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# POTENTIAL APPLICATION OF GRAPE DERIVED POLYPHENOLS IN HUNTINGTON'S DISEASE

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**Abstract**

Huntington's disease (HD) is a progressive neurodegenerative disorder associated with selective neuronal cell death. Abnormal aggregation of huntingtin protein with polyQ expansion has been shown to be causally linked to HD. Grape seed polyphenolic extract (GSPE) is a natural compound that has previously been shown to interfere with aggregations of proteins involved in neurological disorders, such as amyloid beta peptides (Aβ) and Tau protein. In this study we found that GSPE treatment significantly inhibits polyQ aggregation in phaeochromocytoma (PC)-12 cell line containing an ecdysone-inducible protein comprising the first 17 amino acid of huntingtin plus 103 glutamines fused with enhanced GFP. In vivo feasibility studies using the *Q93httexon1* drosophila model of HD, we extended our in vitro evidence and found that flies fed with GSPE had a significantly improved lifespan compared to the control flies. Using the R6/2 rodent model of HD, we found that oral administration of 100 mg/kg/day GSPE (equivalent to 500mg per day in human) significantly attenuated the motor skill decay as well as extended the lifespan in the R6/2 mice relative to vehicle-control mice. Collectively, our studies strongly suggest that GSPE might be able to modulate the onset and/or progression of HD.

**Keywords**

Neurological disorders • Motor function • Abnormal protein aggregation
Huntingtin protein • Drosophila • PC-12 cell • R6/2 mouse



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## 1. Introduction

Huntington's disease (HD) is an autosomal–dominant neurological disorder that is caused by an unstable trinucleotide CAG repeat expansion at the N-terminus of the IT-15 gene, encoding the ~350 kDa huntingtin protein (htt) [1]. The mutation leads to production of htt proteins with an abnormal length of polyglutamine (polyQ) repeats. It is hypothesized that the polyQ repeats causes a gain of function resulting in abnormal protein-protein interaction [2,3]. HD is estimated to affect five to seven people in 100,000 throughout the world [4]. Clinical manifestation of the disease is characterized by severe cognitive and personality changes [5-7], accompanied by coordination decline and motor deficits that eventually lead to immobility [8]. Currently, there is no cure for this devastating neurodegenerative disorder, and treatments are mainly focused on alleviating cognitive and psychological symptoms. Recent evidence indicates that mutant htt (mhtt) aggregation in susceptible brain cells may be responsible for the onset and progression of HD phenotypes, and that the pathology and death of affected neurons are associated with the accumulation of mutant proteins in insoluble aggregates [9-12]. There are still debates regarding the role of aggregation in disease pathogenesis and several studies showed that the formation of aggregation could be a protective/defensive mechanism [10,13,14]. However, therapeutic strategies targeting polyQ aggregation have demonstrated some efficacy in various models of HD [15-18], suggesting that inhibition of abnormal neuronal htt protein aggregation might be a novel approach for HD treatment.

We previously reported that treatment with a select grape seed polyphenolic extract (GSPE) significantly attenuates spatial memory impairment in a mouse model of AD phenotypes [19]. Using both physical and biochemical methodologies, we found that GSPE can directly interfere with abnormal aggregation of proteins involved in neurological disorders, such as Aβ petides [19,20] and Tau proteins [21]. GSPE is also a strong antioxidant and powerful metal chelator that can effectively reduce reactive oxygen species in vivo. Previous studies have demonstrated increased oxidative damage in post-mortem brains of HD patients [22,23], and increased free radical production in animal models of HD [24], indicating the involvement of oxidative stress in HD pathogenesis. Based on these findings, in the present study we explore the role of GSPE in HD-type conditions. We report for the first time that GSPE treatment could significantly extend lifespan in the *UAS-Q93httexon1* drosophila model of HD, and attenuate HD phenotypes in the R6/2 mouse model of HD. Mechanisms through which GSPE may attenuate HD phenotypes include GSPE's strong antioxidant activity and demonstrated ability to attenuate abnormal aggregation of mhtt proteins.

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## 2. Experiment Procedures

### 2.1 GSPE.

In this study, MegaNatural grape seed polyphenolic extract (GSPE) was provided by Polyphenolics, Inc. (Madera, CA). It is a highly purified water-soluble polyphenolic preparation from *Vitis vinifera* seeds [25] which does not contain detectable resveratrol. Typically, MegaNatural GSPE contains approximately 8% monomers, 75% oligomers and 17% polymers. We arbitrarily used the molecular weight of catechin and epicatechin dimer (which is the most abundant form of oligmers in GSPE) to calculate the molarity for GSPE in this study.

