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Presence of links between zinc and melatonin during the circadian cycle in old mice: effects on thymic endocrine activity and on the survival

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Abstract

Links between zinc and melatonin in old melatonin treated mice with a reconstitution of thymic functions have been recently documented. Concomitant increments of the nocturnal peaks of zinc and melatonin, with a synchronization of their circadian patterns, are achieved in old mice after melatonin treatment. A recovery of the nocturnal peaks of thymulin plasma levels and of the number of thymulin-secreting cells with a synchronization of their circadian patterns are also achieved. The existence of significant positive correlations between melatonin and zinc and between melatonin and thymulin or the number of thymulin-secreting cells supports the presence of links between zinc and melatonin also during the circadian cycle with a beneficial effect on thymic functions. The altered circadian pattern of corticosteron in old mice is normalized by melatonin. The existence of inverse correlations between corticosteron and melatonin, between corticosteron and zinc and between corticosteron and thymulin or the number of thymulin-secreting cells during the whole circadian cycle, suggests the involvement of glucocorticoids pathway in the melatonin thymic reconstitution, via zinc. The presence of an interplay among zinc, melatonin, glucocorticoids and thymulin may be, therefore, supported during the circadian cycle. 'In vitro' experiments from old thymic explants show a direct action of zinc, rather than melatonin, on thymulin production, further suggesting that the action of melatonin on the thymic efficiency is mediated by the zinc bioavailability. The beneficial effect of the links between zinc and melatonin on thymic functions during the circadian cycle, may be extended to a prolonged survival in aging, where, however, zinc may be more involved.

Keywords: Zinc; Melatonin; Thymulin; Glucocorticoids; Circadian cycle; Aging; Survival

1. Introduction

Melatonin has an immunomodulatory role (Pierpaoli and Regelson, 1994) with a nocturnal peak during the normal light/dark circadian cycle both in man (Touitou et al., 1985) and rodents (Reiter et al., 1980), mice included (Maestroni and Conti, 1993; Conti and Maestroni, 1996). However a general consensus does not seem to exist for melatonin production in mice (Ebihara et al., 1986; Goto et al., 1989). It has recently been shown that melatonin can modulate plasma zinc levels during the circadian cycle (Morton, 1990). A peak of zinc in the dark period is observed (Hurley et al., 1982). This is of interest because zinc is crucial for a good immune functioning, thymic included (Chandra, 1985).

With advancing age the nocturnal peak of melatonin is lost with nearly undosable plasma levels at 20–22 months of age in rodents (Reiter et al., 1980; Maestroni and Conti, 1993) and at 60–70 years of age in humans (Touitou et al., 1985). Plasma zinc levels also show an age-related decline (Hsu, 1979). An impairment of the immune efficiency occurs in aging (Miller, 1991). Administration of zinc (Dardenne et al., 1993; Mocchegiani et al., 1995a), and melatonin (Pierpaoli and Regelson, 1994) have an immune reconstituting effect in aging. The immune reconstituting effect of melatonin in old mice has been recently shown to be mediated by the zinc pool, via glucocorticoids (Moc-

Abbreviations: FTS, inactive zinc-unbound thymulin; ZnFTS, active zinc-bound thymulin; MEL, melatonin; AAS, atomic absorption spectrophotometry; EIA, enzyme immunoassay; CHX, cycloheximide; ACTH, adreno-corticotropic hormone; TRH, thyreotropic-releasing hormone; FITC, fluorescin isothiocyanate; MoAb, monoclonal antibody; GAM, goat anti-mouse; IF, immunofluorescence

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chegiani et al., 1994) This finding supports the existence of links between melatonin and zinc in aging (Mocchegiani et al., 1994).

With these premises, the aim of the present work was to establish the existence of such links, via glucocorticoids, also during the circadian cycle by testing plasma zinc, melatonin and corticosteron in old melatonin treated mice. Since a circadian cycle of a thymic hormone, such as thymosin alfa-1 has been also documented (McGillis et al., 1983), the circadian variations of thymulin plasma levels, a zinc dependent thymic hormone (ZnFTS) (Dardenne et al., 1982) and the number of thymulin-secreting cells (Savino et al., 1982) were also tested.

Moreover, a direct action of zinc on thymulin production has been suggested (Mocchegiani and Fabris, 1995b; Saha et al., 1995), and specific melatonin receptors have been found into the thymus (Martin-Cacao et al., 1993). Thus 'in vitro' experiments on the effect of zinc and/or melatonin on thymulin production from old thymus were checked. The survival in old mice treated with melatonin or with zinc as compared to normal mice in our housing condition was evaluated.

2. Materials and methods

2.1. Animals

Inbred Balb/c mice are a good animal model to study aging process and survival because of their genetic homogeneity (Finch, 1991). Inbred Balb/c male mice with 3 months (young) and 18 months of age (presenescent) were used for the experiments and for the survival.

2.2. Housing

Inbred Balb/c mice were bred in 'conventional' barrier (Finch, 1991; FELASA, 1994), in plastic non-galvanized cages, 5 mice per cage ($36 \times 20 \times 14$ cm) (Tecnoplast, Italy), and fed with standard pellet food (Nossan, Italy) and tap water in sterilized bottles ad libitum. The animals were maintained on a 12-h light/12-h dark cycle from 7:00 a.m. to 7:00 p.m., at constant temperature (20 ± 1°C) and humidity (50 ± 5%). Darkness and light exposure were controlled by a fixed timer governing two standard fluorescent fixtures (Philips TLD 36 W/84): 7 p.m. light off, 7 a.m. light on. A check-up of environmental factors (temperature, humidity, air ventilation, light, food and sawdust) as well as of housing wholesomeness was performed monthly. Both bacteriological and serological analysis were monthly carried out in order to check the healthy status. The maximum life-span of our inbred Balb/c mice was 30 months. Since the majority of deaths occurred at 22–25 months of age (A. Tibaldi, personal communication), mice with 22 months of age were considered as 'old' and with 18 months of age as 'presenescent' (Russel, 1975).

