Inflamm. Res.
DOI 10.1007/s00011-010-0211-7

Inflammation Research

ORIGINAL RESEARCH PAPER

Inflammatory responses improve with milk ribonuclease-enriched lactoferrin supplementation in postmenopausal women

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Received: 16 October 2009 / Revised: 14 January 2010 / Accepted: 27 April 2010

Abstract
Objective and design A 6-month, randomized clinical study was conducted to evaluate the effect of a ribonuclease-enriched lactoferrin (R-ELF) supplement on the circulating cytokine levels and bone health of postmenopausal women.
Subjects Thirty-eight healthy postmenopausal women, aged 45–60 years, were randomized into placebo and R-ELF groups.
Treatment The R-ELF group was supplemented with R-ELF (2 × 125 mg/day) and calcium (100% RDA), while the placebo group received only the calcium supplement.
Methods Serum levels of receptor activator for NF-κB ligand (RANKL), C-reactive protein (CRP) and various pro- and anti-inflammatory cytokines were determined by ELISA.
Results Pro-inflammatory cytokines IL-6 and TNF-α decreased significantly (−44 and −10%, respectively) while anti-inflammatory IL-10 increased (140%) due to R-ELF supplementation at the end of study. RANKL and CRP were modestly reduced (−50%) relative to their placebo levels, although RANKL elevated initially.
Conclusions R-ELF supplementation showed beneficial effects towards improvement of inflammatory status in postmenopausal women.

Keywords Lactoferrin · Osteoporosis · Postmenopausal · Ribonuclease · Inflammation

Introduction

Normal physiological conditions ensure a balance between bone formation and bone resorption to maintain skeletal homeostasis. An imbalance develops during osteoporosis and age-related osteopenia in postmenopausal women [1]. Excess production of osteoclasts and inadequate generation of osteoblasts could lead to such homeostatic imbalance. Current treatment options for postmenopausal osteoporosis include hormone replacement therapy (HRT), selective estrogen receptor modulators (SERM) and bisphosphonates. HRT is effective in reducing osteoclast activity, however, with certain potential detrimental effects including increased risk of breast cancer and thrombo-embolism as reported in the Women's Health Initiative [2] and the Million Women Study [3]. SERMs such as raloxifene are known to disrupt lymphocyte development, immune cell function, and cytokine secretion [4]. A risk for development of esophageal ulcers has also been reported with the use of bisphosphonates [5]. Emerging anabolic strategies include strontium renelate, isosorbide mononitrate and teriparatide (synthetic parathyroid hormone) that show a positive effect on bone formation as well as a reduction in bone resorption activity [6–8]. However, these new treatments require further safety evaluation [9]. Driven by the

Responsible Editor: Ian Ahnfelt-Rønne.

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Published online: 15 May 2010

imperative to explore and develop strategies for enhancing bone formation by natural products and simultaneously prevent bone loss without side effects, we recently reported the development of a milk-ribonuclease-enriched lactoferrin (R-ELF) supplement and its effect on bone turnover in postmenopausal women [10]. The data revealed that R-ELF reduced bone resorption and increased bone formation significantly. Nonetheless, the effect of R-ELF on general inflammation was not evaluated.

The immune and the skeletal systems share several regulatory factors, such as cytokines, transcription factors, and receptors. The immune cells and osteoclasts are derived from the same hematopoietic precursor cells, which originate in bone marrow and interact with bone cells [11, 12]. Inflammation is the primary defense mechanism of the body, characterized by a 'high alert' state of the immune system, triggered by the release of several pro-inflammatory cytokines. It also affects the processes of bone resorption and formation, due to the presence of pathways that are common to both bone cell maturation and inflammation. Pro-inflammatory cytokine levels are known to rise during aging and stress [13]. Elevated levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β receptors suggest subtle changes in the immune system [14]. Thus, the body is in a state of mild, continuous inflammation, characterized by a rise in the levels of acute phase proteins such as C-reactive protein (CRP), a well-known bio-marker for inflammation. In healthy, elderly individuals higher serum CRP levels were associated with high bone turnover rate, resulting in low bone mineral density [15].

