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Original Article

Buyang Huanwu Tang alleviates inflammation and improves motor endplate functions in DSMA rat models by activating several biological molecules and associated signaling pathways

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Abstract: Denervated-dependent skeletal muscle atrophy (DSMA) is considered to be the neuro-disconnection of skeletal muscle. This study aimed to investigate the protective effects of Buyang Huanwu Tang (BYHWT) on the DSMA and clarify associated molecular and genetic mechanisms. DSMA rat models were established according to the previously published study and divided into Model group and BYHWT group. Meanwhile, normal rats were assigned as Normal control (NC) group. Hematoxylin and eosin (HE) staining was used to examine inflammatory responses. Motor endplate activity was evaluated with wholemount acetylcholinesterase (AChE) staining. Mass-spectrometry analysis was conducted to compare differentially expressed proteins. RNAs were prepared and applied to gene functional analysis. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were employed to analyze biological functions. The results indicated that BYHWT remarkably alleviated inflammatory responses and significantly improved motor endplate function, compared to that in DSMA Model rats ($P<0.05$). In BYHWT group, there were 393 differentially up-regulated and 576 differentially down-regulated molecules compared to that in Model group. Comparing to Model group, the cellular response to interferon-gamma, integral component of plasma membrane and voltage-gated potassium channel activity genes in BYHWT group were the most biological process (BP), cellular component (CC) and molecular function (MF) differential genes, respectively. Fructose/mannose metabolism and glycerolipid metabolism KEGG signaling pathways illustrated the most significant enrichment of differentially expressed genes. In conclusion, BYHWT alleviated the inflammations and improved the motor endplate function of DSMA rats by activating cellular response to interferon-gamma, integral component of plasma membrane and voltage-gated potassium channel activity genes and associated signaling pathways.

Keywords: Denervated-dependent skeletal muscle atrophy, Buyang Huanwu Tang, differentially expressed genes, inflammatory response

Introduction

In recent years, denervated-dependent skeletal muscle atrophy (DSMA) is extensively known as a kind of peripheral neuro-disconnection disorder which also induces the other skeletal muscle associated diseases [1, 2]. Clinically, DSMA is usually caused by the pharmacological or therapeutic factors and could induce the damage or destroy for skeletal muscle and traumatic peripheral nerve injuries [3, 4]. When symptoms or representations of SMA-related factors combining with the muscle denervation, DSMA always demonstrates plenty of irreversible or harmful dysfunction of skeletal muscles [5]. However, all of the above symptoms can't be fully rescued by using any other single therapeutic approach [6, 7]. In spite of the extensively used therapeutic approaches for DSMA, the therapeutic effects or outcomes of which are not satisfy and the pathological mechanisms or reasons of which have not been fully clarified.

Buyang Huangwu Tang (BYHWT) is designed according to the Traditional Chinese Medicine theory, which is incline to the theory of "supple-

menting *qi*" [8]. The previous study [9] reported that BYHWT plays many critical roles in improving human's health, including clearing collaterals, promoting circulation of blood, removing blood stasis, suppressing cerebral ischemia-reperfusion and treating vascular dementia. Moreover, BYHWT could inhibit the apoptosis of cells by regulating mitochondrial functions or via the mitochondrial signaling pathway [10]. Our previous study [11] also reported that BYHWT could improve DSMA by enhancing the ANGPTL4 and modulating NF-kB/MURF1 expression. Thus, BYHWT might also play the anti-neurotoxicity roles and improve the skeletal muscle status by regulating a few genes and the associated signaling pathways.

Therefore, the present study aimed to clarify molecular and genetic mechanisms for the protective effects of BYHWT on the DSMA. In the present investigation, BYHWT was administrated to DSMA rat models for improving the inflammation and motor endplate function and exploring the associated pathogenesis.

Materials and methods

Rats and BYHWT components

Sprague-dawley (SD) rats (weighting from 180 g to 220 g, aging from 8 weeks to 10 weeks, purchasing form Huafukang BioSci. Co. Ltd., Beijing, China) were fed in cages and freely accessed to the food and water at 25°C. All experiments or tests in present investigation were approved by the Ethical Committee of Nanjing University of Traditional Chinese Medicine, Nanjing, China. Meanwhile, this study was conducted according to the Guidelines of Institutional Animal Care.

The BYHWT is compounded according to the Traditional Chinese Medicine recording and includes plenty of components as our previously published study reported [11]. All of the components composing BYHWT were purchased from Tong Ren Tang Co. Ltd. (Beijing, China). Moreover, the other chemical reagents composing the BYHWT, including K_3PO_4 K_2HPO_4, $MgCl_2$, were also used in this study (Kelong Co. Ltd., Chengdu, China).

Establishment of DSMA rat model

In this study, 8 SD rats were selected to establish the DSMA rat models. For establishing DSMA models, the SD rats were anesthetized by intraperitoneally injecting with 7% chloral hydrate at final dosage of 1.0 ml/200 g rat body-weight. In brief, the detailed DSMA establishing procedures were conducted based on the previously published study [11].

BYHWT treatment and trial grouping

Total of 8 DSMA rat models were randomly divided into Model group (n=4) and HYHWT group (n=4, intragastricly administrating with BYHWT stock solution). While, another 4 rats were employed as the normal control (NC, treating without any solution) group.

Hematoxylin and eosin (HE) staining

In order to evaluate the inflammation of aterior cervical muscles, the tissues were isolated, treated and fixed by using 4% formaldehyde (Beyotime Biotech. Shanghai, China) in PBS solution (Beyotime Biotech.). Briefly, the above treated aterior cervical muscle tissues were stained with hematoxylin and eosin, according to the previous study [12]. The inflammation of HE stained tissues was captured by using an Olympus AX70 digital microscope (Olympus, Tokyo, Japan) with magnification of 100 ×.

