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THE ALTERATIONS IN *DROSOPHILA MELANOGASTER* LIFESPAN DUE TO NITRIC OXIDE SYNTHASE DONORSANDINHIBITORS INFLUENCE

N. Mit', A. Amirgalieva, M. Begmanova, A. Tolebaeva, L.Djansugurova

Institute of General Genetics and Cytology Committee of Science
Ministry of Education and Science Republic of Kazakhstan
75A, al-Farabi Ave., Almaty 050060, Kazakhstan
e-mail: nata-mit@yandex.ru

The investigations of nitric oxide influence to *Drosophila melanogaster* lifespan were conducted. Donors and inhibitors of nitric oxide synthase (NOS) were addedto medium throughout *Drosophila* life. It was shown that the average lifespan of *Drosophila* statistically significantincreasesunder the influence ofnitric oxide synthaseinhibitorNω-nitro-L-argininecompared with exposure tolipopolysaccharide and S-Methyl-L-thiocitrulline. Reductionof nitric oxide concentrationin *Drosophila* organism extendsthe lifespan.

Keywords: lifespan, aging, *Drosophila melanogaster*, nitric oxide synthase donors, nitric oxide synthase inhibitors.

In recent years the more and more researchers pay attention to lifespan, aging and longevity problems. Lifespan is one of the most biologically and socially important quantitative traits of the organism. Lifespan of individual is determined by its aging. Aging is a physiological process, accompanied by age-related changes, which are hereditarily programmed. These changes limit the adaptive-compensatory reactions of organism, reduce its resistance to stress, destabilizing vital functions and cause of death. Lifespan is determined by the interaction of genetic and environmental factors and can be regulated by many factors. It was revealed that lifespan and aging can be controlled by the systems of antioxidant defense, DNA repair, tumor suppression, maintenance of telomeres, and other metabolic pathways [16–17]. Also there are many environmental factorsthat can make different effects on aging process, the most significant of which is considered to be oxidative stress.

As it is known, the genetic control of aging and lifespan is highly conserved, so experimental studies are often conducted on model objects. Experimental research in the field of aging and longevity control using fruit fly *D. melanogaster*as a model organism seem to be very promising, because many *Drosophila* geneshavetheir orthologs in other eukaryotes, including human. Therefore, we have analyzed the alterations in *D. melanogaster* lifespan under nitric oxide synthase (NOS) donors and inhibitors influence as factors of oxidative stress [8].

Materials and methods

The *D. melanogaster* virgin flies of wild-type strain *Oregon R* were used for experiments. Three series of experiments were conducted.In the first lipopolysaccharides (LPS) (SIGMA) at a concentration of 0.5mg/1 ml of standard medium have been used as a donor of endogenous NO. In the second 0,01M solution of S-Methyl-L-thiocitrulline (Ltc) (SIGMA) at a concentration of 10μl/1 ml of medium has been used as NOS inhibitor. In the third series of experiment 0,01M solution of Nω-nitro-L-arginine methyl ester (L-NAME) (SIGMA) has been used as NOS inhibitors at a concentration of 10 μl /1 ml of medium. These substances were added to the nutrient medium from the beginning of *Drosophila* imago stage till their death. As a control we used flies maintained on standard medium without any supplements.

Females housed separately from males. During the experiment, every day the dead flies were counted separately in each tube without ether anesthesia. Received data were written in the tables of surviving and mortality, which were used for statistical processing of results. Survived flies were transferred to fresh medium every 5–7 days. The average lifespan was evaluated for entire sample volume and for 10% of long-livers. Also the maximum lifespan, survival and mortality were estimated. The average and maximum lifespan was determined in days, mortality and survival - as a percentage. Calculating the arithmetic mean and its deviation was performed by standard formulas [4]. To determine the significance of differences between groups we used Student's test [4].

