MEGA
JOURNAL OF CASE REPORTS

Research Article

Compiled Date: December 10, 2022

Salvia Miltiorrhiza Attenuates White Matter Injury Induced by Hypoperfusion in Neonatal Rats

Xuewen Su#, Haifeng Yuan#, Wanyu Feng, Ruixia Song, Junlong Chen, Yanyan Liang, Hua Zhu and Zhongxia Dou*

Department of Pediatrics, Inner Mongolia Autonomous Region People's Hospital, China

#Xuewen Su and Haifeng Yuan contributed equally as first authors.

***Corresponding author**: Zhongxia Dou, Department of Pediatrics, Inner Mongolia Autonomous Region People's Hospital, Hohhot, Inner Mongolia 010017, P.R. China, Tel: +86-18047192455; E-mail: s13015203605@163.com

Abstract

Purpose: To evaluate the therapeutic effects of Salvia miltiorrhiza (SM) on preterm white matter injury (WMI).

Methods: A preterm WMI rat model was induced and the rats with WMI were injected with SM (5 mg/kg daily). On the 14th day after modeling, the rats were sacrificed. Brains were observed using an optical microscope, and myelin basic protein (MBP) and CC1 were visualized using immunofluorescence. Furthermore, LC-MS/MS was used to analyze possible pathways of SM action.

Results: WMI rats treated with SM had reduced cellular swelling, reduced lesions, and increased myelin sheaths. In addition, SM significantly induced MBP and CC1 expression. The therapeutic effect of SM on WMI model rats was closely associated with regulation of the complement and coagulation cascades.

Conclusions: These findings indicated that SM may be useful as a therapeutic agent for treating preterm WMI.

Keywords: Salvia miltiorrhiza; White matter injury; Myelin basic protein; Complement and coagulation cascades

Introduction

Preterm birth is a major global health problem that is associated with high mortality and morbidity. The survival rate of preterm infants has increased with advances in neonatal care and intensive care [1]. However, preterm infants often have complications, and these complications contribute greatly to the mortality rate of children and to the occurrence of neurodevelopmental disabilities [2-7]. White Matter Injury (WMI) is the most common brain injury in preterm infants and may be present in up to 50% of very low birth weight infants [8]. The failure of myelination during white matter development is believed to be the cause of preterm WMI [9,10]. Salvia

Miltiorrhiza (SM) is a traditional herbal plant that is commonly used to treat cardiovascular and cerebrovascular diseases in China, Japan, Korea, and other Asian countries [11-13]. Compared to the toxicity of many other herbal medicines, SM has low acute toxicity [14]. The beneficial effects of SM in ischemia-induced symptoms have been demonstrated [15]. In addition, studies have shown that SM has effects on inflammatory responses, such as decreasing IL-6 and IL-8 in the endothelial cells of veins and reducing TNF-α and IL-1β levels in mouse models of liver injury [16,17]. Furthermore, SM also has effects on chilblain, psoriasis, insomnia, neurasthenia, and visceral pain [18]. Since many studies have confirmed that SM has effects on cerebral ischemia, we hypothesized that SM may also have therapeutic effects on preterm WMI. Therefore, in order to examine the effect of SM on preterm WMI, SM was administered to neonatal rats with WMI. We found that SM can attenuate WMI induced by hypoperfusion in neonatal rats.

Materials and Methods

Animals and ethics

Neonatal Sprague-Dawley (SD) rats aged three days were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal experimental procedures were approved by the Animal Ethical Welfare of Inner Mongolia People's Hospital (No. 2018014) and conformed to the Chinese National Institute of Health Guide for the Care and Use of Laboratory Animals.

Induction of WMI and treatment

The rats were immobilized on the operating table following induction of anesthesia using 3% (wt/vol) pentobarbital sodium in an aseptic environment. The neck skin was sterilized using iodophor and a median cervical incision was made. The subcutaneous tissues on the right side were separated and the common carotid artery accompanying the internal jugular vein and vagus nerve was found and dissociated. The right common carotid artery was permanently ligated with an electric coagulation pen and the skin incision was sutured. The rats were then placed on a constant temperature mat at 37 ℃. Following the procedure, the rats were hypoxic for 80 min in a 33 ℃ hypoxia chamber with 6% oxygen and 94% nitrogen. The mice were grouped as follows: Normal group (n=6), WMI group (n=6), WMI + SM (5 mg/kg/d, tail vein injection; n=6). SM was administered continuously for seven days from the first day of modeling.

Hematoxylin and eosin (H&E) staining

On the 14th day after modeling, the rats were sacrificed. Their chests were opened and they were perfused with PBS through the left ventricle (about 50 mL) and then perfused with 4% paraformaldehyde. Their brains were removed and fixed in 4% formaldehyde and then embedded in paraffin. Five-millimeter paraffin sections were stained with H&E. Slides were observed using an optical microscope.

Immunofluorescence

Liver tissues were fixed in 4% formaldehyde and embedded in paraffin. Sections were dewaxed and washed three times with phosphate buffer solution and then blocked with 5% goat serum for 60 min. Following blocking, the sections were incubated with anti-Myelin Basic Protein (MBP) primary antibodies (1:100) (ab40390, Abcam, UK) and anti-CC1 (ab2377, Abcam, UK) overnight at 4°C, followed by incubation with a secondary antibody for 2 h. The sections were visualized with a fluorescence microscope and photographed.

