Effects of zinc-fortified drinking skim milk (as functional food) on cytokine release and thymic hormone activity in very old persons: a pilot study

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Received: 28 November 2013 / Accepted: 8 April 2014 / Published online: 26 April 2014
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Abstract Zinc is a relevant nutritional factor for the whole life of an organism because it affects the inflammatory/immune response and antioxidant activity, leading to a healthy state. Despite its important function, the dietary intake of zinc is inadequate in elderly. Possible interventions include food fortification because it does not require changes in dietary patterns, the cost is low and it can reach a large portion of the elderly population, including very old subjects. Studies evaluating the impact of Zn-fortified foods on functional parameters in elderly, in particular, in very old individuals, are missing. The objective of this study was to evaluate the efficacy of consumption of a zinc-fortified drinking skim milk

(Zn-FMilk) for a period of 2 months in comparison to standard non-fortified milk (No-FMilk) on some biochemical parameters, zinc status, inflammatory/immune response and on a key parameter of the T cell-mediated immunity (thymulin hormone) in healthy very old subjects. The treatment with zinc-fortified milk (Zn-FMilk) is a good omen to increase the cell-mediated immunity in very old age represented by thymulin activity and some cytokine (IL-12p70, IFN- γ) release. At clinical level, a good healthy state occurs in 70 % of the subjects with no hospitalization after 1 year of the follow-up in comparison to very old control subjects that did not participate to crossover design. In conclusion, the Zn-FMilk can be considered a good functional food for elderly, including older people. It might be a good replacement to the zinc tablets or lozenges taking into account the attitude of old people to uptake milk as a preferential food.

Keywords Zinc-fortified milk · Inflammatory/immune response · Thymulin · Cytokines · Ageing

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Introduction

Zinc is one of the most relevant nutritional factors for the whole life of an organism because it affects the inflammatory/immune response, metabolic harmony and antioxidant activity, leading to a healthy state (Rink and Haase 2007; Mocchegiani et al. 2013). Despite its important function, the dietary intake of zinc

is unfortunately inadequate in the elderly (Ames 2006), and several factors contribute to the marginal zinc deficiency. Firstly, the poor socioeconomic status of many elderly individuals can lead to a greater consumption of inexpensive foods deficient of this micronutrient (Kant 2000). The zinc deficiency is then exacerbated by loss of appetite, lack of teeth and decreased energy requirement, leading to frailty, disability and functional dependence (Failla 2003). Moreover, a lower absorptive efficiency has been also reported in the elderly (Meydani 2001), which could justify a dietary requirement higher than that for adults. The old body has limited zinc stores that are easily depleted by the kidney due to the presence of chronic inflammation (Prasad 2009) and to the unbalance in zinc transporter families (Zip and ZnT families) (Cragg et al. 2005; Kahmann et al. 2008; Mocchegiani et al. 2013; Giacconi et al. 2012; Haase and Rink 2014). As such, the old body is unable to compensate long periods of zinc deficiency. Moreover, alterations in zinc uptake, retention, sequestration or secretion can quickly lead to zinc deficiency and affect many zinc-dependent functions in tissues, organs and systems, including the immune system. Additionally, ageing as well as zinc deficiency is associated with dysregulated basal cytokine release (Kahmann et al. 2008; Mariani et al. 2006; Mariani et al. 2008), leading to a status of low-grade inflammation (Franceschi et al. 2000). The latter condition seems to predispose the individuals to increased incidences of some inflammatory age-related diseases, such as Alzheimer's disease, atherosclerosis and infections (Franceschi et al. 2000). Since zinc deficiency is often marginal and asymptomatic, nutritional zinc correction may have a significant impact on different aspects of the human health. Possible interventions include supplementation, fortification and dietary diversification or modification. In this context, zinc supplementation in tablets or lozenges, as "zinc sulphate" or "zinc gluconate" or "zinc aspartate", has been reported to be of benefit in elderly with a relevant impact on inflammatory/immune response (Mocchegiani et al. 2013), psychological parameters (Marcellini et al. 2006) and in restoring T cell functions (Kahmann et al. 2008). However, a particular caution has to be taken into account because zinc supplementation for long periods and with high doses can lead to zinc accumulation in the cells with a possible development of adverse effects on the immune functions (Bozym et al. 2010), worsening the just precarious

