ELSEVIER

Mechanisms of Ageing and Development
97 (1997) 81–91

mechanisms of ageing and development

Effect of melatonin and pineal peptide preparation epithalamin on life span and free radical oxidation in *Drosophila melanogaster*

Vladimir N. Anisimov [a,*], Sergey V. Mylnikov [b], Tatyana I. Oparina [b], Vladimir Kh. Khavinson [c]

[a] *Laboratory of Experimental Tumors, N.N. Petrov Research Institute of Oncology, Pesochny-2, St. Petersburg 189646, Russia*
[b] *Department of Genetics, St.Petersburg State University, St. Petersburg, Russia*
[c] *Institute of Bioregulation and Gerontology, St. Petersburg, Russia*

Received 14 October 1996; accepted 18 February 1997

Abstract

It was shown previously that epithalamin delays age-related changes in reproductive and immune systems and increases the life span of mice and rats. These effects could be mediated by stimulating influences of epithalamin on synthesis and secretion of melatonin and on free radical processes. A comparative study on the effect of epithalamin and melatonin on both the life span of *Drosophila melanogaster* (strain HEM) and on the intensity of lipid peroxidation and activity of antioxidative enzymes in their tissues was the main aim of this work. Melatonin and epithalamin was added to the nutrition medium (100 μg/ml) during 2–3rd age of larvas. For survival analysis the flies were passed (five coupes per vessel) each 3–7 days. Lipid peroxidation was evaluated as the level of ketodienes (KD) and conjugated hydroperoxides (CHP) in fly tissues at the age of 11 days. Activity of Cu, Zn-superoxide dismuatse (SOD) and catalase was evaluated as well. The mean, median and maximum life span (MLS) were estimated. Mortality rate (MR) was calculated as α in the Gompertz equation ($R = R_o$ (exp αt) and mortality rate doubling time (MRDT) as ln $2/\alpha$. These parameters in groups of male and female flies exposed to melatonin and in male flies exposed

* Corresponding author. Tel.: +7 812 4378607; fax: +7 812 4378947; e-mail: anisimov@anisimov.spb.ru

PII S0047-6374(97)01897-6

to epithalamin were no different from the parameters for controls. However, exposure to epithalamin was followed in females by a significant increase in mean life span (by 17%, $P < 0.02$), of median (by 26%), of MLS by 14% and by a 2.12 times decrease of MR ($P < 0.01$) and MRDT (by 32%) compared with female controls. The level of CHP and KD in the tissues of male control flies was 40 and 49% less than that in females and indirectly correlates with male life span. Exposure to melatonin was followed by a decrease in the level of CHP and KD in females and the deletion of sex differences in them. Exposure to epithalamin significantly decreased the level of CHP and KD in female flies compared to controls (2.3 and 3.4 times, respectively, $P < 0.001$). Exposure to melatonin failed to influence the activity of catalase in males but increased it in females by 24% ($P < 0.02$) and failed to influence SOD activity both in males and females. Exposure to epithalamin was followed by a significant increase in activity of catalse, 20% in males and 7% in females and by an increase in SOD activity in males (41%). Thus, it was shown that exposure to epithalamin significantly increases the mean life span and MLS of female *D.melanogaster* and slowed down their aging rate by 2.12 times. This effect is in good agreement with the inhibiting effect of epithalamin in lipid peroxidation processes in fly tissues. © 1997 Elsevier Science Ireland Ltd.

Keywords: Melatonin; Epithalamin; Life span; Free radical oxidation; *Drosophila melanogaster*

1. Introduction

There is significant evidence to support the proposition that oxidative damage may play a significant role in aging [1–3]. According to the free radical theory of aging some active molecules of oxygen, superoxide (O_2^-), H_2O_2, hydroxyl radical ($HO^\cdot$) and possibly singlet oxygen ($\uparrow O_2$) damage cellular macromolecules that lead to mutations, genome instability followed by aging and age-related pathology, including atherosclerosis, immunodepression, brain disfunction, cataracts, cancer and others [1–3]. Some natural endogenous factors (Cu, Zn-superoxide dismutase (SOD), glutathione peroxidase, catalase, coeruloplasmin, β-carotene, α-tocopherol (vitamin E), ascorbic and uric acids) defend macromolecules from oxidative damage [1,2]. Exposure to some natural or synthetic antioxidants increases the life span of laboratory rodents and flies [1,4,5]. It was shown that transgenic flies which overexpressed both catalase and SOD have greater mean and maximum longevity and slowing of aging [6].

