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RESEARCH ARTICLE

Zingiber officinale extends *Drosophila melanogaster* life span in xenobiotic-induced oxidative stress conditions

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BACKGROUND: The possibility of dietary ginger to enhance oxidative stress resistance and to extend life span was studied on *Drosophila melanogaster*.
METHODS: Oxidative stress was induced by a reducing agent dithiothreitol. Experimental groups of male *D. melanogaster* were cultured on media containing: 1) no additive; 2) dithiothreitol, added into the nutritional mixture to the final concentration of 10 mM; 3) 25 mg of ginger powder g^{-1} of the nutritional mixture; and 4) 10 mM of dithiothreitol and 25 mg of ginger powder g^{-1} of the nutritional mixture. The number of alive fruit flies was inspected daily, and mean life span was determined for each experimental group.
RESULTS: The addition of dithiothreitol to *D. melanogaster* nutritional mixture was established to result in an increase in concentration of two markers of oxidative stress conditions (thiobarbituric acid reactive substances as products of lipid peroxidation and carbonylated proteins as products of protein oxidation) in fly tissues. It was followed by significant reduction of mean life span and maximum life span of the last 10% of flies. Plant preparation, being added simultaneously with dithiothreitol, significantly diminished the negative effects of this xenobiotic. In conditions of additional stress load induced by hydrogen peroxide or high temperature, survival of insects treated with dithiothreitol on the background of ginger powder was the highest.
CONCLUSIONS: Thus, the presented data give the evidence that ginger preparations can reduce oxidative stress outcomes and significantly increase the life expectancy of fruit flies in stress conditions.

Keywords ginger, oxidative stress, *Drosophila melanogaster*

Introduction

Current interest in the aging process, both in scientific and public senses, has been stimulated by a number of factors. First of all, advances in medicine and public health have essentially increased average life expectancy over the past 200 years. As an example, the proportion of population aged 65 years and over, which was 8 percent in 1950, has increased to 16 percent today and will most likely increase to a record 26 percent by 2050 (Haub, 2011).

Since the population of elderly people gradually increases, it becomes more important to understand the biological bases of aging as well as morphological and molecular aspects underlying various age-related diseases. This knowledge will result in future pharmacological interventions that can delay many aspects of decline occurring at advanced age and prolong the age of people in a good health.

In spite of intensive research, the endogenous causes of aging remain elusive. One of the most popular modern concepts of aging is based on the hypothesis that accumulation of oxidative deteriorations is responsible for the progressive functional deterioration at advancing in years.

The primary assumption of this concept is that normal antioxidant defense levels are not enough sufficient, in such a way some reactive oxygen species (ROS) can escape elimination and cause molecular damages, some of which are nonrepairable and accumulate with age. This kind of "oxidative aging" results in decreased resistance to multiple forms of stress, as well as in increased vulnerability to numerous diseases (Kregel and Zhang, 2007). If ROS causes aging, then enhanced defense against ROS should reduce

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oxidative stress, slow down aging process, and ultimately extend the life span (Jin, 2010).

As known, plants are rich sources of small molecules with potent antioxidant activities; they continue to attract valuable research focused on elucidation of their biological activities. Just to name a few, ginger (*Zingiber officinale* Rosc., Zingiberaceae) is a perennial reed-like plant with annual leafy stems. Ginger rhizomes are used as vegetables and spices. Ginger root active ingredients are presented by volatile oils and phenol compounds known as gingerols, sesquiterpenoids, shogaols and so on (Oboh et al., 2012). Gingerol, one of the main phenolic compounds in ginger, is known to possess several activities such as hepatoprotective (Ezeonu et al., 2011), antidiabetic (Al-Amin et al., 2006), antioxidant (Oboh et al., 2012), and radioprotective (Baliga et al., 2012).

Despite the extensive studies on the chemical characterization of ginger phytoconstituents and their antioxidant properties, the mechanisms of ginger effect on organism and possibility of ginger to prevent the development of neurodegenerative and other diseases associated with oxidative stress remain poorly understood. In addition, no studies examining the ability of ginger preparations to prolong life or increase stress resistance have been conducted.

On the basis of free radical theory of aging, it is postulated that any substance with a potent antioxidant capacity can be a potential candidate for delaying the aging. Based on *Drosophila* genome similarity and presence of highly conserved metabolic pathways with eukaryotes including humans, as well as fruit fly short life span and easy growing and handling properties in the laboratory, *Drosophila* has taken a position of one of the best model organisms for investigating aging mechanisms, longevity factors and potential life prolongators.

Thus, the objective of the work was to evaluate the effect of dietary ginger on oxidative stress resistance and life span in *D. melanogaster*.

