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Metallothioneins (I + II) and thyroid–thymus axis efficiency in old mice: role of corticosterone and zinc supply

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Abstract

Thymic atrophy or thymus absence causes depressed thyroid–thymus axis (TTA) efficiency in old, young propyl-thiouracil (PTU) (experimental hypothyroidism) and in young-adult thymectomised (Tx) mice, respectively. Altered zinc turnover may be also involved in depressed TTA efficiency. Zinc turnover is under the control of zinc-bound metallothioneins (Zn-MTs) synthesis. Thyroid hormones, corticosterone and nutritional zinc affect Zn-MT induction. Zn-MT releases zinc in young-adult age during transient oxidative stress for prompt immune response. In constant oxidative stress (ageing and liver regeneration after partial hepatectomy), high liver Zn-MTs, low zinc ion bioavailability and depressed TTA efficiency appear. This last finding suggested that MT might not release zinc during constant oxidative stress leading to impaired TTA efficiency. The aim of this work/study is to clarify the role of Zn-MTs (I + II) in TTA efficiency during development and ageing. The main results are (1) Old and PTU mice display high corticosterone, enhanced liver MTmRNA, low zinc and depressed TTA efficiency restored by zinc supply. Increased survival and no significant increments in basal liver Zn-MTs proteins occur in old and PTU mice after zinc supply. (2) Lot of zinc ions bound with MT in the liver from old mice than young (HPLC). (3) Young-adult Tx mice, evaluated at 15 days from thymectomy, display high MTmRNA and nutritional–endocrine–immune damage restored by zinc supply or by thymus grafts from old zinc-treated mice. (4) Young-adult Tx mice, but evaluated at 40 days from thymectomy, display natural normalisation in MTmRNA and nutritional–endocrine–immune profile with survival similar to normal mice. (5) Stressed (constant dark for 10 days) mice overexpressing MT display low zinc, depressed immunity, reduced thymic cortex, high corticosterone, altered thyroid hormones turnover showing a likeness with old mice. These findings, taken altogether, show that corticosterone is pivotal in MTs induction under stress. MTs bind preferentially zinc ions in constant oxidative stress, but with no release of zinc from

Abbreviations: AT, active thymulin; MTs, metallothioneins; MT-I*, metallothioneins overexpressing mice; PTU, propyl-thiouracil; TECs, thymic epithelial cells; TSH, thyroid stimulating hormone; TTA, thyroid–thymus axis; TT, total thymulin; Tx, thymectomy.

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MT leading to impaired TTA efficiency. Zinc supply restores the defect because zinc has no interference in affecting pre-existing Zn-MTs protein concentrations in old and PTU mice. Therefore, free zinc ions are available for TTA efficiency after zinc supply. Thymus from old zinc-treated mice induces the same restoring effect when transplanted in Tx recipients. However, Tx mice display natural normalisation in MTmRNA and in nutritional–endocrine–immune profile in the long run. Therefore, Zn-MTs (I + II) are crucial in zinc homeostasis for endocrine–immune efficiency during the entire life assuming a role of potential and novel ‘biological clock of ageing’. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Zinc; Metallothioneins; Ageing; Inflammation; Thyroid–thymus axis; Corticosterone; Thymus grafts; PTU; Stress; MTs transgenic mice; Biological clock; Survival

1. Introduction

Thymic atrophy leads to depressed thyroid–thymus axis (TTA) efficiency in old and in young propyl-thiouracil (PTU) treated mice (exp. hypothyroidism) (Fabris, 1992; Abou-Rabia and Kendall, 1994). Young-adult thymectomised (Tx) mice display impaired immune and thyroid functions. Neonatal thymus grafts or triiodothyronine (T_3) treatments restore TTA efficiency (Fabris and Mocchegiani, 1985). In this context, the bioavailability of free zinc ions may be pivotal. Indeed, zinc supply restores TTA efficiency in old mice with prolonged survival (Mocchegiani et al., 1998b) as well as in clinical hypothyroidism (Down’s syndrome) (DS) with reduction of infectious episodes (Licastro et al., 1992). The reasons are two. First, zinc confers biological activity to thymulin (Zn-FTS) (AT) (Dardenne et al., 1982), which decreases in ageing and DS (Fabris et al., 1984). The zinc-unbound form (FTS) of thymulin is inactive and increases in the plasma of both conditions. The *in vitro* zinc addition up to plasma samples unmasks FTS showing the total amount of thymulin produced (active Zn-FTS + inactive FTS) (TT). Therefore, the low zinc ion bioavailability allows a lack in saturating all thymulin molecules produced in ageing and DS (Fabris et al., 1984). Second, zinc finger home domains are required in activating T_3 receptors (Darling et al., 1998) on T-cells (Arpin et al., 2000) and on thymic epithelial cells (TECs) believed to secrete thymulin (Villa-Verde et al., 1992). Zinc turnover is affected by metallothioneins (MTs) synthesis (Kagi and Shaffer, 1988) which is, in turn, induced by thyroid hormones (Yeiser et al., 1999), corticosterone and nutritional zinc (Cousins and

Lee-Ambrose, 1992). MTs bind preferentially zinc than copper in ageing (Hamer, 1986) and hypothyroidism (Yeiser et al., 1999). Zn-MTs (I + II) are zinc donors during transient oxidative stress in young-adult age (Jacob et al., 1999) and in young TECs to activate thymulin (Coto et al., 1992). However, enhanced liver Zn-MTs, low zinc, depressed AT, reduced natural killer (NK) activity and hypothyroidism occur in constant oxidative stress, such as in ageing and liver regeneration after partial hepatectomy (Mocchegiani et al., 1997; Maliekal et al., 1997) as well as in constant dark regarding NK cells distribution (Dhabhar et al., 1995). Thus, while, on one hand, MTs capture zinc in zinc deficiency because zinc ions must not be lost (Kelly et al., 1996), on the other hand, MTs might not be zinc donors in ageing. This latter may cause low zinc ion bioavailability for TTA efficiency and NK activity (Mocchegiani et al., 1998a). The aim of this work/study is to clarify the role of Zn-MTs (I + II) in TTA efficiency during development and ageing. NK cells activity is included because it is affected by zinc and TTA efficiency, via Zn-FTS saturation (Muzzioli et al., 1992), and by stress (Dhabhar et al., 1995).

2. Materials and methods

2.1. Animal models used and treatments

Young, adult, old Balb/c inbred H-2 compatible male mice as well as young Tx mice (thymectomised at 2 months of age after puberty) (adult thymectomy) (Fabris and Mocchegiani, 1985) and PTU mice (0.17 mg/g bw/day of propyl-thiouracil

for 30 days in drinking water) (Mocchegiani et al., 1990) were used. Mice were divided in various groups (ten mice/group) according to treatments (zinc sulphate or thymus grafts) (see Table 1). Tx mice were called: Tx1 = evaluated 15 days after thymectomy and Tx2 = evaluated 40 days after thymectomy (Table 1, groups f, g). Tx1 mice were used for treatments (zinc sulphate or thymus grafts) at 15th day from thymectomy (Piantanelli et al., 1978). No treatment in Tx2 mice.

In particular, old, Tx1 and PTU mice were treated with zinc sulphate in drinking water (18 $\mu\text{g/ml Zn}^{++}$) (physiological dose) (Mocchegiani et al., 1998a) for 30 days (Table 1, groups d, h, o). Controls were treated with tap water (Table 1, groups a, b, c, e, n). Other Tx1 mice were grafted with thymus fragments from young, adult, old and old zinc-treated mice [Table 1, groups i, j, k, l; sham-grafted Tx is respective control (group

m)]. The thymus graft was performed after 15 days from thymectomy (Piantanelli et al., 1978).

Young male transgenic MT mice (MT-I*) [C57BL/6J-TgN(Mt1)174Bri] (Jackson Laboratory, Bar Harbor, ME, USA), in which 56 copies of the MT-I gene (Mt1) were transferred into the genome (Palmiter et al., 1993) and controls C57BL/6J were also used (Table 1, groups p, r). Another group of MT-I* and C57BL/6J mice underwent to stress by constant dark for 10 days (Table 1, groups q, s). This model of stress induces high corticosterone in young animals with major peaks post-meridieum during the circadian cycle (Fischman et al., 1988), as it occurs in old ones (Mocchegiani et al., 1998b). MT-I* mice were stressed because of no difference in basal liver MT between MT-I* and controls (Deng et al., 1999). Other mice (PTU, Tx and PTU + zinc) (50/group) (Table 1, groups t, u, v) were exclusively used for survival analysis (see below).