Lactoferrin (LF) is a multifunctional milk protein with anti-inflammatory and immune modulatory properties [16]. LF stimulates lymphocytes and natural killer cells, and is involved in multiple pathways of immune response. Enhanced secretion of the anti-inflammatory cytokines IL-4 and IL-10, along with altered expression of pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6 and TNF-α) have been observed with LF intake [17, 18]. Oral administration of LF in animal models could upregulate anti-inflammatory IL-4 and IL-10 [19] and inhibit TNF-α along with a rise in IL-10 secretion in animal arthritis models [20]. In view of the immuno-modulating and anti-inflammatory properties of LF and the established links between inflammatory cytokines with bone remodeling processes, we set out to investigate the changes in inflammatory status of subjects supplemented with R-ELF. The present study reports the effect of R-ELF supplementation on the levels of inflammatory cytokines and CRP, as indicators of inflammation, and receptor activator for nuclear factor-κB ligand (RANKL) as a marker of osteoclast activity in postmenopausal women.

Materials and methods

Subjects

Selection of subjects was based on the response to a questionnaire on general bone health status, previous injury/disease, current or previous treatment, and consumption of calcium-rich foods. General health was determined by routine standard medical assessment of physical and mental health. Details of the inclusion and exclusion criterion for the study have been described previously [10]. After evaluation, 38 healthy, ambulatory postmenopausal women, 45–60 years old, with no menses for at least 12 months were registered for the study. Three subjects were excluded, one with a history of treatment for bone health and two women had hypothyroidism. The study was approved by the Institutional Review Board of the Western University of Health Sciences, Pomona, CA. Prospective participants were advised of the nature of the study and provided written informed consent before participation.

Study design

R-ELF is a ribonuclease (angiogenin)-enriched LF either co-isolated from bovine milk (50:50 ratio wt/wt) or both proteins (LF and ribonuclease) admixed to obtain required ratios, as previously described [21, 22]. Thirty-five women included in the study were randomly assigned to one of the two groups: placebo group or R-ELF group. Fifteen subjects assigned to the placebo group were supplemented with 100% RDA (Recommended Daily Allowance) of calcium, in a tablet form, whereas 20 subjects of the R-ELF group were given two R-ELF capsules of 125 mg each, along with 100% RDA of calcium administered orally from Day 1 to Day 180. Venous blood samples were collected, by standard venipuncture technique, from each subject on Day 0 (baseline before starting the supplement), Day 30, Day 90 and Day 180 of the study. Further details of design and flow of the study have been described previously [10].

Inflammatory cytokines and markers

The levels of inflammatory cytokines—IFN-γ, IL-6, IL-12+p40, TNF-α, IL-1β, IL-10 and TGF-β were determined by enzyme-linked immunosorbent assay (ELISA) using respective monoclonal antibodies purchased from Invitrogen (Camarillo, CA). High purity CRP and anti-CRP rabbit polyclonal antibody were obtained from Calbiochem/EMD (San Diego, CA, USA) and anti-RANKL rabbit polyclonal antibody was from Abcam Inc. (Cambridge, MA, USA). CRP assay had a sensitivity of 8 pg/mL and detection limit

of 1 ng/mL. All buffers and other reagents were obtained from Invitrogen.

Statistical analysis

Cytokine data from each day was analyzed for measures of central tendency, deviation and distribution of data. Data were considered outliers if they were >1.5 times the inter-quartile range (IQR) above the third quartile or below the first quartile. Outliers were discarded from datasets for statistical tests of significance. In view of the small size of placebo and R-ELF groups, median and standard error of the mean were used as the preferred measures of central tendency for this modest set of data. The Kolmogorov–Smirnov test (KS test) was used as a test for normal distribution of data within each set [23]. Student's unpaired two-sample *t* test was used for comparison of the mean observed change in markers for placebo and R-ELF data sets to establish the effect of R-ELF supplementation. OriginPro Ver. 8 (OriginLab, MA, USA) software was used for data analysis.

Results

Baseline characteristics, compliance and adverse events

The baseline characteristics of placebo and R-ELF groups are shown in Table 1. There is a close match between the health characteristics of the placebo and R-ELF groups, reflecting generally good bone health status. As detailed in our previous study, the bone health status of the two groups were also comparable, as evaluated by measuring the levels of several bone turnover markers [10]. Three participants from the control group dropped out of the study before Day 15 and one from the R-ELF group was dropped due to non-compliance. There was >95% compliance to the supplement regimen among the subjects. The body weight and blood pressure of all the subjects essentially maintained within ±3% of their baseline values. No adverse events were reported during the 6-month study or 3-month post-study follow up.

