Article

Impact of dietary potassium nitrate on the life span on Drosophila Melanogaster

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Abstract: The recently defined and yet rather new topic of Healthy aging is gathering more attention on the global stage. With world's population getting older - it is rapidly becoming very important to develop and maintain functional abilities in older age and develop mechanisms to protect senior population from chronic diseases. One of the most effective components, as well as, one may call - process involved in, and strongly associated with aging is recently discovered and Nobel prize awarded - nitric oxide as a signaling molecule, which followed by later discoveries showed to have a positive metabolic, immune and anti-inflammatory effects. One of the most debated topics of the last decade in the scientific community is nitrates, one of the pathways involved in nitric oxide production. Thus, the objective of this study was to evaluate effect of different potassium nitrate concentrate supplementation on *Drosophila melanogaster* longevity. 0,5-3% potassium nitrate medium was analyzed on the life span and motor function in different groups consisting of 100 females fruit flies each. In this assay, female fly species supplemented with potassium nitrate diet showed life span increase by 18.6% and 5.1% with 1% and 2% KNO_3^- respectively with a positive impact on locomotor function. In conclusion, we found that low concentration of potassium nitrate medium increased lifespan and locomotor function in *Drosophila melanogaster.*

Keywords: nitric oxide; nitrates; dietary potassium nitrate; *Drosophila melanogaster*; longevity; lifespan.

1. Introduction

Aging is a natural although imminent decline of physiological functions that eventually leads to death. Age is a major risk factor for the most common chronic degenerative, metabolic, and cardiovascular diseases [1, 2]. Thus, healthy aging is a relevant and decisive process to develop and maintain functional ability for wellbeing in older age [3].

External environmental factors such as diet, sleep quality, stress management, physical activity, psychosocial aspects have an important impact on healthy aging and longevity [4,5]. Some dietary patterns and supplements and/or drug treatment can slow the aging process [6] and extend lifespan [7]. All of them as well as regular exercising have shown to reduce the risk of neurodegenerative, cardiovascular and metabolic diseases in mammalian models [6].

Dietary nitrate and nitrite supplementation can positively affect production of nitric oxide (NO). NO has been identified as major participant in regulative processes in the role of signaling molecule, relevant to vast variety of different physiological processes including mitochondrial function, blood stream regulation, cardiac and skeletal muscle contraction [8], neural protection and development, immune activation, as well as signal transmission. [9] Scientific data shows that nitrite protects against heart, brain, kidney and liver cells injuries. NO can be produced in two pathways: enzymatic pathway - when special enzyme NO-synthase endogenously converts L-arginin into NO [8]. NO can be made by an non-enzymatic recently discovered nitrate ($NO3^-$) - nitrite ($NO2^-$) – nitric oxide (NO) pathway. The production of NO via the NO-synthase pathway is oxygen depended, while the NO3-NO2-NO pathway is activated under hypoxic conditions [10]. Consumed dietary $NO3^-$ is broken down to bioactive $NO2^-$ by oral bacteria (saliva's anaerobic bacteria) and then further reduced to $NO2^-$ and to NO in the stomach and/or in peripheral blood vessels [8, 10-11]. Aging interferes with NO signaling at every possible biochemical stage, from its production to inactivation [8; 12; 13]. Scientists agree that the bioavailability of NO decreases with age. In young and healthy organisms, the endothelial production of NO through *L*-arginine pathway is effective and adequate, but aging processes inhibits synthesis of endothermic NO production [9,14,15]. Deficiency of NO production increases hypertension, atherosclerosis, peripheral artery disease, heart failure, heart attack and stroke risk [8,12], impaired cognitive function [16]. All of these conditions have been positively affected by dietary nitrite and nitrate intakes, which can boost nitrate-nitrite-nitric oxide pathway and could have cardiovascular and metabolic benefits [12, 17, 18].

In recent decades, various model organisms, including fruit fly *Drosophila melanogaster*, have been used to study the processes of aging and longevity. *Drosophila melanogaster* is an excellent model to analyze age-related decline and has a number of advantages as a specie of short lifespan (60-80 days) [19] as well as great accessibility of different genetics tools [20]. NO plays essential signaling role for these species as well [21, 22]. Nitrite reduction to NO in the fruit fly was demonstrated in the newest trial indicating it's important role in fly's organismal physiology. Assuming that lower NO bioavailability is one of the hallmarks of aging and longevity, and is related to cardiovascular and metabolic diseases as well, boost of dietary nitrate-nitrite-NO pathway could express an effectual option to prevent age-related diseases [23].