Table 1
Animal models used and treatments

Animal model	Age (months)	Number	Treatment
(a) Young	2	10	No treatment (used as controls).
(b) Adult	12	10	No treatment (used as controls).
(c) Old	22	10	No treatment (used as controls).
(d) Old	22	10	Treated with zinc sulphate in drinking water (18 $\mu\text{g/ml Zn}^{++}$) for 30 days.
(e) sham-Tx	2	10	No treatment (used as controls of thymectomised mice).
(f) Tx1	2	10	Thymectomised at 2 months of age and evaluated 15 days after thymectomy.
(g) Tx2	2	10	Thymectomised at 2 months of age and evaluated 40 days after thymectomy.
(h) Tx1	2	10	Treated with zinc sulphate in drinking water (18 $\mu\text{g/ml Zn}^{++}$) for 30 days.
(i) Tx1	2	10	Grafted with thymus fragment (2–3 mg) from young mice of 2 months of age.
(j) Tx1	2	10	Grafted with thymus fragment (2–3 mg) from adult mice of 12 months of age.
(k) Tx1	2	10	Grafted with thymus fragment (2–3 mg) from old mice of 22 months of age.
(l) Tx1	2	10	Grafted with thymus fragment (2–3 mg) from old zinc-treated mice of 22 months of age.
(m) sham-grafted Tx	2	10	No treatment (used as control of Tx1 thymus grafted mice).
(n) PTU	2	10	Treated with PTU (0.17 mg/g bw/day) in drinking water for 30 days. Successively, these mice received tap water.
(o) PTU	2	10	After PTU treatment (see point n), these mice were treated with zinc-sulphate in drinking water (18 $\mu\text{g/ml Zn}^{++}$) for 30 days.
(p) MT-I* mice	2	10	Used as controls of stressed MT-I* mice (group q).
(q) MT-I* mice	2	10	Underwent to constant dark for 10 days (stressed).
(r) C57BL/6J	2	10	Used as controls of stressed C57 BL/6J mice (group s).
(s) C57BL/6J	2	10	Underwent to constant dark for 10 days (stressed).
(t) PTU mice	2	50	Treated with PTU for 30 days and used for analysis survival.
(u) Tx mice	2	50	Thymectomised at 2 months of age and used for analysis survival.
(v) PTU mice	2	50	After 30 days of PTU treatment, mice were constantly treated with zinc sulphate in drinking water (18 $\mu\text{g/ml Zn}^{++}$) and used for analysis survival.

2.2. Housing

Inbred Balb/c, C57BL/6J and MT-I* mice used were bred in our 'conventional barrier' (Mocchegiani et al., 1998b), in plastic non-galvanised cages, five mice per cage (36 × 20 × 14 cm) (Technoplast, Italy), and fed with standard pellet food (Nossan, Italy, containing 185 ppm of zinc) and tap water in sterilised 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. with fixed timer governing two standard fluorescent fixtures (Philips TLD 36 W/84), at constant temperature (20 ± 1 °C) and humidity (50 ± 5%). Check-up of environmental factors and bacteriological and serological analysis were carried out monthly following FELASA (1994) suggestions. The maximum life span of inbred Balb/c mice in our housing breeding condition was of 30 months (Mocchegiani et al., 1998b).

2.3. Surgical procedures

2.3.1. Thymectomy

Thymectomy was performed in young mice by suction under ether anaesthesia. At sacrifice, mice showing thymic remnants were discarded. The successful of thymectomy was of 90% (Fabris and Mocchegiani, 1985). Therefore, thymic remnants occurred in one mouse/ten Tx mice. Following that, additional mice (40) were thymectomised in order to substitute discarded Tx mice (1–2/group considered). Thus, ten successful Tx mice/group were guaranteed (Table 1, groups f, g, h, i, j, k, l).

2.3.2. Thymus graft

Grafts of thymuses from mice of different age was performed using a little lobe of the thymus (2–3 mg) in order to avoid differences in total thymus weight between young and old mice (Piantanelli et al., 1978). Such fragments were transplanted in Tx1 mice under the kidney capsule with ether anaesthesia (see Table 1, groups i, j, k, l) (Piantanelli et al., 1978). At sacrifice, the thymus grafted was analysed by histological analysis of the kidney capsule. Grafted mice with no acceptance of the thymus were discarded. The success of the thymus grafted under the kidney capsule

was 90% (Piantanelli et al., 1978). Therefore, success of the thymus grafted occurred in nine Tx1/ten Tx1 mice that underwent to transplantation. Following that, an additional number of Tx1 mice (20) grafted with thymus fragments from young, adult, old and old zinc-treated mice was taken into account in order to substitute the discarded Tx1 thymus grafted mice (1–2/group considered). Thus, ten successful Tx1 thymus grafted mice/group were guaranteed (see Table 1, groups i, j, k, l).

2.4. Sacrifice

Thymus grafted mice were killed after 30 days from transplantation, because the effects of thymus-grafted were evident after 1 month from transplantation (Piantanelli et al., 1978). All other mice were sacrificed following protocols of the treatments (30 days after treatments with zinc sulphate or PTU) (see Table 1). Animals were sacrificed under ether anaesthesia (law n.86/609 by CEE) at 10–12 h p.m. using a red lamp (Philips) in a separate room to avoid aspecific stress to other animals. The choice of 10–12 h p.m. is because corticosterone displays higher peaks during these hours in the circadian cycle in young and old animals (Fischman et al., 1988; Mocchegiani et al., 1998b). Heparinized blood samples (1 ml) were collected by cardiac puncture. Plasma samples stored at –70 °C until used. Tests were performed in blind.

Taking into account the animal models as well as the lot of mice used (Table 1), in vivo treatments were carried out once a time (Fabris and Mocchegiani, 1985), but with ten mice/group. This number is optimal to achieve statistically significant means of the experimental data (Matthews and Farewell, 1988).

2.5. Active thymulin (ZnFTS) (AT) and total thymulin (ZnFTS + FTS) (TT) determination

Plasma active zinc-bound thymulin (ZnFTS), as extensively described elsewhere (Fabris et al., 1984), was measured using a bioassay based on the ability to restore the inhibitory effect of azathioprine on rosette formation in spleen cells from

young Tx mice. Results were expressed as the \log_{-2} of the reciprocal maximal dilution of tested plasma able to induce this phenomenon (Fabris et al., 1984). This bioassay is still required because questions raised over the specificity of the radioimmunoassay (RIA) (Mocchegiani et al., 1998b). In order to avoid interference due to zinc turnover, the thymulin bioassay was also performed by *in vitro* addition of zinc sulphate at final concentration of 200 nM up to plasma samples. This fact shows the total amount of thymulin produced (active ZnFTS + inactive FTS) (TT) (Fabris et al., 1984).

2.6. Histological and immunocytochemistry studies

2.6.1. Histological studies

Four micrometer cryosections of frozen thymus were fixed in 80% cold acetone for 15 min and stained with haematoxylin–eosin.

2.6.2. Immunocytochemistry studies

Immunocytochemical characterisation of thymic epithelial cells (TECs) was detected *in situ* from thymic sections using anti-thymulin MoAb (kindly supplied by M. Dardenne) which can be revealed by the GAM/IgG2a/FITC diluted 1/20 (Dardenne et al., 1989). Anti pan-cytokeratin IgG1/FITC MoAb (Sigma, USA) diluted 1/25 and anti-keratin MoAb (Sigma, USA) diluted 1/20 were also used. For this latter, guinea pig IgG/FITC (Sigma, USA) diluted 1/60 was used as second antibody. These MoAbs are specific to detect TECs (cortical and medullary) (Itoh et al., 1982; Dardenne et al., 1989; Kurz et al., 1996). *In situ* TECs were assessed at fluorescence microscope by counting 100 microscope fields of 135 000 μm^2 from three or four frozen 2 μm sections obtained at different level of the organ (Savino and Dardenne, 1984; Dardenne et al., 1989). For *in vitro* analysis, percentages of separated TECs (see below) from PTU mice and after stimulating agents were counted in 1000 cells under fluorescence microscope. *In situ* and *in vitro* tests were performed after pre-fixation with cold methanol in the slides. Controls were performed without the primary antibodies.

2.6.3. Whole thymuses *in vitro* cultures

Whole thymuses (n.2) from young and PTU mice were put in plate wells containing M10 zinc-free chelated (Chelex 100) medium, as previously described (Mocchegiani et al., 1990, 1998b). Two whole thymuses were optimal to test *in vitro* kinetic thymulin (Mocchegiani et al., 1990). Supernatant samples (50 μl) were taken at two different times of culture (30 min and 6 h). Maximum thymulin production occurs after 6 h of culture with a plateau maintenance in thymulin production up to 12 h of culture with a progressive decline thereafter (Mocchegiani et al., 1990). Such a production is *de novo* synthesis because completely prevented by preincubation with cycloheximide (Mocchegiani et al., 1990). Zinc sulphate was added at final concentration of 1 μM in the culture medium alone or with T_3 (1 ng/ml) (Mocchegiani et al., 1990, 1998b).