Table 1 Baseline characteristics of the study population

Characteristic	Control group	R-ELF group	*P*
Number of participants (*n*)	15	20	–
Age (years)			
Mean ± SD	51.0 ± 4.4	53.5 ± 5.4	0.10
Range	44–59	45–61	
Weight (lbs)			
Mean ± SD	134 ± 20	141 ± 24	0.36
Range	104–174	107–208	
Blood pressure (mm Hg)			
Mean Systolic ± SD	121 ± 21	127 ± 24	0.47
Mean Diastolic ± SD	78 ± 14	80 ± 15	0.64
Cytokines (pg/mL)[a]			
IFN-γ	12.9 ± 2.1	17.2 ± 4.0	0.37
IL-6	3.3 ± 1.5	4.2 ± 3.2	0.11
TNF-α	243.5 ± 15.8	277.0 ± 7.7	0.07
IL-1β	7.7 ± 2.3	12.8 ± 3.6	0.26
IL-12+p40	66.9 ± 10.7	54.8 ± 8.3	0.38
IL-10	11.9 ± 3.2	9.2 ± 2.1	0.47
TGF-β	743.1 ± 73.1	771.6 ± 21.1	0.68

[a] Mean ± SEM

The mean levels and range of inflammatory cytokines in the serum of study subjects were similar to those reported for generally healthy individuals [24–28]. The levels of TGF-β in postmenopausal women are known to vary widely, ranging from 9,800 [29] to 46,500 [30]. In the analysis of cytokine levels, the median ± SEM values are used to estimate changes with R-ELF supplementation, as the groups were small. Unusual variations in the data of some subjects resulted in high standard errors.

Pro-inflammatory cytokine levels improved towards a better bone health

Variation in the serum levels of IFN-γ, IL-1β, TNF-α, IL-6 and IL-12+p40 for placebo and R-ELF groups during the course of the study are shown in Fig. 1. The median IFN-γ ranged from 14.4 ± 1.7 to 18.3 ± 2.9 pg/mL for the placebo group while the values increased from 28.6 ± 13.7 to 32.4 ± 12.8 pg/mL for the R-ELF group and the difference between them was statistically significant ($P = 0.0007$ for median IFN-γ, placebo vs. R-ELF). IFN-γ levels for the R-ELF group decreased (−6.6%) within 30 days but increased considerably (+42%) for the placebo group. By the end of study, although IFN-γ levels for the R-ELF group were increased (+13.5%), the increment was smaller compared to the placebo group (+27%) (Fig. 1a). Median IL-1β levels ranged from 10.2 ± 3.8 to 15.0 ± 10.2 pg/mL for the placebo and from 22.6 ± 10.9 to 23.3 ± 19.9 pg/mL for the R-ELF group ($P = 0.0131$ for median IL-1β, placebo vs. R-ELF). A large decrease in IL-1β levels was observed initially but the trend reversed by Day 90 and turned out significantly positive (+46.5%) for the placebo group, while it remained close to the baseline (+2.8%) for the R-ELF group (Fig. 1b). TNF-α levels ranged from 239.3 ± 15.8 on Day 0 to 238.3 ± 69.5 pg/mL for placebo, while a decrease from 279.7 ± 31.0 to 251.4 ± 43.2 pg/mL ($P = 0.0341$ for median TNF-α, placebo vs. R-ELF) was observed with R-ELF supplementation. A large initial decline in TNF-α levels (−27.4% for R-ELF vs. −15.5% for placebo) was sustained until