Measurement of nitrite and nitrate quantity was carried out using the Griess reaction [10]. The sodium nitrite (NaNO2) was used for calibration in the concentration range (from 2 mM to 15 mM). Adsorption was measured at a wavelength of 550 nm using spectrophotometer Ultrospec 2000, Pharmacia Biotech.

For sample preparation 50 adult flies (total weight of about 50 mg) were homogenized in 1xPBS (130 mM NaCl, 7mm Na2HPO4, 3 mm NaH2PO4, pH 7,0). The homogenate was centrifuged at 13,000xg for 30 minutes. The supernatant was used as the enzyme sample. The protein amount was measured by the method Loury [13] at a wavelength of 750 nm using the calibration by bovine serum albumin (BSA, Sigma) in a concentration range (from 25 mg/ml to about 6 g/ml).

Results and discussion

For estimation of NO influence on *Drosophila* lifespan donors and inhibitors of NOS were used. LPS acts as a donor of endogenous NO. LPS is an endotoxin for eukaryotic cells. It causes inflammation in the organism, as a result, inducible NOS activates, that results to additional synthesis of NO. In our experiments as NOS inhibitors Ltc and L-NAME were used. S-Methyl-L-thiocitrulline is a specific inhibitor of Ca^{2+}-dependent NOS forms (endothelial NOS, eNOS and neuronal NOS, nNOS) which are constitutive forms and present in a mammalian cell constantly. N^{ω}-nitro-L-arginine methyl ester (L-NAME) inhibits 60% of Ca^{2+}-dependent forms NOS (eNOSand nNOS) activity, and 40% of Ca^{2+}-independent form NOS (iNOS) activity, i.e. L-NAME affects both the constitutive andinducible forms NOS [1].

First, the NOS-activity was measured in *D. melanogaster* adult flies after LPS, Ltc or L-NAME addition to medium and also without any addition. NO moleculeis unstable and lives for only 5–10 seconds, and then itis converted in to nitrates and nitrites. Therefore nitrites and nitrates quantity was measured (Table 1).

Table 1

Nitritesand nitrates quantity in *D. melanogaster*adult flies in control and under NO-donor and NOS inhibitors influence

Investigated groups	NO_2^-/NO_3^- quantity in *D.melanogaster* adult flies, mkM/mkg of protein	Accuracy of the difference compared to control
Without treatment	292,40±8,44	–
LPS influence	308, 20±6,10	t_{st}=1,51 p>0,05
Ltcinfluence	265,20±5,45	t_{st}=2,71 p<0,01
L-NAME influence	135,60±7,02	t_{st}=14,28 p<0,003

According to these data NOS activity does not increases significant under LPS influence, but it decreases statistically significant under Ltcand L-NAME influence.

Then experiments for lifespan determining were conducted. In control experiments 2012 individuals were studied including 1157 females and 855 males. Using LPS, we studied 2253 flies including 1085 males and 1168 females. In experiments with Ltc supplement 2193 flies were investigated, including 1130 females and 1063 males. Using L-NAME, 2115 flies were studied including 973 females and 1142 males.

It was demonstrated that the average lifespan of *Drosophila* females was higher than lifespan of males approximately to 8 days in all series of experiments. This difference was statistically significant ($p<0,05$). The survival rate of males and females were similar within the first month of life, but during the second month survival rate of males was significantly lower than survival rate of females (Table 2).

Table 2

Survival and mortality percentage in *D. melanogaster* studied groups

The studied group	Survival, %		Mortality, %	
	1st month	2nd month	1st month	2nd month
Control	91±0,6	51±1,1	9±0,6	49±1,1
LPS	86±0,7	47±1,1	14±0,7	53±1,1
Ltc	90±0,6	34±1,0	10±0,6	66±1,0
L-NAME	91±0,6	43±1,1	9±0,6	57±1,1

After the experiment has been finished, the curves of survival were built (Fig. 1). Figure 2 illustrates average and maximum lifespan in all series of experiment.