2.6.4. TEC separation

TECs were separated following methods described by Itoh (1979) and Kurz et al. (1996). Briefly, the thymus from young and PTU mice after 6 h of culture was minced into small fragments and incubated with collagenase (1 mg/ml, Sigma, USA) in PBS for 1 h at 37 °C (1 ml of collagenase solution/thymus). The suspension was then centrifuged (2 min, 400 g) and the pellet suspended in 1 ml of Dulbecco's modified Eagle medium/Ham's F12 medium (1:1) (DMEM/F12, Gibco, Germany). The cells were subjected to two-steps trypsin (0.1 and 0.25%, respectively) and 0.001% DNase treatment in order to avoid fibroblasts (Kurz et al., 1996). After three washes in PBS, the cells were dissociated by cautious triturating through Eppendorf tips and incubated in 3 ml of DMEM/F12 medium for 2–3 h at 37 °C in humidified 5% CO_2 -atmosphere in order to make to adhere the cells. The supernatant containing unattached TEC was seeded into another plastic flask containing DMEM/F12 medium supplemented with 10% horse serum and put in culture in humidified 5% CO_2 -atmosphere. The cultures were inspected for morphologically visible fibroblasts (spindle shaped cells). In cases of significant contamination, the cells were washed with PBS and underwent again to trypsin-

isation (Kurz et al., 1996). Separated TECs were washed three times in PBS. An aliquota (10^3) was resuspended in 1 ml of medium and underwent to TEC percentage analysis as described by Dardenne et al. (1989).

2.6.5. TEC proliferation

After TEC separation, another aliquota (40×10^3) was resuspended in 4 ml of zinc-free chelated (Chelex 100) DMEM/F12 medium for TEC proliferation analysis, which was approached using [^3H] thymidine incorporation using 96 microtiter plates (Nunc, Denmark). 40×10^3 TECs were put in 40 wells ($100 \mu\text{l}/\text{well} = 10^3$ TECs/well). Ten wells were used as controls; ten were added zinc ($1 \mu\text{M}$); ten were added T_3 (1 ng/ml); ten were added zinc + T_3 . Concomitantly, $1 \mu\text{Ci}$ [^3H]-thymidine/well (Amersham, UK) was also added. The plates were incubated in humidified 5% CO_2 atmosphere for 6 h. Automatic harvester collected the samples and the amount of incorporated radioactivity was determined in a liquid scintillation beta-counter (Perkin–Elmer, USA).

2.6.6. TEC assessment and proliferation in pure rat TEC cell line (IT-45R1)

The same TEC proliferation radioactivity procedure as well as TECs percentage and thymulin production (described above) were tested in 10^3 pure rat TEC cell line (IT-45R1) (HSRRB, Japan), suggested to produce thymulin (Itoh et al., 1982), in standard culture condition and after zinc ($1 \mu\text{M}$) and/or T_3 (1 ng/ml) addition for 6 h in the culture chelated medium (DMEM/F12) at 37°C in CO_2 -atmosphere. In this last contest, corticosterone ($0.5 \mu\text{g/ml}$) (Kurz et al., 1996) was also added to IT-45R1 cells for 6 h of culture.

2.6.7. Crude zinc balance and tissue zinc content

Crude zinc balance was determined during a metabolic period (7 days before sacrifice). Food, water intake, urinary and faecal excretion were measured every day for each mouse in metabolic non-galvanised cages (Technoplast, Italy). The faecal weight was determined in humid faeces. The measure of zinc in urine, faeces, water and food was performed with methods extensively described elsewhere (Mocchegiani et al., 1997). The

crude zinc balance is the difference between zinc intake (food and water) and zinc excretion (urine and faeces). The negativity implies a zinc loss from the body (Mocchegiani et al., 1998a). Plasma and tissue zinc content were determined in Atomic Absorption Spectrophotometry (AAS) against zinc references standard (Sigma, USA), as extensively described elsewhere (Mocchegiani et al., 1997, 1998b).

2.6.8. NK cells assay

The target cells for NK activity were murine lymphoma cell line YAC-1. NK activity, as described elsewhere (Muzzioli et al., 1992), was tested in splenocytes using 2×10^6 cells as target and $100 \mu\text{Ci}$ of ^{51}Cr (Na_2CrO_4 Amersham, UK). Splenocytes were used at the final concentrations of 10×10^6 cells. Results are expressed in Lytic Unity (L.U. $20/10^7$ cells) (Mocchegiani et al., 1997).

2.6.9. Thyroid hormones and corticosterone determination

T_3 , thyroxine (T_4) and thyroid stimulating hormone (TSH) were detected in the plasma using RIA commercial kits (Byk-Mallinckrodt, Germany). Plasma corticosterone level (ng/ml) was determined by RIA rat-corticosterone- ^3H kit (ICN Biomedicals, CA, USA) and 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 corticosterone.

2.6.10. RNA isolation and RT-PCR analysis

Total RNA was extracted from frozen liver using Tri-Reagent according manufacture's instructions (Sigma, USA). Total RNA ($0.1 \mu\text{g}$) was reverse transcribed using a reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM DTT, 150 ng oligo dT, 20 units of Rnase inhibitor, 0.5 mM deoxynucleotide triphosphates and 200 units M-MLV Reverse Transcriptase (Sigma, USA). PCRs were performed using sense and antisense primers as follows:

MT-I 5'-ATGGACCCCAACTGCTCCTGCTCCACC-3', 5'-GGGTGGAAGTGTATAGGAA-GACGCTGG-3' (259 bp), β actin 5'-GGACTCCTATGTGGGTGACGAGG-3' 5'-GGGAG

AGCATAGCCCTCGTAGAT-3' (366 bp). Conditions for amplification were as follows: for MT-I each cycle consisted of 94 °C 0.30 min, 50 °C 0.30 min, 72 °C 0.30 min with 30 cycles; for β -actin each cycle consisted of 94 °C 1 min, 61 °C 1 min, 72 °C 1 min, with 28 cycles. The products of the RT-PCR reactions were size-fractionated by 2% agarose gel electrophoresis and visualised by staining with ethidium bromide. Semi-quantitative analysis of the amplified products was performed by an image analyser (Geldoc 2000 instrument, BIO-RAD, USA). The result was evaluated as a relative unit determined by normalisation of the density of each band to that of the β -actin band.

2.6.11. Tissue MTs (I + II) protein concentrations detection

Liver MTs concentrations were detected by silver-saturation method as extensively described elsewhere (Mocchegiani et al., 1997). Ag bases this technique in the saturation of MTs. Ag + concentration was measured in AAS using Ag + standard solution (Sigma, USA). MTs (I + II) amount was calculated from the obtained Ag + concentrations assuming those 17 mol of Ag + bind 1 mol of MT (Mocchegiani et al., 1997). This methodological procedure has the same sensitivity of radioimmunoassay MTs (I + II) protein detection as previously demonstrated (Leibrant et al., 1991).

2.6.12. Liver MT (I + II) isolation

The peritoneal cavity was opened and the excised livers were kept frozen at -70 °C until analysed. Suzuki's method (1980) was performed. Briefly, livers (0.4–0.7 g) from young and old mice were homogenised in three volumes (w/v) of 20 mM Tris–HCl buffer (pH 8.6 at 25 °C) in presence or in absence of 10 mM mercaptoethanol. The homogenate was centrifuged at 100 000g for 1 h at 4 °C. The resulting supernatants (0.5 ml) were used for MT isolation on a Sephadex G-75 column (0.8 cm \times 30). This was equilibrated with 1 mM Tris–HCl buffer solution in presence or in absence of the reducing agent mercaptoethanol (Suzuki, 1980). The flow rate was 0.4 ml/min. Fractions of 0.5 ml were collected

and rabbit MT was used as a calibration marker. Rabbit MT peak eluted in correspondence of fractions 13–17, which were recognised as containing MT (I + II) (Suzuki, 1980). Zinc and copper concentrations were determined by AAS in each fraction.

