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Dose-dependent effect of melatonin on life span and spontaneous tumor incidence in female SHR mice

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Abstract

From the age of 3 months until their natural death, female Swiss-derived SHR mice were given melatonin with their drinking water (2 or 20 mg/l) for 5 consecutive days every month. Intact mice served as controls. There were 54 mice in each group. The results of this study show that the treatment of melatonin did not significantly influence food consumption, but its administration at lower doses did decrease the body weight of mice; it slowed down the age-related switching-off of estrous function; it did not influence the frequency of chromosome aberrations in bone marrow cells; it did not influence mean life span; and it increased life span of the last 10% of the survivors in comparison to controls. We also found that treatment with low dose melatonin (2 mg/l) significantly decreased spontaneous tumor incidence (by 1,9-fold), mainly mammary carcinomas, in mice whereas higher doses (20 mg/l) failed to influence tumor incidence as compared to controls. For this reason, we conclude that the effect of melatonin as a geroprotector is dose-dependent.

Keywords: Melatonin; Aging; Longevity; Biomarkers of aging; Spontaneous tumors; Mice

1. Introduction

During the past decade, a number of reports, sometimes contradictory, appeared concerning the role of the pineal gland in aging (Armstrong and Redman, 1991; Anisimov, 1995; Reiter, 1995; Reppert and Weaver, 1995; Pierpaoli, 1998). Melatonin (N-acetyl-5-methoxytriptamine) is the main pineal hormone synthesized from tryptophan, predominantly at night (Arendt, 1995). It has a wide spectrum of physiological effects on endocrine and reproductive functions (Arendt, 1995; Reiter, 1995; Vanecek, 1998). With advancing age the nocturnal production of melatonin decreases in various species of animals, including humans (Reiter, 1995; Waldhauzer et al., 1998; Touitou, 2001). The performance of a pinealectomy on rats reduced life span (Malm et al., 1959; Reiter et al., 1999). The administration of melatonin to mice, rats, fruit flies, or planaria extended life, (Pierpaoli and Regelson, 1994; Oakin-Bendahan et al., 1995; Anisimov et al., 1997a; Thomas and Smith-Sonneborn, 1997; Mocchegiani et al., 1998; Izmaylov and Obukhova, 1999; Oxenkrug et al., 2001; Bonilla et al., 2002). Many studies show melatonin inhibits tumor growth in vivo and in vitro (Blask, 1993; Bartsch et al., 2001). Interest in all of these observations significantly increased after the discovery of the in vitro and in vivo antioxidant activity of melatonin (Reiter et al., 1995; Tan et al., 2002). At the same time, in several studies, melatonin failed to show effects on life span (Pierpaoli et al., 1991; Izmaylov and Obukhova, 1999; Lipman et al., 1998). Moreover, long-term treatment with melatonin was followed by increased tumor incidence in some mouse strains (Romanenko, 1983; Pierpaoli et al., 1991; Lipman et al., 1998; Anisimov et al., 2001). A critical review of data on the effect of melatonin on the life span and tumor incidence in rodents showed that most studies did not follow guidelines for long-term testing

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of chemicals for carcinogenic safety (Gart et al., 1986; Freedman and Zeizel, 1988; Vainio et al., 1992) or principles of gerontological experiments (Warner et al., 2000). This aspect has been discussed elsewhere (Anisimov, 2001).

Here we present the results of a study of the effects of different doses of melatonin on life span, some biomarkers of aging (estrous function, body temperature, frequency of chromosome aberrations) and spontaneous tumor incidence.

2. Material and methods

2.1. Animals

One hundred sixty two female Swiss-derived SHR 2-month-old mice were purchased from the Rappolovo Animal Farm of the Russian Academy of Medical Sciences (St. Petersburg) (Anisimov, 1987). The mice were kept in polypropylene cages (30 × 21 × 9 cm), 5 mice to a cage at a temperature of 22 ± 2 °C. A regimen of 12 h of light and 12 h of dark, was followed. The animals received sterilised standard laboratory chow (Baranova et al., 1986) and tap water *ad libitum*.Mice were checked daily by animal care personnel and weekly by a veterinarian. The study was carried out in accordance with the regulations for ensuring the humane treatment of animals under the approval of the Committee on Animal Research of the N.N. Petrov Research Institute of Oncology.

2.2. Experiment

At the age of 3 months, the mice were randomly divided into 3 groups, each of 54 animals, and were individually marked. Two groups of mice were given melatonin (Sigma, St.Louis; MS) with tap water (2 mg/l-group 2; and 20 mg/l-group 3) during the night (from 6 PM to 9 AM), 5 consecutive days each month with 4-weeks intervals between treatments, until their natural death. Calculations showed that mice in group received an average melatonin dose of 0.25-0.32 mg/kg/day in the group 2 and 2.5-3.2 mg/kg/day in group 3. Melatonin was dissolved in several drops of 96% ethanol and diluted with sterile tap water to the relevant concentration. A fresh melatonin solution was prepared each third day. All bottles containing melatonin were made from dark glass. 54 mice were kept intact to serve as controls (group 1) and were given tap water with the same concentration of ethanol (<0.004%) as in the groups 2 and 3.

Four intact female SHR mice were euthanized at the age of 3 months for evaluation of the initial level of chromosome aberrations. Additionally four mice from each group were euthanized at the age of 12 months for a cytogenetic study of chromosome aberrations in bone marrow cells (see below). Once every 3 months, simultaneously with weighting, the amount of drinking water and food consumed was measured. Thirty grams of food were given in each cage after cleaning and in 24 h after the food which not be consumed was collected from each cage and weighted. The mean amount of food (grams) consumed per mouse during this day was calculated for each group.

Once every 3 months, vaginal smears taken daily for 2 weeks from the animals were cytologically examined to estimate the phases of their estrous functions. In the same period, rectal body temperatures of the mice were measured with an electronic thermometer, TPEM (KMIZ, Russia). Animals were observed until natural death. The date of each death was registered, and mean life span, the age by which 90% of the animals died, and maximal life span estimated.

