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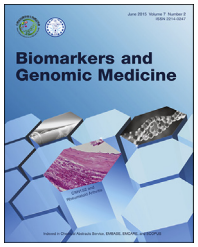

ORIGINAL ARTICLE

The anti-osteoporosis effects of CSN1S2 protein of goat milk and yoghurt on a complete Freund's adjuvant-induced rheumatoid arthritis model in rats

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Abstract This study aimed to evaluate the anti-osteoporosis effect of CSN1S2 protein from goat milk and yoghurt on a complete Freund's adjuvant (CFA)-induced rheumatoid arthritis (RA) model in rats. Twenty-four rats were randomly divided into six groups: control (untreated) group (C), group treated with CSN1S2 (CM) protein from goat milk, group treated with CSN1S2 protein from goat yoghurt (CY), RA group, RA group treated with CSN1S2 protein from goat milk (RAM), and RA group treated with goat yoghurt (RAY). Mineral elements and mesostructure were analyzed using X-ray fluorescence and scanning electron microscopy. Bone histomorphometry was analyzed using the BoneJ software. One way analysis of variance and Tukey post hoc tests were used to analyze and compare the means of all variables between groups. The phosphorus levels were not significantly different between treatment groups relative to the control group ($p > 0.05$), but were significantly higher in the CM and RAY groups relative to that observed in the CY group ($p < 0.05$). CSN1S2 protein of goat milk repaired the collagen structure in the femur trabecular bone. The trabecular thickness and volume were significantly lower in CM and CY groups relative to the control group ($p < 0.05$). The trabecular volume also decreased significantly in the CM group relative to the control group ($p < 0.05$). The trabecular thickness was significantly lower in the CY group relative to the CM group ($p < 0.05$), but the

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trabecular separation and trabecular volume were significantly greater ($p < 0.05$). The trabecular volumes were significantly elevated in the RAM and RAY groups relative to the CM group ($p < 0.05$). The trabecular thickness was significantly higher in the RAY group relative to the CM and CY groups ($p < 0.05$). CSN1S2 protein of goat milk is better than goat yoghurt in repairing femur crystallization and mesostructure in CFA-induced rheumatoid arthritis in rats.

Introduction

Rheumatoid arthritis (RhA) is a chronic inflammatory disease with multiple risk factors. The pathomechanism of RhA is associated with chronic soft-tissue inflammation often followed by bone and cartilage destruction.[1] Bone complications are the major extra-articular complications of RhA and can be described as three different forms: periarticular bone loss, adjacent to the inflamed and swelling joints; bone erosions, which share common mechanisms with the periarticular osteopenia; and systemic osteoporosis.[2] Studies indicated that there is higher frequency of osteoporosis and reduced bone mass in RhA patients, in which the largest effect is found on the hip, as compared to healthy controls.[3–6] There is also a two-fold increase in osteoporosis in female RhA patients ranging in age from 20 years to 70 years,[7] with osteoporosis being one of the determinants of fracture risk. RhA patients have an increased risk of fractures of the hip, vertebrae, and pelvis.[7–9] Studies also suggested that women with early-onset RhA have significantly lower femoral neck and whole-body bone-mineral density (BMD), but have similar lumbar spine BMD as compared with controls. The frequency of bone loss at all sites is significantly greater in women with RhA than that of the controls.[10]

Nutritional disorders affect the pathological condition and long-term outcome of physical functions in RhA. Many studies have reported that malnutrition is a well-recognized symptom associated with RhA.[11] The values of nutritional markers, such as albumin, are low in patients with RhA, owing to malnutrition and excessive protein catabolism triggered by high levels of inflammatory substances.[12] Therefore, the nutritional status may be deeply involved in RhA outcome or symptoms.