2.6.13. Survival analysis

Fifty mice are sufficient for survival analysis (Mocchegiani et al., 1998b). Fifty PTU mice were continuously treated with zinc sulphate (18 μ g/ml Zn^{++}) in drinking water (Table 1, group v). 50 PTU mice treated with tap water served as controls (Table 1, group t). Fifty Tx mice (adult thymectomy) were also used for survival analysis (Table 1, group u). Mice were censored for 2 days and individually weighted at time intervals. Health status of mice was monitored with bacteriological analysis at bronchoalveolar and gastrointestinal levels using standard laboratory methods. Serological analysis (Sendai virus, mouse hepatitis virus, mycoplasma pulmonis and corona virus) (ELISA kits) following FELASA suggestions (1994), was carried out (INRCA Veterinary Service).

2.6.14. Statistical methods

Two-tailed Student's *t*-test, ANOVA test (one-way) and Bonferroni test evaluated differences between means. In particular, Bonferroni test was also used to simultaneously compare all experimental groups before and after treatments. Correlations were determined by linear regression analysis by the least square method. Differences were evaluated by analysis of covariance. Survival analysis was performed using Kaplan–Meier method. Differences were significant when $P < 0.05$.

3. Results

3.1. Nutritional–endocrine–immune profile in Tx, PTU and old mice: effect of zinc supply

Taking into account the neuroendocrine–immune damage in Tx, PTU and old mice (Fabris, 1992; Abou-Rabia and Kendall, 1994) and the

Table 2
Parameters studied in Tx1, PTU and old mice before and after zinc supply

Parameters	Animal models									
	Young (a)	sham-Tx (e)	Tx1 (f)	Tx2 (g)	Adult (b)	PTU (n)	Old (c)	Tx1+Zn (h)	PTU+Zn (o)	Old+Zn (d)
Crude Zn balance ($\mu\text{g/day/mice}$)	+1.5 ± 0.5	+1.2 ± 0.4	-0.8 ± 0.4*	+0.5 ± 0.2	+0.8 ± 0.5	-1.6 ± 0.5*	-1.3 ± 0.3*	+1.0 ± 0.4	+1.0 ± 0.3	+1.2 ± 0.4
Plasma Zn ($\mu\text{g/dl}$)	110 ± 11	98 ± 8	80 ± 6*	90.1 ± 3.2	90 ± 7	82 ± 7*	81 ± 6.3*	90 ± 4.5	95 ± 6	91.3 ± 3.1
A.T. (\log_{-2})	5.2 ± 0.3	5.0 ± 0.4	1.0 ± 0.4*	1.5 ± 0.2*	4.2 ± 0.4	1.0 ± 0.2*	1.0 ± 0.2*	2.0 ± 0.3*	4.0 ± 0.3	4.5 ± 0.4
T.T. (\log_{-2})	5.2 ± 0.2	5.1 ± 0.3	1.0 ± 0.4*	1.5 ± 0.2*	4.5 ± 0.3	4.1 ± 0.3	4.0 ± 0.3	2.0 ± 0.3*	4.2 ± 0.3	4.6 ± 0.3
NK (L.U. $20/10^7$)	31 ± 4	28 ± 7	12 ± 5*	21 ± 2	20 ± 6	10 ± 2*	7 ± 5*	26 ± 6	20 ± 4	19 ± 5
T3 (ng/dl)	103 ± 6.4	102 ± 7.1	60 ± 4.1*	87 ± 4.5	85 ± 3.4	60 ± 10*	45 ± 5.3*	92 ± 6.1	90 ± 9	90 ± 3.7
T4 ($\mu\text{g/dl}$)	6.2 ± 2.2	6.0 ± 1.7	4.0 ± 1.0*	5.6 ± 1.3	5.3 ± 1.4	4.0 ± 1.3	4.3 ± 1.4*	5.0 ± 1.1	5.0 ± 1.0	5.0 ± 1.1
TSH ($\mu\text{U/ml}$)	2.3 ± 0.7	2.1 ± 0.8	3.7 ± 1.4**	3.3 ± 0.7	3.4 ± 1.0	6.0 ± 3.1*	3.8 ± 1.6**	2.5 ± 0.8	2.3 ± 1.0	2.4 ± 0.6
Corticost. (ng/ml)	153 ± 18.3	161 ± 16.4	277 ± 22.4*	155 ± 16	177 ± 14.4	225 ± 17.1*	265 ± 23.2*	166 ± 13.3	144 ± 15	152 ± 12.2

Ten mice/group. Mean ± SD.

* $P < 0.01$.

** $P < 0.05$ when compared PTU, Old and Tx1 mice with respective controls (young-adult for old and PTU mice and sham-Tx for Tx1 mice) (two-tailed Student's *t*-test, ANOVA test, Bonferroni test). A restoration in nutritional-endocrine-immune profile occurs after zinc supply in Tx1, PTU and old mice when compared simultaneously with young, sham-Tx and adult mice ($P > 0.05$) (Bonferroni test).

Table 3
Parameters studied after thymus grafts in Tx1 recipients

Parameters	Animal models						
	sham-Tx graft (m)	Tx1 (f)	Tx2 (g)	Tx1 + old thymus (k)	Tx1 + old thymus + Zn (l)	Tx1 + young thymus (i)	Tx1 + adult thymus (j)
Crude Zn bal. (µg/day/mice)	+1.1 ± 0.3	-0.8 ± 0.4*	+0.5 ± 0.2	-0.9 ± 0.3*	+1.0 ± 0.3	+1.3 ± 0.4	+0.7 ± 0.2
Plasma Zn (µg/dl)	95 ± 7	80 ± 6*	90 ± 3.2	80 ± 3.4*	89 ± 2.7	91 ± 4.5	84 ± 4.0
A.T. (log ₋₂)	5.2 ± 0.4	1.0 ± 0.4*	1.5 ± 0.2	1.0 ± 0.3*	4.6 ± 0.3	5.0 ± 0.4	4.5 ± 0.3
T.T. (log ₋₂)	5.3 ± 0.3	1.5 ± 0.2*	4.0 ± 0.3	2.7 ± 0.2*	5.3 ± 0.4	5.0 ± 0.4	4.7 ± 0.4
NK (L.U. 20/10 ⁷)	26 ± 5	12 ± 5*	21 ± 2	11.7 ± 2.2*	24 ± 3	26 ± 3.5	22 ± 4.0
T3 (ng/dl)	100 ± 6.7	60 ± 4.1*	87 ± 4.5	61 ± 3.8*	95 ± 4.6	103 ± 5.8	91 ± 4.8
T4 (µg/ml)	6.1 ± 1.9	4.0 ± 1.0*	4.6 ± 1.3	4.5 ± 1.0*	5.7 ± 1.3	5.8 ± 1.4	5.5 ± 1.3
TSH (µU/ml)	2.3 ± 0.9	3.7 ± 1.4**	2.8 ± 0.7	3.5 ± 1.3**	2.8 ± 0.8	2.6 ± 0.9	3.5 ± 1.3
Corticosterone (ng/ml)	163 ± 15.4	277 ± 22.4	155 ± 16	267 ± 16*	168 ± 15	165 ± 13.7	171 ± 16.2

Ten mice/group. Mean ± SD.

* $P < 0.01$.

** $P < 0.05$ when compared to Tx1 thymus grafted mice (groups l, i, j), sham-Tx graft and Tx2 mice (Bonferroni test).

role of zinc in affecting neuroendocrine–immune network (Fabris, 1994), some endocrine–immune parameters are tested and related to zinc pool.

Crude zinc balance is negative and AT, NK cells activity, T_3 , T_4 levels are decreased in Tx1, PTU and old mice as compared to sham-Tx and young-adult controls ($P < 0.01$) (Table 2). TT is within the adult normal range in old and PTU mice (Table 2). TSH and corticosterone increase in Tx1, PTU, old mice as compared to young-adult controls ($P < 0.05$ and < 0.01 , respectively). By contrast, Tx2 mice display crude zinc balance, NK cells activity, thyroid hormones and corticosterone levels similar to those ones found in adult controls (Table 2). Therefore, zinc supply is carried out in Tx1, old and PTU mice. Zinc supply restores crude zinc balance and endocrine–immune parameters in Tx1, PTU and old mice (Table 2). No difference exists between AT and TT in old and in PTU zinc-treated mice (Table 2). Significant correlation exists between crude zinc balance and NK activity or T_3 or T_4 or TSH or corticosterone before and after zinc supply in Tx1, PTU and old mice (data not shown).

3.2. Nutritional–endocrine–immune profile in Tx1 mice: effect of thymus grafts

Taking into account: (i) the relevance of zinc supply in recovering endocrine–immune response (Table 2) and thymic efficiency in old mice (Fabris et al., 1997); (ii) the importance of young thymus grafts in restoring endocrine–immune response in Tx mice (Piantanelli et al., 1978; Fabris and Mocchegiani, 1985), a relevant question arises: is thy-

mus from old zinc-treated mice also able to restore endocrine–immune response when transplanted in Tx mice? Thus, thymus grafts from old zinc-treated mice are also carried out in Tx1 mice.

