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Qi-Wei-Bai-Zhu powder improves inflammatory response *via* the myd88 pathway in neonatal mice infected with human rotavirus.

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Abstract

Qi-Wei-Bai-Zhu Power (QWBZP), as one of the classic herbal prescription, has been proved to decrease diarrhea incidence, reduce mortality and regulate cytokines secretion in both human and rodents infected with Rotavirus (RV). We conducted the present study to elucidate the specific mechanism how QWBZP exerts its beneficial effects on HRV infection in neonatal mice. Cytokine contents and expression of proteins in Toll-Like Receptor 3 (TLR3) pathway were determined in HRV and QWBZP treated neonatal mice and CD 8 T cells. The results showed that QWBZP decreased diarrhea incidence and fecal virus shedding in HRV-infected mice. Moreover, QWBZP decreased interleukin (IL)-1β, Tumor Necrosis Factor-α (TNF-α), interferon (IFN)-α and IFN-β contents and increased IL-10 content in both serum and intestine in HRV-infected mice. QWBZP, as well as myeloid differentiation factor 88 (MyD88) inhibitor peptide, downregulated MyD88-NFκB pathway and improved cytokines secretion, however, they did not affect TLR3 expression in HRV-treated CD 8 T cells. In addition, neither QWBZP nor MyD88 inhibitor peptide exerted any effects on NFκB expression and cytokines secretion in poly (I: C)-treated CD8 T cells. In conclusion, our results indicated that HRV activated TLR3 pathway and its downstream target NFκB dependent on MyD88, while QWBZP selectively inhibited MyD88 which further inactivated NFκB pathway and subsequently improved the production of cytokines, without affecting TLR3.

Keywords: Qi-Wei-Bai-Zhu power, Rotavirus, Cytokines, Toll-like receptor 3, MyD88.

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Introduction

The double-stranded Rotavirus (RV) is the world's leading cause of severe diarrhea in infants and young children, where it can lead to gastrointestinal tract damage [1]. According to previous reports, the RV disease burden is greatest in developing countries, where RV results in nearly 500,000 deaths per year [2]. It is estimated that 81 countries have introduced rotavirus vaccine into their national immunization program until May 2016 [3]. However, vaccine efficacy in developing countries is remarkably lower than that observed in developed countries [4]. As in some regions, poor nutritional status and chronic infection such as helminths are supposed to suppress the immune response to RV vaccine [2]. In addition, high titers of antibody and breast milk IgA from maternal nutrition have the potential to neutralize RV vaccine and lower RV antigen exposure. These factors warranted the need for alternative treatments for RV and several previous studies had proved the possibility of curing the RV disease without RV vaccine. Bacterial flagellin has been reported to prevent Rotavirus (RV) infection in mice and cure chronically RV-infected mice [5]. Neonatal pigs fed dietary calrose rice bran exhibited reduced diarrhea after administration with HRV [6,7]. Moreover, diarrhea was completely diminished when the rice bran feed were added with *L. rhamnosus* GG and *E. coli* Nissle [8].

Qi-Wei-Bai-Zhu Power (QWBZP), as one of the classic herbal prescription, has been used to treat infantile diarrhea for centuries [9]. Our previous study showed that QWBZP decreased diarrhea incidence, reduced mortality, improved absorptive function and alleviated the pathological changes in small intestine in both children and rodents infected with RV [10]. Moreover, we also found that QWBZP significantly regulated the production of inflammatory cytokines such as

TNF-α and IL10 [9]. Based on these facts, we speculated that QWBZP may alleviate RV infection-induced pathological change through certain pathways involved in inflammatory response. Since mammalian Toll-Like Receptor 3 (TLR3) recognizes double-stranded RNA, and that activation of the receptor activates NF-κB and induces the production of type I interferons [4], the present study was conducted to elucidate whether TLR3-activated myeloid differentiation factor 88 (MyD88) pathway was involved in the beneficial effects of QWBZP on RV infection.

