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ORIGINAL ARTICLE

Milk ribonuclease-enriched lactoferrin induces positive effects on bone turnover markers in postmenopausal women

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Abstract
Summary Current treatments for postmenopausal osteoporosis suffer from side effects. Safe and natural milk proteins, ribonuclease, and lactoferrin promote formation of new capillaries and bone formation. A ribonuclease-enriched lactoferrin supplement studied here, demonstrates significant reduction in resorption and increase in formation, towards restoring the balance of bone turnover within 6 months.
Introduction Osteoporosis, a major health issue among postmenopausal women, causes increased bone resorption and reduced bone formation. A reduction in angiogenesis could also contribute to this imbalance. Current treatments such as hormone replacement therapy and bisphosphonates have drawbacks of severe side effects. Milk ribonuclease (RNase) is known to promote angiogenesis and lactoferrin (LF) to stimulate bone formation by osteoblasts. We examine the effect of ribonuclease-enriched lactoferrin supplement on the bone health of postmenopausal women.
Methods A total of 38 healthy, postmenopausal women, aged 45 to 60 years were randomized into placebo or RNAse-enriched-LF (R-ELF) supplement groups. The bone health status was monitored by assessing bone resorption markers, serum N-telopeptides (NTx), and urine deoxypyridinoline (Dpd) crosslinks and serum bone formation markers, bone-specific alkaline phosphatase (BAP), and osteocalcin (OC).
Results R-ELF supplementation demonstrated a decrease in urine Dpd levels by 14% (19% increase for placebo) and serum NTx maintained at 24% of the baseline (41% for placebo), while serum BAP and OC levels showed a 45% and 16% elevation (25% and 5% for placebo).
Conclusions R-ELF supplementation demonstrated a statistically significant reduction in bone resorption and increase in osteoblastic bone formation, to restore the balance of bone turnover within a short period.

Keywords Lactoferrin · Osteoporosis · Postmenopause · Ribonuclease

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Introduction

Osteoporosis is a bone and joint disorder that causes a significant reduction in bone density and alteration of the bone microstructure that primarily affects elderly women. These chronic changes in the interior of bone tissue lead to fractures that could have adverse influence on the quality of life of the elderly. The US National Osteoporosis Foundation has estimated that by 2010, about 12 million people over the age of 50 are expected to have osteoporosis and another 40 million to have low bone mass (osteopenia). In addition, osteoporosis also affects one in eight men worldwide [1, 2]. Bones undergo a continuous remodeling process through repeated cycles of destruction and

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rebuilding. In healthy young adults, the amount of new bone formation approximately balances the amount of bone resorption. As the age increases, however, the balance shifts to favor bone resorption. Among the numerous factors that contribute to the development of osteoporosis in women, postmenopausal estrogen deficiency accelerates bone loss of 1–5% per year during the first decade after menopause. Current efforts to treat bone diseases have primarily concentrated on the development of drugs to block bone resorption, which decrease the formation or activity of osteoclasts. Present treatment options for postmenopausal osteoporosis include hormone replacement therapy (HRT) and bisphosphonates. HRT is known to significantly reduce osteoclast activity but is prone to adverse effects, such as increased risk of breast cancer [3, 4], and bisphosphonates have a risk of development of esophageal ulcers [5]. Therefore, it is imperative to explore and develop strategies for enhancing the bone formation and simultaneously prevent the bone loss without side effects.

Milk, a superior source of bioavailable calcium, has pronounced effects on bone metabolism. The basic protein fraction of milk contains several active components that have been shown to suppress osteoclast-mediated bone resorption and osteoblast differentiation. Studies on milk basic protein (MBP) intake have demonstrated bone-strengthening effects in cell culture studies and animal experiments [6]. A limited number of studies report the influence of MBP supplementation on bone metabolism and radial bone mineral density in healthy adult women [7, 8]. Recently, the component of MBP responsible for the inhibition of osteoclastic resorption has been shown to be identical to milk angiogenin, a 14-kDa milk basic protein that exhibits both angiogenic and RNAse properties [9]. Angiogenesis or formation of new capillaries within bone tissue could facilitate supply of nutrients and removal of waste products and may be responsible for healing of fractures, remodeling and regeneration [10, 11].