### 2.2 Drosophila strains and GSPE treatment

Expression of polyQ-containing peptides is driven by the bipartite expression system upstream activator sequence (UAS)-GAL4 in transgenic flies [26]. w; *UAS-Q93httexon1* flies were obtained from Dr. Ivana Delalle. *elav-GAL4* flies (Flybase ID FBti0072910) were obtained from the Bloomington Stock Center. Elav-GAL4 flies were crossed to *UAS-Q93httexon1* flies to generate *elav>Q93httexon1* flies. Lifespans were generated by collecting newly enclosed animals, placing them at low density 10 animals per vial, either on control food (fly medium formula 4-24) or GSPE food (fly medium formula 4-24 supplemented with 2.8 µg/ml GSPE). The animals were counted each day and transferred to fresh vials every few days. Lifespans were generated by calculating the percentage of survivors daily and plotting this as a function of time in days.

### 2.3 R6/2 mice and GSPE treatment

R6/2 ovarian transplant female mice [27] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). R6/2 ovarian transplanted female mice were randomly divided into two groups (the 100 mg/kg/day GSPE treatment group and the $H_2O$ treated control group), and mated with wild type male mice. The weaning pups were continuously fed on the same treatment regimen as their parents. The resulting R6/2 mice and age-, strain-, and gender-matched wild type littermates were both included in the study. All procedures and protocols were approved by the Mount Sinai School of Medicine's Institutional Animal Care and Use Committee (IACUC) through the Center for Comparative Medicine and Surgery.

### 2.4 Rotarod behavior test

We used rotarod to assess motor functions in the mice. HD transgenic mice were trained to stay on a narrow rod in an accelerating rotarod apparatus (4 rpm- 40 rpm in 10 minutes) at 6 weeks of age, and rotarod performance was monitored once a week starting at 8 weeks of age. Three trials were conducted for each test given, and the average of the three trials was recorded. Loss of motor function is reflected by reduced latency time before the animals fall off the apparatus.

### 2.5 Rat adrenal pheochromocytoma PC-12 cells

PC-12 cells stably transfected with an ecdysone-inducible protein comprising the first 17 amino acids of huntingtin plus 103 glutamines fused with enhanced GFP [17] were used for the in vitro cell based study. Cells were grown in humidified 5% $CO_2$ incubators at 37°C. The medium comprises DMEM supplemented with 10% FBS, 1 mg/ml geneticin, zeocin and penicillin-streptomcycin (all drugs are from Invitrogen). At 50% cell confluence level, 0.2 µM of muristerone A (Invitrogen) was used to induce the expression of the transgene.

### 2.6 Western blot analysis.

48 hours after muristerone A induction, cells were washed once with cold phosphate buffered saline (PBS, pH 7.4) and harvested with lysis buffer and boiled. 25 µg protein was loaded on the 4-16% Bis-Tris gel and transferred on the nitrocellulose membrane. The western procedure was performed as previously described [28]. The antibodies used were: mouse monoclonal anti-polyQ-expansion antibody (Millipore); rabbit anti-GFP antibody (Calbiochem); rabbit anti-actin antibody, HRP-conjugated anti-mouse antibody and HRP-conjugated anti-rabbit antibody (Santa Cruz Biotechnology).

### 2.7 Oxidation assay and antioxidant capacity assay

PC-12 *htt103Q-EGFP* cells were lysed with sucrose isolation buffer (0.32 M sucrose, 0.125 M Tris pH 8.0, 0.1 mM EDTA, and 0.6 mM $MgCl_2$ with protease inhibitors) supplemented with 50 mM DTT and the supernatants were collected. Carbonylated protein level was determined using commercially available OxyBlot protein oxidation detection kit (Chemicon International, MA) according to the manufacturer's instructions. Lipid oxidation and protein nitration were determined by western blot analysis using the protocol described by Owen *et al.* [29]. Antioxidant capacity was analyzed using a commercially available antioxidant assay kit (Cayman's Chemical, MI) according to manufacturer's instruction.

## 3. Statistical Analyses

2-tailed student t-tests were used for analyzing rotarod tests. For surviving studies in drosophila subjects, the results of the four mortality analyses on drosophila survival were compared by a chi-square test for the differences among the respective z statistics that were obtained as square roots of the respective chi-squares on 1 degree of freedom from the Kaplan-Maier analyses. For surviving studies in mice, the log-rank test was used.