Motor endplates examination

In this study, in order to identify the motor endplate activities, the wholemount acetylcholinesterase (AChE) was employed to stain the aterior cervical muscles of DSMA rat modes. The isolated and dissected aterior cervical muscles were stained with AChE to identify the distribution and localization for motor endplates of DSMA rat models. In brief, staining procedures for wholemount AChE were conducted depended on the previous study reported [13].

Two-dimensional (2D) electrophoresis and mass spectrometry

The 2D gel electrophoresis and mass spectrometry were used to identify the molecules or proteins in the aterior cervical muscles of BYHWT group, Model group and NC group. The concentrations of proteins extracted from aterior cervical muscles were determined by using commercial Amersham Biosciences 2-D quant kit (GE health Care, Piscataway, NJ, USA). Then, the extracted proteins were applied for the 2D

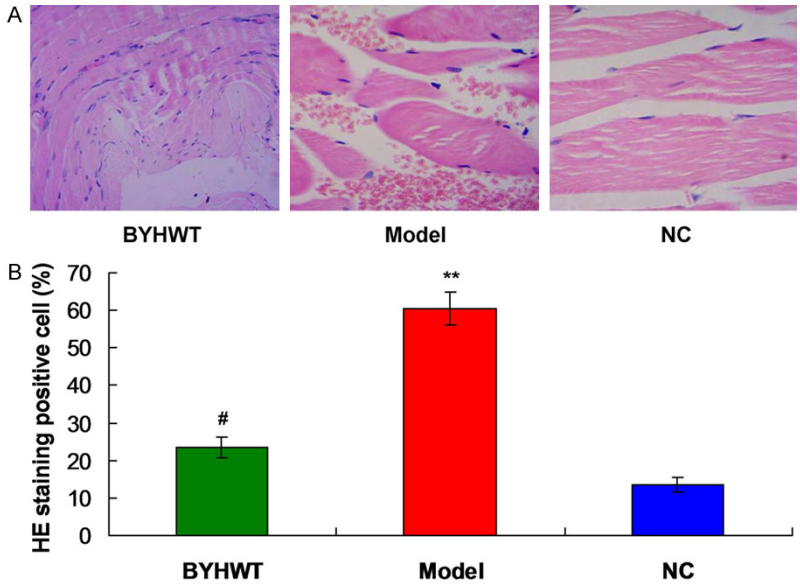

Figure 1. HE staining for evaluating inflammation in aterior cervical muscle tissues of DSMA rat models. A. HE staining images for inflammation in aterior cervical tissues. B. Statistical analysis for the HE staining results. $^{**}P<0.01$ *vs.* Model group, $^{\#}P<0.05$ *vs.* NC group.

electrophoresis and mass spectrometry according to the previously published report [14].

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

The molecular functions of the proteins separated by 2D electrophoresis and the molecules identified by the MS spectrometry in different groups were evaluated by using GO analysis. GO annotations were conducted by utilizing the PANTHER and QuickGO to search the homologies manually in this study, according to the previous studies [15, 16]. The lists for all identified molecules were reduced to the list that assigns as non-redundant molecules (or proteins) for the aterior cervical muscles in each group. GI accession number for the above molecules (or proteins) was up-loaded to the PANTHER to categorize the molecules depending on the molecular or biological functions in aterior cervical muscles in each group. The un-annotated molecules (or proteins) identified from the PANTHER were analyzed with a further step by employing QuickGO and by searching manually with the GO website [17].

Meanwhile, the KEGG analysis was also conducted to analyze the biological functions, according to the previously published study [18].

Statistical analysis

Data in this study were assigned as mean ± SD and analyzed by using the professional SPSS software 19.0 (SPSS Inc., Chicago, Ull, USA). Student's *t* test was used for comparing the data between two groups. The Tukey's post-hoc test was employed to validate the analysis of variance (ANOVA) for comparing data among multiple groups. $P<0.05$ was assigned as statistical significance.

Results

BYHWT inhibited inflammation in DSMA models

The previous study [19] reported that there are obvious inflammatory factors in muscle tissues of DSMA rat models. Therefore, the inflammations were evaluated by using HE staining. HE staining results indicated that there were many inflammatory cells appeared in DSMA rat Model group, however, there were only a few amounts of inflammatory cells in NC and BYHWT treatment group (**Figure 1A**). The statistical analysis findings also showed that BYHWT treatment significantly reduced the amounts of inflammatory cells compared to that in the Model group (**Figure 1B**, $P<0.01$).

BYHWT increased acetylcholinesterase levels

To evaluate function for the motor endplates, the activity of AChE was evaluated in this study (**Figure 2A**). The acetylcholinesterase staining results illustrated that only limited regions were positively stained in the Model group. However, the plenty part of tissues were positively stained in the BYHWT group and NC group. The statistical analysis results showed that levels of AChE were decreased significantly in DSMA rat Model group compared to that in the NC group (**Figure 2B**, $P<0.05$). However, BYHWT treatment significantly enhanced the levels of AChE compared to that in the Model rat group (**Figure 2B**, $P<0.01$).

