol-cytochrome-c reductase activity

P-value of the difference test was corrected by Bonferroni correction method.

that occurs in the pathological process (Muller et al., 2014).

In addition, genes associated with oxidation process and cell apoptosis were up-regulated. GO enrichment analysis showed that genes reflecting inhibition of oxidative stress by MP were mainly involved in positively regulating the biosynthesis of inducible nitric oxide synthase (iNOS) and negatively regulating the binding activities of riboflavin and glutathione. We speculated that MP could regulate oxidation in damaged tissue and play a role in the inhibition of oxidative stress. The *Rfk* gene [8CFV9, log₂(Fold change) = 2.54, $P = 0.00695$], encoding riboflavin kinase that catalyzes riboflavin to produce a coenzyme of antioxidant, regulates the biological oxidation process to ensure normal metabolism (Schramm et al., 2014). *Uqcrh* [P99028, log₂(Fold change) = 3.34, $P = 0.00742$] is a target gene of peroxisome proliferator activated receptor-1 α (PPAR-1 α) in normal mitochondrial metabolism. The expression of *Uqcrh* was significantly down-regulated by oxidative stress and other pathological states (Zaza et al., 2013). The regulation of apoptosis by MP was reflected in the negative regulation of myeloid cell apoptosis and necrosis.

Compared with the SCI group, the genes down-regulated in the MP group were associated with neuronal differentiation and regeneration, inflammatory reaction and biological oxidation (Table 5). The genes participating in the regulation of neuronal differentiation and regeneration were involved in neural genesis and dendritic morphogenesis. The down-regulation of this kind of gene might reflect MP therapy having inhibitory effects on nerve regeneration; however, the ultimate effect on the restoration of neural function is the sum of positive and negative effects. The down-regulated genes associated with inflammatory

Table 6 Examples of up-regulated GO items of differentially expressed genes associated with secondary damage mechanisms in methylprednisolone and methylprednisolone + methotrexate groups

Classification number	<i>P</i>	Term
Nervous system		
GO:0048168	1.75E-03	Regulation of neuronal synaptic plasticity
GO:0042552	3.28E-03	Myelination
GO:0031133	6.76E-03	Regulation of axon diameter
GO:0050885	1.01E-02	Neuromuscular process controlling balance
GO:0048712	2.27E-02	Negative regulation of astrocyte differentiation
GO:0007422	3.13E-02	Peripheral nervous system development
GO:0048680	3.94E-02	Positive regulation of axon regeneration
GO:0043005	1.61E-05	Neuronal projection
Inflammation		
GO:0070664	1.79E-02	Negative regulation of leukocyte proliferation
GO:0071638	2.27E-02	Negative regulation of monocyte chemotactic protein-1 production
GO:0032691	3.34E-02	Negative regulation of interleukin-1 beta production
GO:0050778	4.58E-02	Positive regulation of immune response
GO:0050870	4.58E-02	Positive regulation of T cell activation
Biological oxidation		
GO:0016209	3.00E-02	Antioxidant activity
GO:0051771	2.78E-02	Negative regulation of nitric-oxide synthase biosynthetic process
GO:0004602	2.87E-03	Glutathione peroxidase activity
GO:0047066	7.81E-03	Phospholipid-hydroperoxide glutathione peroxidase activity
Cell apoptosis		
GO:0060547	6.44E-05	Negative regulation of necrotic cell death
GO:0043524	3.80E-02	Negative regulation of neuronal apoptosis
GO:2001237	6.76E-03	Negative regulation of extrinsic apoptotic signaling pathway

P-value of the difference test was corrected by Bonferroni correction method.

reaction mainly reflected the activation of chemotactic factors. *Pf4* [P06765, $\log_2(\text{Fold change}) = -2.31$, $P = 2.93E-05$] is an important marker gene of the inflammatory reaction (Liu et al., 2014). It encodes platelet factor-4, which can increase the chemotaxis of immune cells, and promote their migration to inflammatory sites, so as to aggravate the inflammatory reaction. The down-regulation of this kind of gene further illustrated that MP had an anti-inflammatory effect. Compared with the SCI group, there were associated oxidation processes highlighted in the MP group, but they did not directly affect oxidative stress.