immune picture of the old individuals (Mocchegiani et al. 2013). A particular attention needs also to be addressed to the age of the old subjects that need zinc supplementation because of the extreme cellular frailty present in older individuals (Collerton et al. 2012) that can lead to no significant biochemical modifications even with low doses of zinc in tablets (Hininger-Favier et al. 2007) with also adverse effects and toxicity (Hsu and Guo 2002). In this context, fortified foods (functional foods) are an emerging field in food science with the intention of increasing the life expectancy and improving human health conditions (Villalpando et al. 2006; Sazawal et al. 2010; Vinodkumar et al. 2009). Food fortification holds promise, because it does not require changes in dietary patterns, the cost is low and, as such, it can reach a large portion of the elderly population, including very old subjects. Taking into account the attitudes to the milk consumption in old people (Elbon et al. 1996) and the low frequency of lactose intolerance and lactose maldigestion in ageing (Carroccio et al. 1998), the zinc-fortified milk could be an excellent way to provide a nutrient-rich meal in elderly people and to compensate the reduced dietary zinc intake, which in turn shows a further progressive decline up to very old age (Kanoni et al. 2010). Studies evaluating the impact of Zn-fortified foods on functional parameters in elderly, in particular, in very old individuals, are missing. The objective of this study was to evaluate the efficacy of consumption of a zinc-fortified drinking skim milk (Zn-FMilk) for a period of 2 months in comparison to standard non-fortified milk (No-FMilk) on biochemical parameters, zinc status (plasma zinc, intracellular zinc, zinc release by metallothioneins (MT)), inflammatory/immune response (pro-, anti-inflammatory cytokine release) and on a key parameter of the T cell-mediated immunity (thymic hormone activity (thymulin)) in healthy, very old subjects. Such a thymulin activity is fundamental because it is closely related to the zinc ion bioavailability within the thymus (Mocchegiani and Fabris 1995). The Cu/Zn ratio, which is a strong index of the inflammatory status (Malavolta et al. 2010), will be also tested in order to check the effect of Zn-FMilk consumption on the degree of inflammation. A period of follow-up (1 year) is considered to evaluate the impact of Zn-FMilk consumption at clinical level in comparison to very old control subjects that did not participate to crossover design.

Materials and methods

Subjects and study design

A total of $n=21$ elderly subjects, 6 men and 15 women (mean age 87 ± 5.3 years), were enrolled from two nursing homes in Region Marche (Central Italy) for crossover design. Each participant was fully informed about the characteristics of the study, and a written informed consent was obtained and signed. The study protocol was approved by the Ethical Committee of the INRCA Hospital. Eligible subjects (aged ≥ 82 years) were apparently in healthy state, with no lactose intolerance and no use of drugs known to influence the immune system, i.e. steroids and nonsteroidal anti-inflammatory drugs (NSAID). Some subjects ($n=15$; 3 males and 12 females) made use of some diuretic drugs also for the whole period of the zinc trial. Elderly with degenerative age-related diseases (cancer, severe infections, diabetes, severe cardiopathies and neurodegenerative diseases) were excluded from the study. A control group ($n=23$ subjects, 15 females and 8 males, age >80 years and apparently in healthy state) that did not participate in the crossover study is considered as comparison for clinical events during the period of the follow-up (1 year).

A crossover design was used, in which each participant served as their own control. Our sample size of $n=21$ subjects may be a reliable number for a crossover design for a small pilot study in nutrition (Garaiova et al. 2007) as well as in elderly despite the presence of heterogeneity in old people (DeSure et al. 2013). Briefly, each eligible elderly received 250 ml/day of each of the two treatments [non-fortified milk (No-FMilk) and zinc-fortified milk (Zn-FMilk)] for a 60-day period (with a 15-day wash out period between). The No-FMilk corresponded to regular drinking skim milk distributed under the brand name “Cooperlat” (Jesi, Italy) and provided ~ 4 mg/l of zinc. Zn-FMilk corresponded to the same product plus the addition of 12 mg/l of zinc gluconate, for a total zinc content of 16 mg/l. Therefore, the uptake of the Zn-FMilk was in total of 4 mg/250 ml/each day/each individual for 2 months. The amount of added zinc to milk was calculated considering the following: (a) the minimum dietary zinc intake in old subjects, that is of 10 mg/day for Italy (Kanoni et al. 2010); (b) the normal zinc dietary intake in adults (7 mg/day for females and 9.5 for males) by RDA (Harper 2003; Murphy and Barr 2006); (c) the

safety of the zinc dose that has to be below 30 mg/day to avoid zinc toxicity (Murphy and Barr 2006). Therefore, the addition of 4 mg/day of zinc to the milk and to the minimum dietary zinc intake is in line with the dose used in the ZINCAGE project (10 mg/day in tablets for 45 days) in order to obtain an improvement of immune and antioxidant performances in old people (Mocchegiani et al. 2008, Mocchegiani and Zincage Consortium 2008).

At the baseline (No-FMilk) and after the 60-day treatment (Zn-FMilk), a heparinised blood withdrawal (20 ml) was performed and immediately collected to extract peripheral blood mononuclear cells (PBMCs). Plasma was stored at -80 °C until used for haematological, biochemical and immunological analyses. Psychological profiles (Barthel index, abbreviated mental test score (AMTS) and Geriatric Depression Scale (GDS)) were also evaluated from each patient at the baseline and after the 60-day treatment. The clinical status of the subjects was evaluated by a geriatric doctor at the time of the recruitment (baseline (No-FMilk)), after 60-day treatment (Zn-FMilk) and after 1 year of the follow-up from the end of the treatment.

