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Melatonin Increases Both Life Span and Tumor Incidence in Female CBA Mice

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From the age of 6 months until their natural deaths, female CBA mice were given melatonin with their drinking water (20 mg/l) for 5 consecutive days every month. Intact mice served as controls. The results of this study show that the consumption of melatonin did not significantly influence food consumption, but it did increase the body weight of older mice; it did not influence physical strength or the presence of fatigue; it decreased locomotor activity and body temperature; it inhibited free radical processes in serum, brain, and liver; it slowed down the age-related switching-off of estrous function; and it increased life span. However, we also found that treatment with the used dose of melatonin increased spontaneous tumor incidence in mice. For this reason, we concluded that it would be premature to recommend melatonin as a geroprotector for long-term use.

THE search for an effective drug that prevents premature aging and increases active life span is an important direction of research in modern gerontology (1,2). The difficulties of this search are related to the lack of theoretical background as well as to insufficient information regarding the safety and adverse effects of existing geroprotectors. Some of these geroprotectors, for example, antioxidants, chelating agents, and growth hormone, which are capable of increasing the average life span in animals, can promote cancer development or suppress the reproductive function under certain conditions (3,4).

During the past decade, a number of reports, sometimes contradictory, appeared concerning the role of the pineal gland in aging (5–10). Melatonin (*N*-acetyl-5-methoxytriptamine) is the main pineal hormone synthesized from tryptophan, predominantly at night time (11). It has a wide spectrum of physiological effects on endocrine and reproductive functions (8,11,12). As age advances, the nocturnal production of melatonin decreases in animals of various species, including humans (8,13,14). The performance of a pinealectomy on rats produced a reduced life span (15,16), whereas the administration of melatonin to mice, rats, fruit flies, or planaria led in some cases to life extension (17–24). The grafting of a pineal gland from young donors into the thymus of old mice or in situ into pinealectomized old mice prolonged the life span of the recipients (18,25). Many studies show that melatonin inhibits tumor growth both in vivo and in vitro (26,27,28). The interest in all these observations was significantly increased after the discovery of the antioxidant activity of melatonin, both in vitro and in vivo (8,29). At the same time, in several studies, melatonin failed to show any effect on life span (18,24,30,31). Moreover, long-term treatment with melatonin was followed by an increase of tumor incidence in some mouse strains (18,31,32).

There are strong critical comments on the antiaging effects of melatonin in mice. These comments mainly relate to the observation that murine strains used in some studies do not synthesize melatonin as a result of a genetic defect (BALB/c, NZB, and C57BL/6). In fact, a shortening of the life span was observed in melatonin-producing mouse strain C3H/He (9,33). Later it was shown that the pineal gland did produce melatonin in the above-mentioned mouse strains with genetic defects, but the production night peak was very short, so its presence was difficult to detect (34). At the same time, a critical review of available data on the effect of melatonin on the life span and tumor incidence in rodents has shown that practically all studies are invalid from the point of view of the current guidelines for long-term testing of chemicals for carcinogenic safety (35,36) and, to some extent, from the point of view of the correctness of the gerontological experiment (37).

Risk assessment is a set of decision rules widely used in the United States and other countries for identifying and quantifying the risk of chemicals, causing adverse effects in humans, primarily cancer (38). According to the International Agency for Research on Cancer and the U.S. National Toxicology Program, exposure must start a few weeks after animals are weaned, and surviving animals must be sacrificed at 18 months (mice and hamsters) or at 24 months (rats). Some problems inherent to this protocol are currently under discussion (39–42). The 2-year assay is significant for

observation of the carcinogenic potential of the majority of agents. However, in some cases, if animals have been exposed to a tested agent from a young age and are sacrificed at the age of 2 years, an underestimation of the carcinogenic potential of nongenotoxic agents and tumor promoters is possible (43). It may be important to reevaluate standard 2-year protocols for the long-term assay for carcinogenicity, and it may be recommended to keep animals until their natural deaths. Although this is not profitable from an economical point of view, it is more reliable scientifically (1,43), particularly for studies that evaluate the life extension potential of pharmacological interventions (1,37,43).

Often in experiments with rodents, melatonin is given to a small number of animals (10–20); the treatments start when the animals are old; the observations stop when 50% of the animals have died or at some other voluntary time, but not at the natural death of the last survivor; an autopsy and a correct pathomorphological examination sometimes are not performed; the body weight gain and food consumption of the animals are not monitored; and so on.

In this paper we present the results of a study of the effects of melatonin on life span and spontaneous tumor incidence. The study was designed to avoid the drawbacks and limitations mentioned above. Some additional biomarkers of aging (estrous function, body temperature, locomotor activity, physical strength, and fatigue) were monitored as well. The effect of melatonin on free radical processes was also evaluated in the same study. Female CBA mice were selected for the study because of unequivocal evidence of pineal melatonin production in this mouse strain (33).

Methods

Animals

One hundred twenty female CBA 4-month-old mice were purchased from the Rappolovo Animal Farm of the Russian Academy of Medical Sciences (St. Petersburg). 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 hours of light and 12 hours of dark was followed. The animals received sterilized standard laboratory chow (44) 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.

Experiment

At the age of 6 months, all the mice were randomly divided into 2 groups, 60 animals in each, and they were individually marked. Mice of one group were given melatonin (Sigma, St. Louis, MS) with tap water (20 mg/l) during the night (from 6 PM to 9 AM), 5 days a week each month, until their natural deaths. This dose of melatonin has been used for life extension and carcinogenesis inhibition in several studies (19,27). Melatonin was dissolved in several drops of 96% ethanol and diluted with sterile tap water to a relevant concentration. A fresh melatonin solution was prepared each third day. All bottles containing melatonin were made from dark glass. The other 60 mice were kept intact and served as a control group.

Ten mice from each group were euthanized in 24 hours after the first course of melatonin administration for biochemical study. Their blood sera, livers, and brains were immediately frozen with liquid nitrogen and kept at −20°C. The remainder of the animals were weighed monthly with an electronic balance. For the animals of each group, a mean body weight and its standard errors, as well as the slope of the linear regression of age-related body weight gain and its standard errors, were defined. Additionally, the mice were divided into 3 classes: lean (≤28 g), medium, (29–33 g) and fat (≥34 g). The mice in each group that were operationally defined as fat, medium, and lean were defined on the 6th, 12th, and 15th months of the experiment. Once every 3 months, simultaneously with weighing, the amount of drinking water and consumed food was measured and the rate of consumed tap water (milliliters) and food (grams) per mouse and per body weight unit was calculated.