In recent years it has been reported that the pineal indole hormone melatonin is a highly potent hydroxyl radical and peroxyl radical scavenger both in vitro and in vivo [7–10]. Like some other antioxidants, melatonin increased the life span of mice and rats [11,12], however some serious criticism of the results emerged [13–15].

The pineal peptide preparation epithalamin [16,17] has been shown effectively to increase the life span of mice and rats, slow down the aging of their reproductive and immune systems [16–20] and revealed significant antioxidative potential [21–

23]. Because exposure to epithalamin increased pineal synthesis and secretion of melatonin [17,20] it was suggested that some effects of epithalamin are mediated by this indole hormone.

It is worthy of note that melatonin is present in mammals as well as in many invertebrate taxa and insects [24,25]. The comparative study on effects of melatonin and epithalamin on life span, lipid peroxidation and activity of antioxidative enzymes, SOD and catalase, was the main aim of the present work.

2. Materials and methods

2.1. Animals

The HEM strain of *Drosophila melanogaster*, selected for a high rate of embryonal mortality from a wild population Lerick and then passed through about 300 generations of strict inbreeding [26], was used in the experiments.

2.2. Chemicals

Melatonin was from Sigma, stored at −4°C and officinal pineal peptide preparation epithalamin [16,17] was from the St. Petersburg Plant of Medical Preparations.

2.3. Experiment

Melatonin and epithalamin were dissolved 'ex tempore' in 0.01% ethanol and were added to nutrient medium at a concentration 100 μg/ml, being fed on by larve of 2–3rd stages that most effectively resulted in life span modification in the adult flies [27]. Flies were kept, five couples per vessel, passing every 3–7 days. Control groups of larvas were exposed to the solvent.

2.4. Biochemical study

Some of the adult flies from each group was sacrificed at the age of 11 days. Products of lipid peroxidation were extracted with heptan–isopropanol (1:1, v:v) containing 0.1% butylated hydroxytoluene as antioxidant. Intensivity of lipid peroxidation was estimated at an optical density of wave length 274 nm (ketodiene, KD) and 232 nm (conjugated hydroperoxides, CHP) [28] with a Beckman spectrofotometer DU-65. Obtained values were related to the flies weight at sampling. There were 3–5 samples from each group containing 100 flies per sample. Activity of catalase (EC 1.11.1.6) and SOD (EC 1.15.1.1) was evaluated in flies according to [29]. There were 4–7 samples from each group containing 40–50 flies per sample.

Table 1
Parameters of life span in *D.melanogaster* exposed to melatonin or to epithalamin

Exposure group	Sex	No. of flies	Life span (days)			Mortality rate ($\alpha \times 10^2$, days^{-1})	MRDT ($\ln 2/\alpha$)
			Mean	Median	Maximum		
Controls	Females	199	24.7 ± 1.21	23	80	7.0 ± 1.29	3.7
	Males	189	28.3 ± 1.24*	23	82	6.5 ± 1.29	4.3
Melatonin	Females	206	23.7 ± 1.19	23	75	6.2 ± 1.56	4.3
	Males	190	26.4 ± 1.24	23	75	9.0 ± 1.18	3.9
Epithalamin	Females	207	28.8 ± 1.19**	29	91	3.3 ± 0.33***	4.9
	Males	186	26.0 ± 1.25	23	86	7.2 ± 1.11**	4.2

The difference with corresponding parameter in females is significant: * $P<0.05$; ** $P<0.01$.
The difference with sex-matched controls is significant: *** $P<0.02$.