Materials and Methods

Preparation of QWBZP extract. QWBZP prescription is composed of the following dried herbal medicines: 7.5 g roots of *Panax ginseng* C. A. Mey (Polyporaceae), 15 g roots of *Atractylodes macrocephala* Koidz (Compositae), 15 g sclerotium of *Poria cocos* Wolf (Polyporaceae), 15 g roots of *Pueraria lobata* Ohwi (Leguminosae), 15 g leaves of *Agastache rugosa* (Labiatae), 6 g roots of *Costustroot* (Rosaceae), and 3 g roots of *Glycyrrhiza uralensis* Fisch (Leguminosae). These herbs above were supplied by the First Affiliated Hospital of Hunan University of TCM (Hunan, China), and their authenticity was confirmed by College of Pharmacy of Hunan University of TCM (Hunan, China). Voucher specimens (PC06-2) have been deposited in the Herbarium of College of Pharmacy of Hunan University of TCM. QWBZP extraction was prepared according to previous study [9]. Briefly, the dried crude herb mixture (76.5 g) was boiled in a flask in 1000 ml of distilled water for 30 min and this procedure was repeated three times. The aqueous extract of three successive extractions was collected, combined, filtered and concentrated under vacuum and then lyophilized. The extract was dissolved in distilled water to a concentration of 1 g/ml, and then stored at 4°C and used within three days. The extract final concentration here is referred to the total weight of crude herb mixture used for the preparation.

HRV titration

Human rotavirus (HRV, Wa strain) and MA-104 cells derived from African Green monkey were both supplied by Chinese Center for Disease Control and Prevention. MA-104 cells (1×10^5/well) were plated in 96-well microtiter plates and incubated overnight. Serial 10 fold dilutions of HRV were added into cells after removing the growth medium. Wells were scored for the presence or absence of infection with either a positive or a negative symbol. Replicates of 7 were performed for each virus dilution. After incubated for 5 d, the $TCID_{50}$ value of HRV was calculated by the Reed-Muench method [11] and the $TCID_{50}$ of HRV in this study was 10^{-7}/ml.

Experimental animals, viral inoculation and drug treatment

All mice were obtained from the Experimental Animal Department of Hunan University of TCM. Neonatal C57BL/6 mice aged 4 d were separated from their mother and randomly assigned to 4 groups: normal mice (CON), HRV infected mice (HRV), HRV and QWBZP treated mice (QWBZP), and HRV and Ribavirin treated mice (RIB). All the animals used in this study were confirmed to be seronegative for rotavirus antibody. Mice were inoculated with 100 μL of HRV (the $TCID_{50}$ value is 10^{-7}/ml) according to previous study [9]. And 24 h after the inoculation, feces were collected for the detection of HRV with ELISA kit (Cusabio, Wuhan, China) and then mice were administrated with QWBZP extract (1 g/ml), ribavirin (1.4 mg/ml, Hubei Yiyao Co. Ltd., China) and physiological saline for 5 d (twice a day and 0.05 ml every time), respectively. All the mice received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Hunan University of TCM.

Diarrhea incidence, sample collection, and isolation and treatment of CD 8 T cells from intestine

During the 5 d period, mice were monitored for incidence of diarrhea daily. On d 5, mice were killed, and serum and intestine samples were collected for the analysis of inflammatory cytokines. In addition, small intestine samples were collected for lymphocytes isolation according to previous procedures [12]. CD 8 T cells were further isolated using CD 8 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and were used for analysis of gene expression of inflammatory cytokines. In addition, 1×10^5 CD 8 T cells were incubated at 37°C for 24 h with HRV in RPMI containing 10% heat inactivated FBS. QWBZP (0.1 mg/ml), MyD88 inhibitor peptide (100 nmol/ml) (Novus Biologicals, Littleton, CO, USA) and/or polyinosine-polycytidylic acid (poly (I: C)) (25 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) were added for the final 6 h of incubation, respectively. Then cells were collected for the analysis of gene and protein expression.