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), and the physical activity of each mouse was estimated. After 12 months of treatment, an assessment of muscular strength and fatigability was performed as well. The animals were observed until their natural deaths. The date of each death was registered, and the mean life span, the age at which 90% of the animals died, and the maximal life span were determined.

Method of estimating physical activity of mice in the "open field" test.—Animals of each group were placed one by one in a plastic chamber measuring 30 × 21 × 9 cm, at the bottom of which squares (5 × 5 cm) were drawn: 5 squares in length and 4 squares in breadth. Each mouse was observed moving in the cage, and its locomotor parameters were estimated: (1) the number of crossed squares in the field (a square was considered crossed if the animal stepped over its border at least with 2 paws); (2) the number of vertical sets (when the animal rose to its hind paws); and (3) the duration of grooming reaction of muzzle, body, and genita-

Table 1. Body Weight Gain Dynamics in Female CBA Mice Treated With Melatonin

	Body Weight (g)					
Group	6 mo	9 mo	12 mo	15 mo	18 mo	21 mo
Control	21.4 ± 0.25	24.5 ± 0.32	29.0 ± 0.56	28.9 ± 0.60	29.7 ± 0.78	30.8 ± 1.18
Melatonin	20.9 ± 0.66	24.5 ± 0.29	29.6 ± 0.43	30.4 ± 0.47*	32.2 ± 0.66*	32.7 ± 0.77

*The difference with controls is significant: $p < .05$.

Table 2. The Distribution of Mice According to the Body Weight in Groups Treated and Not Treated With Melatonin

Group	Number of Mice in Body Weight Classes (%)		
	Lean (≤28 g)	Medium (29–33 g)	Fat (≥34 g)
At the Age of 12 Mo			
Control	38	54	8
Melatonin	24.5	59.2	16.3
At the Age of 18 Mo			
Control	38	58	4
Melatonin	21.7	60.9	17.4
At the Age of 21 Mo			
Control	25	67	8
Melatonin	11.9	54.8	33.4*

*The difference with controls is significant: $p < .05$.

lia. As a way to exclude the possibility of smell-associated orientation reaction, the chamber floor was wiped with a wet cloth after each animal. The mice were tested at the age of 6, 9, 12, and 18 months in the daytime from 10 AM to 5 PM.

Method of studying muscular strength and physical fatigability of the mice.—The mice were suspended on a string stretched to an altitude of 80 cm, so that they would hang by the string, clutching at it with their front paws. The time until the moment of their fatigue and fall was registered in seconds. In 20 minutes the mice were suspended again and the time for which they managed to hold on was measured. A discrepancy between these 2 indices was regarded as a parameter of physical restoration.

Pathomorphological Examination

All the animals that died or that were sacrificed when moribund were autopsied. At autopsy their skin and all the internal organs were examined. Revealed 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 as "incidental" (for the cases in which the animal died of a different cause) (35). All the tumors, as well as the tissues and organs with suspected tumor development, were excised and fixed in 10% neutral formalin. After routine histological processing, the tissues were embedded in paraffin. Thin, 5–7 μm histological sections were stained with hematoxylin-eosine and were microscopically examined; regarding the experimental group to which the mice belonged, this was a blind process. Tumors were classified in accordance with IARC recommendations (45).

Biochemical Tests

The generation of reactive oxygen species (ROS) was studied in brain and liver homogenates and in blood serum according to the method of peroxide luminol-dependent chemiluminescence (46) on an Emilite EL-1105 chemiluminometer (BioChimMac, Moscow, Russia). The intensity of peroxide lipid oxidation was estimated by the content of diene conjugates and Schiff's bases (47). Total antioxidant activity (AOA) of the tissues was evaluated by the method of riboflavin chemoluminescence registration (48). Simultaneously, the activity of Cu-, Zn-superoxide dismutase (SOD) was estimated by means of suppressing Nitro Blue Tetrasodium with biological material (49).

Statistics

Experimental results were statistically processed by the method of variation statistics (50). Parameters of the regression equation for the curves of age-related body weight dynamics were calculated. The significance of the discrepancies was defined according to Student's *t* criterion, Fischer's exact method, a chi-square analysis, and a nonparametric criterion of Wilcoxon-Mann-Whitney (50). For discrepancies in neoplasm incidence to be estimated, an IARC method of combined contingency tables calculated individually for the fatal and incidental tumors (35) as well as a prevalence analysis (51,52) were applied. For survival analysis, Cox's method (53) was used for testing two groups' equality, and Tarone's (54) life table test was used. All reported *p* values for the survival analyses are two sided.

Mathematical Models and Estimations

Two mathematical models were used to describe survival under the treatment. The first model is the traditional Gompertz model with survival function

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

where parameters α and β are associated with the aging rate and the initial mortality rate, respectively. Parameter α is often characterized by the value of mortality rate doubling time (MRDT), calculated as ln(2)/α.

The second model was used to estimate changes in the aging rate in the two-stage model. The two-stage model uses

Table 3. Food Consumption Dynamics in CBA Mice Treated and Not Treated With Melatonin

Group	Unit	Daily Food Consumption (g)					
		6 mo	9 mo	12 mo	15 mo	18 mo	21 mo
Control	g	2.5 ± 0.21	3.4 ± 0.21	3.1 ± 0.12	3.1 ± 0.23	2.8 ± 0.14	3.2 ± 0.08
	g/100 g of body weight	11.7 ± 0.98	13.8 ± 0.86	10.8 ± 0.41	10.6 ± 0.80	9.6 ± 0.47	10.3 ± 0.26
Melatonin	g	2.7 ± 0.11	4.0 ± 0.10	3.3 ± 0.19	3.2 ± 0.11	2.7 ± 0.06	2.9 ± 0.04
	g/100 g of body weight	12.8 ± 0.39	16.5 ± 0.33	11.1 ± 0.86	10.5 ± 0.44	8.5 ± 0.22	9.0 ± 0.24