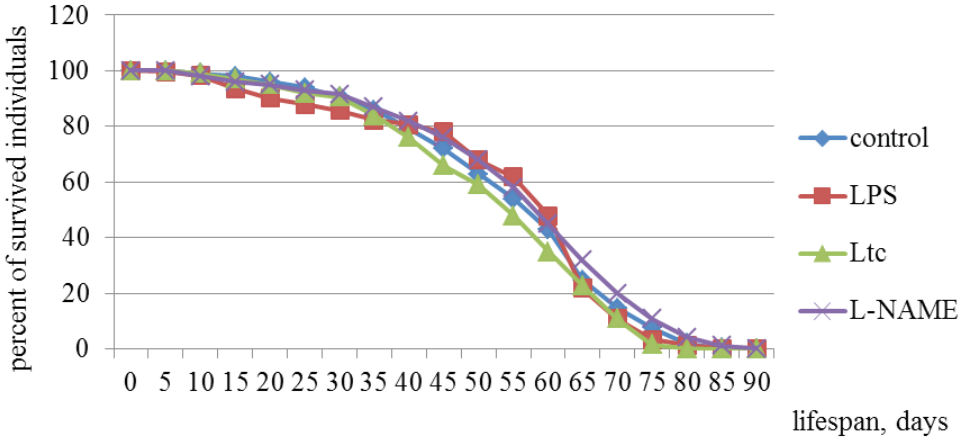

Fig. 1. Survival curves of *D.melanogaster* imagos in control and experimental groups.

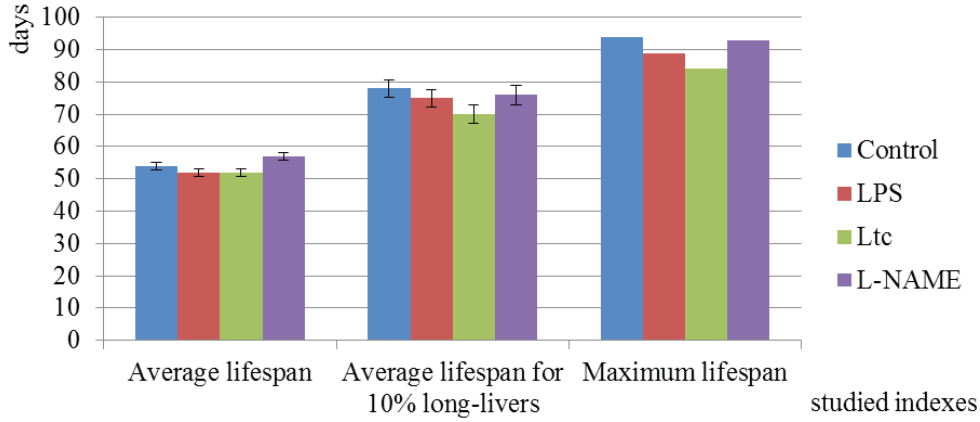

Fig. 2. Dynamics of *D. melanogaster* lifespan indexes under the influence of NO donorsand NOS inhibitors and in control group.

We expected the average lifespan in LPS group would be less than in control group, because LPS can stimulate the expression of NOS gene. In turn high level of NO can stimulate the

oxidative stress. In the contrary, it was expected that average lifespan in LtcandL-NAME groups should be higher, than in the control because of NO deficit. However average lifespan in Ltc group was similar with average lifespan in LPS group.

We carried out a statistical analysis of the differences between the studied groups using Student's test. The difference between the average life span in each of the experimental groups compared with the control was unreliable. However, the differences in average lifespan between LPSand L-NAME groups, as well as between LtcandL-NAMEgroups were statistically significant (Table 3).