The endocrine–immune profile is altered and the crude zinc balance is still negative in Tx1 mice grafted with thymus from old mice as compared to Tx1 and old mice (Table 3). Grafts with thymus from old zinc-treated mice restore zinc pool and the endocrine–immune profile in Tx1 mice, as it occurs with thymus grafts from young and adult mice (Table 3). No significant difference exists between AT and TT in Tx1 mice grafted with thymus from old zinc-treated mice (Table 3). Significant correlation exists between crude zinc balance and AT or NK activity or T_3 or T_4 or TSH or corticosterone in Tx1 mice before and after thymus grafts from old zinc-treated mice (data not shown).

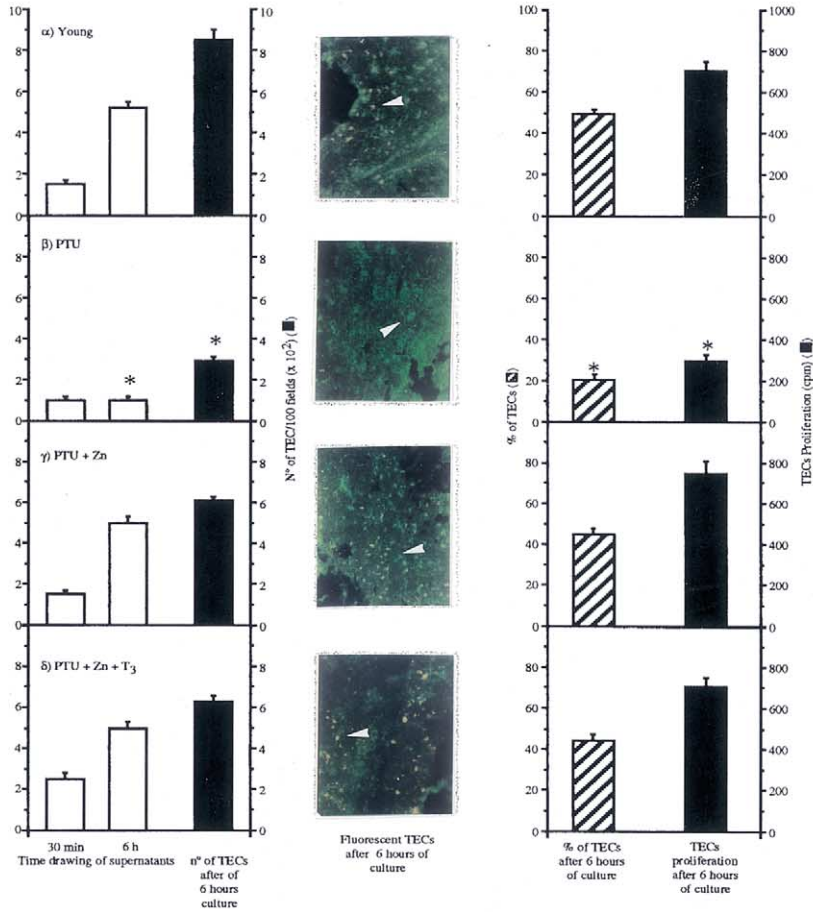
3.3. TECs assessment and proliferation: effect of *in vitro* zinc

In order to confirm *in vivo* data (Table 2), *in vitro* experiments on thymic epithelial cells (TECs) assessment and proliferation by adding zinc and/or T_3 in the culture medium are carried out.

AT production, TEC proliferation, as well as TEC assessment (percent and number) are reduced after 6 h of culture in PTU mice as compared to young ($P < 0.01$) (Fig. 1A). *In vitro* zinc restores AT production, TEC proliferation and assessment (after 6 h of culture) (Fig. 1A). No synergistic increment occurs with *in vitro* zinc + T_3

Fig. 1. (A) *In vitro* effect of zinc and zinc + T_3 on active thymulin production from thymic explant of PTU mice during two times of culture (30 min and 6 h). TECs number detected after 6 h of culture. Photos of fluorescent TECs (white arrow) are reported. Lecture ($40\times$); photos (magnitude $10\times$). [Note: yellow spots are aspecific fluorescence impossible to avoid due to fraying of the thymus in the medium after 6 h of culture with new perivascular spaces imbued in the medium (Mocchegiani et al., 1998b)]. * $P < 0.01$ vs. young (ANOVA, Bonferroni test). TECs proliferation and percentages are also reported. Significant decrements in PTU mice (β) are observed as compared to young (α) ($P < 0.01$). Zinc restores them (γ) with no synergism after zinc + T_3 addition (δ) as compared to zinc alone (γ). Mean \pm SD of four cultures with two thymuses each. Number of TECs and photos were performed using anti-thymulin MoAb. Anti-keratin MoAb for testing TEC percentage. No differences in *in situ* TECs number were observed between anti-thymulin and anti-keratin MoAbs (data not shown). (B) Active thymulin production, TEC proliferation and percentage in pure murine TEC cell line 9IT-45R1) and after the addition of zinc, T_3 , zinc + T_3 and corticosterone. Mean \pm SD of four experiments each on triplicate cultures. Active thymulin was evaluated by the rosette assay, TEC percentage by using anti pan-cytocheratin FITC MoAb. * $P < 0.05$ as compared to none (control culture) or T_3 . ** $P < 0.01$ as compared to none (ANOVA, Bonferroni test).

A) Murine TECs



B) Pure murine TECS cell line (IT - 45R1)

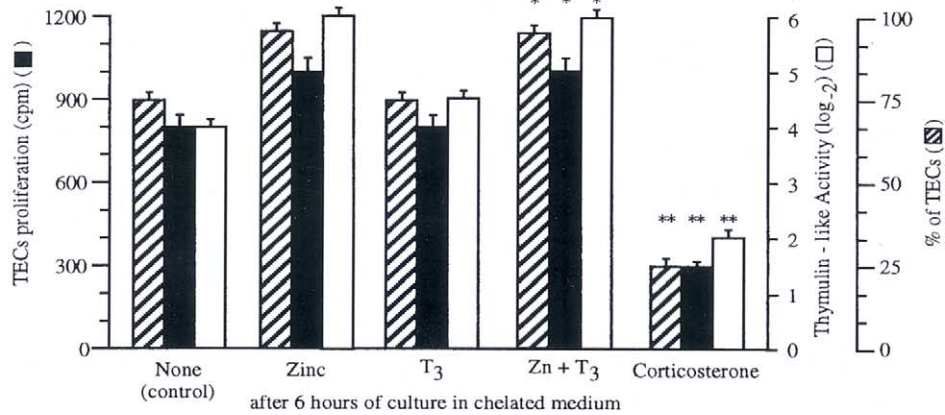


Fig. 1.

(Fig. 1A). A quota of zinc is present in thymic tissue of PTU mice after 6 h of culture ($93.7 \pm 6.4 \mu\text{g/g}$ vs. $153.3 \pm 9.7 \mu\text{g/g}$ in young controls; $P < 0.05$). It justifies the presence of TECs in PTU because zinc affects TECs assessment and proliferation, as shown after BrdUrd incorporation in the thymus of old zinc-treated mice (Fabris et al., 1997). But, this quota of zinc is not sufficient to induce the activation of all thymulin molecules produced after 6 h of culture, as it occurs in old thymic tissue (Mocchegiani et al., 1998b).

Taking into account 6 h of culture plus the essential times in TECs separation technical procedure, TEC percentage is performed after 16–18 h from the beginning of whole thymuses cultures. Thymulin production progressively declines after 13–24 h of culture (Mocchegiani et al., 1990). Therefore, these percentages are in agreement with in vitro TEC percentages after 1 day of murine culture (Dardenne et al., 1989), suggesting that a quota of TECs are in resting status.

Zinc also significantly increases active thymulin production, TEC percentage and proliferation in pure rat TEC cell line (IT-45R1) as compared to control culture ($P < 0.05$) (Fig. 1B). T_3 has no effect with any synergism in TECs assessment and proliferation by in vitro addition of T_3 + zinc (Fig. 1B). Corticosterone decreases IT-45R1 cell assessment and proliferation as compared to control culture ($P < 0.01$) (Fig. 1B).

3.4. Zinc and copper bound to MT in the liver of young and old mice

Zinc ions affect immune efficiency (Table 2) and MTs bind zinc and copper (Kagi and Shaffer, 1988). Thus, it is interesting to explore the quota of zinc or copper ions bound with MTs in ageing. We have used the liver for two reasons. First, because MTs are mainly produced in the liver (Kagi and Shaffer, 1988); second, because the liver is a site of extrathymic T-cell pathway prominent in ageing (Abo et al., 2000), and zinc is also required for extrathymic T-cell pathway (Mocchegiani et al., 1998a). Thus, separation of MTs is carried out from the liver of young and old mice using Sephadex G-75 columns (see Section 2).