2.3. Cytogenetic study

Chromosomal aberrations in bone marrow cells was studied by modified Ford's method described in Rosenfeld et al. (2001). Mice were sacrificed with ether anaesthesia. Both femurs of each mouse were dissected and bone marrow cells flushed gently with 0.56% KCl solution into a centrifuge tube. Cells were treated for 20 min with hypotonic solution and fixed with ethanol: acetic acid mixture (3:1). Slides were stained with 4% acetoorseine. 20–30 well spread anaphases were analyzed for each animal and cells with chromosome breaks, acentric fragments, and other aberrations were evaluated on 1,000x magnification with a light microscope (Leitz, Germany).

2.4. Pathomorphological examination

All animals that died, or were sacrificed when moribund, were autopsied. At autopsy their skin and internal organs were examined. Neoplasia were classified according to the recommendations of the International Agency of Research on Cancer (IARC) as 'fatal' (i.e. those, that directly caused the death of the animal) or 'incidental' (for cases where the animal died of a different cause) (Gart et al., 1986). All tumors, as well as tissues and organs with suspected tumors, were excised and fixed in 10% neutral formalin. After routine histological processing, tissues were embedded in paraffin. Thin, 5–7 μm histological sections were stained with hematoxylin-eosine and microscopically examined. The experimental group to which the mouse belonged was blinded. Tumors were classified according to IARC recommendations (Turusov and Mohr, 1994).

2.5. Statistics

Experimental results were statistically processed by methods of variation statistics (Goubler, 1978). The significance of discrepancies was defined according to Student's t-criterion, Fischer's exact method, χ^2-analysis, and the non-parametric criterion of Wilcoxon-Mann-Whitney (Goubler, 1978). For discrepancies in neoplasm incidence to be estimated, an IARC method of combined

Table 1
Body weight gain dynamics in female SHR mice treated and not treated with different doses of melatonin

Group	Body weight (g)							
	3 mo	5 mo	7 mo	9 mo	11 mo	13 mo	15 mo	17 mo
Control	23.9 ± 0.42	27.9 ± 0.37	30.2 ± 0.80	33.0 ± 1.10	33.3 ± 1.10	35.6 ± 1.00	34.0 ± 1.00	32.5 ± 1.3
Melatonin, 2 mg/l	25.6 ± 0.30[b]	28.1 ± 0.51	28.1 ± 0.52[a]	30.2 ± 0.65[a]	31.9 ± 0.61	31.6 ± 0.68[b]	30.5 ± 0.53[b]	31.4 ± 0.88
Melatonin, 20 mg/l	24.0 ± 0.45	26.4 ± 2.50	29.0 ± 0.54	32.2 ± 0.63	32.7 ± 0.49	31.9 ± 1.04	32.9 ± 0.83	31.0 ± 1.23

The difference with the controls is significant, [a]$p < 0{,}05$; [b]$p < 0{,}01$; Student's t test.

contingency tables calculated individually for the fatal and incidental tumors (Gart et al., 1986). For survival analysis, Cox's method (Cox and Oakes, 1996) was used. All reported test values for survival analyses are two sided.

2.6. Mathematical models and estimations

The mathematical model used to describe survival is the Gompertz model with the survival function

$$S(x) = \exp\left\{-\frac{\beta}{\alpha}[\exp(\alpha x) - 1]\right\}$$

where parameters α and β are associated with the aging, and initial mortality rate, respectively. Parameter α is often characterized by the value of mortality rate doubling time (MRDT), calculated as $\ln(2)/\alpha$. Parameters for the model were estimated from data using the maximum likelihood method implemented in the Gauss statistical system (Gauss System and Graphic Manual, 1994). Confidence intervals for the aging rate parameter estimates were calculated using log-likelihood functions (Cox and Oakes, 1996).

3. Results

3.1. Age-related body weight dynamics

Mean values of body weight for mice at different ages in the control and melatonin-treated groups are displayed in Table 1. The body weight of the mice in all groups increased with age. The body weight of 15-month-old controls was exceed their weight at the age of 3 months by 42.3% ($p < 0.001$, Student's t test). For mice exposed to melatonin at the dose of 2 mg/l this gain was 23.4%, whereas mice given 20 mg/l melatonin have a body weight 37.1% higher than that at the age of 3 months. The mean body weight of mice exposed to low dose melatonin was decreased 7–11% compared to controls at the ages of 7,9,13 and 15 months ($p < 0.05$, Student's t test). The group exposed to high dose melatonin did not differ in body weight compared to in controls. At the age of 17 months, the mean body weight of mice was similar in all groups (Table 1).

3.2. Age-related dynamics of water and food consumption

The amount of drinking (tap) water consumed was stable during observation, at 5.3 ± 1.2 ml/mouse per 24 h; 3.8 ± 1.1 ml/mouse per night. There were no differences in water consumption between control and melatonin-treated animals. Measurements shows that the amount of food consumed by the mice varied with age. Periods of increased and decreased food consumption were recorded. These were similar in both groups. Animals exposed to 2 mg/l and to 20 mg/l of melatonin eat less food in comparison to controls at the age of 15 months ($p < 0{,}05$, Student's t test), however it was equal in all groups at the age of 3 to 13 months and at the age of 17 months (Table 2).

3.3. Age-related dynamics of estrous function in mice

Investigations of estrous function in animals were performed every 3 months starting when at 3 months of age. The parameters of estrous function estimated were: length of estrus, the relative rate of estrous cycle phases (in percent); and the relative number of short (<5 days) and long (>5 days) estrous cycles. The relative number of animals with regular cycles and irregular cycles (persistent estrus and anestrus) were calculated. Judging by the data presented in Table 3, the length of estrous cycle in the control female mice was stable with the advance in age.

Table 2
Food consumption dynamics in female SHR mice treated and not treated with different doses of melatonin

Group	Daily food consumption (g)							
	3 mo	5 mo	7 mo	9 mo	11 mo	13 mo	15 mo	17 mo
Control	4.3 ± 0.23	3.4 ± 0.43	3.5 ± 0.47	3.6 ± 0.51	3.1 ± 0.24	3.8 ± 0.42	5.1 ± 0.40	5.5 ± 0.15
Melatonin, 2 mg/l	4.1 ± 0.10	3.1 ± 0.15	3.3 ± 0.17	3.2 ± 0.14	3.4 ± 0.16	3.8 ± 0.25	3.9 ± 0.14[a]	5.1 ± 0.29
Melatonin, 20 mg/l	4.8 ± 0.32	3.4 ± 0.46	3.1 ± 0.2 3	3.0 ± 0.43	2.8 ± 0.53	3.7 ± 0.28	3.7 ± 0.26[a]	4.5 ± 0.29

The difference with the controls is significant, [a]$p < 0{,}01$; Student's t test.

