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Original Article

Molecular pathways related to the longevity promotion and cognitive improvement of *Cistanche tubulosa* in *Drosophila*

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ABSTRACT

Background: The aging process, including physical dysfunction and age-related memory impairment (AMI), are considered to be correlated with cumulative oxidative damages and insulin/IGF-1 signaling pathway.
Purpose: The present study was to elucidate the *in vivo* effects on delaying aging and ameliorating AMI and underlying molecular mechanisms of *Cistanche tubulosa* (CT), a herb used in traditional Chinese medicine to improve sexual function and treat kidney dysfunction.
Methods: The flies, treated and untreated with CT, were observed for lifespan, resistance to oxidative stress with H_2O_2 or paraquat, starvation assay, cognitive behaviors with T-maze, and transcript levels of target genes with quantitative RT-PCR.
Results: Administering CT extended the mean and maximum lifespan and increased resistance to oxidative stress in flies. CT supplementation also enhanced memory formation in young flies and suppressed AMI upon aging. Several genes and signaling pathways, including the mechanistic target of rapamycin (mTOR) and Notch networks, have been identified as causing these pharmacological effects and alterations in the gene expression of glutamate receptors.
Conclusion: Our results indicate that CT supplementation may contribute to slowing aging phenotypes and alleviating cognitive behavioral decline in flies, indicating potential applicability for enhancing human health and reducing susceptibility to age-related disorders.

Introduction

As the aged population increases worldwide, facilitating healthy aging and longevity is an increasingly crucial public health concern for science, society, and the medical care field. The aging process has been proposed as being controlled by complex mechanisms, driven by a broad interplay of genetic and environmental factors. Because of gradual damages at the cellular and tissue levels, age is a risk factor for and highly correlated with the susceptibility to chronic diseases such as cancer as well as cardiovascular and neurodegenerative diseases (Kenyon, 2005). Aging also reduces synaptic activity, new protein synthesis, and cognitive function. These functional changes contribute to age-related memory impairment (AMI) (Burke and Barnes, 2010; Schimanski and Barnes, 2010). Therefore, developing drugs for delaying age-related pathology and cognitive aging might be beneficial for the quality of human life. In this study, we used *Drosophila melanogaster*, which is an ideal organism for *in vivo* aging studies because of its short lifespan, to evaluate drug efficacy and explore the molecular mechanisms underlying the amelioration of aging and AMI.

Herba cistanche, also known by its Chinese name *Rou Cong Rong*, is a parasitic plant that is mainly distributed in the deserts of Northwest China. Two *Cistanche* species, *Cistanche deserticola* Y. C. Ma and *Cistanche tubulosa* (Schrenk) Wight, are included in the Chinese Pharmacopoeia (2005 edition) for their similar chemical ingredients and pharmacological activities. Their stem extracts

Abbreviations: AMI, age-related memory impairment; mTOR, mechanistic target of rapamycin; H_2O_2, hydrogen peroxide; $OH^{\cdot -}$, hydroxyl radical; $O_2^{\cdot -}$, superoxide anions.

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have been widely used for thousands of years as a tonic remedy, mainly for treating various symptoms, including deficient kidney function, impotence, infertility, and for facilitating catharsis, as described in ancient Chinese medical books. Numerous chemical constituents, including essential oils, phenylethanoid glycosides (PhGs), iridoids, lignans, and saccharides, have been isolated from *Cistanche* species, and two major PhG components, acteoside and echinacoside, are typically selected as markers for the quality evaluation of *Herba cistanche* because of their significant pharmacological actions (Wang et al., 2012).

C. tubulosa (CT) extracts possess with various biological functions that include modulating immune activity, enhancing sexual function, promoting hair growth, and other benefits on antioxidation, hepatoprotection, antiosteoporotic activity and anti-tumor (Li et al., 2016; Seok et al., 2015; Wang et al., 2012). Additionally, *in vitro* study revealed that *Herba cistanche* extract can protect dopamine-like neurons from H_2O_2-induced oxidative damage and significantly increase the levels of nerve growth factor and brain-derived neurotrophic factor (Lin et al., 2013). Echinacoside prevented dopamine neuron death in MPTP-lesioned mouse model of Parkinson's disease (Zhao et al., 2010). Moreover, CT extract ameliorated the cognitive dysfunction through decreasing amyloid deposition, and increasing acetylcholine and dopamine levels in a rat model of Alzheimer's disease (AD) (Wu et al., 2014). Although the therapeutic effect of *Herba cistanche* are still required to confirm, a small size, non-placebo-controlled clinical trial on CT glycoside extracts showed that the drug has a potential to treat patients with mild to moderate AD and is with relatively mild adverse reactions, even after long-term administration (Guo et al., 2013). These data suggest that glycoside extracts in *Herba cistanche* have neuroprotective and anti-dementia effects. Despite various benefits of CT, its molecular mechanisms and effects on delaying aging and ameliorating AMI have not been elucidated. In this study, the effects of CT on a significant extension of lifespan, improved resistance to environmental stress, and suppressed age-related cognitive dysfunction were examined to determine the possible clinical utility of CT in slowing the aging process.

