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Original Research Article

Hot water extract from spent mushroom substrate of *Ganoderma lucidum* improves immune function in immune-deficient mice

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Abstract

Purpose: *To investigate the effects of hot water extract of the spent mushroom substrate from Ganoderma lucidum (HWE) on the immune functions of immune-deficient mice in the presence or absence of cyclophosphamide.*
Methods: *The C57BL/6 mice were treated with varying doses of HWE and cyclophosphamide, and the spleen transcriptome was evaluated by high-throughput sequencing. Pathway enrichment analysis was conducted to generate an overview of differentially-expressed genes (DEGs) functions and interactions.*
Results: *Significant DEGs were observed among the control (CK), normal control (HWE), model (CY), and high-dose HWE (CY + HWEH) groups ($p < 0.05$). Compared with CK group, HWE and CY + HWEH groups showed upregulation of genes (Alas2, CCNE1, and CCNA2), whereas genes encoding major histocompatibility complex components, costimulatory factors, proinflammatory chemokines, and inflammatory chemokines were significantly downregulated ($p < 0.05$). Compared with CY group, multiple tumor suppressor and tumorigenesis genes, such as CDKN1A, CDKN1B, MAPK10, and TN-C, were downregulated in CK, HWE, and CY + HWEH groups.*
Conclusion: *This study highlights changes in the spleen transcriptomic profiles of C57BL/6 mice treated with HWE and CY, indicating that HWE improves immunomodulation in a mouse model with immune deficiency. Hot water extract of the spent mushroom substrate from Ganoderma lucidum (HWE) has potentials as an immune enhancer in immunocompromised patients.*

Keywords: *Spleen transcriptome, Hot water extract, Ganoderma lucidum, Cyclophosphamide, High-throughput sequencing, Immunomodulation*

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INTRODUCTION

Lingzhi (*Ganoderma lucidum*) is highly valued as a traditional medicinal fungus in Asia and has long been known to act as an immunomodulating agent with applications in the treatment of gastric cancer, hypertension, arthritis, chronic hepatitis, diabetes, asthma, nephritis, arteriosclerosis, and immunological disorders [1]. The effects of Lingzhi have been attributed to polysaccharides,

which are thought to stimulate the immune system [2] and activate both innate and adaptive immune responses [3]. In Chinese herbal medicine, polysaccharides have been shown to regulate the immune system through multiple mechanisms [4]. Previous studies suggest that treatment with the hot water extract of a spent mushroom substrate from Lingzhi (HWE) enhances the immune functions of normal mice [5]. In addition, HWE treatment improved the recovery of cyclophosphamide-suppressed immune functions in mice. However, the mechanisms underlying the immunomodulatory effects of HWE remain unknown.

This study investigated the effects of HWE on gene expression in the spleen of an immune-deficient mouse model by high throughput sequencing using Illumina Genome Analyzer Ⅱx. Then differentially-expressed genes (DEGs) were screened for different treatments. The deep analyses of DEGs, such as Gene Ontology analysis and Pathway enrichment analysis, were determined to better examine the specific effects of HWE treatment in immune-deficient mice and better understand the genes and pathways involved in regulating the immune response to HWE and cyclophosphamide.

EXPERIMENTAL

Animals

Sixty pathogen-free male C57BL/6 mice (7 weeks old, weighing 18 – 20 g) were purchased from Charles River Laboratories, Beijing, China (animal license number: SCXK (Beijing) 2012-0001). All animal protocols were approved by the Animal Care Committee of Peking University Health Center (approval no. LA2015055). All experiments were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals [16]. All procedures described below were conducted in accordance with the Inspection and Evaluation of Health Food [17].

Treatments

Mice were randomly allocated to six groups, ten for each group. The experimental period lasted for 28 days. Mice received injections on days 1, 7, 14, and 21 and were sacrificed on day 28. As shown in Table 1, mice in the cyclophosphamide model (CY), low-dose HWE (CY + HWE_L), medium-dose HWE (CY + HWE_M), and high-dose HWE (CY + HWE_H) groups were intraperitoneally injected with cyclophosphamide (100 mg/kg) (MACKLIN Shanghai Inc., Shanghai, China) on days 1, 7, 14, and 21, whereas the mice in the control (CK) and HWE groups were injected with normal saline. Mice in the HWE (1 g/kg body weight (BW)), CY + HWE_L (0.5 g/kg BW), CY + HWE_M (1 g/kg BW), and CY + HWE_H (2 g/kg BW) groups were orally administered HWE daily, whereas water was given to the mice in the CK and CY groups.