Materials and methods

Chemicals

All chemicals and reagents were obtained from Sigma-Aldrich (UK), Merck (Germany) and Ukrainian chemical plants.

Test organisms

Oregon strain of *Drosophila melanogaster* was used in the study. Flies were raised on a standard medium, containing 5% sucrose, 5% cornmeal, 5% yeast, 1.2% agar-agar, and 0.18% nipagin. Nipagin (methyl-4-hydroxybenzoate) was added into the nutritional medium as an antifungal and antibacterial agent.

Immediately after hatching from pupae, male flies were placed into vessels (not more than 20 flies per vessel) containing food of full value. They were kept under standard laboratory conditions at 24°C, relative humidity 55%–60% and 12 h/12 h light/dark cycle. The flies were replaced onto fresh nutritional medium every two days.

Experimental design

For the control flies, the food media used were without addition of any supplement. While in case of treatments, only food mixture for larvae (not for adult flies) contained ginger powder (GP) and/or dithiothreitol (DTT).

The concentration of GP ginger powder in the nutritional medium (25 mg g^{-1} of media) was chosen on the base of experimental condition optimization conducted in our laboratory. The GP was prepared on the grinded mixture of *Zingiber officinale* rhizome, obtained from the local market of Kharkiv, Ukraine. Briefly, the inedible parts of the fresh rhizomes were removed from the edible parts; the edible parts were thoroughly washed in distilled water to remove any contaminants, chopped into small pieces and air-dried before grinding. The rhizome was grinded into a fine powder using an electrical blender and kept in tightly closed container until needed.

Dithiothreitol was introduced into the nutritional mixture only at the stage of larvae at the final concentration of 10 mM. Higher concentrations of this xenobiotic were unfavorable for viability of the flies and their larvae.

In conditions of additional temperature stress, insects were subjected to 37°C (while the optimum temperature for keeping them is 24°C) within one hour once a week for four weeks. In conditions of additional oxidative stress, insects were subjected to 30% hydrogen peroxide introduction in food media (6% glucose), and fly survival data were recorded.

Longevity experiments were initiated by collecting newly enclosed adults on day 1. Adults were transferred to new vials three times weekly and fly survival data were recorded for each vial. The data for each treatment group were compiled, and survival curves were plotted using Excel software.

The mean life span ($X_m = \Sigma x_i/n$, where X_m is the mean life span, x_i is the life span of the i^{th} fly, and n is the total number of flies in the sample) and the maximum life span (average of the last 10% of surviving flies) were calculated in each case.

Preparation of crude extract of fly tissues

The *Drosophila* fly tissue crude extract was prepared as follows. The flies were immobilized by cooling on ice and homogenized (teflon/glass) in medium containing 100 mM Tris-HCl, pH 7.4, at 4°C. A weighed sample of the flies and the medium volume were taken at the ratio of 1:5. The resulting homogenate was filtered through two layers of nylon tissue and used in the experiments.

Biochemical and physiologic analyses

Lipid peroxidation intensity assay
As an index of lipid peroxidation intensity, thiobarbituric acid reactive substances (TBARS) content was measured spectrophotometrically at 532 nm according to the standard method proposed by (or just of) Ohkawa et al. (1979). The TBARS concentration was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol/mg protein.

Protein oxidation assay
As an index of protein oxidation, carbonylated protein (CP) content was determined in fly tissue homogenates by spectrophotometric method of Levine et al. (1990). Briefly, carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine, and the amount of carbonylated proteins was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22000 M^{-1} cm^{-1} and expressed as nmol/mg protein.

Protein concentration assay
Total protein concentration was determined in fly tissue homogenates by the spectrophotometric method of Lowry et al. (1951) in Miller's (1959) modification to determine large numbers of samples. Briefly, proteins were detected by reaction with Folin phenol reagent, and the total protein concentration was measured spectrophotometrically at 540 to 600 nm. A calibration curve was obtained using bovine serum albumin (BSA) as a standard.

The study of the motor activity (negative geotaxis)
Vertical climbing ability of male flies from different treatment bottles was assessed according to Seuge et al. (1985). Briefly, 20 male flies per each group were collected and transferred to the empty 0-20 cm graduated vial. The vial was gently tapped and placed in a vertical position. The number of flies that crossed the 20-cm mark in 10 s was counted. Three trials were conducted on each set of 20 flies. The data were expressed as the percentage of flies that crossed the 20-cm mark.

Statistical analysis

The means in the samples with normal distribution were compared using Student's *t*-test. Values were considered statistically significant at $P < 0.05$. All the data were expressed as mean±SD of number of experiments (n = 10 and more).