Determination of inflammatory cytokines

The concentration of interleukin (IL)-1β, IL-10, Tumor Necrosis Factor-α (TNF-α), Interferon (IFN)-α and IFN-β in serum and culture supernatants of CD 8 T cells were determined using microplate reader (Infinite M200 PRO) with ELISA kits (Wuhan Huamei Biotech co., LTD, Wuhan, china) in accordance with the manufacturer's instructions.

RT-qPCR analysis

The total RNA was extracted from intestine and CD 8 T cell samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed to obtain cDNA. The primers (synthesized by Sangon Biotech (Shanghai) Co., Ltd) were designed with Primer 5.0 and primer sequences are as follow (5' → 3'): IFN-α: forward, TGCCCAGCAGATCAAGAAGG; reverse, TCAGGGGAAATTCCTGCACC; IFN-*γ*: forward, CTGTGATTGCGGGGTTGTAT; reverse, GAGTTATTTGTCATTCGGGTGT; IL-10: forward, GGACCAGCTGGACAACATACTGCTA; reverse, CCGATAAGGCTTGGCAACCCAAGT; IL-1β: forward, TGCCACCTTTTGACAGTGATG, reverse, AAGGTCCACGGGAAAGACAC; TNF-α; forward,

ATGAGAAGTTCCCAAATGGC; reverse, CTCCACTTGGTGGTTTGCTA; GAPDH: forward, CCCTTAAGAGGGATGCTGCC; reverse, ACTGTGCCGTTGAATTTGCC. The mRNA expression of genes in CD 8 T cells were analysed by real-time quantitative PCR as described previously [13]. Briefly, 1 μL cDNA template was added to a total volume of 10 μL assay solution containing 5 μL SYBR Green mix (Takara, Tokyo, Japan), 0.2 μL Rox, 3 μL deionized H_2O, and 0.4 μmol/L each of forward and reverse primers. The following protocol were performed using Roche LightCycler480 II : (i) pre-denaturation (10 s at 95°C); (ii) amplification and quantification, repeated 40 cycles (5 s at 95°C, 20 s at 60°C); (iii) melting curve construction (60-99°C with heating rate of 0.1°C S-1 and fluorescence measurements). Relative gene expression was expressed as a ratio of the target gene to the control genes using the formula $2^{-\Delta\Delta Ct}$, where ΔΔ Ct=(Ct target-Ct GAPDH) treatment-(Ct target-Ct GAPDH) control [14].

Western blotting analysis

Intestine samples were ground in liquid nitrogen and the CD 8 T cells were washed three times with ice-cold PBS and centrifuged for 5 min at 300 g to get cell samples for protein extraction. The total protein was extracted using RIPA (KeyGen BioTech, Nanjing, China) according to the manufacturer's protocol and protein content was determination by BCA assay (Pierce Biotechnology, Rockford, IL, USA). Then western blotting analysis was carried out as previous study did [15]. In brief, 20 μg proteins per lane were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were blocked with skim milk. Then primary antibody against TLR3, NFκB and MyD88 (Cell Signaling, Danvers, MA, USA), and GAPDH (Boster, Wuhan, China) was applied overnight at 4°C. After incubating with the secondary antibody overnight, the membrane was detected using BIORAD/ChemiDocTM XRS^+ with image LabTM software with the EZ-ECL (Biological Industries, Cromwell, CT, USA).

Statistical analysis

When comparing two groups of data a paired samples t-test was performed. When comparing multiple groups a one-way analysis of variance followed by a post-hoc Tukey's test was used. All statistical analysis was performed using SPSS version 22.0 (IBM SPSS, Armonk, NY, USA) And data were presented as means ± SEM. Mean values were considered to be significantly different when $P<0.05$.

Results

Effects of QWBZP on diarrhea incidence and fecal virus shedding in HRV-infected mice

Oral inoculation of neonatal C57BL/6 mice with HRV resulted in RV antigen becoming detectable in feces 1 d after inoculation (Figure 1A). RV shedding and diarrhea incidence peaked on d 2 and lasted on d 5 (Figures 1A and 1B). Repeated administration of QWBZP or ribavirin significantly decreased RV shedding and diarrhea incidence.

