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Lifespan extension by cranberry supplementation partially requires SOD2 and is life stage independent

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ABSTRACT

Many nutraceuticals and pharmaceuticals have been shown to promote healthspan and lifespan. However, the mechanisms underlying the beneficial effects of prolongevity interventions and the time points at which interventions should be implemented to achieve beneficial effects are not well characterized. We have previously shown that a cranberry-containing nutraceutical can promote lifespan in worms and flies and delay age-related functional decline of pancreatic cells in rats. Here we investigated the mechanism underlying lifespan extension induced by cranberry and the effects of short-term or life stage-specific interventions with cranberry on lifespan in *Drosophila*. We found that lifespan extension induced by cranberry was associated with reduced phosphorylation of ERK, a component of oxidative stress response MAPK signaling, and slightly increased phosphorylation of AKT, a component of insulin-like signaling. Lifespan extension was also associated with a reduced level of 4-hydroxynonenal protein adducts, a biomarker of lipid oxidation. Moreover, lifespan extension induced by cranberry was partially suppressed by knockdown of SOD2, a major mitochondrial superoxide scavenger. Furthermore, cranberry supplementation was administered in three life stages of adult flies, health span (3–30 days), transition span (31–60 days) and senescence span (61 days to the end when all flies died). Cranberry supplementation during any of these life stages extended the remaining lifespan relative to the non-supplemented and life stage-matched controls. These findings suggest that cranberry supplementation is sufficient to promote longevity when implemented during any life stage, likely through reducing oxidative damage.

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1. Introduction

Aging is modulated by both genetic and environmental factors (Fontana et al., 2010). A number of interventions using pharmaceuticals and nutraceuticals have been identified to promote healthspan and lifespan in model organisms (Dong et al., 2012; Frankel et al., 2011; Hada et al., 2013; Lee et al., 2010; Lucanic et al., 2013). However, aging is a complex biological process and many roadblocks remain to be overcome before any of these interventions can be applied to humans. On one hand, lifespan is modulated by multiple genetic pathways, including insulin-like, target-of-rapamycin (TOR) and sirtuin signaling pathways (Fontana et al., 2010; Hall et al., 2013). On the other hand, an adult life can be divided into three separate life stages, health span, transition span and senescence span from young to old, based on the age-specific mortality rate of the population of an organism (Arking, 2006; McDonald et al., 2013). Age-specific mortality rate is negligible in the health span, gradually rises in the transition span and exponentially increases in the senescence span. At the molecular level, the three life stages are characterized by different gene expression patterns and increasing levels of oxidative damage to macromolecules with age. At the physiological level, the three life stages are associated with gradual disruption of organelle function, declining metabolic capacity and sensitivity to stress with age. At the behavioral and cognitive level, the three life stages are associated with gradual declines in locomotor activity and learning and memory capacity with age.

The complex nature of life stages poses a major challenge to develop effective interventions for promoting healthspan and lifespan. Many interventions tested so far have been initiated in very young adults and continued throughout adult life in model organisms (Lucanic et al., 2013; Roth et al., 2005). Any intervention starting in young adults can be costly and impractical to implement in humans Moreover, it may miss interventions effective in certain life stages. A few studies have started to address the effects of life stage-specific interventions on healthspan and lifespan. For example, some investigators have tested the prolongevity effect of drugs by initiating the intervention in middle-aged mice. The median lifespan of mice under standard lab conditions is approximately two years. The effects of rapamycin and resveratrol on lifespan and healthspan have been revealed by feeding these two drugs to one-year old mice, respectively (Baur et al., 2006;

Abbreviations: 4-HNE, 4-hydroxynonenal; cran, cranberry; IR, inverted repeat; MAPK, MAP kinase; RNAi, RNA interference; S6K, S6 kinase; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; SY, sugar-yeast diet; TOR, target of rapamycin.

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Harrison et al., 2009). More systematic life stage-specific interventions have been tested for curcumin and sodium butyrate in *Drosophila*, which has mean lifespan of a couple of months, depending on culture conditions. Curcumin supplementation at a modest level throughout adult life can extend lifespan in *Drosophila* (Lee et al., 2010; Shen et al., 2013). However, supplementation with a high concentration of curcumin extends lifespan when implemented during the health span, but decreases lifespan when administered chronically throughout adult life in *Drosophila* (Soh et al., 2013). Supplementation of sodium butyrate can extend the lifespan when implemented during the transition or senescent span, but decrease the lifespan when applied during the health span in flies (McDonald et al., 2013). These studies point out the importance to test the effect of any intervention during different life stages on healthspan and lifespan. Such studies are still very limited in the number of cases.

Cranberry is a popularly consumed berry rich in numerous kinds of bioactive phytochemicals (He and Liu, 2006; Howell, 2007; Neto, 2007). Cranberry derived nutraceuticals have been shown to extend lifespan in worms and flies (Guha et al., 2013; Wang et al., in press). Cranberry feeding also delays age-related functional decline of pancreatic β-cells in rats (Zhu et al., 2011). Here we investigated the mechanisms underlying the prolongevity effect of cranberry and determined the effect of short-term cranberry supplementation in three life stages on lifespan in *Drosophila*. We found that cranberry reduces accumulated oxidative damage and does not substantially increase lifespan of *sod2* knockdown flies. We also found that implementation of cranberry supplementation during any of the health, transition or senescence span is sufficient to extend the remaining lifespan of flies. Our findings suggest that cranberry supplementation promotes healthy aging at least partially through reducing oxidative damage and is effective even when implemented for a short-term.

