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# *Lycium barbarum* polysaccharide attenuates the cytotoxicity of mutant huntingtin and increases the activity of AKT

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ABSTRACT

Huntington's disease (HD) is an inherited neurodegenerative disease that is caused by the abnormal expansion of CAG repeats in the gene encoding huntingtin (Htt). Reduced AKT phosphorylation and inhibited AKT activity have been shown to be involved in mutant Htt (mHtt)-induced cell death. *Lycium barbarum* polysaccharide (LBP), the main bioactive component of *Lycium barbarum*, reportedly has neuroprotective roles in neural injuries, including neurodegenerative diseases. Here, we report that treatment with LBP can increased the viability of HEK293 cells that stably expressed mHtt containing 160 glutamine repeats and significantly improved motor behavior and life span in HD-transgenic mice. Furthermore, we found that in LBP-treated HEK293 cells expressing mHtt, mHtt levels were reduced and the phosphorylation of AKT at Ser473 (p-AKT-Ser473) was significantly increased. We also found that treatment with LBP increased p-AKT-Ser473 and decreased mHtt in the cortex, hippocampus and striatum in HD-transgenic mice. The level of phosphorylation of p-GSK3β-Ser9 remained unchanged in both cultured cells and HD-transgenic mice. Our findings suggest that LBP alleviates the cytotoxicity of mHtt by activating AKT and reducing mHtt levels, indicating that LBP may be potentially useful for treating HD.


## 1. Introduction

*Lycium barbarum* (also known as fructus lycii or wolfberry) has been used as a traditional Chinese herbal medicine for thousands of years. *Lycium barbarum* polysaccharide (LBP) is the main component of *Lycium barbarum* extracts, and it has many bioactivities, including antioxidant, hypoglycemic, immunological and neuroprotective effects (Chen et al., 2008; Gan et al., 2003; He et al., 2014b; Ho et al., 2007; Luo et al., 2004; Wang et al., 2014).

Huntington's disease (HD) is a fatal neurodegenerative disorder that is caused by an expanded polyglutamine tract, above a threshold of approximately 35 or more repeats, encoded by a CAG repeat sequence in the huntingtin (Htt) gene which is located on chromosome 4 (Ha and Fung, 2012). HD classically manifests with a triad of signs and symptoms that include motor, cognitive and behavioral features. In HD, AKT plays an important role in protecting neurons from death (Colin et al., 2005; Humbert et al., 2002; Ribeiro et al., 2014). HD alters the abundance and activity of AKT. In a rat model of HD, AKT was downregulated during neuronal dysfunction prior to the observation of any signs of degeneration. Finally, in postmortem brains of patients with HD, AKT is cleaved, and this cleavage appears to be the final step that ensures the irreversible deactivation of AKT and abolishes the ability of AKT to promote survival in cells expressing mutant Htt (mHtt) (Colin et al., 2005). Decreased levels of AKT and/or activated AKT reduced Htt phosphorylation, which enhances the toxicity of mutant Htt (mHtt) (Humbert et al., 2002).

In this study, we used HEK293 cells that stably express 20Q or 160Q variants of Htt (HEK293-20Q and HEK293-160Q, respectively) to detect changes after LBP treatment. We also confirmed the function of LBP using the transgenic mouse model B6C3-Tg(HD82Gln)81Dbo/J Tg, which expresses an N-terminally truncated human Htt cDNA that encodes 82 glutamines and encompasses the first 171 amino acids (Schilling et al., 1999a).

## 2. Materials and methods

### 2.1. Materials and chemicals

*Lycium barbarum* fruits, which were produced in the Ningxia province of China, were dried at 60 °C. The dried fruit was ground

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to a fine powder, refluxed twice using chloroform:methanol solvent (2:1) to remove lipids, and then refluxed with 80% ethanol solvent at 80 °C to remove oligosaccharides. After the solution was filtered, the residues were extracted 4 times in a 4-fold volume of hot water, filtered at 60 °C, and precipitated with 95% ethanol, 100% ethanol and acetone (Luo et al., 2014, 1999). This process yielded 4.65 g of vacuum-dried LBP powder from 100 g *Lycium barbarum* fruits for an extraction ratio of 4.65%. The LBP powder was freshly dissolved in distilled water (for mice) or DMEM (for cells) prior to use. Other chemicals were obtained from the following companies: protease inhibitor cocktail, phosphatase inhibitor cocktail, and anti-GAPDH monoclonal antibodies were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA); rabbit monoclonal antibodies against phosphorylated-AKT (Ser473), AKT (pan), phosphorylated GSK-3β (Ser9), and GSK-3β were purchased from Cell Signaling Technology (Beverly, MA, USA); rabbit polyclonal Htt and mouse monoclonal Htt antibodies were obtained from Professor Xiao-jiang Li (Department of Human Genetics, Emory University School of Medicine, USA); horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); nitrocellulose membranes were purchased from Amersham Biosciences (GE Healthcare Life Sciences, NJ, USA); the enhanced chemiluminescence (ECL) detection kit, TRIzol® Reagent, PageRuler™ Prestained Protein Ladder and BCA Protein Assay Kit were obtained from Thermo (Waltham, MA USA); high-glucose Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Waltham, MA USA); and the Cell Counting Kit-8 (CCK8), caspase-3 activity assay kit and LY 294002 (inhibitor of PI3K/AKT) were obtained from Beyotime Biotechnology (Jiangsu, China); biotinylated goat anti-rabbit antibody and avidin-biotin complex kit (Vector ABC Elite) were purchased from Vector Laboratories (Burlingame, CA, USA).