Bioactive peptides from milk proteins were included within the primary sequence of native proteins that are hydrolyzed by digestive enzymes and then cleaved and modified to produce an active product as a fragment of specific proteins. Bioactive peptides play important roles as signaling molecules in physiological functions and pathogenesis-related chronic diseases, such as hypertension, diabetes, cancer, and osteoporosis, as well as immunomodulatory, anti-hypertension,[13,14] anti-inflammation,[15] anti-oxidative,[16] and anti-glycosylation[17] molecules. Milk proteins are major suppliers of amino acids and micronutrients in young mammals or humans. Milk is also rich in biologically active peptides necessary for health. Bioactive peptides are products of enzymatic fermentation that is an important aspect of dietary proteins and offer adequate nutritional effects. Many researchers have reported that peptide-derived milk fermentation products, such as yogurt, cheese, or other fermented-foods, display more beneficial biological functions related to cell signaling pathways as compared to peptides from fresh milk.[13,14,18,19] However, the bioactive peptides from fresh milk and yogurt that regulate pathways associated with osteoporosis and RhA remain unclear.

Food-derived bioactive peptides represent a source of health enhancing components that may be incorporated into functional foods and/or used as nutraceuticals.[20] Moreover, milk protein-derived bioactive peptides are health-enhancing components that can be used to reduce the risk of disease or to enhance certain physiological functions.[21] Whole casein and electrophoretic-casein fractions have been shown to exhibit different biological activities, such as immunomodulation.[22,23] Our recent study reported that goat milk has the alpha-S2 casein (CSN1S2), a protein with a molecular weight of 36 kDa, which is not present in bovine milk. We have also determined that this protein contains eight peptides analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry[24]; however, there is no study to evaluate the ability of CSN1S2 protein isolated from goat milk and yoghurt to inhibit femur osteoporosis in RhA. Therefore, this study aimed to evaluate the anti-osteoporosis properties of CSN1S2 protein from goat milk or yoghurt in RhA-associated osteoporosis.

Materials and Methods

Animals

Twenty-four 12-week old adult male Wistar rats, weighing 150—200 g, were acclimatized for 1 week to the laboratory conditions prior to experimental manipulation. These animals were purchased form Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta, Indonesia. The animals were exposed to a 12-hour light and 12-hour dark cycle at room temperature of 24°C. They had free access to a standard laboratory diet and water *ad libitum*. The animals were randomly divided into six groups ($n = 4$ each): control (untreated) group (C), group receiving CSN1S2 protein from goat milk (CM), group receiving CSN1S2 protein from goat yoghurt (CY), RhA group (RA), RhA group receiving CSN1S2 from goat milk (RAM), and RhA group receiving CSN1S2 from goat yoghurt (RAY).

Freund's adjuvant-induced arthritis in rats

The induction of arthritis in rats was performed according to previous studies with modifications.[19] Adjuvant-induced arthritis in rats was established by a single subcutaneous injection of 100 μL of complete Freund's adjuvant (CFA, Sigma–Aldrich, St. Louis, MO, USA). Fourteen days after the first injection, the rats were intradermally injected with 50 μL CFA in the lower extremity. Arthritic rats were divided into RA, RAM, and RAY groups, while the rats that did not receive CFA injections were divided into C, CM, and CY groups.

Preparation and administration of casein

The casein was isolated from milk samples from Etawah goats (250 mL). The milk was heated to a temperature of 40°C prior to addition of glacial acetic acid (5 mL) while stirring until precipitate formation. The precipitate was separated by filtration through a nylon mesh membrane. The protein content was measured using a NanoDrop machine (NanoDrop; Thermo Scientific, Wilmington, DE, USA) and then stored at −20°C if not immediately used. The CSN1S2 protein from goat milk and yoghurt was then orally administrated to the rats by gavage at doses of 2 mL/kg body weight according to a previous study with modification.[25]

Tissue preparation

At the end of the treatment, all rats were anesthetized with diethyl ether. Both femurs were cut using a bone saw, collected, weighed, and rinsed and cleaned with physiological saline. All femur samples were stored in glutaraldehyde until analysis.

Femoral bone mineral content

Femoral bone mineral content was analyzed by X-ray fluorescence (XRF) by placing bones in a bone tube and scanning according to manufacturer instructions. The bones were analyzed at 20 kV accelerating voltage (MiniPAL 4; PANalytical, Almelo, The Netherlands).[26]

Femoral bone hydroxyapatite crystals

Characterization of the X-ray diffraction results was performed using PANalytical X'Pert PRO-MPD (PANalytical B.V., Almelo, Netherlands). Subsequent analysis was performed using the software programs as follows: High Score Plus (PANalytical), Crystal Maker (CrystalMaker Software, Ltd., Begbroke, UK), and DDVIEW (http://www.icdd.com), complemented with the latest version of PDF2 (http://www.icdd.com). Diffraction spectra were recorded at an angle of 2θ, from 20° to 60°, with a Cu-K α radiation source (wave length = 1.54056 Å, 40 mA, 40 kV) and step size of 0.05°.[26] The lattice parameter of each mineral atomic coordinate was visualized with crystal maker.