Sample collections

Venous peripheral blood samples were collected after an overnight fast. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (density (d)=1,077 g/ml) (Biochrom, Berlin, Germany) gradient centrifugation ($400\times g$ for 30 min at 20 °C), collected, washed with D-PBS (Invitrogen, San Giuliano Milanese, Milan, Italy) and counted. Cell viability was checked with trypan blue staining under the microscope. Plasma, useful for biochemical, zinc and copper determinations as well as to test the thymic endocrine activity (thymulin), was separated after centrifugation at $2,000$ – $3,000\times g$ for 10 min at room temperature and frozen at -80 °C until used. Haematological and biochemical parameters were determined with standard laboratory procedures at INRCA Lab. Analysis (Ancona, Italy). Blood cell and haemoglobin counts were performed by standard automated procedures (Sysmex XE-2100). Erythrocyte sedimentation rate (ESR) was measured by TEST 1 Alifax Analyzer. Blood concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, glucose, azotemia and albumin were measured by an enzymatic colorimetric or kinetic tests on

modular automated clinical chemistry analyzers (Roche-Hitachi). The normal reference values are referred to INRCA Lab. Analysis.

Ex vivo LPS stimulation of PBMCs

Freshly isolated PBMCs were adjusted to 2.5×10^6 cells/ml in Rosewell Park Memorial Institute (RPMI 1640) medium plus 10 % heat-inactivated low-endotoxin foetal calf serum, 25 mM HEPES, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin (all obtained from Invitrogen, San Giuliano Milanese, Milan, Italy). Cells were cultured in 24-well tissue culture dishes (Nunc, Sigma-Aldrich, Milan, Italy), stimulated in duplicate with 100 ng/ml lipopolysaccharides (LPS) (*E. coli* serotype O26:B6, Sigma-Aldrich, Milan, Italy) and incubated at 37 °C in a 5 % humidified CO₂ atmosphere. For detection of the basal cytokine production rate, one aliquot remained unstimulated and received 10 µl/ml of the culture medium. After 24.0 ± 0.25 h of incubation, the supernatants were harvested and stored at –80 °C until measuring cytokine concentrations by enzyme-linked immune-absorbent assays (ELISA). Cells cultured were recovered, washed three times with RPMI medium and used for the determination of intracellular available zinc by flow cytometry. Maximum storage time for all supernatants was 12 months.

Cytokine assays

Concentrations of IL-1 α , IL-1 β , IL-2, IL-6, IL-10, IL-12p70, IFN γ and TNF α in the samples were measured using the SearchLight® Human Inflammatory Cytokine Array (Aushon Biosystems, Tema Ricerca Srl, Bologna, Italy). All samples from each elderly patient were analysed on the same plate. All ELISA assays were carried out using the manufacturer's instructions.

Plasma trace element concentrations, analysis of intracellular labile zinc and NO-induced zinc release by MT

Plasma zinc and copper concentrations were determined with Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA, USA), following the manufacturer's instructions (AN_EO604) with slight modifications (Malavolta et al. 2006).

Zinc intracellular availability (iZnL) was determined in thawed PBMCs, divided into two equal aliquots of

2×10^5 cells, at least. One aliquot was incubated with 20 µM Zinpyr-1 (ZP-1) (Neurobiotex, Galveston, TX, USA) for 30 min at 37 °C, 5 % CO₂ in HEPES-buffered zinc-free RPMI medium containing 1 mM EDTA, as extracellular chelator of free zinc eventually still present in the medium and/or adsorbed to the cell membrane. The second aliquot was always incubated in the same conditions plus 50 µM N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (Sigma-Aldrich, Milan, Italy), in order to detect the autofluorescence of the zinc-free ZP-1 probe. After incubation, the aliquots were immediately analysed by flow cytometry (Coulter Epics XL). After selecting the lymphocyte population according to the forward light and side scatters, the mean fluorescence intensity (MFI) for ZP-1 was detected (excitation wavelength 488 nm and detection at 525 ± 15) in the two aliquots. Data were reported as the ratio of ZP-1 fluorescence/ZP-1 autofluorescence and represented the intracellular labile Zn (iZnL) (Malavolta et al. 2006). To investigate the intracellular NO-induced release of Zn by MT (iZnR), another aliquot was incubated with 20 µM ZP-1 plus 100 µM diethylamine NOoate acetoxymethylated (AcOM DEA/NO) (Calbiochem, VWR International, Milan, Italy) (Misra et al. 1996). In fact, AcOM-DEA/NO is a cell-permeable acetoxymethylated diazeniumdiolate compound that donates NO "intracellularly" following the action of intracellular esterases (Saavedra et al. 2000). Once the incubation period was terminated, all aliquots were immediately read by the flow cytometer. The difference between iZnL in the presence and absence of the NO donor was used to estimate the intracellular release of Zn (iZnR), as previously reported (Malavolta et al. 2006).