Table 3
3Age-related dynamics of estrous functional parameters in mice treated and not treated with melatonin

Age (mo)	No. of mice	Length of estrous cycle (days)	Rate of separate phases of estrous cylce (%)			Rate of estrous cylces (%)			No. of mice with regular cycles	No. of mice with irregular cycles
			E	D	P + M	<5 d.	5–7 d	>7 d		
Controls										
3	30	5.96 ± 0.21	35.8	54.6	9.6	17.8	60.8	21.4	93.3	6.7
6	26	6.08 ± 0.29	42.4	47.7	9.9	20.4	61.2	18.4	100	0
9	24	6.20 ± 0.37	31.0	46.0	23	17.6	61.8	20.6	83.3	16.7
12	23	5.32 ± 0.30	48.8	47.8	3.4	28.8	58.0	13.2	87.0	13.0
15	18	7.12 ± 0.50	51.4	46.8	1.8	8.0	56.0	36.0	83.3	16.7
Melatonin, 2 mg/l										
3	30	4.8 ± 0.26[a]	43.8*	53.0	3.2**	50.0***	44.7	5.3*	90.0	10.0
6	25	4.3 ± 0.23[a]	41.2	56.5*	2.3**	65.5***	31.0**	3.5*	88.0	12.0
9	25	4.7 ± 0.27[a]	47.3**	51.8	0.9***	51.4**	40.0	8.6	96.0	4.0
12	20	4.6 ± 0.53	53.8	46.2	0	54.5	36.4	9.1	85.0	15.0
15	16	3.4 ± 0.22[a]	49.4	46.1	4.5	100***	-	-	100	0
Melatonin, 20 mg/l										
3	30	5.95 ± 0.17	43.1	54.0	2.9**	20.0	60.0	20.0	100	0
6	26	5.39 ± 0.27	45.1	51.8	3.1**	36.9	47.9	15.2	96.2	3.8
9	25	4.90 ± 0.27[b]	51.7**	44.4	3.9**	41.3*	52.2	6.5	92.0	8.0
12	24	5.94 ± 0.16	48.2	48.8	3.0	13.9	69.4	16.7	87.5	12.5
15	21	5.77 ± 0.24[a]	45.0	53.1	1.9	19.2	69.3	11.5*	66.7	33.3

Note: E = estrus; D = diestrus; P = proestrus; M = metaestrus; The difference with the corresponding age in the control group: [a]$p < 0.05$; [b]$p < 0.01$ (Student's t test); *$p < 0,05$; **$p < 0,01$; ***$p < 0,001$ (Fischer's exact and χ^2 tests).

No essential age-related alterations in the rate of estrous cycle phases were observed in the control group. However, the relative number of short estrous cycles significantly decreased with age (20.4% at the age of 6 months, 28.8% at 12 months and 8% at 15 months; the difference with the parameter at the age of 12 months is significant, $p < 0.05$; Fischer's exact test), whereas the number of long cycles rose (18.4%, 13.2% and 36.0%, respectively; the difference with the parameter at the age of 12 months is significant, $p < 0.05$; Fischer's exact test). Only regular cycles were observed in the control group at the age of 6 months, however 16,7% of control females had irregular cycles at the age of 9 and 15 months.

In mice exposed to low dose melatonin (2 mg/l) the length of estrous cycles was stable between the age of 3 and 12 month and decreased at the age of 15 months ($p < 0.05$, Student's t test). It was decreased in comparison to the control group at the age of 3, 6, 9 and 17 months ($p < 0.05$, Student's t test).

There was no age-related decrease in number of short cycles or increase in the number of long cycles between the age of 3 and 12 months. Short cycles were observed in all mice of this group at the age of 15 months. It is worthy of note that the relative number of mice with short estrous cycles were higher in mice exposed to 2 mg/l melatonin compared to controls during the period of observation (from age 3 to 15 months). The number of mice with regular cycles did not change significantly with age in the group (Table 3).

In mice treated with melatonin at the high dose (20 mg/l), no age effects were observed on the length of the estrous cycle and the rate of separate phases of the estrous cycle compared to controls. However, the number of short and long estrous cycles was practically the same at ages 3 and 15 months (Table 3). The number of mice with long estrous cycles was less in this group as compared to the controls at the age of 15 months ($p < 0.05$, Fischer's exact test). The number of mice with irregular cycles in the group treated with higher dose of melatonin has a tendency to an increase compared with the control group at the age 15 months.

These data suggest that the long-term administration of 2 mg/l melatonin slows age-related changes in estrous function whereas the higher dose of melatonin failed significantly influence the estrous function.

3.4. Age-related dynamics of body temperature in mice

Data on body temperatures of the control mice and mice exposed to melatonin are in Table 4. Control mice revealed significant decreases in body temperature with age, both on the whole (irrespective of estrous cycle phases), and in any phase. No cyclic alterations in rectal body temperature during estrus phases or its age-related changes, were observed in mice treated with 2 mg/l melatonin. The average body temperature in this group of mice at 7 months was lower, and at the age of 12 and 15 months higher, than in controls during phases of estrus and diestrus (Table 4). There was significant decrease in the average body temperature in diestrus in 19-month-old mice compared to 7–17-month-old mice. In mice exposed to the higher dose of melatonin (20 mg/l) the pattern of age-related changes in

Table 4
Body temperature dynamics in SHR mice treated and not treated with melatonin