### 2.2. Cell culture and stable cell lines establishment

The HEK293 cell line was cultured as previously described (Huang et al., 2010). HEK293 cells were stably transfected with plasmid for expressing EGFP-exon-1 htt-20Q or EGFP-exon-1 htt-160Q respectively, using Lipofectamine® 2000, and the stable lines were then selected as previously described (Li et al., 2010). The cells were treated with 9.6 μg/μL LBP and/or 40 μM LY294002.

### 2.3. Cell viability assay and caspase-3 activity assay

The Cell Counting Kit-8 (CCK8)-based cell viability assays were performed as follows: $1 \times 10^4$ cells were seeded into 96-well plates in DMEM containing 10% FBS for 12 h. The cells were then treated with LBP and/or LY294002 for 24 h. Next, CCK8 was added according to the manufacturer's instructions, and the cells were incubated for 1 h at 37 °C. The optical density was read at 450 nm using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Each experiment was performed in triplicate and was repeated 3 times.

The activity of caspase-3 was assessed based on an observation of the specific protease-peptide substrate chromogenic reaction (Liang et al., 2015; Liu et al., 2007; Yang et al., 2008; Zhu et al., 2012). The cells were cultured in 6-well plates at $1 \times 10^6$ cells/well in DMEM containing 10% FBS for 12 h. The cells were then treated with LBP and/or LY294002 for 48 h. After that, the cells were harvested, lysed, and centrifuged. Then, aliquots of supernatants were collected and incubated with the peptide substrates of caspase 3. Caspase 3 activity was determined based on the absorbance at 405 nm, which was read using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Each experiment was performed in triplicate and was repeated 3 times.

### 2.4. Animals and drug treatment

HD-N171-82Q-81 (also known as B6C3-Tg(HD82Gln)81Dbo/J Tg) mice (Schilling et al., 1999a) that were obtained from Jackson Laboratories were nicknamed TG mice. The mice were bred in SPF-grade animal houses. Genotyping was performed using polymerase chain reaction (PCR) with DNA obtained from tail biopsies. The genotyping primers were obtained from Jackson Laboratories (oIMR1239: GTGGATACCCCCTCCCCCAGCCTAGACC, oIMR1240: GAACTTTCAGCTACCAAGAAAGA CCGTGT).

All mice were housed with a 12 h light/dark cycle with ad libitum access to rodent chow and water at normal room temperature (22–25 °C) and humidity. Corn-husk bedding and 2 small bedding pads were provided in each cage. Food pellets and autoclaved water were suspended from a feeding cage and a drinking bottle, respectively. The mice were observed twice per day, at 9:00 a.m. and 9:00 p.m. Mouse body weight was monitored weekly, beginning when the mice were 12 weeks old.

The mice were divided into 4 groups: a wild-type mouse group that received distilled water (WT), a wild-type mouse group that received LBP (WT + LBP), a positive mouse group (POS), and a positive mouse group that received LBP (POS + LBP). For the survival tests, motor function performance assessments and body weight tests, 6 mice per group were sacrificed from the WT and WT + LBP groups, 11 mice were sacrificed from the POS group, and 7 mice were sacrificed from the POS + LBP group (TG mice died early, before they were 18 weeks old, and we therefore examined 11 and 7 mice in the POS and POS + LBP groups, respectively, because they unexpectedly died). For Western blotting analysis and RT-PCR tests, 16 mice were sacrificed (4 per group). For histological analysis and Nissl staining, 8 mice were sacrificed (2 from each group). The animals were intragastrically administered LBP (40 mg/kg body weight per day at a volume of 0.1–0.2 mL/g) (Wang et al., 2014) or an equal volume of distilled water daily beginning when they were 8 weeks old and continuing until they were 18 weeks old (to test life span, the mice were treated until they were sacrificed). Oral gavage was performed in the mice using ball-tip stainless steel feeding needles that were slightly bent and inserted along the back of the throat. If the mouse was observed to struggle, the needle was withdrawn, and the mouse was given a brief rest before the needle was reinserted to administer the remaining solution (this situation usually happened at the beginning of the study, and the mice later adapted to the procedure) (Jiang et al., 2015). The Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology, approved all animal experiments. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

### 2.5. Survival tests

For the lifespan studies, animals were monitored until they exhibited a moribund appearance (based on poor movement or grooming). At this time, they were either euthanized or discovered already dead. The endpoint was reached when a sudden weight drop of ~20% over a week was coupled with severe tremor, weakness, or abnormal gait. When the mice reached this point, they were sacrificed using the cervical dislocation method. In the POS and POS + LBP groups, 14 mice were sacrificed this way, and the other 4 mice were died before we could ease their suffering.