Lactoferrin (LF), a multi-functional protein, present in neutrophils and exocrine secretions like milk and tears, is also present in synovial fluid of bone joints. LF is a major constituent of colostrum responsible for the rapid growth and development of skeletal and immune system of the newborn. Recent studies have established LF as a bone growth factor as it stimulates osteoblast differentiation and new bone formation; and reduces bone resorption in vitro and in animal models [12, 13]. LF is also a transport protein that could facilitate absorption of many essential minerals and nutrients that bind to it; however, the effect of LF on bone health in humans is not known. The objective of the present study is to investigate the benefits of milk RNAse-enriched LF (R-ELF) supplement along with calcium on bone health of postmenopausal women in comparison to an appropriate age-matched control that received only calcium supplement. The bone health status was assessed by well-established assays for markers of bone resorption and bone formation. Our studies have, for the first time, demonstrated that R-ELF significantly reduced the bone resorption markers and simultaneously increased the bone formation markers; and suggest possible utilization of R-ELF in natural supplements for osteoporosis without significant side effects.

Materials and methods

Subjects

More than 50 women responded to the announcement about this study made through notification in a local community organization. All of the prospects completed 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. The exclusion criterion for the study included potential risk factors for osteoporosis, dietary calcium intake, and medical history. Women who had history of any illness (active Paget's disease, hyperthyroidism, hypothyroidism, type I diabetes mellitus, calcium intolerance, kidney problems; cancer diagnosis for solid malignancies, and inflammatory bowel disease) that affect bone mineral metabolism were excluded. Women that received treatment with estrogens, progesterone, raloxifene, or tamoxifen and antiresorptive agents like calcitonin or bisphosphonates were also excluded. We also excluded women with life expectancy of less than 2 years; current and ongoing use of methotrexate, phenytoin, phenobarbital, or inhaled corticosteroids at a dose greater than 800 μg/day; and a body mass index above 32 or below 20. On evaluation, 38 healthy, ambulatory postmenopausal women, 45–60 years old, with no menses for at least 12 months were registered for the study. Every effort was made to recruit only those women who fulfilled the inclusion and exclusion criteria. Nonetheless, there were three exclusions, one with a history of treatment for bone health and other two with hypothyroidism. The study was approved by the appropriate Institutional Review Board of the Western University of Health Sciences in Pomona, CA, USA. Prospective participants were advised of the nature of the study and provided written informed consent before participation.

Study design

Thirty-five women included into the study were randomly assigned to one of the two groups: placebo group or R-ELF

group. R-ELF is a ribonuclease (angiogenin)-enriched lactoferrin either co-isolated from bovine milk (50:50 ratio wt/wt) or both proteins admixed to obtain required ratios, as previously described [14, 15]. There were 15 subjects in the placebo group and were supplemented with 100% recommended daily allowance (RDA) of calcium, in tablet form, whereas 20 subjects of the R-ELF group were administered with two R-ELF capsules of 125 mg each, along with 100% RDA of calcium administered orally from day 1 to day 180. Venous blood (by standard venipuncture technique) and urine samples were collected from each subject on day 0 (baseline before the commencement of supplementation), day 15, day 30, day 60, day 90, and day 180 of the study. Standard systolic/diastolic blood pressures as well as the body weight were also monitored at baseline and the aforementioned days. All the participants were interviewed at each visit for adherence to the regimen and occurrence of adverse events. A flow diagram of the study is shown in Fig. 1.