2.3. Experimental design: circadian rhythmicity

A total of 35 presenescent inbred Balb/c male mice were treated with melatonin in drinking water. Four months later the animals underwent the circadian cycle test for melatonin, zinc, thymulin and corticosteron plasma levels at various time intervals (9:00 a.m.; 12:00 a.m.; 7:00 p.m.; 11:00 p.m.; 2:00 a.m.; 7:00 a.m. and 9:00 a.m;) according to known crucial circadian times for melatonin (Reiter et al., 1980; Conti and Maestroni, 1996) and for zinc (Hurley et al., 1982). The timing of 4 months of treatment was chosen on the basis of previous works on the immune-reconstituting effects by melatonin in aging mice (Pierpaoli and Regelson, 1994; Mocchegiani et al., 1994). Thirty-five old (22 months of age) and 35 young (3 months of age) untreated mice served as controls.

Five 'old' mice treated with melatonin, five 'old' and five 'young' control mice were sacrificed in a separate room from other animals under ether anaesthesia in each time interval considered during the circadian cycle. Mice were sacrificed in the sequence: 1 young control, 1 old control and 1 old treated mice till to 15th mice for each time interval, in accordance to principles of chronobiology in animals (Haus et al., 1974). Heparined blood samples (1 ml) were collected by cardiac puncture. Blood samples for thymulin, corticosteron and melatonin tests were centrifuged at $1500 \times g$ for 15 min at 4°C and stored at −70°C until tested. Blood samples for zinc test were subjected to a different procedure (see below). We have used red lamp (Philips) for night blood withdrawals. After the sacrifice the thymuses was removed and frozen in liquid nitrogen for immunofluorescence studies. For the replication, melatonin treatment was performed during spring and summer. The sacrifice occurred in September.

2.4. Treatments

2.4.1. Melatonin oral administration

About 100 mg of melatonin (MEL), purchased from Sigma (St. Louis, MO), were dissolved in 1 ml absolute ethanol and further diluted to 100 ml distilled water. Five milliliters of the MEL stock solution were diluted to 500 ml tap water. The final concentration of MEL in the drinking tap water was 10 μg/ml. MEL was administered in the drinking tap water with a fixed darkness cycle: both control and melatonin-containing opaque bottles were administered from 6:00 p.m. to 8:00 a.m. of the next day (Mocchegiani et al., 1994; Pierpaoli and Regelson, 1994). Water with and without melatonin was changed twice a week. The melatonin-water and tap-water intake were similar (4–5 ml/day/mouse) in old treated and untreated mice (young and old controls), respectively, corresponding to 40–50 μg melatonin/day/mouse in treated mice.

The control and melatonin-containing water bottles were removed from 8:00 a.m. to 6:00 p.m. No drinking water was given during that period in order to homogenize the experimental design of melatonin (Pierpaoli and Regelson, 1994). The control mice received drinking water plus 0.0001% of ethanol.

2.4.2. Zinc oral administration

Oral zinc supplementation was performed from the age of 18 months with zinc sulphate dissolved in tap water at final concentration of 22 mg/l (Mocchegiani et al., 1995a). Presenescent mice treated with tap water served as controls.

2.5. Survival

Fifty mice are sufficient for analysis survival (Piantanelli et al., 1994). Fifty presenescent male Balb/c mice were treated with night-melatonin (10 μg/ml) in the drinking water. Fifty male Balb/c mice were treated with zinc (22 mg/l) in the drinking water for the whole day. Fifty male Balb/c mice treated with tap water served as controls. Melatonin was administered during the night (Pierpaoli and Regelson, 1994), because the effect of light-day melatonin administration on the survival is contradictory (Pierpaoli et al., 1991; Lenz et al., 1995). Zinc was administered for the whole day because no side or toxic effects were observed in previous studies (Dardenne et al., 1993; Mocchegiani et al., 1995a). The choice of 18 months of age was for two reasons. Firstly because no significant differences in the survival existed between melatonin or zinc treated mice and control groups before this age in our housing condition (A. Tibaldi, personal communication). Secondly because 18 months of age was the onset of the immune age-related decline (Pierpaoli and Regelson, 1994). The healthy status of 150 mice used at the age of 18 months was within the 'conventional housing' normal range (Sebesteny, 1991). Mice were censored each two days. All mice used were also individually weighted at time intervals.

2.6. Thymulin determination

This bioassay, as extensively described elsewhere (Bach et al., 1975; Mocchegiani et al., 1994), is specific for zinc-bound active thymulin (ZnFTS), since it is unaffected by other thymic hormones. The rosette-inducing activity is completely removed by passing plasma samples through an antithymulin immunoadsorbent (Bach et al., 1975). The sensitivity of the bioassay allows detection of 1 pg/ml synthetic thymulin (Sigma, USA). The assay is reliable since in two consecutive blind assays, no difference of more than $1/\log_2$ was found in all samples (Bach et al., 1975). In order to evaluate possible interferences by zinc bioavailability, thymulin measurements have been concomitantly performed with the 'in vitro' addition of zinc–sulphate to the plasma samples at a final concentration of 200 nM. This zinc concentration is the optimal for unmasking zinc-unbound inactive thymulin (FTS) showing the total amount of thymulin (zinc-bound ZnFTS plus zinc-unbound FTS) produced (Mocchegiani et al., 1995a).

The apparently low molar concentration of zinc required may be explained by the fact that the available free zinc is not more than 2–3% of total plasma zinc, the major quota being bound to proteins which are retained by the 50.000 mol. wt. cut-off membranes (Mocchegiani et al., 1995a). This bioassay is still required because thymulin radioimmunoassay recently developed is unable to discriminate between zinc-bound and zinc-unbound thymulin (Safieh et al., 1990).

2.7. Zinc determination

Heparined blood samples for zinc test were collected into fluorinated tubes (no. 115317, LP, Italy) in order to avoid contamination and centrifugated 20 min later at $3000 \times g$ for 10 min at 4°C. Plasma samples were then frozen at -70°C until tested. Zinc was determined by atomic absorption spectrophotometry (AAS) against zinc references standard (Mocchegiani et al., 1995a).