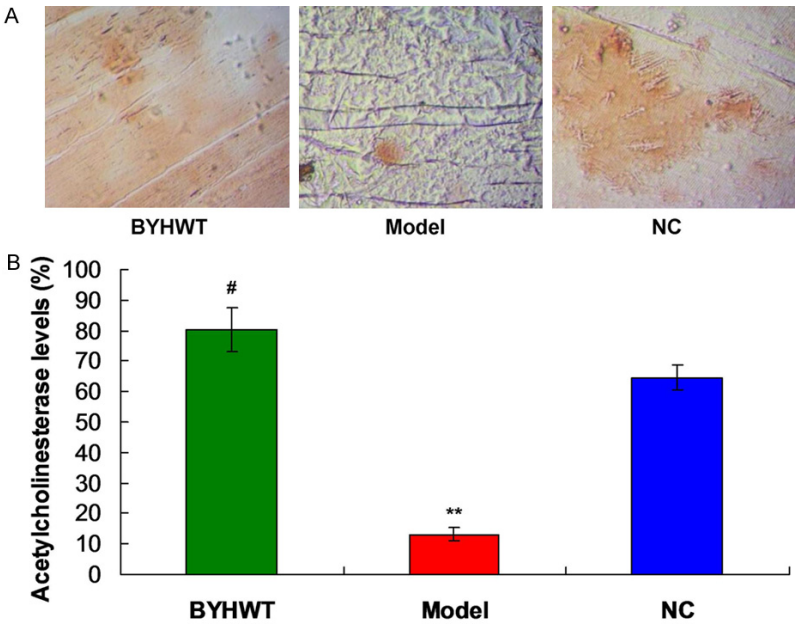

Figure 2. Evaluation for improving effects of BYHWT on motor endplate activity by testing the acetylcholinesterase levels. A. Images for acetylcholinesterase stained aterior cervical muscle tissues. B. Statistical analysis of the acetylcholinesterase stained images. $^{**}P<0.01$ *vs.* Model group, $^{\#}P<0.05$ *vs.* NC group.

BYHWT activated differentially expressed genes comparing with Model rats

In order to investigate the mechanism for BYHWT triggered inflammation inhibition and AChE activity enhancement, the differentially expressed genes that triggered by BYHWT were identified in the present study (**Figure 3**). These results showed that comparing with the Model group, there were 393 genes differentially up-regulated and 576 genes differentially down-regulated in BYHWT group, with total of 969 changed genes. Comparing with NC group, there were 1351 genes up-regulated and 1062 genes down-regulated in BYHWT group, with total of 2413 changed genes. Meanwhile, comparing with NC group, there were 1771 genes up-regulated and 1545 genes down-regulated in Model group, with total of 3316 changed genes.

Identification for the data distribution

The data distribution was evaluated by using Box-whisker Plot, Scatter Plot and PCA approaches according to the previous study [20]. The Box-whisker Plot results showed that there were no significant differences for the normalized intensity values among BYHWT, Model and NC group (**Figure 4A**). The above results suggests that all data characterizing by good symmetry. Scatter Plot findings illustrated better overall distribution tendency comparing the BYHWT *vs.* Model, BYHWT *vs.* NC and Model *vs.* NC (**Figure 4B**). PCA results indicated that the data among all groups exhibited good bio-repeatability (**Figure 4C**).

BYHWT regulated biological processes, cellular components and molecular functions

The GO analysis recordings showed that there were 20 biological processes (**Figure 5A**, especially for the cellular response to interferon-gamma and cellular response to mechanical stimulus) up-regulated and 20 biological processes (**Figure 5B**, especially for the collagen fibril organization, chromosome segregation, mitotic cytokinsis) down-regulated in BYHWT group, when comparing with Model group. There were 20 cellular components (**Figure 6A**, especially for the integral component of plasma membrane, dendrite, voltage-gated potassium channel complex and neuronal cell body) up-regulated and 20 cellular components (**Figure 6B**, especially for the extra-cellular matrix, proteinaceous extra-cellular matrix and extra-cellular space) down-regulated in BYHWT group, when comparing with Model group. Also, there were 20 molecular functions (**Figure 7A**, especially for the voltage-gated potassium channel activity, glutathione transferase activity) up-regulated and 20 molecular functions (**Figure 7B**, especially for the heparin binding, extra-cellular matrix structural constituent and integrin binding) down-regulated in BYHWT group, when comparing with the Model group.

BYHWT triggered KEGG pathways with significant enrichment of differentially expressed genes

We used KEGG pathway enrichment analysis to observe BYHWT treatment triggered differen-