We hypothesized that stimulating *Drosophila* via potassium nitrate supplementation, could extend life span of this specie. To test this hypothesis, we performed 39 days duration experiment with fruit flies supplemented by medium of different potassium nitrate concentration.

2. Materials and Methods

2.1.Fruit Flies culture

The fruit flies *Drosophila melanogaster* (female species only) Berlin-K wild strain kindly provided by Vilnius University originally obtained from Bloomington Drosophila Stock Center (catalogue line Nr. 8522) were used in this study. Flies were cultivated under standard laboratory conditions. Unmated female species were transferred into the standard Drosophila vial (25x95mm) with oxygen permeable plug/cap, kept in vertical position at all times (plug side up). Test vials were placed on the standard tray in incubators for the whole duration of experiment. Each tube contained 10 food flies, each group consisted of 10 vials, totaling 100 food flies in each group. All flies were randomly divided into 5 groups: Control group, 0.5% potassium nitrate (KNO_{3^-}), 1% KNO_{3^-}, 2% KNO_{3^-} and 3% KNO_{3^-} solution groups. The solutions were prepared immediately before use.

2.2.Feeding assay

Flies were maintained on the media consisting of: water 750 g., agar-agar 15 g., dry yeast 150 g., razins 40 g., melisa 50 g., corn fluor 50 g. Flies were separated 10 h after hatching and reared under standard laboratory conditions with a 12-hour light/dark cycle at 25°C. All experimental vials contained on average 20 ml of media, supplemented with 0.5%, 1%, 2% or 3% of KNO_{3^-}, which was added after cooling the media to 50 °C. Distilled water was used in preparation of corresponding solution for all experimental groups, which served as addition to regular diet. Dead flies as well as locomotion assessment was carried out on the daily basis (same time or the day 10:00 CET).

2.3. RING protocol (locomotor behavior)

Locomotor behavior of fruit flies was evaluated using a rapid iterative negative geotaxis (RING) protocol [24]. Female fruit flies were placed in the same conditions test tubes containing ADY diet without or with the addition of 0.5% KNO_{3^-}, 1% KNO_{3^-}, 2% KNO_{3^-}, 3% KNO_{3^-} solutions. A RING assay was repeated 7, 14 and 20 days after the ADY diet was started. 20 fruit flies without anesthetizing and randomly were transferred to each of prepared 5 tubes, forming all them into the RING apparatus on the experimental day. Fruit flies were allowed to acclimate in the new environment for 15 min. During this time, a digital camera was placed 1 meter in front of RING apparatus. Digital camera was focused, zoomed and set a timer to 3.0 s. RING apparatus with fruit flies was sharply tapped three times on the surfaces of the bench, ensuring that all the flies knocked down to the bottom of the tubes. A picture was taken at three seconds after the last (third) tap of apparatus. 1 min brake for flies before repeating again the same procedure. The whole experiment was performed in five sets (for control and four different potassium nitrate groups), and total of five pictures was done for each set of the experiment. All the pictures were analyzed and a mean value was calculated form the pictures.

2.4. Statistical analysis

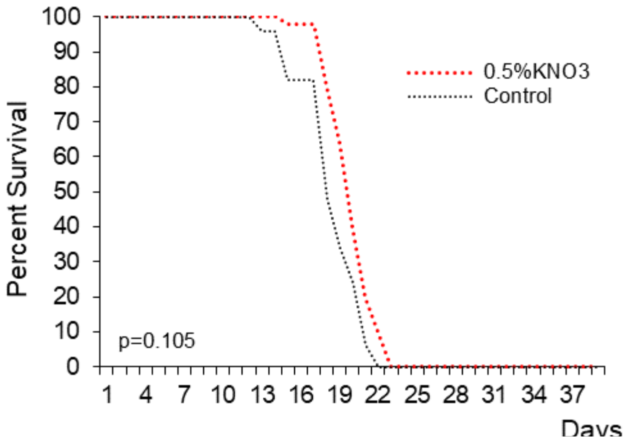
Survival days estimates were expressed as the median ± percentage difference from Control group. Data normality is checked using the Shapiro-Wilk criterion. Based on the results the distribution of survival days does not follow normal distribution, so the non-parametric Kruskal-Wallis (KW) criterion is used because we compare more than 2 independent samples. The results of KW are found to be statistically significant, so the post-hoc Mann-Whitney criterion was applied to determine the impact of the salts on the survived population lifespan. Test results were statistically significant when probability $p<0.05$. Survival assays for all conditions were done once.