Biochemical markers of bone turnover

Venous blood was drawn into a vacutainer (BD Biosciences, NJ, USA) by standard venipuncture technique. The blood was allowed to clot and the serum was separated by centrifugation. The serum specimens were stored at −80°C until analysis. Urine was collected after an overnight fast and frozen at −20°C until analysis. On the day of analysis, serum and urine samples were thawed, mixed, centrifuged, and the clear supernatant was used for the assay. Bone formation rates were assessed by measuring two well-established markers of osteoblast activity: (1) change in levels of bone-specific alkaline phosphatase in the serum (sBAP) [16] and (2) change in serum levels of intact bone gla protein or osteocalcin (sOC) [17]. Bone resorption was also investigated by identifying the change in levels of two resorption markers, N-telopeptides in the serum (sNTx) [18] and free deoxypyridinoline crosslinks in urine (uDPD) [19]. These four bone turnover markers were monitored in the urine and blood samples collected from each subject, before R-ELF supplementation (baseline) and with R-ELF supplementation at the end of 15, 30, 60, 90, and 180 days. Metra-BAP, Metra-OC, and Metra-DPD antibody immunoassay kits used for the study were obtained from Quidel, San Diego, CA, USA. Osteomark NTx enzyme immunoassay kit was purchased from Inverness, Princeton, NJ, USA. Urinary creatinine was determined using standard colorimetric assay (Oxford Biomedical Research, Oxford, MI, USA) based on well-known Jaffe reaction [20].

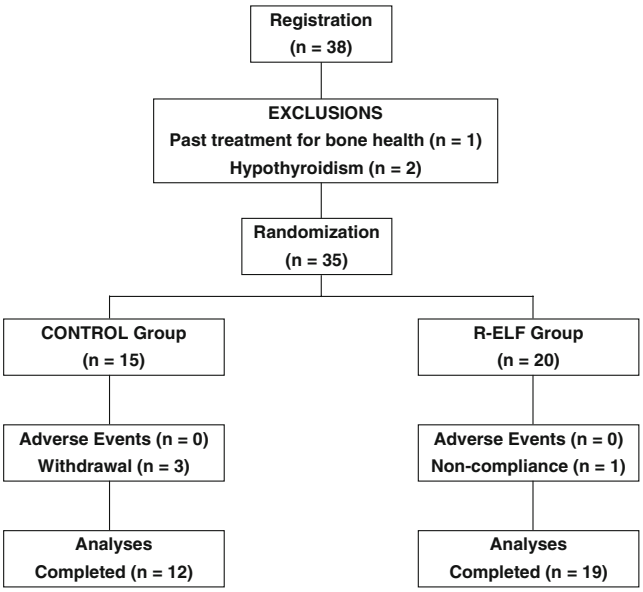

Fig. 1 Flow-chart of the study

Statistical analysis

Biochemical bone turnover marker data for each observational 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 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 throughout this study, as it is least influenced by the extremes of data. The Kolmogorov–Smirnov test (KS test) was used for normal distribution of data within each set [21]. Non-linear least-squares regression of the marker data to a logistics curve was performed to evaluate the peak levels of bone turnover markers at the end of the study. Student's unpaired two-sample *t* test was used for comparison of the mean observed change in markers for placebo and R-ELF datasets in order 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. The data shows a comparable match between the placebo and R-ELF groups, i.e. generally good bone health status and distribution of the bone turnover marker levels. The median and range of markers are within the accepted levels for generally healthy, postmenopausal women [22–32]. Three participants from the control group

Table 1 Baseline characteristics of the study population

Characteristic	Typical values for PM women[a]	Control	R-ELF
Number of participants (*n*)		15	20
Age (years)			
Mean±SD		51.0±4.4	53.5±5.4
Range		45–59	45–60
Weight (lbs)			
Mean±SD		134±20	141±24
Range		104–174	107–208
Blood pressure (mm Hg)			
Mean systolic		121	127
Mean diastolic		78	80
Bone resorption status			
Serum NTx (nM BCE[b])	15.9 (8–28)	15.8±2.6[c] (5.3–29.1)	12.9±3.3 (7.8–41.6)
Urine Dpd (nM/mM CR[d])	7.5 (4–12)	6.6±0.7 (5.2–11.7)	8.2±0.6 (6.0–13.2)
Bone formation status			
Serum BAP (U/L)	25 (14–43)	25.0±1.3 (18.9–30.6)	19.7±2.4 (13.1–44.8)
Serum OC (ng/mL)	−3–10	4.1±0.5 (2.1–7.0)	4.2±0.4 (2.6–9.8)