Age (mo)	Number of mice	Total cycle (without phase sub-division)	Mean body temperature (°C)		
			Estrus	Diestrus	Metaestrus + Proestrus
Control					
7	25	40.23 ± 0.15	40.41 ± 0.2	40.2 ± 0.25	39.67 ± 0.5
12	23	38.77 ± 0.17[b]	38.96 ± 0.3[c]	38.67 ± 0.22[c]	–[d]
15	20	38.48 ± 0.13[b]	38.8 ± 0.2[c]	38.37 ± 0.2[c]	–
17	18	38.58 ± 0.20[b]	–[d]	38.58 ± 0.2[c]	–
19	12	37.66 ± 0.12[c]	37.7 ± 0.1[c]	37.7 ± 0.3[c]	–
Melatonin, 2 mg/l					
7	25	38.51 ± 0.18**	38.81 ± 0.27***	38.42 ± 0.27***	38.43 ± 0.54
12	22	39.42 ± 0.11[a]**	39.43 ± 0.12[a]	39.48 ± 0.15[b]**	–
15	16	39.10 ± 0.24*	39.08 ± 0.68	39.11 ± 0.12[a]**	–
17	14	39.07 ± 0.22[a]	38.27 ± 0.32	39.0	–
19	10	38.33 ± 0.19**	37.43	37.19 ± 0.39[b]	–
Melatonin, 20 mg/l					
7	25	39.22 ± 0.2**	39.2 ± 0.4*	39.49 ± 0.2*	38.35 ± 0.21*
12	24	39.22 ± 0.16*	39.0 ± 0.3	39.2 ± 0.19*	–
15	21	39.47 ± 0.06**	39.4 ± 0.1*	39.6 ± 0.11**	–
17	16	38.31 ± 0.2[b]	38.5 ± 0.3	38.4 ± 0.2[b]	–
19	14	37.88 ± 0.1[a]	37.8 ± 0.1[a]	37.9 ± 0.14[c]	–

The difference with the age of 7 mo is significant:[a]$p < 0{,}05$; [b]$p < 0{,}01$; [c]$p < 0{,}001$ (Student;s t test). The difference with the corresponding age in the control group is significant: *$p < 0{,}05$; **$p < 0{,}01$; ***$p < 0{,}001$ (Student's t test).
[d] The absence of data means that at the day of the vaginal smears study there was no mice with this stage of the estrous cycle.

body temperature was similar to controls both in estrus and diestrus.

3.5. Chromosome aberrations in mouse bone marrow cells

Incidence of chromosome aberrations in bone marrow cells of 3-month-old female SHR was 2.1 ± 0.29%. At 12 months this increased to 8.2 ± 0.41% ($p < 0.001$, Student's t test) in intact controls. In mice treated with melatonin in 2 and 20 mg/l doses the incidence of chromosome aberrations at 12 months was 7,5 ± 0.25 (− 8.5%) and 7.2 ± 0.42 (− 12.2%), respectively (the difference with the controls is non-significant for both group exposed to melatonin, $p > 0.05$, Wilcoxon-Mann-Whitney test) (Fig. 1).

3.6. Survival and longevity of female SHR mice

Survival in mice treated with melatonin are in Table 5. Survival was similar in all groups up to 14 months. It is worthy to note that mortality in the group treated with low dose melatonin increased between the 16th and the 18th months compared to controls. Afterward, survival in controls and melatonin-treated groups were similar until 25 months (Fig. 2).

The last mouse in the control group died at the age of 772 days (25,4 months). In groups treated with melatonin at the doses 2 and 20 mg/l 6% and 12% of mice survived this age, correspondingly. The maximum life span was 881 days (29 months) and 890 days (29.3 months) in these groups. The mean life span of mice treated with melatonin was not increased compared to controls. Mean life span of the last 10% of survivors increased with melatonin treatment as compared with the control (by 55 days at the dose 2 mg/l, $p > 0.05$, and 85 days at the dose 20 mg/l, $p < 0.05$, student's t test) (Table 6).

3.7. Spontaneous tumor development in female shr mice

Total tumor incidence in the control female mice was 42%. Mammary carcinomas and leukemias developed most

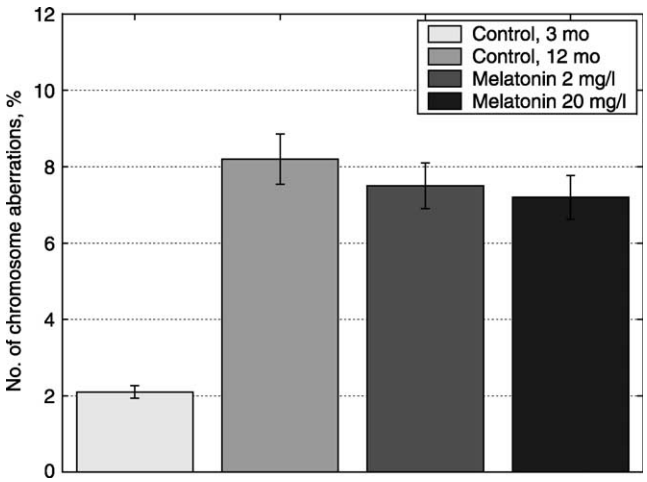

Fig. 1. Frequency of chromosome aberrations in the bone marrow cells in 12-month-old female SHR mice treated and not treated with melatonin. Ordinate, number of chromosome aberrations, %.

Table 5
Survival distribution of female SHR mice exposed and not exposed to melatonin

Group	No. of survivors																							
	5 mo	6 mo	7 mo	8 mo	9 mo	10 mo	12 mo	14 mo	16 mo	18 mo	20 mo	22 mo	24 mo	25 mo	26 mo	27 mo	28 mo	29 mo	30 mo					
Control	50	46	38	38	38	34	34	33	27	22	15	14	10	3	0	0	0	0	0					
Melatonin, 2 mg/l	50	47	43	43	43	43	41	38	18	13	12	10	6	3	3	3	2	2	0					
Melatonin, 20 mg/l	50	44	42	42	41	41	36	33	27	22	16	12	6	6	6	6	3	2	0					

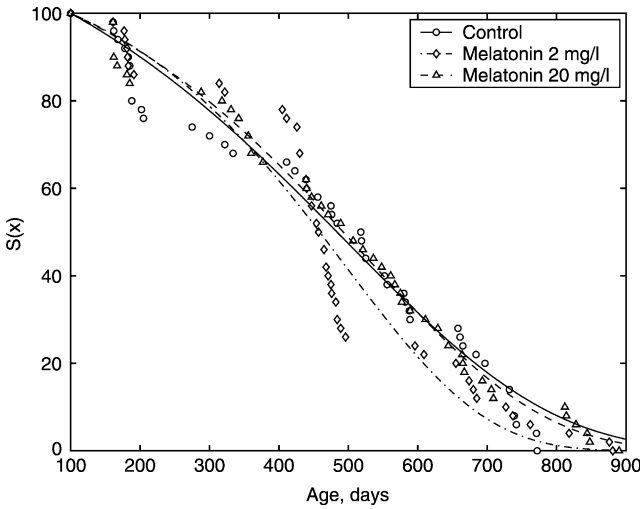

Fig. 2. Effect of melatonin on the survival curves of female SHR mice. Ordinate, number of mice,%.