2. Materials and methods

2.1. Fly stocks and culture

Fly stocks were maintained on the standard cornmeal-yeast-agar medium at 25 ± 1 °C, 60 ± 5% humidity and a 12:12 h light/dark cycle (Ashburner et al., 2005). The wild-type strain *Canton S*, Gal4 driver da-Gal4 (w^{1118}; P{w[+mW.hs] = GAL4-da.G32},3) and UAS line UAS-*sod2 inverted repeat* (*IR*) (w^1; P{UAS-Sod2.dsRNA.K}15/SM5) were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). *sod2* knockdown flies were generated from crossing UAS-*sod2*IR with da-Gal4 flies. Two isocaloric sugar-yeast (SY)-agar based diets, SY9:1 and SY1:1, described previously were used for assays (Lee et al., 2008; Skorupa et al., 2008; Sun et al., 2012). The SY9:1 diet contained 18% sugar and 2% autolyzed yeast (MP Biochemicals, Solon, Ohio), which has SY at 9:1 ratio, and the SY1:1 diet had 10% sugar and 10% yeast. The cranberry supplemented diets were prepared as previously described (Wang et al., in press). Briefly, proanthocyanidin-enriched cranberry extract, kindly provided by Decas Botanical Synergies (Carver, MA), was added to the cooled SY diet to the final concentrations of 2% by weight/volume. The main ingredients of the cranberry extract include ≥4.0% proanthocyanidins, 37.9% sugars, 4.1% dietary fiber, 0.5% protein, ≤5.0 moisture, 7.2% ash and ≤4.0% total vitamin and minerals as described by Decas Botanical Synergies (http://www.decasbotanical.com).

2.2. Lifespan assay

Lifespan assay was performed as previously described (Sun et al., 2012). For life stage-specific interventions, adult flies were transferred to the SY1:1 diet in bottles within 24 h after eclosion. After mating for 24 h, female flies were sorted out and placed in SY1:1 vials each with approximately 20 flies. After another 24 h on SY1:1, cranberry supplementation was initiated in three life stages. For supplementation during the health span, flies were fed SY9:1 with and without 2% cranberry from the age of day 3 to 30; for supplementation during the transition span, cranberry supplementation was implemented from the age of day 31 to 60; for intervention during the senescence stage, cranberry supplementation was administered from the age of day 61 to the end when all flies died. For measuring the lifespan of *sod2* knockdown flies, sorted females of 2-day old were transferred to SY9:1 or SY1:1 with and without 2% cranberry. All flies were transferred to fresh food once every 2–3 days. The number of dead flies was recorded at each transfer until all flies were dead. Each lifespan assay used approximately 100–200 flies in 5–8 vials.

2.3. Western blot analysis

The heads or whole bodies of 14-d old flies were collected for protein preparation with tissue extraction reagent 1 (Cat # FNN0071, Life Technologies Inc.). The flies were fed the SY9:1 diet with and without 2% cranberry starting at the age of day 3 for 11 days before sample collection. Protein samples were separated by electrophoresis in a 10% Bis-Tris NuPAGE gel (Cat. # NP0303BOX, Life Technologies Inc.) and transferred to a polyvinylidene difluoride membrane. Proteins of interest were probed with primary antibodies against ERK and pERK (Cat # 4695 and 9101, Cell signaling, Danvers, MA), 4-HNE-protein adducts (Cat # 393206, CalBiochem), AKT and pAKT (Cat # 9272 and 4691, Cell signaling), and β-Actin (Cat #8224, Abcam) at a dilution from 1000 to 5000. Secondary antibodies were HRP goat anti-rabbit IgG (Abcam Cat # ab6721), HRP goat anti-mouse IgG (Abcam Cat # ab6789) or HRP donkey anti-goat IgG (Santa Cruz Cat # sc-2020) at a dilution of 5000–20,000. The signals from Western blot analysis were quantified by usihe Image Quant TL software in the Typhoon TRIO + Variable Mode Imager (GE Healthcare life sciences). Each assay was repeated with 5–6 biologically independent replicates.

2.4. Statistical analysis

Statistical analyses were performed using StatView version 5.0 software (SAS, Cary, NC), SPSS version 18 software (IBM) and R program. Lifespan data were analyzed by Mantel–Cox logrank tests and a likelihood ratio test. All other measurements were analyzed by Student's *t*-test. Maximum lifespan was calculated as mean lifespan of the 10% longest surviving flies of a population. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cranberry supplementation affects insulin-like and MAPK signaling

We previously found that 2% cranberry can extend lifespan of female flies fed a high sugar-low protein SY9:1 diet (Wang et al., in press). To understand the underlying mechanisms, we measured changes in the phosphorylation of proteins in three key metabolism and stress response pathways using the head protein samples of flies fed the SY9:1 diet supplemented with and without 2% cranberry for 11 days. The proteins tested are S6 kinase (S6K) in TOR signaling (Kim and Guan, 2011; Zoncu et al., 2011), AKT in insulin-like signaling (Broughton and Partridge, 2009) and ERK1/2 in stress response MAP kinase (MAPK) signaling (Rubinfeld and Seger, 2005). We chose head samples to reduce the complexity of protein samples since it is known that gene expression patterns are different among tissues. Cranberry supplementation did not significantly change the ratio of phosphorylated S6K (pS6K) to the total S6K in fly heads (Fig. 1A). However, cranberry supplementation slightly but significantly increased the ratio of pAKT/AKT ($p < 0.05$, Fig. 1B), while it significantly reduced the ratio of phosphorylated ERK1/2 to the total ERK1/2 ($p < 0.05$, Fig. 1C), when compared to the non-supplemented controls, respectively. These findings suggest