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Comparative approaches to facilitate the discovery of prolongevity interventions: Effects of tocopherols on lifespan of three invertebrate species

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Summary

Many compounds hold promise for pharmacologic manipulation of aging. However, such claims are difficult to investigate due to time and budget constraints. Here, we took a comparative approach, using short-lived invertebrate species, to directly test the effects of two tocopherols (vitamin E) on longevity. γ -tocopherol represents the most abundant tocopherol in the Western diet, while α -tocopherol is selectively enriched in human plasma. Both isoforms demonstrate antioxidant activity and are proposed to have anti-aging activities. We compared the effects of α - and γ -tocopherol supplementation on lifespan in three invertebrate species. γ -, but not α -, tocopherol slightly extended lifespan in nematodes, but neither significantly affected lifespan in two fly species. This study shows that a comparative approach, utilizing multiple invertebrate species, can increase the robustness of invertebrate-based pilot screens for longevity interventions.

Pharmacological and nutritional approaches to retard aging and increase longevity have been proposed (Floyd, 2006, Ingram et al., 2006, Joseph et al., 2005). Large-scale screens are being considered for a number of model organisms to accelerate discovery of compounds with the greatest potential for extending lifespan (Bauer et al., 2004, Gill et al., 2003, Sampayo et al., 2003, Wang et al., 2004). One class of compounds with potentially beneficial effects on aging are the tocopherol isomers found in Vitamin E and which are found naturally in plant oils (Ames et al., 2005). Vitamin E is a mixture of several related tocopherol and tocotrienol isomers, including the α - and γ -tocopherols. The Western diet is enriched in γ -tocopherol, but α -tocopherol is selectively retained in human plasma (Jiang et al., 2001, Tucker and Townsend, 2005). Both tocopherols are derived from fatty acids and exist within membranes, where they affect membrane fluidity and can act as antioxidants.

The high antioxidant capacity of vitamin E makes it a candidate for a longevity therapy, since aging is correlated with increased oxidative stress in cells (Ames et al., 1993, Harman,

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2006). However, numerous clinical trials have found little benefit from vitamin E (α -tocopherol) supplementation for outcomes related to cardiovascular disease, cancer or neurodegenerative disease (Pham and Plakogiannis, 2005, Pham and Plakogiannis, 2005, Poston et al., 2006). To date, most studies have focused on α -tocopherol supplementation, based on the rationale that this is the most abundant isoform in the body. However, several investigators have raised the possibility that γ -tocopherol may have beneficial properties worthy of investigation (Devaraj and Jialal, 2005, Jiang et al., 2001).

One efficient strategy for directly examining whether these compounds have any effect on lifespan is to determine whether supplementation can affect lifespan in short-lived invertebrate species, which offer models for aging in humans (Kenyon, 2001). Here, we have directly examined the effect of both α - and γ -tocopherols on lifespan of the nematode *Caenorhabditis elegans*, the fly *Drosophila melanogaster* and the Mexican fruit fly *Anastrepha ludens*. *C. elegans* and *D. melanogaster* have been widely used for studies of aging and longevity, due to their relatively short lifespans (2-3 week and 2-3 months, respectively). The Mexican fruit fly, *A. ludens*, has proven to be an excellent model for demographic studies of aging (Vaupel et al., 1998).

For *C. elegans* aging assays, synchronous populations were obtained by allowing 5-10 hermaphrodites to lay eggs for 4 hours to overnight. For ease of analysis, we used the adult sterile strain, *fem-1(hc17)*, to avoid progeny overgrowth in lifespan assays. Lifespan scoring was initiated after hermaphrodites completed the final larval molt, on the first day of adulthood. For *D. melanogaster* aging assays, wild type *Canton-S* flies were cultured on cornmeal food to lay eggs. Within 24 hours after eclosion, adult flies were transferred to bottles with 10% yeast extract, 10% sugar and 2% agar food (SY medium) and allowed to mate overnight (Magwere et al., 2004). Subsequently flies were separated by sex and transferred to vials containing SY medium and various concentrations of tocopherols for lifespan experiments. The lifespan assays for *A. ludens* were conducted in population cages (Carey et al., 2005). Each cage housed approximately 2000 adult flies with roughly equal number of males and females. Two Petri dishes with yeast extract, sugar and various amounts of tocopherols were provided to the flies in each cage once a week. The cages were kept under dim light to minimize the exposure of tocopherol to light. Dead flies were removed from the cages, sorted by sex and counted daily until all the flies in the cage had died.