3. Results

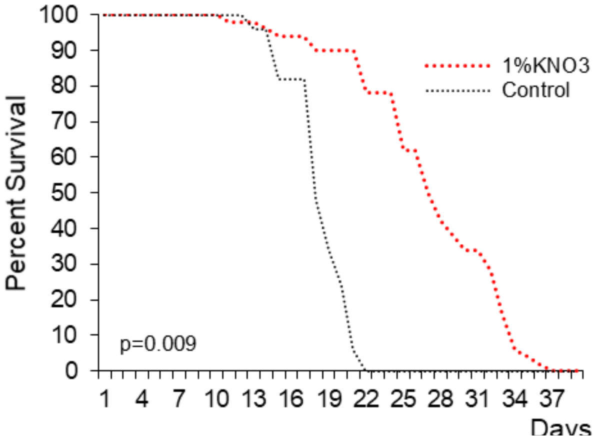
The aim of this study was to assess whether dietary potassium nitrate affects the lifespan of Drosophila melanogaster. Accordingly, we performed a 39 days duration study in female fruit flies fed an agar–dextrose–yeast media supplemented with increasing concentrations of potassium nitrate (0%, 0.5%, 1%, 2%, 3%). This experiment showed the 1% and 2% concentrations of potassium nitrate significantly extended lifespan in female flies (Fig. 1 B; C). Precisely, median lifespan has significantly increased by 18.6% and 5.1% with 1% and 2% KNO_3^- respectively (Fig. 1 B; C; E; Table 1).

In the control group, the median lifespan was 29.5 days for female fruit flies. Different KNO_3^- supplementation diversely changed fruits flies' lifespans 0.5% and 3% KNO3 had no significant effect on the lifespan in fruit flies (Fig. A; D; E). The median lifespan appeared to extend by 1.7% with 0.5% nitrate ($p>0.05$), while 3% KNO_3^- medium concentration shortened lifespan ($p>0.05$) if compared to the control group (Fig. A; D; E and Table 1).

A.

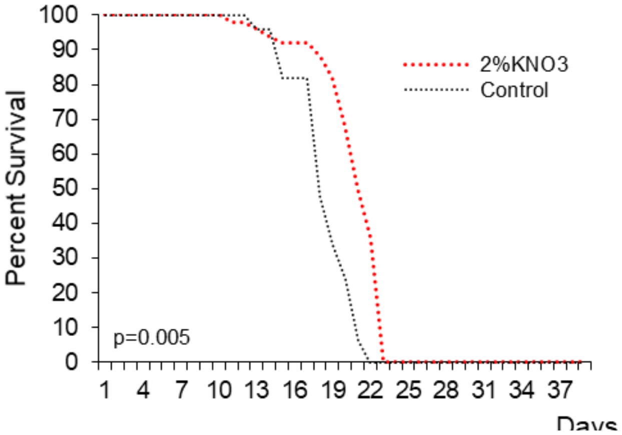


B.



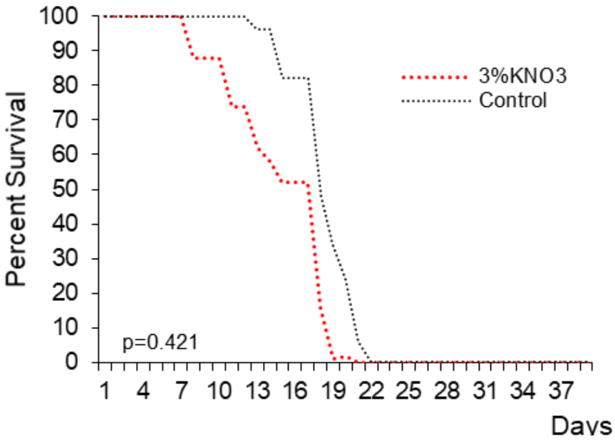
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
1 4 7 10 13 16 19 22 25 28 31 34 37
Days
1%KNO3
Control
p=0.009

C.