Gelfiltration chromatography of all liver cytosol

reveals the presence of two main peaks of zinc. Typical chromatograms of liver preparations from a young and old mouse are reported in Fig. 2. A major peak of zinc, bound to high molecular weight proteins (HP), elutes between fractions 8 and 12. A minor one, bound to MT, is present in fractions 13–17. As regard to copper only one peak, in correspondence of MT elution volume, is observed. The amount of zinc bound to MT is significantly higher ($P < 0.05$) in old mice ($0.79 \pm 0.1 \mu\text{g}$; $n = 3$) respect to young ones ($0.65 \pm 0.01 \mu\text{g}$; $n = 3$). On the contrary zinc sequestered by HP is higher in young mice ($1.5 \pm 0.011 \mu\text{g}$) respect to old mice ($1.05 \pm 0.013 \mu\text{g}$). However, this differences is not significant ($P > 0.05$) (Fig. 2). As regard to copper, more reduced peak exists as compared to zinc, with no significant differences in Cu-MTs between young and old mice ($0.26 \pm 0.013 \mu\text{g}$ in young vs. $0.29 \pm 0.011 \mu\text{g}$ in old; $P > 0.05$) (Fig. 2). Similar results are obtained in presence of mercaptoethanol (data not shown).

3.5. MTmRNA expression, Zn-MTs levels and zinc content in the liver of old, Tx, PTU mice

Following data obtained from Table 2 and Figs. 1 and 2 and taking into account that zinc affects

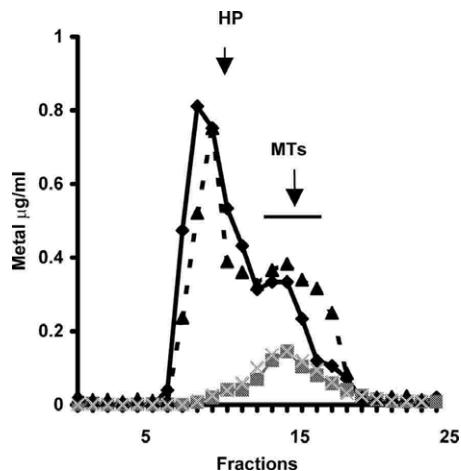


Fig. 2. Sephadex G-75 elution profile of liver cytosol from young (black line) and old (hatched line) mice. The column was equilibrated with 20 mM Tris-HCl, pH 8.6. (Black triangle and rhombus for zinc; grey square and symbol X for copper.) Peak (fractions 7–12) for high molecular weight proteins (HP). Peak (fractions 13–17) for MT.

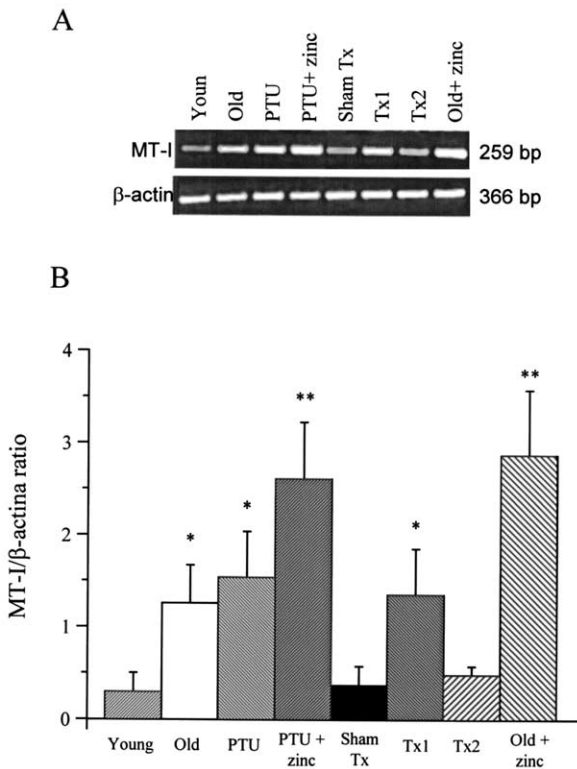


Fig. 3. (A) RT-PCR analysis of liver mRNA using specific primers for murine MT-I and β -actin under conditions described in Materials and Methods. (B) After mRNA isolation and cDNA synthesis, the amount of mRNA for MT-I was determined by semi-quantitative PCR. Results of densitometry analysis are shown in the histograms and are expressed as MT-I/ β -actina ratio. Results are the mean \pm SD of 10 mice/group. * $P < 0.01$ as compared to young, sham-Tx and Tx2 mice; ** $P < 0.01$ as compared to PTU and old mice (ANOVA, Bonferroni test).

MTmRNA expression (Cousins and Lee-Ambrose, 1992), MTmRNA and Zn-MTs protein concentrations are tested before and after zinc supply in old and PTU mice.

Liver MTmRNA expression (RT-PCR) and its quantification are reported in Fig. 3A and B, respectively. The semi-quantitative analysis shows that MTmRNA is increased in old, PTU and Tx1 mice as compared to young, sham-Tx and Tx2 mice ($P < 0.01$) (Fig. 3B). Zn-MTs protein concentrations and zinc content are increased in the liver from old, Tx1 and PTU mice as compared to young, sham-Tx and Tx2 mice ($P < 0.01$ and $P <$

0.05, respectively) (Table 4) because AAS tests zinc-bound and zinc-unbound (Mocchegiani et al., 1998a). Zinc supply increases liver MTmRNA expression in old and PTU mice (Fig. 3A and B), with no significant modifications in Zn-MTs protein levels as compared to old and PTU controls ($P > 0.05$) (Table 4). Zinc content is normal in the liver of Tx2, PTU and old mice after zinc supply (Table 4). Significant inverse correlation exists between Zn-MTs proteins and corticosterone concentrations before and after zinc supply in old and PTU mice ($r = -0.61$, $P < 0.05$ and $r = -0.54$, $P < 0.05$, respectively).

3.6. Nutritional–endocrine–immune profile in MT-I* mice in absence/presence of stress

In order to confirm that high constant corticosterone may induce abnormal MTs induction with no subsequent release of zinc from MT and endocrine–immune damage, stressed mice overexpressing MT (MT-I*) are good models because MTmRNA is similar between MT-I* and controls in absence of stress (Deng et al., 1999). This fact suggests that MTs are in quiescent status in absence of stress.

No stressed MT-I* mice show quite normal nutritional–endocrine–immune parameters with, however, a trend to alterations in comparison to respective controls (C57BL/6J) (Table 5). Thymic cortex is quite similar to young controls (Fig. 4a and c). Such a trend becomes significant in stressed MT-I* mice. Indeed, AT levels and NK cells activity are reduced in stressed MT-I* as compared to respective controls ($P < 0.01$), despite plasma zinc concentrations are within the normal range (Table 5). TT levels are within the normal range in stressed MT-I* mice as compared to respective controls (Table 5). The thickness of the thymic cortex is reduced in stressed MT-I* and old mice as compared to MT-I* and young controls ($P < 0.001$) (Table 6). Thyroid hormones turnover is altered. Plasma corticosterone and liver Zn-MTs concentrations are higher in stressed MT-I* mice in comparison with MT-I* and young controls ($P < 0.01$) (Table 5). With regard to stressed C57BL/6J mice, the nutritional–endocrine–immune profile is altered and the thick-

ness of the thymic cortex is reduced as compared to no stressed C57BL/6J ($P < 0.01$) (Table 6). However, stressed MT-I* mice display a more significant damage in nutritional–endocrine–immune profile and in thymic cortex than stressed C57BL/6J mice (Tables 5 and 6 and Fig. 4). No differences exist among C57BL/6J and inbred Balb/c (Table 5).

3.7. Survival in PTU and Tx mice

PTU and old mice display nutritional–endocrine–immune damage (Table 2) and increased MTmRNA (Fig. 3A and B). Zinc supply prolongs the survival in old mice (Mocchegiani et al., 1998b) with no interference in basal MT levels (Table 4). Tx2 mice display natural normalisation in nutritional–endocrine–immune profile and in MTmRNA (Tables 2 and 4). Thus, the survival in PTU mice during zinc supply and in Tx ones is assessed.