2.5. Statistics

Two way analysis of variance and regression analysis were used for statistical treatment of the results [30]. The mortality rate was evaluated as a in Gompertz equation $R = Ro\ (\exp \alpha t)$ [31] and as a mortality rate doubling time (MRDT) $\ln 2/\alpha$. Differences in the parameters of life span and biochemical parameters were evaluated with Mann-Whitney and Student's t criteria [30].

3. Results

3.1. Effect of melatonin and epithalamin on the life span of D. melanogaster

Mean life span of control male flies was 14.6% shorter than that of control females ($P < 0.05$) whereas the sex difference in both median and aging rate calculates as α in Gompertz equation were statistically insignificant (Tables 1 and 2). None from these parameters were statistically different from those for exposure to melatonin flies. Melatonin treatment abolished sex differences in the mean life span of the flies.

Exposure to epithalamin failed to change significantly any life span parameter in male flies, however in females it significantly increased the mean life span (17%, $P < 0.02$), median (26%) and maximum longevity (14%), and decreased both the mortality rate of the population (2.12 times, $P < 0.01$) and MRDT (32%) as compared with controls (Tables 1 and 3). The survival curve was shifted to the right and the slope of the Gompertz plot decreased only in female flies exposed to epithalamin (Fig. 1).

Table 2
Effect of melatonin and epithalamin on sex ratio (females:males) of parameters of life span and free radical oxidation in *D. melanogaster*

Parameters	Controls	Melatonin	Epithalamin
Life span			
Mean	0.87*	0.90	1.11
Mediana	1.00	1.00	1.26
Maximum	0.98	1.00	1.13
Mortality rate (α)	1.08	0.69	0.46*
MRDT ($\ln 2/\alpha$)	0.86	1.10	1.17
Conjugated hydroperoxides	1.67*	1.08	1.37
Ketodiene	1.97*	1.18	1.10
SOD	1.01	1.04	0.78
Catalase	0.46*	0.59*	0.52*

* The sex diffrence was estimated as significant, $P < 0.05$.

Table 3
Comparative effect of melatonin and epithalamin on parameters of life span and free radical oxidation in *D.melanogaster*

Parameters	Change (%) to control value			
	Melatonin		Epithalamin	
	Female	Male	Female	Male
Life span				
Mean	−4.0	−6.7	+16.6**	−8.1
Mediana	0	0	+26.1	0
Maximum	6.3	−8.5	+13.8	+4.9
Mortality rate (α)	−11.4	+38.5	−52.9**	+10.8
MRDT ($\ln 2/\alpha$)	+16.2	−9.3	+32.4	−2.3
Conjugated hydroperoxides	−52.7**	−27.1	−57.4**	−47.9*
Ketodienes	−61.1**	−41.7	−70.7**	−47.9
SOD	−16.1	−18.3	+8.1	+41.2*
Catalase	+24.1**	−1.7	+19.6**	+6.9*

The difference with sex-matched controls is significant, * $P<0.05$; ** $P<0.02$.

3.2. Effect of melatonin and epithalamin on lipid peroxidation and antioxidant enzyme activity in D.melanogaster

The tissue level of CHP and KD was significantly higher in control females than males (by 40 and 49%, respectively, Table 4), that inversely correlated with the mean life span of the flies. Exposure to melatonin significantly reduced lipid peroxidation in females and abolished sex differences between the groups. Most significantly the content of CHP and KD was decreased in female flies exposed to epithalamin (by 2.3 and 3.4 times, respectively, $P < 0.001$) compared to controls. Sex differences in the content of the products of lipid peroxidation were diminished as well.

The activity of catalase was 2-fold higher in control males than that in control females, whereas the activity of SOD was the same in both sexes. Exposure to melatonin failed to influence the activity of catalase in males but increased it in females (by 24%, $P < 0.02$) and failed to influence the activity of SOD both in males and females. Exposure to epithalamin was followed by a significant increase in the activity of catalase by 20% in males and by 7% in females and by the increase in SOD activity in males (41%).