2.8. Corticosteron determination

Plasma corticosteron levels were determined by using RIA rat-corticosteron-^{3}H kit (ICN Biomedicals, CA, USA) which used a specific anti-corticosteron antibody. The data (ng/ml) were referred against a standard curve. The percentage of cross reaction with other steroids was < 0.01. The sensitivity was of 0.05 ng/ml of corticosteron.

2.9. Melatonin determination

Plasma melatonin concentrations were determined by using melatonin enzyme immunoassay (EIA) kit (Buhlmann Laboratories, Switzerland). This melatonin EIA kit was intended for the direct quantitative determination of melatonin in human plasma, but it can be used for other specimen, including mice. The samples were extracted prior the EIA assay by using centrifugation procedure and specific columns (B-MEC) purchased by Buhlmann Laboratories (Switzerland) in order to avoid high background values. After extraction procedure, the extracts obtained with methanol as eluant were evaporated under a stream of nitrogen and frozen at -70°C until assayed. The extraction recovery was validated by spiking of a small amount (50 μl) of ^{3}H-melatonin in the specimen. The recovery of melatonin from plasma samples using this method was 92% (± 4) in the range 19–287 pg/ml. The melatonin ELISA kit was based on a capture second antibody technique. A polyclonal antibody specific for rabbit immunoglobulin was coated into the microtiter plate provided in the kit. Melatonin present in the extracts bound to a rabbit

anti-melatonin antibody. The melatonin-antibody complex and free anti-melatonin antibodies were then captured from the second antibody on the coated well. An enzyme–melatonin–peroxidase complex was then added in the wells. A coloured product was formed in inverse proportion to the amount of melatonin present in the extracts. The data obtained (expressed in pg/ml) were referred against a standard curve. The lecture of samples was performed at 492 nm (Multiskan). The antiserum used was highly specific for melatonin. The percentage of the cross-reactivities of the melatonin antiserum with precursors of melatonin were between 0.0% for 5-Methoxytryptamine and 0.5% for 5-Methoxytryptophol. The sensitivity of the kit was 2.6 pg/ml (11.3 pmol/l) of melatonin.

2.10. Thymic 'in vitro' cultures

The thymuses from young and old mice were put into plate wells (Cook, UK), containing 1 ml of zinc-free M10 medium plus 5% chelated heat inactivated bovine foetal serum The thymic cultures were put in a CO_2 incubator at 37°C. Supernatant samples (50 μl) were taken at different culture times (0′, 15′, 30′, 1 h, 2 h, 4 h, and 6 h) and the culture volume was reconstituted with fresh zinc-free M10 medium. Previous studies have shown that thymulin appears in the supernatants with a maximum thymulin production after 6 h of culture. Such an appearance may be due to 'de novo' synthesis, since the preincubation with cycloheximide (CHX), a potent inhibitor of protein synthesis, completely prevents the appearance of thymulin in supernatants (Mocchegiani and Fabris, 1995b). Zinc sulphate was used at the final concentration of 1 μM (Dardenne et al., 1982). Melatonin was used at a final concentration of 50 μg/ml (Mocchegiani et al., 1994).

2.11. Immunofluorescence studies

Thymulin containing cells were detected by means of an anti-thymulin MoAb (kindly supplied by Dr. M. Dardenne, Paris, France), which can be revealed by the GAM/IgG2a/FITC diluted 1/20. The number of thymulin containing cells in the thymus of each mouse was assessed by counting 100 microscopic fields of 135,000 μm^2 from three or four frozen two micrometers sections obtained at different levels of the organ (Savino et al., 1982).

2.12. Statistical analysis

Analysis of melatonin, zinc and corticosteron rhythmicity was performed according to the cosinor method (Nelson et al., 1979). With this procedure it is possible to determine whether there is a rhythm within a 24-h period and to evaluate the following parameters with their 95% confidence limits: (1) mesor (midline estimating statistic of rhythm), rhythm-adjusted 24-h average; (2) amplitude, the difference between the maximum value measured at acrophase and the mesor in the cosine curve; (3) acrophase, lag between reference time (midnight) and time of highest value of the cosine function used to approximate the rhythm. Paired Student's *t*-test and ANOVA (two-way) were used where appropriate. Correlations were determined by linear regression analysis by the least square method. The differences between the various regression lines were evaluated by covariance analysis. Differences were considered significant when $p < 0.05$.

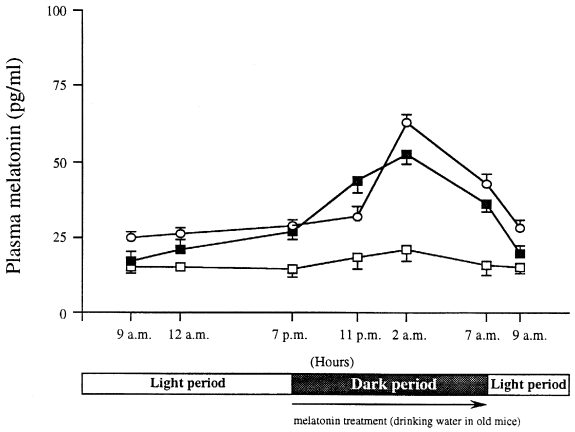


Fig. 1. Circadian plasma melatonin levels in young (○), old (□), and old + MEL (■). Means ± S.D.