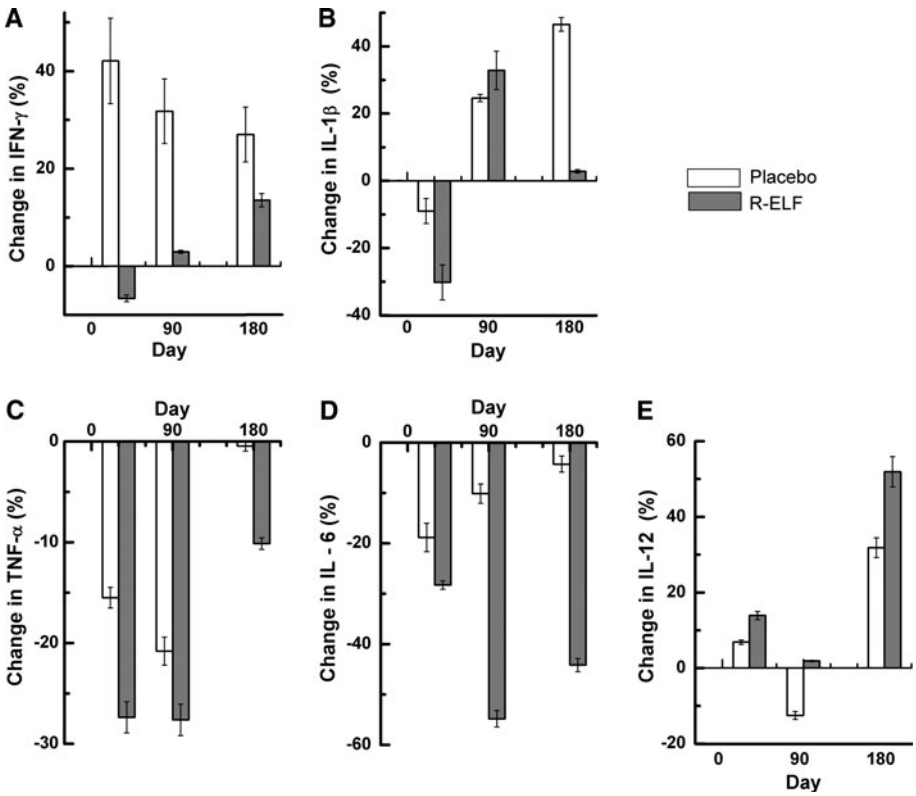

Fig. 1 Effect of R-ELF supplementation on pro-inflammatory cytokines. Median change in the serum levels of pro-inflammatory cytokines. IFN-γ (**a**), IL-1β (**b**), TNF-α (**c**), IL-6 (**d**) and IL-12 (**e**)—in subjects from placebo (*open bars*) and R-ELF (*filled bars*) supplemented groups

Day 180 for the R-ELF group (−10.1%), but not with the placebo (−0.4%) (Fig. 1c). Furthermore, median IL-6 levels slightly decreased for the placebo group from 2.1 ± 1.5 on Day 0 to 2.0 ± 2.1 pg/mL by the end of study, while IL-6 decreased from 5.5 ± 3.9 to 3.0 ± 4.1 pg/mL for the R-ELF group. As shown in Fig. 1d, by the end of study, the decrease in IL-6 was significant ($P = 0.0338$ for median IL-6, placebo vs. R-ELF) for the R-ELF group (−44.1%) compared to the placebo (−4.3%). Levels of IL-12+p40 (also referred to as IL-12) were similar for both groups; placebo ranging from 68.5 ± 1.6 to 90.2 ± 1.6 pg/mL and R-ELF group from 73.1 ± 4.0 to a slightly higher 111.1 ± 5.6 pg/mL. R-ELF supplementation significantly raised IL-12 levels by 51.9% by Day 180 compared to placebo, with an increase of 31.8% from their respective baseline levels ($P < 0.0005$ for mean IL-12, placebo vs. R-ELF) (Fig. 1e). These results demonstrate that R-ELF supplementation induced down-regulation of pro-inflammatory TNF-α and IL-6, and a moderate elevation of IL-1β and IFN-γ levels within 6 months.