Before PTU treatment, the health status is within the FELASA ‘conventional housing’ normal range (INRCA Veterinary Service). After PTU treatment, PTU mice show shorter survival (15 months) as compared to normal mice (30 months) ($P < 0.001$, Log-rank test) (Fig. 5). Degenerative diseases and lung infections with frequency of 42 and 58%, respectively, are the main causes of death in PTU mice. Zinc supply increases the survival in PTU mice up to 28 months (Fig. 5) with decrements from 58 to 32% in infections and from 42 to 20% in degenerative diseases. The survival is similar between Tx and normal mice (27 vs. 30 months) ($P > 0.05$, Log-rank test) (Fig. 5). No variations in food intake and body weight are observed during treatments (data not shown).

Table 4

Zn-MTs (I+II) protein concentrations and zinc content in the liver of mice

Parameters	Animal models							
	Young	Old	PTU	PTU+zinc	sham-Tx	Tx1	Tx2	Old+zinc
Zn-MTs (I+II) ($\mu\text{g/g ww}$)	4.8 ± 1.4	$8.5 \pm 1.8^*$	$7.3 \pm 2.1^*$	11.3 ± 3.5	5.7 ± 1.5	$9.3 \pm 2.6^*$	4.5 ± 1.9	11.1 ± 4.1
Zinc tissue ($\mu\text{g/g}$)	17.4 ± 2.7	$21.3 \pm 4.7^{**}$	$22.7 \pm 4.4^{**}$	15.6 ± 4.3	16.2 ± 2.0	$19.4 \pm 2.9^{**}$	13.8 ± 1.2	18.0 ± 4.1

Ten mice/group. Mean \pm SD.

* $P < 0.01$.

** $P < 0.05$ when compared to young or sham-Tx control (ANOVA, Bonferroni test).

4. Discussion

Zinc-bound MTs I + II (Zn-MTs), via corticosterone turnover, is crucial in zinc homeostasis for TTA efficiency during development and ageing. Negative crude zinc balance, depressed thymic and NK activities, high liver MTs, enhanced corticosterone and altered thyroid hormones turnover occur in old and PTU mice (Tables 2 and 4). The likeness between old and PTU mice is largely due to the thymic atrophy in both strains of mice (Abou-Rabia and Kendall, 1994; Fabris et al., 1997). As such, degenerative diseases rise in PTU mice (Rittenhouse and Redei, 1997) affecting the survival, despite good ‘health status’ observed before PTU treatment (F. Orlando, unpublished observation). Physiological zinc supply restores thymic and NK activities as well as the survival in old (Mocchegiani et al., 1998a) and PTU mice with no modifications in basal liver MT concentration (Table 4). Young-adult Tx1 mice (evaluated 15 days after thymectomy) display high liver MTs, increased corticosterone, negative crude balance, altered thyroid hormones turnover and impaired NK cells activity, which are restored by zinc supply or by thymus grafts from old zinc-treated mice (Table 3). In contrast, young-adult Tx2 mice (evaluated 40 days after thymectomy) display positive crude zinc balance, normal plasma corticosterone and liver MT level, satisfactory thyroid–immune profile and survival similar to normal mice (Tables 2 and 4 and Fig. 5). In vitro zinc restores TEC assessment and proliferation in PTU mice (Fig. 1A), as it occurs in old ones (Mocchegiani et al., 1998b). Significant inverse correlation exists between corticosterone and Zn–

Table 5
Parameters studied in stressed MT-I* and C57BL/6J

Parameters	Animal models				
	str. MT-I*	MT-I*	str.C57BL/6J	C57BL/6J	Inbr. Balb/c
Plasma Zn (µg/dl)	95 ± 8.7	108 ± 6.7	100 ± 6.4	116 ± 9	110 ± 11
A.T. (log ₋₂)	1.3 ± 0.2**	3.2 ± 0.2	2.5 ± 0.2	5.7 ± 0.3	5.2 ± 0.3
T.T. (log ₋₂)	5.0 ± 0.3	5.1 ± 0.3	4.8 ± 0.3	5.6 ± 0.2	5.2 ± 0.2
NK (L.U. 20/10 ⁷)	15.7 ± 2.0*	30.6 ± 1.8	24.6 ± 2.8	31.8 ± 4.2	31 ± 4
T3 (ng/dl)	75.4 ± 4.2**	95.2 ± 5.7	85.6 ± 4.7	108 ± 6.4	103 ± 6.1
T4 (µg/dl)	4.4 ± 1.3**	5.3 ± 1.0	5.0 ± 1.1	6.3 ± 2.3	6.2 ± 2.2
TSH (µU/dl)	4.0 ± 0.9**	3.0 ± 0.6	2.9 ± 0.5	2.2 ± 0.5	2.3 ± 0.7
Corticosterone (ng/ml)	290 ± 15.2*	180 ± 12.4	240 ± 16.6	145 ± 21.2	153 ± 18.3
Liv. Zn-MTs (µg/g ww)	13.5 ± 1.3**	6.2 ± 1.6	8.5 ± 1.5	5.0 ± 1.2	4.8 ± 1.4

Ten mice/group. Mean ± SD.

* $P < 0.01$.

** $P < 0.05$ as compared to MT-I*, str. C57BL/6J and controls (ANOVA and Bonferroni test).

MTs before and after zinc supply in old and PTU mice. High corticosterone decreases TEC proliferation in pure TEC cell line (IT-45R1) (Fig. 1B).

Immune damage (Khansari et al., 1990) and zinc loss occur during stress (King 1990). A synchrony exists between high TSH and enhanced adrenocorticotropin (ACTH) within the anterior pituitary in stress condition (Childs, 1992). Therefore, our findings confirm that high corticosterone is associated with low zinc ion bioavailability and thyroid-immune alteration in old and PTU mice. But, at the same time, they pin-point that high constant corticosterone affects abnormal MTs induction with no release of zinc from MT. Zinc is relevant for endocrine-immune response (Mocchegiani et al., 2000b) and MT binds preferentially zinc ions in ageing (Fig. 2). Therefore, the no release of zinc by MT leads to few free zinc ions for TTA efficiency with subsequent TTA impairment. Zinc supply restores nutritional-endocrine-immune profile and prolongs survival in old (Mocchegiani et al., 1998a) and PTU mice (Fig. 5). Thus, MTs synthesis, via corticosterone, is crucial in zinc homeostasis for TTA efficiency during development and ageing. Such an interpretation is much more relevant and, at the same time, very intriguing because zinc is also required for liver extrathymic T-cell pathway (Mocchegiani et al., 1998a), which is gradually prominent in Tx and old mice in order to compensate thymus absence or thymic atrophy, respectively (Abo et al., 2000). Indeed, Tx2 mice display

natural normalisation in MTmRNA, in nutritional-endocrine-immune profile and in survival. Therefore, the thymus gland may be more useful in TTA efficiency during development than in ageing in which, on the contrary, zinc turnover may become prominent. The restoration in nutritional-endocrine-immune profile by thymus grafts from old zinc-treated mice or by zinc supply in Tx1 recipients is on line with this interpretation. The discovery showing multiple neonatal thymus grafts ineffective on survival in old mice (Hirokawa, 1997), in comparison with zinc supply (Mocchegiani et al., 1998b), supports this interpretation.

Zn-MTs release zinc in young TECs to activate thymulin (Coto et al., 1992) and in the cytosol of endothelial cells for antioxidant enzyme activity, via glutathione reductase or NO nitrosylation (Jacob et al., 1998; Maret et al., 1999; Zangger et al., 2001). Such a release occurs in young-adult age during transient oxidative stress (Jacob et al., 1999). Oxidative stress and zinc deficiency are persistent in ageing (Fabris, 1994) and hypothyroidism (Licastro et al., 1992; Thoei et al., 1997). MTs capture zinc in zinc deficiency because zinc ions must not be lost (Kelly et al., 1996). But, the task of Zn-MTs as zinc donors (Coto et al., 1992; Kelly et al., 1996; Jacob et al., 1999) may be questioned in constant oxidative stress. Indeed: (i) exogenous zinc is necessary in the plasma samples from old (Mocchegiani et al. 1998a) and PTU mice as well as in the plasma from Down's syn-

drome subjects to activate thymulin, despite plasma zinc values are within the normal range for age (Fabris et al., 1984); (ii) *in vitro* zinc, rather than T_3 , restores TECs assessment and proliferation in PTU thymic cultures and increases TEC proliferation in pure rat TEC cell line (IT-45R1) (Fig. 1A and B). In this context, direct mechanisms of zinc involving protein-kinase-C (PKC) activation (Coto et al., 1992; Saha

et al., 1995) or phase S stimulation of the cell cycle (Dreosti, 2001) have been proposed; (iii) high liver Zn-MTs (I + II), low zinc and depressed TTA efficiency are observed in young partial hepatectomised mice during liver regeneration (model of persistent oxidative stress and great inflammation) (Mocchegiani et al., 1997); (iv) lot of zinc ions bound with MT in the liver from old mice than young (Fig. 2); (v) mice overexpressing MTs