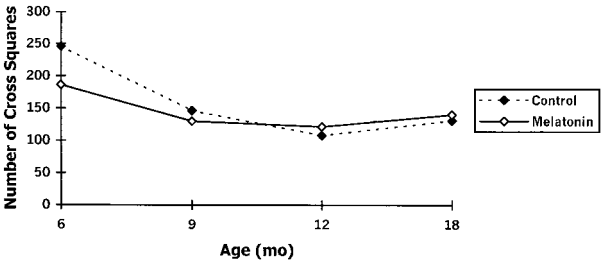

Figure 1. Effect of melatonin on age-related dynamics of square crossing ("open field" test) in female CBA mice.

two different values of aging rate during the life span. The model is constructed from two Gompertz models as follows:

$$S(x) = \exp\left\{-\frac{\beta_1}{\alpha_1}[\exp(\alpha_1 x) - 1]\right\} \qquad x \le x^*$$

$$= \exp\left\{-\frac{\beta_1}{\alpha_1}[\exp(\alpha_1 x^*) - 1]\right\}$$

$$\exp\left(-\frac{\beta_2}{\alpha_2}\{\exp[\alpha_2(x - x^*)] - 1\}\right), \qquad x > x^*$$

where x^* is the day separating the first and the second phases of the life span, and $S(x)$ is a survival function. It is worth noting that our two-stages model provides a description of the survival of a single organism, not a population.

Parameters for the models were estimated from empirical data by use of the maximum likelihood method implemented in the Gauss statistical system (55). Confidence intervals for the aging rate parameter estimates were calculated by profiling the log-likelihood function (53).

Results

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. As demonstrated in the table, the body weight of the mice in both groups increased with age, exceeding by 21 months the body weight of 6-month-old animals by 43.9% in the control group and by 56.5% in the group that received

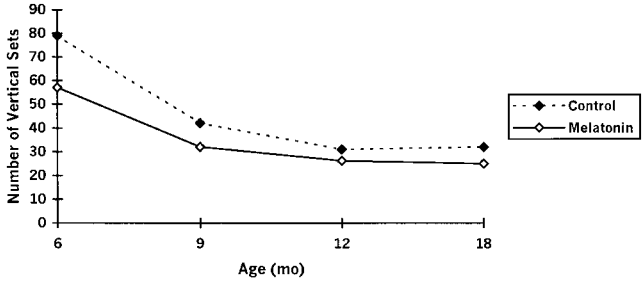

Figure 2. Effect of melatonin on age-related dynamics of vertical sets ("open field" test) in female CBA mice.

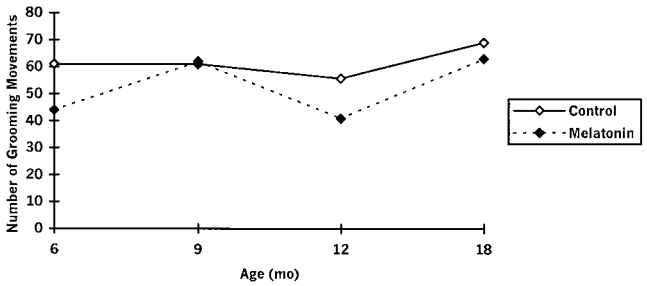

Figure 3. Effect of melatonin in age-related dynamics of the grooming reaction in female CBA mice.

melatonin. The mean body weight of mice exposed to melatonin was increased in comparison with those in the control group at the age of 15 and 18 months ($p < .05$). Parameters of the regression equation for body weight gain in the controls were 1.108 ± 0.062; those in the group that received melatonin were -0.906 ± 0.033 ($p < .01$).

It must be emphasized that the individual body weights of the animals of all groups varied considerably, and the number of mice weighing much less or more than the mean indices for the group differed with age. After the subdivision of mice of each group into 3 body weight classes—lean (≤28 g), medium (29–33 g), and fat (≥34 g)—it appeared that at every age the number of medium-weighing mice did not differ among the groups. At the same time, the number of lean mice treated with melatonin was relatively smaller than that in the control group, whereas the number of fat experimental animals exceeded that in the control group (Table 2).

Age-Related Dynamics of Water and Food Consumption

The amount of drinking (tap) water was stable during the entire period of observations, that is 5.3 ± 1.2 ml/mouse per 24 hours and 3.8 ± 1.1 ml/mouse per night. There were no differences in this parameter between the control and melatonin-treated animals. Regular measurements did not reveal any significant differences in the amount of consumed food between groups of controls and melatonin-treated animals, both at a rate per mouse and per unit of its body weight (Table 3). The amount of food consumed by the mice varied in different age periods. Thereby, both the periods of increased and decreased food consumption were registered. These variations were similar in both groups. The obtained data indicated that the greater weight of mice treated with melatonin was not caused by the hormone's impact on food consumption by the animals.

Effect of Melatonin

Physical activity of mice.—In the initial test, physical activity of the animals (or locomotor activity, which would be more appropriate in this case) was evaluated. The test consisted of defining the number of crossed squares in the field and appeared to be most energy consuming. The obtained data (Figure 1) demonstrate that the control mice revealed the highest activity according to this parameter at the age of 6 and 9 months. In the primary test, mice treated with

Table 4. Parameters of Muscular Strength and Fatigue Measured in 18-Month-Old CBA Mice Treated and Not Treated With Melatonin

Group	Relative Body Weight Held by the Mice in a Unit of Time (g/s)			
	Measurement 1	Measurement 2	Sum 1 + 2	Difference 2 − 1
Body Weight ≤ 28 g				
Control	0.52 ± 0.16	0.26 ± 0.06	0.78 ± 0.19	−0.21 ± 0.15
Melatonin	0.56 ± 0.21	0.18 ± 0.04	0.75 ± 0.23	−0.36 ± 0.20
Body Weight 29–33 g				
Control	0.54 ± 0.15	0.38 ± 0.06	0.92 ± 0.17	−0.17 ± 0.15
Melatonin	0.54 ± 0.15	0.33 ± 0.05	0.89 ± 0.15	−0.22 ± 0.1
Body Weight ≥ 34 g				
Control	4.97 ± 2.04	3.18 ± 1.22	8.15 ± 0.82	−1.79 ± 3.26
Melatonin	1.64 ± 1.01	1.54 ± 0.83	3.18 ± 1.83	−0.15 ± 0.26

melatonin crossed a significantly fewer number of squares in comparison with the controls ($p < .001$). Subsequently, when the mice reached the age of 12 and 18 months, the discrepancies in the number of crossed squares in the field became statistically insignificant. A study of the dynamics of this parameter revealed a rather predictable age-related decrease in locomotor activity. This decrease was especially pronounced at the age of 9 months—in comparison with the age of 6 months, a significant lessening of activity was observed, by 41% in the control group and by 30% in the group exposed to melatonin. At the age of 12 months, only the control mice demonstrated a significantly smaller number of crossed squares in the field in comparison with 9-month-old animals ($p < .05$).