LC-MS/MS analysis

Protein extraction and digestion, 20 μg of protein for each sample were mixed with 5X loading buffer respectively and boiled for 5 min. The proteins were separated on a 12.5% SDS-PAGE gel (constant current 14 mA, 90 min). Protein bands were visualized with Coomassie Blue R-250 staining. A total of 100 μg of the peptide mixture of each sample was labeled using iTRAQ and TMT reagent according to the manufacturer's instructions. Labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE Healthcare). LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 60/90 min. MS data was acquired using a data-dependent top 10 method that dynamically choose the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Identification and quantitation of proteins. The MS raw data for each sample were searched using the MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4 software for identification and quantitation analysis. Then cluster analysis, subcellular localization, domain annotation, Gene Ontology (GO) annotation, KEGG annotation, enrichment analysis, and protein-protein interaction analyses were carried out.

Results

SM attenuated WMI

On the 14th day after modeling, the rats were sacrificed, and their brains were removed, fixed, and stained with H&E. In the normal group, the corpus callosum structure was clearly stained and the cells were observed as neatly arranged and evenly distributed. There were no swollen cells or pathological changes **(Figure 1a).** In the WMI group, the structure of the corpus callosum was irregular and the cells appeared swollen and necrotic. In addition, induction of WMI resulted in significantly reduced myelin formation, with irregular and loosely arranged nerve fibers and significantly decreased myelin sheaths **(Figure 1a).** Interestingly, WMI rats treated with SM had reduced cellular swelling, reduced lesions, and increased myelin sheaths **(Figure 1a).** MBP was expressed by oligodendrocytes before the formation of myelin sheaths. MBP is closely related to myelin sheath formation. We evaluated MBP expression by analyzing immunofluorescence. In the WMI group, significant MBP loss was observed in the brains **(Figure 1b).** However, MBP expression in WMI rat brains was increased after treatment with SM **(Figure 1b).** CC1 is a marker of myelinating oligodendrocytes and we evaluated the expression of CC1. As shown in **Figure 2b,** the expression of CC1 in WMI rat brains was increased after treatment with SM **(Figure 1b).** These data indicated that SM alleviated white matter damage.

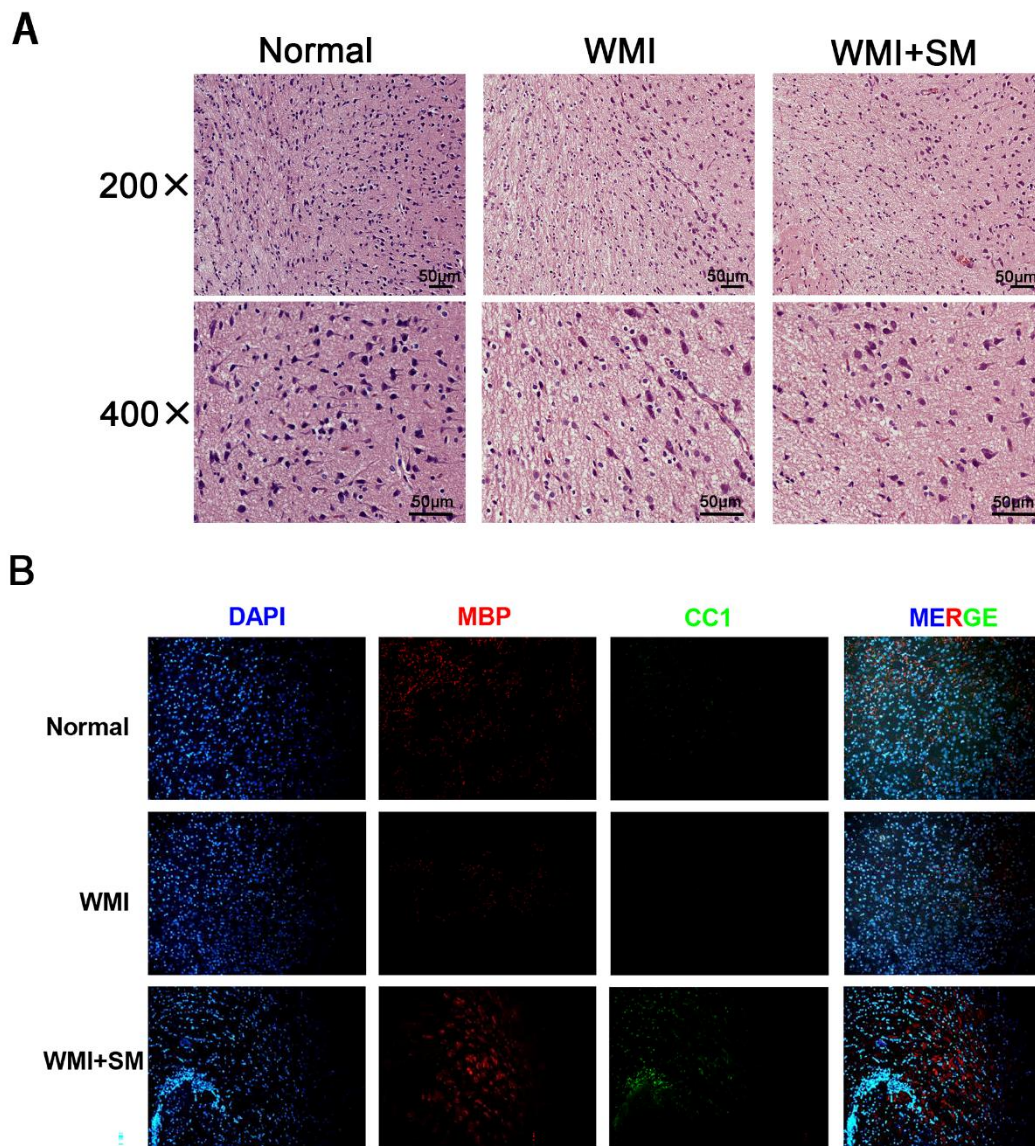

Figure 1: Salvia Miltiorrhiza (SM) attenuated white matter injury. (a) On the 14th day after modeling, the rats were sacrificed, and their brains were removed, fixed and stained with H&E. (b) Immunofluorescence was used to evaluate the expression of MBP and CC1 protein in the brains.