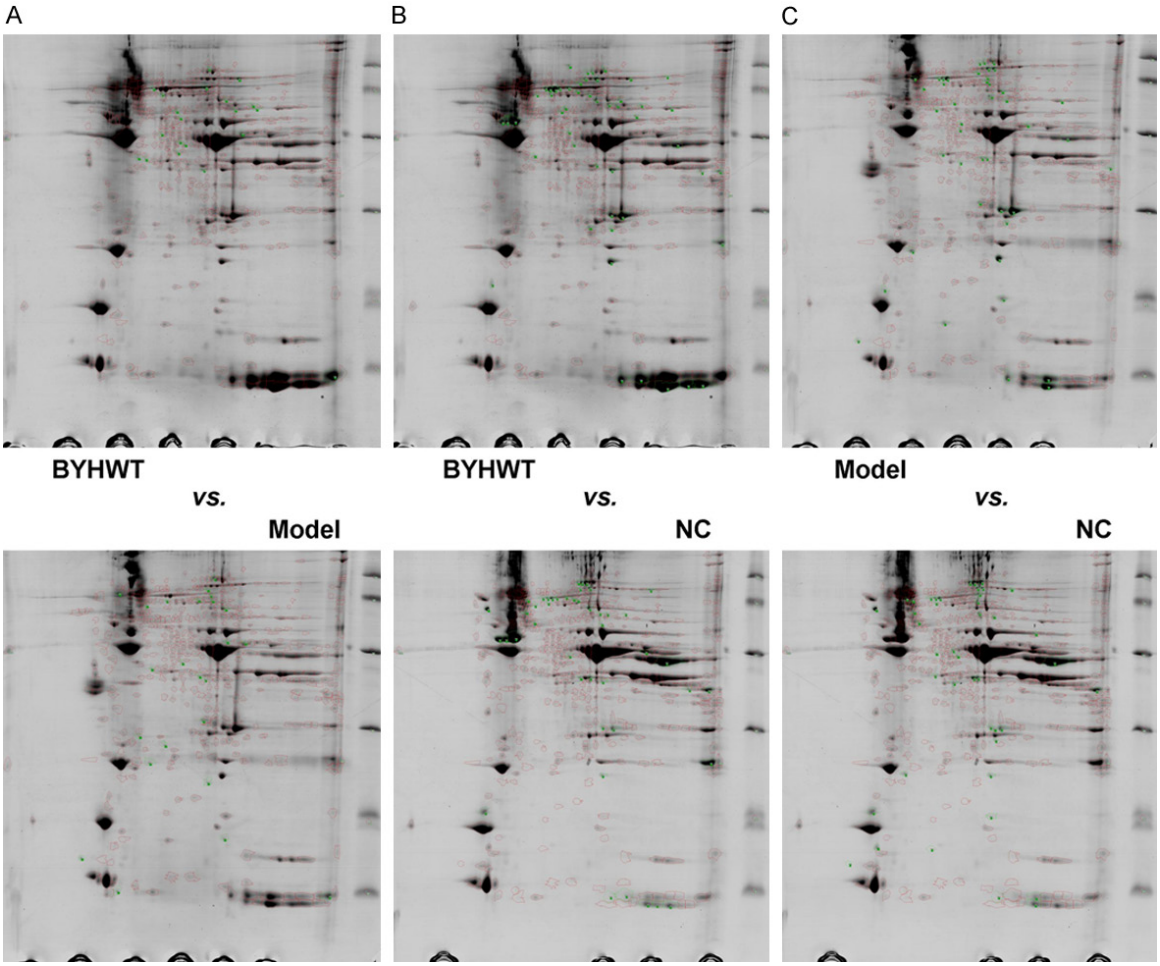

Figure 3. Representative 2D gel images for the aterior cervical muscle tissues isolating from rats in different groups. A. Images for BYHWT vs. Model. B. Images for BYHWT vs. NC. C. Images for Model vs. NC.

tially expressed genes in aterior cervical muscles and to explore the associated cellular signaling pathways. The findings illustrated that there were 3 KEGG signaling pathways with the most significant enrichment of differentially up-regulated genes in BYHWT group comparing with Model group, including fructose and mannose metabolism signaling pathway, glycerolipid metabolism signaling pathway and galactose metabolism signaling pathway (**Figure 8A**). There were 4 KEGG signaling pathways with the most significant enrichment of differentially down-regulated genes in BYHWT group comparing with Model group, including cell-cycle signaling pathway, ECM-receptor interaction signaling pathway, protein digestion and absorption signaling pathway and malaria signaling pathway (**Figure 8B**).

Moreover, there were 10 KEGG signaling pathways with the most significant enrichment of differentially up-regulated genes in Model group comparing with NC group (**Figure 8C**). There were 5 KEGG pathways with the most significant enrichment of differentially down-regulated genes in Model group comparing with NC group (**Figure 8D**).

Discussion

In the present study, we discovered that BYHWT treatment could relieve the denervation inflammation and enhance motor endplates activity of DSMA rat models. Also, this study discovered the molecules and signaling pathways that involve in the protective effects of BYHWT on DSMA rat models.

The previous studies [11, 21] reported that BYHWT is considered to be a Traditional Chinese Medicine and plays potential neuro-protective roles, anti-ischemic stroke roles and

