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Effect of synthetic thymic and pineal peptides on biomarkers of ageing, survival and spontaneous tumour incidence in female CBA mice

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Abstract

Fifty female CBA mice were injected s.c. either with 0.1 ml saline, or with synthetic thymic dipeptide Lys–Glu or with synthetic pineal tetrapeptide Ala–Glu–Asp–Gly both in a single dose of 0.1 μg/animal monthly for five consecutive days from the age of 6 months until natural death. Lys–Glu did not significantly influence the body weight and food consumption, free radical processes and estrus function in mice and did increase their physical activity with the subsequent decrease in spontaneous lung adenomas incidence. The pineal peptide treatment was failed to modify the food consumption and physical strength of mice, and was followed by the increase in the body weight, mean survival (by 5.3%, $P < 0.05$) and maximum (by 10 months), by slow down of the ageing of estrus function, by the decrease in body temperature, physical activity, free radical processes and spontaneous tumor incidence (mainly, lung adenomas) in mice. These data suggest the geroprotector potential of the pineal peptide.

Keywords: Thymic dipeptide; Pineal tetrapeptide; Survival; Reproductive function; Body temperature; Physical activity; Behaviour; Spontaneous tumours; CBA mice

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1. Introduction

Studies of ageing and longevity which may help to increase active life span, avoid premature ageing and postpone senescent take priority in the modern gerontology (Anisimov and Soloviev, 1999; Bezrukov et al., 1999; Hayflick, 2000). However, the absence of unifying theories of ageing, as well as insufficient information on the safety and side effects of existing preparations hamper the search for effective geroprotectors. Some of them, 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 (Anisimov, 1987, 1998a).

According to the immunologic theory of ageing, immune dysfunction develops with age to cause a decrease in resistance to infections and enhance the risk of tumour and autoimmune disease development (Walford, 1974; Kay and Makinodan, 1986; Miller, 1998). In compliance with the theory, efforts have been made to inhibit ageing of the immune system in old animals by means of grafting lymphocytes and thymus or administering various immunomodulators and thymus preparations (Zatz and Goldstein, 1985; Kay and Makinodan, 1986; Anisimov, 1987; Ghanta et al., 1991).

In a series of investigations carried out in the course of 25 years the ability of Thymalin® – a thymus polypeptide preparation – to increase the life span of mice and rats has been revealed (Anisimov et al., 1982, 1989, 1994; Morozov and Khavinson, 1996a). A number of other peptide preparations of thymus, such as α-thymosin, have been shown to produce immunomodulating and slight geroprotective effects (Zatz and Goldstein, 1985; Kay and Makinodan, 1986; Anisimov, 1987; Ghanta et al., 1991). It must be noted that despite Thymalin efficacy the potential of its clinical application is limited by the insufficient raw material supply for its manufacture. On the contrary, synthesis of biologically active thymic peptides provides a greater opportunity to integrate such preparations with clinical practice. We have recently revealed the geroprotective effect of synthetic thymic dipeptide Thymogen® (Glu–Trp) in rats (Anisimov et al., 2000a). Dipeptide L-Lys–L-Glu (Lys–Glu) has been structured on the basis of the amino acid analysis of thymus preparation Thymalin, amino acid sequences of thymic peptides and cytokines (Morozov and Khavinson, 1978, 1996b, 1997; Kuznik et al., 1998; Khavinson et al., 1999; Kiseleva et al., 1999).

During the last decade, a number of sometime contradictory reports appeared on the role of the pineal gland in ageing (Armstrong and Redman, 1991; Treintini et al., 1991; Reiter, 1995; Reppert and Weaver, 1995; Anisimov, 1996; Pierpaoli, 1998). Melatonin (*N*-acetyl-5-methoxy-triptamine) is the main pineal hormon synthesized from tryptophan predominantly at night time (Arendt, 1995). As age advanced, the nocturnal production of melatonin decreases in animals of various species as well as in human (Reiter, 1995; Touitou et al., 1997; Waldhauzer et al., 1998). Pinealectomized rats showed a reduced life span (Malm et al., 1959; Reiter et al., 1999) whereas an administration of melatonin to mice, rats, fruit flies or planaria leads in some cases to the life extension (Pierpaoli et al., 1991; Pierpaoli

and Regelson, 1994; Oakin-Bendahan et al., 1995; Anisimov et al., 1997b; Thomas and Smith-Sonneborn, 1997; Mocchegiani et al., 1998; Izmailov and Obukhova, 1999). The grafting of the pineal gland from young donors into the thymus of old mice or in situ into pinealectomized old mice prolonged the life span of the donors (Pierpaoli et al., 1991; Lesnikov and Pierpaoli, 1994). There are a lot of data which shown that melatonin inhibits tumor growth both in vivo and in vitro (Blask, 1993). The interest to all these observations was significantly increased after the discovery of antioxidant activity of melatonin both in vitro and in vivo (Reiter et al., 1995). At the same time, in several studies melatonin failed to show any effect on the life span (Pierpaoli et al., 1991; Lipman et al., 1998; Izmailov and Obukhova, 1999). Moreover, long-term treatment with melatonin was followed by an increase of tumor incidence in some mouse strains (Romanenko, 1983; Pierpaoli et al., 1991; Lipman et al., 1998; Anisimov et al., 2000b).

At the same time, there is an evidence of production of some peptides by pineal gland (Yuwiler and Brammer, 1993; Anisimov, 1998b). It was shown that pineal polypeptide preparation Epithalamin® increases the life span of mice, rat and fruit flies and inhibits the development of spontaneous, induced by chemical and ionizing radiation and transplantable tumors in mice and rats (Anisimov et al., 1982, 1989, 1994, 1997b, 1998). It is worth noting that clinical use of Epithalamin was shown as effective for treatment of ovarian disturbances and cancer in cancer patients (Morozov and Khavinson, 1996b) but rather restricted due to difficulties in the availability of calf pineal gland for it production. The synthesis of the pineal tetrapeptide Ala–Glu–Asp–Gly with high biologival activity (Khavinson and Morozov, 1999) gives a new opportunity for its implementation in clinical practice. It was recently shown that the pineal peptide increased the life span in two strains of fruit flies (Khavinson et al., 2000; Mylnikov and Lyubimova, 2000).

In this article we present the results of a comparative complex study of newly synthesized thymic peptide Lys–Glu and pineal peptide Ala–Glu–Asp–Gly effect upon the life span, physical and locomotor activity, reproductive function, body temperature, spontaneous tumour development and free radical processes in female mice of CBA strain.

2. Material and methods

2.1. Animals

One hundred and eighty 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 ($30 \times 21 \times 9$ cm^3) at 22 ± 2°C under the 12-h light/dark regimen. The animals received standard laboratory chow (Baranova et al., 1986) and tap water ad libitum.