WMI treatment with SM showed changes in the expression of proteins in the brain

To better understand the effects of SM on WMI, we analyzed the expression of specific proteins of the Normal group (n=3), WMI group (n=3), and WMI + SM group (n=3). Our comparative assessments of the global proteins showed they were significantly differentially expressed **(Figure 2a).** Analysis of the protein expression profiles of the WMI group and Normal group revealed that 87 proteins (52 up-regulated and 35 down-regulated) were differentially expressed between these groups **(Figure 2b).** Moreover, analysis of the global protein expression profiles of the WMI group and WMI + SM group showed that 113 proteins (34 up-regulated and 79 down-regulated) were differentially expressed between these groups **(Figure 2b),** and analysis of the global protein expression profiles of the WMI + SM group and Normal group showed that 79 genes (37 up-regulated and 42 down-regulated) were differentially expressed between these groups **(Figure 2b).** The proteins that were

differentially expressed (based on fold changes) among the Normal group (n=3), WMI group (n=3), WMI + SM group are shown as heatmaps in Figure 3.

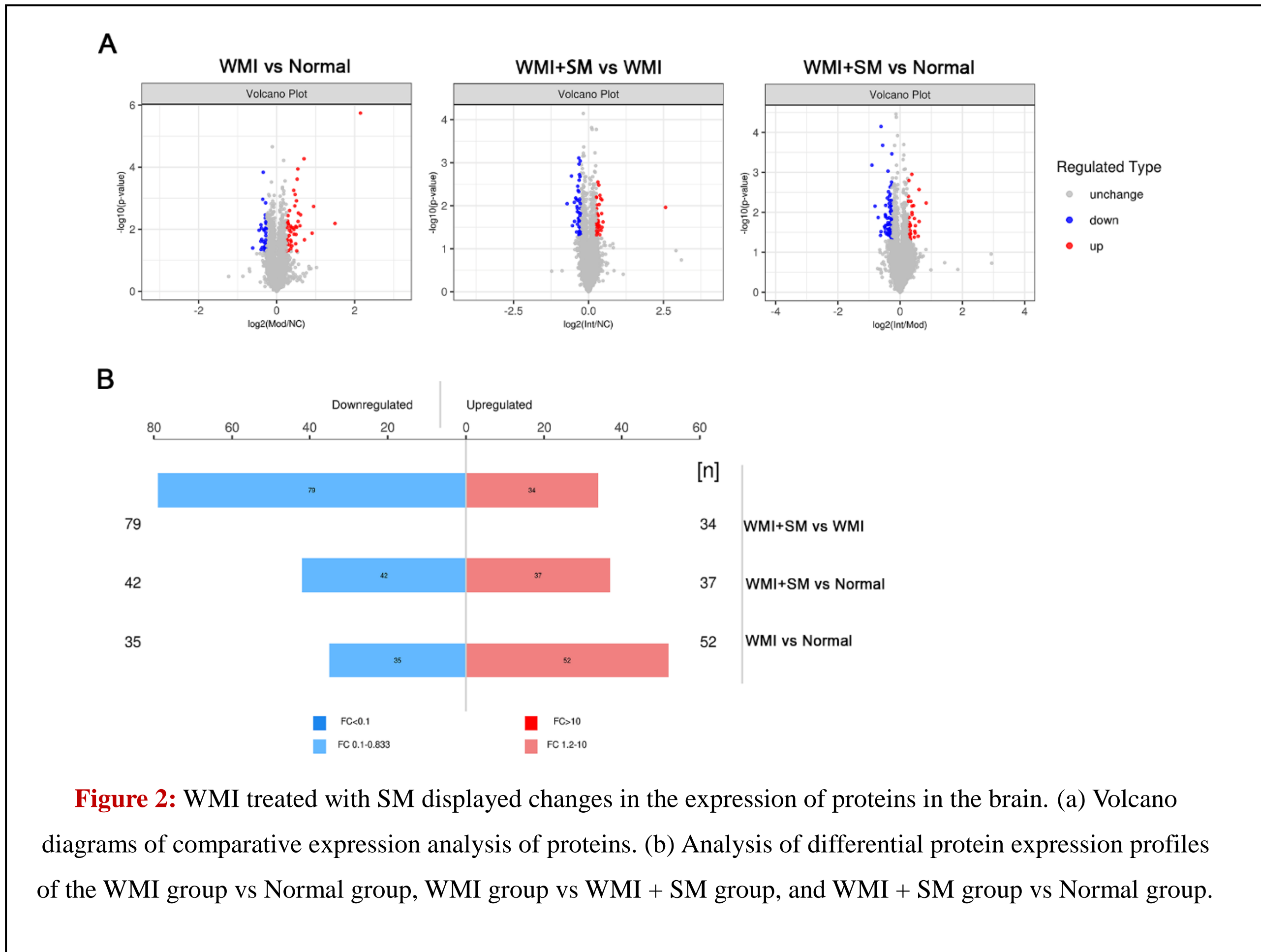

Figure 2: WMI treated with SM displayed changes in the expression of proteins in the brain. (a) Volcano diagrams of comparative expression analysis of proteins. (b) Analysis of differential protein expression profiles of the WMI group vs Normal group, WMI group vs WMI + SM group, and WMI + SM group vs Normal group.