Results on the second parameter of locomotor activity—the number of vertical sets—were similar to those on the previous parameter: the control animals showed the maximum activity (Figure 2). However, in the melatonin-treated group these discrepancies were significant only at the age of 6 months. Age-related dynamics of the number of vertical sets were analogous to those of the number of crossed squares in the field: in both tests, a significant decrease by the age of 9 months was registered.

Grooming reaction should refer not to locomotor activity (progressive movement) but rather to physical activity, because the animal moved, though without changing its place. The test on this reaction was not too energy consuming. The control mice showed the highest activity according to this parameter and a number of other parameters studied separately (Figure 3). Time of grooming in the case of melatonin administration significantly differed from that in the control only at the age of 6 months; subsequently (at the age of 9 and 12 months) the duration of the studied reaction did not significantly differ from that in the control. Age-related dynamics according to this parameter appeared to differ from those according to the above-regarded parameter to a certain extent. In particular, by the age of 9 months, a certain increase in grooming duration was observed; in the last test, 18-month-old animals were washing themselves a little longer in comparison with 12-month old mice.

The conducted observations prompted a conclusion that locomotor activity in the control mice decreased with age, whereas melatonin caused a regular lessening in locomotor activity over the course of life. On the whole, these data suggest a slight decrease in the physical activity of mice exposed to melatonin.

Table 5. Age-Related Dynamics of Estrous Functional Parameters in Mice Treated and Not Treated With Melatonin

Age (mo)	No. of Mice	Length of Estrous Cycle (d)	Rate of Separate Phases of Estrous Cycles (%)			No. of Estrous Cycles					No. of Mice	
						Total	Short (<4 d)		Long (>4 d)			
			P + M	E	D	No.	No.	%	No.	%	PE	AE
Control												
6	30	4.86 ± 0.31	18	39	43	57	28	49	29	51	1	0
9	30	4.59 ± 0.24	12	42	46	60	31	52	29	48	0	0
12	30	4.86 ± 0.25	10	43	47	59	27	46	32	54	0	0
15	28	5.48 ± 0.43	8	41	51	31	13	42	18	58	0	1
18	25	5.71 ± 0.39	13	36	51	31	9	29*	22	71*	0	4
Melatonin												
6	30	4.35 ± 0.25	18	36	46	53	29	55	24	45	0	0
9	30	4.57 ± 0.22	7	36	57	54	30	56	24	44	0	0
12	30	4.65 ± 0.31	6	45	49	51	28	55	23	45	0	0
15	30	5.23 ± 0.29	7	44	49	40	14	35	26	65	0	0
18	27	5.13 ± 0.38	15	31	54	38	17	45	21	55	1	4

Note: P = proestrus; E = estrus; M = metaestrus; D = diestrus; PE = persistent estrus; AE = anestrus.
*The difference with the age of 6 mo is significant; $p < .05$.

Table 6. Body Temperature Dynamics in CBA Mice Treated and Not Treated With Melatonin

Age (mo)	Total Cycle (w/out Phase Subdiv.)	Mean Body Temperature (°C)		
		Estrus	Diestrus	Metaestrus + Proestrus
Control				
9	37.42 ± 0.062	35.8 ± 1.600	37.48 ± 0.112	37.4 ± 0.207
12	37.67 ± 0.092*	37.7 ± 0.200	37.73 ± 0.080	37.31 ± 0.234
15	37.37 ± 0.120	34.96 ± 2.469	37.37 ± 0.183	37.26 ± 0.219
18	37.13 ± 0.077	37.11 ± 0.167	37.18 ± 0.111	37.12 ± 0.136
Melatonin				
9	38.23 ± 0.033†	38.21 ± 0.049	38.24 ± 0.054***†	38.25 ± 0.061†
12	37.70 ± 0.046***	37.83 ± 0.103**	37.70 ± 0.048***	37.47 ± 0.115***
15	36.04 ± 0.088***†	36.04 ± 0.146***	36.08 ± 0.130***†	35.96 ± 0.214***†
18	37.01 ± 0.104***	37.04 ± 0.107***	36.97 ± 0.181***	37.05 ± 0.206**

*The difference with the age of 12 mo is significant: $p < .05$; **$p < .01$; ***$p < .001$.
†The difference with the controls of the corresponding age: $p < .01$.

Muscular strength and physical fatigability of mice.— A study of the muscular strength, fatigability, and ability to recover strength in both the experimental and control mice was carried out on 18-month-old animals, 12 months after the start of the experiment. Measurements indicated a great individual variability of the mice. Treatment with melatonin failed to modify these parameters (Table 4).

Age-related dynamics of estrous function in mice.— Investigations of the estrous function in the animals of both age groups were performed every 3 months, starting when the mice were 6 months of age. The following parameters of estrous function were estimated: the length of estrus, the relative rate of estrous cycle phases (in percent); and the relative number of short (<4 days) and long (≥4 days) estrous cycles. The relative number of animals with regular cycles, persistent estrus and anestrus were calculated as well. Judging by the data presented in Table 5, the length of estrous cycle in the control female mice was stable with the advance in age. Thus, no essential age-related alterations in the rate of estrous cycle phases were observed. However, the relative number of short estrous cycles significantly decreased with age (49% at the age of 6 months and 29% at the age of 18 months; $p < .05$), whereas the number of long cycles rose (51% and 71%, respectively). In the controls of the oldest age groups, anestrus was registered, which was not observed in younger animals. In the group of mice treated with melatonin, no effects were observed on age-related dynamics of both the length of the estrous cycle and the rate of separate phases of the estrous cycle as compared with the control group. However, the number of both short and long estrous cycles was practically same at the age of 6 and 18 months (Table 5). Thus, these data suggest that the long-term administration of melatonin slows down age-related changes in estrous function.