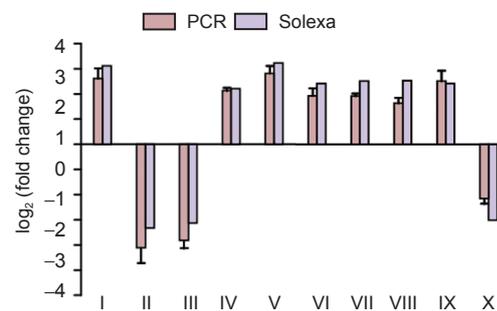
(2) Analysis of differential gene expression between MP + MTX and MP groups

There were 316 genes that were differentially expressed between MP + MTX and MP groups (275 genes were up-regulated and 41 were down-regulated). GO enrichment analysis showed

Table 7 Examples of down-regulated GO items of differentially expressed genes associated with secondary damage mechanisms in methylprednisolone and methylprednisolone + methotrexate groups

Classification number	<i>P</i>	Term
Inflammation		
GO:0072610	5.73E-03	Interleukin-12 secretion
GO:0032735	3.39E-02	Positive regulation of interleukin-12 production
GO:0038112	5.73E-03	Interleukin-8-mediated signaling pathway
GO:0002552	1.14E-02	Serotonin secretion by mast cells
GO:0010759	1.90E-02	Positive regulation of macrophage chemotaxis
GO:0050718	2.46E-02	Positive regulation of interleukin-1 beta secretion
GO:0090023	2.64E-02	Positive regulation of neutrophil chemotaxis
GO:0042007	3.04E-03	Interleukin-18 binding
GO:0004918	4.55E-03	Interleukin-8 receptor activity
GO:0031732	4.55E-03	CCR7 chemokine receptor binding
GO:0042088	1.14E-02	T-helper 1 type immune response
Cell apoptosis		
GO:0016505	7.58E-03	Peptidase activator activity involved in apoptotic process

P-value of the difference test was corrected by Bonferroni correction method.

**Figure 4** qPCR analysis of 10 genes shown to be differentially expressed by Solexa sequencing.

PCR was performed on 10 genes randomly selected from differentially expressed genes identified by Solexa sequencing. Data are expressed as the mean \pm SD ($n = 4$; one-way analysis of variance followed by Dunnett's *t*-test). Each data point represents the average from three tests, and error bars represent the standard deviations. *Ftl1*: Ferritin light chain 1; *Cdk5r2*: cyclin-dependent kinase 5 activator 2; *Omg*: oligodendrocyte-myelin glycoprotein; *Mif*: macrophage migration inhibitory factor; *Uqcrh*: ubiquinol-cytochrome c reductase hinge protein; *Ncdn*: neurochondrin; *Syp*: synaptophysin; *Gstp1*: glutathione S-transferase P; *Tgfb3*: transforming growth factor beta-3; *Thbs1*: thrombospondin-1. The experiment was repeated three times. qPCR: Quantitative polymerase chain reaction. I: *Ftl1*; II: *Cdk5r2*; III: *Omg*; IV: *Mif*; V: *Uqcrh*; VI: *Ncdn*; VII: *Syp*; VIII: *Gstp1*; IX: *Tgfb3*; X: *Thbs1*.

that there were a large number of associated items that promote nerve restoration among the up-regulated genes that also involve anti-inflammation, anti-oxidation and anti-apoptosis (Table 6). The down-regulated genes were mainly involved in pro-inflammatory and pro-apoptotic processes (Table 7).

Compared with the MP group, the up-regulation of genes that promote neural restoration in the MP + MTX group was related to the regulation of neuronal synaptic plasticity, myelin

formation, control of neuromuscular balance, and regeneration of neurons and axons. The *Ndcn* gene [O35095, \log_2 (Fold change) = 2.47, $P = 0.0161$], encoding neural cartilage protein, and the *Syp* gene [P07825, \log_2 (Fold change) = 2.72, $P = 0.0143$], encoding synaptic vesicle protein, play neurotrophic effects on promoting axonal growth and modulating synaptic plasticity in the central nervous system (Oku et al., 2013; Park et al., 2015). The *Mal* gene [Q64349, \log_2 (Fold change) = 2.39, $P = 0.00377$], encoding myelin and lymphocyte protein, is mainly produced by oligodendrocytes and Schwann cells. It regulates the peripheral nervous system to produce myelin. It might promote myelin wrapping by regulating the formation of membrane components (Buser et al., 2009).