Effect of R-ELF supplementation on anti-inflammatory cytokines

Enhanced production of anti-inflammatory cytokines, such as IL-10 and TGF-β, would complement the beneficial effects of R-ELF as these cytokines promote formation of new bone matrix. Changes in the serum levels of IL-10 and TGF-β were determined for both groups and are shown in Fig. 2. Levels of IL-10 for the placebo group were close to the baseline median value of 17.4 ± 2.7, until Day 180 (16.3 ± 3.6 pg/mL), while the levels increased from 7.7 ± 9.2 to 18.8 ± 4.2 pg/mL for the R-ELF group. A major positive change was observed in IL-10 with R-ELF supplementation (+150%) compared to placebo (−6.2%) ($P = 0.0235$ for change in median IL-10, placebo vs. R-ELF) by the end of study (Fig. 2a). Median TGF-β levels

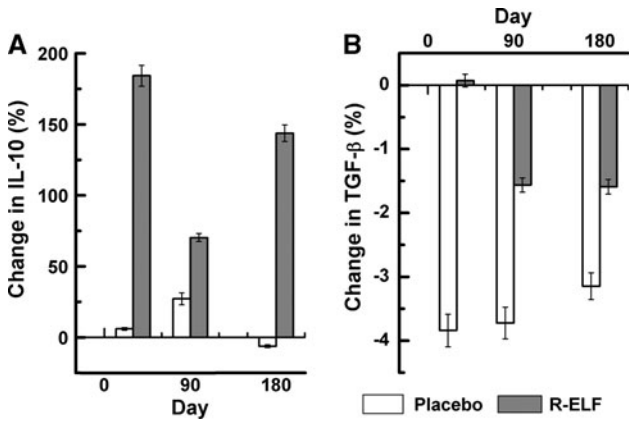

Fig. 2 Variations in serum levels of anti-inflammatory cytokines with R-ELF supplementation. Median change in the serum levels of anti-inflammatory cytokines. IL-10 (**a**) and TGF-β (**b**) in subjects from placebo (*open bars*) and R-ELF (*filled bars*) supplemented groups

slightly decreased for both the groups, 786.3 ± 73.1 to 761.5 ± 85.1 pg/mL for the placebo, compared to 739.4 ± 58.9 to 727.6 ± 51.9 pg/mL for the R-ELF ($P = 0.0072$ for median TGF-β, placebo vs. R-ELF). However, the decrease was smaller for the R-ELF group (−2%) than the placebo (−3%) (Fig. 2b). These results indicate a relatively positive change in anti-inflammatory cytokines with R-ELF supplementation, compared to calcium supplementation alone.

The correlation between pro- and anti-inflammatory cytokines was analyzed and the results are presented in Table 2. The correlation matrix for placebo and R-ELF groups were generally maintained from Day 0 to Day 180 and improved with R-ELF supplementation. Most cytokine correlations also remained statistically significant at 95% confidence interval. The correlation of IL-6 versus IL-10 improved with R-ELF supplementation. In general, TNF-α, IL-12+p40 and TGF-β did not show any correlation, neither with the progress of study nor with supplementation. IL-10 was highly correlated with IFN-γ and IL-6 at the beginning of the study for both groups, which improved by the end of the study. Interestingly, IFN-γ was not correlated with IL-10 at baseline but demonstrated a high degree of correlation after R-ELF supplementation. These positive correlations suggested a moderate elevation of anti-inflammatory cytokines that could reduce pro-inflammatory response. Inverse correlation is a favorable outcome of reduction in pro-inflammatory and elevation of anti-inflammatory cytokines, in which could diminish inflammation. An improved negative correlation was observed between IL-6 and TGF-β with R-ELF supplementation, although not statistically significant.

Table 2 Correlation between pro- and anti-inflammatory cytokines

Cytokine	Day 0		Day 180	
	IL-10	TGF-β	IL-10	TGF-β
PLACEBO Group				
IFN-γ	0.89 (<0.005)	nc	0.97 (<0.0005)	nc
IL-6	0.79 (0.05)	nc	0.97 (<0.0005)	nc
TNF-α	nc	nc	0.86 (0.05)	nc
IL-1β	0.67[a] (0.066)	nc	0.98 (<0.0005)	nc
IL-12	nc	nc	nc	nc
R-ELF Group				
IFN-γ	nc	nc	0.95 (<0.005)	nc
IL-6	0.82[a] (0.086)	−0.41[b] (0.271)	0.92 (< 0.05)	−0.50[b] (0.167)
TNF-α	nc	nc	nc	nc
IL-1β	nc	nc	nc	nc
IL-12	nc	nc	nc	nc