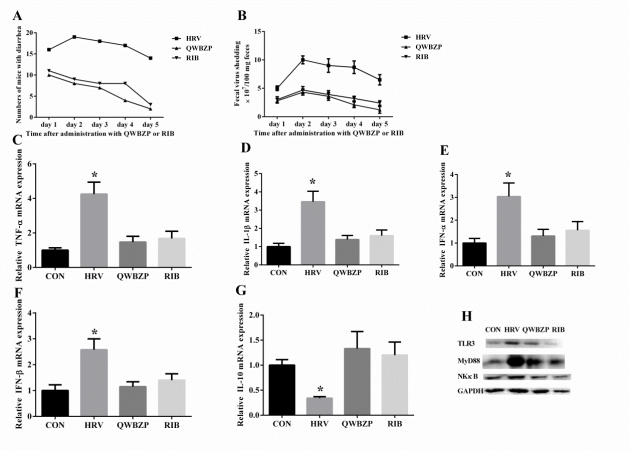

Figure 1. *Effects of QWBZP on diarrhea incidence, fecal virus shedding and intestinal expression of cytokines and proteins in TLR3 pathway in HRV-infected mice. CON: Normal Mice; HRV: HRV Infected Mice; QWBZP: HRV and QWBZP treated mice; RIB: HRV and Ribavirin treated mice. Values are expressed as mean ± SEM, n=7. *Mean values were significantly different among groups (P<0.05).*

Effects of QWBZP on contents of serum cytokines in HRV-infected mice

As shown in Table 1, serum contents of TNF-α, IL-1β, IFN-α and IFN-β were significantly increased, while IL-10 were significantly decreased in HRV-induced mice when compared with control mice. QWBZP and ribavirin administration significantly alleviated these changes in serum.

Effects of QWBZP on expression of cytokines and proteins in TLR3 pathway in intestine of HRV-infected mice

Gene expression of TNF-α, IL-1β, IFN-α and IFN-β were significantly increased (Figures 1C-1F), while IL-10 were significantly decreased (Figure 1G) in HRV-induced mice when compared with control mice. However, no such changes were observed in mice administrated with QWBZP or ribavirin. Protein expression of TLR3, MyD88 and NFκB were significantly increased in HRV-induced mice when compared with control mice, while no such changes were observed in mice administrated with QWBZP or ribavirin (Figure 1H).

Effects of QWBZP or MyD88 inhibitor peptide on cytokines contents and expression of proteins in TLR3 pathway in HRV-induced CD 8 T cells

According to Figure 2, concentrations of TNF-α, IL-1β, IFN-α and IFN-β secreted from CD 8 T cells were significantly increased (Figures 2A-2D), while IL-10 concentration was significantly decreased (Figure 2E) after they were treated with HRV. However, addition of QWBZP significantly alleviated these changes. Protein expression of TLR3, MyD88 and NFκB

were significantly increased after the cells were treated with HRV. Addition of QWBZP significantly decreased expression of MyD88 and NFκB while it had no effects on TLR3 expression (Figure 2F). MyD88 inhibitor peptide significantly alleviated HRV-induced increase of TNF-α, IL-1β, IFN-α and IFN-β concentrations (Figures 2G-2J) and decrease of IL-10 concentration (Figure 2K). MyD88 inhibitor peptide decreased protein expression of MyD88 and NFκB, while it had no effects on TLR3 expression (Figure 2L).