3. Results

3.1. Effect of age on melatonin circadian rhythm and recovery by melatonin treatment in old mice

Fig. 1 shows the disappearance of the nocturnal peak of melatonin in old mice as compared to young mice ($p < 0.01$). Melatonin treatment restores the nocturnal peaks of

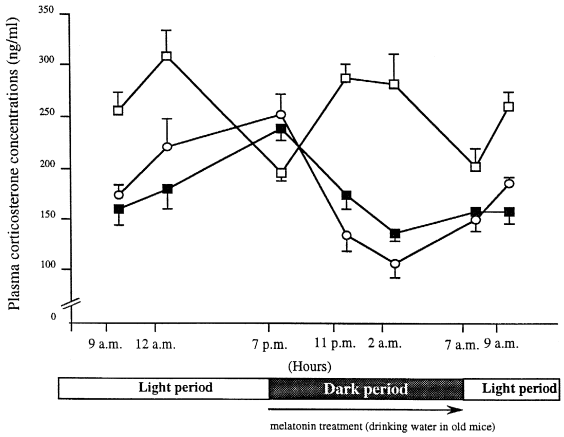


Fig. 2. Circadian plasma corticosteron concentrations in young (○), old (□), and old + MEL (■). Means ± S.D.

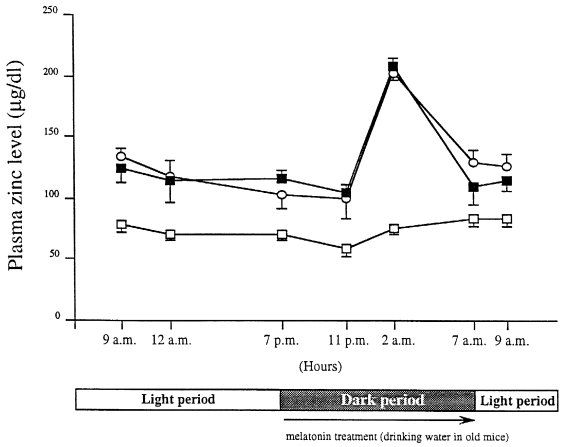


Fig. 3. Circadian plasma zinc concentrations in young (○), old (□), and old + MEL (■) Means ± S.D.

melatonin in old mice. (Fig. 1). No differences exist in melatonin values between 11 p.m. and 2 a.m. in old melatonin treated mice (Fig. 1), therefore the effect of melatonin may be related with the dark period of melatonin administration in the drinking water.

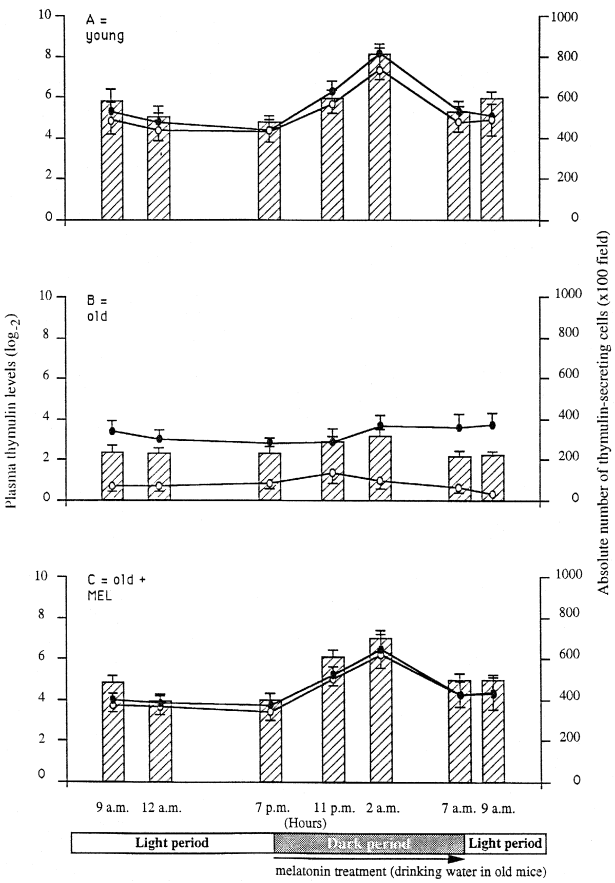


Fig. 4. Circadian plasma active thymulin (○) and total thymulin (●) levels and number of thymulin secreting cells (□) in young (A), in old (B), in old + MEL (C). Means ± S.D.

3.2. Effect of age on corticosteron and zinc circadian rhythm and recovery by melatonin treatment in old mice

The circadian rhythm of corticosteron shows a loss of the fall of plasma corticosteron concentrations during the night in old mice as compared to young mice ($p < 0.01$) (Cosinor analysis) (Fig. 2). Melatonin treatment restores the circadian pattern of corticosteron in old mice with no significant differences in amplitude in the expected peaks as compared to young controls (Fig. 2).

A nocturnal peak of zinc is observed in young mice. It disappears in old mice (Fig. 3). The cosinor analysis shows a significant decrement of circadian plasma zinc levels in old mice as compared to young mice ($p < 0.01$; Fig. 3). Melatonin treatment restores the circadian pattern of zinc in old mice with a reappearance of the nocturnal peak (Fig. 3).

3.3. Effect of age on the circadian rhythm of thymulin and thymulin-secreting cells and recovery by melatonin treatment in old mice

Fig. 4 shows the circadian rhythm of active (ZnFTS), total (ZnFTS + FTS) thymulin plasma levels and the num-