2.2. Peptides

Thymic dipeptide Lys–Glu and pineal tetrapeptide Ala–Glu–Asp–Gly were synthesized in Laboratory of Bioorganic Chemistry of St. Petersburg Institute of Bioregulation and Gerontology. Lys–Glu has been structured on the basis of the amino acid analysis of thymus preparation Thymalin, amino acid sequences of thymic peptides and cytokines (Morozov and Khavinson, 1978, 1991, 1996b; Kuznik et al., 1998; Khavinson et al., 1999; Kiseleva et al., 1999). On the base of Lys–Glu a medicine Vilon® has been designed (Kiseleva et al., 1999). Pineal tertapeptide Ala–Glu–Asp–Gly has been structured on the basis of the amino acid analysis of pineal peptide preparation Epithalamin (Morozov and Khavinson, 1996a,b) and on the basis of the tetrapeptide a medicine Epithalon® has been designed (Khavinson and Morozov, 1999). Vilon and Epithalon have been used in this study.

2.3. Experiments

At the age of 6 months all the mice were randomly divided into three groups, 60 animals in each. Mice of the control group were subcutaneously injected with 0.1 ml of 0.9% normal saline monthly in the courses of 5 consecutive days, while each mouse of the second and third group received either 0.1 μg of Lys–Glu or 0.1 μg the pineal peptide dissolved in 0.1 ml of saline. Ten mice from each of the three groups were decapitated in 24 h after the first course of the preparation administration for biochemical study. Their blood serum, liver and brain were immediately frozen with liquid nitrogen and kept at -20°C. Rest of the animals was monthly weighed in the electronic balance. For the animals of each group there were defined the average value of their body weight and its standard errors, slope of the linear regression of age-related body weight gain and its standard errors. Additionally, the mice were divided into three classes: of lean (weighing up to 28 g), medium (from 29 to 33 g) and fat (over 34 g) animals. The proportion (%) of fat, medium and lean mice in each group was defined on the sixth, 12th and 15th months of the experiment. Once in every 3 months, simultaneously with weighing, the amount of consumed food was measured and the rate of the consumed food mass (g) per mouse and per body weight unit was calculated.

Once in every 3 months, daily for 2 weeks vaginal smears of the animals were cytologically examined to estimate the estrus function. In the same period rectal body temperature of the mice was measured with electronic thermometer TPEM (KMIZ, Russia) and their physical activity was estimated. In 12 months upon the experiment onset muscular strength and fatigability were estimated as well. The animals were observed till their natural death. Date of their death was registered, the average life span, age at which 90% of the animals died and the maximal life span were calculated.

2.4. Method of estimating physical activity of mice in the "open field" test

Animals of each experimental group were placed one by one in a plastic chamber measuring 30 × 21 × 9 cm^3, at the bottom of which squares (5 × 5 cm^2) were drawn: 5 squares lengthways and 4 squares in breadth. The mice were observed moving in an "open field" and the following behavioural 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) Number of vertical sets (when the animal rose to its hind paws); (3) Duration of grooming reaction of muzzle, body and genitalia. 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 10:00 to 17:00 h.

2.5. Method of studying muscular strength and physical fatigability of mice

The mice were suspended on a string stretched at an altitude of 75–80 cm, so that they would hang by the string clutching at it with their front paws. The time till the moment of their fatigue and fall was registered in seconds. In 20 min the mice were suspended again and the time, during which they managed to hold on was measured. Discrepancy between these two indices was regarded as a parameter of physical restoration.

2.6. Pathomorphologic examination

All the died animals or sacrificed when moribund were autopsied. At the 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, which directly caused death of the animal) or as "incidental" (in cases, when the animal died of a different cause) (Gart et al., 1986). All the tumours, as well as the tissues and organs with suspected tumor development were excised and fixed in 10% neutral formalin. After the routine histological processing the tissues were embedded into paraffin. 5–7 μm thin histological sections were stained with haematoxylin and eosine and were microscopically examined. Tumors were classified in according to IARC recommendations (Turusov and Mohr, 1994).

2.7. Biochemical tests

Generation of reactive oxygen species (RAS) was studied in brain and liver homogenates and in blood serum according to the method of peroxide luminol-dependent chemiluminescence (Prokopenko et al., 1995) on Emilite EL-1105 chemiluminometer (Russia). The intensity of peroxide lipid oxidation was estimated by the content of diene conjugates and Schiff's bases (Stalnaya, 1974). Total antioxidant activity (AOA) of the tissues was evaluated by the method of riboflavin chemolu-

minescence registration (Burmistrov et al., 1997). Simultaneously, the activity of Cu, Zn-superoxide dismutase (SOD) was estimated by means of suppressing nitroblue tetrasolium with biological material (Agostini et al., 1980).

2.8. Statistics

Experimental results were statistically processed by the methods of variation statistics with the use of STATGRAPH statistic programme kit. Parameters of regression equation for the curves of age-related body weight dynamics were calculated. The significance of the discrepancies was defined according to the Student t-criterion, Fischer's exact method, χ^2, non-parametric criterion of Wilcoxon–Mann–Whitney (Goubler, 1978). To estimate the discrepancies in the neoplasm incidence there was applied IARC method of combined contingency tables calculated individually for the fatal and incidental tumours (Peto's test) (Gart et al., 1986).

2.9. Mathematical models and estimations

Different mathematical models were used to describe survival under the treatment: Gompertz model and two-stage model. Gompertz model describes the survival function as

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

where parameters α and β are associated with the "ageing rate" and the "initial mortality rate", respectively.

The two-stage model considers two separate phases of the life span with different values for the "ageing rate". The model is composed from two Gompertz models in form

$$S(x) = \begin{cases} \exp\left(-\frac{\beta_1}{\alpha_1}(\exp(\alpha_1 x) - 1) \right), & x \leqslant x^*, \\ \exp\left(-\frac{\beta_1}{\alpha_1}(\exp(\alpha_1 x^*) - 1) \right) \exp\left(-\frac{\beta_2}{\alpha_2}(\exp(\alpha_2 (x - x^*)) - 1) \right), & x > x^*, \end{cases}$$

where x^* is the day separating the first and the second phases of the life span. Parameter α is often characterised by the value of "mortality rate doubling time" (MRDT) calculated as $\ln(2)/\alpha$. Parameters for the models were estimated from empirical data by maximum likelihood method implemented in GAUSS statistical system (Gauss System and Graphic Manual, 1994). Confidence intervals for the "ageing rate" parameter estimates were calculated by profiling the log likelihood function (Cox and Oakes, 1996).

3. Results

3.1. Age-related body weight dynamics

Mean values of the animals' body weight at different age in the experimental and control groups are displayed in Table 1. As it is demonstrated in the table, 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 control, by 62.3% in the group which received Lys–Glu ($P < 0.05$) and by 52.9% in the group treated with the pineal peptide. The mean body weight of mice exposed to the thymic peptide was increased in comparison to that in the control group at the age of 18 and 21 months ($P < 0.01$) and in group treated with the pineal peptide – at the age of 18 months ($P < 0.05$). The slope parameters in the regression equation for the body weight gain in the controls constituted 1.108 ± 0.062, while in the group, which received Lys–Glu, it was 0.913 ± 0.025 ($P < 0.01$) and in the group exposed to Ala–Glu–Asp–Gly it was 0.954 ± 0.028 ($P < 0.05$).