Age-related dynamics of body temperature in mice.— Data on body temperature alterations in the mice exposed to melatonin are presented in Table 6. The control mice revealed a pronounced increase in rectal body temperature during diestrus in comparison with estrus from the 9th to the 15th month of life, which was caused by the functioning of the corpora lutea in the ovaries during diestrus. Rectal body temperature increase during diestrus was not observed in 18-month-old control mice: the body temperature indices remained constant irrespective of the cycle phase. The control mice did not reveal any significant alterations in body temperature with age, both on the whole (irrespective of the estrous cycle phases) and in any of the phases. No cyclic alterations in rectal body temperature during the estrous phase or its age-related changes were observed in mice treated with melatonin. It should be noted that the average body temperature in the mice of this group at the age of 9 months was higher, and at the age of 15 and 18 months was lower, than that in the controls during the phase of diestrus of the estrous cycle (Table 6).

Survival and longevity of female CBA mice.— Survival rate dynamics in the mice treated with melatonin are provided in Table 7. As shown in the table, the survival rate dynamics were similar in both groups up to the age of 22 months. Afterward, a pronounced decrease in mortality rate was observed under the effect of melatonin. Under the influence of melatonin the number of mice that reached the age of 24 months increased 5.4-fold in comparison with the controls ($p < .001$). Thus, the survival curve of mice treated

Table 7. Survival Distribution of Female CBA Mice Exposed and Not Exposed to Melatonin

Group	No. of Survivors														
	12 mo	14 mo	16 mo	17 mo	18 mo	19 mo	20 mo	21 mo	22 mo	23 mo	24 mo	25 mo	26 mo	28 mo	29 mo
Control	50	50	49	48	47	45	43	42	40	33	7	0	0	0	0
Melatonin	50	49	49	49	49	46	46	46	41	41*	38*	12*	1	1	0

*The difference with the control group is significant: $p < .001$.

Table 8. Parameters of Life Span in Female CBA Mice Treated and Not Treated With Melatonin

Parameters	Controls $n = 50$	Melatonin $n = 50$
Mean life span, d (mean ± SE)	685 ± 9.2	722 ± 12.6*
Median	705	747
Mean life span of last 10% of survivors	738 ± 1.1	793 ± 18.6*
Maximum life span	740	867

*The difference with control is significant: $p < .05$.

with melatonin was shifted to the right as compared with that in the control animals. The mean life span of mice treated with melatonin was slightly increased compared with controls (+5.4%, $p < .05$). The life span in the last 10% of the mice increased as a function of the duration of melatonin treatment (by 2 months). The maximum life span expanded by almost 4 months under the effect of melatonin (Table 8).

Spontaneous tumor development in female CBA mice.—The total tumor incidence in the control female mice was 30%. Lung adenomas and mammary carcinomas developed most frequently, which corresponded to the oncological characteristics of CBA mice (56). The treatment with melatonin was followed by a 20% increase in malignant tumor incidence in comparison with that of the control group ($p < .001$) (Table 9). Five cases of lymphomas and 5 cases of lung adenocarcinomas were observed in the group treated with melatonin, whereas no cases of similar tumors were found in the control group. At the same time, the development of lung adenomas in animals treated with melatonin was decreased in comparison with that of the controls ($p < .01$). No essential impact of melatonin upon the neoplasia of other localization was registered (Table 9). It is worth noting that the mean life span of fatal tumor-bearing mice in the group treated with melatonin was increased by

Table 9. Incidence, Localization, and Type of Tumors in Female CBA Mice Treated and Not Treated With Melatonin

Parameters	Control $n = 50$	Melatonin $n = 50$
No. of Tumor-Bearing Mice	15 (30%)	17 (34%)
No. of Malignant Tumor-Bearing Mice	3 (6%)	13 (26%)*
Total no. of Tumors	20	22
Total no. of Malignant Tumors	3	15
Localization and Type of Tumors:		
Mammary gland		
Adenoma	1	0
Adenocarcinoma	5 (3†)	4
Lungs		
Adenoma	11 (10‡)	4
Adenocarcinoma	0	5*
Uterus		
Leiomyosarcoma	0	1
Lymphoma	0	5*
Vessel wall: hemangioma	3	3

Note: Parenthetical information denotes the number of mice with this tumor.
*The difference with the control group is significant: $p < .001$.
†Two mice developed 2 tumors of this site.
‡One mouse had 2 lung adenomas.

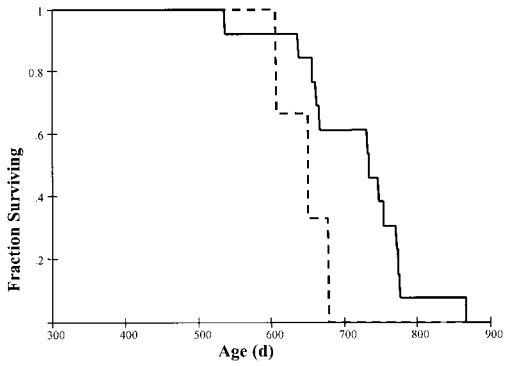

Figure 4. Proportion of survived mice for fatal tumor (melatonin treated, —; control group, ---).

2.3 months as compared with that of the control group (Figure 4; Table 10).