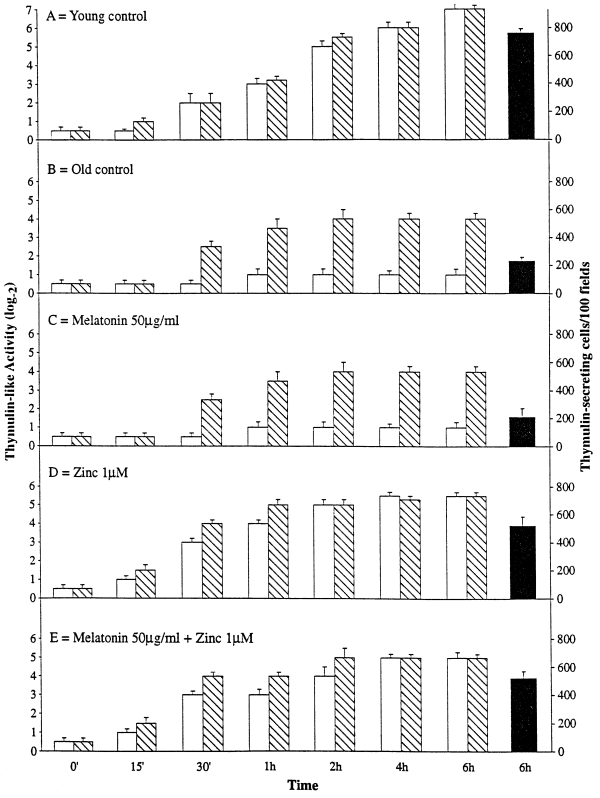
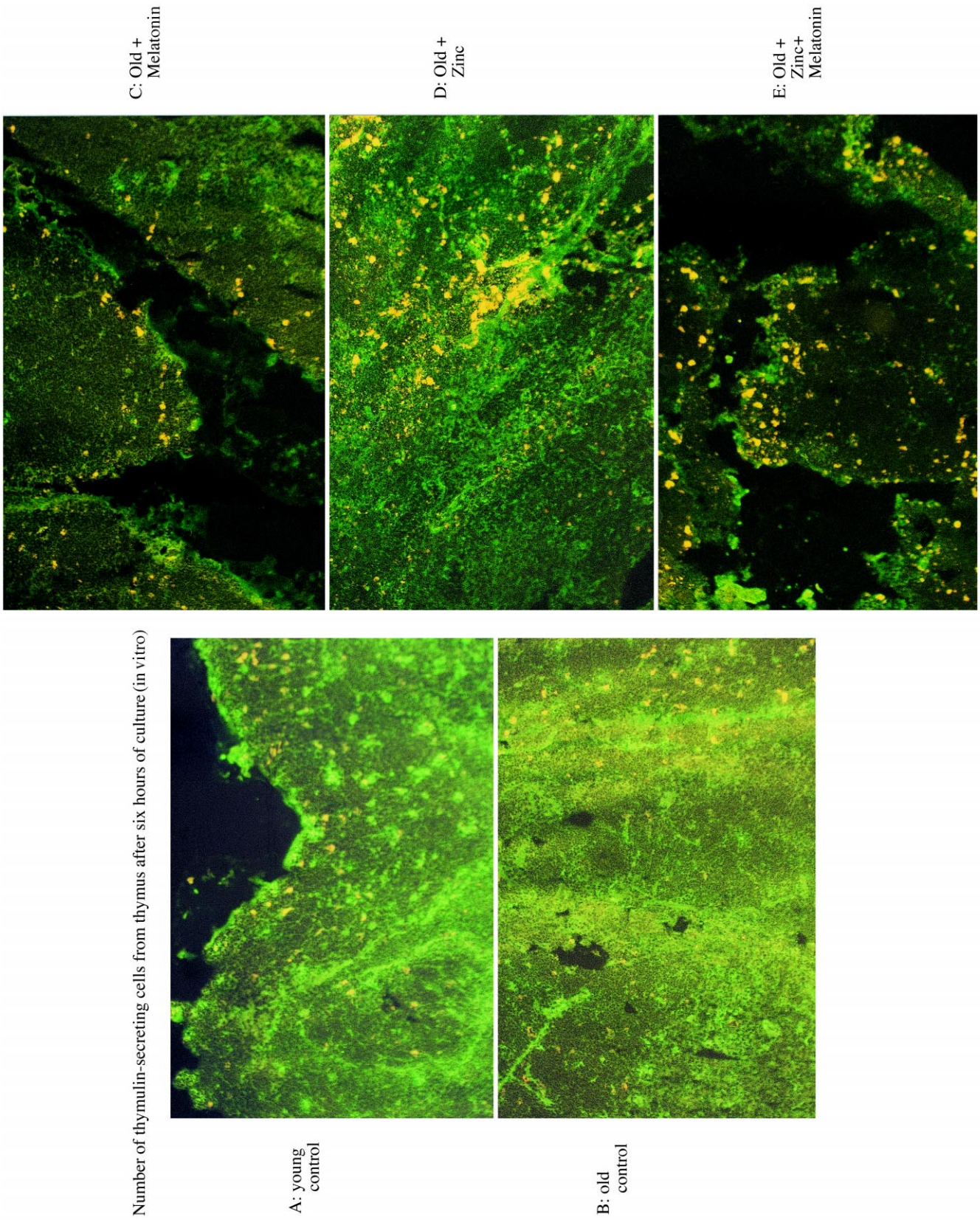


Fig. 5. Kinetics of 'in vitro' active (□) and total (□) thymulin synthesis by thymuses from young (A) and old (B) Balb/c mice cultivated for a short period (6 h). Black columns indicate the number of thymulin-secreting cells tested at the end of culture. Active and total thymulin values and number of thymulin-secreting cells by thymuses after 'in vitro' addition of melatonin (C), melatonin + zinc (D) and zinc alone (E) to the cultures are reported. Data are expressed as means ± S.D. of six cultures (two thymuses of young mice and of old mice for each culture).


Number of thymulin-secreting cells from thymus after six hours of culture (in vitro)
A: young control
B: old control
C: Old + Melatonin
D: Old + Zinc
E: Old + Zinc+ Melatonin

ber of thymulin-secreting cells. Young mice show nocturnal peaks of active and total thymulin fractions as well as of the number of thymulin-secreting cells (Fig. 4A). Reductions of the number of thymulin secreting cells and active thymulin fraction are observed in old mice during the whole circadian cycle with a disappearance of the nocturnal peaks as compared to young mice ($p < 0.01$; Fig. 4B). Total thymulin shows a pattern similar to the active fraction in old mice, but the overall concentrations are reduced when compared to young mice ($p < 0.05$; Fig. 4B). Melatonin treatment restores the nocturnal peaks of active and total thymulin as well as the number of thymulin-secreting cells in old mice (Fig. 4C).

3.4. Correlations among the various parameters tested for circadian rhythmicity

Significant inverse correlations exist between corticosteron and melatonin circadian values ($r = -0.85$, $p < 0.01$), between corticosteron and zinc ($r = -0.85$, $p < 0.01$), between corticosteron and thymulin ($r = -0.80$, $p < 0.01$) and between corticosteron and the number of thymulin-secreting cells ($r = -0.78$, $p < 0.01$) (Cosinor analysis and Cross Regression Model).

