The Proteomics Analysis of the Effects of Zhishi Rhubarb Soup on Ischemic Stroke

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Research

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Abstract

Background: Stroke has always been a big threat around the world, but most severe in China with 2.5 million new stroke cases each year and 7.5 million stroke survivors, which also has laid a heavy burden on social and national healthcare system. The Zhishi Rhubarb Soup (ZRS) is a Traditional Chinese Medicine (TCM) that has been used clinically for many years in China. In order to explore the potential mechanism of ZRS as the pathogen of stroke. In this study, liquid chromatography mass spectrometry (LC-MS) was employed to uncover the mechanism underlying the effects of ZRS on stroke.

Methods: In this study, the quantitative proteomics method LC-MS was used to analyze the proteomic differences of middle cerebral artery occlusion MCAO between normal and after ZSR treatment.

Results: Further liquid chromatography mass spectrometry (LC-MS) analysis could observe a total of 3,5006 peptide were identified, 5160.0 proteins identified, of which 4094.0 can be quantified. Differentially expressed proteins with significance were extracted by data analysis and subject to Gene Ontology (GO) analysis showed the overviews of dysregulated proteins in the biological process (BP), cell component (CC), and molecular function (MF) categories. Proteins related to brain repair was found to change significantly, including BDNF, IL-10, IL-6, TGF- β partly demonstrating the effects of ZRS on improving the tissue injury.

Conclusion: ZRS was found to contribute to brain repair caused by ZRS treatment, and this could be partly mediated through the anti-inflammation effect by upregulating the vitamin transport. The results confirmed that ZRS presented a unique protein profile that indicated the adaptive mechanisms in acute stroke.

Introduction

Stroke has always been a big threat around the world¹, but it's most severe in China with 2.5 million new stroke cases each year and 7.5 million stroke survivors². The number of stroke patients in China has risen steeply, and the death from stroke accounts for roughly one third of worldwide stroke mortality¹, which also has laid a heavy burden on social and national healthcare system. With the aging of populations advancing, situation could get worse. Limited therapies are accessible to treat the stroke patients, with tPA therapy and mechanical thrombectomy³⁻⁶, are currently used in clinic⁷. But, only a small percentage of ischemic stroke patients benefit from them due to the narrow therapeutic time window and absolute contraindications⁷.

Stroke can stimulate the growth and differentiation of endogenous neural stem cells (NSCS) in the adult hippocampus as a defense response to damage^{8,9}. Then the newborn neurons can migrate to the brain injury site and replace the damaged neurons¹⁰. However, the newborn neurons can be killed in a short time likely thoroughly apoptosis^{8,11-13}. Anti-neuroinflammation is an important aspect for neuroprotection^{10,14}. However, relationships between immune response and neurogenesis have always

been controversial^{14, 15}. Some studies showed that activated microglia cells could slow the neurogenesis and hamper the brain amelioration^{16, 17}, and neuro-inflammation have also been suggested as a target for treatment of stroke^{14, 18}; but immune response has been shown to promote the neurogenesis^{19, 20}, like transforming growth factor- β (TGF- β) and IL-10 are all anti-inflammatory cytokines and neurotrophic mediators^{21–23}, which suppress inflammation and facilitate axonal outgrowth and angiogenesis²⁰. The complex and multi-face of immune response in ischemic stroke constitutes a major challenge to the development of immunomodulatory therapies, if not impossible.

ZRS is a kind of Chinese herb medicine. In our research, it was previously shown that ZRS could promote the amelioration of brain damage caused by infarction stroke, but the mechanisms underlying this still remained unresolved. In this research, LC-MS was employed to uncover the mechanism underlying the effects of ZRS on stroke. Differentially expressed proteins were screened and classified, enriched, and compared among three groups. And it was found that ZRS could inhibit the inflammation and promote the neurogenesis and brain repair. And this effect is possibly related to the upregulation of vitamin transport-related proteins.