Table 3

The Student's test results between studied groups

Compared groups	Average life span, days	t_{st}	p
LPS and control	52±1,1 and 54±1,1	1,29	p>0,05
Ltc and control	52±1,1 and 54±1,1	1,31	p>0,05
L-NAME and control	57±1,2 and 54±1,1	1,84	p>0,05
LPS and Ltc	52±1,1 and 52±1,1	0	p>0,05
LPS and L-NAME	52±1,1 and 57±1,1	3,29	p<0,05
Ltc and L-NAME	52±1,1 and 57±1,1	3,26	p<0,05

The datain Table 3 mean, that flies, housed on medium containing L-NAME, live longer, than flies housed on medium with LPS or Ltc. We suggest, that these changes can be explained with different NO concentration in *Drosophila* organism due to NOS donors and inhibitors influence. Adding LPS to medium does not change significantly NO concentration in adult flies and the lifespan does not change compared to control. Adding Ltc or L-NAME decreases NO concentration in flies, but L-NAME decreases it much stronger. It is known, LtcandL-NAME are widely used in mammals, both inhibit NOS by binding to enzyme instead of L-arginine. But Ltcisa specific inhibitor of Ca^{2+}-dependent forms NOS, while L-NAME affects all NOS isoforms, so it can inhibit NOS activity stronger.

As for *Drosophila*, there is only one *NOS* gene (*dNOS*) encodes a family of at least 10 different transcripts that may code seven different proteins [15]. Only one of them, *dNOS1*, encodesan enzymatically active protein DNOS1, that bears the strong resemblance to all three NOS isoforms of mammals. The majority of *dNOS* alternative transcripts encode truncated proteins that lack the crucial C-terminal reductase domain, but retain the N-terminal oxygenase domain [15]. All NO synthases are catalytically active as homodimers. The truncated proteins are capable to suppress the enzymatic activity of the full-length DNOS1 protein, perhaps by disrupting the dimerization of DNOS1 molecules [14]. The majority of *dNOS* transcripts are expressed throughout *Drosophila* development in embrio, larva, and imago at the same level. But dNOS4 transcript has different transcriptional levels: maximum in embrio, minimum in larva and close to maximum in adult flies [15]. This may be one more mechanism of endogenic NO regulation.

Previous experiments conducted in our laboratory have shown that microinjections of nitric oxide donors are able to increase NO concentration in *Drosophila* organism and enhance apoptosis in eye-antennal discs, while microinjections of NOS inhibitors reduce NO concentration and slow down the apoptotic processes [5]. In addition, data were obtained about the effect of NO on the stress response and activation of apoptosis in *Drosophila* [2, 11].The effect of nitric oxide donors and inhibitors on proliferative processes in *Drosophila* also was demonstrated by B.Kuzinet all [12]: NOS inhibitors stimulated proliferative processes in developing imaginal discs, while NO donors have accelerated the process of differentiation in imaginal discs. The authors concluded that NO acts as an antiproliferative agent in the development of *Drosophila*, controlling the balance between cell proliferation and cell differentiation [12].

In the literature, there are convincing data about the nitric oxide role in oxidative stress processes. Several studies [7] demonstrated that NO is able to enhance the negative effect of superoxide radicals and other ROS. Thus the reaction of superoxide radical with nitric oxide is able to generate highly toxic peroxynitrite (ONOO-). ONOO- is more stable than the NO molecular and is able to diffuse at a relatively long distance and cause cell membrane damages, lipid oxidational DNA breaks. Overproduction of NO may be the reason of morphological and functional abnormalities in vascular endothelium due to NO free radical ability to generate peroxynitrite with strong oxidizing effects. It was shown [3] that in the blood plasma of rats treated with LPS, the concentration of nitrates and nitrites – stable metabolites of NO was increased, that indicates an increasing of NO production in rats under endotoxemia condition and leads to endothelial dysfunction of blood vessels.