[a] Reference [20–30]
[b] Bone collagen equivalents
[c] Median±SEM given for all markers. Range shown in parenthesis
[d] Creatinine

dropped out of the study before day 15 and one from the R-ELF group was dropped out from the study due to non-compliance. There was >95% compliance to the supplement regimen among the subjects. The body weight and blood pressures 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.

Bone resorption markers

The median sNTx and uDpd changed gradually to a peak level for both placebo as well as R-ELF groups, as shown in Fig. 2. The resorption markers for each observational day were also normally distributed (p=0.34–0.99). sNTx for the placebo group increased from 15.8±2.6 nM bone collagen equivalents (BCE) on day 0 to 22.1±1.7 nM BCE on

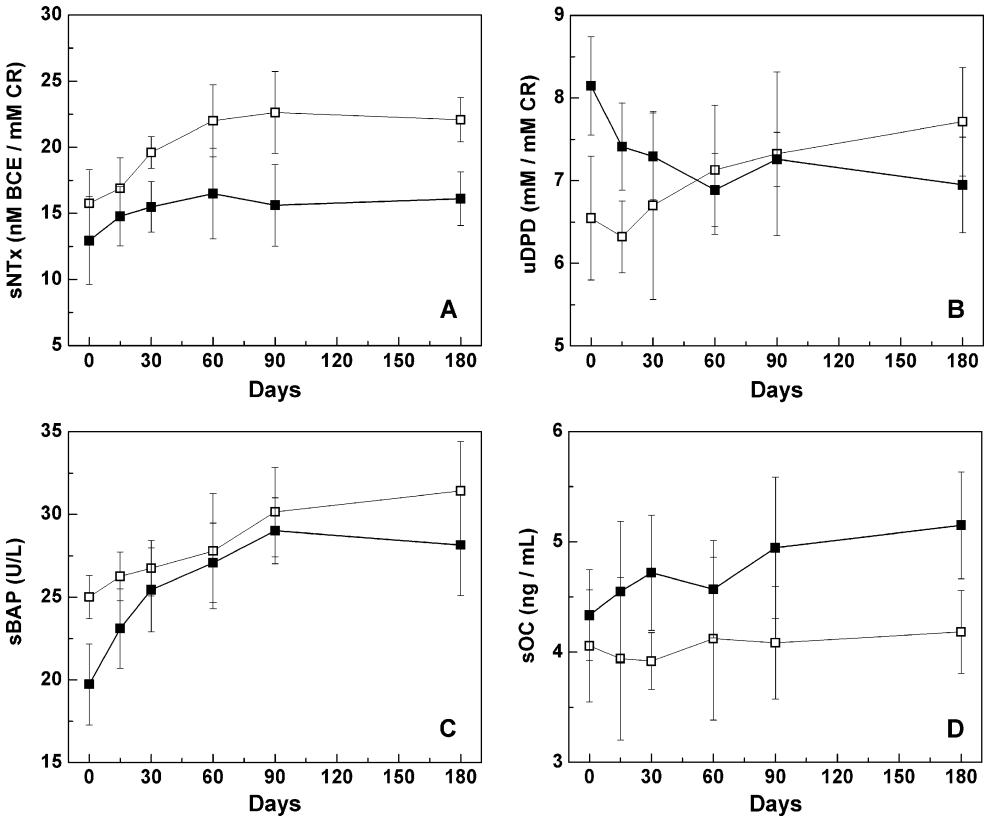

Fig. 2 Variation of bone resorption markers—sNTx (**a**) and uDpd (**b**) and bone formation markers—sBAP (**c**) and sOC (**d**) with the progress of the study. Median±SEM data are shown for placebo (*unfilled square*) and R-ELF (*filled square*) groups