All mice were sacrificed on day 28, and spleen samples were collected and immediately frozen in liquid nitrogen for later use. Extraction of HWE was performed as described previously [13].

Transcriptome sequencing

Allwegene Tech. (Beijing, China) performed RNA sequencing using an Illumina Genome Analyzer Ⅱx (Illumina, Inc., California, USA). The details of RNA extraction, RNA sequencing, differential expression analysis, and functional gene annotation are described in previous studies [8,13].

Differential expression analysis

For each sequenced library, differentially-expressed gene (DEG) analysis was performed using IDEG 6. The *p*-value corresponds to the differential gene expression test. The FDR value ≤ 0.001 and the absolute value of $\log_2$ (fold change) ≥ 1 were used as the threshold criteria for determining significant differential expression.

Table 1: Treatment scheme for establishing immune-deficient mouse models

Group	Treatment	Cyclophosphamide (mg/kg)
Control (CK)	Water	–
Normal control (HWE)	1 g/kg HWE	–
Cyclophosphamide model (CY)	Water	100
Low-dose HWE (CY+HWE_L)	0.5 g/kg HWE	100
Medium-dose HWE (CY+HWE_M)	1 g/kg HWE	100
High-dose HWE (CY+HWE_H)	2 g/kg HWE	100

***Key**: CK, blank control; CY, cyclophosphamide model; HWE, hot water extract of Ganoderma lucidum spent mushroom substrate*

RESULTS

Significant differentially-expressed genes and significant pathway enrichment analysis

Significant DEGs among the groups are shown in Figure 1. Compared with the CK group, 2,155 genes were significantly downregulated, and 1,318 genes were significantly upregulated in the CY + HWE_H group, whereas 216 genes were significantly downregulated, and 500 genes were significantly upregulated in the HWE group.

When compared with the CY group, there were 3869 genes significantly downregulated, and 786 genes were significantly upregulated in the CY + HWE_H group. In the CK group, 2647 genes were significantly downregulated and 574 genes were significantly upregulated in the CK group. Moreover, 2798 genes were significantly downregulated and 567 genes were significantly upregulated in the HWE group. The DEGs were classified into categories, with the largest proportion of DEGs clustered into cellular components, followed by biological processes and molecular functions.

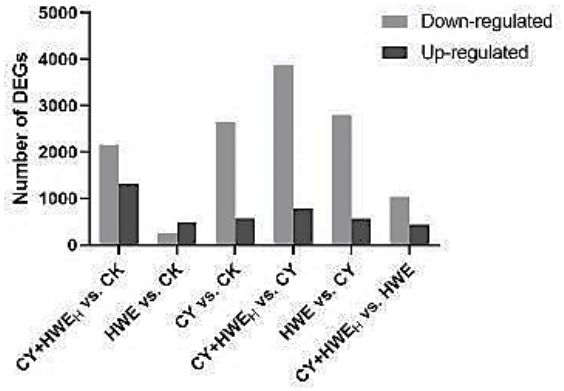

Figure 1: Differentially-expressed genes. CK, control; CY, cyclophosphamide; HWE: hot water extract of *Ganoderma lucidum* spent mushroom substrate; HWEH: high-dose HWE

Pathway enrichment analysis of differentially expressed genes

The pathway enrichment analysis was conducted to generate overviews of DEG functions and interactions, particularly for the HWE vs. CK, CY + HWE_H vs. CY, and HWE vs. CY group comparisons. To determine the functional distribution of DEGs, Gene ontology analysis was performed for the following group comparisons: CY + HWE_H vs. CK; HWE vs. CK; CY + HWE_H vs. HWE; CY + HWE_H vs. CY; CK vs. CY; and HWE vs. CY.