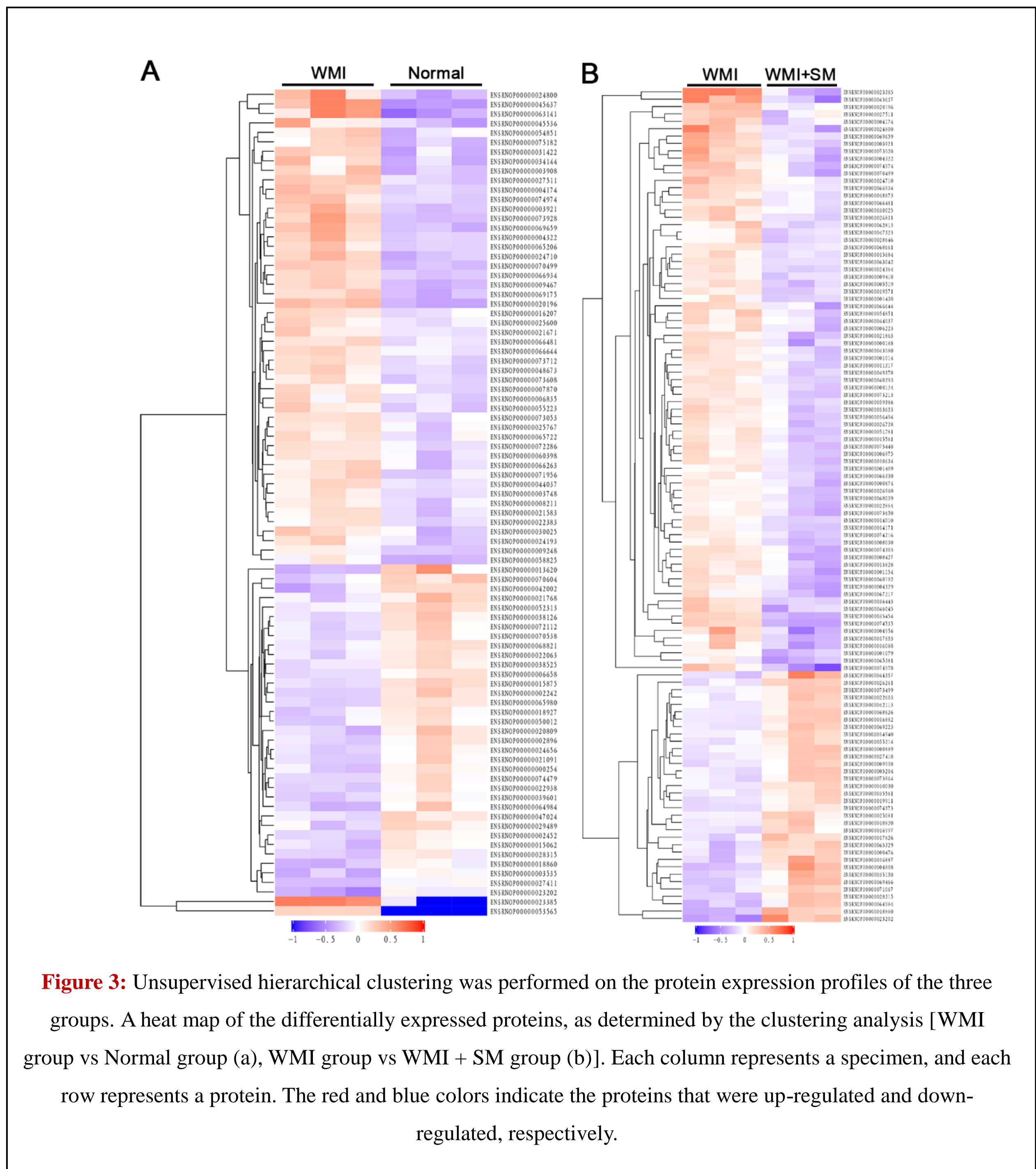

Figure 3: Unsupervised hierarchical clustering was performed on the protein expression profiles of the three groups. A heat map of the differentially expressed proteins, as determined by the clustering analysis [WMI group vs Normal group (a), WMI group vs WMI + SM group (b)]. Each column represents a specimen, and each row represents a protein. The red and blue colors indicate the proteins that were up-regulated and down-regulated, respectively.

GO was used to analyze the differentially expressed proteins

GO analysis showed that the differentially expressed proteins could be classified into the following three categories: biological process, cellular component, and molecular function. Compared with the normal group, differential proteins in biological processes in the WMI group were mainly enriched in acute inflammatory response, negative regulation of endopeptidase activity, and negative regulation of peptidase activity **(Figure 4a);** the differential proteins in cellular components in the WMI group were mainly enriched in extracellular space, extracellular region parts, and extracellular region **(Figure 4b);** and the differential proteins in molecular function in the WMI group were mainly enriched in serine−type endopeptidase inhibitor activity, endopeptidase inhibitor activity, peptidase inhibitor activity, endopeptidase regulator activity, and peptidase regulator activity

(Figure 4c). In addition, compared with the WMI group, differential proteins in biological processes in the WMI + SM group were mainly enriched in wound healing, acute−phase response, and acute inflammatory response **(Figure 5a);** the differential proteins in cellular components in the WMI + SM group were mainly enriched in extracellular space, extracellular region parts, and the extracellular region **(Figure 5b);** and the differential proteins in molecular functions in the WMI + SM group were mainly enriched in nucleosomal DNA binding, nucleosome binding, and endopeptidase regulator activity **(Figure 5c).**