frequently, corresponding to the oncological characteristics of female shr mice (Anisimov, 1987). Treatment with melatonin at the dose of 2 mg/l was followed by a 1.9 fold decrease in total, and a 2.2-fold decrease in malignant tumor incidence as compared to the controls ($p < 0.01$; Fischer's exact and χ^2 tests; iarc method) (Table 7). The incidence of mammary carcinomas in mice exposed to the lower dose of melatonin was decreased 4.3-fold ($p < 0.01$, fischer's exact and χ^2 tests) as compared with the control group. Four cases of lung metastases of mammary carcinomas was observed in the control group whereas only 1 case of metastazing was detected in the group treated with mealtonin at the dose of 2 mg/l. The mean latent period of mammary carcinomas was significantly increased (by 2 months, $p < 0.01$, Student's t test) in mice of this group as compared to the control group. There was no significant difference in the incidence of any other tumor between mice treated with the lower melatonin dose and controls. There was no effect of treatment with higher dose of melatonin (20 mg/l) on the total incidence of tumors or on the incidence of tumor at any site (Table 7). While the treatment with lower dose of melatonin the total tumor yield curve to the right, the treatment with higher did not (Fig. 3), showing that melatonin at the dose of 2 mg/l inhibited spontaneous carcinogenesis in female SHR mice whereas the hormone at the higher dose (20 mg/l) did not.

Treatment with both doses of melatonin postponed development of mammary carcinoma (Fig. 4).

3.8. Mathematical model and estimation of survival of tumor-free and tumor-bearing mice

A mathematical analysis of the survival of mice from the control and melatonin-treated groups was done for: (1) all animals in each group; (2) fatal tumor-bearing mice, and (3) fatal tumor-free mice. We compared groups without

Table 6
Parameters of life span in female SHR mice treated and not treated with melatonin

Parameters	Controls	Melatonin, 2 mg/l	Melatonin, 20 mg/l
Number of mice	50	50	50
Mean life span, days (M ± S.E.)	479 ± 30	479 ± 25	495 ± 29
Median, days	519	461	507
Mean life span of last 10% of survivors, days	759 ± 8	815 ± 29	845 ± 13*
Maximum life span, days	772	881	890

*The difference with control is significant: $p < 0.05$ (Student's t test).

considering effects caused by dependence of groups. The analysis of Gompertz curves failed to show significant differences in the population aging rate (α) due to either dose of melatonin. It has showed a significant increase in α in 'fatal tumor-bearing mice' (by 17,7%, $p < 0.05$) and 'fatal tumor-free mice' (by 2.14 fold, $p < 0.05$) in the group treated with 2 mg/l melatonin. The MRDT in controls calculated for total cases, 'fatal tumor-bearing mice' and 'fatal tumor-free mice' was higher than in the group treated with low dose melatonin (2 mg/l) (Table 8). This could be interpreted as an acceleration in fatal tumor growth rate. Survival for 'fatal tumor-bearing mice' in mice exposed to the low dose of melatonin shifted to the right compared to controls like 'rectangulation' of the Gompertz curve (Fig. 5).

Estimates of α in the total number of cases and the 'fatal tumor-bearing mice' contexts in groups treated with high dose melatonin (20 mg/l) showed small but significant decrease in the value of this parameter and the significant increase in MRDT as compared with the controls. There was a significant acceleration of the aging rate and reduction of MRDT in this group in the fatal tumor-free mice in comparison to the controls (both 1.7-fold) ($p < 0.05$). In Figs. 2 and 5 at ages 100–200 days and 400–600 days there was a dramatic increase in mortality in all groups, the highest with in the group treated with low dose melatonin. The mortality spike at 400–600 days may depend on mortality related to fatal tumors (Fig. 6). Tumors were not observed before 318 days in any group.

4. Discussion

Our results show long-term nightly administration of melatonin at doses of 2 or 20 mg/l in drinking water

Table 7
Incidence, localization, and type of tumors in female SHR mice treated and not treated with melatonin

Parameters	Controls	Melatonin, 2 mg/l	Melatonin, 20 mg/l
Number of mice	50	50	50
Number of tumor-bearing mice	21(42%)	11(22%)*	19(38%)
Number of malignant tumor-bearing mice	20(40%)	9(18%)*	15(30%)
Total number of tumors	26	17	23
Total number of malignant tumors	22	11	18
Time of the death of the 1st fatal tumor-bearing mice, days	411	457	431
Localisation and type of tumors			
Mammary gland			
Adenocarcinoma	13(26%)	3(6%)	15(12) [a](24%)
Nos. of metastases	4	1	6
Latency of the 1st mammary carcinoma	379	527	272
Mean latency of mammary carcinomas	486 ± 14.9	549 ± 13.1**	522 ± 33.7
Leukemia	7	6	3
Mean survival of mice with leukemia	606 ± 44.9	604 ± 47.4	626 ± 34.2
Lung			
adenoma	–	1	3
Adenocarcinoma	1	3	–
Utery			
Polyp	2	1	–
adenocarcinoma	1	–	–
Ovary			
Cyst	1	3	1
Granuleso-cell tumor	–	–	1
Skin			
Papilloma	1	–	–

The difference with control is significant: *$p < 0.01$ (Fischer's exact and χ^2 tests); **$p < 0.01$ (Student's t test).
[a] a-Three mice have two mammary carcinomas each.