Thymulin determination

Thymulin is a specific thymic hormone secreted by the thymic epithelial cells with an action on T cell maturation and differentiation and with the peculiarity of its binding of zinc ion (in the ratio 1:1) to fully express its biological activity. The zinc unbound form of thymulin (FTS), as in ageing and zinc deficiency, is inactive, exerting an inhibitory action on the active zinc-bound form (ZnFTS) named thymulin (Mocchegiani and Fabris 1995). Thymulin activity is closely dependent on the age of the subjects up to very low or undetectable levels at the age ≥ 70 years (Mocchegiani and Fabris 1995). We have here tested thymulin in its zinc-bound form using enzyme immune assay kit (EIA kit) (Immunodiagnostik AG, Bensheim, Germany) and

following manufacturer's instructions. The values are expressed in nanograms per milliliter. Each test was performed in triplicate. The normal value of the zinc-bound thymulin herein tested with EIA kit and referred to the old age of the subjects is 0.5–1.5 ng/ml. These values fit with those ones obtained in very old age by using thymulin rosette bioassay (Mocchegiani and Fabris 1995).

Psychological assessment

The psychological assessment was addressed in (a) Barthel index, (b) abbreviated mental test score (AMTS) and (c) Geriatric Depression Scale (GDS).

- a) Barthel index is an ordinal scale used to measure performance in activities of daily living (ADL). Each performance item is rated on this scale with a given number of points assigned to each level or ranking (O'Sullivan and Schmitz 2007). It uses ten variables describing ADL and mobility. A higher number is associated with a greater likelihood of being able to live at home with a degree of independence (Mahoney and Barthel 1965). For each variable, the score is 0–20. The maximum score is 100 and shows independence in all basic activities of daily living.
- b) The abbreviated mental test score (AMTS) was introduced by Hodkinson in 1972 to rapidly assess elderly patients for the possibility of dementia and cognitive impairment. It uses ten questions. For each question, the score is 0–1 without a half mark. A total score of 6 or less suggests cognitive impairment at the time of testing.
- c) The Geriatric Depression Scale (GDS) validated in different studies consists of a 15-item self-report assessment used to identify depression in the elderly (Yesavage et al. 1982). The GDS questions are answered by “yes” or “no”. This simplicity enables the scale to be used with ill or moderately cognitively impaired individuals. The scale is commonly used as a routine part of a comprehensive geriatric assessment. A relative point (0 or 1) is assigned to each answer and the cumulative score is rated on a scoring grid. The grid sets a range of 0–4 as “normal”, 5–8 as “mild”, 9–11 as “moderate” and 12–15 as “severe” depressed.

Statistical analysis

Statistical analysis was performed using the SPSS software (version 11.5, SPSS Inc. Chicago, IL, USA). Values are shown as mean experimental $\pm$ standard error (SE). Univariate analysis was performed to analyse the differences between the two groups studied using as covariates the age and gender variables and Pearson's χ^2 test for categorical variables. Comparison between variables was performed using partial correlation coefficients after controlling for age and gender values. Results were considered significantly different when $p < 0.05$.

Results

Haematological, biochemical and psychological parameters

Table 1 shows the haematological, biochemical and psychological profiles of the 21 participants at the baseline (non-Fortified milk (No-FMilk)) and after the 60-day treatment (zinc-fortified drinking skim milk (Zn-FMilk)) with the respective normal reference values. No statistically significant differences between the baseline and the ending blood, clinical or psychological tests are observed ($p > 0.05$). Noteworthy, all the parameters considered are in the respective normal range, suggesting the presence of a satisfactory healthy state in the recruited subjects, with also good ADL and cognitive functions as well as no presence of depression. In this last case, a slight trend, but not significant ($p > 0.05$), to diminish in GDS after the 60 days of treatment by Zn-FMilk occurs (Table 1).

Trace element assessment and zinc status

No significant differences exist in plasma zinc (Zn) and copper (Cu) before and after treatment with, however, a trend to increase in plasma zinc leading to a slight decrement in the Cu/Zn ratio (Table 2). No significant differences also exist in intracellular zinc content (iZnL) before and after treatment both in the presence and absence of LPS stimulation. By contrast, significant differences in the zinc release by MT (iZnR) are observed both in the presence/absence of LPS stimulation between the No-FMilk and Zn-FMilk treatment ($p < 0.05$) (Table 2).