It must be emphasised that the individual body weight of the animals of all groups varied considerably and the number of mice weighting much less or more than the mean indices for the group differed with age. After subdividing of mice in each group into three body weight classes – "lean" (weighing less than 28 g), "medium" (weighting from 29 to 33 g) and "fat" (exceeding 34 g) – it appeared that at every age the number of medium-weighted mice did not essentially differ between the groups (from 49% to 67%). At the same time, the number of lean mice treated with the peptides was relatively smaller than that in the control group, while the number of fat experimental animals exceeded that in the control (Table 2). This discrepancy became especially vivid on the 15th month of the experiment, when the mice reached the age of 21 months.

Table 1
Effect of Lys–Glu and Ala–Glu–Asp–Gly on the age-related body weight dynamics in female CBA mice

Group	Age (months)					
	6	9	12	15	18	21
Body weight (g)						
Saline	21.4 ± 0.25	24.5 ± 0.32	29.0 ± 0.56	28.9 ± 0.60	29.7 ± 0.78	30.8 ± 1.18
Lys–Glu	20.7 ± 0.19	24.2 ± 0.32	29.1 ± 0.46	29.6 ± 0.46	32.3 ± 0.51**	33.6 ± 0.66*
Ala–Glu–Asp–Gly	20.8 ± 0.22	24.4 ± 0.28	29.8 ± 0.44	30.0 ± 0.50	31.6 ± 0.57*	31.8 ± 0.74

* Significant in comparison with the control: $P<0.05$.
** Significant in comparison with the control: $P<0.01$.

Table 2
Effect of Lys–Glu and Ala–Glu–Asp–Gly on the distribution of mice according to their body weight

Group	Number of mice in body weight classes, in %		
	'Lean' (<29 g)	'Medium' (29–33 g)	'Fat' (>33 g)
At the age of 12 months			
Control	38	54	8
Lys–Glu	29.2	52.1	18.7
Ala–Glu–Asp–Gly	16.3	63.3	20.4*
At the age of 18 months			
Control	37.8	57.8	4.4
Lys–Glu	14	67.4	18.6
Ala–Glu–Asp–Gly	22.8	56.3	20.8
At the age of 21 months			
Control	25	66.7	8.3
Lys–Glu	8.1	48.7	43.2*
Ala–Glu–Asp–Gly	10.5	63.2	26.3*

* Significant in comparison with the control: $P<0.05$.

3.2. Age-related dynamics of food consumption

Regular measurements did not reveal any significant differences in the amount of consumed food between the control and Lys–Glu-treated animals, both at a rate per mouse and per unit of its body weight, whereas mice treated with the pineal peptide consumed more food than the controls at the age of 18 months (Table 3). As it is demonstrated in 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 increase in weight of mice treated with the peptides was not caused by peptides impact upon the consumption of food by the animals.

3.3. Effect of the peptides on physical activity of mice

The first studied parameters was that of physical activity of the animals (or locomotor activity, which would be more adequate in this case). The test consisted in defining the number of crossed squares in the field and appeared to be most energy consuming. The obtained data (Fig. 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 the thymic or pineal peptide crossed a significantly less number of squares in comparison with the controls ($P < 0.001$). Subsequently, at the age of 12 and 18 months, the discrepancies in the number of crossed squares in the field smoothed down and were not significant. Study of the age-related dynamics according to this parameter revealed a rather predictable age-related locomotor activity decrease in the animals. This decrease was especially

Table 3
Food consumption dynamics in female CBA mice treated with Lys–Glu or with Ala–Glu–Asp–Gly

Group	Unit	Age (months)					
		6	9	12	15	18	21
Daily food consumption							
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 the b.w.	11.7 ± 0.98	13.8 ± 0.86	10.8 ± 0.41	10.6 ± 0.80	9.6 ± 0.47	10.3 ± 0.26
Lys–Glu	g	2.8 ± 0.08	3.8 ± 0.08	3.1 ± 0.24	3.1 ± 0.13	2.8 ± 0.07	3.0 ± 0.08
	g/100 g of the b.w.	13.5 ± 0.39	15.8 ± 0.33	0.8 ± 0.86	10.4 ± 0.44	8.7 ± 0.22	9.0 ± 0.24
Ala–Glu–Asp–Gly	g	2.9 ± 0.15	3.6 ± 0.19	3.6 ± 0.12*	3.1 ± 0.30	2.7 ± 0.10	3.2 ± 0.14
	g/100 g of the b.w.	13.9 ± 0.71	14.9 ± 0.79	12.0 ± 0.40	10.3 ± 1.00	8.6 ± 0.32	10.2 ± 0.44

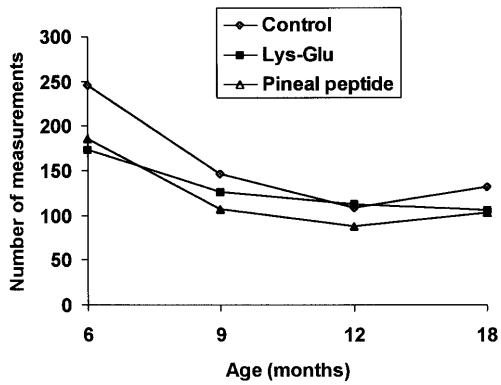

Fig. 1. The effect of Lys–Glu and the pineal peptide on the age-related dynamics of crossed squares in the field.

pronounced at the age of 9 months – in comparison with the age of 6 months a significant activity lessening was observed: by 41% in the control group and by 27% in the group subjected to Lys–Glu administration and by 43% in the group treated with Ala–Glu–Asp–Gly. At the age of 12 months only the control mice demonstrated a significantly less number of crossed squares in the field in comparison with 9-month old animals.

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 (Fig. 2). However, in Lys–Glu-administered group these discrepancies were significant only at the age of 6 months and in the group treated with Ala–Glu–Asp–Gly – at the age of 6 and 9 months. Age-related dynamics of the number of vertical sets was analogous to that of the number of crossed squares in the field: in both tests a significant decrease by the age of 9 months was registered.

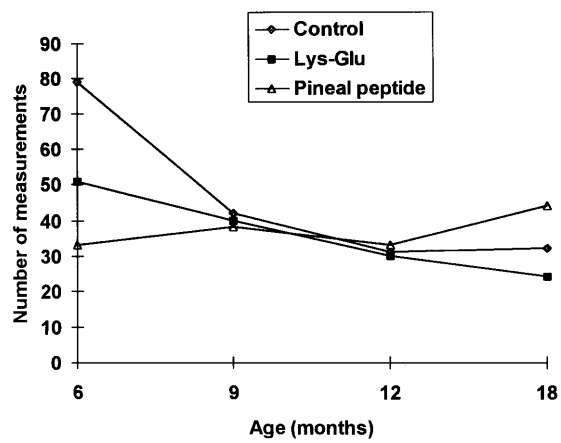

Fig. 2. The effect of Lys–Glu and the pineal peptide on the age-related dynamics of vertical sets number.