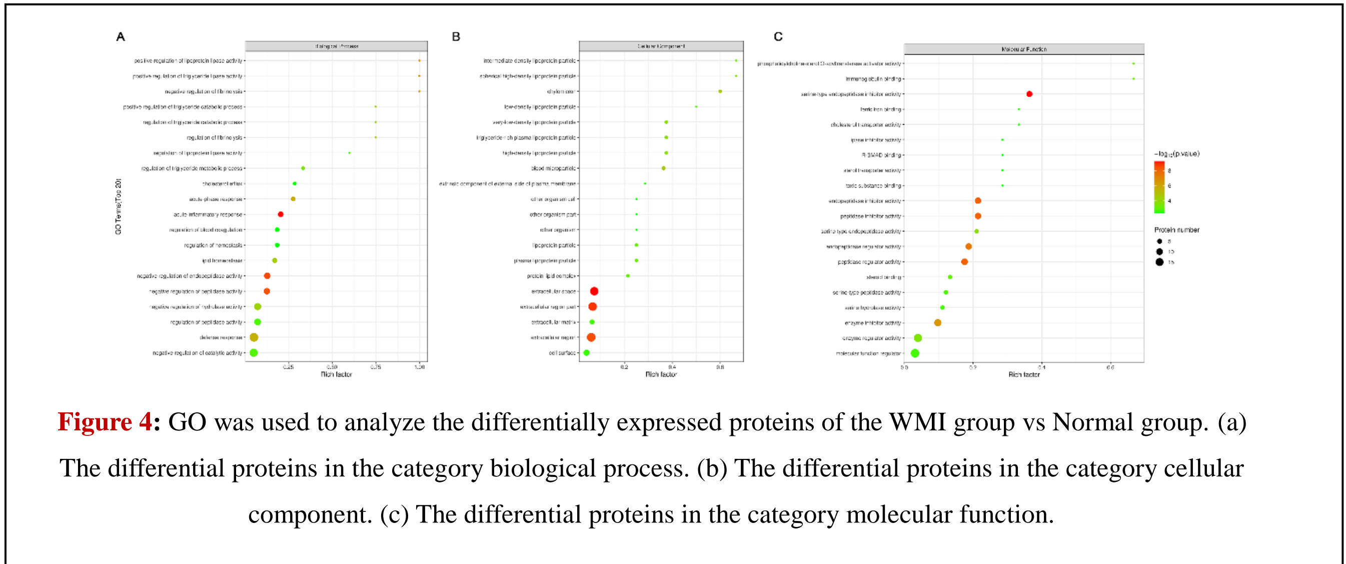

Figure 4: GO was used to analyze the differentially expressed proteins of the WMI group vs Normal group. (a) The differential proteins in the category biological process. (b) The differential proteins in the category cellular component. (c) The differential proteins in the category molecular function.

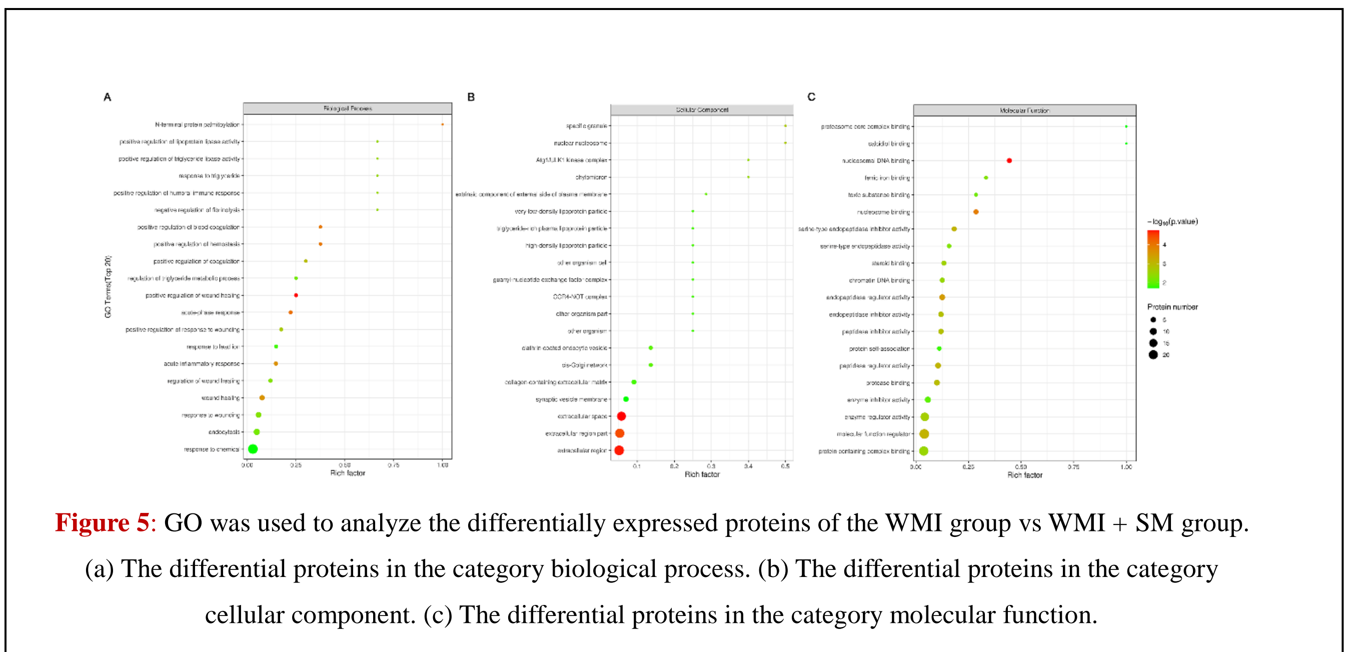

Figure 5: GO was used to analyze the differentially expressed proteins of the WMI group vs WMI + SM group. (a) The differential proteins in the category biological process. (b) The differential proteins in the category cellular component. (c) The differential proteins in the category molecular function.