Significant positive correlations exist between melatonin and zinc ($r = 0.82$, $p < 0.01$), between melatonin and thymulin ($r = 0.88$, $p < 0.01$) and between melatonin and the number of thymulin-secreting cells ($r = 0.83$, $p < 0.01$) (Cosinor analysis and Cross Regression Model).

These correlations are obtained from data of all experimental groups of mice considered (old treated and young and old controls).

3.5. 'In vitro' effect of zinc or melatonin on thymic endocrine activity from thymic explants of old mice

Thymic explants from old mice show a strong reduction of active thymulin during the time intervals considered as compared to young mice (Fig. 5A) ($p < 0.001$). The total thymulin is also significantly reduced in old thymic cultures as compared to young thymic cultures ($p < 0.01$). The number of thymulin-secreting cells (determined at the end of the culture period), is reduced in old thymic explants as compared to young ($p < 0.001$) (Fig. 5A,B). The 'in vitro' addition of melatonin (50 μg/ml) shows a pattern of both active and total thymulin and the number of thymulin-secreting cells similar to the pattern observed in old thymic culture (Fig. 5C). In contrast, the 'in vitro' addition of zinc (1 μM) induces significant increments of active thymulin fractions as compared to old thymic culture ($p < 0.001$). The number of thymulin-secreting cells are also restored after 'in vitro' zinc addition (Fig. 5D). The total thymulin is unaffected by the 'in vitro' zinc addition (Fig. 5D). The 'in vitro' zinc + melatonin addition does not induce any further increment of active and total thymulin as well as of the number of thymulin-secreting cells in old thymic culture, showing a pattern similar to 'in vitro' zinc alone (Fig. 5E).

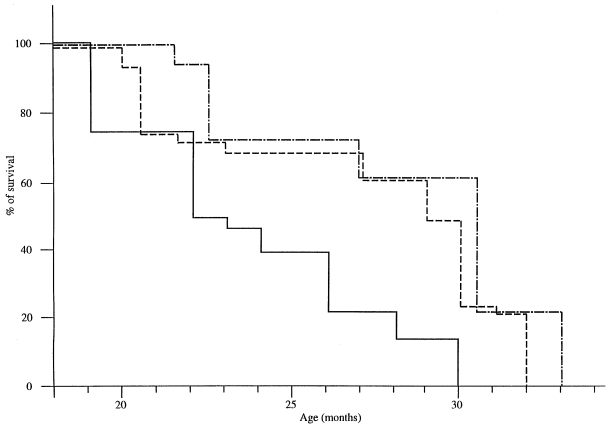


Fig. 7. Survival curves (Kaplan–Meier) from the age of 18 months in mice treated with melatonin (— — —), treated with zinc (—·—·—) and in control (————) in our housing 'conventional' condition.

A quota of zinc is always present in thymic tissue in old mice after 6 h of culture (86.5 ± 5.8 μg/g of zinc in old thymus vs. 293.4 ± 10.4 μg/g of zinc in young thymus) (E. Mocchegiani, unpublished data). It might justify the presence of thymulin secreting cells in old thymus measured with the anti-thymulin monoclonal antibody (Fig. 6B), which recognizes intracellular thymulin in its zinc-bound active form (Savino et al., 1982), as it occurs in young thymus (Fig. 6A). However such thymic zinc content in old thymus may not be sufficient to induce the activation of all thymulin molecules produced during the 6 h of culture either in old thymic culture (Fig. 6B) or in old thymic culture plus melatonin (Fig. 6C). The addition of exogenous zinc is required (Fig. 6D,E), as previously demonstrated in old thymic 'in vitro' models (Mocchegiani and Fabris, 1995b).

3.6. Effect of melatonin or zinc treatment on the survival in old mice

Fig. 7 shows the survival curve (Kaplan–Meier) of control mice (from 18 months of age) in our housing condition with a maximum life-span of 30 months. A melatonin treatment increases the maximum life span to 32 months ($p < 0.05$, Log-rank test). A more significant in-

Fig. 6. (right) Thymulin-secreting cells from thymus after 6 h of culture. A = Young control; B = Old control; C = Old + Mel; D = Old + Zinc; E = Old + Mel + Zinc. Magnitude 10 ×. Lecture of the number of thymulin-secreting cells 40 ×.

Table 1

A. Body weight (g) of presenescent mice (from 18 months of age) performed at various time of intervals during the survival analysis

	Age 18 months	Age 22 months	Age 28 months
Control ($n = 50$)	27.6 ± 1.3	27.5 ± 1.3	24.3 ± 1.4
Old + Melatonin ($n = 50$)	27.5 ± 1.4	26.8 ± 1.4	24.5 ± 1.3
Old + Melatonin ($n = 50$)	27.6 ± 1.3	26.9 ± 1.4	25.6 ± 1.4
	Age 30 months	Age 32 months	Age 33 months
Control ($n = 50$)	22.4 ± 1.5	–	–
Old + Melatonin ($n = 50$)	21.8 ± 1.3	22.5 ± 1.4	–
Old + Melatonin ($n = 50$)	22.4 ± 1.3	22.6 ± 1.4	22.5 ± 1.6

1B. Measure of the food intake (g/day/mouse) in 30 mice in metabolic cages for 1 week at each interval of age considered

	Age 18 months	Age 22 months	Age 28 months
Control ($n = 10$)	4.15 ± 0.71	4.27 ± 0.54	4.51 ± 0.73
Old + Melatonin ($n = 10$)	4.05 ± 0.63	4.15 ± 0.63	4.27 ± 0.65
Old + Zinc ($n = 10$)	4.10 ± 0.61	4.18 ± 0.57	4.15 ± 0.53

crement of the survival is observed for the middle age as compared to controls ($p < 0.01$, Log-rank test) (Fig. 7).