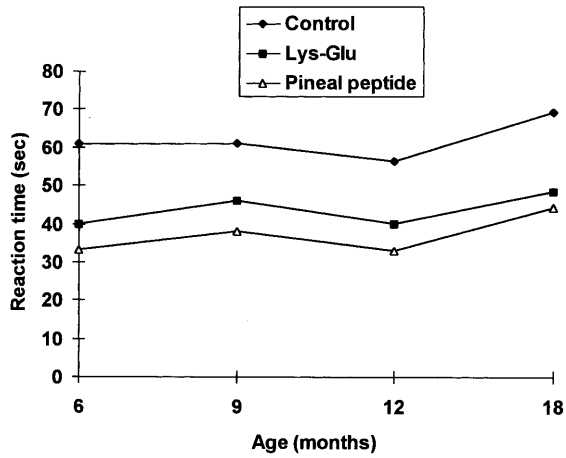

Fig. 3. Effect of Lys–Glu and the pineal peptide on the age-related dynamics of grooming duration.

Grooming reaction should be referred not to the locomotor (progressive movement), but to physical activity, since the animal moved, though without changing place thereby. 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 (Fig. 3). Time of grooming in case of Lys–Glu administration significantly differed from that in the control only at the age of 6 months, subsequently (9 and 12 months) the duration of the studied reaction did not significantly differ from that in the control. In mice treated with the pineal peptide Ala–Glu–Asp–Gly the decrease in the grooming length was significant at the age of 6, 9 and 12 months ($P < 0.05$). Age-related dynamics according to this parameter appeared to differ from that according to the above regarded parameter at 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, while the thymic peptide and, in particular, the pineal peptide caused a regular lessening in locomotor activity in the course of life. On the whole, the results of observations obtained in the "open field" test evidenced a decrease in the locomotor activity of mice exposed to long-term treatment with the peptide preparations in comparison to the control.

3.4. Effect of the peptides on muscular strength and physical fatigability of mice

Study of the muscular strength, fatigability and ability to strength recovery both in the experimental and control mice was carried out on 18-month old animals in 12 months after the experiment start. Measurements indicated a great individual variability of the mice. However, it was observed that the index, which reflected the

ratio of the animal body weight to the time between their suspension on a string and fall was significantly lower in Lys–Glu-administered mice with excessive body weight (over 34 g). Thus, the fat experimental mice hung longer both during the first and second suspensions than the fat control animals, i.e. they appeared to be physically stronger. This parameter in the fat mice exposed to the thymic peptide almost did not vary from that in the medium-weighed mice, while the fat control mice injected with saline fell nearly right after their suspension (Table 4). A similar tendency was registered in the lean animals as well. Treatment with the pineal peptide failed to modify these parameters. Thus, a prolonged administration of the thymic peptide enhanced the muscular strength of the animals and decreased their fatigability whereas the pineal peptide was not active in this relation.

3.5. Effect of the peptides on the age-related dynamics of estrus function in the mice

Investigations of the estrus function in the animals of both age groups were repeatedly performed every 3 months since the age of 6 months. The following parameters of the estrus function were estimated: duration of the estrus cycle, correlation between the estrus cycle phases; the relative number of short (less than 4 days) and long (over 4 days) estrus cycles, the relative number of animals with regular cycles, persistent estrus and anestrus were calculated. Judging by the data

Table 4
Parameters of muscular strength and fatigability measured in 18-month old female CBA mice treated with Lys–Glu or with Ala–Glu–Asp–Gly

Group	Relative body weight held by the mice in a unit of time (g/s)			
	First measurement	Second measurement	Sum (1+2)	Difference (2−1)
Body weight up to 29 g				
Control	0.52 ± 0.16	0.26 ± 0.06	0.78 ± 0.19	-0.21 ± 0.15
Lys–Glu	0.28 ± 0.09	0.21 ± 0.11	0.49 ± 0.18	-0.07 ± 0.06
Ala–Glu–Asp–Gly	0.41 ± 0.11	0.30 ± 0.09	0.71 ± 0.16	-0.12 ± 0.13
Body weight from 29 to 33 g				
Control	0.54 ± 0.15	0.38 ± 0.06	0.92 ± 0.17	-0.17 ± 0.15
Lys–Glu	0.47 ± 0.09	0.37 ± 0.07	0.84 ± 0.14	-0.10 ± 0.08
Ala–Glu–Asp–Gly	0.43 ± 0.08	0.39 ± 0.08	0.82 ± 0.12	-0.01 ± 0.12
Body weight over 34 g				
Control	4.97 ± 2.04	3.18 ± 1.22	8.15 ± 0.82	-1.79 ± 3.26
Lys–Glu	0.87 ± 0.36**	1.04 ± 0.32*	1.91 ± 0.56***	0.17 ± 0.38
Ala–Glu–Asp–Gly	1.46 ± 0.76	1.22 ± 0.38	2.69 ± 1.10	0.58 ± 0.46

* Significant in comparison with the control of the same weight: $P<0.05$.
** Significant in comparison with the control of the same weight: $P<0.01$.
*** Significant in comparison with the control of the same weight: $P<0.001$.

presented in Table 5 the length of estrus cycle in the control female mice slightly increased with age. Thereby, no essential age-related alterations in the correlation between the estrus cycle phases were observed. However, the relative number of short estrus cycles significantly decreased with age, while the number of long cycles rose. In the controls of older age groups, anestrus was registered, which was not observed in younger animals.

Long-term administration of the thymic peptide did not exert an essential effect upon the age-related dynamics of the estrus function in the mice. At the same time the treatment with the pineal peptide slow down the age-related changes in estrus function. Thus, under the influence of the preparation there was no increase in the percent of long estrus cycles and no decrease in the rate of short estrus cylces (Table 5).

3.6. Effect of the peptides on the age-related dynamics of body temperature in mice

Data on body temperature alterations in the mice exposed to the thymic and pineal peptides are presented in Table 6. The control mice revealed a pronounced increase in the 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 yellow bodies 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 the body temperature with age, both on the whole (irrespective of the estrous cycle phases) and in any of its phases. No cyclic alterations in the rectal body temperature during estrus phase or its age-related changes were observed in mice treated with the thymic peptide. It should be noted that the average body temperature in the mice of this group was lower than that in the controls during diestrus, in the period from the 12th to the 18th month of life. The mean body temperature of mice treated with Ala–Glu–Asp–Gly the pineal peptide was decreased in comparison to the controls (Table 6).