Among the up-regulated genes, the anti-inflammatory GO genes mainly negatively regulate the proliferation of leukocytes, and the generation of IL-1 β and chemokines. Among them, the *Mif* gene [P34884, \log_2 (Fold change) = 2.84, $P = 0.014$] is involved in inhibiting excessive phagocytosis of macrophages. The *Gstp1* gene [P04906, \log_2 (Fold change) = 2.70, $P = 0.000151$], encoding glutathione-S-transferase, is also an important marker of the inflammatory reaction (Iorio et al., 2015).

Among the up-regulated genes, the antioxidant genes were mainly related to the activation of antioxidase activity and inhibition of peroxidase activity. *Prdx1* [P0CB50, \log_2 (Fold change) = 2.60, $P = 0.0484$], encoding peroxide reductase-1, is an important antioxidase that scavenges for free radicals and protects against oxidative damage. *Gpx4* [O70325, \log_2 (Fold change) = 2.40, $P = 0.00914$], encoding glutathione peroxidase, is also a major endogenous antioxidant enzyme in animals. It catalyzes the disproportionation reaction of superoxide anions (Storey, 1996). *Gstp1* [P04906, \log_2 (Fold change) = 2.79, $P = 0.00468$] was an up-regulated gene. Its expression is inversely proportional to that of iNOS in the pathological process (Raposo et al., 2013).

Among the up-regulated genes, the anti-apoptosis GO genes were associated with the negative regulation of apoptosis and apoptosis signaling pathways. Among them, *Tgfb3* [Q07258, \log_2 (Fold change) = 2.57, $P = 0.0114$] is important for cell morphology genesis, differentiation and tissue reconstruction. It can enhance the protective effects of various neurotrophic factors and is an important cytokine for neuronal survival (Kriegelstein et al., 2002).

Compared with the MP group, proinflammatory genes were down-regulated in the MTX + MP group. These were mainly related to interleukin secretion, mast cells, macrophages and neutrophils, and inflammatory factor chemotaxis. Among them, *Cxcr1* [P70612, \log_2 (Fold change) = -2.99, $P = 0.0447$], *Il18bp* [Q9Z0M9, \log_2 (Fold change) = -2.01, $P = 5.49E-05$] and *Ccl19* [O70460, \log_2 (Fold change) = -2.63, $P = 0.00230$] can regulate secretion of IL-8, IL-18 and IL-19 and binding with their respective receptors. These interleukins can promote the chemotaxis of macrophages in pathological processes and induce inflammation (Galindo et al., 2009; Bishayi et al., 2015; Xuan et al., 2015). *Thbs1* [P35441, \log_2 (Fold change) = -2.99, $P = 0.00771$] can promote macrophage chemotaxis and its expression was confirmed to be increased in inflammation-associated pathological processes (De Luna et al., 2010). In addition, pro-oxidant and pro-apoptotic genes also appeared among the down-regulated genes. *Gapdh* [P16858, \log_2 (Fold change) = -2.57, $P = 0.0146$] encodes glyceraldehyde-3-phosphate de-

hydrogenase, an important enzyme in glycolysis and aerobic metabolism. However, a recent study found its expression was strongly associated with the apoptosis of neurons in neurodegenerative diseases (El Kadmiri et al., 2014).

Quantitative PCR results

Ten genes shown to be significantly differentially expressed by Solexa sequencing were randomly selected and their relative mRNA expression determined. The results were consistent with the results of Solexa sequencing (Figure 4). These results indicated that the Solexa sequencing data reflected the real situation of gene expression in each experimental group; *i.e.*, the information obtained by gene expression profile sequencing was of high reliability.

Discussion

The present study showed that the combination of MP with MTX significantly improved damaged motor function caused by SCI in rats. Both compounds and MP combined with other drugs have been widely studied; however, to our knowledge, this is the first study to show motor function recovery in rats after traumatic SCI by a combination of MP and MTX at the level of the transcriptome. Our study illustrates that the restorative effects of this combination therapy may be through the anti-inflammatory reaction, inhibition of oxidative stress and apoptosis and other common gene regulation pathways.