A zinc treatment induces the same significant increment of the survival both in the middle age and in maximum life-span (33 months) as compared to controls ($p < 0.01$ and $p < 0.05$, respectively, Log-rank test). No significant differences in the survival curves exist between melatonin and zinc treatments. The average weight of control mice changes from 18 months to 30 months of age (from 27.6 ± 1.3 g to 22.4 ± 1.5 g) (Table 1A). No significant differences exist between melatonin or zinc treated mice as compared to controls (Table 1A). The food intake is constant in all experimental groups of mice during survival analysis period (Table 1B).

4. Discussion

The thymic-reconstituting effect of melatonin in old mice may be mediated by the zinc pool, via glucocorticoids (Mocchegiani et al., 1994). Zinc is crucial for good thymic endocrine activity (Dardenne et al., 1982, 1993; Mocchegiani et al., 1995a). The thymic-reconstituting effect of melatonin mediated by the zinc pool is also present during the circadian cycle. A predominant action of zinc, rather than melatonin, on thymic functions may, however, occur because of 'in vitro' studies from old thymic cultures show a direct action of zinc on thymulin production. The existence of a good homeostasis between zinc and melatonin on thymic endocrine activity rises the rate of survival in aging.

A circadian cycle of melatonin with a nocturnal peak has been documented in young man (Touitou et al., 1985) and in young rodents (Reiter et al., 1980; Cassone, 1990; Reiter, 1992). No melatonin production has been reported in inbreed mice (Ebihara et al., 1986; Goto et al., 1989). Recently a circadian cycle of melatonin has been found in inbred Balb/c mice.with an acute nocturnal peak of melatonin (Maestroni and Conti, 1993; Conti and Maestroni, 1996). A nocturnal peak of melatonin is also found in our young Balb/c mice with, however, larger peaks and lower melatonin values. Different melatonin tests used may cause this discrepancy. However, without discarding the hypothesis of mutant genes for some precursors of melatonin synthesis in mice (Ebihara et al., 1986), nocturnal peak of melatonin in young mice is present perhaps because also of melatonin synthesis in organs and tissues other than the pineal gland (Huether, 1993).

With advancing age the melatonin nocturnal peak is lost both in man (Iguchi et al., 1982; Touitou et al., 1985) and in mammals, rodents included (Reiter et al., 1980; Reiter, 1992). The disappearance of the nocturnal peak is also observed in old mice. Melatonin treatment restores melatonin nocturnal peaks. In this context, a contradictory point with other laboratories is related to the absence of a melatonin peak one h later (7 p.m.) from the beginning of treatment (6 p.m.). The half-life of melatonin is very short (12–30 min) even when administered at pharmacological dose (Lang et al., 1983; Barlow-Walden et al., 1995) with, however, a peak of melatonin 1–2 h after, and subsequent progressive disappearance within 24 h (Lang et al., 1983; Pevet and Pitrosky, 1997). A possible explanation may be related that mice don't drink immediately melatonin because of drinking-water melatonin bitter taste (W. Pierpaoli, personal communication), despite the deprivation in the light-day period. A subsequent habit to melatonin taste may induce the mice to drink and, consequently, peaks of circulating melatonin (11 p.m. and 2 a.m.). Such an hypothesis may be supported because of no significant differences in melatonin values between 11 p.m. and 2 p.m in old melatonin treated mice. In addition, the intake of melatonin and tap water in treated and in young control, respectively, for the whole night is similar (4–5 ml). Further experiments are, however, required to constantly measure the exact amount of water-melatonin intake for the whole dark period. Nevertheless, these observations

may suggest the model of melatonin drinking water in mice useful only when the first blood withdrawal is performed 3–4 h after the beginning of the nocturnal treatment to study kinetics of circulating melatonin.

Links between melatonin and glucocorticoids may suggest the presence of melatonin peaks in old treated mice. Indeed, the circadian rhythmicity of corticosteron is dependent by melatonin rhythmicity having melatonin a suppressive role on corticosteron (Oxenkrug et al., 1984; Gupta, 1990) with different mechanisms. A down-regulation of ACTH release (Vaughan et al., 1980) because of specific melatonin receptors in the hypothalamus (Vanecek et al., 1987), or direct actions of melatonin on the adrenals (Persengiev et al., 1989) have been suggested. The reduced nocturnal peak of melatonin can be restored after melatonin treatment in adrenalectomized rats (Reiter et al., 1982). Moreover, the increased adrenal weight in pinealectomized mice can be restored by melatonin treatment (Mocchegiani et al., 1996). Thus the nocturnal peak of melatonin in old treated mice may be linked to the restoration of the nocturnal peak of corticosteron, even if corticosteron has been found normal in pinealectomized rats (Reiter et al., 1980). Melatonin may be produced by other organs with a circadian rhythmicity (Tosini and Menaker, 1996). Reduced melatonin and normal corticosteron levels were observed in blinded humans (Arendt, 1992). These findings give a further rationale for a link between melatonin and corticosteron, whatever the organ of melatonin production may be. The presence of an inverse correlation during the circadian cycle between corticosteron and melatonin may be, therefore, supported with, however, no interference of stress (anaesthesia or sacrifice of mice) because of significant differences in corticosteron circadian rhythmicity between treated or young controls and old control mice.