HWE vs CK

Significant DEGs between the CK and HWE groups were associated with various pathways, including *Staphylococcus aureus* infection, porphyrin, and chlorophyll metabolism, phagosomes, malaria, hypertrophic cardiomyopathy, dilated cardiomyopathy, and glycerolipid metabolism (Figure 2). The DEGs associated with immune functions among the top 20 significantly enriched pathways identified in the comparison between the CK and HWE groups.

In the pathway for porphyrin and chlorophyll metabolism (as shown in Table 2), *Alas2,* which encodes 5-aminolevulinic acid synthase, was upregulated by 2.49 folds in the HWE group, whereas *Ugt1a6b* (UDP glucuronosyltransferase 1 family, polypeptide A6B precursor) was downregulated when compared with the CK group.

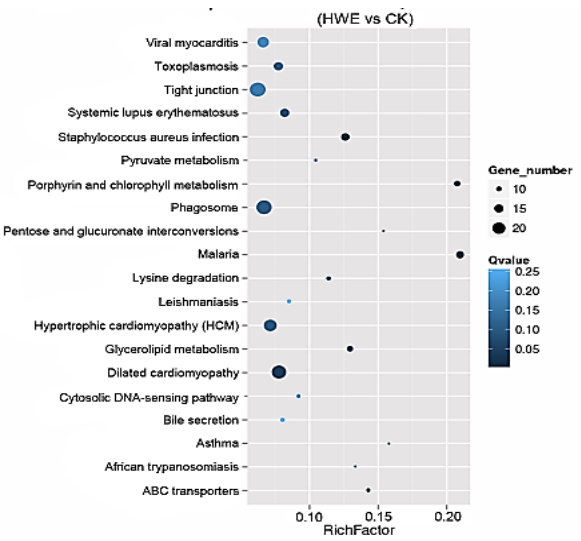

Figure 2: Top 20 statistics of pathway enrichment for HWE vs CK

CY + HWE_H vs CY

The top 20 pathways associated with significant DEGs between the CY and CY + HWE_H groups included pathways in cancer, T-cell receptor signaling, mitogen-activated protein kinase (MAPK) signaling, human T-cell lymphotropic virus type 1 (HTLV-1) interaction, focal adhesion, cell adhesion molecules (CAMs), extracellular matrix (ECM)–receptor interactions, toxoplasmosis, and axon guidance (Figure 3). Some DEGs associated with immune function among the top 20 significantly enriched pathways identified in the comparison between the CY + HWE_H and CY groups.

Table 2: Differential expressions of genes associated with immune function (HWE vs. CK)

Pathway	Gene	Gene ID	Encoded Protein	Fold (log_2 ratio)	*P*-value	FDR
Porphyrin and chlorophyll metabolism	*alas2*	11656	5-aminolevulinic acid synthase (EC 2.3.1.37), partial (*Mus musculus*)	2.49	0	0
	UGT1A6B	394435	UDP glucuronosyltransferase 1 family, polypeptide A6B precursor (*Mus musculus*)	−9.80	5.60E−07	3.38E−06

***Key**: CK, control: FDR, false-discovery rate: HWE: hot water extract of Ganoderma lucidum spent mushroom substrate*

Table 3: Differential expressions of genes associated with immune function (CY+HWE_H vs. CY)

Gene Style	Gene	Gene ID	Encoded Protein	Fold (log_2 ratio)	*P*-value	FDR
Pathways in cancer	**CCNE1**	12447	cyclin E (*Mus musculus*)	2.93	7.16E−166	3.06E−164
	CCNA2	12428	cyclin-A2 (*Mus musculus*)	1.86	0	0
	CDKN1A	12575	cyclin-dependent kinase inhibitor 1A (P21), isoform CRA_b (*Mus musculus*)	−4.24	0	0
	CDKN1B	12578	cyclin-dependent kinase inhibitor 2A, isoform 3 isoform 2 (*Mus musculus*)	−2.93	1.70E−05	3.88E−05
Focal adhesion	*MAPK10*	26414	mitogen-activated protein kinase 10, isoform CRA_a (*Mus musculus*)	−7.23	0.00045	0.00089
	TRNP1	69539	TMF-regulated nuclear protein 1 (*Mus musculus)*	−4.63	5.66E−07	1.46E−06