Materials And Methods

Materials

Protein kinase inhibitor was purchased from Calbiochem; trypsin was purchased from Promega; acetonitrile, Ultra-pure water (H₂O) from Fisher Chemical; trifluoroacetic acid, formic acid, Fluka, iodoacetamide, dithiothreitol, urea, Triethylammonium bicarbonate (TEAB) from Sigma. Zhishi Rhubarb Soup (ZRS), Chinese herbal regimen was Purchased from the Department of Pharmacy, Nanjing University of Traditional Chinese Medicine Affiliated to Nanjing University of Traditional Chinese Medicine, decocted according to the conventional method, concentrated to 2.5g/ml and stored at 4°C for later use. Zhishi Rhubarb Soup is consisted of cooked rhubarb 10g, citrus aurantium 10g, magnolia root 10g, scutellaria 10g, woody 3g, licorice 3g.

Ethics statement

All animal procedures and protocols were performed in accordance with The Guide for the Care and Use of Laboratory Animals (NIH publication, 85–23, revised 1996) and were reviewed and approved by the Animal Research Committee at National Research Institute of Chinese Medicine. IACUC protocol no: P-99-11; IACUC Approval No: A-99-1. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

The animal Model building:

The MCAO (middle cerebral artery occlusion) model was built using the thread bolt method²⁴. The method refers to the modified Longa suture method. Briefly, the experimental animals were anesthetized with 10% chloral hydrate (0.3ml/100g) in the abdominal cavity, fixed on the operating table supine (the

rectal temperature was controlled at $37.3\pm0.5^{\circ}\text{C}$), and the common carotid artery and vagus nerve were quickly exposed, separated and the proximal end of the common carotid artery and the external carotid artery were connected, thread the internal carotid artery for use, and cut a small opening at the upper end of the common carotid artery ligation from the bifurcation of the common carotid artery, Insert the prepared fishing line into the internal carotid artery along the common carotid artery, and ligate the internal carotid artery when it reaches the specified length (about 18 mm). The tether of different diameters was chosen according to the animal's weight and nutrition, then the incision sutured. After the operation, the body temperature was maintained at $37\pm0.5^{\circ}\text{C}$ with an irradiation lamp, and the rectal temperature, respiration and heart rate were monitored. The animal was kept in a cage until awake for further use.

Nerve function score: After the animal is awake for 2 hours, the rat's behavior and neurological symptoms were observed, and a score was given. According to Longa's 5-level standard scoring method: 0: normal, without any neurological deficits; 1: The front paw cannot be straight when lifted vertically; 2: Lean to the right and rotate to the right when walking; 3: The body falls to the right side while walking; 4: not walk spontaneously or have a consciousness disorder. According to the first score, animals with no neurological deficit, 4 points, dyspnea, early death, and subarachnoid hemorrhage found at the time of execution were discarded. Animals excluded from the group will be supplemented in subsequent experiments.

Animal grouping and administration

Group A (sham group): normal feeding, free drinking water.

Group B (MCAO group): Normally reared after modeling, with free drinking water.

Group C (ZRS group) (effective dose was screened and set at 10g crude drug/kg): start gavage 3h after model building, once a day for 7 days. And the hippocampus tissue was collected after treatment at day 7.

Protein extraction and trypsin treatment

An appropriate amount of tissue (rat hippocampus) was weighed into a mortar pre-cooled with liquid nitrogen, and liquid nitrogen is added to fully grind the tissue to powder. Then samples of each group were added with 4 times the volume of powdered lysis buffer (8 M urea, 1% protease inhibitor, 3 μM TSA, 50 mM NAM and 2 mM EDTA) and lysed by ultrasound. The cell debris is removed after centrifuge, the supernatant was transferred to a new centrifuge tube, and the protein concentration is determined using the BCA kit.

Dithiothreitol was added to the protein supernatant to a final concentration of 5 mM, and reduced at 56°C for 30 min. Then iodoacetamide was added to make the final concentration 11 mM, and supernatant was then incubated for 15 min at room temperature in the dark. The urea concentration of the sample is diluted to less than 2 M. pancreatin was added at a mass ratio of 1:50 (pancreatin: protein), and protein

was digested overnight at 37°C. Finally, the protein was subject to second enzymatic hydrolysis for 4h after with pancreatin added at a mass ratio of 1:100 (pancreatin: protein).