Table 1 Haematological, biochemical and psychological profiles of healthy elderly subjects at the baseline (non-fortified milk (No-FMilk)) and after 60-day treatment (zinc-fortified drinking skim milk (Zn-FMilk))

Parameter	Normal reference intervals	No-FMilk (mean $\pm$ SE) <i>n</i> =21	Zn-FMilk (mean $\pm$ SE) <i>n</i> =21
WBC ($\times 10^3/\mu\text{l}$)	4.00–9.00	6.01 $\pm$ 0.42	6.21 $\pm$ 0.35
RBC ($\times 10^6/\mu\text{l}$)	3.90–5.60	4.20 $\pm$ 0.09	4.20 $\pm$ 0.10
Hemoglobin (g/dl)	11.5–15.5	12.27 $\pm$ 0.29	12.10 $\pm$ 0.33
Hematocrit (%)	36.0–48.0	35.33 $\pm$ 1.76	36.90 $\pm$ 0.93
Platelet number ($\times 10^3/\mu\text{l}$)	140–400	235.00 $\pm$ 16.50	221.05 $\pm$ 14.86
Glucose (mg/dl)	70–110	86.67 $\pm$ 3.90	99.62 $\pm$ 6.67
Total cholesterol (mg/dl)	<200	178.76 $\pm$ 8.10	174.57 $\pm$ 7.40
HDL cholesterol (mg/dl)	<40	47.10 $\pm$ 2.88	48.05 $\pm$ 2.54
LDL cholesterol (mg/dl)	<100	133.00 $\pm$ 4.00	97.42 $\pm$ 7.85
Triglycerides (mg/dl)	<150	113.10 $\pm$ 9.47	114.29 $\pm$ 8.47
Azotemia (mg/dl)	10–50	38.24 $\pm$ 2.04	42.67 $\pm$ 2.70
ESR (mm/h)	up to 12	14.86 $\pm$ 2.78	16.00 $\pm$ 2.50
Albumin (g/dl)	3.4–4.8	3.85 $\pm$ 0.42	3.87 $\pm$ 0.41
Barthel Index	50–100	69.62 $\pm$ 6.09	68.19 $\pm$ 6.65
AMTS	>6 (no dementia)	8.33 $\pm$ 0.24	8.19 $\pm$ 0.48
GDS	0–4 (normal)	3.81 $\pm$ 0.51	2.95 $\pm$ 0.50

Analyses of covariance were used to compare the variables between No-FMilk and Zn-FMilk. Mean values were adjusted using age and gender as covariates

WBC white blood cells, RBC red blood cells, ESR erythrocyte sedimentation rate, AMTS abbreviated mental test score, GDS Geriatric Depression Scale, SE standard error

LPS-stimulated cytokine release from PBMCs

The spontaneous and stimulated cytokine productions from PBMC cultures before (No-FMilk) and after the 60-day treatment (Zn-FMilk) are shown in Table 3. In the presence of no stimulation by LPS (w/o condition), a significant percent change ($p<0.05$ or $p<0.01$) of cytokine release is evident and is related to a good functioning of the cell-mediated immunity (63.9 % IL-12p70 and 29.72 % IFN- γ) coupled with increase of anti-inflammatory cytokines (43.54 % IL-10) and decrease of pro-inflammatory cytokines (27.08 % IL-1 α) after treatment with Zn-FMilk when compared before treatment (No-FMilk) (Table 3). The other cytokines seem to be not affected by the treatment in w/o condition (Table 3). In the presence of LPS stimulation, the release of all the cytokines herein considered are not affected by the treatment with the exclusion of IFN- γ that shows a

significant increment (25.7 %) after treatment (Zn-FMilk) when compared to No-FMilk ($p<0.01$) (Table 3). In this context, it has to be considered that LPS stimulation is not the optimal mitogen to test the cytokine release related to T cell-mediated immunity, such as IL-2 and IFN- γ . However, the presence of significant correlation between IL-2 or IFN- γ and thymulin activity (see below), which is in turn an index of the T cell-mediated immunity (Savino and Dardenne, 2000), after LPS stimulation before and after zinc trial may be an index of the positive effect of LPS on T cell-mediated immunity.

Thymulin activity

The measure of the thymulin activity in the plasma before treatment (No-FMilk) is included in the normal range for the age of the subjects herein considered (0.5–1.5 ng/ml) (Fabris et al. 1984). The treatment with zinc-fortified milk is able to induce a significant increment (33.34 %) of the thymulin activity when compared to No-FMilk (1.52 $\pm$ 0.07 vs 1.14 $\pm$ 0.07 ng/ml, respectively) ($p<0.05$).