Mathematical Models and Estimations on Survival of Tumor-Free and Tumor-Bearing Mice

A mathematical analysis of the survival data of the mice from the control and melatonin-treated groups has been done separately for five different contexts: (1) for all animals in each group (total cases); (2) for total tumor-free animals; (3) for total tumor-bearing mice; (4) for fatal tumor-free mice, and (5) for fatal tumor-bearing mice. We composed the groups of animals without consideration of possible effects caused by dependence between these groups. The one-stage model (the traditional Gompertz model) shows a slight slow down in the aging rate (calculated as α in the Gompertz equation) under the influence of melatonin and a significant reduction in parameter α for "fatal tumor-bearing mice" context (by three-fold; $p < .05$). This could be interpreted as a slow down in the growth rate of fatal tumors. The limitations of the Gompertz model are demonstrated in Figure 5, presenting the observed and modeled survival for total tumor-free mice. Applying the same aging rate value to the whole life span, we obviously underestimated the mortality rate for ages between 725 and 775 days. The two-stage model allows us to use two different values of aging rate during the life span. Figure 6 shows that this model gives good approximation for the mortality process. We used the two-stages model to give a numerical interpretation for observed survival curves. The simple Gompertz model fails to fit the curves, with a rather long initial plateau. We interpret this as the presence of two stages: a "negligible mortality" stage and a "Gompertz-like mortality" stage. The "join point" was estimated by the use of the maximum likelihood method, together with other parameters.

Calculations of α and MRDT in the two-stage model show that at the first phase of the life span (before the age of 700 days) melatonin treatment has no significant effect on the aging rate parameter, α, which also renders insignificant the seeming increase of the MRDT, calculated as $\ln(2)/\alpha$. However, at the second phase of life, the aging rates for the total cases and the total tumor-free mice have a clear tendency to increase in the case of melatonin treatment. The parameters for the fatal tumor-free mice were significantly

Table 10. Parameters of Life Span in Female CBA Mice Treated and Not Treated With Melatonin

Group	Total No. of Cases	Total No. Tumor-Free Mice	Total No. Tumor-Bearing Mice	Fatal No. Tumor-Free Mice	Fatal No. Tumor-Bearing Mice
Number of Mice					
Controls	50	35	15	47	3
Melatonin	50	33	17	37*	13*
Mean life span (days)					
Controls	685 ± 9.2	688 ± 10.6	678 ± 18.8	688 ± 9.6	645 ± 20.6
Melatonin	722 ± 12.6*	722 ± 17.0	722 ± 17.7	724 ± 15.1*	716 ± 23.0*
Mean Life Span of the Last 10% of Survivors (days)					
Controls	738 ± 1.1	736 ± 2.4	725 ± 4.6	738 ± 1.1	645 ± 20.1
Melatonin	793 ± 18.6*	770 ± 2.2**	789 ± 19.8**	770 ± 2.2**	788 ± 20.0**
One-Stage Model					
Aging Rate $\alpha \times 10^3$ ($days^{-1}$)					
Controls	30 (23; 38)	32 (23; 36)	27 (17; 37)	31 (24; 39)	42 (22; 48)
Melatonin	19 (15; 26)	43 (32; 47)	15 (11; 21)	41 (30; 45)	14 (9; 20)*
MRDT (days)					
Controls	23.1	21.7	25.7	22.4	16.5
Melatonin	36.5	16.1	46.2	16.9	49.5
Two-Stage Model					
Aging Rate $\alpha \times 10^3$ ($days^{-1}$), Stage I					
Controls	6 (0; 13)	3 (0; 13)	10 (0; 25)	2 (0; 10)	42 (12; 120)
Melatonin	4 (0; 12)	1 (0; 9)	14 (3; 50)	1 (0; 9)	15 (4; 30)
MRDT (days), Stage I					
Controls	115.5	231.0	69.3	346.6	16.5
Melatonin	173.3	593.1	49.5	593.1	46.2
Aging Rate $\alpha \times 10^3$ (day^{-1}), Stage II					
Controls	34 (6; 61)	34 (3; 62)	35 (0; 93)	34 (6; 61)	—
Melatonin	76 (56; 97)	83 (60; 108)	8 (0; 20)	90 (65; 118)*	10 (0; 22)
MRDT (days), Stage II					
Controls	20.4	20.4	19.8	20.4	—
Melatonin	9.1	8.3	86.6	7.7	69.3

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: $p < .05$; **$p < .001$.

($p < .05$) modified: α was increased by 2.6-fold whereas MRDT was decreased by 2.6-fold. A comparison of Figures 5A and 5B shows that mortality of the elder melatonin-treated mice directly related to the development of fatal tumors in the second half of their life.

Effect of Melatonin on Free Radical Processes in Mice

The comparison of the data on free radical processes generated in various organs revealed the most intensive generation of ROS in blood serum, where it twice exceeded the corresponding indices in liver and brain. Thus, the level of antioxidant activity in blood sera was significantly lower than that found in livers and brains (Table 11). The treatment with melatonin was followed by a significant decrease in luminol-induced chemiluminescence and a significant increase of total antioxidant activity in the serum, but not in the brain or liver of mice. Melatonin treatments inhibited lipid peroxidation (decreased the level of both diene conjugates and Schiff's bases) in brain and liver tissue. In the serum, melatonin treatment was followed by a decrease in the level of diene conjugates, but not Schiff's bases. Melatonin treatment failed to modify the activity of SOD in any tested tissue.

Discussion

The results of our study show that long-term night administration of melatonin at a daily dose of 20 mg/l in drinking water significantly increases both the survival and malignant tumor incidence in female CBA mice. The effect of melatonin on survival does not relate to its influence on food consumption. No reduction in food consumption was observed. Moreover, body weight was increased in mice treated with melatonin as compared with that in the control group. These results are in concert with other observations (23,31). A positive correlation between excessive body weight and tumor incidence is observed in rodents and in human females (57,58). Melatonin failed to modify physical strength and fatigue and slightly decreased the locomotor activity of mice. The sedative (sleep) effect of melatonin is well known (11,14). Long-term treatment with melatonin was followed by a slowing down of the aging of the reproductive system in female CBA mice. A similar observation in female rats was recently reported (59).

It is worth noting that, in our study, melatonin induced a decrease of body temperature in mice (Table 6). It has been shown in the literature that a decline of body temperature caused by the slowing down of metabolic processes in an organism is followed by an extension of life (60–62). Other effects of melatonin on life span could be related to its antioxidant potential (8,29). Our results confirm these observations.