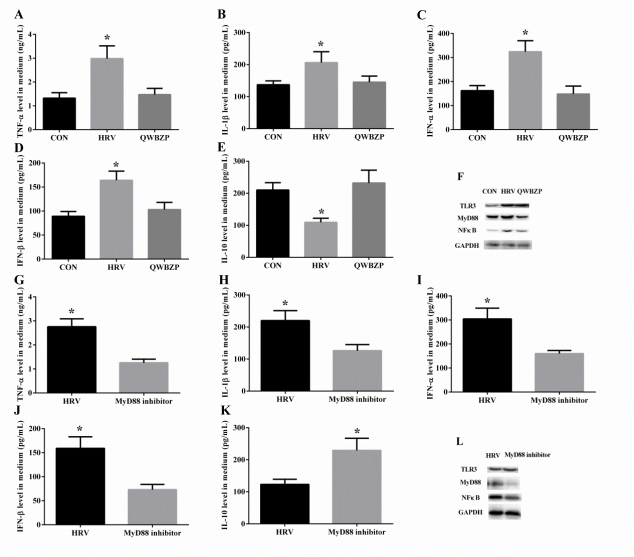

Figure 2. *Effects of QWBZP or MyD88 inhibitor peptide on cytokine contents and expression of proteins in TLR3 pathway in HRV-induced CD 8 T cells. CON: CD 8 cells without treatment; HRV: HRV treated CD 8 T cells; QWBZP: QWBZP and HRV treated CD 8 T cells; MyD88 inhibitor: MyD88 inhibitor peptide treated CD 8 T cells. Values are expressed as mean ± SEM, n=4. *Mean values were significantly different among groups (P<0.05).*

Effects of QWBZP or MyD88 inhibitor peptide on cytokines contents and expression of proteins in TLR3 pathway in poly (I: C)-treated CD 8 T cells

As shown in Figure 3, QWBZP treatment had no effects on TNF-α, IL-1β, IL10, IFN-α and IFN-β concentrations (Figures 3A-3E) secreted from CD 8 T cells treated with poly (I: C). QWBZP treatment decreased MyD88 protein expression, while it had no effects on protein expression of TLR3 and NFκB (Figure 3F). MyD88 inhibitor peptide had no effects on TNF-α, IL-1β, IL10, IFN-α and IFN-β concentrations (Figures 3G-3K) secreted from CD 8 cells treated with poly (I: C). MyD88 inhibitor peptide decreased MyD88 protein expression, while it had no effects on protein expression of TLR3 and NFκB (Figure 3L).

Table 1. *Contents of inflammatory cytokines in serum.*

Cytokines	CON	HRV	QWBZP	RIB
IFN-α, pg/ml	155.3 ± 18.2^b	232.1 ± 20.3^a	169.8 ± 16.1^b	183.9 ± 20^b
IFN-γ, pg/ml	208.6 ± 19.9^b	287.8 ± 21.5^a	223.4 ± 13.9^b	235 ± 27.6^b
IL-10, pg/ml	310.8 ± 28.7^a	128.4 ± 12.4^b	279.5 ± 27.6^a	238.8 ± 26.4^a
IL-1β, pg/ml	87.7 ± 8.1^b	290.4 ± 8.7^a	100.7 ± 9.4^b	128.9 ± 17.7^b
TNF-α, pg/ml	582.6 ± 34.4^b	853.4 ± 57.2^a	666.7 ± 49.2^b	678.2 ± 88.7^b

Values are expressed as mean ± SEM, n=7. Mean values with different letters differ (P<0.05). CON, normal mice; HRV: HRV infected mice; QWBZP: HRV and QWBZP treated mice; RIB: HRV and Ribavirin treated mice.