Correlations

Significant positive correlation exists without (w/o) LPS stimulation between IFN- γ release and iZnR before and after zinc trial ($r=0.65$; $p<0.05$). The same positive correlations exist in w/o condition between IFN- γ release and thymulin activity ($r=0.62$; $p<0.05$) and between thymulin activity and iZnR ($r=0.70$; $p<0.05$) before and after zinc trial. Positive correlations exist also between IL-2 and thymulin activity ($r=0.66$; $p<0.05$) and between IFN- γ and thymulin activity before and after zinc trial ($r=0.61$; $p<0.05$) after LPS stimulation.

Clinical conditions after 1 year of the follow-up

Table 4 reports the clinical condition of the very old subjects after 1 year of the follow-up from the end of the zinc trial with Zn-FMilk, in comparison to a control group. Taking into account the very old age of the subjects and their subsequent frailty condition (Collerton et al. 2012), an interesting result is the presence of only 10 % of deaths during 1 year of the follow-up involving two females. The cause of death was due to myocardial infarction. The 10 % (two males) had hospitalization for falls (head trauma or rupture of the

Table 2 Plasma trace element concentrations and intracellular zinc ion availability of healthy elderly subjects after 60-day treatment of non-fortified milk (No-FMilk) and zinc-fortified drinking skim milk (Zn-FMilk)

Parameter	No-FMilk (mean $\pm$ SE) $n=21$	Zn-FMilk (mean $\pm$ SE) $n=21$
Cu ($\mu\text{mol/l}$)	17.71 $\pm$ 0.76	18.61 $\pm$ 0.72
Zn ($\mu\text{mol/l}$)	13.66 $\pm$ 1.13	15.49 $\pm$ 1.36
Cu/Zn	1.48 $\pm$ 0.13	1.39 $\pm$ 0.13
iZnL–LPS (MFI)	3.02 $\pm$ 0.44	2.79 $\pm$ 0.21
iZnR–LPS (MFI)	4.21 $\pm$ 0.72	8.71 $\pm$ 0.52*
iZnL+LPS (MFI)	3.54 $\pm$ 0.40	3.32 $\pm$ 0.16
iZnR+LPS (MFI)	4.47 $\pm$ 0.53	5.99 $\pm$ 0.46**

Statistical analysis was performed by analyses of covariance, and analyses of covariance were used to compare the variables among No-FMilk and Zn-FMilk. Mean values were adjusted using age and gender as covariates

iZnL intracellular labile zinc, *iZnR* intracellular NO-induced release of Zn, *MFI* mean fluorescence intensity, *No-FMilk* standard non-fortified milk, *Zn-FMilk* zinc-fortified drinking skim milk, *+LPS* PBMC stimulated with lipopolysaccharides, *–LPS* PBMC unstimulated with lipopolysaccharides

* $p<0.001$; ** $p<0.05$ when compared to No-FMilk

femur) and another 10 % (two females) had hospitalization for influenza virus. No severe clinical events occurred in the remaining recruited subjects (70 %) (4 males and 11 females) with no hospitalization during 1 year of the follow-up. This result is compared to a control group during the same period that did not participate in the crossover study but with the same age and clinical condition of the subjects included in the present study. As shown in Table 4, a major number of deaths occurred in the control group (52 %) (ten females and two males) as well as the presence of infections with the consequent reduced number of subjects in healthy status (17 %) (two females and two males). The 22 % (three females and two males) had hospitalization for influenza virus and 9 % (two males) for falls (Table 4).

Discussion

From the data herein reported, the treatment with zinc-fortified milk (Zn-FMilk) is a good omen to increase the cell-mediated immunity in very old age represented by thymulin activity and some cytokine (IL-12p70 and

Table 3 Basal (w/o) and stimulated cytokine productions of PBMCs of healthy very old subjects after 60-day treatment of non-fortified milk (No-FMilk) and zinc-fortified drinking skim milk (Zn-FMilk)

Stimulant	Cytokine	No-FMilk (mean $\pm$ SE) (pg/ml)	Zn-FMilk (mean $\pm$ SE) (pg/ml)	Changes (%)
w/o	IL1- α	2.88 $\pm$ 0.23	2.10 $\pm$ 0.28*	↓ 27.08
	IL1- β	4.90 $\pm$ 2.06	4.48 $\pm$ 1.15	↓ 8.57
	IL-2	18.30 $\pm$ 4.19	18.42 $\pm$ 4.66	↑ 0.65
	IL-10	10.38 $\pm$ 1.52	14.90 $\pm$ 1.50*	↑ 43.54
	IL-12p70	1.46 $\pm$ 0.20	2.39 $\pm$ 0.35*	↑ 63.69
	IL-6	167.85 $\pm$ 28.89	164.76 $\pm$ 23.71	↓ 1.84
	IFN- γ	4.61 $\pm$ 0.40	5.98 $\pm$ 0.31**	↑ 29.72
	TNF- α	18.00 $\pm$ 4.70	17.49 $\pm$ 3.70	↓ 2.83
LPS	IL1- α	164.29 $\pm$ 25.19	175.84 $\pm$ 24.99	↑ 7.03
	IL1- β	761.46 $\pm$ 187.34	834.94 $\pm$ 184.04	↑ 9.64
	IL-2	82.92 $\pm$ 14.89	98.37 $\pm$ 10.96	↑ 18.7
	IL-10	1,784.69 $\pm$ 307.22	1,830.75 $\pm$ 299.24	↑ 2.58
	IL-12p70	12.23 $\pm$ 0.76	13.87 $\pm$ 1.40	↑ 1.64
	IL-6	70,973.31 $\pm$ 9,250.25	75,791.00 $\pm$ 8,030.73	↑ 6.78
	IFN- γ	17.00 $\pm$ 0.50	21.37 $\pm$ 0.37**	↑ 25.7
	TNF- α	315.09 $\pm$ 74.31	308.96 $\pm$ 42.99	↓ 1.95