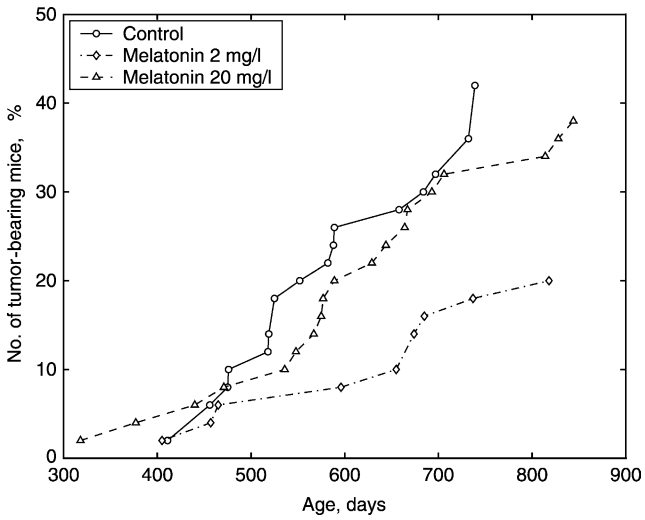

Fig. 3. Effect of melatonin on total tumor yield curves in female SHR mice.

influenced the survival and malignant tumor incidence in female SHR mice in different manners. The effect of melatonin on survival does not relate to its influence on food consumption. No reduction in food consumption was observed between the age of 3 and the 13 months in groups treated with both doses of melatonin. Only at the age of 15 months the decrease in food consumption was recorded in treatment groups. Body weight was not changed in mice treated with higher dose of melatonin as compared to the controls during the observation. In our experiment with female CBA mice treated with the same dose and regimen of melatonin a mean body weight was increased at ages 15 and 18 months as compared with the control group (Anisimov et al., 2001). Treatment with low dose melatonin decreased body weight between the age of 7 and 15 months. This observation is in agreement with the data on a positive correlation between excessive body weight and tumor

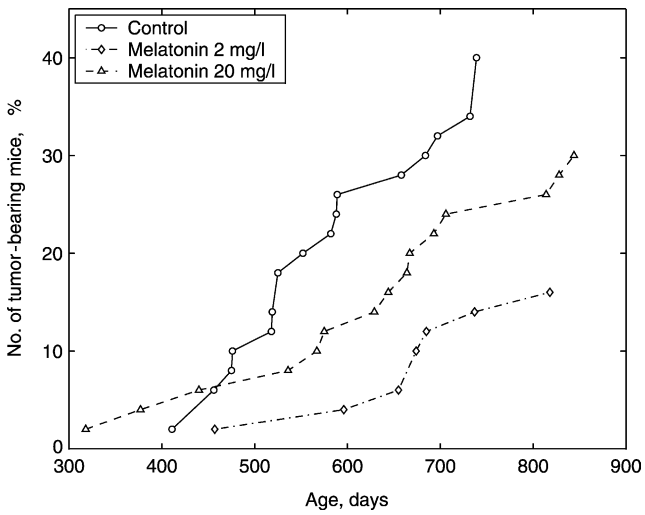

Fig. 4. Effect of melatonin on mammary carcinoma yield curves in female SHR mice.

incidence in rodents and human females (Weindruch and Walford, 1988; Dilman, 1994).

Long-term treatment with melatonin was followed by a slowing of the aging of the reproductive system in female SHR mice. Similar observations in female rats and CBA mice were reported (Meredith et al., 1998; Anisimov et al., 2001).

In our study, both doses of melatonin decreased body temperature in mice at 7 months followed by increases of temperature at 12 and 15 months and then decreased to the level of controls (Table 4). In CBA mice, we observed an increase in the average body temperature at 9 months; a decrease at 15 and 18 months (Anisimov et al., 2001). Average body temperature was increased in SHR mice treated with melatonin at the dose of 2 mg/l as compared to the group treated with higher dose at 17 and 19 months ($p < 0.05$). A decline of body temperature caused by the slowing of metabolic processes is followed by life extension (Lane et al., 1996; Weindruch and Sohal, 1997. Increased body temperature reflects increased metabolism in an organism and could decrease life span. In the present experiment, mice treated with low dose melatonin had increased temperature that correlated with decreased body weight and accelerated aging rate evaluated as constant α and MRDT compared to controls (Table 8).

An important result of our previous study was the increase of malignant tumor incidence under the influence of melatonin in female CBA mice (Anisimov et al., 2001). In female SHR mice we failed to observe any carcinogenic effect of melatonin at either doses. In the low dose, melatonin inhibited the development of spontaneous tumors due to selective inhibition of mammary carcinogenesis (Table 7; Fig. 4). Our observation is in agreement with data on inhibitory effects of melatonin on mammary carcinoma growth in vitro as well as on the development of transplantable, spontaneous in inbred or in HER-2/neu transgenic mice, or induced by 7,12-dimethylbenz[a]-anthracene, N-nitrosomethylurea or γ-rays mammary carcinomas in vivo (Subramanian and Kothari, 1991; Blask, 1993; Anisimov et al., 1999; Cos and Sanchez-Barcelo, 2000; Mockova et al., 2000; Bartsch et al., 2001; Baturin et al., 2001).

In Table 9 we summarized available data on survival and tumor incidence in mice exposed to long-term treatment with melatonin.

Melatonin did not induce malignancies in male C57BL/6 mice when administered at 10 mg/l (1.5–2.0 μg/kg) in the night drinking water from 19 months (Pierpaoli and Maestroni, 1987; Pierpaoli et al., 1991). Lipman et al. (1998) observed lymphomas in 77.9% of male C57BL/6 mice that received melatonin with food (11 ppm or 68 mg/kg) from the age of 18 months and survived to 50% mortality (26.5 months). In controls only 28.6% of mice developed lymphomas. Leukemia was detected in 70–98% of C57BL/6 mice and 78% of CC57BR mice (both males and females) treated subcutaneously with melatonin at a

Table 8
Parameters of life span in female SHR mice treated and not treated with melatonin

Group	Total no. of cases	Fatal tumor-bearing mice	Fatal tumor-free mice
Number of mice			
Control	50	20	30
Melatonin, 2 mg/l	50	9[a]	41[a]
Melatonin, 20 mg/l	50	15	35
Mean life span (days)			
Control	479 ± 30	583 ± 24	410 ± 43
Melatonin, 2 mg/l	479 ± 25	644 ± 38	443 ± 27
Melatonin, 20 mg/l	495 ± 29	620 ± 40	442 ± 35
Mean life span of the last 10% of survivors (days)			
Control	759 ± 8	739 ± 0	772 ± 0
Melatonin, 2 mg/l	815 ± 29	818 ± 0	812 ± 39
Melatonin, 20 mg/l	845 ± 13*	836 ± 8*	815 ± 39
Aging rate $\alpha \times 10^3 (days^{-1})$			
Control	4.24(4.04; 4.37)	9.44(9.28; 10.1)	1.89(1.82; 2.16)
Melatonin, 2 mg/l	4.38(4.34; 4.88)	11.11(10.3; 15.3) #	4.04(4.01; 5.36) #
Melatonin, 20 mg/l	3.96(3.94; 4.30)#	7.10(6.94; 8.25) #	3.18(3.08; 3.76) #
MRDT (days)			
Control	163.50	73.39	367.1
Melatonin, 2 mg/l	158.13	62.27#	171.68#
Melatonin, 20 mg/l	175.06#	97.69#	218.05#