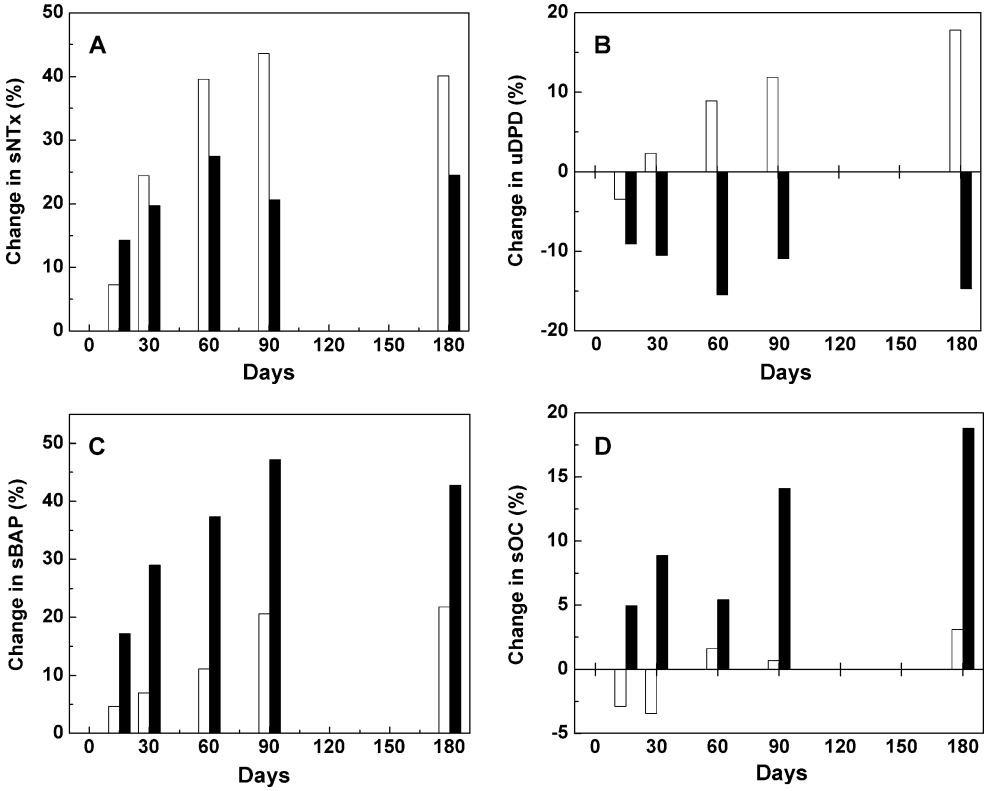

Fig. 3 Change from the baseline of median bone turnover markers with the progress of the study—sNTx (**a**), uDpd (**b**), sBAP (**c**), and sOC (**d**). *Open bars* denote the placebo group and *filled bars* denote R-ELF group in all the panels. In the case of sOC, change in mean from baseline level is shown

day 180, while R-ELF group displayed a relatively smaller rise (12.9±3.3 to 16.1±2.0 nM BCE). The change in bone turnover markers, calculated as a percent of the corresponding baseline levels are represented in Fig. 3. Median sNTx for the placebo group showed an increase of 40.1% in 180 days, reflecting significant bone resorption while the R-ELF group showed a relatively smaller rise of 24.5% during the same period ($p<0.001$, 95% CI). The duration to achieve 80% of the peak change in sNTx is about 25 days for R-ELF group compared to 45 days for the placebo group, indicating that R-ELF supplementation induces its effects within a short time. The calculated parameters for the bone markers and the results of statistical tests are summarized in Table 2.