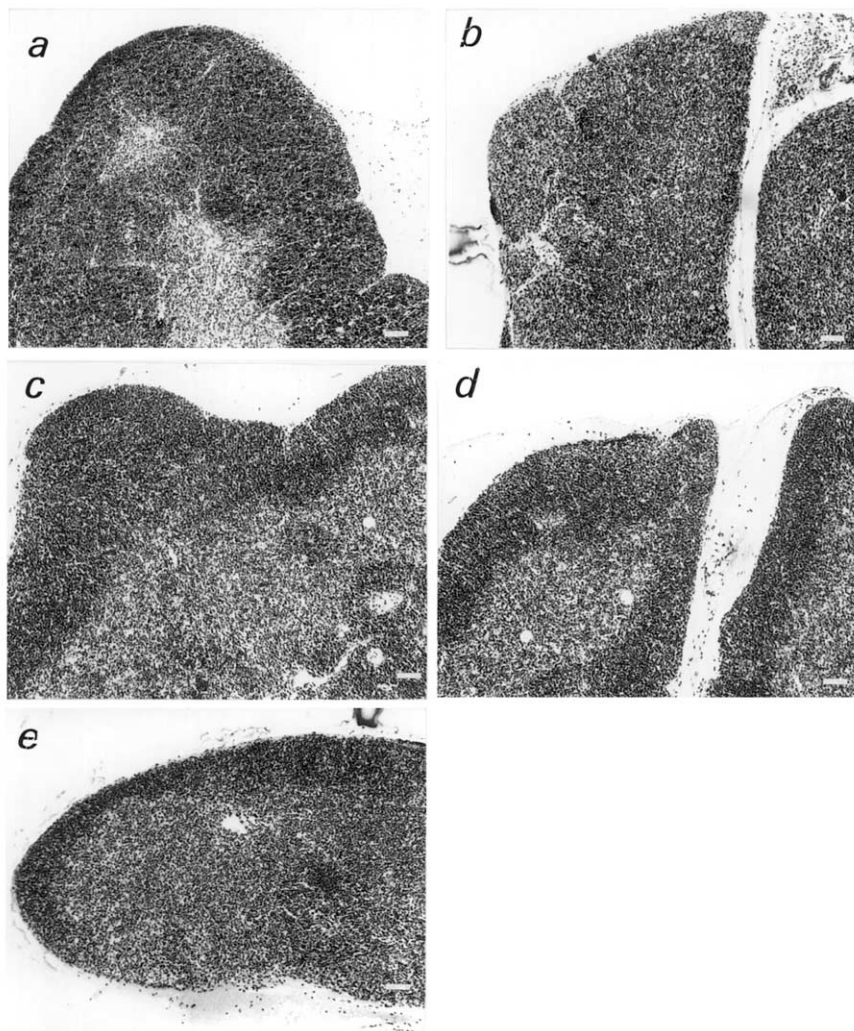


Fig. 4. Thymic histology in young (a), old (b), MT-I*(c), young stressed C57BL/6J (d) and stressed MT-I* (e) mice. Young (2 months of age) and old (22 months of age) are C57BL/6J controls (magnitude $10\times$). The thickness of the thymic cortex (more dark area of thymic cellularity) [measured in various fields of thymic sections by computer assisted image analyser (KS 300, Kontron, USA)], is reported in Table 5. Thymic cortex is absent in old mice (b) and very limited in stressed MT-I* mice (e). Scale bar in white = 125 μm .

Table 6

Thickness of thymic cortex in MT-I* mice and controls exposed to stress

Animals	Thickness (μm)
Young controls (C57BL/6J)	450.3 \pm 19.6
Old controls (C57BL/6J)	78.2 \pm 13.4*
Young-stressed controls (C57BL/6J)	253.2 \pm 21.1**
MT-I*	315.8 \pm 28.6
Stressed MT-I*	138.7 \pm 17.6*

Ten mice/group. Mean \pm SD.

* $P < 0.001$ as compared to MT-I* and young controls.

** $P < 0.01$ as compared to young controls (ANOVA and Bonferroni test).

(MT-I*) underwent to stress (constant dark for 10 days) display altered nutritional–endocrine–immune profile looking like old mice. The likeness is especially in reduced thymic cortex and in adding exogenous zinc in the plasma to reactivate thymulin, despite plasma zinc levels are within the normal range (Tables 5 and 6). All these points suggest no or very limited release of zinc from MT during high constant corticosterone production leading to low zinc ion bioavailability for endocrine–immune response.

Some direct and indirect evidences support this interpretation. The most important direct evidences are: (i) the presence of high Zn-MTs con-

centrations and low zinc ion bioavailability in the atrophic thymus of old mice (Mocchegiani et al., 1998a), and high corticosterone decreases TEC proliferation (Fig. 1B) and increases thymocytes apoptosis in old (Fabris et al., 1997) and stressed MT-I* mice (Deng et al., 1999); (ii) the persistence of high liver MTmRNA, enhanced corticosterone, low zinc and depressed liver NK cells activity during the whole circadian cycle in old mice (Mocchegiani et al., 2000a); (iii) the presence of high MTmRNA in lymphocytes from Down's syndrome subjects and old people in comparison to young (Mocchegiani et al., 2001). The most relevant indirect evidence is the failure of zinc-finger protein (A20) in protecting liver cells by apoptosis induced by TNF- α (Lee et al., 2000), which is in turn augmented in ageing (Fagiolo et al., 1993) and involved in MT induction (Andrews, 2000).

Zinc supply may provoke the release of zinc because zinc supply induces a faster degradation of Zn-MTs in lysosomes with complete release of zinc from MT (Klaassen et al., 1994). In agreement with Cousins and Lee-Ambrose (1992), zinc supply increases liver MTmRNA in old and PTU mice (Fig. 3A and B) with, however, no significant modifications in basal liver Zn-MTs (I + II) concentrations after zinc supply (Table 4). These findings confirm that Zn-MTs (I + II) are saturated by pre-existing zinc ions during constant oxidative stress, as shown in old hepatectomised mice during liver regeneration (Mocchegiani et al., 1997). But, at the same time, they also suggest that increased MTmRNA by zinc may be related to faster Zn-MTs degradation with a balance between Zn-MTs production and degradation. As a consequence, a plateau in Zn-MTs protein production is maintained. Therefore, zinc supply preserves this balance and, as such, free zinc ions are always available for TTA efficiency. The presence of significant inverse correlation between Zn-MTs and corticosterone before and after zinc supply in PTU and old mice is on line with this assumption, which is reinforced by low MTmRNA in lymphocytes from very old mice (30 months of age) and human exceptional individuals (centenarians) (Mocchegiani et al., 2001).

In conclusion, abnormal Zn-MTs (I + II) production, via high corticosterone, are not of benefit

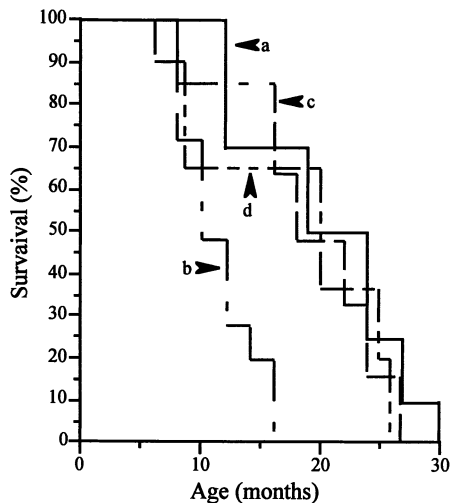


Fig. 5. Rate of survival (Kaplan–Meir) in normal (a), PTU (b), PTU zinc-treated (c) and Tx mice (d).

in TTA efficiency in ageing because of the inability to release zinc. Zinc supply, rather than thymus grafts, restores the defect. Thus, zinc-bound MTs (I + II) may be pivotal in maintaining zinc homeostasis and, consequently, TTA efficiency during the entire life with a possible role of potential and novel ‘biological clock of ageing’.

The trafficking of Zn-MTs within the cytosol and the nucleus involving chaperones activity (Cherian and Apostolova, 2001) may be related to no release of zinc from MT in ageing. Chaperones activity is required in the correct folding of proteins, is zinc-dependent (Jakob et al., 2000) and decreases in old age (Cuervo and Dice, 2000). Therefore, the different task of Zn-MTs as zinc donors between young and old age may be related to modified chaperones activity, representing, as such, an interesting future tool to understand the role of Zn-MTs in immune–neuroendocrine senescence.

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