3.7. Effect of the peptides on survival and longevity of female CBA mice

Survival rate dynamics in the mice injected with saline, the thymic or pineal peptides is shown in Table 7. As it is shown in the table, the survival rate dynamics was similar in all groups up to the age of 19 months. Afterwards, a pronounced decrease in mortality rate was observed under the effect of the peptides. Under Lys–Glu influence the number of mice, which reached the age 23 months, increased 2.57-fold in comparison to the controls ($P < 0.01$) and under the influence of Ala–Glu–Asp–Gly-increased 4.0-fold ($P < 0.01$). Thus, the survival curves of mice treated with the peptide preparation were shifted to the right compared to that in the control animals (Fig. 4). The mean life span of mice treated with the thymic peptide was not changed compared to the control, whereas the treatment with the pineal peptide was followed by slight (by 5.3%) but significant ($P < 0.05$) increase

Table 5
Age-related dynamics of the estrus parameters in female CBA mice treated with Lys–Glu or Ala–Glu–Asp–Gly[a]

Age (months)	Number of mice	Length of estrus cycles (days)	Rate of separate phases of estrus cycles (%)			Number of estrus cycles					Number of mice	
			P+M	E	D	Total number	Short (<4 days)		Long (>4 days)		PE	AE
							No.	%	No.	%		
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
Lys–Glu												
6	30	4.54 ± 0.23	17	38	45	61	30	49	31	51	0	0
9	30	4.96 ± 0.20	9	46	45	53	24	45	29	55	0	0
12	28	4.66 ± 0.30	5	40	55	41	21	51	20	49	0	0
15	27	6.06 ± 0.41	9	40	51	32	7**	22	25**	78	0	2
18	26	5.16 ± 0.36	14	35	51	37	14	38	23	62	0	2
Ala–Glu–Asp–Gly												
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	28	4.65 ± 0.31	6	45	49	51	28	55	23	45	0	0
15	27	5.23 ± 0.29	7	44	49	40	14	35	26	65	0	0
18	26	5.13 ± 0.38	15	31	54	38	17	45	21	55	1	5

[a] P, proestrus; E, estrus; M, metaestrus; D, diestrus; PE, persistent estrus; AE, anestrus.
* Significant in comparison with the parameter at the age of 6 months: $P<0.05$.
** Significant in comparison with the parameter at the age of 6 months: $P<0.01$.

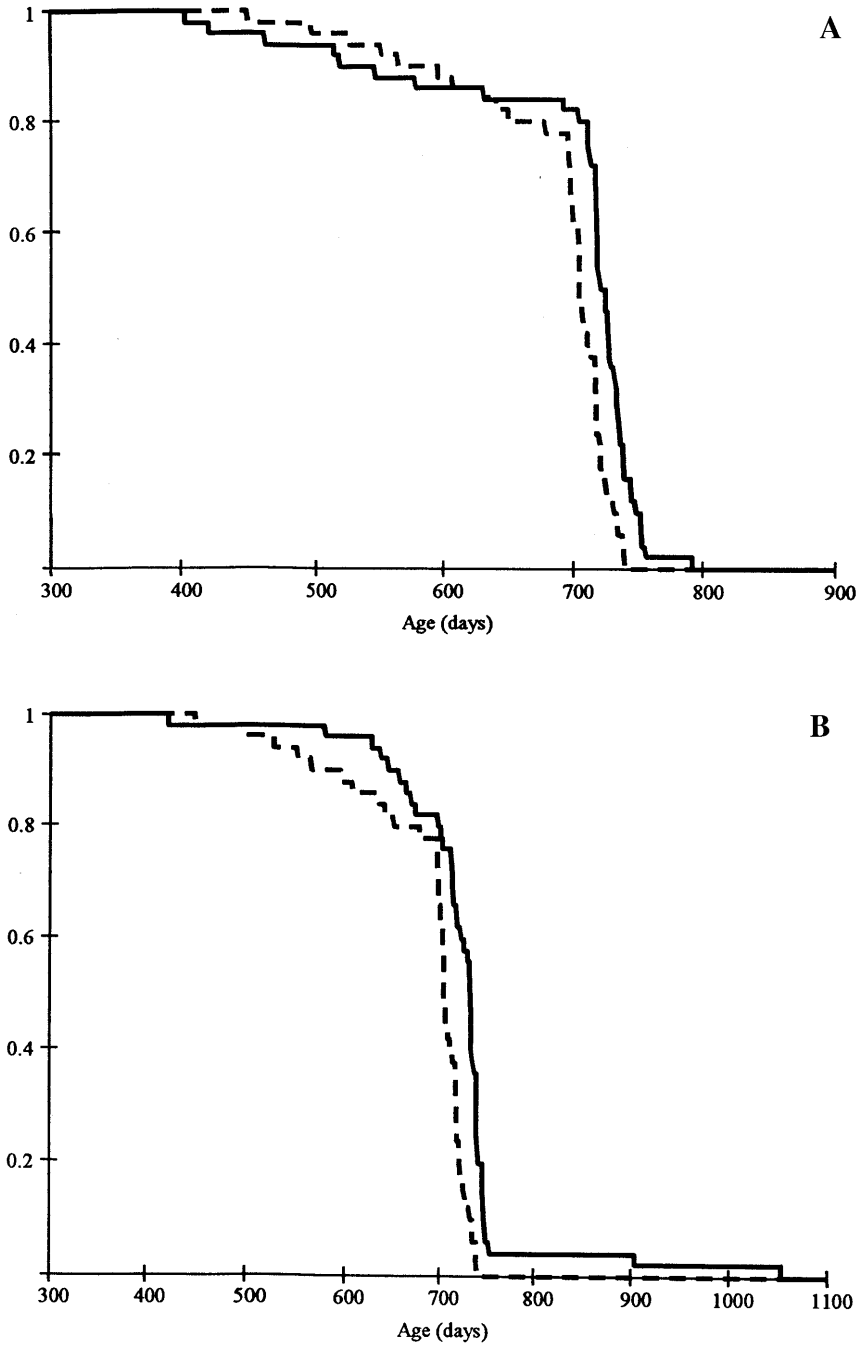

Fig. 4. Proportion of survived mice for all causes of death under Lys–Glu (A) and pineal (B) treatment (peptid treated, solid line, control group, dashed line).

Table 6
Age-related body temperature dynamics in female CBA mice treated with Lys–Glu or Ala–Glu–Asp–Gly

Age (months)	Mean body temperature (°C) Phase of estrus cycle			
	Irrespective of the estrus cycle phase	Estrus	Diestrus	Meta-estrus or proestrus
Control				
9	37.42 ± 0.062	35.8 ± 1.600	37.48 ± 0.112	37.4 ± 0.207
12	37.67 ± 0.092^{a}	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
Lys–Glu				
9	37.27 ± 0.076	37.30 ± 0.116^{f}	37.33 ± 0.096^{d}	36.92 ± 0.319
12	$36.90 \pm 0.085^{b,f}$	$36.94 \pm 0.136^{a,e}$	$36.96 \pm 0.122^{c,f}$	36.61 ± 0.229^{c}
15	$36.00 \pm 0.079^{c,f}$	$36.94 \pm 0.153^{c,f}$	$35.99 \pm 0.107^{c,f}$	$36.24 \pm 0.146^{c,d}$
18	$36.65 \pm 0.144^{c,f}$	37.03 ± 0.085	$36.43 \pm 0.212^{c,f}$	$36.92 \pm 0.384^{b,d}$
Ala–Glu–Asp–Gly				
9	37.66 ± 0.074^{d}	37.56 ± 0.114^{f}	37.71 ± 0.110	37.85 ± 0.096
12	$37.39 \pm 0.071^{a,d}$	37.38 ± 0.113	$37.38 \pm 0.113^{a,e}$	–*
15	$36.35 \pm 0.085^{b,f}$	$36.41 \pm 0.166^{c,f}$	$36.41 \pm 0.166^{c,f}$	$36.43 \pm 0.224^{b,d}$
18	$36.38 \pm 0.084^{c,f}$	36.94 ± 0.108^{b}	$36.94 \pm 0.108^{a,e}$	–*