## 4. Results

### 4.1 GSPE treatment extends lifespan in the elav>httQ3 fly model of HD

In this study, *elav-GAL4* flies were crossed with *UAS-Q93httexon1* male flies to generate *elav>Q93httexon1* flies. Lifespan experiments were only performed in male flies, since mating can affect female lifespan. Lifespan studies in the male *elav>Q93httexon1* flies revealed that GSPE significantly increased lifespan (Figure 1, $\chi 2 = 21.73$, $df = 1$, $p = 0.0001$). In control lifespan studies using wild type flies, we found that GSPE had no effect (data not shown). Four independent lifespan trials and a total of 114 flies per group were used in this test.

### 4.2 GSPE treatment improves motor function and extends lifespan in the R6/2 mouse model of HD

The R6/2 mouse model of HD generated by Dr. Bates and colleagues [27] was used for in vivo feasibility studies. Animals were treated with 100 mg/kg/day GSPE and motor coordination tests (by accelerating rotarod) showed no significant behavior difference between the treatment and control groups at 6 weeks of age, when these animals are mostly asymptomatic. However, at 9 and 11 weeks of age, at which time HD phenotypes have developed in this mouse model, the GSPE treated group performed significantly better on motor coordination test than the control mice (Figure 2A). In control studies in wild type mice, we found that GSPE treatment had no effect on motor function (data not shown).

In addition, we found that GSPE treatment significantly extended lifespan in R6/2 mice compared with water-treated control mice (Figure 2B, Log-rank test, p= 0.0220). The median lifespan for non-treated control R6/2 mice was 90.5 days, while GSPE treated mice had a median lifespan of 98 days.

### 4.3 GSPE treatment reduces protein aggregation in PC-12 htt103Q-EGFP cells

Given the anti-protein aggregation role of GSPE in other neurological disorders [19-21], and our finding that GSPE could significant reduce motor deficits and increase lifespan in two independent in vivo HD models, we next tested whether GSPE exerts its beneficial effect by interfering with the formation of polyQ-containing aggregates. Using the PC-12 cell line that contains an ecdysone-inducible protein comprised of the first 17 amino acids of huntingtin protein, plus 103 glutamines fused with enhanced GFP (Htt103Q-EGFP) [17], we found that, consistent with previous reports, induction of polyQ protein expression with 0.2 μM muristerone A did not significantly affect cell viability. Induction of polyQ protein expression resulted in a range of polyQ antibody reactive species by western blot analysis, with molecular weight ranging from 55 kDa to 250 kDa. There was a visible smear of very high molecular weight polyQ reactive species in control cells, while treatment with 12.5 or 25 μM GSPE significantly reduced the accumulation of high molecular weight htt aggregates in a concentration-dependent manner by western blot analysis, and treatment with 25 μM GSPE completely diminished high molecular weight species (Figure 3A). This reduction in aggregates was also evident by reduced GFP aggregates under fluorescent microscope (Figure 3B). We also noted the smear between 36 kDa and 55 kDa in control cells which are much less in the 12.5 μM GSPE treated cells and almost absent in the 25 μM GSPE treated cells, which might be reflecting the degradation product of the high molecular weight species.

### 4.4 GSPE treatment reduces protein oxidation in PC-12 htt103Q-EGFP cells

Increased oxidative damage has been reported in HD brains for both human cases and experimental models of HD. Given that GSPE is a strong antioxidant, we tested whether treatment with GSPE might reduce oxidative stress in PC-12 cells expressing the mhtt protein. We first measured the antioxidant capacity of PC-12 cells upon GSPE treatment. The antioxidant capacity of the cells 48 hours after mhtt protein induction was 9.6 ± 0.3 mM/mg protein, while 12.5 μM GSPEtreated cells with mtt protein induction had increased levels of antioxidant capacity (12.8 ± 1.9 mM/mg protein, $p<0.05$). As protein carbonylation and nitration and lipid peroxidation are standard

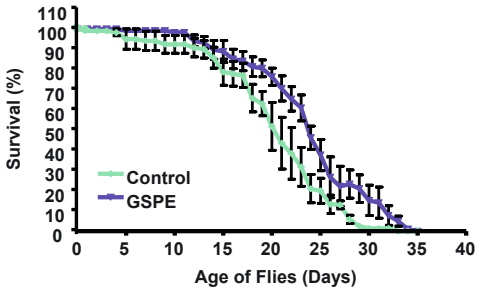


Figure 1. GSPE extends lifespan in male HD flie. elav> *Q93httexon1* male flies derived from crossing *elav-GAL4* and *UAS-Q93httexon1* flies were collected within one day of eclosion and placed 10 per vial on control food (n=30) or GSPE food (n=30). Lifespan was generated by calculating the percentage of surviving flies daily. Four independent lifespan trials were conducted in this manner.