4. Discussion

Our results have shown significant sex differences in some parameters studied in control flies. The mean life span of the HEM strain of *D. melanogaster* was inversely correlated with levels of conjugated hydroperoxides and ketodienes that is

in agreement with data published earlier [32]. Contrary, the mean life span directly correlated with activity of catalase. These findings are in a good agreement with the postulates of the free radical theory of aging [1–3,33].

The exposure to epithalamin significantly increased in the mean and maximum life span of female *D. melanogaster* and slowed the aging rate of the population by more than 2. These effects are in a good agreement with the strong inhibiting effect

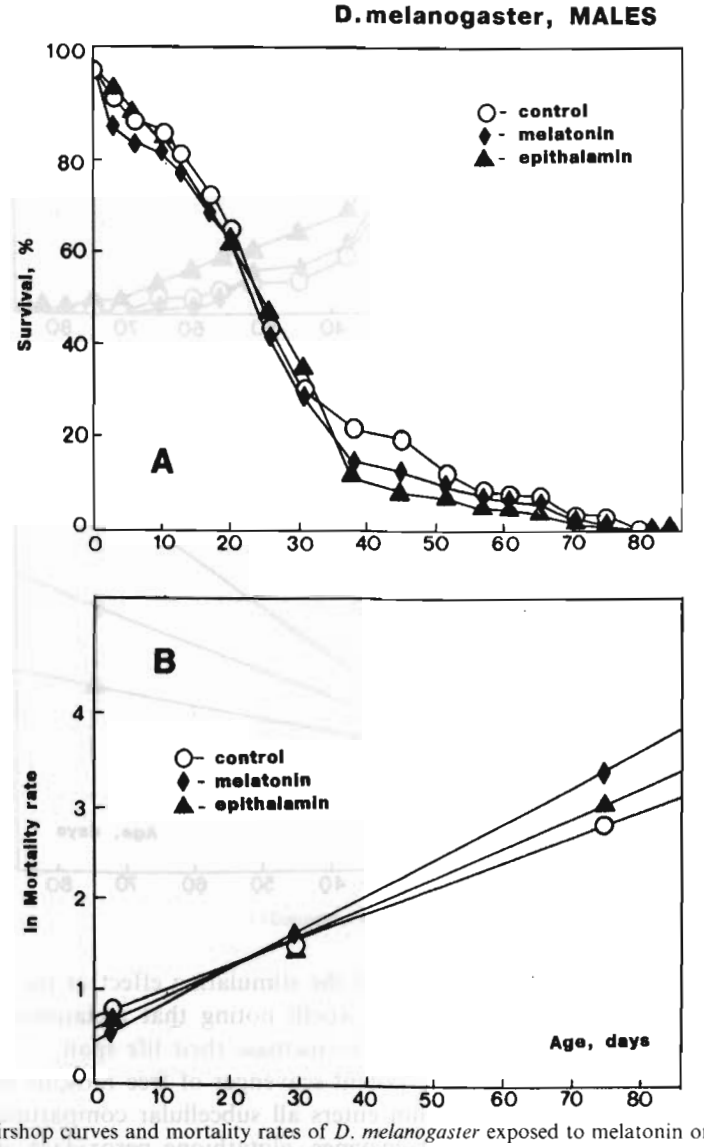

Fig. 1. Survivirshop curves and mortality rates of *D. melanogaster* exposed to melatonin or epithalamin. (a) Survivorship curves; (b) mortality rates of flies are graphed on a semilogarothmic scale (Gompertz plots).