[a] The difference with the age of 12 months is significant, $P<0.05$.
[b] The difference with the age of 12 months is significant, $P<0.01$.
[c] The difference with the age of 12 months is significant, $P<0.01$.
[d] Significant in comparison with the controls of the corresponding age: $P<0.05$.
[e] Significant in comparison with the controls of the corresponding age: $P<0.01$.
[f] Significant in comparison with the controls of the corresponding age: $P<0.001$.
* There was a small number of animals for estimation of a mean value.

in this parameter. The life span in the last 10% of the animals significantly increased under the influence of the peptide preparations (from 737 ± 1.1 days in control to 761 ± 7.7 days in cases of Lys–Glu administration, $P < 0.05$, and to 793 ± 18.6 days in the case of the pineal peptide). At the same time, the maximum life span expanded almost by 2 months under the effect of Lys–Glu and by 10 month under the influence of the pineal peptide (Table 8), which could evidence a certain inhibition of the ageing rate in the animals in the second half of their life caused by the peptide preparation.

3.8. Effect of the peptides on spontaneous tumour development in female CBA mice

The total tumor incidence in the control female mice was 30%. Thereby, lung adenomas and mammary carcinomas developed most frequently, which corresponded to the oncologic characteristics of CBA mice (Zurcher et al., 1982; Staats, 1985). The treatment with the peptide bioregulators exerted a certain inhibitory

Table 7
Survival distribution of female CBA mice treated with Lys–Glu or with Ala–Glu–Asp–Gly

Treatment	Age (months)																			
	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	29	30	32	34	35
	Number of mice, which reached the above given age																			
Saline	50	50	49	49	48	47	45	43	42	40	33	7	0	0	0	0	0	0	0	0
Lys–Glu	50	48	48	47	46	44	44	43	42	42	41*	18**	1	1	0	0	0	0	0	0
Ala–Glu–Asp–Gly	50	49	49	49	49	49	49	48	46	43	40*	28**	2	2	2	2	1	1	1	0

* Significant in comparison with the control: $P<0.5$.
** Significant in comparison with the control: $P<0.001$.

Table 8
Parameters of life span in female CBA mice treated with Lys-Glu or with Ala-Gu-Asp-Gly

Parameters	Saline	Lys-Glu	Ala-Gu-Asp-Gly
Number of mice	50	50	50
Mean life span, days (M ± S.E.)	685 ± 9.2	694 ± 12.5	721 ± 11.1*
Mediana	705	725	732
Mean life span of last 10% survivors	738 ± 1.1	761 ± 7.7*	842 ± 60.7
Maximum life span	740	792	1053

* The difference with control is significant: $P<0.05$.

effect upon the spontaneous carcinogenesis in the mice, which was manifested in a decreased incidence of total tumour development ($P < 0.05$ in Peto's test) and their multiplicity (Table 9, Fig. 6). Development of lung adenomas in animals treated with Lys–Glu decreased two-fold, $P < 0.05$, and in animals treated with the pineal peptide – by 5 time ($P < 0.01$). At the same time a tendency to a decrease in the incidence of mammary adenocarcinomas was observed in mice treated with Lys–Glu and a tendency to an increase in these tumor rate in mice treated with

Table 9
Incidence, localization and type of tumors in female CBA mice treated with Lys–Glu or with Ala–Gu–Asp–Gly[a]

Parameters	Saline	Lys–Glu	Ala–Gu–Asp–Gly
Number of mice	50	50	50
Number of tumor-bearing mice	15 (30%)	10 (20%)	9 (18%)[b]
Number of malignant tumor-bearing mice	3 (6%)	4 (8%)	7 (14%)
Total number of tumors	20	11	11
Localization and type of tumors:			
Mammary gland:			
Adenoma	1	0	0
Adenocarcinoma	5 (3*)	1	5
Lungs:			
Adenoma	11 (10**)	5[c]	2[c]
Adenocarcnioma	0	1	1
Uterus:			
Leiomyosarcoma	0	0	1
Haematopoietic system			
Lymphoma	0	2	2
Vessel wall:			
Haemangioma	3	1	0
Skin:			
Papilloma	0	1	0

[a] Number of mice with this tumors are given in brackets.
[b] The difference with the control group is significant, $P<0.05$, in Peto's test.
[c] The difference with the control group is significant, $P<0.001$, exact Fischer test.
* Two mice developed 2 tumors of this site.
** One mouse had 2 lung adenomas.

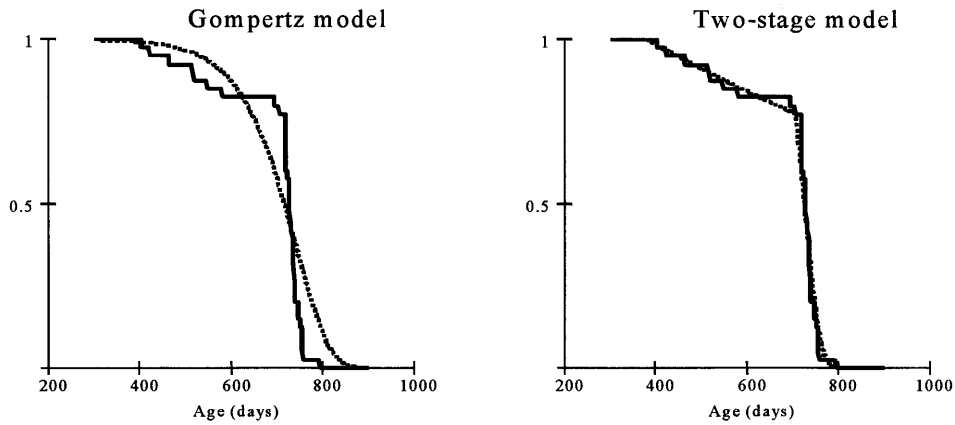

Fig. 5. Gompertz and two-stage models for survival among total tumor-free mice death Glu treatment (proportion survived mice, solid, modeled survival, dots).

Ala–Glu–Asp–Gly (both tendency were insignificant). No essential impact of both peptide preparations upon the neoplasia of other localisation was registered (Table 9).