TMT labelling

The peptides digested by trypsin were desalted with Strata X C18 (Phenomenex), freeze-dried in vacuo, and then dissolved with 0.5 M TEAB and labelled according to the TMT kit operating instructions. briefly: thaw the labeling reagent and dissolve it with acetonitrile, mix it with the peptide and incubate at room temperature for 2 hours, mix the labeled peptide, remove the salt, and freeze-dry in vacuum.

HPLC fractionation

The peptides were fractionated by high pH reverse HPLC, and the column was Agilent 300Extend C18 (5µm particle size, 4.6 mm inner diameter, 250 mm length). The peptides were subject to a step gradient of 8%-32% acetonitrile, pH 9, and 60 components are separated in 60 minutes, and then the peptides are combined into 9 components, and the combined components are vacuum freeze-dried for subsequent operations.

LC-MS analysis

The peptides were dissolved in the mobile phase A of liquid chromatography (0.1% (v/v) formic acid aqueous solution) and separated using the EASY-nLC 1000 ultra-high-performance liquid-system. Mobile phase A is an aqueous solution containing 0.1% formic acid and 2% acetonitrile; mobile phase B is an aqueous solution containing 0.1% formic acid and 90% acetonitrile. Liquid gradient setting: 0-30 min, 12%~26%B; 30-52 min, 26%~40%B; 52-56 min, 40%~80%B; 56-60 min, 80%B. The flow rate was maintained at 320 nL/min.

The peptides are separated by the ultra-high-performance liquid system and injected into the NSI ion source for ionization and then analyzed by Orbitrap Fusion Lumos mass spectrometry. The ion source voltage is set to 2.0 kV, and the peptide precursor ions and their secondary fragments are detected and analyzed by high-resolution Orbitrap. The scanning range of the primary mass spectrum is set to 350-1550 m/z, and the scanning resolution is set to 60,000; the scanning range of the secondary mass spectrum is set to a fixed starting point of 100 m/z, and the secondary scanning resolution is set to 15,000. The data acquisition mode uses the data-dependent scanning (DDA) program, that is, the first 20 peptide precursor ions with the highest signal intensity are selected to enter the HCD collision cell and use 32% fragmentation energy for fragmentation after the first scan. Grade mass spectrometry analysis. In order to improve the effective utilization of the mass spectrometer, the automatic gain control (AGC) is set to 5E4, the signal threshold is set to 50000 ions/s, the maximum injection time is set to 70 ms, and the dynamic rejection time of the tandem mass spectrometry scan is set to 30 seconds to avoid precursor ions.

Database search

The secondary mass spectrum data was searched using Maxquant (v1.5.2.8). Search parameter settings: The database is Rattus_Uniprot_10116 (29955 sequences), an anti-database is added to calculate the false positive rate (FDR) caused by random matching, and a common contamination library is added to the database to eliminate the contaminating protein in the identification results Impact; set the restriction enzyme digestion method to Trypsin/P; set the number of missed cleavage sites to 2; set the minimum peptide length to 7 amino acid residues; set the maximum modification number of peptides to 5; first search and main search primary precursor ion The mass error tolerance is set to 20 ppm and 5 ppm, respectively, and the mass error tolerance of the secondary fragment ion is 0.02 Da. The cysteine alkylation is set as a fixed modification, and the variable modification is the oxidation of methionine, the acetylation of the N-terminus of the protein, and the deamidation (NQ). The quantitative method is set to TMT-6plex, and the FDR for protein identification and PSM identification is set to 1%.

Western Blot

Twenty micrograms of protein/well was loaded onto 10% gels for separation using sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE). The gels were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 or 0.20 μm pore size; Millipore, Billerica, MA, USA). The blotted membranes were blocked with 5% nonfat dry milk in a Tris-buffered saline solution (25 mM Tris, pH 7.5, and 150 mM NaCl) containing 0.05% Tween 20 (TBST) for 2 h at room temperature, followed by incubation with the diluted primary antibody against target protein for 4 h at room temperature. After washing for 10 min in TBST solution, the membranes were incubated with properly diluted secondary antibody conjugated with horseradish peroxidase for 2 h at room temperature. Western signals were developed using ECL chemiluminescent reagents from Thermo Scientific (Waltham, MA, USA). The β -actin levels were used as loading controls.