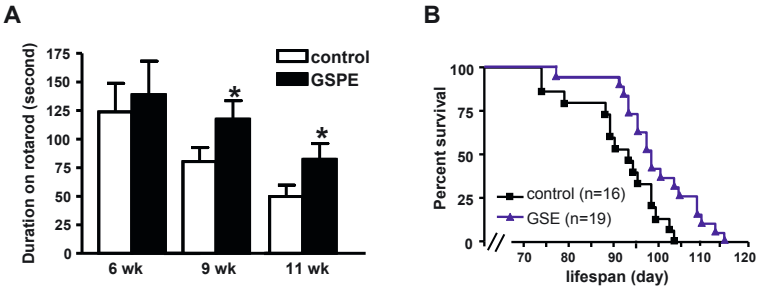


Figure 2. The impact of GSPE on the motor function and survival in R6/2 mice (A) Motor functions of R6/2 mice were assessed using accelerating rotarod at 6 weeks, 9 weeks and 11 weeks. The average of three trials conducted for each test was recorded. Duration on the rotarod is defined as latency time taken the animals to fall off the apparatus. (B) R6/2 transgenic mice were treated either with 100 mg/kg/day GSPE or $H_2O$ (as control group) and the survival rate was recorded daily.

markers for oxidative stress, we next measured levels of protein carbonylation, 3- nitrotyrosine (3NT) for protein nitration, and protein-bound 4-hydroxynonenal (HNE) for lipid oxidation upon GSPE treatment. We found that levels of protein carbonyls were significantly elevated 48 hours after mhtt protein induction, compared to non-induced control cells (Figure 4, lane 3 vs lane 1), confirming that polyglutamine aggregates could potentially induce oxidative stress. Further, treatment with 12.5 μM GSPE significantly reduced the level of carbonyls (Figure 4, lane 4 vs lane 3), consistent with the antioxidant capacity analysis. Treatment with GSPE significantly reduced the amount of aggregated proteins in PC-12 cells, which could have in turn reduced the level of oxidative stress (although it is plausible that GSPE could be directly involved in oxidative stress reduction). No significant alteration was found in the levels of 3NT or protein-bound 4-HNE (data not shown) in GSPE-treated cells.

## 5. Discussion

HD is a devastating neurological disease caused by abnormal trinucleotide CAG repeats. It is associated with neuronal intranuclear inclusions (NIIs) as well as cytoplasmic inclusions containing truncated polyQ fragments [2,30], resulting in progressive neuronal loss in the caudate and putamen [31]. Currently there is no cure.

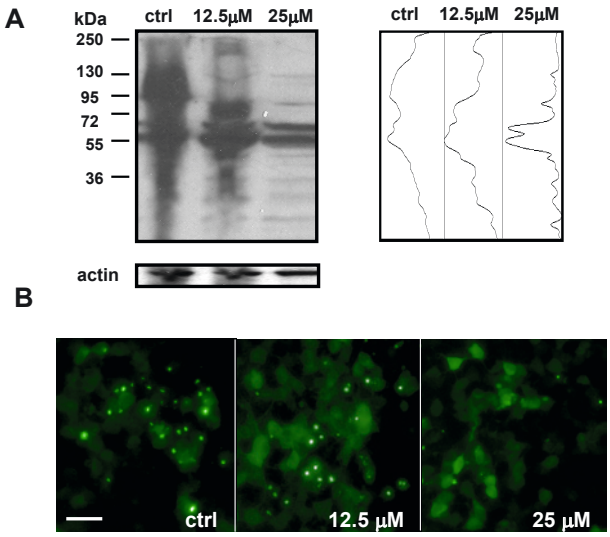


Figure 3. GSPE reduces Htt103Q-EGFP aggregation in PC-12 cell- based assay (A) Western blot analysis of PC12 cells expressing Htt103Q-EGFP in the absence (control) and presence of 12.5 μM and 25 μM 48 hours after 0.2 μM muristerone A induction. Left panel: western blot probed with anti-GFP antibody; right panel: densitometrical analysis of the western blot. (B) Representative fluorescent microscopy images of vehicle-treated control cells and cells treated with 12.5 μM and 25 μM GSPE after muristerone A induction; scale bar: 50 μm.