Mean values and standard error (SE) of 21 independent experiments are represented. Analyses of covariance were used to compare the variables between No-FMilk and Zn-FMilk. Mean values were adjusted using age and gender as covariates

↓, decrement; ↑, increment

* $p<0.05$; ** $p<0.01$ when compared with No-FMilk

Table 4 Clinical events during 1-year of follow-up after the zinc trial in treated group (Zn-FMilk) and control group

Clinical events	Treated group (Zn-FMilk), (%)	Control group, (%)
Deaths	2/21 (10)	12/23 (52)
Hospitalization for influenza virus infection	2/21 (10)	5/23 (22)
Hospitalization for falls (head trauma or rupture of femur)	2/21 (10)	2/23 (9)
No significant clinical events and no hospitalization	15/21 (70)	4/23 (17)

Pearson chi-square=14.737;
df=3; $p<0.01$

IFN- γ) release that are known to affect either the thymulin activity or the T cell maturation and differentiation within the thymus, via a good thymulin activity (Savino and Dardenne 2000). Such a treatment is also capable to keep under better control the inflammatory status and, consequently, to provoke again the zinc release by MT, as it occurs in healthy adult age (Mocchegiani et al. 2007), coupled with a trend to decrease in the Cu/Zn ratio, taking into account that a high ratio is an index of the chronic inflammatory status and mortality in elderly (Malavolta et al. 2010). The better control on the inflammation by Zn-FMilk is also evident in the cytokine releases after LPS stimulation especially for IFN- γ , being this cytokine both involved in a correct inflammatory/immune response and in affecting the T cell-mediated immunity (O'Garra and Murphy 1994). At the same time, the IFN- γ production is also under the control of zinc ion bioavailability, as shown in old and in very old mice and in centenarian subjects (Mocchegiani et al. 2002). Thus, the Zn-FMilk may be a good functional food to increase the T cell-mediated immunity and to keep under better control the inflammation in very old age.

On the other hand, similar results in increased IFN- γ release have been also reported in old zinc-supplemented individuals using tablets of zinc aspartate (Kahmann et al. 2008), suggesting the peculiar role played by zinc in affecting inflammatory/immune response and the cell-mediated immunity in elderly regardless by the different forms of zinc used. In this context, many papers report a benefit of zinc supplementation using zinc tablets or lozenges in elderly but with contradictory data (Haase and Rink 2009; Mocchegiani et al. 2013). However, a general consensus exists in a positive role of zinc in affecting the T cell response and in decreasing the inflammation in ageing with a specific effect in restoring the altered balance of Th1/Th2/Th3 paradigm (Prasad 2000). Anyway, no specific data in literature report the benefit of zinc supplementation in very old age (>80 years) in tablets or

lozenges. The use of these forms of zinc might be a limitation due to their bitter taste (Farr and Gwaltney 1987) and by the possible interference with the concomitant uptake of some common drugs (aspirin or diuretics) in elderly leading to a possible loss of zinc by urine (Braun and Rosenfeldt 2013). The uptake of Zn-FMilk might instead be a pleasure food in ageing and in very old age due to the attitudes to the milk consumption in old people (Elbon et al. 1996) and to the low frequency of lactose intolerance and lactose maldigestion in ageing (Carroccio et al. 1998). Moreover, a lower risk in the zinc loss might occur due to the concomitant uptake of some common drugs usually used in elderly. Although the zinc content in the urine (zincuria) is not herein tested, such an assumption is supported by the maintenance of a satisfactory intracellular zinc status after the treatment with Zn-FMilk, despite the uptake of some diuretic drugs by the majority of the very old subjects during the zinc trial. Moreover, the fractional intestinal absorption of zinc gluconate in healthy adult people is satisfactory using ^{70}Zn -Glu isotope (Malavolta M. unpublished results). Such an addition of zinc gluconate in the milk leads to a decrease in some pro-inflammatory cytokines (IL-1 α) with the concomitant significant increase in zinc ion release by MT, as it occurs in healthy adult people (Mocchegiani et al. 2007). Such a phenomenon is of relevance because a major zinc ion bioavailability provokes a significant increase of the zinc-dependent thymic hormone (thymulin) activity as well as augmented IFN- γ production and, consequently, a better T cell-mediated immunity. The significant correlation between zinc release by MT (iZnR) and thymulin activity and between thymulin activity and IFN- γ production before and after zinc trial both in without (w/o) and LPS stimulation support this assumption. A significant correlation exists also between IL-2 and thymulin activity before and after zinc trial after LPS stimulation. These data suggest that LPS mitogen may be also considered to test T cell-mediated immunity, even if the LPS mitogen is not the optimal for