Percent Survival
100
90
80
70
60
50
40
30
20
10
0
1 4 7 10 13 16 19 22 25 28 31 34 37
Days
2%KNO3
Control
p=0.005

D.



Percent Survival
100
90
80
70
60
50
40
30
20
10
0
1 4 7 10 13 16 19 22 25 28 31 34 37
Days
3%KNO3
Control
p=0.421

E.

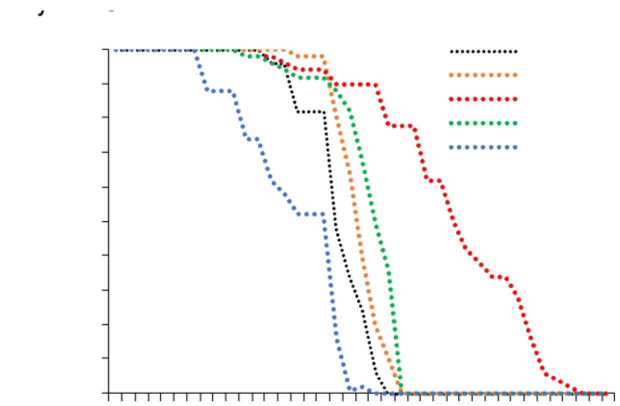


Figure 1. Potassium nitrates impact on lifespan in female flies. Female flies supplemented with 0.5% (A; n = 50; p = 0.105), 1% KNO_3^- (B; n = 50; p = 0.009), 2% KNO_3^- (C; n = 50; p=0.005) and 3% KNO_3^- (D; n = 50 p = 0.421). Cumulative survival of female (E).

Table 1. Potassium Nitrate extends median and mean lifespan *Drosophila melanogaster*.

Potassium Nitrate concentration (%)	Survival days			Median lifespan change (% vs control)
	Median	Mean	Max	
Control	29.5	20.00	22	
0,5	30	21.60	23	+1.7
1	35	31.80	37	+18.6
2	31	23.00	23	+5.1
3	29	19.20	21	-1.7

To assess the potassium nitrate impact on fruit flies lifespan, we additionally used a negative geotaxis assay to measure locomotor ability in fruit flies [23]. The same groups of Drosophila were tested 7, 14 and 20 days from the whole experiment beginning. Aging significantly decreased climbing height in all groups of fruit flies ($p<0.05$). 1% KNO_3^- solution significantly maintain higher climbing height in 7, 14 and even 20 days old flies comparing to the control group ($p<0.05$) (Fig.2).

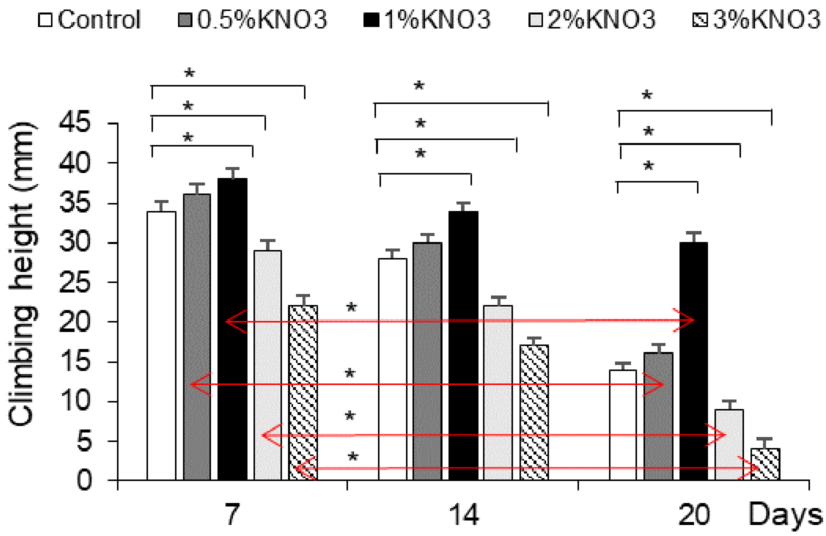


Figure 2. Potassium nitrate impact on fruit flies locomotor decline.
**p* < 0.05 comparison between control vs different potassium nitrate groups, and in each group between 7, 14 and 20 days.