Two previous studies had assessed the effect of tocopherols on *C. elegans* lifespan and found that α -tocopherol either had no effect (Adachi and Ishii, 2000) or a slightly beneficial effect (Harrington and Harley, 1988). In our study, we compared the effect of α - or γ -tocopherols on *C. elegans* lifespan at either 20 or 200 $\mu\text{g/mL}$. Treatment with α -tocopherol at the higher dose slightly reduced mean *C. elegans* lifespan, although neither dose affected maximum lifespan (Fig 1A). In contrast, γ -tocopherol increased mean lifespan at both doses tested (Fig. 1B). Interestingly, when α - and γ -tocopherol were mixed in equivalent ratios, there was no significant effect on lifespan (Fig. 1C), suggesting that the detrimental effect of α -tocopherol supplementation could suppress any benefits of γ -tocopherol supplementation in this model.

To determine if these findings in *C. elegans* could be confirmed in other invertebrates, we examined the effects of α - or γ -tocopherol supplementation on lifespan of *D. melanogaster*. Several previous studies had assessed the effect of vitamin E abundant with α -tocopherol on lifespan of *D. melanogaster*, but the results were inconsistent. One study found that a low concentration of vitamin E (20 $\mu\text{g/mL}$) but not higher concentrations extended mean lifespan (Driver and Georgeou, 2003). Other studies found that medium (100 $\mu\text{g/mL}$), but not higher or lower, concentrations of Vitamin E extended mean lifespan (Baker, 1993, Miquel et al., 1982). Here we tested α - or γ -tocopherol at concentrations of 20, 100 or 200 $\mu\text{g/mL}$ of food. Supplementation with α - or γ -tocopherol at these three doses had a negligible effect on lifespan

of *D. melanogaster* males. In contrast, supplementation with α - or γ -tocopherol at the higher concentration (200 γ g/mL), but not the lower concentration (20 γ g/mL), slightly reduced mean lifespan in females by 6.9% or 12.5% respectively ($p < 0.01$) (Fig. 2, Table 1).

To further explore the effects of tocopherols on lifespan of invertebrates, we supplemented another insect species, *A. ludens*, with exogenous α - or γ -tocopherol. Large-scale cultivation of *A. ludens* Mexican fruit flies has greatly facilitated demographic studies of aging in this species (Carey et al., 2005). For lifespan assays, both males and females were housed together, which allowed mating and reproduction throughout the treatment. At a concentration of 100 γ g/mL, supplementation with α - or γ -tocopherol had little effect on both mean and maximum lifespan of females (Fig. 3). Supplementation with α - or γ -tocopherol had little effect on the lifespan of males, although α -tocopherol supplementation slightly reduced mean lifespan of males by about 10% (but not statistically significant) (Fig. 3). To examine the temporal effects of tocopherols, we calculated the daily mortality rate of *A. ludens* fed tocopherols. Age-specific mortality rates were similar for both females and males fed either α - or γ -tocopherol compared to the controls across their lifespan (data not shown).

In summary, we have compared the effects of α - and γ -tocopherol supplementation on lifespan of three different species representing readily available model organisms used for aging research. While γ -tocopherol was slightly beneficial for longevity for *C. elegans* nematodes, there was no statistically significant benefit detected for *D. melanogaster* or *A. ludens* flies. In fact, slightly detrimental effects of α -tocopherol were detected for *C. elegans* and of both isoforms for *A. ludens* or for *D. melanogaster*.

The supplementation doses used in this study were reasonable for testing bioactivity in these organisms. Based on the estimated weight and daily food consumption for *D. melanogaster* flies (weight 0.5-1.0 mg, daily food consumption 0.5-1 γ l) and Mexican fruit flies (weight 10 mg, daily food consumption 10-40 γ l), we estimate that daily tocopherol intake was at least 5 γ g/g of fly body weight at the lowest concentration of tocopherol (20 γ g/mL) (Carvalho et al., 2005). Thus, it would appear that this dose would be higher per gram body weight than the US RDA for Vitamin E for humans, (15-30 mg/adult, approximate adult weight of 80 kg).

One possible explanation for the failure of tocopherol supplementation to extend lifespan in this study may be that this compound did not sufficiently alter redox status or cellular oxidative damage. Alternatively, these negative results may contribute to the increasing evidence that reductions in macromolecular oxidative damage do not always correlate with increased lifespan. For example, although mice with reduced mitochondrial superoxide dismutase activity exhibited increased levels of oxidative damage to nucleic acids, they did not have shortened lifespans (Bokov et al., 2004). Thus, the levels of cellular oxidative damage do not always clearly correlate with lifespan.