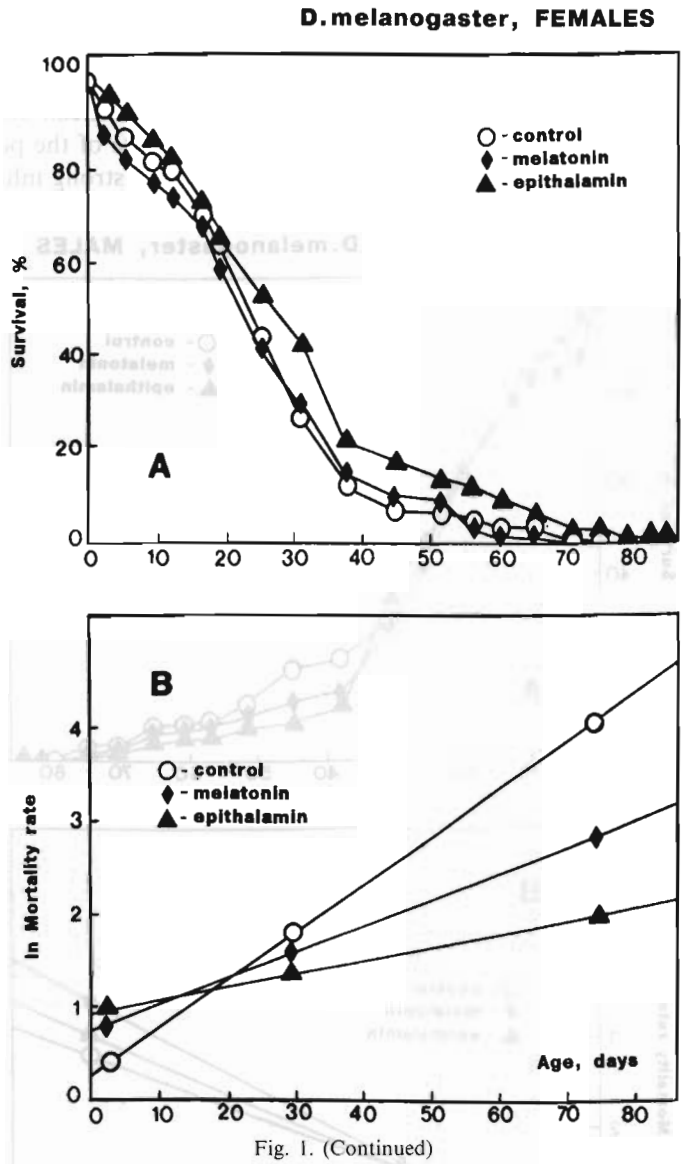

Fig. 1. (Continued)

of epithalamin on lipid peroxidation and the stimulating effect of the drug on the activity of antioxidative enzymes. It is worth noting that melatonin which also inhibits lipid peroxidation in flies failed to increase their life span.

It was shown that melatonin is a potent scavenger of free radicals both in vivo and in vitro [8–10,21–23]. Melatonin enters all subcellular compartments, stimulates one of the potent antioxidant enzymes, glutathione peroxidase, in rat brain and liver [8–10] but, however, failed to influence the activity of serum SOD and

Table 4
Parameters of free radical processes in *D. melanogaster* exposed to melatonin or epithalamin

Parameters	Controls	Melatonin	Epithalamin
Females			
Catalase[a]	41.3 ± 1.46	51.4 ± 1.58**	49.4 ± 1.76**
SOD[b]	135.8 ± 11.24	114.0 ± 12.14	146.8 ± 13.30
Conjugated HP[c]	0.976 ± 0.079	0.462 ± 0,137**	0.416 ± 0.079**
Ketodiene[c]	0.519 ± 0.044	0.181 ± 0.075**	0.152 ± 0,044*
Males			
Catalase[a]	89.3 ± 1.94*	87.8 ± 1.94*	95.5 ± 1.94*,****
SOD[b]	134.1 ± 13.30	109.6 ± 14.87	189.4 ± 14.87***
Conjugated HP[c]	0.584 ± 0.097*	0.426 ± 0.097	0.304 ± 0.079***
Ketodiene[c]	0.26 ± 0.053*	0.154 ± 0.053	0.138 ± 0.044

[a] mM H_2O_2 min.mg of protein;
[b] Units/mg of protein;
[c] nM/g of tissue.
The differnce with corresponding parameter in females is significant, * $P < 0.001$.
The differnce with sex-matched controls is significant, ** $P < 0.01$; *** $P < 0.05$.