An interesting reversal of trend was observed with median uDpd levels although the placebo group showed an increase from 6.6±0.8 to 7.7±0.7 by day 180, the R-ELF group decreased from 8.2±0.6 to 6.9±0.6 nM Dpd/mM Creatinine. The overall rise of 17.8% indicated a significant level of bone resorption in the placebo group. In contrast,

Table 2 Progress of biochemical bone turnover markers and statistical analysis

Marker	Change from baseline (%)		Time to reach 80% of peak level in days	*t* test[c]
	Median[a]	Peak[b]		
Bone resorption markers				
Serum NTx				
Placebo	32.0±7.6	41.6	45	15.18
R-ELF Group	20.2±4.0	24.1	25	<0.001
Urine Dpd				
Placebo	5.6±3.3	19.2	95	4.11
R-ELF Group	−10.7±2.2	−14.0	31	<0.01
Bone formation markers				
Serum BAP				
Placebo	9.1±3.9	25.4	115	4.10
R-ELF Group	33.2±7.2	44.9	45	<<0.001
Serum OC				
Placebo	0.4±1.0	5.1	–[d]	−20.3
R-ELF Group	7.2±2.8	16.4	–	<0.001

[a] Median±SEM
[b] Peak change in the marker obtained from a non-linear least squares fit to logistics curve
[c] Value of the *t* statistic. Significance *p* (95% CI) is given in parenthesis
[d] Not determined as there was no significant change in serum OC for the control group

Dpd levels of the R-ELF group showed a reduction in resorption by ~10% within 30 days and continued to fall to 14.6% by the end of the study (p=0.0021, 95% CI). The R-ELF group achieved 80% of peak change in Dpd levels in about 31 days, compared to 95 days taken by the placebo group.

Bone formation markers

The two markers of bone formation, sBAP and sOC, were determined at the same pre-defined intervals during the study. Figure 2 shows the measured variation of median sBAP and sOC levels for both groups. The range of observed sBAP and sOC values are similar to that reported in other recent studies [22–32]. As with the bone resorption markers, the sBAP and sOC datasets for each observational day were normally distributed (p=0.39–0.98).

The variation of sBAP and sOC from their respective baseline levels is depicted in Fig. 3. Median sBAP gradually increased to a peak level for both the groups (25.0±1.3 to 31.4±3.0 U/L for placebo and 19.7±2.4 to 28.1±3.1 U/L for the R-ELF group). Although median levels for R-ELF group are lower than those for the placebo group, the percentage change from baseline was better for the R-ELF group. The 42.7% elevation for R-ELF group compared to 25.7% for the placebo was also statistically significant (p<0.001, 95% CI). The peak level attained was about twice that of the placebo group and 80% of peak change was achieved within ~45 days of R-ELF supplementation. In the case of sOC, mean marker levels maintained within ±3% of baseline for placebo (4.1±0.5 to 4.2±0.4 ng/mL), while those for the R-ELF group increased linearly by 18.8% from 4.3±0.4 to 5.2±0.5 ng/mL. The results summarized in Table 2 indicate that change in sOC with supplementation was statistically significant (p<0.001, 95% CI).

Bone formation markers were observed to be highly correlated with bone resorption markers for both groups, with Pearson correlation coefficient (r) values in the range 0.58–0.93 for the placebo and 0.66–0.90 for the R-ELF group, respectively. The correlation plots along with the statistical parameters are shown in Fig. 4. The positive correlation observed for placebo group is generally improved with R-ELF supplementation, as seen from the higher r and smaller p values for sNTx with sBAP as well as sOC. In case of uDpd, the positive correlation seen with the placebo group is changed to negative for the R-ELF group—r changing from 0.93 to −0.87 for sBAP and from 0.83 to −0.66 for sOC, respectively. This change reflects the effect of R-ELF supplementation to reduce resorption markers while increasing the formation markers to restore balance of bone turnover.