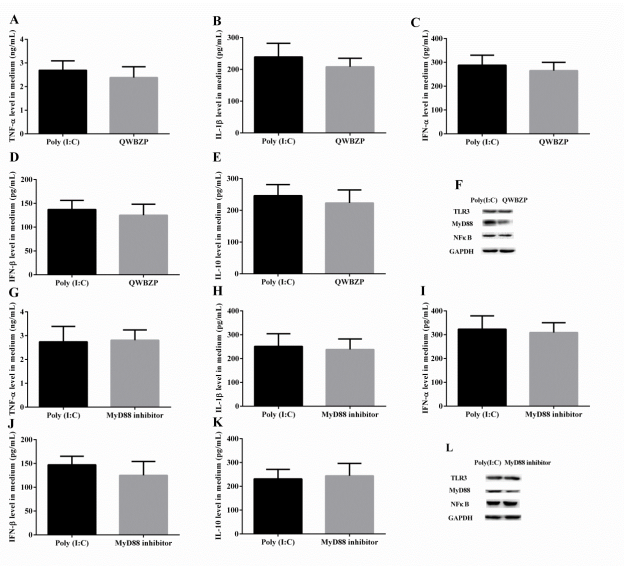

Figure 3. *Effects of QWBZP or MyD88 inhibitor peptide on cytokine contents and expression of proteins in TLR3 pathway in poly (I: C)-treated CD 8 T cells. Poly (I: C): Poly (I: C) treated CD 8 T cells; QWBZP: QWBZP and poly (I: C) treated CD 8 T cells; MyD88 inhibitor: MyD88 inhibitor peptide and poly (I: C) treated CD 8 T cells. Values are expressed as mean ± SEM, n=4.*

Discussion

Based on our previous results that QWBZP could alleviate HRV-induced pathological changes through modulating the production of inflammatory cytokines [9], we in the present study further elucidated the specific mechanisms how QWBZP alleviated inflammatory responses. The results showed that HRV activated TLR3 pathway and its downstream target NFκB dependent on MyD88, while QWBZP selectively inhibited MyD88 which further inactivated NFκB pathway and subsequently decreased the production of cytokines, without affecting TLR3.

Innate immunity, which is the first arm of host immunity system, is activated through recognizing pathogens by the germ-line-encoded Pattern-Recognition Receptors (PRRs) [16]. Toll-Like Receptors (TLRs), as one of the PRRs, recognize molecular patterns related to microbial pathogens, and induce antimicrobial immune responses. Among the TLRs, mammalian TLR3 was proved to recognize viral double-stranded RNA [17]. As expected, HRV which is a double-

stranded RNA virus induced higher expression of TLR3 protein both *in vivo* and *in vitro*. After receptors activation, signaling pathways are activated and then induce the production of cytokines in virus-infected subjects. TLR3 activation stimulates NFκB pathway which promotes the expression of IFN-α and IFN-β, and the secretion of proinflammatory cytokines. In the TLR3-NFκB signaling pathway, TLR3 could induce cytokines production either dependent on MyD88 or independent MyD88 [18], which is an adaptor protein for TLR3 [19].

In the present study, we found that HRV activated the TLR3-MyD88-NFκB signaling pathway which then promoted the production of cytokines in neonatal mice. Previous study demonstrated that CD8 T cells mediate clearance of rotavirus infection in mice [20]. Consequently, we isolated CD8 T cells from the intestine of neonatal mice to further confirm these mechanisms. MyD88 inhibitor peptide, as well as QWBZP, downregulated MyD88 and NFκB expression and alleviated the increased secretion of type I IFNs and proinflammatory cytokines. These results indicated that QWBZP may alleviate inflammation responses to HRV through targeting MyD88 in CD8 T cells. To further confirm this, we used poly (I: C) to activate the TLR3 pathway. Poly (I: C) could mimic the effects of double-stranded RNA [21] and has been proved to activate TLR3- NFκB signaling pathway and promote cytokines production either dependent on MyD88 or independent on MyD88 [18]. The results showed that poly (I: C) activated TLR3-MyD88-NFκB pathway and induced inflammatory responses as HRV did. However, neither QWBZP nor MyD88 inhibitor peptide had any effects on NFκB expression and cytokines secretion, although MyD88 expression was downregulated. In all, we in the present study further confirmed the beneficial effects of QWBZP on HRV infection-induced diarrhea and inflammatory responses. Our results also suggested that QWBZP played its important role in alleviating HRV infection mainly by targeting MyD88.

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Disclosures

All authors declare no financial competing interests. All authors declare no non-financial competing interests.

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