3.9. Mathematical models and estimations on survival of tumor-free and tumor-bearing mice

Mathematical analysis of the survival data of mice from the control and the peptides-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. The traditional Gompertz model shows slight acceleration of ageing rate (calculated as α in Gompertz equation) under the influence of Lys–Glu. The similar estimation gives a calculation of MRDT parameters for the same contexts (Table 10, one-stage model). The limitations of this model are demonstrated in Fig. 5. Applying the same "ageing rate" value to the whole life span one obviously underestimates the mortality process for ages between 700 and 750 days.

Calculations of α and MRDT have shown that at both phases of the life span (before and after the age of 720 days) Lys–Glu has no any significant effect on the ageing rate parameter α, that make also insignificant the seeming increase of the parameter MRTD, calculated as ln (2)/α. However, the treatment with the pineal peptide at the second phase of life the ageing rate for the "total cases", the "total tumor-free mice" and the "fatal tumor-free mice" contexts have a clear tendency to the increase, whereas the parameter α was increased whereas MRDT was decreased in comparison to the control values.

Table 10
Parameters of life span in female CBA mice treated and non-treated with Lys–Glu and Ala–Glu–Asp–Gly (one-stage and two-stages models)[a1]

Group	Total cases	Total tumor-free mice	Total tumor-bearing mice	Fatal tumor-free mice	Fatal tumor-bearing mice
Number of mice					
Saline	50	35	15	47	3
Lys–Glu	50	40	10	46	4
Ala–Glu–Asp–Gly	50	41	9[*1]	43	7
Mean life span, days (M ± S.E.)					
Saline	685 ± 9.2	688 ± 10.6	678 ± 18.8	688 ± 9.6	645 ± 20.6
Lys–Glu	694 ± 12.5	691 ± 15.5	710 ± 9.1	694 ± 13.5	695 ± 21.0
Ala–Glu–Asp–Gly	721 ± 11.1[*1]	726 ± 13.1[*1]	696 ± 13.3	726 ± 12.5[*1]	689 ± 16.0
Mean life span of the last 10% survivors, days (M ± S.E.)					
Saline	738 ± 1.1	736 ± 2.4	725 ± 4.6	738 ± 1.1	645 ± 20.1
Lys–Glu	761 ± 7.7[*1]	761 ± 7.7[*1]	723 ± 3.2	761 ± 7.7[*1]	695 ± 21.1
Ala–Glu–Asp–Gly	842 ± 60.7	842 ± 60.6	725 ± 3.9	842 ± 60.7	710 ± 11.7[*1]
One-stage model					
Ageing rate $\alpha \times 10^3$ ($days^{-1}$)					
Saline	30 (23; 38)	32 (23; 36)	27 (17; 37)	31 (24; 39)	42 (22; 48)
Lys–Glu	23 (18; 29)	20 (15; 26)	72 (53; 84)[*1]	23 (17; 27)	47 (29; 51)
Ala–Glu–Asp–Gly	16 (13; 26)	16 (12; 26)	37 (20; 45)	16 (13; 36)	32 (16; 40)
MRDT (mortality rate doubling time) (days)					
Saline	23.1	21.7	25.7	22.4	16.5
Lys–Glu	30.1	34.6	9.6	30.1	14.7
Ala–Glu–Asp–Gly	43.6	44.6	18.6	44.0	21.5
Two-stage model					
Ageing rate $\alpha \times 10^3$ ($days^{-1}$), I stage					
Saline	6 (0; 13)	3 (0; 13)	10 (0; 25)	2 (0; 10)	42 (12; 120)
Lys–Glu	1 (0; 8)	0.1(0; 7)	12 (0; 24)	1 (0; 7)	13 (0; 40)
Ala–Glu–Asp–Gly	16 (8; 26)	13 (4; 25)	26 (8; 41)	13 (4; 26)	1 (0; 52)
MRDT (days), I stage					
Saline	115.5	231.0	69.3	346.6	16.5

Table 10 (*Continued*)

Group	Total cases	Total tumor-free mice	Total tumor-bearing mice	Fatal tumor-free mice	Fatal tumor-bearing mice
Lys–Glu	693.1	6931.5	57.8	693.2	53.3
Ala–Glu–Asp–Gly	43.3	53.3	26.7	53.3	693.2
Ageing rate $\alpha \times 10^3$ *(day*$^{-1}$*), II stage*					
Saline	34 (6; 61)	34 (3; 62)	35 (0; 93)	34 (6; 61)	–
Lys–Glu	25 (9; 39)	29 (13; 44)	6 (0; 102)	51 (22; 79)	299 (0; 840)
Ala–Glu–Asp–Gly	93 (64; 123)	100 (69; 135)*[1]	157 (30; 300)	95 (64; 129)*[1]	95 (5; 191)
MRDT (days), II stage					
Saline	20.4	20.4	19.8	20.4	–
Lys–Glu	27.7	23.9	115.5	23.6	2.3
Ala–Glu–Asp–Gly	7.5	6.9	4.4	7.3	7.3

* The difference with controls is significant, $P<0.05$.

[a] In brackets 95% confidential limits are given. **The difference with controls is significant, $P<0.001$.

3.10. Effect of the peptides on free radical processes in mice

The comparison of the data obtained on various organs revealed the most intensive generation of ROS in blood serum, where it twice exceeded the corresponding indices in liver and brain. Thereby, the level of antioxidant activity in blood serum was significantly lower in comparison with that in liver and brain (Table 11). The treatment with the thymic peptide did not exert any notable effect upon any of the studied indices. At the same time, the treatment with the pineal peptide effectively inhibited ROS formation in the brain tissue. This peptide preparation significantly inhibited lipid peroxidation in the brain and in the liver of

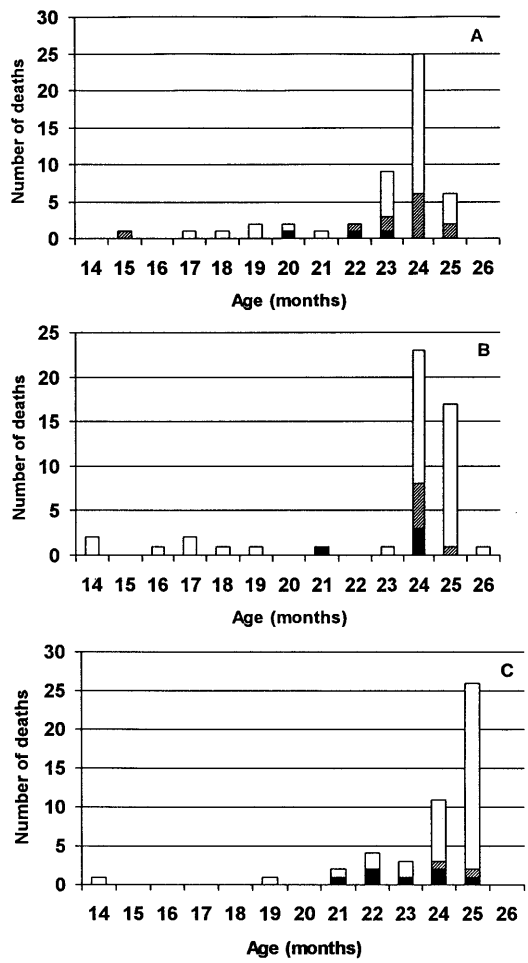

Fig. 6. Number of deaths among control (A), Lys–Glu-treated (B) and pineal (C) treated mice (tumor free, white; non-fatal tumor, textured; fatal tumor, black). Data for deaths later than at the age of 26 months are not shown (see Table 7) 18.