coeruloplasmin in rats [23]. Long-term treatment with melatonin increases the life span of mice and rats [11,12]. Exposure to a constant light regimen inhibiting melatonin production was followed by a decrease in the survival time of fruit flies [24] and pinealectomy reduced the life span of rats [34]. The pineal peptide drug epithalamin stimulates synthesis of melatonin in the rat pineal gland in vitro and in vivo and increased it secretion in the blood of adult and old rats [17,20]. Like melatonin, epithalamin inhibits lipid peroxidation and increases catalase activity in rats and in flies, but unlike melatonin, epithalamin increased serum activity of SOD and coeruloplasmin in rats and SOD in flies [21–23]. Only overexpression of both SOD and catalase was followed by an increase in life span of transgenic flies, whereas the life span of flies with overexpression of SOD or catalase alone had no different to controls [6]. It was shown that long-term exposure to epithalamin increased the mean life span and slowed the aging rate of mice and rats and inhibited the development of spontaneous and chemically induced or ionizing radiation carcinogenesis [16,17,19]. The results of our experiments with *D. melanogaster* are in agreement with the above mentioned data and suggest that both melatonin-mediated antioxidative and SOD and other antioxidative enzyme-mediated effects of epithalamin play a significant role in the mechanism of it's geroprotective potential.

Acknowledgements

This work was supported by grants Nos. 04/95 and 05/95 from the Institute of Bioregulation and Gerontology.

References

[1] D. Harman, Free-radical theory of aging: increasing the functional life span. *Ann. N.Y. Acad. Sci.*, *717* (1994) 1–15.

[2] M.K. Shigenaga, T.M. Hogen and B.N. Ames, Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci. USA*, *91* (1994) 10771–10778.

[3] G.M. Martin, S.N. Austad and T.E. Johnson, Genetic analysis of aging: role of oxidative damage and environmental stresses. *Nat. Genet.*, *13* (1996) 25–34.

[4] N.M. Emanuel and L.K. Obukhova, Types of experimental delay in aging patterns. *Exp. Gerontol.*, *13* (1978) 25–29.

[5] S.P. Sharma and R. Wadha, Effect of butylated hydroxytoluene on the life span of *Drosophila bipenctinata*. *Mech. Aging Dev.*, *23* (1983) 67–71.

[6] W.C. Orr and R.S. Sohal, Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science*, *263* (1994) 1128–1130.

[7] B. Poeggeler, R.J. Reiter, D.-X. Tan, Chen L.-D. and L.C. Manchester, Melatonin hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. *J. Pineal Res.*, *14* (1993) 151–168.

[8] R.J. Reiter, The pineal gland and melatonin in relation to aging: a summary of the theories and of the data. *Exp. Gerontol.*, *30* (1995) 199–212.

[9] R.J. Reiter, D. Melchiorri, M.D. Sewerinek et al., A review of the evidence supporting melatonin's role as an antioxidant. *J. Pineal Res.*, *18* (1995) 1–11.

[10] E. Sewerynek, M. Abe, R.J. Reiter et al., Melatonin administration prevents lipopolysaccharide-induced oxidative damage in phenobarbital-treated animals. *J. Cell. Biochem.*, *58* (1995) 436–444.

[11] W. Pierpaoli and W. Regelson, Pineal control of aging: effect of melatonin and pineal grafting on aging mice. *Proc. Natl. Acad. Sci. USA*, *91* (1994) 787–791.

[12] S. Oakin-Bendahan, Y. Anis, I. Nir and N. Zisappel, Effects of long-term administration of melatonin and a putative antagonist on the ageing rat. *Neuroreport*, *6* (1995) 85–788.

[13] S.M. Reppert and D.R. Weaver, Melatonin madness. *Cell*, *83* (1995) 1059–1062.

[14] F.W. Turek, Melatonin hipe hard to swallow. *Nature*, *379* (1996) 295–296.

[15] D. Bonn, Melatonin's multifarious marvels: miracle or myth? *Lancet*, *347* (1996) 184.

[16] V.N. Anisimov, V.Kh. Khavinson and V.G. Morozov, Carcinogenesis and aging. IV. Effect of low molecular-weight factors of thymus, pineal gland and anterior hypothalamus on immunity, tumor incidence and life span of C3H/Sn mice. *Mech. Ageing Dev.*, *19* (1982) 245–258.