Note: Mean life spans are given as mean ± standard error; 95% confidence limits are given in parentheses; MRDT = mortality rate doubling time.The difference with controls is significant: [a]$p < 0.05$ (Fischer's exact test); *$p < 0.05$ (Student's t test); #$p < 0.05$ (Cox's method).

dose of 2.5 mg/mouse (~80 mg/kg) twice a week for of 2.5–5 months (Romanenko, 1983, 1985). Thus, being administered in significantly higher dose (80 mg/kg) melatonin induced lymphomas and leukemias in C57BL/6 mice. At low dose (1.5–2.0 mg/kg) it did not induce them. In a previous our study with CBA mice, melatonin given in night drinking water in an interrupted (course) regimen at a relatively low dose (3–3.5 mg/kg) was carcinogenic. Lymphomas and lung adenocarcinomas developed in CBA mice treated with melatonin whereas no such malignancies developed in controls (Anisimov et al., 2001). In female SHR mice, melatonin given approximately in the same dose (20 mg/l; 2.7–3.3 mg/kg) failed significantly increase the total incidence of tumors or tumors of any localization. Strain differences in susceptibility to chemical carcinogens is well known (Turusov et al., 1982; Vainio et al., 1992).

The aging process predisposes cells to accumulate mutations; some necessary for initiation of tumor growth in target tissues (Tuker, 1998; Vijg, 2000). The incidence of chromosome aberrations increases with age in different

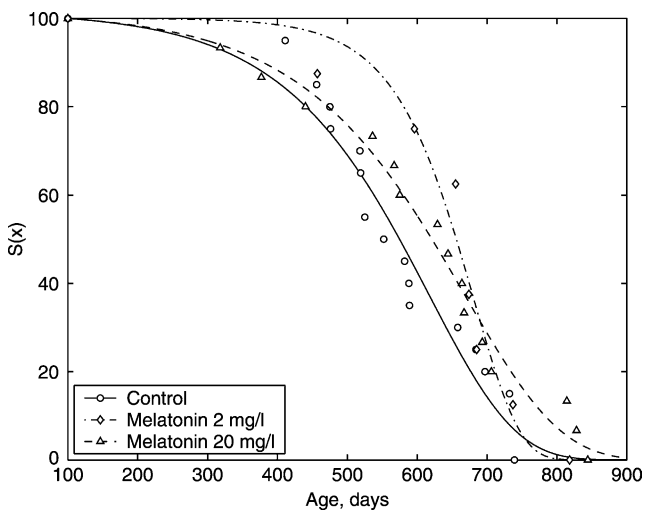

Fig. 5. Effect of melatonin on the survival curves of fatal tumor-bearing SHR mice. Ordinate, number of mice, %.

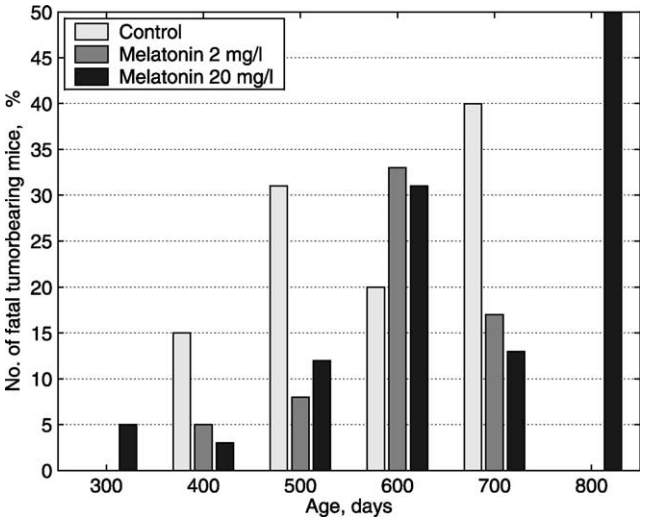

Fig. 6. Number of deaths among fatal tumor-bearing mice. Ordinate, number of fatal tumor-bearing mice,%; Abscissa, age, days.

Table 9
Summary of experiments on the effect of melatonin on life span and spontaneous tumor incidence in mice