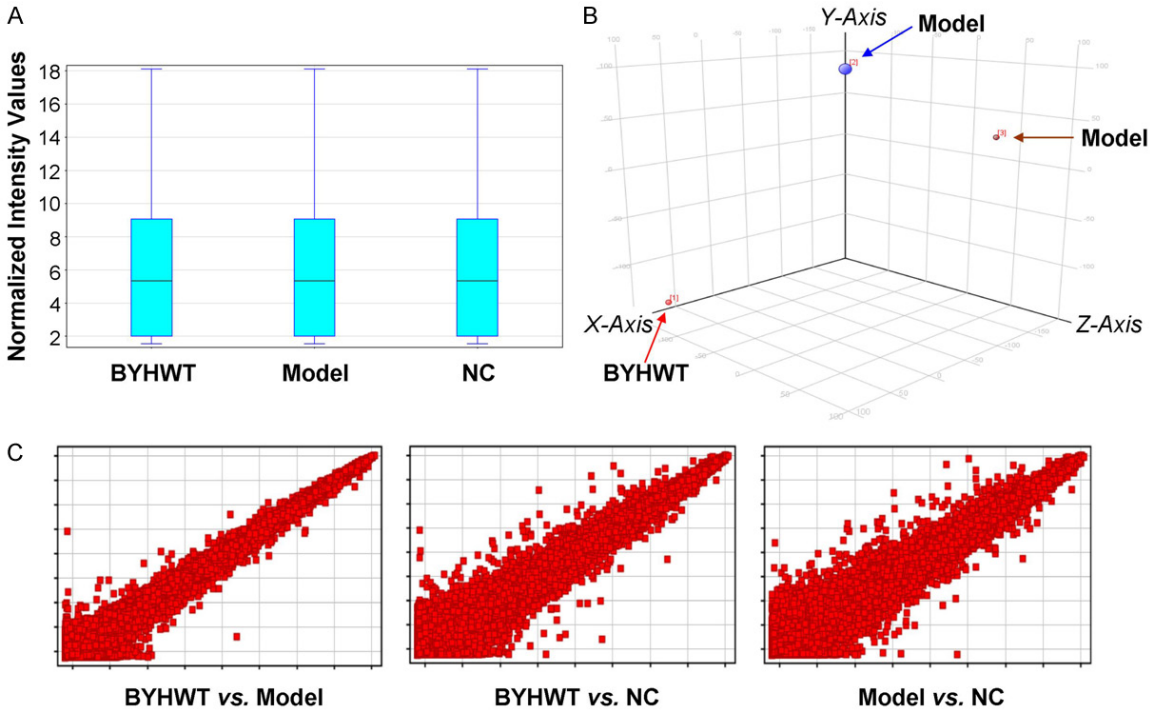

Figure 4. Identification for the data distribution by using Box-whisker Plot (A), Scatter Plot (B) and PCA (C) approaches, respectively.

protective effects on muscle atrophy. Therefore, to evaluate the anti-inflammation effects of BYHWT on DSMA rat models, HE staining was conducted firstly. The results illustrated that BYHWT significantly decreased the inflammations comparing with the Model group, which suggests that BYHWT effectively suppressed nerve damage related inflammation in DSMA rat models. This result is consistent with the previous study [11].

According to the previous study, the activity of motor endplates could reflect functions of skeletal muscle, and which is usually evaluated by detecting wholemount acetylcholinesterase (AChE) levels [22]. Our results demonstrated that AChE activity in Model group was significantly enhanced compared to that in the Model group, which suggests that BYHWT remarkably increased activity of motor endplates and significantly improve the functions of skeletal muscle. In the following study, application of BYHWT might become a potential therapeutic tool for treating the DSMA.

In the previous studies [23-25], although the functions of BYHWT have been investigated, the specific mechanisms and associated molecular signaling pathways have not been fully clarified. In the present study, the gene microarray results showed that comparing with Model group, there were 393 genes up-regulated and 576 genes down-regulated in BYHWT group, with total of 969 changed genes. Therefore, the above 393 up-regulated molecules and 576 down-regulated molecules were subjected to the following GO analysis and KEGG analysis, all of which might participate in the protective effects of BYHWT on the DSMA. A few former reports [26, 27] utilized the GO and KEGG analysis for investigating the differentially expressed genes or molecules, which are critical for predicting the signaling pathways for the pathogenesis. The GO analysis demonstrated that there were 20 biological processes, especially for cellular response to interferon-gamma, cellular response to mechanical stimulus, were up-regulated and 20 biological processes, especially for the collagen fibril organization, chromosome segregation, mitotic cytokines, were down-regulated in BYHWT group, when comparing with Model group. These findings suggest that the BYHWT might impact the DSMA by affecting the intracellular biological processes, even in the chromosome levels. Meanwhile, there were 20 cellular components, especially for the integral component of plasma membrane, dendrite, voltage-gated potassium