Statistical analysis

The two-sample two-tailed The T test method were employed to calculate the p-value. When p-value<0.05, the change of differential expression exceeding 1.2 is used as the change threshold for significant up-regulation, and the change threshold for significant down-regulation is less than 1/1.2. For biological or technical replicate samples, we use principal component analysis (PCA), relative standard deviation (RSD) and Pearson's Correlation Coefficient to evaluate protein quantitative repeatability.

Results

1. ZRS promoted the brain injury amelioration and improved the nerve function

The MCAO model was built and treated with or without ZRS accordingly. At day 7 after treatment, the behavior of rats was monitored to measure the nerve function, and the hippocampus was collected and subjected to TTC (Triphenyl tetrazolium chloride) stain. It was found that ZRS notably decrease the infarct volume (Fig. 1A and Fig. 1C). Besides, the nerve severity improved significantly (Fig. 1B).

2. The overview of results of proteomics analysis

At day 7 after treatment, the hippocampus was collected, protein extracted for MS test (Fig. 2A), and the results were compared among three groups. For each comparison group, two repeated experiments were conducted, and the shared up-regulation and down-regulation proteins are selected as the final differentially expressed protein of the comparison group. More attention will be paid to C/B compare group so as to better elucidate the mechanism of ZRS.

In the experiment, a total of 3,5006 peptide were identified through spectral analysis, 5160.0 proteins identified, of which 4094.0 can be quantified (Fig. 2B). When data from group C was compared to the group B (C/B), it was found that 37 proteins were upregulated, 16 proteins found to downregulate. In the B/A group, 172 proteins were elevated, expression of 187 proteins were decreased. In the C/A group, 200 proteins were elevated, 193 proteins were downregulated (Fig. 2C).

3. Analysis of Gene Ontology (GO), protein domains, KEGG pathways

In order to thoroughly understand the proteins identified and quantified, the functions and characteristics of these proteins were classified in terms of Gene Ontology (GO), protein domains, KEGG pathways, as well as the location of subcellular structures, then detailed annotations are made. Consistent with the mixture nature of ZRS in C/B groups, various pharmacological effects were observed, including energy metabolism, cell proliferation and development, general signal transduction (Fig. 3A). However, most of the upregulated protein located extracellularly (Fig. 3B).

After the screen and classification of differentially expressed protein, each comparable group was subject to the functional enrichment analysis in terms of GO analysis, KEGG levels. The aim is to detect whether these differentially expressed proteins have a significant enrichment trend in certain functional types. And it was found the highest enrichment of up-regulated proteins resides in the extracellular region (Fig. 4A); proteins related to vitamin transport are most highly enriched; proteins about ensheathment of neurons can also be observed to enrich to a high degree (Fig. 4B and 4C); in contrast to what's been observed in increased protein group, proteins response to TNF was found to decrease (Fig. 4D).

4. Heatmap of the Cluster analysis based on GO classification

To find the correlation between the functions of differentially expressed proteins in the comparison group, cluster analysis was performed. In the C/B comparison group (A), in contrast to the C/A comparison group (B), BP analysis showed that the degree of protein enrichment related to neurogenesis, repair, nervous system development was not high (Fig. 5A), but the upregulated protein related to vitamin transport exhibited a rather high degree of enrichment (Fig. 5A). Besides, some proteins related to immunosuppression, such as TNF, showed signs of downregulation and enrichment (Fig. 5A and 5B). In the CC analysis, consistent with the previous protein location analysis (Fig. 3B), extracellular proteins also showed a strong trend of enrichment (Fig. 5C).

5. Confirmation of differentially expressed proteins with western blot

To reconfirm what's observed in MS test, western blot was performed. BDNF is a marker of nerve regeneration^{25, 26}, it was observed to decreased in MCAO model, but recovered after ZRS treatment(Fig. 6A). While TNF- α and IL-6 are both factors that promote inflammation^{27, 28}, which were up-regulated in the vehicle group and down-regulated in the ZRS group (Fig. 6B). IL-10 is a factor that inhibits inflammation^{21, 22}, which was inhibited in treatment group(Fig. 6B). but the transforming nerve growth factor (TGF), which was thought to be involved in tissue remodeling and matrix deposition²⁹, was shown to elevated in the ZRS group(Fig. 6B).