Strain [a]	Sex	Nos. of mice C/M	Age at the start of treat-ment,mo	Treatment with melatonin	Age at the end of observation	Effects of melatonin on:		References
						Mean life span	Tumor incidence	
Balb/c	Female	26/12	15	10 mg/l in night drinking water	ND	+18%	No data	Pierpaoli and Regelson (1994)
Balb/c	Male	50/50	18	10 mg/l in night drinking water	ND	Shift to right of the survival curve	No data	Mocchegiani et al. (1998)
C57BL/6	Male and female	25/45	1.5	2.5 mg/mouse s.c. twice a wk × 5 mo	22 mo	−13%	Increases	Romanenko (1983)
C57BL/6	Male and female	29/57	1.5	2.5 mg/mouse s.c. twice a wk × 2.5 mo	22 mo	−20.6%	Increases	Romanenko (1985)
C57Br	Male and female	26/57	1.5	2.5 mg/mouse s.c. twice a wk × 2.5 mo	22 mo	−12%	Increases	Romanenko (1985)
C57BL/6J	Male	10/10	19	10 mg/l in night drinking water	ND	+20%	No data	Pierpaoli and Maestroni (1987)
C57BL/6	Male	20/15	19	10 mg/l in night drinking water	ND	+17%	No data	Pierpaoli et al. (1991)
C57BL/6	Male	1:20/20 2:7/ 13 3:38/30	18	11 ppm (68 mg/kg) with lab chow ad libitum	1: 24 mo; 2: 50% survival 3: died < 2 y	No effect	1: No effect; 2: Increaess; 3: No effect	Lipman et al., 1998
CBA	Female	50/50	6	20 mg/l in night drinking water	ND	+5%	Increases	Anisimov et al. (2001)
C3H	Male Female	20/20 20/20	1 8	2.5 mg/kg/day in night drinking water	23 mo 27 mo	+20% No effect	No data	Oxenkrug et al. (2001)
C3H/He	Female	14/15	12	10 mg/l in night drinking water	ND	No effect	Increases	Pierpaoli et al. (1991)
C3H/Jax	Female	16/39	3 wk	25 − 50 mkg/ mouse/d with drinking water	12 mo	No data	Decreases	Subramanian and Kothari (1991)
FVB/N (HER-2 /neu)	Female	30/27 /22	2	2.5 mg/kg/ day in night drinking water 5 d/w monthly or constantly	ND	M1: No effect M2: -13%	M1: No effect; M2: Decreases	Anisimov et al., (in press)
NOD	Female	25/30	1	4 mg/kg s.c. at 4:30 PM, 5 times a wk, 4-38 wk	50 wk	C:32% survivors M:90% survivors	No data	Conti and Maestroni (1998)
NOD	Female	29/17	1	10 mg/l in night drinking water, 5 times a wk, 4-38 wk	50 wk	+17%	No data	Conti and Maestroni, 1998
NZB	Female	10/10	4	10 mg/l in night drinking water	20 mo	Survivors: C:0; M: 40%	No data	Pierpaoli et al. (1991)
NZB/W	Female	15/15/ 15	8	2 − 3.5 mg/kg s.c.daily at 8–10 hrs (M1) or at 17–19 hrs (M2) × 9 mo	34 wk	Survivors: C: 20%; M1: 60%; M2: 60%	No data	Lenz et al. (1995)
SAMP-1	Female	20/20	3	20 mg/l in night drinking water	ND	No effect	No effect	Rosenfeld (2002)
SAMR-1	Female	10/12	3	20 mg/l in night drinking water	ND	−11%	No effect	Rosenfeld (2002)
SHR	Female	50/50/50	3	2 or 20 mg/l in night drinking water	ND	No effect; M1, M2: +3 mo. MLS	M1: ↓ 1,9-fold M2: No effect	Anisimov et al. (present study)

Note: C = control group; M = melatonin-treated group; MLS = maximum life span; ND = animals were survived until natural death; NOD = non-obese diabetic; SAMP-1 = senescence accelerated mouse-prone; SAMR-1 = senescence accelerated mouse-resistant.

[a] *Nomenclature for the strains of mice see: Staats, 1985; Klein and Klein, 1987;

strains of mice (Crowley and Curtis, 1963; Sato et al., 1995). Earlier we found age-related increases in chromosome aberrations in bone marrow cells and in primary spermatocytes in male SHR mice (Rosenfeld et al., 2001). In this paper we observed a significant increase in the frequency of chromosome aberrations in the bone marrow cells in 12-month-old female SHR mice compared to 3-month-old ones. Treatment with both doses of melatonin revealed a slight tendency to alleviate the age-associated increase in chromosome aberrations in female SHR mice. It was shown melatonin inhibits mutagenesis induced by some mutagens, cytostatics and ionizing irradiation (Reiter et al., 1995; Musatov et al., 1997; 1998; Vijalaxmi et al., 1999). Thus, inhibitory effect of melatonin on mammary carcinogenesis in female SHR mice is not directly related to it influence on the incidence of chromosome aberrations in blood marrow cells and could depend on anti-estrogen potential, on it capacity to inhibit a serum level of prolactin and free radical processes in the serum in tissues (Bartsch et al., 2001; Cos and Sanchez-Barcelo, 2000).

To identify molecular events regulated by melatonin, gene expression profiles were evaluated using cDNA gene expression arrays (15,247 cDNA clone set, NIA, USA) in the heart of female CBA mice exposed to melatonin at 20 mg/l (Anisimov et al., 2002a). Comparative analysis of cDNA gene expression arrays hybridized with heart RNA samples from controls and treated with melatonin 233 clones (1,53% of the total number of clones) with >2-fold change in expression for melatonin. The most important biological targets for melatonin were genes regulating cell cycle, adhesion, membrane transport and mitochondrial genes. These findings are in agreement with data on the influence of melatonin on cell proliferation, apoptosis and cell adhesion (Arendt, 1995; Bartsch et al., 2001). Melatonin inhibited expression of oncogene-like myeloblastosis gene Mybl 1 (by 2.32-fold) but stimulated expression of gene of myeloid/lymphoid leukemia MLLT3 (by 2.14-fold) and protooncogene A-Raf serine/treonine proteinkinase (by 4.2-fold) (Anisimov et al., 2002a, Recently we showed that melatonin treatment (20 mg/l in night drinking water) resulted in a 2.5-fold reduction in the expression of *HER-2/neu* mRNA in mammary tumors from *HER-2/neu* transgenic mice (Baturin et al., 2001). These finding are in accordance with inhibitory effect of melatonin on development of mammary carcinomas and its capacity to stimulate development of lymphomas and lympholeukemias (see Table 9). It is worthy to note that treatment with melatonin inhibits development not only mammary tumorigenesis. There are data on suppressive effect of melatonin on development of spontaneous endometrial adenocarcinomas in BDII/Han rats (Deerberg et al., 1997), on colon carcinogenesis induced by 1,2-dimethylhydrazine in rats (Anisimov et al., 1997b) and on DMBA-induced carcinogenesis of the uterine cervix and vagina in mice (Anisimov et al., 2000).

Thus, melatonin has two faces - it is both a potent anticarcinogen and inhibitor of tumor growth in vivo and in vitro (Blask, 1993; Bartsch et al., 2001) and in some models may induce tumors and promote tumor growth (Romanenko, 1983,1985; Pierpaoili et al., 1991; Anisimov et al., 2001; Catrina et al., 2001). There is no contradictions between data on the carcinogenic and anticarcinogenic potential of melatonin. Some antioxidants, including natural ones (e.g. α-tocopherol) have both geroprotector and tumorigenic potential and could be potent anticarcinogens as well (see Anisimov, 1998, 2001).

In conclusion, it is important to stress that the effect of melatonin on life span and tumor development depends on it dose. At relative small doses, melatonin treatment inhibits carcinogenesis, however at others (relative big), it stimulates tumor development. We believe that the study of long-term effects of melatonin at a variety of doses in different strains and species (e.g. in rats) will be useful for making a conclusion about it safety.

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