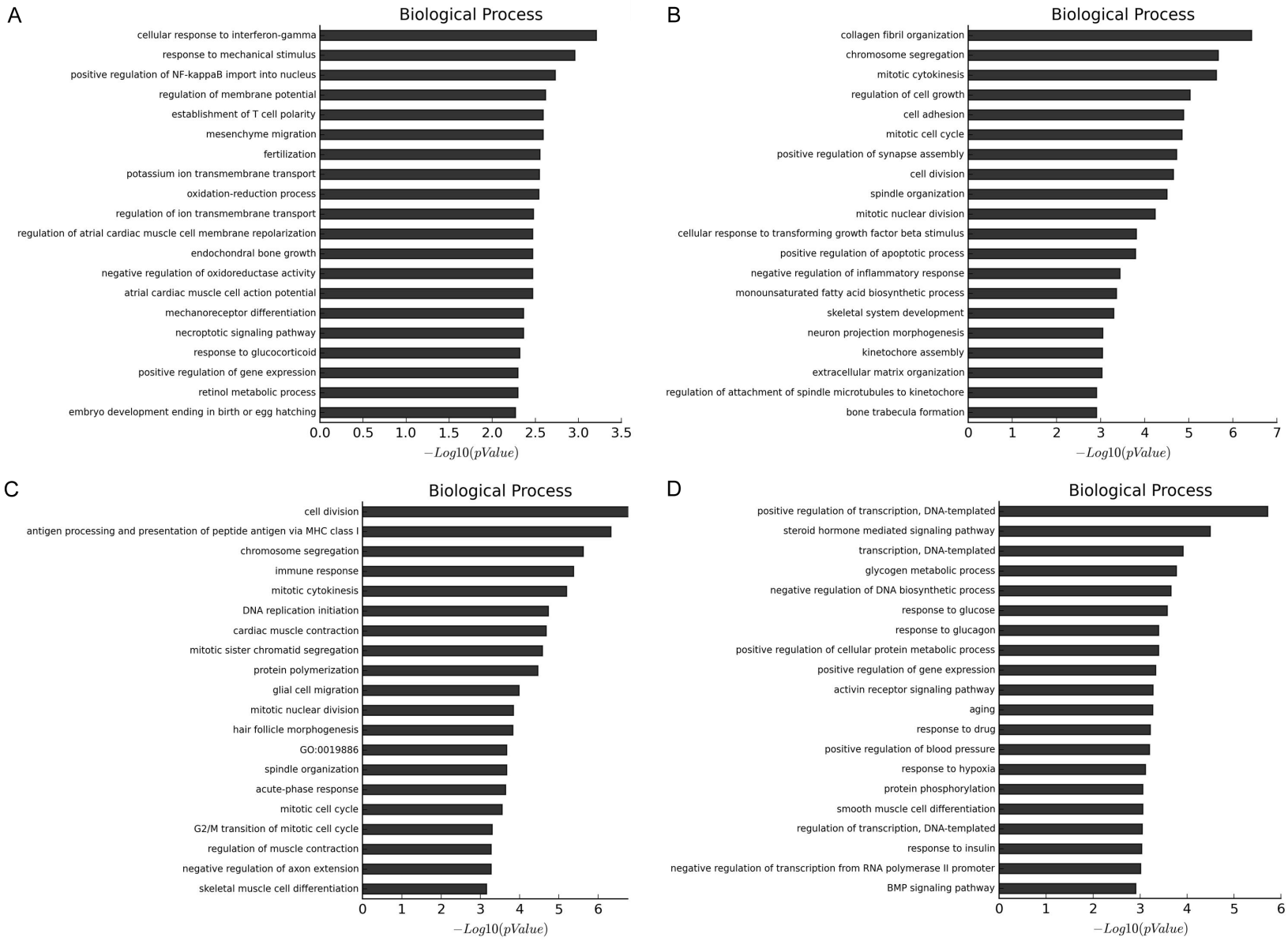

Figure 5. Identification for the BYHWT triggered biological processes involving in neuro-protection using GO analysis. A. Differentially up-regulated biological processes in BYHWT group comparing with Model group. B. Differentially down-regulated biological processes in BYHWT group comparing with Model group. C. Differentially up-regulated biological processes in Model group comparing with NC group. D. Differentially down-regulated biological processes in Model group comparing with NC group.

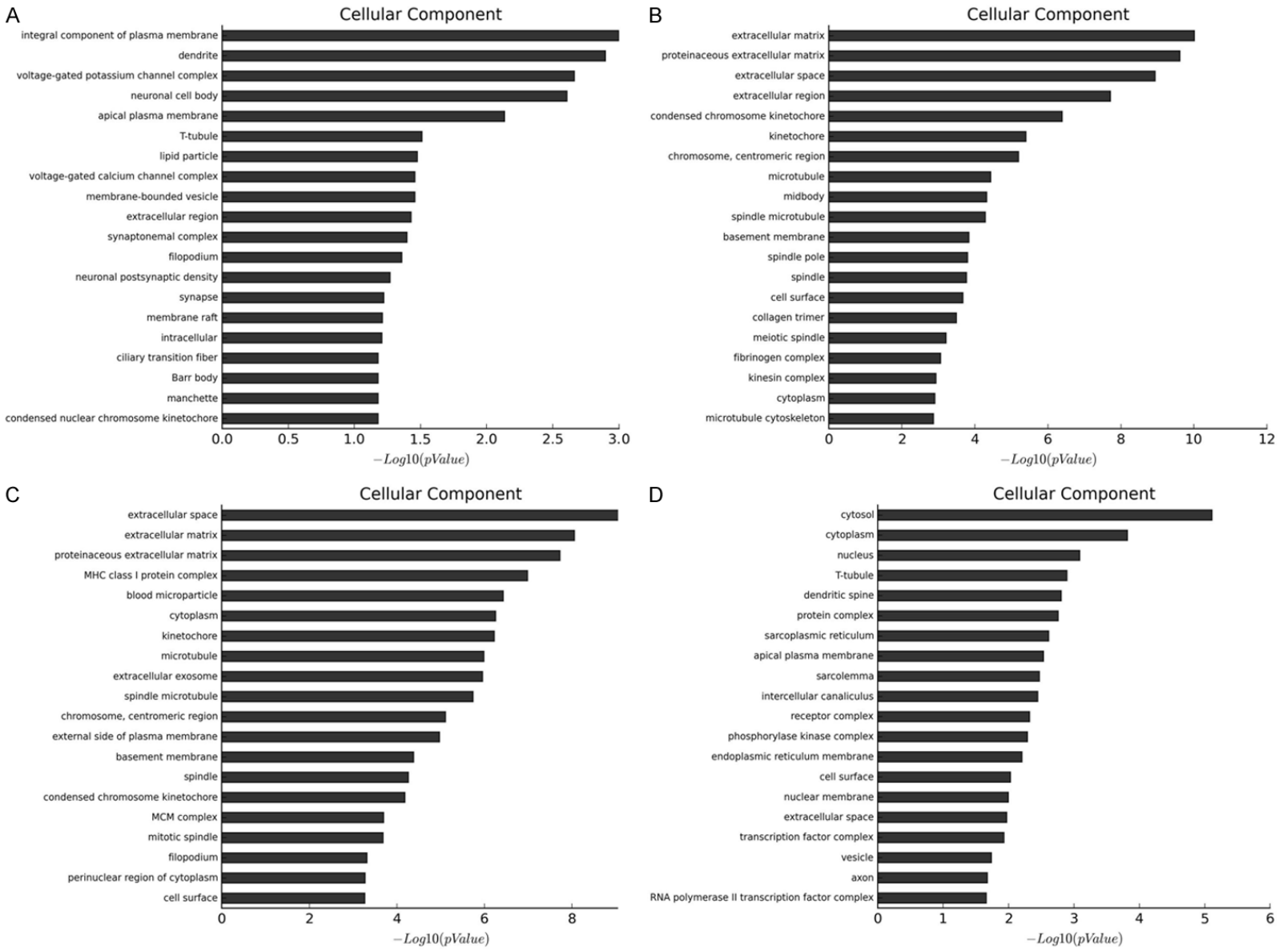

Figure 6. Screening for BYHWT triggered cellular components involving in protective effects using GO analysis. A. Differentially up-regulated cellular components in BYHWT group comparing with Model group. B. Differentially down-regulated cellular components in BYHWT group comparing with Model group. C. Differentially up-regulated cellular components in Model group comparing with NC group. D. Differentially down-regulated cellular components in Model group comparing with NC group.