Moreover, there are some reports in the literature that high doses of NO are able to activate the p53 protein in cell [18] or heat shock proteins [9], thus inducing the cell death by apoptosis. In vitro investigation of NO-mediated macrophage cytotoxicity showed that the addition of NOS inhibitors, such as a substrate analogue NG-monomethyl-L-arginine (L-NMMA), suppresses the cytotoxic effect of macrophages for tumor cells. It was shown that phagocytosis and reactive oxygen forms production is strongly suppressed in rat macrophages cultured under conditions that allow to produce NO. Macrophages expressing iNOS or treated with nitric oxide have condensed nucleus and cytoplasm. Thus, the secretion of NO by activated macrophages leads to the suppression of their function and eventually to apoptosis. These phenomena are clearly related to NO, as it is warned by the addition of NOS inhibitors [6]. Conversely low NO doses prevent cell death, i.e. possess cytoprotective action.

Thus, the most probable mechanism of NO influence on aging and lifespan seems to be NO participation in the reactions of free radicals formation, in some cases leading to oxidative damage of key cell molecules, disruption of intracellular signaling and running the apoptosis program. In further experiments we are going to investigate the role of endogenous nitric oxide in *Drosophila melanogaster* longevity and aging using transgenic lines containing extra copies of the NOS-genes.

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ЗМІНА ТРИВАЛОСТІ ЖИТТЯ *DROSOPHILA MELANOGASTER* ПІД ВПЛИВОМ РЕЧОВИН-ДОНОРІВ ТА ІНГІБІТОРІВ СИНТАЗИ ОКСИДУ АЗОТУ

Н. Міть, А. Аміргалієва, М. Бегманова, А. Толебаєва, Л. Джансугурова

Інститут загальної генетики і цитології Комітету науки
Міністерства освіти і науки Республіки Казахстан
пр. аль-Фарабі, 75А, Алмати 050060, Казахстан
e-mail: nata-mit@yandex.ru

Проведено дослідження впливу оксиду азоту на тривалість життя *Drosophila melanogaster*. Речовини-донори та інгібітори синтази оксиду азоту додавали в живильне середовище протягом усього життя дрозофіли. Показано, що середня тривалість життя дрозофіли достовірно збільшується під впливом інгібітора синтази оксиду азоту Nω-nitro-L-arginine порівняно з впливом ліпополісахаридів і S-Methyl-L-thiocitrulline. Зменшення концентрації оксиду азоту в організмі дрозофіли подовжує тривалість життя.

Ключові слова: тривалість життя, старіння, *Drosophila melanogaster*, донори синтази оксиду азоту, інгібітори синтази оксиду азоту.

ИЗМЕНЕНИЕ ПРОДОЛЖИТЕЛЬНОСТИ ЖИЗНИ *DROSOPHILA MELANOGASTER* ПОД ВЛИЯНИЕМ ВЕЩЕСТВ-ДОНОРОВ И ИНГИБИТОРОВ СИНТАЗЫ ОКСИДА АЗОТА

Н. Мить, А. Амиргалиева, М. Бегманова, А. Толебаева, Л. Джансугурова

Институт общей генетики и цитологии Комитета науки Министерства образования и науки Республики Казахстан
пр. аль-Фараби, 75А, Алматы 050060, Казахстан
e-mail: nata-mit@yandex.ru

Проведены исследования влияния оксида азота на продолжительность жизни *Drosophila melanogaster*. Вещества-доноры и ингибиторы синтазы оксида азота добавляли в питательную среду на протяжении всей жизни дрозофилы. Показано, что средняя продолжительность жизни дрозофилы достоверно увеличивается под влиянием ингибитора синтазы оксида азота Nω-nitro-L-arginine по сравнению с воздействием липополисахаридов и S-Methyl-L-thiocitrulline. Уменьшение концентрации оксида азота в организме дрозофилы увеличивает продолжительность жизни.

Ключевые слова: продолжительность жизни, старение, *Drosophila melanogaster,* доноры синтазы оксида азота, ингибиторы синтазы оксида азота.