nc No correlation observed

[a] 90% Confidence interval

[b] Not statistically significant

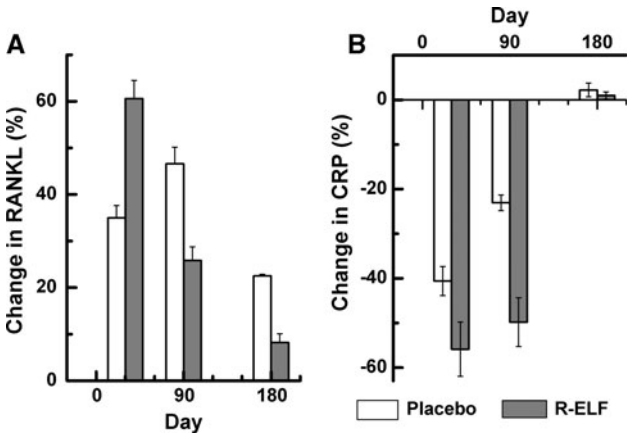

Fig. 3 Changes in serum levels of RANKL and CRP with R-ELF supplementation. Median change in the serum levels of RANKL, a marker of osteoclast formation (**a**) and CRP, an inflammatory marker (**b**) in subjects from placebo (*open bars*) and R-ELF (*filled bars*) groups

Reduced levels of CRP and RANKL with R-ELF supplementation

RANKL is a well established marker for the onset of osteoporosis in postmenopausal women. RANKL, a mediator of osteoclast formation, indicated a sharp rise by Day 30 followed by a steady decline for both placebo and R-ELF groups (Fig. 3a). However, in response to R-ELF supplementation there was a larger and rapid decline by −34.8% (60.6% on Day 30 to 25.8% on Day 90) compared to an 11.6% increase (35.0% on Day 30 to 46.6% on Day 90) with calcium supplementation alone. By the end of the study, RANKL levels were further decreased to 8.2% for R-ELF group and remained at 22.5% for placebo ($P = 0.0202$ for median change in RANKL, placebo vs. R-ELF). Similarly, CRP levels showed a sharp decrease for both the groups by Day 30, but the trend reversed and CRP increased to baseline levels by the end of the study (Fig. 3b). A relatively large reduction in CRP levels was observed for the R-ELF group (−55.9% by Day 30), which sustained for a long time (−49.8% by Day 90) ($P = 0.0286$ for mean change in CRP, placebo vs. R-ELF). On the other hand, the placebo group regained CRP levels rapidly, −40.6 to −23.1% of its baseline CRP level in 60 days. These results suggest an improvement in the inflammatory status and reduced bone resorption in postmenopausal women with R-ELF supplementation.

Discussion

In our previous study, R-ELF supplementation reduced bone resorption and significantly elevated bone formation, measured as changes in the levels of several bone turnover

markers [10]. Bone metabolism is known to be influenced by inflammatory cytokines. Development and activation of osteoclasts and subsequent bone resorption is regulated by the RANKL pathway [31, 32]. Anti-resorptive therapies, such as denosumab, targeted towards RANKL, are currently being developed [33]. Several cytokines influence the expression of RANKL, which in turn, affects bone remodeling [34]. Estrogen deficiency in postmenopausal women causes elevation of pro-inflammatory cytokines such as TNF-α and IL-6, which increases RANKL levels, leading to increased osteoclast maturation, activity and subsequent risk for osteoporotic fractures [35–37]. Other treatments, such as HRT and raloxifene, resulted in either increased or unchanged levels of IL-6, although TNF-α levels showed a considerable decline. [38–40]. In the present study, we have observed a significant reduction in TNF-α and IL-6 levels, after six months of R-ELF supplementation, with substantial favorable changes in TNF-α (−27%) and IL-6 (−44%) occurring within the first 90 days. Based on this decrease in TNF-α and IL-6 levels, a change in RANKL was expected in R-ELF treated subjects. However, median change in RANKL levels varied significantly from baseline and was reduced for the R-ELF group relative to placebo by the end of study. It is noteworthy that RANKL levels have not shown a consistent behavior with the progress of anti-resorptive therapy. Similar inconsistent variations in RANKL levels have been well documented [41–43]. In serum, RANKL is present in free form as well as bound to osteoprotegerin (OPG). The increase in RANKL levels has been interpreted as an increase in the OPG-bound form, which is a favorable outcome, indicating reduced osteoclast activity [43]. In addition, RANKL levels were undetectable for several subjects in a raloxifene study due to limitation of the assay [42]. Therefore, our preliminary observations considering the results of other investigators, suggest that R-ELF intervenes with RANK/RANKL pathway, directly or indirectly, to down-regulate osteoclast formation and subsequently reduce bone resorption (as has been observed by changes in serum NTx and urine DPD markers in our previous study [10]).