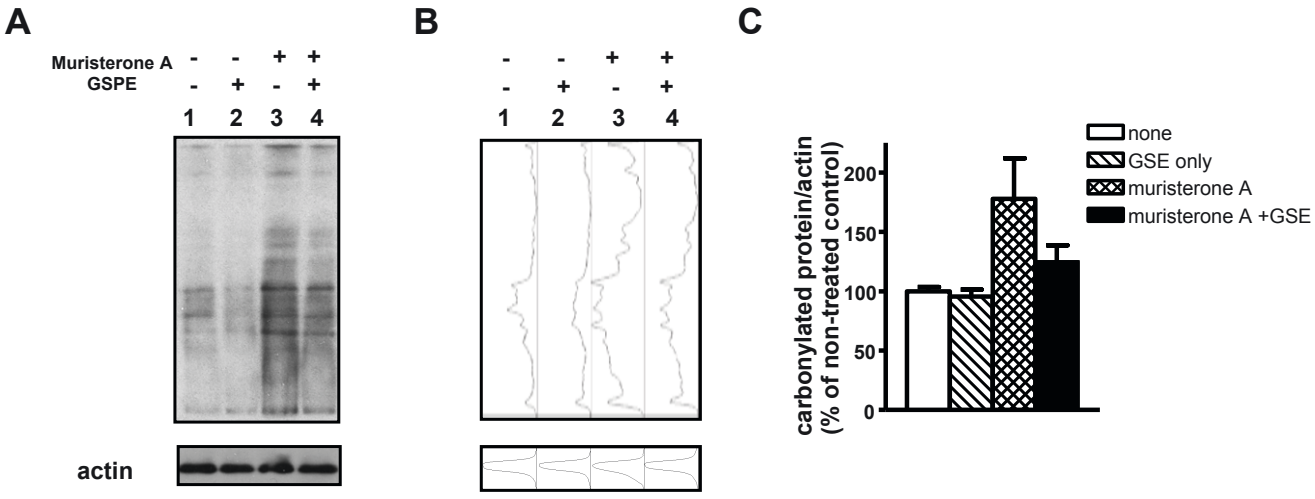


Figure 4. GSPE reduces protein oxidation in PC-12 htt103Q-EGFP cells (A) Western blot assessment of carbonylated protein level in PC-12 cells in the presence or absence of 12.5 μM GSPE with or without muristerone A induction. (B) Densitometrical analysis of the western blot. (C) Quantitative measure of carbonylated protein content as percentage of control (average of 3 independent experiments)

Overexpressing disease-associated aggregation-prone proteins has been used previously to model aspects of HD in drosophila [32-35]. Expression of Gal4 in a pan-neural pattern (*elav-Gal4*) in transgenic lines containing *UAS-Q93httexon1* results in adult-onset neurodegeneration, including the disruption of photoreceptor cells of the eye, impairments of climbing ability, and reduced lifespan. In this study, we demonstrated that GSPE could effectively improve survival in the Drosophila models of HD. To further assess the in vivo efficacy of GSPE, we also used a well-established mouse model of HD, specifically the R6/2 mice which exhibit a very aggressive neurological phenotype and provide clear experimental endpoints, which are ideal for preclinical feasibility study [27,36]. Treatment of R6/2 mice with 100 mg/kg/day GSPE significantly alleviated the deterioration of motor function and extended lifespan.

We have previously shown that GSPE could physically interfere with Aβ and Tau peptide aggregation [19-21]. Others have demonstrated that green tea polyphenol epigallocatechin-gallate was able to inhibit htt exon 1 aggregation and reduce motor impairment in HD flies [18]. Using PC-12 cells overexpressing *htt103Q-EGFP*, we demonstrated that GSPE could inhibit polyQ-htt aggregation in an in vitro tissue culture system. Moreover, GSPE could also increase antioxidant capacity and reduce the amount of carbonylated protein content in cells expressing mhtt aggregates, which is consistent with their strong antioxidant activity.

A unique feature of GSPE is that it is readily absorbed through the intestinal mucosa due to the modification of the constituent polyphenols [37]. Moreover, GSPE is well-tolerated in both humans and rodents [38]. One of the main obstacles for applying polyphenolic compound in the treatment of neurological disorders is the limited understanding of their bioactivity and whether they can pass the blood brain barrier and reach the central nervous system. In a recent bioavailability studies, we showed that GSPE and its metabolites can be detected in the brain when delivered at doses in the equivalent range of that employed in our feasibility studies using the R6/2 mouse model of HD [39]. In summary, our study demonstrated that GSPE could inhibit polyQ-htt aggregation in PC-12 cells expressing mhtt protein and reduce the oxidative stress induced by the expression of mhtt protein. Most importantly, oral treatment in both drosophila and mouse models of HD with GSPE effectively reduced motor deficits and extended lifespan. Our studies demonstrated strong evidence of preclinical efficacy of GSPE in HD treatment, and provide impetus for the application of GSPE for human clinical testing.

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