Discussion

Extensive efforts have been made to uncover the pathophysiology of stroke but without many achievements. Worldwide it is still a major cause of mortality and disability due to the limited accessible treatment choices. There had previously been reported Chinese herbs could promote neurogenesis by inhibiting inflammation³⁰. In this research, we found that ZRS could ameliorate the brain injury caused by stroke. In treatment group, the improvement of brain infarction was observed after the ZRS treatment when compared to the MCAO group (Fig. 1A), which shows that ZRS does promote nerve regeneration. And this was further confirmed by the score improvement in behavior and neurological function (Fig. 1B). Following that, a series of experiments and analysis were performed to find out the mechanisms underlying this. To begin with, the differentially expressed proteins were identified, classified and subject to Gene Oncology analysis in terms of biological process, cellular component, and molecular function (Fig. 2). We found that functions of proteins were highly focused on binding and transporting activities (Fig. 3A), and located mostly extracellularly (Fig. 3B), which is consistent with the following enrichment analysis (Fig. 4). It was found extracellular proteins have the most highly degree of enrichments, including the ensheathments of neurons, which may indicate the process of neurogenesis. Finally, proteins related to brain repair was found to change significantly, including BDNF, IL-10, IL-6, TGF- β , partly demonstrating the effects of ZRS on improving the tissue injury.

In addition, the proteins of vitamin transport were also found to enrich in a high degree (Fig. 4B). Noteworthy is that it was previously found that vitamin, including vitamin D and vitamin A, could have a neuroprotective effect, which could be mediated through various signaling pathways³¹. Interestingly, vitamins are found to be related to immune responses^{32, 33}. Therefore, interesting questions could be raised whether ZRS could have some effects on the vitamin transportations and thus modulate the immune system to contribute the brain repair. Studies showed that activation of vitamin receptors could affect various processes including immune modulation, inflammation and detoxification³¹. Vitamin D hormone could stimulate transforming growth factor TGF β -1 and interleukin 4 (IL-4) production, which in turn may suppress inflammatory T cell activity³². It could possibly be that the activation of vitamin receptors triggers transport of vitamins, and suppress the immune response.

The relationship between vitamins and immune response is very complicated³⁴. On one hand vitamin may enhance immunity³⁵, on the other hand it may suppress it^{36, 37}. With regard to the stroke, it is very

likely that at the beginning of brain repair vitamins serve to enhance immunity and eliminate infarcted nerve cells, and then turn to suppress the immune response to make new neural stem cells survive for a longer time. An interesting question is that whether the high expression and enrichment of surface membrane proteins is related to the transportation of vitamins, which also need further research. In MS data analysis, some plasma proteins like albumin³⁸, apoa1³⁹ were found to be upregulated and enriched (data not shown), which were found to correlate with lipid transport. What's more interesting was that vitamin and lipid transport share some transporters⁴⁰.

In the heatmap of cluster analysis(Fig. 5), the degree of protein enrichment related to neurogenesis and repair and nervous system development is not high. This could probably be attributed to the tissue collection timepoint. It was at day 7 after treatment the brain tissue was collected, when the brain repair process could have come to an end⁴¹. The acute phase is generally considered to last for 24 hours to 1 week, but the subacute phase 1 to 3 weeks^{41, 42}, which could varied depending on the specific circumstances. Therefore, studies of different time-points are also needed to better track the mechanism of ZHS amelioration on infarction stroke.

Though the positive results observed, the pharmacological effects of ZRS were far too diverse due to the mixture nature inherent in the regimen that many side effects could also been dug out in MS data analysis but truth had been buried underneath. Thus, to more clearly pinpoint the exact role of each ingredient in ZRS, the isolation and purification of botany methodology is needed to obtain the pure component.