The pro-inflammatory cytokine IFN-γ has a dual role in bone metabolism. IFN-γ functions both as a pro-resorptive and an anti-resorptive cytokine [44]. IFN-γ blocks RANKL-induced osteoclast differentiation by direct interaction with osteoclast precursors but indirectly stimulates osteoclast formation. Accordingly, the modest 13.5% increase in IFN-γ levels observed for the R-ELF group can potentially be attributed to this dual property. It is well known that pro-inflammatory cytokines IL-6, IL-1β and TNF-α are increased in post-menopausal estrogen deficiency [45–47]. In our study, R-ELF supplementation greatly reduced IL-6 and TNF-α levels compared to placebo, indicating a beneficial effect. IL-1β, a potent stimulator of osteoclastogenesis [48], however, showed an initial decrease followed by an increase by Day 90, then settled close to baseline by the end of study despite R-ELF supplementation. Nevertheless, IL-1β levels were increased by >40% by Day 180 in the placebo group, whereas R-ELF supplementation prevented such an increase, indicating that long term supplementation of R-ELF may be necessary to observe positive effects. In contrast, IL-12 inhibits the RANKL-induced maturation and activation of osteoclasts on its own or in synergy with IL-18 [49]. R-ELF increased the serum levels of IL-12 by more than 50% in 6 months, which may be responsible for the observed decrease in RANKL levels after Day 30. Further, IL-12 has been shown to inhibit TNF-α induced osteoclastogenesis via T cell dependent mechanism in mice [50]. Therefore, the increased levels of IL-12 with R-ELF supplementation are a favorable outcome in this study.

Anti-inflammatory cytokines, TGF-β and IL-10, tend to suppress osteoclastogenesis [37, 51–53]. A reduction in TGF-β levels results in inflammation, autoimmunity and increased T cell production of IFN-γ, TNF and RANKL. In the present study, TGF-β levels for the placebo group exhibited slight decrease (−3%) whereas R-ELF supplementation limited the loss to −2%. The change in TGF-β levels is minimal, however, serum TGF-β levels were significantly lowered by HRT (>50%) while raloxifene treatment had no effect [54]. The marked increase in IL-10 levels (+150%) observed with the R-ELF group appears also to be a beneficial outcome. In view of the detrimental effects of IL-10 deficiency, IL-10 elevation by R-ELF could be responsible for the increased bone formation (as measured by bone specific alkaline phosphatase and osteocalcin levels) observed in our previous clinical study [10].

The kinetics of acute phase marker CRP synthesis are influenced by several cytokines involved in the inflammatory process. The serum concentration of CRP has been shown to correlate with TNF-α and IL-6 with minor effect from other cytokines [55]. R-ELF supplement, therefore, was expected to bring down inflammation considerably with reduction of TNF-α and IL-6 levels. In agreement, the R-ELF group demonstrated significantly lower CRP levels by Day 30 and Day 90 than calcium supplementation alone.

In summary, R-ELF supplementation reduced osteoclast generation, as indicated by decreased levels of RANKL, and improved inflammatory status of the subjects, as indicated by the decline in CRP levels. These changes are attributed to diminished pro-inflammatory and increased anti-inflammatory cytokine levels. Despite being a preliminary investigation with a small number of subjects monitored for a short duration, the results are promising and warrant further investigation by a placebo-controlled

study in a larger population. R-ELF, a milk protein-based bone health supplement, could provide a safe and natural alternative to synthetic hormone and drug therapy limited by serious side effects.

Acknowledgments We thank Tiffani Davis (phlebotomist), Natver Patel, and Sreus AG Naidu for coordinating with the clinical study. This project was funded by N-terminus Research Laboratory, Pomona, CA, USA.

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