***Keys:** CY: cyclophosphamide; FDR, false-discovery rate; HTLV-1: human T-cell lymphotropic virus type 1; HWE_H: high-dose hot water extract of Ganoderma lucidum spent mushroom substrate; MAPK: mitogen-activated protein kinase*

As shown in Table 3, in the pathways in cancer, cyclin E (*CCNE1*) and cyclin A2 (*CCNA2*) were upregulated 2.93-fold and 1.86-fold, respectively, in the CY + HWE_H group when compared with the CY group, whereas *CDKN1A* and *CDKN1B* were downregulated in the CY + HWE_H group, with a 4.24-fold increase in *CDKN1A* expression observed in the CY group. In the focal adhesion pathway, mitogen-activated protein kinase 10, isoform CRA (*MAPK10*), and TMF-regulated nuclear protein 1 (*TRNP1*) were upregulated 7.23-fold and 4.63-fold, respectively, in the CY + HWE_H group compared with the CY group.

HWE vs CY

The top 20 enriched pathways associated with significant DEGs between the CY and HWE groups included regulation of the actin cytoskeleton, pathways in cancer, focal adhesion, cytokine–cytokine receptor interaction, dilated cardiomyopathy, CAMs, ECM-receptor interaction, axon guidance, and amoebiasis (Figure 4). Some DEGs associated with immune functions were among the top 20 significantly enriched pathways identified in the comparison between the HWE and CY groups.

As shown in Table 4, *TN-C* was upregulated by 3.06-fold in the CY group when compared with the HWE group. Cyclophosphamide injection results in injury, leading to increased expression of the protein encoded by *TN-C*. Likewise, *GLI2* was also upregulated by 3.02-fold in the CY group when compared with the HWE group, whereas *CTLA4* expression in the HWE group was 2.04-fold than in the CY group.

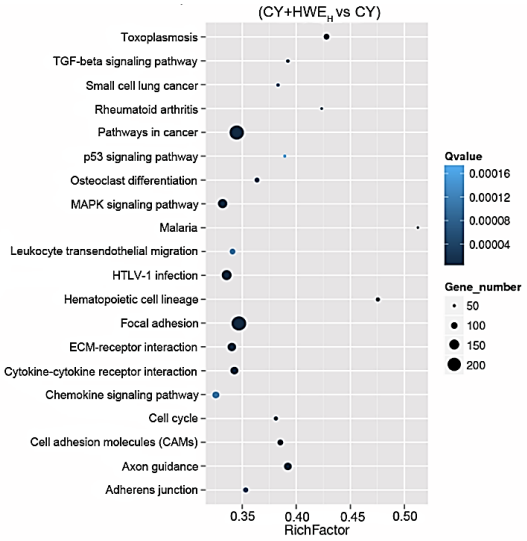

Figure 3: Top 20 statistics of pathway enrichment for CY + HWE_H vs CY

The HWE group showed more downregulated genes than the CY group. Genes such as *MGST3*, *CPNE5*, *FAM132A*, and *HMMR* were upregulated, whereas *GLI1*, *GLI2*, *TN-C*, *VEGFA*, *PDCD1*, and *CDKN1A* were downregulated in the HWE group when compared with the CY group.

DISCUSSION

Ganoderma lucidum polysaccharide is the major biologically active component in *G. lucidum* [6] and regulates specific immune cell functions, including T lymphocytes, B lymphocytes, macrophages, dendritic cells, and natural killer cells [7]. A previous study showed that HWE can enhance murine immune functions [5].

Differential pathway enrichment analysis between the HWE and CK groups identified significant differences in pathways related to systemic lupus erythematosus, dilated cardiomyopathy, and hypertrophic cardiomyopathy, and DEGs were also concentrated in pathways associated with pyruvate metabolism, porphyrin and chlorophyll metabolism, and other metabolic pathways.