4. Discussion

In this experiment, we found a supporting effect of potassium nitrate on fruit fly *Drosophila melanogaster* lifespan together with their physical activity assessment.

Our principal findings were that different mediums of KNO_3^- had a significant effect on the fruit flies longevity. It was mentioned that median lifespan duration of healthy and well-overseen fruit flies is approximately 70 days and has a maximum of approximately 90 days at 25 °C [25, 26]. The experiment performed by our scientific team lasted only 39 days, which in comparison to other experiments carried out with *Drosophila melanogaster that* averaged 70-90 days would seem rather short. It is important to stress that most experiments use synthetic, very balanced medium such as instant drosophila medium 4-24. In our case a natural medium from ingredients widely available in practically any food store was used, which in our opinion best represents products used in human diet. This leads to possible interpretations of balanced and unbalanced diets and potassium nitrate influence on oxidative stress caused by consumed food and/or lifestyle.

In our study, low doses of potassium nitrate medium – 1% and 2% - significantly prolonged lifespan of female fruit flies, but no significant effect was seen of 0.5% and 3% medium. We could explain this based on the U-shaped effect of nitrite. It was reported that low doses of nitrite, but not higher doses, had protective effects in vivo and vitro models on vascular dysfunctions, myocardial ischemic injury [27, 28] and liver ischemia-reperfusion injury as well [27, 29].

Plenty of various aging-related markers can be observed in the Drosophila population from genetic to environmental factors. One of the most relevant aging-related factors is physiological decline with the changes of metabolism, behavior, reproductive and immune capacity, physical activity, neuronal, gut and cardiac functions [30]. In the present study physical activity or more detailed - negative geotaxis of fruit flies was evaluated. Experiment showed that climbing was significantly higher of those fruit flies, which were fed 1% $KNO3^-$ medium.

Aging is related with the organism functions capacity decline and impairment. Aging is the main risk factor for life-menacing conditions such as cardiovascular diseases, neurodegeneration, cancer, sarcopenia – which demonstrate a significant increase in the amount of cases [31]. Sarcopenia is one of the most principal health problems associated with the aging [32; 33], described as age-related muscle mass decline and associated with the physical disability, metabolic impairment, and frailty in older persons [34; 35]. *Drosophila* and human muscles despite their differences demonstrate morphological and functional changes due to the aging process [35]. Locomotor activity significantly decreased with aging in *Drosophila* [37-39]. With age, heart muscle functional and performance decline is also observable in food flies [40] as in humans [41]. Simultaneously, this dysfunction in humans increases a risk of cardiovascular diseases, and this impairment conventionally associated with a risk of cognitive impairment in later life [42, 43]. So, we need to understand precisely aging mechanisms and how this process enhances the risk of diseases in order to help this growing problem. As one of the possible solutions to the problem one might consider nitrate consumption threw available salts (e.g. $NaNO_3^-$, KNO_3^-) or green leafy, or root vegetables (e.g. beetroots concentrate or their juice, red spinach, etc.) [44,45]. It is known that nitrate and nitrite supplements and their consumption at therapeutic strategies are effective in enhancing NO concentrations in vivo [46-48]. NO is a signaling molecule crucial to the regulation of various physiological processes and preservation for the physiological functions and health with advancing age [9]. NO pathway through the numerous biochemical and physiological cascades regulates vascular, metabolic, immune and cognitive functions [49]. And we support the opinion that NO pathway by boosting nitrate-nitrite supplementation may be an acceptable option to respond directly to age-relates impairments [23, 49].

5. Conclusions

We presented results of increased lifespan by low concentration of potassium nitrate supplement in *Drosophila melanogaster.* Additionally, locomotor function of fruit flies in low potassium nitrate medium was noticeable higher.

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