[17] V.N. Anisimov, V.Kh. Khavinson and V.G. Morozov, Twenty years of study on effect of pineal peptide preparation: epithalamin in experimental gerontology and oncology. *Ann. N.Y. Acad. Sci.*, *719* (1994) 483–493.

[18] V.M. Dilman, V.N. Anisimov, M.N. Ostroumova, V.Kh. Khavinson and V.G. Morozov, Increase in life span of rats following polipeptide pineal extract treatment. *Exp. Pathol.*, *17* (1979) 539–545.

[19] V.N. Anisimov, A.S. Loktionov, V.Kh. Khavinson and V.G. Morozov, Effect of low molecular-weight factors of thymus and pineal gland on life span and spontaneous tumour development in female mice of different age. *Mech. Ageing Dev.*, *49* (1989) 245–257.

[20] V.N. Anisimov, L.A. Bondarenko and V.Kh. Khavinson, Effect of pineal peptide preparation (epithalamin) on life span and pineal and serum melatonin level in old rats. *Ann. N.Y. Acad. Sci.*, *673* (1992) 53–57.

[21] V.N. Anisimov, V.M. Prokopenko and V.Kh. Khavinson, Melatonin and epithalamin inhibit process of free radical oxidation in rats. *Proc. Russian Acad. Sci.*, *343* (1995) 557–559.

[22] V.N. Anisimov, A.V. Arutyunian and V.Kh. Khavinson, Melatonin and epithalamin inhibit process of lipid peroxidation in rats. *Proc. Russian Acad.Sci.*, *348* (1996) 265–267.

[23] V.N. Anisimov, A.V. Arutyunian and V.Kh. Khavinson, Effect of melatonin and epithalamin on activity of antioxidant defense system in rats. *Proc. Russian Acad. Sci.*, 352 (1997) 831–833.

[24] J. Arendt, *Melatonin and the Mammalian Pineal Gland*. Chapman Hall, London, 1995, p. 183.

[25] B. Vivien-Rocls and P. Pevet, Melatonin: presence and formation in invertebrates. *Experientia*, *49* (1993) 6420–647.

[26] S.V. Mylnikov and A.N. Smirnova, Adult mortality in the inbred selected *Drosophila melanogaster* stocks and their hybrids. *Ontogenez, 25 (4)* (1994) 7–11.
[27] L.K. Obukhova N.Sh. Nakaidze, A.M. Serebryany and L.D. Smirnov, Experimental analysis of the mechanismx of aging in *Drosophila melanogaster. Exp. Gerontol., 14* (1979) 335–342.
[28] T.F. Slater, Overview of methods used for detecting lipid peroxidation. *Methods Enzymol., 195* (1994) 283–293.
[29] A. Agostini, G.C. Gerli, L. Berletta and M. Bianchi, Superoxide dismutase, catalase and glutathione peroxidase activities in maternal and cord blood erythrocytes. *J. Clin. Chem. Clin. Biochem., 18* (1980) 772–773.
[30] E.V. Gubler, *Calculating Methods of Analysis and Recognition of Pathological Processes.* Meditsina, Leningrad, 1978.
[31] C.E. Finch and L. Hayflick (eds), *Handbook of the Biology of Aging.* Van Nostrand Reinhold, New York, 1977.
[32] S.V. Mylnikov, A.N. Smirnova and T.I. Oparina, Comparative genetic analysis of interlinear differences in the rate level of lipid peroxidation and longevity in *Drosophila melanogaster. Genetika, 39* (1994) 1466–1466.
[33] S. Agarwal and R.S. Sohal, DNA oxidative damage and life expectancy in houseflies. *Proc. Natl. Acad. Sci. USA, 91* (1994) 12332–12335.
[34] O.J. Malm, O.E. Skaug and P. Lingjaerde, The effect of pinealectomy on bobily growth, survival rate and 32p uptake in the rat. *Acta Endocrinol., 30* (1959) 22–28.