An important result of our study is the increase of malignant tumor incidence under the influence of melatonin in female CBA mice. In Table 12 we summarized the available

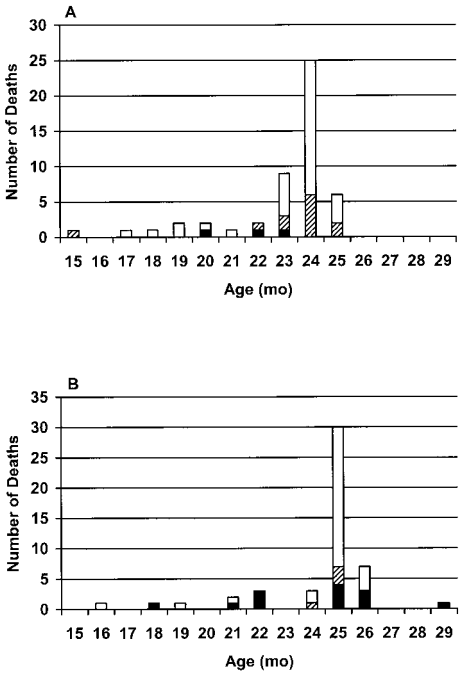

Figure 5. Number of deaths among control, **A**, and melatonin-treated, **B**, mice (tumor free: □; non-fatal tumor: ▨; fatal tumor: ■).

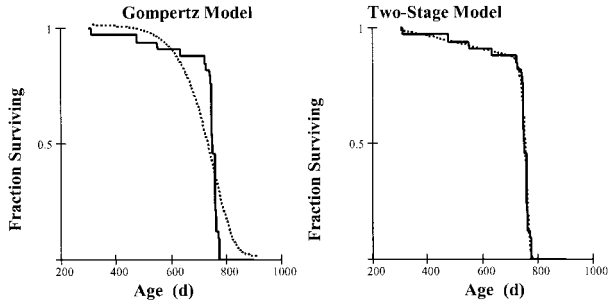

Figure 6. Gompertz and two-stage models for survival among total tumor-free mice (proportion survived mice, —; modeled survival, . . .).

data on survival and tumor incidence in mice and rats exposed to long-term treatment with melatonin. There are findings of lymphosarcomas and reticulosarcomas and ovarian carcinomas in female C3H/He mice that have received melatonin at night hours starting at the age of 12 months (18). However, Subramanian and Kothari (63) reported a suppressive effect of melatonin on the development of spontaneous mammary carcinomas in female C3H/Jax mice treated from the age of 3 weeks until the age of 12 months. Pierpaoli and colleagues (18) started the treatment with melatonin in mice that were older than 12 months. The daily dose of melatonin in these experiments could be estimated as 0.8–2.0 mg/kg of body weight. It is worth noting that Pierpaoli and colleagues (18) did not report any cases of spontaneous mammary adenocarcinomas in C3H/He mice observed at 12 months of age. Subramanian and Kothari (63) reported that 62.5% of intact female C3H/Jax mice developed mammary adenocarcinomas at the age of 12 months. A similar incidence of spontaneous mammary adenocarcinomas was observed in female C3H mice of different sublines, for example, C3H/He and C3H/Sn (3,64,65).

Melatonin did not induce any malignancies in male C57BL/6 mice when it was administered at a dose of 10 mg/l (∼1.5–2.0 mg/kg) in the night drinking water from the age of 19 months (17,18). However, Lipman and colleagues (31) 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 to 50% survival at 26.5 months, whereas in control groups only 28.6% mice developed lymphomas. Leukemias were detected in 70–98% of C57BL/6 mice and in 78% of CC57Br mice (both males and females) that were treated subcutaneously with melatonin at a dose of 2.5 mg/mouse (∼80 mg/kg) twice a week for a duration of 2.5–5 months (32,66). The number of C57BL/6 mice in the study of Pierpaoli and Maestroni (17) and in the study of

Table 11. Effect of Melatonin on Free Radical Processes in Female CBA Mice

Parameters	Control	Melatonin
Brain		
Luminol-induced chemiluminescence (× 10^4 units/mg of protein)	94.1 ± 7.9	76.9 ± 7.7
Total antioxidant activity (units/mg of protein)	3.1 ± 0.19	3.56 ± 0.25
Diene conjugates (nM/g of tissue)	22.29 ± 0.87	19.02 ± 0.65*
Schiff's bases (units/g of tissue)	388 ± 25	276 ± 13*
SOD activity (units/mg of protein)	23.4 ± 1.8	23.5 ± 1.7
Liver		
Luminol-induced chemiluminescence (× 10^4 units/mg of protein)	78.4 ± 10.4	79.4 ± 6.8
Total antioxidant activity (units/mg of protein)	4.72 ± 0.45	4.81 ± 0.20
Diene conjugates (nM/g of tissue)	66.37 ± 2.17	59.58 ± 1.52*
Schiff's bases (units/g of tissue)	543 ± 14	459 ± 19**
SOD activity (units/mg of protein)	35.5 ± 1.6	34.5 ± 1.7
Serum		
Luminol-induced chemiluminescence (× 10^4 units/mg of protein)	224.0 ± 29.8	126.3 ± 23.8*
Total antioxidant activity (units/mg of protein)	1.40 ± 0.15	1.86 ± 0.07***
Diene conjugates (nM/g of tissue)	4.36 ± 0.43	2.47 ± 0.52*
Schiff's bases (units/g of tissue)	29.2 ± 3.2	26.2 ± 2.5

Note: SOD = superoxide dismutase.
*The difference with control is significant: $p < .05$; **$p < .01$; ***$p < .001$.