Materials and methods

Herbal medicine

The origin identification of *Cistanche tubulosa* (Schrenk) Wight (Orobanchaceae) (CT) was verified in the Ko Da Pharmaceutical Co. Ltd. (Taoyuan, Taiwan), where voucher specimens (10,603,205) have been kept and the CT concentrated herbal medicine is manufactured. Briefly, the dried fleshy stem of CT was extracted in 12-fold boiled water for 1 h followed by filtration. The filtrates were collected and subjected to vacuum and reduced-pressure concentration for 1 h to obtain extracts. The CT extracts were mixed with starch at 1:1 ratio (w/w) and subjected to spray granulation for obtaining CT powder.

Fly stock maintenance and lifespan measurement

The wild-type fly line used in this study was of the *Canton S*, obtained from the Bloomington *Drosophila* Stock Center. Flies were raised and maintained in cornmeal standard media at 25 °C in 50–60% relative humidity under a 12-h light-dark cycle. Emerging adult flies were collected within 24 h and separated by sex. From early adulthood, flies were supplemented with CT. CT powder was dissolved in water and added to cornmeal media at 5.4, 10.8, and 21.6 mg/ml concentrations, which were proportional to the effective daily dosage for human oral administration. In a lifespan analysis, twenty to thirty flies were raised in a food vial with or without CT; at least 250 flies were conducted for each treatment. Food vials were replaced every 3 to 4 days, and dead flies were counted at that time.

Stress resistance assays

Adult flies were collected, and then pretreated with control diet without CT and experimental diets supplemented with 5.4, 10.8, and 21.6 mg/ml of CT for 20 days at 25 °C. For starvation analysis, flies were kept in vials with wet papers only and evaluated their stamina against starvation-induced oxidative stress. For oxidative stress assays, the flies were transferred to food-free vials containing a filter paper with hydrogen peroxide (H_2O_2) (Sigma-Aldrich) in a 5% glucose solution to gauge the flies' resistance against H_2O_2-induced hydroxyl radical ($OH^{\cdot -}$) stress. Similarly, flies were transferred to vials containing a filter paper saturated with 10 mM paraquat (Sigma-Aldrich), which generates superoxide anions ($O_2^{\cdot -}$) that cause oxidative damage to animals. Flies were scored for mortality at least three times daily until all were dead. At least 250 flies per treatment were performed. For climbing assay, flies exposed to paraquat for 0 and 16 h were placed in a plastic vial and gently tapped to the bottom. The number of flies at the top of the vial was counted after 18 sec of climbing.

Fecundity assessment

Both virgin males and females fed 5.4 and 10.8 mg/ml of CT for 20 days served as experimental groups. Ten experimental females were mated with an equal number of virgin males, which were 9-day-old and had not received CT. Similarly, the experimental males were mated with virgin females in the same condition. The mated female flies were then transferred to a new vial for egg laying for 24 h, and the total eggs laid in the vials were counted manually. An index of fecundity was calculated as the mean daily egg production per female. At least five vials per treatment were performed, and total flies n = 50–80.

Olfactory associative learning and memory

Flies were collected after eclosion and pretreated with 5.4, 10.8, and 21.6 mg/ml of CT served as experimental groups, and the control flies had not received CT for 5 and 20 days at 25 °C. A single training trial was performed according to a previous study (Dubnau et al., 2001). Briefly, approximately 100 flies were exposed sequentially to two odors, either 3-octanol (OCT, Sigma-Aldrich) or 4-methylcyclohexanol (MCH, Sigma-Aldrich). Flies exposed to the first odor (conditioned stimulus, CS+) were paired with 80 V electric shocks and then received a second odor (CS-) without shocks. Learning ability was determined immediately after training and the abilities of 3- and 24-h memory were conducted the tests after training procedure for 3 and 24 h, respectively. To perform the test, the trained flies were trapped into the choice point of a T-maze in which they were exposed simultaneously to OCT and MCH. A performance index (PI) was calculated to represent the conditioned odor avoidance. A PI of zero indicated a 50:50 distribution, and a PI of 100 showed a 0:100 distribution away from the CS+ odor.

Measurements of transcript levels through real-time quantitative PCR (qPCR)

Flies were collected after eclosion and pretreated with 5.4 and 10.8 mg/ml of CT for 20 days at 25 °C served as experimental groups, and the control flies had not received CT. The total RNA was extracted using an RNeasy Mini kit (Qiagen), and cDNA was synthesized from the total RNA through reverse transcription reaction by using a High-Capacity cDNA Reverse Transcription Kit