this purpose. Consequently, benefits on the T cell-mediated immunity occur, via thymulin and IFN- γ release, coupled with new maturation and differentiation of naive T cells within the thymus, as shown by increased thymic output (Wong et al. 2009) and by a restoration of the CD4/CD8 ratio (Dardenne et al. 1993) after zinc supplementation in ageing. Moreover, the major zinc release also occurs in the presence of LPS stimulation coupled with augmented IFN- γ release after the zinc trial (Table 3). In this context, the zinc release from MT might be underestimated due to presence of altered zinc transporter gene expression on cell membrane in old age (Cragg et al. 2005; Kahmann et al. 2008; Mocchegiani et al. 2013, Haase and Rink, 2014), leading to a possible leakage of zinc from the cells. However, ZIP1 messenger RNA expression decreases in the elderly after zinc supplementation, suggesting a greater retention of zinc in their body (Andree et al. 2004; Kahmann et al. 2008). Although further experiments are required to better clarify the precise benefit of the zinc release from MT in ageing, the Zn-FMilk-treated older individuals, via more zinc release, may have a prompt and satisfactory inflammatory/immune response against external noxae with maintenance of the healthy state. Such a hypothesis is supported by the still good clinical conditions with no hospitalization after 1 year of the follow-up in the 70 % of the subjects despite of their frailty condition, in comparison to a control group (17 %) that did not participate to crossover design (Table 4). On the other hand, high levels of IFN- γ occurs in centenarians (Miyaji et al. 2000) coupled with satisfactory zinc ion bioavailability (Mocchegiani et al. 2002). In addition, increased IFN- γ has been also reported after LPS stimulation in old people supplemented with zinc aspartate lozenges at physiological doses (Kahmann et al. 2008). Altogether, these findings corroborate the assumption that a good zinc ion bioavailability by means of a physiological zinc supplementation is capable of making the elderly more prone for a prompt immune response against external noxae, as it occurs in centenarians (Mocchegiani et al. 2002; Franceschi et al. 2000). In this context, the dose of zinc added to the milk of 4 mg/250 ml is a safety dose for two reasons. Firstly, since the minimum intake of zinc by foods in elderly is of 4 mg/day up to 10 mg/day in Italian population (Kanoni et al. 2010), the addition of 4 mg in 250 ml of milk reaches the dose recommended by the RDA (7–8 mg/day) and is in line with the dose used for zinc supplementation (10 mg/day) in the

ZINCAGE project (Mocchegiani et al. 2008a, b). Secondly, even if in the nursing homes the old subjects eat foods containing zinc, the addition of 4 mg of zinc in 250 ml of milk does not reach the zinc dose that can be considered toxic (30 mg/day) leading to adverse effects on the immunity (Plum et al. 2010) or no effect on immunity in elderly, as shown in the Zenith project using 20–30 mg/day of zinc (Hodkinson et al. 2007). Such an assumption is supported by the beneficial effects of Zn-FMilk on some immune parameters herein tested despite the body of the very old subjects is very fragile (Collerton et al. 2012), in which even low doses of zinc can be dangerous because leading to apoptosis of the immune cells (Fraker et al. 2000). Anyway, the dose of zinc added to the milk is useful to maintain, at least, also a good psychological performance (see Table 1). In conclusion, the data herein reported, as a pilot study in a small sample of subjects, show that Zn-FMilk can be considered a good functional food for the elderly taking into account the attitude of the old people to uptake milk as a preferential food, even in presence of the uptake of some diuretic drugs.

Acknowledgments This study was supported by INRCA and Regione Marche, Department of Agriculture and Fisheries (Project “Il latte bovino come alimento funzionale per l’anziano” no. 361/S-10). The authors are indebted to the nursing homes of Cupramontana (AN) and Filottrano (AN) for their availability in the recruitment of the very old subjects. The authors are also indebted to the company “Cooperlat”, Jesi (AN), for the preparation of the milk added with zinc. The authors thank Mrs. Nazzarena Gasparini, Mr. Romeo Pierandrei and Mr. Enzo Cerusico for the excellent technical assistance.

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