Conclusion

This study is the first to conduct quantitative proteomics using LC-MS/MS to identify differentially expressed proteins in stroke with ZRS. The results confirmed that ZRS presented a unique protein profile that indicated the adaptive mechanisms in acute stroke. ZRS was found to contribute to brain repair caused by ZRS treatment, and this could be partly mediated through the anti-inflammation effect by upregulating the vitamin transport. However, much more detail and evidence are needed from further research to uncover mechanism underlying the ZRS effect on stroke.

Abbreviations

MCAO, middle cerebral artery occlusion, TNF- β , tumor necrosis factor; tPA, tissue plasminogen activator; VDR, vitamin D receptor; TGF- β , transforming growth factor- β ; ZRS, Zhishi Rhubarb Soup; EDTA, ethylenediaminetetraacetic acid; NSCS, endogenous neural stem cells; LC-MS, liquid chromatography mass spectrometry; NIH, national institutes of health; TMT, tandem mass tags; BDNF, brain-derived Neurotrophic factor, IL-6, interleukin-6; Apoa1, ATP-Binding Cassette Transporter A1.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

JHZ, and YZ contributed conception and design of the study; JHZ and YJS performed the experiment and analyzed the data, YJS wrote the initial draft of the manuscript; JHZ and YJS created and arranged the Figs; ZH, SLW and CH arranged the study funds; All authors contributed to manuscript revision, read and approved the submitted version.

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Availability of data and materials.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The MCAO model was built and treated with or without ZRS accordingly. At day 7 after treatment, the behavior of rats was monitored to measure the nerve function, and the hippocampus was collected and subjected to TTC (Triphenyl tetrazolium chloride) stain. It was found that ZRS notably decrease the infarct volume (figure 1A and figure 1C). Besides, the nerve severity improved significantly (figure 1B).

figure2 the workflow and results of proteomics analysis

Figure 2

At day 7 after treatment, the hippocampus was collected, protein extracted for MS test (Figure2A), and the results were compared among three groups

figure3

In order to thoroughly understand the proteins identified and quantified, the functions and characteristics of these proteins were classified in terms of Gene Ontology (GO), protein domains, KEGG pathways, as well as the location of subcellular structures, then detailed annotations are made. Consistent with the mixture nature of ZRS in C/B groups, various pharmacological effects were observed, including energy metabolism, cell proliferation and development, general signal transduction (Figure3A). However, most of the upregulated protein located extracellularly (Figure3B).

A cellular component

B biological process

C molecular function

D biological process

The aim is to detect whether these differentially expressed proteins have a significant enrichment trend in certain functional types. And it was found the highest enrichment of up-regulated proteins resides in the extracellular region (figure4A); proteins related to vitamin transport are most highly enriched; proteins about ensheathment of neurons can also be observed to enrich to a high degree (figure4B and 4C); in contrast to what's been observed in increased protein group, proteins response to TNF was found to decrease (figure4D).

figure5

图16 基于GO分类、KEGG通路和蛋白结构域富集的聚类分析热图，几个不同的角度都展现出同样的趋势

Figure 5

To find the correlation between the functions of differentially expressed proteins in the comparison group, cluster analysis was performed. In the C/B comparison group (A), in contrast to the C/A comparison group (B), BP analysis showed that the degree of protein enrichment related to neurogenesis, repair, nervous system development was not high (figure5A), but the upregulated protein related to vitamin transport exhibited a rather high degree of enrichment (figure5A). Besides, some proteins related to immunosuppression, such as TNF, showed signs of downregulation and enrichment (figure5A and 5B). In the CC analysis, consistent with the previous protein location analysis (figure3B), extracellular proteins also showed a strong trend of enrichment (figure5C).

Figure 6

To reconfirm what's observed in MS test, western blot was performed. BDNF is a marker of nerve regeneration^{25, 26}, it was observed to decreased in MCAO model, but recovered after ZRS treatment (figure 6A). While TNF- α and IL-6 are both factors that promote inflammation^{27, 28}, which were up-regulated in the vehicle group and down-regulated in the ZRS group (figure 6B). IL-10 is a factor that inhibits inflammation^{21, 22}, which was inhibited in treatment group (figure 6B). but the transforming nerve growth factor (TGF), which was thought to be involved in tissue remodeling and matrix deposition²⁹, was shown to elevated in the ZRS group (figure 6B).