Gait analysis

Treadscan gait analysis can provide an all-around gait analysis for the assessment of motor function in SCI rats (Beare et al., 2009; Myers et al., 2012; Schira et al., 2012). In our study, the effects of MP and MTX, alone and in combination, were compared by Treadscan gait analysis. Measures included hind limb swing time, stride time, minimum longitudinal deviation, instant run speed, hindlimb footprint area and regularity index. Forgione showed that footprint area was significantly decreased in incomplete spinal cord contusion model rats (Forgione et al., 2014). The results of this study also showed that SCI significantly reduced the area of the posterior footprint, and the treatment of MP combined with MTX significantly improved the posterior surface area after SCI; the regularity index was decreased in the SCI group compared with sham group, which was consistent with a previous study (Chiang et al., 2014).

Gene sequencing

The possible mechanism of different combinations of drugs that promote recovery of motor function in rats was explored through expression profile sequencing analysis. Here, differential gene expression analysis in SCI and sham groups showed that neural development- and function-associated genes were down-regulated after SCI, directly reflecting the damage to nerve structure and function. Moreover, genes associated with energy metabolism, transcription, catalysis, ion exchange, and signal transduction were differentially expressed. These results, at the genetic level, are consistent with a previous study (Tang et al., 2014).

MP treatment

In this study, gene expression profile analysis after MP treat-

ment showed that the expression of genes associated with axon regeneration, synaptic transportation, and oligodendrocyte genesis were up-regulated, confirming that MP had an effect on nerve function recovery after injury. In addition, the expression of genes regulating neurogenesis and dendritic morphogenesis in the central nervous system were down-regulated, indicating that MP treatment had certain side effects on neurological recovery. This may contribute to the reason why severe SCI fails to be completely cured by MP. However, the specific mechanism of action remains to be further examined.

The inflammatory reaction is an important mechanism of secondary SCI. A large number of experimental results show that high-dose MP treatment in the acute stage can decrease the infiltration of inflammatory cells, and effectively control the inflammatory reaction (Alibai et al., 2010; Zhang et al., 2014). Our gene expression profile analysis showed that MP treatment can up-regulate the expression of anti-inflammatory genes and down-regulate the expression of genes promoting the inflammatory reaction, which demonstrated the anti-inflammatory effect of MP. These results explained the anti-inflammatory effects of MP in three ways: (1) negative regulation of immune cell activation and immune response: genes that negatively regulate leukocyte and T cell proliferation were up-regulated. Genes positively regulating the inflammatory and immune responses were down-regulated. (2) Negative regulation of the pro-inflammatory effect of chemokines: The negatively regulating *Mcp-1* was up-regulated and genes activating chemokine activity were down-regulated (Hassanshahi et al., 2013; White et al., 2009). (3) Negative regulation of inflammatory factor expression: The genes negatively controlling IL-1 β and TNF generation were up-regulated. IL-1 β and TNF were mainly produced by activated monocytes/macrophages, and could promote neutrophil phagocytosis, cause an inflammatory reaction, participate in fever, blood coagulation and other pathological processes (Linton and Thoman, 2014). Gene expression analysis, therefore, indicated a trend against the inflammatory reaction after MP treatment.

Oxidative stress plays an important role in secondary spinal cord injury. Boyaci et al. (2015) reported that MP treatment could effectively reduce oxidative stress products after SCI and improve the level of antioxidants. In our study, many oxidation-related genes were differentially expressed after MP treatment. The genes directly reflecting oxidative stress inhibition by MP, such as iNOS (negative regulation of NO biosynthesis), and positive regulation of glutathione binding activity were up-regulated. iNOS overexpression can catalyze the generation of peroxynitrite after injury (Maggio et al., 2012). Glutathione itself is an important antioxidant (Horiyama et al., 2014) and endogenous glutathione can be consumed in a few hours or even a few minutes after injury (Lucas et al., 2002).

Apoptosis of neurons is an important factor in irreversible damage and secondary injury. Neuronal apoptosis can not only lead to local nerve degeneration, but can also cause stop nutrition of distal nerve fibers, leading to necrosis. Wang et al. (2013) reported that intrathecal injection and intravenous injection of MP could effectively inhibit the apoptosis of nerve cells and promote the recovery of motion function in the hind limbs of SCI rabbit models. The results of this study showed that the anti-apoptotic effect of MP was the up-regulation of genes that negatively regulate necrosis and myeloid cell apoptosis-associated genes.