Results

Thiobarbituric acid reactive substances (TBARS) content in control and experimental *Drosophila melanogaster*

The addition of DTT (10 mM) to *Drosophila* nutrient mixture was found to result in increase in TBARS content in fly tissues (Table 1). DTT treatment caused a rise in TBARS content in fly tissues by 26% and 14% in 5 and 33-day flies, respectively.

Ginger powder, being added simultaneously with DTT, significantly diminished the negative effects of this xenobiotic. Thus, TBARS content was reduced almost to the control level The essential thing is the fact that the protective effect of ginger preparation was found in both young (5 days) and especially in adult (33 days) insects (Table 1).

Carbonylated protein (CP) content in control and experimental *Drosophila melanogaster*

DTT exposure increased the CP content in fly tissues, especially in adult insects (by 39 and 53% in 5 and 33-day flies, respectively) (Table 1). Ginger powder, being added simultaneously with DTT, significantly reduced the negative effects of DTT leading to the decrease of CP content almost to the control level. The protective effect of ginger preparation was found in both insects group, being more expressed in this case in young (5 days) insects (Table 1).

Thus, the intensity of the effects caused by the studied plant preparation had certain age-specific features. The ability of GP to decrease CP level was somewhat more expressed in young flies, while its efficacy in lowering of TBARS content

Table 1 Thiobarbituric acid reactive substances (TBARS) and carbonylated protein (CP) content in control and experimental *Drosophila melanogaster* tissue crude extract.

Experimental group	Fly age	
	5 days	33 days
	TBARS content (nmol/mg protein)	
Control	0.103±0.004	0.157±0.006
DTT	0.130±0.006*	0.179±0.008*
DTT with GP	0.103±0.009	0.141±0.003**
	CP content (nmol/mg protein)	
Control	8.33±0.14	12.18±1.88
DTT	11.61±0.22*	18.69±0.73*
DTT with GP	3.67±0.50*,**	12.79±2.37

Data are presented as mean±SD; n = 10 – 12; DTT – dithiothreitol; GP – ginger powder; * – $P < 0.05$ compared to control flies; ** – $P < 0.05$ compared to DTT exposed flies.

was slightly higher in adults (Table 1).

Effect of DTT treatment on the flies life span on standard diet or diet, containing ginger powder

The addition of DTT to *Drosophila* nutrient mixture was found to result in significant reduction of mean life span (MLS) and maximum life span of the last 10% of flies (MLS10) (Table 2). For example, MLS of control fruit flies maintained on media with no additive was 59.7 days. In contrast, significant decrease of this parameter to 35.8 days was noted in fruit flies maintained on media supplemented with DTT. This is well manifested by survival curves of *Drosophila* males (Fig. 1).

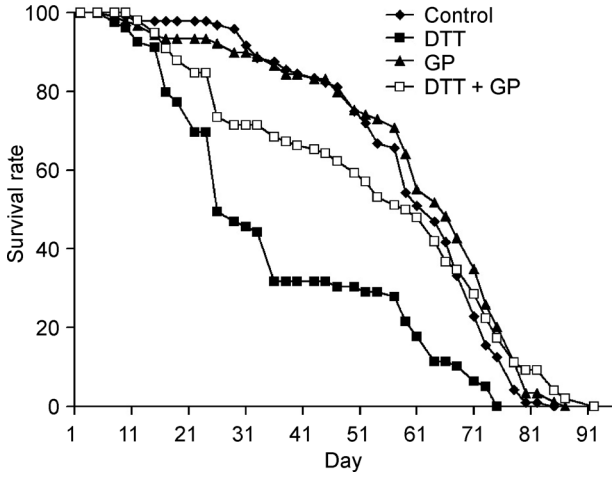

Figure 1 Survival curves of *Drosophila melanogaster* males fed basal diet (control), ginger powder (GP) containing diet and basal diet with dithiothreitol chronic exposure (DTT) or DTT containing diet with addition of ginger powder (DTT + GP). The survival of flies on the basal diet and in the presence of the various additives was determined on samples of 20 males per vessel. The living flies were counted daily; the food was changed every two days. The initial number of flies was taken as 100%.

Ginger powder, being added simultaneously with DTT, significantly diminished the negative effects of this xenobiotic. Life span of insects which consumed xenobiotic in conjunction with ginger preparation was almost equal to that of control insects (MLS) or even slightly higher (MLS10) (Fig. 1, Table 2).