Table 12. Summary of Experiments on the Effect of Melatonin on Life Span and Spontaneous Tumor Incidence in Rodent Models

Strain	Sex	No. of Animals, C/M	Age at the Start of Treatment, mo	Treatment With Melatonin	Age at the End of Observation	Effects of Melatonin on:		References (Authors, year, number)
						Mean Life Span	Tumor Incidence	
Mice								
CC57Br	Male & female	26/57	1.5	2.5 mg/mouse s.c. twice a wk × 2.5 mo	22 mo	C: 17 mo; M: 15 mo (−12%)	C: 38%; M: 78%	Romanenko, 1985 (66)
C57BL/6	Male & female	29/57	1.5	2.5 mg/mouse s.c. twice a wk × 2.5 mo	22 mo	C: 17 mo; M: 13.5 mo (−20.6%)	C: 14%; M: 70%	Romanenko, 1985 (66)
C57BL/6	Male & female	25/45	1.5	2.5 mg/mouse s.c. twice a wk × 5 mo	22 mo	C: 22 mo; M: 19 mo (−13%)	C: 32%; M: 98%	Romanenko, 1983 (32)
C57BL/6J	Male	10/10	19	10 mg/l in night drinking water	ND	C: 752 ± 81 d; M: 931 ± 80 d (+20%)	No data	Pierpaoli and Maestroni, 1987 (17)
C57BL/6	Male	20/15	19	10 mg/l in night drinking water	ND	C: 743 ± 84 d; M: 871 ± 118 d (+17%)	No data	Pierpaoli, Dall'Ara, Pedrinis, and Regelson, 1991 (18)
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 on survival in 3 cohorts	Lymphomas: 1: C (0); M (0) 2: C (28.6%); M (77.9%); 3: C (21.1%); M (23.3%)	Lipman and colleagues, 1998 (31)
Balb/c	Female	26/12	15	10 mg/l in night drinking water	ND	C: 715 d; M: 843 d (+18%)	No data	Pierpaoli and Regelson, 1994 (19)
Balb/c	Male	50/50	18	10 mg/l in night drinking water	ND	Shift to right of the survival curve; MaxLS: +2 mo	No data	Mocchegiani and colleagues, 1998 (23)
NZB	Female	10/10	4	10 mg/l in night drinking water	ND	No control mice survived to the age 20 mo; M: 4 mice were alive at age 20 mo	No data	Pierpaoli, Dall'Ara, Pedrinis, and Regelson, 1991 (18)
NZB/W	Female	15/15/15	8	2–3.5 mg/kg s.c. daily at 8–10 PM (M1) or at 5–7 PM (M2) × 9 mo	44 wk	C: 20% survivors at 34 wk; M1: 60% survivors at 34 wk, 20% at 44 wk; M2: 60% survivors at 34 wk, 20% at 37 wk	No data	Lenz, Izui, Benediktsson, and Hart, 1995 (67)
NOD (nonobese diabetic)	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 (34)
NOD (nonobese diabetic)	Female	29/17	1	10 mg/l in night drinking water, 5 times a wk, 4–38 wk	50 wk	C: 34.5% survivors; M: 58.8% survivors	No data	Conti and Maestroni, 1998 (34)
C3H/Jax	Female	16/39	3 wk	25–50 mkg/ mouse/d with drinking water	12 mo	No data	C: 62.5%; M: 23.1%	Subramanian and Kothari, 1991 (63)
C3H/He	Female	14/15	12	10 mg/l in night drinking water	ND	No effect on the life span	Increase in tumor incidence (lympho- and reticulosarcomas, ovarian Ca)	Pierpaoli, Dall'Ara, Pedrinis, and Regelson, 1991 (18)

(*Continued*)

Table 12. (*Continued*).

Strain	Sex	No. of Animals, C/M	Age at the Start of Treatment, mo	Treatment With Melatonin	Age at the End of Observation	Effects of Melatonin on:		References (Authors, year, number)
						Mean Life Span	Tumor Incidence	
CBA	Female	50/50	6	10 mg/l in night drinking water	ND	C: 685 ± 9.2 d; M: 722 ± 12.6 d (+5%); MaxLS: C: (740 d); M: (867 d)	Total/Malignant tumor incidence: C (35/6%); M (34/26%)	Anisimov and colleagues, present study
Rats								
CD	Male	16/15	11–13	4 mg/l in drinking water	26–29 mo	C: 44% survivors; M: 87% survivors	No data	Oakin-Benhadan, Anis, Nir, and Zisappel, 1995 (20)
BDII/Han	Female	40/40	1	0.1 mg/rat/d in night drinking water	ND	M: increased survival	No effect	Deerberg, Bartsch, Pohlmeyer, and Bartsch, 1997 (68)

Note: C = control group; M = melatonin-treated group; MaxLS = maximum life span; ND = animals were survived until natural death.

Pierpaoli and colleagues (18) was small, and no data on tumor development were reported. Romanenko (32,66) and Lipman and colleagues (31) used many more animals in their studies. Assuming that the body weight of mice was 16–30 g in the studies of Romanenko (32,66), we could estimate the dose of melatonin as 80–120 mg/kg of body weight. In our study, melatonin that was given in night drinking water in an interrupted (course) regimen in a relatively low dose (3–3.5 mg/kg) was carcinogenic as well. We believe that the testing of an effect of melatonin in a variety of doses (including much smaller doses that have been used in our study and other studies) will be useful in coming to a more exact conclusion on the carcinogenicity of melatonin.

A clastogenic effect of melatonin may be involved in the mechanism of its carcinogenic effect. It was shown that in pharmacological doses melatonin induces DNA damage and cleavage in hamster ovarian cells in a Single Cell Gel Electrophoresis (COMET) assay (69). In contrast, melatonin has not been shown to be mutagenic using the Ames test (69,70) and inhibits mutagenesis induced by some cytostatics and ionizing irradiation (29,71,72). A treatment with melatonin inhibits development of spontaneous endometrial adenocarcinomas in BDII/Han rats (68), mammary carcinogenesis induced by 7,12-dimethylbenzo(a)anthracene (DMBA) or *N*-nitrosomethylurea in rats (26,28), colon carcinogenesis induced by 1,2-dimethylhydrazine in rats (27) and DMBA-induced carcinogenesis of the uterine cervix and vagina in mice (73).

There are no principal controversies between the 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 (for a review, see 3,4). It is worth noting that melatonin treatment accelerated aging rate parameters (α and MRDT) in the second phase of life as estimated in the two-stage model. In our previous observations it was shown that there is a positive correlation between the aging rate (estimated as α) of the population and the malignant tumor incidence in the same population (3,4). In other words, those geroprotectors that increase the rate of aging induce an increase in malignant tumor incidence; however, those that decrease the aging rate inhibit spontaneous carcinogenesis. These results make it premature to recommend melatonin for wide use as an anti-aging drug.

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