KEGG analysis showed that differential proteins after SM treatment were mainly enriched in complement and coagulation cascades

KEGG was used to identify the biological pathways that were enriched most significantly in the above groups. KEGG pathway analysis revealed that the proteins that were differentially expressed between the WMI group and normal group were those associated with complement and coagulation cascades, cholesterol metabolism, vitamin digestion and absorption, and fat digestion and absorption **(Figure 6a),** and the proteins that were most

significantly differentially expressed between the WMI group and normal group were associated with complement and coagulation cascades **(Figure 6a)**. In addition, the proteins that were differentially expressed between the WMI + SM group and WMI group were those associated with complement and coagulation cascades, vitamin digestion and absorption, cholesterol metabolism, and neutrophil extracellular trap formation **(Figure 6b),** and the proteins that were most significantly differentially expressed between the WMI + SM group and WMI group were associated with complement and coagulation cascades **(Figure 6b).**

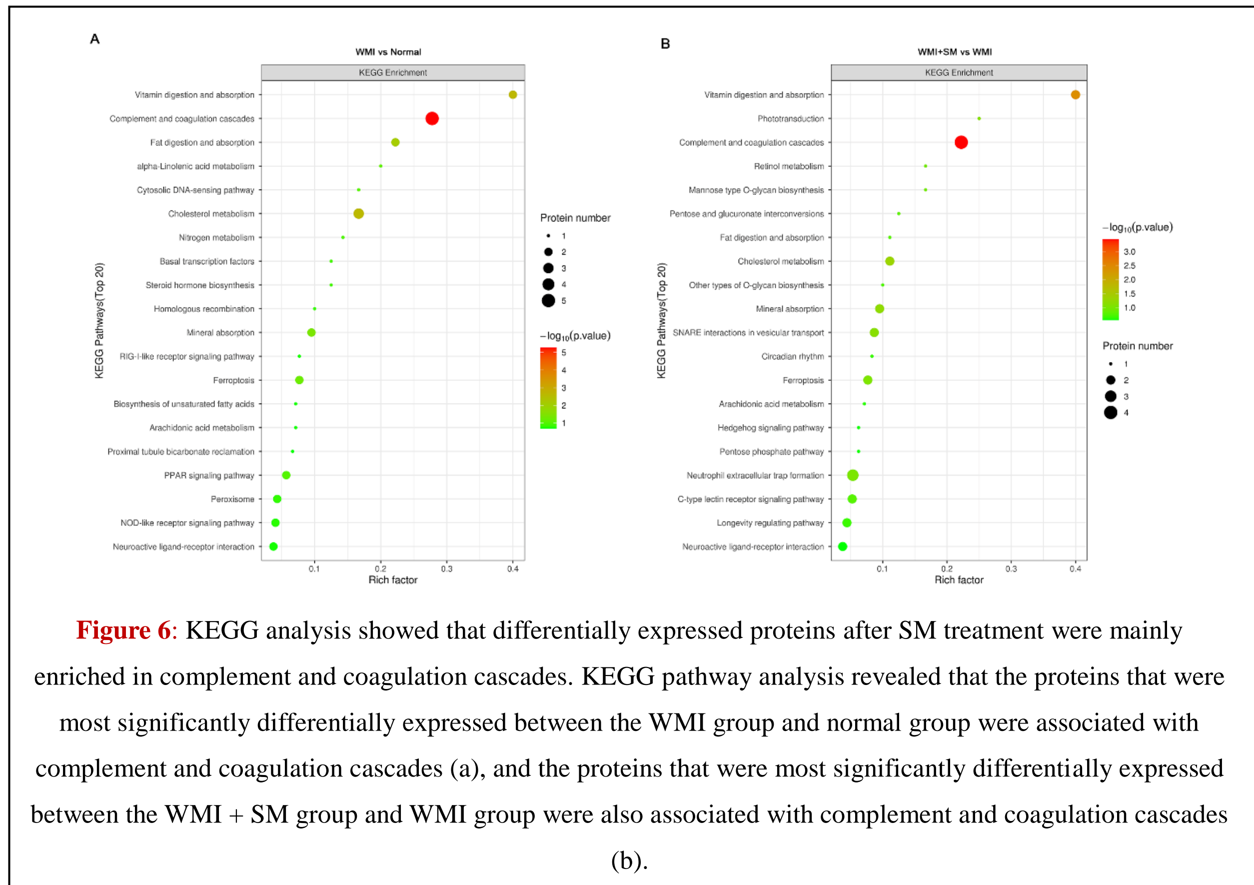

Figure 6: KEGG analysis showed that differentially expressed proteins after SM treatment were mainly enriched in complement and coagulation cascades. KEGG pathway analysis revealed that the proteins that were most significantly differentially expressed between the WMI group and normal group were associated with complement and coagulation cascades (a), and the proteins that were most significantly differentially expressed between the WMI + SM group and WMI group were also associated with complement and coagulation cascades (b).