Protective effect of ginger preparation in conditions of additional oxidative and environmental stresses

Insects were subjected to 37°C within one hour once a week for four weeks. The lowest viability was in flies which were given only DTT, while this parameter was practically of the same level in the control group and the group received DTT and ginger preparation (Table 3).

In case of additional oxidative stress induced by hydrogen peroxide, survival of insects treated with DTT on the background of the plant preparation was the highest. Thus, at 48 h after addition of hydrogen peroxide, 20% of the initial quantity of flies in the group consuming ginger preparation along with DTT have survived, whereas only 6% and 8% of individuals in the control group and the group consuming only DTT have survived. Further, at 96 h after addition of hydrogen peroxide, only 4% of the initial quantity of flies in the group consuming ginger preparation and DTT have survived, whereas all individuals in other groups have died.

Negative geotaxis of *Drosophila* flies

The study of the motor activity (negative geotaxis) of *Drosophila* flies in the presented work has shown that there was a significant decline in the physical activity in DTT exposed flies (the percentage of flies that crossed the 20-cm mark, was 74 and 56% in control and DTT groups, respectively). In contrast, diet supplemented by ginger powder led to a significant increase in the physical activity of the flies (69% of DTT and ginger powder exposed flies crossed the 20-cm mark).

Discussion

As is known, dithiothreitol is a reducing agent that strongly induces endoplasmic reticulum stress (Gaddam et al., 2013) leading to accumulation of ROS and induction of oxidative stress response (Higa and Chevet, 2012).

Dithiothreitol (DTT) was introduced into the fly nutritional mixture at the final concentration of 10 mM. This concentration of DTT in culture media was selected on the base of previous experiments conducted in our laboratory.

First, pilot experiments were carried out with small fly numbers (50 flies per sample) and several DTT concentrations

Table 2 Effect of DTT treatment on the survival time of the flies fed the standard diet or diet, containing ginger powder.

Experimental group	Mean lifespan (day)	Maximum lifespan (average of the last 10% of survived flies) (day)
Control	59.7±1.6	79.3±0.7
DTT	35.8±2.3*	72.3±0.7*
GP	60.8±2.0	81.9±0.9
DTT with GP	53.4±2.4**	86.3±1.1**

Data are presented as mean±SD; $n = 80 – 100$; DTT – dithiothreitol; GP – ginger powder; * – $P < 0.05$ compared to control flies; ** – $P < 0.05$ compared to DTT exposed flies.

Table 3 Effect of temperature stress (exposure to 37°C within one hour once a week for four weeks) on life span of *Drosophila* flies in the control group and DTT exposed groups with or without addition of ginger powder.

Experimental group	Mean life span	Maximum lifespan (average of the last 10% of survived flies) (day)
Control	49.35±2.85	79.33±0.42
DTT	39.87±2.27*	73.00±1.95*
DTT with GP	47.94±3.14**	81.40±0.98**

Data are presented as mean±SD; $n = 60 – 70$; DTT – dithiothreitol; GP – ginger powder; * – $P < 0.05$ compared to control flies; ** – $P < 0.05$ compared to DTT exposed flies.

(from 5 to 100 mg/ml) in order to determine whether the DTT treatment had any negative effect on life span. We observed a dose-dependent effect of DTT on life span, at concentrations higher than 10 mM this xenobiotic was deleterious for viability of the flies and their larvae (results are not presented).

As evidenced by the results presented, the addition of DTT to *Drosophila* nutrient mixture was found to result in rise in TBARS and CP content in fly tissues and in significant reduction of life span. Ginger powder, being added simultaneously with DTT, significantly diminished the negative effects of this xenobiotic.

Additionally to the investigation of the impact of ginger and DTT on TBARS and CP content and fly life span, the effect of these additives on locomotor activity as a biomarker of health span was also tested. From a great number of locomotor behaviors that can be evaluated in adult flies, negative geotaxis ("an innate escape response elicited by banging flies to the bottom of a container; the flies respond to the mechanical stimulation by walking up the container wall" (Grotewiel et al., 2005)) is one of the most often used assays. Particularly, the association between locomotor activity and aging has been established in a variety of species, including *D. melanogaster* (Le Bourg E., 1987).

The study of the negative geotaxis of *Drosophila* flies in the presented work has shown that there was a significant decline in the physical activity in DTT exposed flies, and diet supplemented by ginger powder led to a significant increase in the physical activity of the flies (see subsection Negative geotaxis of *Drosophila* flies).

Investigation of living organism viability under the influence of various stresses is known to be an appropriate approach for the elucidation of aging mechanisms. For example, it was shown that long-lived animals often show elevated resistance to various environmental stresses, including starvation and oxidative stress (Lithgow et al., 1995; Broughton et al., 2005).