Discussion

Clinical studies of large populations of postmenopausal women have established that biochemical markers of bone metabolism such as serum osteocalcin, bone-specific alkaline phosphatase, and the urinary excretion of cross-linked collagen N-telopeptides are indicators of loss of bone mineral density, which in turn is a risk for osteoporosis and

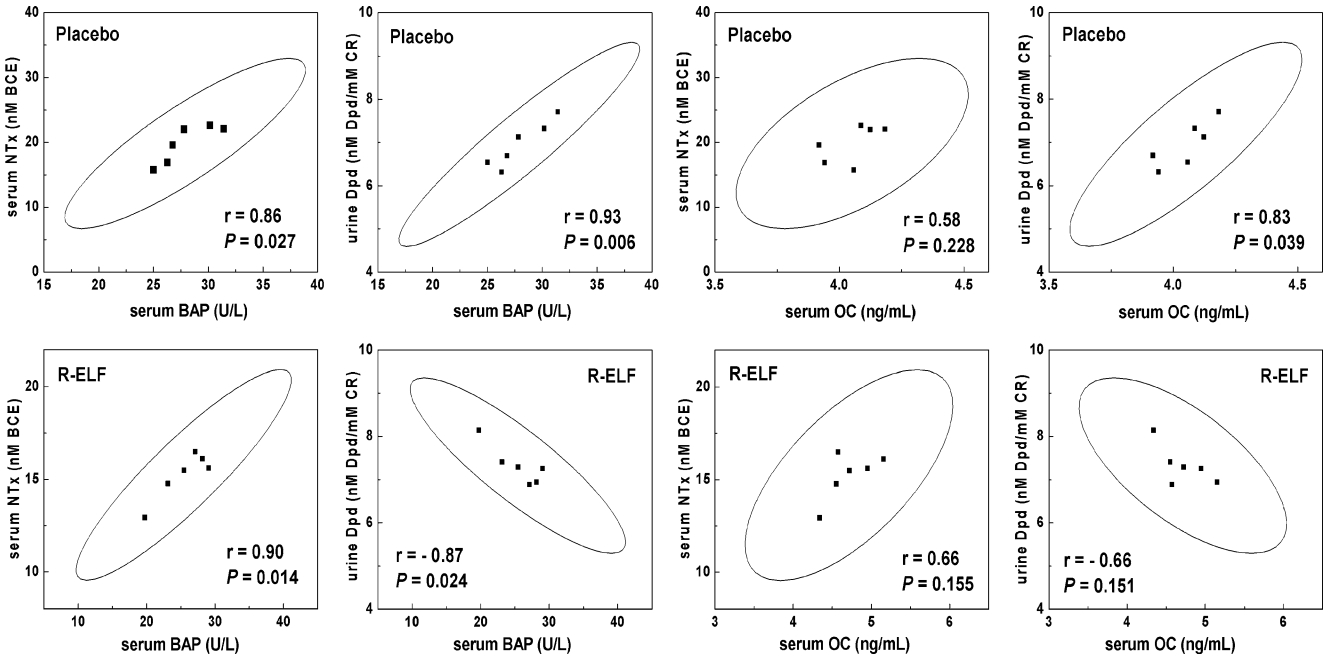

Fig. 4 Correlation plots of bone formation markers vs. bone resorption markers. *Top row* corresponds to the placebo group and the *bottom row* to the R-ELF group. The Pearson correlation coefficient (r) and significance (p) are shown for each plot

fractures. In the present study, R-ELF supplementation has been shown to improve bone formation markers, while reducing the bone resorption in postmenopausal women. In addition, R-ELF supplementation is able to achieve this significant change in bone turnover markers within a short duration. The ability of R-ELF supplementation to improve bone metabolism is attributed to the characteristics of its key components, RNAse and LF. Firstly, LF is a transport protein and present in all exocrine secretions in the body including synovial fluid in the joints. Specific LF receptors have been described in mammalian cell types and tissues including monocytes, lymphocytes, platelets, liver, mammary epithelial cells, and intestine [33]. LF also interacts with low-density lipoprotein receptor-related protein 1 in osteoblasts and increases the mitogenic activity, indicating that LF enhances the bone growth. [34]. It can also form complexes with many nutrients like the glucosaminoglycans, due to its highly positive charge. Therefore, LF is able to interact with cells and may deliver the nutrients to key locations in bones and joints. Furthermore, LF is also known to stimulate osteoblastic activity [12, 13]. Secondly, formation of new capillaries and supply of nutrients is essential for the rejuvenation of the aging bone tissue. Milk RNAse present in the R-ELF may accomplish this function, as it is known to stimulate angiogenesis [35].