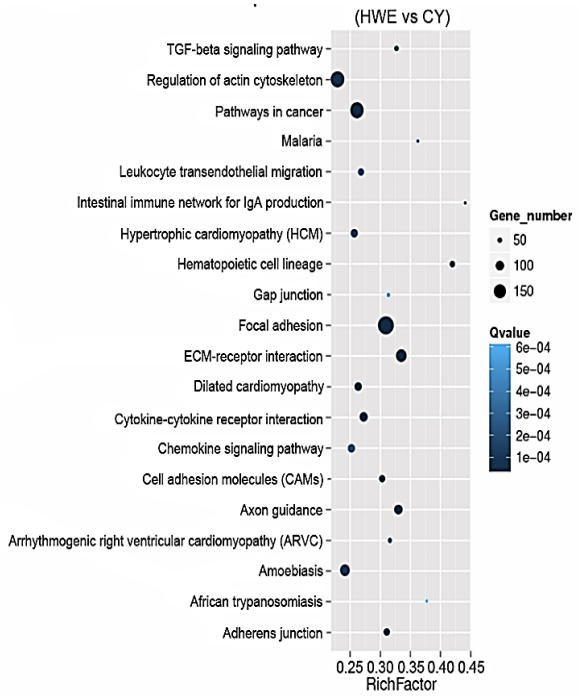

Figure 4: Top 20 statistics of pathway enrichment for HWE vs CY

Table 4: Differential expression of genes associated with immune function (HWE vs. CY)

Gene Style	Gene	Gene ID	Encoded Protein	Fold (log_2 ratio)	*P*-value	FDR
Pathways in cancer	*MGST3*	66447	microsomal glutathione S transferase 3 (*Mus musculus*)	3.12	5.22E−176	3.17E−174
	FAM132A	67389	protein FAM132A precursor (*Mus musculus*)	2.14	1.57E−77	3.78E−76
	CDKN1A	12575	cyclin-dependent kinase inhibitor 1A (P21), isoform CRA_b (*Mus musculus*)	−4.23	0	0
	GLI1	14632	zinc finger protein GLI1 (*Mus musculus*)	−3.19	5.29E−06	1.57E−05
	GLI2	14633	zinc finger protein GLI2 (*Mus musculus*)	−3.02	2.06E−08	7.62E−08
	VEGFA	22339	vascular endothelial growth factor A isoform 6 precursor (Mus musculus)	-2.59	5.44E-229	4.24E-227
ECM-receptor interaction	*FAM132A*	67389	protein FAM132A precursor (*Mus musculus*)	2.14	1.57E−77	3.78E−76
	HMMR	15366	hyaluronan mediated motility receptor (*Mus musculus*)	1.08	1.68E−25	1.38E−24
	TN-C	21923	tenascin precursor (*Mus musculus)*	−3.06	3.32E−10	1.39E−09
Cell adhesion molecules (CAMs)	***CTL4***	12477	cytotoxic T-lymphocyte-associated protein 4 (*Mus musculus*)	2.04	2.03E−13	1.02E−12
Focal adhesion	*FAM132A*	67389	protein FAM132A precursor (*Mus musculus*)	2.14	1.57E−77	3.78E−76

***Keys**: CY: cyclophosphamide; ECM: extracellular matrix; FDR: false-discovery rate; HWE: hot water extract of Ganoderma lucidum spent mushroom substrate*

The porphyrin and chlorophyll metabolism pathway was associated with DEGs encoding metabolic enzymes, such as *Alas2*, one of two genes that encode aminolevulinic acid synthase, the first regulatory enzyme in the heme biosynthetic pathway [8]. Expression of *Ugt1a6b* (UDP glucuronosyltransferase 1 family, polypeptide A6B precursor), which encodes a glucuronyltransferase, was downregulated in the HWE group compared with the CK group. In mice, 14 Ugt genes (including five pseudogenes) and 10 Ugt2 genes have been identified. The function of *UGT1A6* is to catalyze simple phenolic glucuronidation, and the neurotransmitter serotonin (5-hydroxytryptamine) is a typical endogenous substrate [9].