These cross-species experiments suggest that both α - and γ -tocopherol have marginal effects on longevity. However, the overall weak effects of these compounds on lifespan in these three species are consistent with the data from human supplementation trials, at least for α -tocopherol (Pham and Plakogiannis, 2005, Pham and Plakogiannis, 2005). While genetic approaches for examining longevity have been relatively well-defined in invertebrate and rodent species, the usefulness of invertebrate models for identifying compounds that may promote longevity in humans has not been determined. Several potential problems exist, including the inability to determine precise causes of death in small invertebrate species and the problem of determining drug target specificity. In addition, a significant caveat of utilizing invertebrates for pharmacological testing is the limited ability to determine a compound's bioavailability in these species.

Nevertheless, several groups have successfully identified and begun to mechanistically describe compounds that promote increased longevity in the invertebrates, *C. elegans* and *D. melanogaster* (Evason et al., 2005, Gill et al., 2003, Sampayo et al., 2003, Wilson et al., 2006, Wood et al., 2004). The next step in such a screening process would be to determine whether the longevity benefits of these treatments can be generalized to different mammalian species, such as mice, and whether such effects are relevant for human clinical testing. We consider the use of several species in examining the effects of potential compounds necessary. This will enable comparison of results using different methodologies. More importantly, the compounds that have robust beneficial effects on aging and lifespan across several invertebrate species with distinct physiology may be more likely to have generalized beneficial effects on aging in many mammalian species, including humans.

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Fig. 1.

Effect of α - and γ -tocopherol on *C. elegans* lifespan.

Survival analysis of adult sterile *fem-1(hc17)* animals at 25°C in the presence of indicated doses of (A) α -, (B) γ - or (C) α + γ -tocopherols. Animals were placed on tocopherol treatments as young adults and survival scored throughout adulthood. Results are shown for one representative of two independent trials with similar results. Statistical analysis of each treatment: vehicle control, mean lifespan, 12.6 days; α -tocopherol, 20 µg/mL, 13.1 days, 200 µg/mL, 10.8 days; γ -tocopherol, 20 µg/mL, 14.7 days, 200 µg/mL, 15.2 days; α + γ -tocopherols, 20 µg/mL combined, 14.6 days; 200 µg/mL combined, 12.9 days; n=48-60 animals/treatment. For both concentrations of γ -tocopherol, $p < 0.0001$ vs vehicle control (Log-

Rank). An intermediate concentration of γ -tocopherol, 100 $\mu\text{g/mL}$, did not statistically significantly extend lifespan, although there was a slight increase in mean lifespan at this dose compared to controls.

Fig. 2.

Effect of α - and γ -tocopherol on lifespan of *Drosophila melanogaster*.

Canton-S flies were placed on tocopherol treatments at indicated doses as young adults. Each experiment was conducted at 25°C using 5-7 vials with ~20 males or females per vial. Flies were transferred to a new SY medium vial once every 2-3 days. The vials were covered with cardboards to minimize light damage to tocopherols. At the time of transfer, the number of dead flies was counted until all the flies died. Mean lifespan of flies under each treatment is listed in Table 1.

Fig. 3.
Effect of α - and γ -tocopherol on lifespan of *Anastrepha ludens*.

A. ludens flies were placed on tocopherol treatments at 100 $\mu\text{g/mL}$ of α -, or γ -tocopherols as young adults. Each treatment was conducted at 25°C using two cages with approximately 2000 mixed males and females in each cage. Flies in each cage were provided with two new food dishes once a week. The cages were kept at dim light to minimize light damage to tocopherols. Statistical analysis presented in Table 1.

Table1Mean lifespan of *D. melanogaster* and *A. ludens* fed tocopherols.

Treatment	Mean lifespan of males (Number of flies)	Mean lifespan of females (Number of flies)
<i>D. melanogaster</i>		
Control	38.6 (n=150)	23.2 (n=138)
α -tocopherol, 20 μ g/mL	39.1 (n=147)	22.1 (n=131) *
α -tocopherol, 100 μ g/mL	35.6 (n=89)	23.6 (n=145) **
α -tocopherol, 200 μ g/mL	37.9 (n=152)	21.6 (n=114) **
γ -tocopherol, 20 μ g/mL	41.2 (n=91)	21.8 (n=133) *
γ -tocopherol, 100 μ g/mL	34.2 (n=115)	20.9 (n=131) *
γ -tocopherol, 200 μ g/mL	30.9 (n=121)	20.3 (n=140) **
<i>A. ludens</i>		
Control	60.4 (n=1,553)	59.2 (n=1,471)
α -tocopherol, 100 μ g/mL	53.3 (n=1,707)	57.9 (n=1,275)
γ -tocopherol, 100 μ g/mL	56.8 (n=1,466)	60.1 (n=1,328)
* $p < 0.05$		
** $p < 0.01$		