Discussion

SM has been widely used for the treatment of various microcirculatory disturbance related diseases, including cardiovascular disease, cerebrovascular disease, renal deficiency, liver dysfunction, and diabetic vascular complications **[19].** However, there have been no reports on using SM to treat preterm WMI. In this study, we confirmed that SM can attenuate WMI induced by hypoperfusion in neonatal rats, and the therapeutic effect of SM is closely related to the regulation of the complement and coagulation cascades. We established a preterm WMI model in 3-day-old neonatal SD rats by ligating the right common carotid artery and subjecting the rats to hypoxia, and then treating them with SM. Interestingly, WMI rats treated with SM showed reduced cellular swelling, reduced lesions, and increased the numbers of myelin sheaths. In the central nervous system, adhesion of the cytoplasmic surfaces of multilamellar internodal compact myelin is maintained by MBP **[20,21].** MBP is expressed by oligodendrocytes before formation of a myelin sheath and is closely related to myelin sheath formation [22]. MBP has been recognized as an essential structural component of mature compact myelin in the central nervous system. Therefore, we evaluated MBP expression and found the MBP expression in WMI rat

brains was increased after treatment with SM. The results indicated that SM protected against preterm WMI. Oligodendrocyte damage is one of the causes of WMI. The death of oligodendrocytes results in failure to repair myelin sheaths, and this leads to severe demyelination disease [23]. Reduction in myelin loss and promotion of myelin sheath formation are two primary methods of treatment for demyelination diseases. Previous studies have shown that promoting the growth of endogenous oligodendrocyte precursor cells or transplanting exogenous oligodendrocyte precursor cells could regenerate myelin sheaths [24,25]. CC1 is a marker of myelinating oligodendrocytes. Therefore, we evaluated the expression of CC1 and found the expression of CC1 in WMI rat brains was increased after treatment with SM. The results further indicated that SM protected against preterm WMI.

To better understand the effects of SM in WMI, we analyzed the expressions of specific proteins in the Normal group, WMI group, and WMI + SM group. We found WMI treated with SM displayed changes in the expression of proteins in the brain. After SM treatment, differential proteins in biological processes were mainly enriched in wound healing, acute−phase responses, and acute inflammatory responses. The complement system is an important factor participating in neurodegenerative, inflammatory, and cerebrovascular diseases [26]. Primary rat astrocytes in culture synthesize many components, including C2, C3, C4, C5 to C9, and factor B, and regulatory proteins actor H, factor I, C4bp, S-protein, and clusterin [27,28]. Sublytic assembly of C5b-9 on oligodendrocytes reduced the levels of myelin protein, encoding MBP, and proteolipid protein [29]. Oligodendrocytes and myelin activated the classical pathway of complement ***in vitro*** in the absence of antibodies [30]. Interestingly, in this study, KEGG analysis showed that differential proteins after SM treatment were mainly enriched in complement and coagulation cascades. These results suggested that the therapeutic effect of SM is closely related to the regulation of complement and coagulation cascades.

Conclusion

Our findings showed that SM can attenuate WMI induced by hypoperfusion in neonatal rats, and the therapeutic effect of SM is closely related to regulation of the complement and coagulation cascades. These findings indicated that SM may be useful as a therapeutic agent for treating preterm WMI.

Acknowledgements

The study was supported by a grant from the Natural Science Foundation of Inner Mongolia autonomous region (grant no. 2018MS08067).

Authors' Contributions

Xuewen Su, Haifeng Yuan, and Zhongxia Dou conceived and planned the study; Xuewen Su, Haifeng Yuan, Wanyu Feng, Ruixia Song, Junlong Chen, Yanyan Liang, and Hua Zhu carried out the acquisition of data; Zhongxia Dou contributed to the analysis and interpretation of the results; Xuewen Su, Haifeng Yuan, and Zhongxia Dou contributed in drafting the manuscript. All authors read and approved the final version of the manuscript.