Table 11
Indices of free radical processes in various tissues of mice treated with Lys–Glu or with Ala–Glu–Asp–Gly

Parameters	Saline	Lys–Glu	Ala–Glu–Asp–Gly
Brain			
Intensity of chemoluminescence (10^4 conv. U/mg of protein)	94.1 ± 7.9	109.4 ± 15.2	$72.2 \pm 10.1^{*1}$
Value of the general anti-oxidation activity (AOA, conv. U/mg of protein)	3.1 ± 0.19	3.0 ± 0.24	3.8 ± 0.31
Diene conjugates (nM/g of tissue)	22.29 ± 0.87	25.51 ± 2.23	21.85 ± 1.31
Schiff bases (conv. U/g of tissue)	388 ± 25	327 ± 28	$281 \pm 16^{**1}$
Activity of superoxide dismutase (conv. U/mg of protein)	23.4 ± 1.8	24.5 ± 0.7	27.4 ± 2.27
Liver			
Intensity of chemoluminescence (10^4 conv. U/mg of protein)	78.4 ± 10.4	62.4 ± 5.2	79.1 ± 11.3
Value of the general anti-oxidation activity (AOA, conv. U/mg of protein)	4.72 ± 0.45	3.87 ± 0.23	4.13 ± 0.25
Diene conjugates (nM/g of tissue)	66.37 ± 2.17	66.52 ± 0.58	$57.11 \pm 1.41^{**1}$
Schiff bases (conv. U/g of tissue)	543 ± 14	563 ± 31	494 ± 29
Activity of superoxide dismutase (conv. U/mg of protein)	35.5 ± 1.6	34.1 ± 2.4	35.9 ± 1.2
Serum			
Intensity of chemoluminescence (10^4 conv. U/mg of protein)	224.0 ± 29.8	189.7 ± 40.4	$141.8 \pm 19.6^{*1}$
Value of the general anti-oxidation activity (AOA, conv. U/mg of protein)	1.40 ± 0.15	1.60 ± 0.15	$1.77 \pm 0.07^{***1}$
Diene conjugates (nM/g of tissue)	4.36 ± 0.43	4.33 ± 0.78	3.77 ± 0.46
Schiff bases (conv. U/g of tissue)	29.2 ± 3.2	29.7 ± 2.7	31.0 ± 3.4

* The difference with the control group is significant, $P<0.05$.
** The difference with the control group is significant, $P<0.01$.
*** The difference with the control group is significant, $P<0.001$.

mice. It worthy to note that in the brain it inhibits late steps in lipid peroxidation (formation of Schiff's bases) whereas in the liver – first steps of the process formation of diene conjugates).

4. Discussion

The results of our experiments evidenced that long-term administration of newly synthesized thymic peptide Lys–Glu and of pineal peptide Ala–Glu–Asp–Gly did not produce any adverse effect on the development of the animals, state of their reproductive function, life span and spontaneous tumor development in the female

CBA mice. On the contrary, Lys–Glu slightly increased the life span of the animals and suppressed spontaneous lung adenomas development. The thymic peptide administration positively effected the indices of physical activity and enhanced endurance of the animals. At the same time, a body weight increase, locomotor activity lessening and, which appeared most important, a body temperature decrease were revealed. Body temperature decrease and the concomitant inhibition of metabolic processes are known to expand the life span of animals (Finch, 1990; Weindruch and Sohal, 1997; Lane et al., 1996). The treatment with Lys–Glu failed infuence of free radical processes in tissues of mice.

The pineal peptide Ala–Glu–Asp–Gly as well as the thymic peptide Lys–Glu slightly increased the life span of CBA mice and suppressed spontaneous neoplasm development. The pineal preparation slows down ageing of reproductive function, decreased physical activity, failed to modify a physical strength and fatigue and inhibits free radical processes in female mice.

It must be noted that the geroprotective and anti-tumor effects of Lys–Glu and of the pineal peptide were not associated with its impact upon the body weight of the animals and the amount of food consumed by them. Calorie-intake restriction and a therewith-associated decrease in the animal body weight are known to considerably inhibit ageing of experimental rodents and suppress spontaneous tumour development in the animals (Weindruch and Walford, 1988; Weindruch and Sohal, 1997). Inhibition of free radical processes, which play an important role in the processes of ageing (Cutler, 1995; Anisimov et al., 1999; Anisimov and Soloviev, 1999) is regarded as the key mechanism of action of calorie-restricted food (Weindruch and Sohal, 1997). In our experiments, Lys–Glu administration did not exert any considerable effect upon free radical processes in the CBA mice. Presumably, the immunomodulating properties of the thymic preparation (Khavinson et al., 1999; Morozov and Khavinson, 1991, 1996a,b, 1997; Morozov et al., 1994) played the principal role in its geroprotective and anti-tumour effects.

The results of this study have shown also that the pineal peptide leads to the increase a mean and maximum life span of female CBA mice, slows down the ageing of estrus function, inhibits of free radical processes and spontaneous tumorigenesis. These data are in agreement with observations on the similar activities of Epithalamin in mice and rats (Anisimov et al., 1982, 1989, 1994, 1995, 1996, 1997a). The effective concentration of Ala–Glu–Asp–Gly was 1000–5000 times less than of Epithalamin. In experimens with two strains of D. melanogaster Epithalon (Ala–Glu–Asp–Gly) treatment was followed by increase in their mean life span (Khavinson et al., 2000; Mylnikov and Lyubimova, 2000). It is worthy of note that effective concentrations of the pineal tetrapeptide was 1000 times less than of Epithalamin and 16 000–80 $\times$ 10^6 times less than that of melatonin (Khavinson et al., 2000; Mylnikov and Lyubimova, 2000). Epithalon increased catalasa activity and decreased level of conjugated hydroperoxides in the fruit flies (Mylnikov and Lyubimova, 2000). The pineal peptide did not influence a physical strength and fatigue in mice and slow down their locomotor activity. Last effect could be related to the sedative (sleep) effect like Epithalamin (Anisimov et al., 1994). It was shown that Epithalamin increases the night synthesis and secretion of melatonin in the

pineal gland (Anisimov et al., 1992). Thus, some effects of the pineal peptide could be realized by its stimulatory influence on melatonin secretion. The studies on this important question are in the progress.

Results of this investigation correspond to the data obtained in the prior observations on the safety of long-term administration of peptide preparations isolated from the thymus and on their geroprotective and anti-tumour effects (Anisimov et al., 1982, 1989, 1994; Morozov and Khavinson, 1996a). Thus, the obtained results suggest a geroprotective potential of the pineal peptide preparation Epithalon (Ala–Glu–Asp–Gly).

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