However the discovery of a link between zinc and melatonin during development and aging (Mocchegiani et al., 1994; Mocchegiani et al., 1996) suggests also an involvement of zinc in melatonin rhythmicity. Indeed, the complete disappearance of the nocturnal peak of zinc in old mice can be restored by melatonin treatment. The interpretation of this last finding is intriguing, but it might, at least, give a further justification of nocturnal peak of melatonin in old treated mice. Concomitantly with the nocturnal peak of melatonin, a nocturnal peak of zinc occurs in young mice. A modulation of zinc cation levels by melatonin during the light/dark cycle has been reported (Morton, 1990). In addition, a nocturnal peak of zinc has been found in young rats (Hurley et al., 1982). A direct modulation of zinc turnover by melatonin may find support by the presence of melatonin receptors in gut cells (Lee and Pang, 1991), suggesting a possible active transport of zinc at this level (Mills, 1989). An alternative explanation may be related that increased glucocorticoids in aging have a zinc depleting effect (Prasad, 1985), which can be reversed by melatonin treatment (Mocchegiani et al., 1994). A restoration of glucocorticoids pattern by melatonin may be, therefore, a further source of zinc bioavailability. Moreover the circadian cycle of some zinc-dependent hormones, such as prolactin, growth hormone, TRH, gonadotropins (Bunce, 1989), are altered in aging (Sowers and Felicetta, 1988), and they, in turn, can be modulated by melatonin (Blask, 1981). An another source of zinc bioavailability may be, therefore, represented by the modulation of the above cited hormones. The presence of a significant inverse correlation between corticosteron and zinc during the whole circadian cycle may, therefore, suggest an action of glucocorticoids on zinc turnover. Thus the possible existence of an interplay among melatonin, zinc and glucocorticoids, other than in development and aging (Mocchegiani et al., 1994; Mocchegiani et al., 1996) may be also suggested during the circadian cycle with a positive influence on thymic efficiency.

Indeed also thymulin plasma levels (active and total) and the number of thymulin-secreting cells show a nocturnal acrophase in young mice concomitantly when nocturnal acrophase of zinc and melatonin occurs. These findings are in agreement with the observed circadian rhythm of thymosin alfa-1 (McGillis et al., 1983) and with old studies showing an increment of thymic cells proliferation during the night (Scheving and Pauly, 1973). Together with the fall of zinc acrophase, the acrophases of thymulin-secreting cells and of total and active thymulin plasma levels are lost in aging. These data are in line with previous 'in vitro' (Mocchegiani and Fabris, 1995b; Saha et al., 1995) and 'in vivo' studies (Dardenne et al., 1993; Mocchegiani et al., 1995a) showing that in old thymuses the main defect in thymulin production does not reside in the capacity to synthesize the nonapeptide but at the periphery in its binding to zinc ions.

A melatonin treatment synchronizes also the circadian pattern of thymic functions in old mice. Thus taking into account the pivotal role of zinc for thymic efficiency (Dardenne et al., 1982; Mocchegiani et al., 1995a; Saha et al., 1995; Hadden, 1995) and the finding showing the involvement of the zinc pool, via glucocorticoids, on the immune-reconstituting effect of melatonin in old mice (Mocchegiani et al., 1994), is suggestive to interpret the restoration of the thymic functions by melatonin mediated by the zinc turnover, via glucocorticoids, also during the circadian cycle. The existence of significant positive/negative correlations between melatonin and zinc or corticosteron, respectively, and between melatonin or corticosteron and thymulin secreting cells, respectively, for the whole circadian cycle is in line with this interpretation. This may be supported because thymic hormones with a feed-back mechanism affect the functional status of the adrenals (Labunetes, 1996), which are, in turn, under the control of melatonin (Reiter, 1988). Thus an interplay including, other than zinc, melatonin and glucocorticoids, also thymic hormones may be suggested during the circadian cycle.

The presence of melatonin receptors into the thymus (Martin-Cacao et al., 1993), raises the question whether it is zinc or melatonin or both to act on the thymic endocrine activity. 'In vitro' experiments from old thymic explants suggest a direct action of zinc on the number of thymic epithelial cells, rather than melatonin. These data are in agreement with previous 'in vitro' findings showing a direct action of zinc on thymulin production (Mocchegiani and Fabris, 1995b; Saha et al., 1995). Thus, without discarding direct (Martin-Cacao et al., 1993) or indirect (Gupta, 1990; Pierpaoli and Yi, 1990; Maestroni et al., 1987; Vaughan et al., 1987) mechanisms of action of melatonin on the thymus so far proposed, these 'in vitro' and 'in vivo' findings, suggest that the action of melatonin on the thymus may occur by means of the zinc pool also during the circadian cycle. Such an assumption may be further supported by recent findings showing zinc able to prevent thymocytes apoptosis in old age (Provinciali et al., 1995). Melatonin prevents that 'in vivo' (Sainz et al., 1995), and less in 'in vitro' models (Sainz et al., 1995; Provinciali et al., 1996). In this context, intriguing points are related with increased apoptosis of neural cells in the darkness (Schlingensiepen et al., 1994), and with recent 'in vivo' findings showing the nocturnal peak of melatonin associated with decreased NF-kB-DNA binding activity (Chuang et al., 1996). NF-kB, in turn, is under the control of zinc by means of TNF-alfa (Driessen et al., 1994), and involved in preventing apoptosis (VanAntwerp et al., 1996).

From a clinical point, the good homeostasis between zinc and melatonin, via glucocorticoids, on thymic functions during the circadian cycle induces a significant increment of the survival in old mice. Old mice treated with night-melatonin show a prolonged survival as found by others (Pierpaoli and Regelson, 1994). Zinc also prolongs the survival. Nevertheless an increment of the middle age (range 22–28 months) may be more relevant. Although the causes of death were not examined after treatments, the majority of normal mice dies for infectious, degenerative and neoplastic diseases in the middle age (A. Tibaldi, personal communication). Taking into account the involvement of the zinc pool in the immune-reconstituting effect by melatonin, and the relevance of zinc for the efficiency of the immune system (Chandra, 1985), is suggestive to interpret the increased middle age because of a possible reduction of infectious, degenerative and neoplastic diseases. In this context, zinc, rather than melatonin, may play the major role. The existence of no differences in the survival curves after two treatments supports this interpretation. However it does not exclude an action of melatonin on the survival as antioxidant agent (Pieri et al., 1994; Reiter, 1995). Furthermore the food intake and, consequently, the zinc content in the food, is always constant in control and treated mice suggesting no interference of a possible food restriction for the increment of the survival itself (Masoro et al., 1982). Thus, the existence of a good homeostasis between zinc and melatonin, via glucocorticoids, with a beneficial effect on thymic functions during the circadian cycle in old mice may induce a prolonged survival, where, however, zinc may be more involved.

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