References

1. Liu S, Zhang X, Liu Y, Yuan X, Yang L, Zhang R, et al. Early application of caffeine improves white matter development in very preterm infants. Respir Physiol Neurobiol. 2020;281:103495.
2. Liu L, Oza S, Hogan D, Chu Y, Perin J, Zhu J, et al. Global, regional, and national causes of under-5 mortality in 2000-15: an updated systematic analysis with implications for the Sustainable Development Goals. Lancet. 2016;388(10063):3027-35.
3. Song J, Sun H, Xu F, Kang H, Gao L, Guo J, et al. Recombinant human erythropoietin improves neurological outcomes in very preterm infants. Ann Neurol. 2016;80(1):24-34.
4. Song J, Xu F, Wang L, Gao L, Guo J, Xia L, et al. Early amplitude-integrated electroencephalography predicts brain injury and neurological outcome in very preterm infants. Sci Rep. 2015;5:13810.
5. Volpe DSJ, Oliveira NCAC, Santos AC, Linhares MBM, Carlotti APCP. Neuropsychological outcome of children with traumatic brain injury and its association with late magnetic resonance imaging findings: A cohort study. Brain Inj. 2017;31(12):1689-94.
6. Bauer SC, Msall ME. Optimizing neurodevelopmental outcomes after prematurity: lessons in neuroprotection and early intervention. Minerva Pediatr. 2010;62(5):485-97.
7. Maxwell JR, Yellowhair TR, Oppong AY, Camacho JE, Lowe JR, Jantzie LT, et al. Cognitive development in preterm infants: multifaceted deficits reflect vulnerability of rigorous neurodevelopmental pathways. Minerva Pediatr. 2017;69(4):298-313.
8. Agut T, Alarcon A, Cabañas F, Bartocci M, Martinez-Biarge M, Horsch S, et al. Preterm white matter injury: ultrasound diagnosis and classification. Pediatr Res. 2020;87(1):37-49.
9. Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, Kinney HC. Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. J Neurosci. 2001;21(4):1302-12.
10. Khwaja O, Volpe JJ. Pathogenesis of cerebral white matter injury of prematurity. Arch Dis Child Fetal Neonatal Ed. 2008;93(2):F153-61.
11. Qian X, Tang J, Chu Y, Chen L, Chen Z, Li L. A prospective study of Salvia miltiorrhiza and Rhizoma chuanxiong preparation in the treatment of hypoparathyroidism after total thyroidectomy. Pak J Pharm Sci. 2021;34(6):2385-9.
12. Wei Z, Jinguo D. Effectiveness of Radix astragali and Salvia miltiorrhiza injection in treatment of skeletal muscle injury of aerobics athletes. Pak J Pharm Sci. 2018;31(4):1767-71.
13. Liang K, Zhai S, Zhang Z, Wang G, Fu X, Li X. Study on the small intestine absorptive kinetics characters of tanshinol and protocatechualdehyde of Salvia miltiorrhiza extracts in rats in vivo. Pak J Pharm Sci. 2016;29(4):1415-20.
14. Kim MS, Bang JH, Lee J, Kim HW, Sung SH, Han JS, et al. Salvia miltiorrhiza extract protects white matter and the hippocampus from damage induced by chronic cerebral hypoperfusion in rats. BMC Complement Altern Med. 2015;15:415.
15. Liu J, Shen HM, Ong CN. Salvia miltiorrhiza inhibits cell growth and induces apoptosis in human hepatoma HepG(2) cells. Cancer Lett. 2000;153(1-2):85-93.
16. Stumpf C, Fan Q, Hintermann C, Raaz D, Losert S, Daniel WG, et al. Anti-inflammatory effects of danshen on human vascular endothelial cells in culture. Am J Chin Med. 2013;41(5):1065-77.

17 Song YH, Liu Q, Lv ZP, Chen YY, Zhou YC, et al. Protection of a polysaccharide from Salvia miltiorrhiza, a Chinese medicinal herb, against immunological liver injury in mice. Int J Biol Macromol. 2008;43(2):170-5.

18 Su CY, Ming QL, Rahman K, Han T, Qin LP. Salvia miltiorrhiza: Traditional medicinal uses, chemistry, and pharmacology. Chin J Nat Med. 2015;13(3):163-82.

19 Han JY, Fan JY, Horie Y, Miura S, Cui DH, Ishii H, et al. Ameliorating effects of compounds derived from Salvia miltiorrhiza root extract on microcirculatory disturbance and target organ injury by ischemia and reperfusion. Pharmacol Ther. 2008;117(2):280-95.

20 Harauz G, Ladizhansky V, Boggs JM. Structural polymorphism and multifunctionality of myelin basic protein. Biochemistry. 2009;48(34):8094-104.

21 Agoston DV, Shutes-David A, Peskind ER. Biofluid biomarkers of traumatic brain injury. Brain Inj. 2017;31(9):1195-203.

22 Vassall KA, Bamm VV, Harauz G. MyelStones: the executive roles of myelin basic protein in myelin assembly and destabilization in multiple sclerosis. Biochem J. 2015;472(1):17-32.

23 Crescitelli R, Lasser C, Jang SC, Karimi N, Hoog JL, Iva J, et al. Subpopulations of extracellular vesicles from human metastatic melanoma tissue identified by quantitative proteomics after optimized isolation. J Extracell Vesicles. 2020;9(1):1722433.

24 Chen J, Zuo S, Wang J, Huang J, Zhang X, Liu Y, et al. Aspirin promotes oligodendrocyte precursor cell proliferation and differentiation after white matter lesion. Front Aging Neurosci. 2014;6:7.

25 Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. Stem Cells. 2010;28(1):152-63.

26 Rus H, Niculescu F. The complement system in central nervous system diseases. Autoimmunity. 2006;39(5):395-402.

27 Rus HG, Kim LM, Niculescu FI, Shin ML. Induction of C3 expression in astrocytes is regulated by cytokines and Newcastle disease virus. J Immunol. 1992;148(3):928-33.

28 Gasque P, Fontaine M, Morgan BP. Complement expression in human brain. Biosynthesis of terminal pathway components and regulators in human glial cells and cell lines. J Immunol. 1995;154(9):4726-33.

29 Shirazi Y, Rus HG, Macklin WB, Shin ML. Enhanced degradation of messenger RNA encoding myelin proteins by terminal complement complexes in oligodendrocytes. J Immunol. 1993;150(10):4581-90.

30 Wren DR, Noble M. Oligodendrocytes and oligodendrocyte/type-2 astrocyte progenitor cells of adult rats are specifically susceptible to the lytic effects of complement in absence of antibody. Proc Natl Acad Sci U S A. 1989;86(22):9025-9.

Citation of this Article

Xuewen S, Haifeng Y, Wanyu F, Ruixia S, Junlong C, Yanyan L, Hua Z and Zhongxia D. Salvia Miltiorrhiza Attenuates White Matter Injury Induced by Hypoperfusion in Neonatal Rats. Mega J Case Rep. 2022; 5: 2001-2011.

Copyright

© 2022 Zhongxia D. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cite.