Femur mesostructure

The femur head was cut with a scalpel. For scanning electron microscopy (SEM) evaluation, femurs from all groups were cut vertically at the femoral head, and then the femoral bones were fixed in phosphate-buffered formalin, dehydrated in a graded concentration of ethanol, and coated with gold and palladium. The processed bones were then analyzed at 20 kV accelerating voltages by SEM (Inspect TM S50; FEI, Hillsboro, OR, USA).[26]

Femur histomorphometry

Histomorphometric measurements were carried out according to methods from a previous study with modifications.[27] The measurements at the femur were performed at 500× objective magnification using a SEM (FEI), and then the data were analyzed by an image analyzer BoneJ (http://bonej.org/). The parameters measured in this study were trabecular number, trabecular separation, trabecular bone volume, and trabecular thickness. Trabecular number (Tb.N, expressed per millimeter) and trabecular separation (Tb.Sp, expressed per micrometer) were calculated assuming that the trabecular bone could be modeled by the parallel plates and bar model (Tb.N = Tb.Ar 10/Tb.Th; Tb.Sp = 1000/Tb.N − Tb.Th). Trabecular bone volume (BV/TV) is the amount of trabecular bone within the spongy space (expressed as a percentage). Trabecular thickness (Tb.Th, expressed in micrometers) was derived from trabecular perimeter (B.Pm) and trabecular area (B.Ar) (Tb.Th = 1.99 B.Ar/2/B.Pm).[28]

Ethics

This study has been evaluated and approved by the research ethics committee of Faculty of Sciences, Universitas Brawijaya, Malang, East Java, Indonesia (Registration number, KEP-90-UB).

Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using one way analysis of variance and the Tukey post hoc test with SPSS 16.0 statistical package (SPSS, Inc., Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

Results

Mineral composition of CSN1S2 protein isolated from goat milk and yoghurt

There were several inorganic compounds found in the CSN1S2 protein from goat milk and yoghurt, including phosphorus, sodium, calcium, iron, nickel, copper, and ytterbium. The CSN1S2 protein from goat yoghurt had lower levels of calcium and nickel, but higher levels of phosphorus as compared to the CSN1S2 protein from goat milk (Table 1).

Table 1 Mineral content in the CSN1S2 protein from milk and yoghurt.

Mineral contents (%)	CSN1S2-milk	CSN1S2-yoghurt
Phosphorus	5.1 ± 0.3	4.6 ± 0.1
Sodium	15.0 ± 0.4	16.0 ± 0.3
Calcium	39.0 ± 0.8	52.4 ± 0.5
Iron	6.6 ± 0.07	7.0 ± 0.1
Nickel	3.0 ± 0.2	7.1 ± 0.4
Copper	12.0 ± 0.3	13.0 ± 0.2
Ytterbium	19.0 ± 1	0.0 ± 0.0

Data are presented as mean ± standard deviation.

Femoral bone mineral content

Table 2 presents the femoral bone mineral content for each group. The levels of calcium, chromium, iron, copper, zinc, nickel, and ytterbium were not significantly different between groups ($p > 0.05$). The phosphorus level in the CY group was significantly lower as compared to the CM group ($p < 0.05$). The phosphorus level was also significantly higher in the RAY group relative to the CY group ($p < 0.05$).

Femoral bone hydroxyapatite crystal

Figure 1 shows the crystal size and lattice parameters. The crystal size was increased in the RA group and all treated groups as compared to the control (untreated) group. The lattice parameters were different between groups. The crystallinity was significantly higher in the CM, CY, RAM, and RAY groups relative to the control (untreated) group ($p < 0.05$). However, the crystallinity was not significantly different in the RA group as compared to the control group ($p > 0.05$). Inclusion of the CSN1S2 protein from goat milk and yoghurt significantly increased the crystallinity relative to the control (untreated) or RA groups ($p < 0.05$; Figure 2).