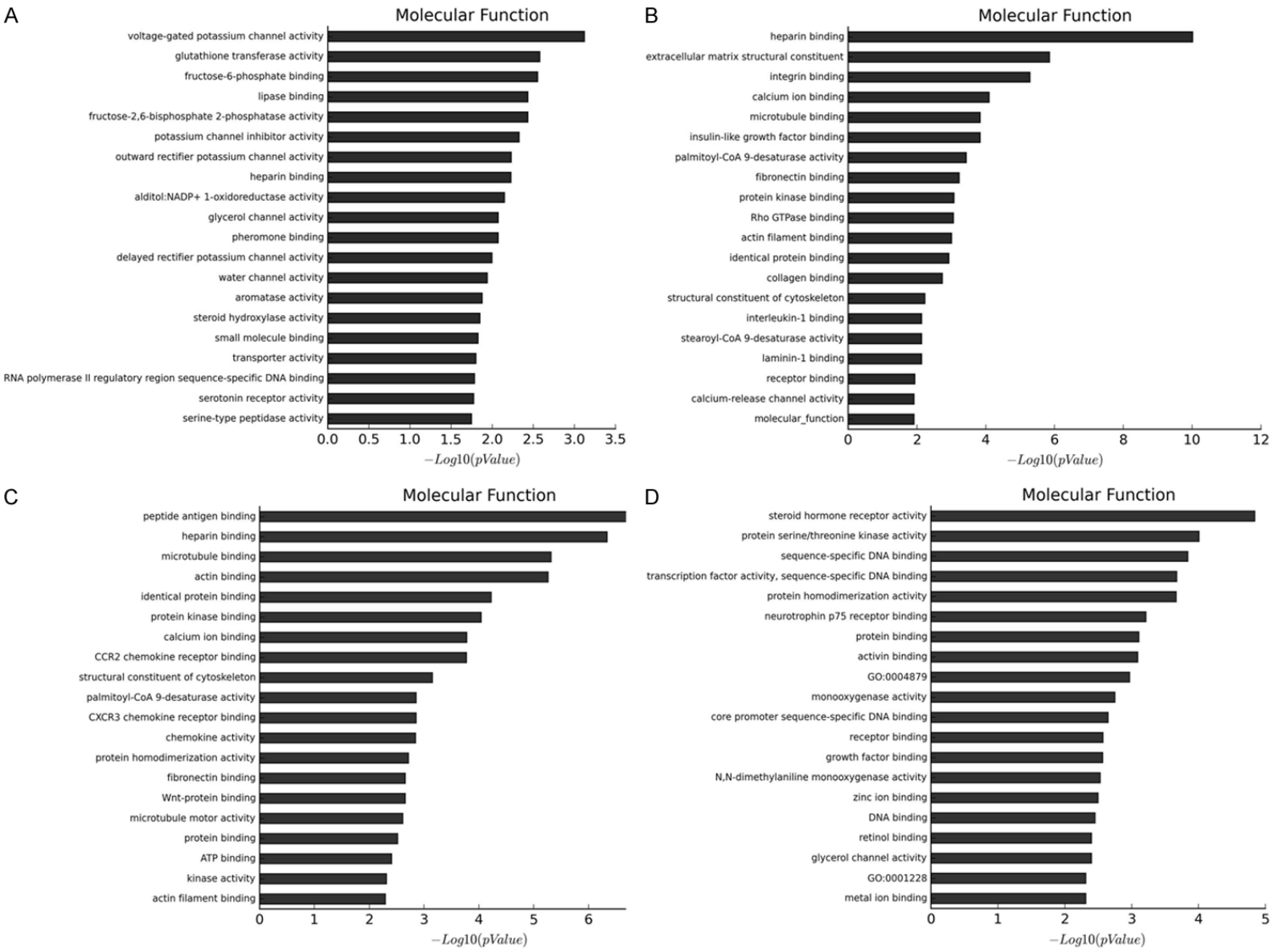

Figure 7. Determination for the molecular functions involving in protective effects of BYHWT using GO analysis. A. Differentially up-regulated molecular functions in BYHWT group comparing with Model group. B. Differentially down-regulated molecular functions in BYHWT group comparing with Model group. C. Differentially up-regulated molecular functions in Model group comparing with NC group. D. Differentially down-regulated molecular functions in Model group comparing with NC group.