Several strategies for the restoration of postmenopausal bone turnover have been developed and established with the support of bone mineral density data over the last decade, and have been approved for therapy. Bisphosphonates, like alendronate and its variants, have been used for treatment of age-related bone loss in postmenopausal women and have consistently shown substantial reduction in bone resorption. However, this reduction in resorption is also accompanied by up to 40% decrease in bone formation as reflected by sBAP and sOC markers in general, and as high as 52% reduction in one trial [29, 30, 36]. In HRT or estrogen therapy trials, a reduction by 30–50% of resorption has been observed along with 20–45% reduction in bone formation makers [26, 36]. Selective estrogen receptor modulator such as raloxifene hydrochloride has shown a gradual 25–30% decrease of resorption markers in 3 years along with 10–20% decrease in formation markers [37, 38]. In contrast to above treatment options, R-ELF not only reduced the resorption markers by about 20% but also affected bone formation rate positively (up to 40% increase), within 3 months of supplementation.

Strontium renelate, isosorbide mononitrate, and teriparatide (synthetic parathyroid hormone (PTH)) are among the few other therapeutic agents that show a positive effect on bone formation as well as a reduction in bone resorption activity [39–41, 32]. Organic nitrates stimulate osteoclasts and osteoblasts via the production of NO and have been observed to elevate bone formation levels. In a recent study, isosorbide mononitrate (ISMO) showed a 45% decrease in resorption markers and a 23% increase in formation markers compared to the placebo group [40]. However, ISMO induced mild to moderately severe headaches in some participants in that study. In cardiovascular health studies, nitrates have been known to cause this side effect in as many as 36–52% of participants [42]. In a recent clinical study of PTH [32, 41], there was a 92% enhancement of sBAP, but was accompanied by 72% increase in bone resorption as measured by uDpd. Although teriparatide has been approved, PTH treatment is known to have adverse effects, at least in animal model studies [43, 44] and requires further long-term evaluation [41]. Of note, similar elevation of bone formation marker was observed by daily oral supplementation of R-ELF in the present study, compared to subcutaneous injection of PTH. Therefore, R-ELF supplementation would provide alternative treatment without significant adverse effects.

Correlations between bone turnover markers for some studies of interventions with HRT, prednisone and teriparatide [26, 28, 32] were compared with our results. Similar to the present study, a generally improved correlation between resorption and formation markers with the intervention was observed. However, the correlation did not change from positive (for placebo) to negative for the treatment group. This is so because reduction in resorption by HRT and bisphosphonate treatments is accompanied by significant decrease in bone formation marker like BAP [45]. Although teriparatide treatment resulted in favorable increase in BAP, it was associated with increased resorption levels [41]. In both these cases, the correlation between the formation and resorption markers remained positively correlated even after intervention, leading away from a balance of bone turnover. A negative correlation between the markers is a condition of increased bone formation and reduced resorption leading to attainment of the balanced bone turnover and is the preferred state of outcome. In the present study, uDpd is negatively correlated with formation markers, sBAP and sOC, after R-ELF supplementation; and therefore, appears to lead towards restoring the balance.

In summary, R-ELF supplementation in preliminary human clinical trials, for the first time, has shown promising and favorable effect on biomarkers of bone turnover in postmenopausal women. Despite the small number of subjects, short duration and lack of bone density data in the present study; and in view of the several shortcomings of drug therapy for postmenopausal bone loss, the results of R-ELF supplementation are very significant, as it is based on safe and natural milk proteins.

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Conflicts of interest S. Bharadwaj, A. G. Tezus Naidu, and A. S. Naidu declares conflict of interest – all are employed by the N-terminus Research Laboratory. All other authors have no conflict of interest.

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