Significant differences between the CY + HWE_H and CY groups were observed for the expression of *CCNE1* and *CCNA2,* which encode cyclin family member cyclin E and cyclin A2, respectively. Expression of *CCNE1* and *CCNA2* was upregulated by 2.93-fold and 1.86-fold, respectively, in the CY + HWE_H group compared with the CY group. Amplification of *CCNE1*, observed in many patients, is critical for cancer cell survival [10]. High *CCNE1* expression is a significant and independent predictor for prolonged overall survival in patients with grade III/IV epithelial ovarian cancer [11]. In the present study, cyclin-associated gene expression was upregulated following high-dose HWE treatment when compared with cyclophosphamide alone, indicating that HWE may attenuate the adverse reactions and damage induced by cyclophosphamide and improve prognosis.

Expression of *MAPK10* was upregulated by 7.23-fold in the CY group when compared with the CY + HWE_H group. Mitogen-activated protein kinase and c-Jun N-terminal kinase (JNK) family members participate in multiple signaling pathways, including apoptosis, differentiation, and proliferation [12], and mitogen-activated protein kinase 10 (MAPK10), also known as JNK3, promotes apoptosis and plays a role in tumor suppression. Compared with the CY group, lymphotoxin A (*LTA*), which encodes an inflammatory interleukin primarily synthesized by lymphocytes, and *Matn1* expression were downregulated in the CY + HWE_H group. The protein encoded by *Matn1* is a cartilage matrix protein precursor that has been reported to be necessary for cerebellar granule neuron production [13] and hair cell production [14].

Glycoprotein tenascin C, an adhesion-regulating protein responsible for various functions within cells that is dysregulated under pathological conditions, such as inflammation, infection, and tumors [15], is encoded by *TN-C*. In this study, *TN-C* expression in the CY group was 3.06-fold that in the HWE group. Cyclophosphamide injection induced injury in mice, leading to an upregulation in the protein encoded by *TN-C*. Tenascin C is a member of the ECM glycoprotein family, and *TN-C* is upregulated during development and embryogenesis. Upregulation of *TN-C* has been reported during tissue repair, remodeling, and pathological conditions, including inflammation, infection, neovascularization, wound healing, and tumorigenesis [16]. The expression of *GLI2* in the CY group was 3.02-fold that in the HWE group, and *GLI2* encodes a C2H2-type zinc finger transcription factor that belongs to the GLI family, which is involved in the Hedgehog (Hh) signaling pathway that plays an important role in regulating embryonic tissue differentiation and development [17].

A previous study showed that HWE improved the recovery of cyclophosphamide-impaired immune function in mice by increasing serum concentrations of superoxide dismutase and catalase and total antioxidative capacity [5]. When utilized as a feed additive, *G. lucidum* HWE enhanced the immunity and antioxidant capacity of dairy cows, thereby improving milk quality [18], milk yield, and hematology parameters, suggesting that HWE may be useful and functional as a feed additive.

Compared with the CK group, the tumor-associated antigen-encoding gene *CHP2* is upregulated in the HWE and CY + HWE_H groups, whereas genes encoding co-suppressors, major histocompatibility complex molecules, costimulatory factors, proinflammatory chemokines, and some inflammatory chemokines were downregulated by HWE treatment. In addition, compared with an immunocompromised mouse model, some tumor suppressive and tumorigenic genes were downregulated in the CK, HWE, and CY + HWE_H groups, such as *CDKN1A*, *CDKN1B*, *MAPK10*, *VASH1*, and *TN-C*.

CONCLUSION

Spleen transcriptomics analysis in mice has shown significant differences in gene expression among the CK, CY, HWE, and CY + HWE_H groups. These results indicate that HWE improves immune function in low-immunity mice. Hot water extract of the spent mushroom substrate from *Ganoderma lucidum* (HWE) has potentials for further investigation as an immune enhancer in immunocompromised patients.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yanling Liu and Zhanxi Lin conceived and designed the study. Tong Meng, Wei Zhang, and Jing Li conducted the experiments and performed the biochemical analysis and part of the histological study. Biaosheng Lin and Jihui Chen analyzed the data, and Yanling Liu wrote the manuscript. All the authors read and approved the manuscript.

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