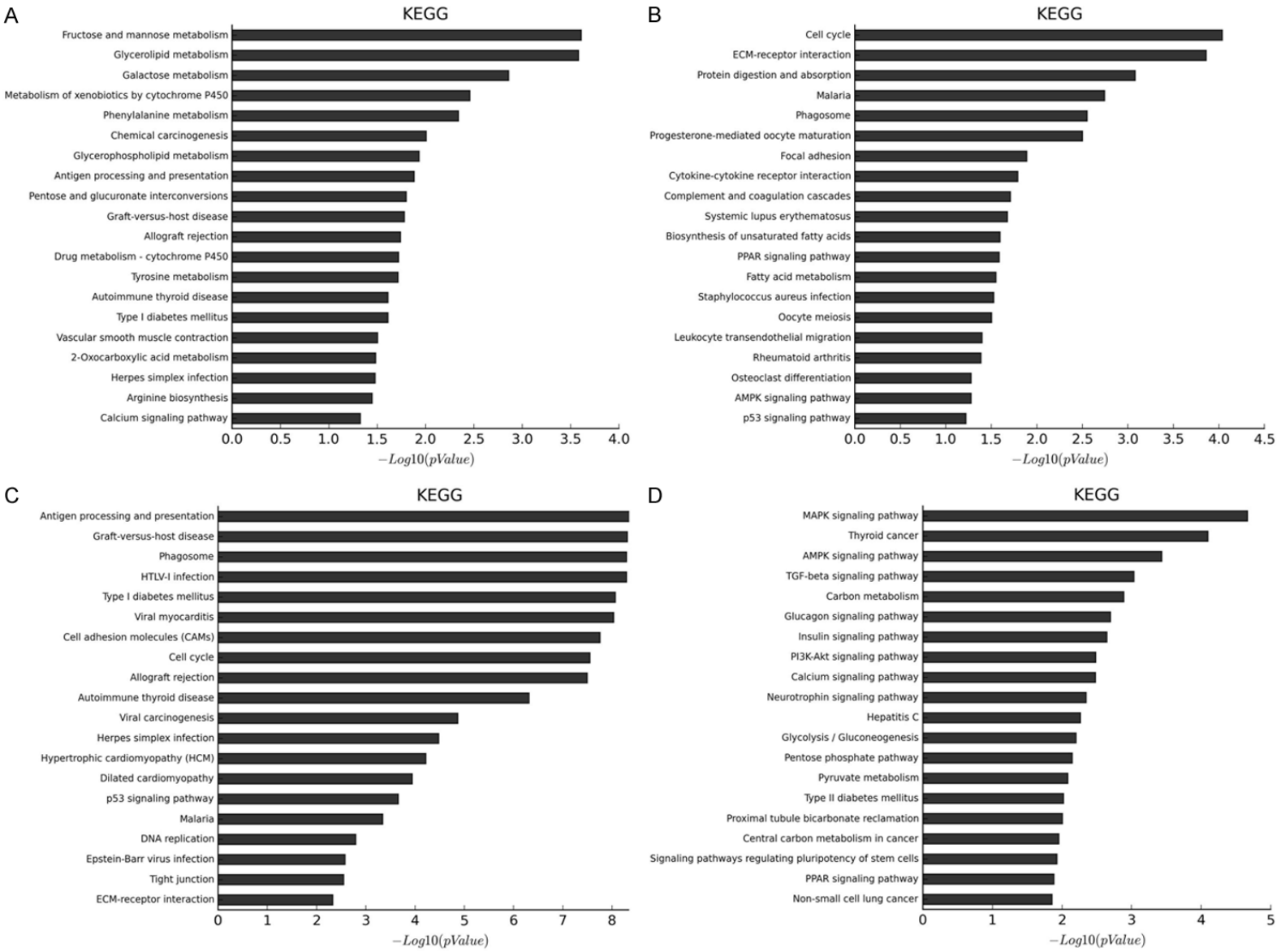

Figure 8. Evaluation for the BYHWT triggered KEGG signaling pathways with significant enrichment of differentially expressed molecules. A. Evaluation for the up-regulated signaling pathways in BYHUW group comparing with Model group. B. Evaluation for the down-regulated signaling pathways in BYHUW group comparing with Model group. C. Evaluation for up-regulated signaling pathways in Model group comparing with NC group. D. Evaluation for the down-regulated signaling pathways in Model group comparing with NC group.

channel complex and neuronal cell body, were up-regulated and 20 cellular components, especially for the extra-cellular matrix, proteinaceous extra-cellular matrix and extra-cellular space, were down-regulated in BYHWT group, when comparing with Model group. These findings suggest that BYHWT plays the neuro-protective roles by activating the extra-cellular components and it's associated signaling pathways. Therefore, the above extra-cellular components are critical for improving the motor endplates activity of muscle tissues. Moreover, there were 20 molecular functions, especially for the voltage-gated potassium channel activity, glutathione transferase activity, were up-regulated and 20 molecular functions. Especially for the heparin binding, extra-cellular matrix structural constituent and integrin binding, which were down-regulated in BYHWT group, when comparing with Model group. These results suggest that the protective effects of BYHWT involve many molecular functions, all of which influence the growth of muscle tissues of DSMA rat models.

Furthermore, the BYHWT also triggered KEGG signaling pathways with significant enrichment of differentially expressed genes. Our findings illustrated that there were 3 KEGG signaling pathways including the fructose and mannose metabolism signaling pathway, glycerolipid metabolism signaling pathway and galactose metabolism signaling pathway, and 4 KEGG pathways, including cell-cycle signaling pathway, ECM-receptor interaction signaling pathway, protein digestion and absorption signaling pathway and malaria signaling pathway, involved in the neuro-protective effects of BYHWT. Among the above signaling pathways, the amino acid metabolism signaling pathways, cell cycle signaling pathway and ECM-receptor interaction signaling pathway played the most critical role.

Although this study received a few interesting results, there are also some limitations. Firstly, this study didn't evaluate the sample size and the power analysis multiple testing correction has not been conducted. Secondarily, the sample sizes were relative small for validating the results in this study. Thirdly, the differentially expressed genes have not been analyzed by using the statistical analysis methods. In the following study, we would conduct the statistical analysis for confirming the differences of differentially expressed genes.

In conclusion, BYHWT alleviated the inflammations and improved the motor endplate functions of DSMA rats by activating cellular response to interferon-gamma, integral component of plasma membrane and voltage-gated potassium channel activity genes and associated signaling pathways.

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Disclosure of conflict of interest

None.

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