As already mentioned earlier, DTT in our studies was introduced into the insect nutritional mixture at the final concentration of 10 mM and higher concentrations of this xenobiotic were unfavorable for viability of the flies and their larvae. Therefore, we could not provoke additional oxidative stress just by simply increasing the DTT concentration. That's why we used another well-known way for activation of oxidative stress – addition of hydrogen peroxide into the insect habitat. This was also interesting as these xenobiotics provoke oxidative stress by different pathways: DTT through the activation of condition known as "ER/oxidative stress," whereas H_2O_2 is a prominent ROS that causes free radical oxidation of different biomolecules.

To assess whether ginger protected flies against oxidative and environmental stresses, we subjected ginger and DTT-fed flies to hydrogen peroxide (H_2O_2), and high temperature.

Our experiments on keeping insects in conditions of additional stress load have also revealed a high level of viability of *Drosophila* flies treated with DTT on the background of plant preparation consumption (subsection Protective effect of ginger preparation in conditions of additional oxidative and environmental stresses, Table 3).

Significant capacity of plant preparations to improve the viability of insects in conditions of oxidative and environmental stresses was also noted in other studies. For example, dietary supplementation of apple phenols (Peng et al., 2011) have been shown to extend adult life span in wild type flies, and supplementation with a resveratrol complex was able to rescue from the locomotor behavior defects and reduction of life span of the alpha-synuclein induced Drosophila Parkinson's disease model (Long et al., 2009).

Other authors (Rawal et al., 2014) used *Drosophila melanogaster* as a model and fed them orally with different concentrations of two commonly used Indian medicinal plant products, *Curcuma longa* (rhizome) and *Emblica officinalis* (fruit). The results showed significant increase in life span of *Drosophila* flies at exposure to both plant products, *C. longa* being more efficient than *E. officinalis*. Interestingly, the results support the free radical theory of aging as both these plant derivatives show high ROS scavenging activities.

In this way, the presented literature data and our own results show that dietary ginger extends life span of *D. melanogaster* in stress conditions, suggesting that more effective inhibition of oxygen radicals resulting from ginger diet is associated with life span extension and stress resistance. In other words, these data are consistent with the idea that consumption of food antioxidants is capable of inhibiting the processes which limit life span.

Indeed, it was previously shown that ginger has a positive effect on the survival of not only insects, but also of more highly organized animals as well. Thus, Ajith (2010) has shown that *Zingiber officinale* significantly prevented intensification of lipid peroxidation in different tissue homoge-

nates and mitochondria of rats. However, the extract could only partially alleviate the DNA damage. The protective mechanism, according to the author, can be related to the free radical scavenging properties of *Zingiber officinale*. The other authors (Ahmed et al., 2008) showed the protective effect of dietary feeding of ginger against lindane-induced oxidative stress in male albino rats. Their findings confirm the possibility of diets containing naturally occurring compounds to exert protective effects by mitigating oxidative stress. Antioxidant and anti-inflammatory effect of ginger was also detected in case of ethanol induced kidney abnormality related to oxidative DNA damage and oxidative stress (Shirpoor et al., 2016).

Thus, numerous literature data indicate that many plant antioxidants, consumed as a component of daily diets or plant-derived dietary supplements, are able to prevent free radical-related diseases by impeding the development of cell oxidative stress. At the same time, antioxidant capability is not the only factor determining their in vivo beneficial effects. Some of plant antioxidants possess hormetic properties: being added in low doses, they act as mild stressors that prepare cells to resist more severe stress (reviewed by Speciale et al., 2011). The available data suggest that these properties are also present in ginger as well.

Probably, it may be the reason of a larger value of maximum life span of the last 10% of flies in the group of insects that received DTT with GP, compared with other groups of flies (Table 2). This assumption needs a detailed verification that will be done in the further studies.

In general, the results presented and available literature data outline that the antioxidant and possible hormetic properties of ginger might be successfully employed for realizing health-promoting dietary interventions.

Conclusion

Generally, the presented data give the evidence that ginger preparations reduce the oxidative stress outcomes and significantly increase life expectancy of fruit flies in conditions of DTT-induced stress. These results and scarce literature data allow to suggest that consumption of ginger will support an organism, which is forced to live in oxidative stress conditions. While no specific studies in humans have examined the association between a diet rich in ginger and organism stress resistance, the data presented here support the idea that ginger consumption is a healthy behavior. Therefore, further studies are needed for its clinical application.

Compliance with ethics guidelines

Volodymyr Padalko, Viktoriya Dzyuba, Olena Kozlova, Hanna Sheremet and Olena Protsenko declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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