MP + MTX combined therapy

MTX is applied in clinical practice as an anti-inflammatory and antitumor drug. Previous studies have shown that low-dose MTX has a protective effect on nerves after SCI (Lee et al., 2008; Sanli et al., 2012; Bakar et al., 2012). The present study also found that MTX might play a neuroprotective role in SCI repair.

The results showed that MTX could reduce the levels of myeloperoxidase and malondialdehyde in injured tissue, suggesting that MTX has a dual function of anti-inflammation and inhibition of oxidative stress. We also demonstrated that MTX plays an important role in protecting axon and myelin integrity, and inhibiting the ultrastructural changes of injured spinal cord. Long-term application of MTX could increase uric acid levels in blood and urine (Chen et al., 2012; Aoyama et al., 2011). Uric acid has a neuroprotective effect, suggesting that MTX inhibited the development of secondary injury and protected nerve tissue from SCI.

Combined therapy of two or more drugs has become a hot topic in clinical research. Many studies have adopted other drugs in combination with MP (Luo et al., 2013; Yin et al., 2013; Cavus et al., 2014; Li et al., 2016), which achieved better effects. From our results, MP combined with MTX improved treatment of SCI in several aspects compared with MP alone. First, the expression of many nerve restoration promoting genes was up-regulated, such as genes regulating neuronal synaptic plasticity, myelogenesis, control of neuromuscular balance, and regeneration of neurons and axons, indicating that combined therapy with two drugs showed significant advantages in the treatment of SCI in protecting the integrity of neuronal structures and in promoting neurons to rebuild functional connectivity compared with MP treatment alone. Second, there were two anti-inflammatory advantages: (1) negative regulation of the proliferation and binding activity of immune cells. Genes negatively regulating leukocyte proliferation were up-regulated, while genes positively regulating mast cell secretion, and macrophage and neutrophil chemotaxis were down-regulated. (2) Negative regulation of the production and activity of pro-inflammatory factors. The genes negatively regulating IL-1 β and chemotactic factor production were up-regulated. However, genes that positively regulate positively regulating interleukins (IL-8, IL-18, and IL-19), their receptors, and chemotactic factor activities were down-regulated. These inflammatory factors can promote neutrophil migration to the site of injury and release a series of active products, resulting in local inflammation (Kwon et al., 2011; Khalatbary and Zarrinjoei, 2012). Third, the inhibition of oxidative stress reactions has advantages. Differential gene expression analysis showed that the combined administration could positively regulate the activity of antioxidant enzymes (glutathione peroxidase and peroxiredoxin 1) and negatively regulate the activity of iNOS. Endogenous antioxidant is rapidly depleted after SCI. Therefore, the up-regulation of genes regulating antioxidant activity could replenish the antioxidant and inhibit peroxidation. In addition, genes regulating iNOS activity were down-regulated, also suggesting that the combined therapy had a better inhibitory effect on oxidative stress. Nitric oxide is produced from L-arginine by iNOS, and can react with hydroxyl free radicals to produce highly toxic free radicals (Yadav et al., 2014). Studies have shown that high-levels of nitric oxide can also promote cell apoptosis. Finally, the inhibition of nerve

cell apoptosis reflected the negative regulation of genes promoting neuronal apoptosis enzymes.

In summary, the effect of MP and MTX combined therapy on the recovery of SCI was verified by gait analysis. The possible inherent mechanism of this treatment was inferred from gene expression profiling. This study selected the anti-inflammatory drug, MTX, with a flexible administration time, in combination with the traditional SCI treatment drug, MP. The results showed that MP and MTX combination therapy could improve the deficiency of MP treatment alone, enhance the neuroprotective effect, inhibit the activities of inflammatory cytokines, strengthen the anti-oxidative and anti-apoptotic effects and increase the clinical therapeutic effect, which could provide new strategies for the control of SCI. However, in-depth research into the mechanism of these therapeutic effects is required owing to the complexity and variety of the differentially expressed genes.

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Conflicts of interest: None declared.

Research ethics: The study protocol was approved by the Animal Ethics Committee of Jiangxi University (approval number A20150107). The experimental procedure followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1986).

Data sharing statement: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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