Femur mesostructure

The SEM results for the femur from the control (untreated) group showed a step-ladder pattern of collagen with granule formations covering the fibrillar collagen. In RA rats, there were irregular step-ladder patterns of collagen with resorption cavities and several hole sizes. The CSN1S2 protein from goat milk repaired the collagen structure in trabecular bone, as indicated by the reduction in holes and the rearrangement of fibrillar collagen into step-ladder patterns (smooth surfaces). In groups administered CSN1S2 protein from goat yoghurt, the fibrillar collagen and irregular topography of the trabecular bone surface were still observed (Figure 3).

Femur histomorphometry

Table 3 shows the characteristics of femoral trabecular bone among treated supplementation and control groups. The trabecular thickness and volume were significantly lower in the CM and CY groups as compared to the control group ($p < 0.05$). The trabecular volume was significantly decreased in the CM group relative to the control group ($p < 0.05$). All parameters were not significantly different in the RA, RAM, and RAY groups as compared to the control group ($p > 0.05$). The trabecular thickness was significantly lower, but the other parameters were significantly larger in the CY group relative to the CM group ($p < 0.05$). The trabecular volume was significantly elevated in the RAM and RAY groups relative to the CM group ($p < 0.05$). The trabecular thickness was significantly higher in the RAY group relative to the CM and CY groups ($p < 0.05$). However, all parameters were not significantly different in the RAM or RAY groups as compared to the RA group ($p < 0.05$).

Discussion

The fermentation process may help improve nutritional profiles. Foods produced through biotechnological processes (such as fermentation) may prevent or at least minimize the risk of lifestyle-related disease.[29] Our findings showed that the CSN1S2 protein from yoghurt (fermentation product) had lower levels of calcium and nickel, but higher phosphorus as compared to the CSN1S2 protein from goat milk. In the scope of bone health, although calcium is one of the main minerals in bones, previous human studies

Table 2 Femoral-bone mineral content in treated and control (untreated) groups.

Mineral (%)	C	CM	CY	RA	RAM	RAY
Calcium	84.25 ± 1.71	84.50 ± 0.34	82.85 ± 5.83	81.60 ± 3.11	82.95 ± 0.17	83.65 ± 3.05
Phosphorus	10.30 ± 4.06	9.94 ± 1.33	4.95 ± 1.21*	10.85 ± 0.98	11.80 ± 0.92	9.80 ± 1.96**
Chromium	0.65 ± 0.63	0.25 ± 0.14	0.41 ± 0.09	0.66 ± 0.61	0.57 ± 0.48	0.58 ± 0.37
Iron	0.98 ± 0.10	0.85 ± 0.39	1.65 ± 0.63	1.26 ± 0.98	1.16 ± 0.62	1.15 ± 0.39
Nickel	1.05 ± 1.74	0.95 ± 0.76	2.32 ± 2.16	1.54 ± 0.39	1.66 ± 0.83	2.41 ± 0.39
Copper	0.34 ± 0.06	0.22 ± 0.03	0.47 ± 0.05	0.48 ± 0.31	0.43 ± 0.21	0.47 ± 0.09
Zinc	0.75 ± 0.11	0.98 ± 0.60	1.42 ± 0.77	0.75 ± 0.51	0.56 ± 0.30	0.67 ± 0.27
Ytterbium	0.25 ± 0.17	0.15 ± 0.17	0.05 ± 0.05	0.15 ± 0.17	0.35 ± 0.40	0.50 ± 0.57

Data are presented as mean ± standard deviation.
C = control (untreated) group; CFA = complete Freund's adjuvant; CM = group receiving CSN1S2 protein from goat milk; CY = group receiving CSN1S2 protein from goat yoghurt; RA = RhA group; RAM = RhA group receiving CSN1S2 from goat milk; RAY = RhA group receiving CSN1S2 from goat yoghurt; RhA = rheumatoid arthritis.
* $p < 0.05$ in comparison with CM group.
** $p < 0.05$ in comparison with CY group.

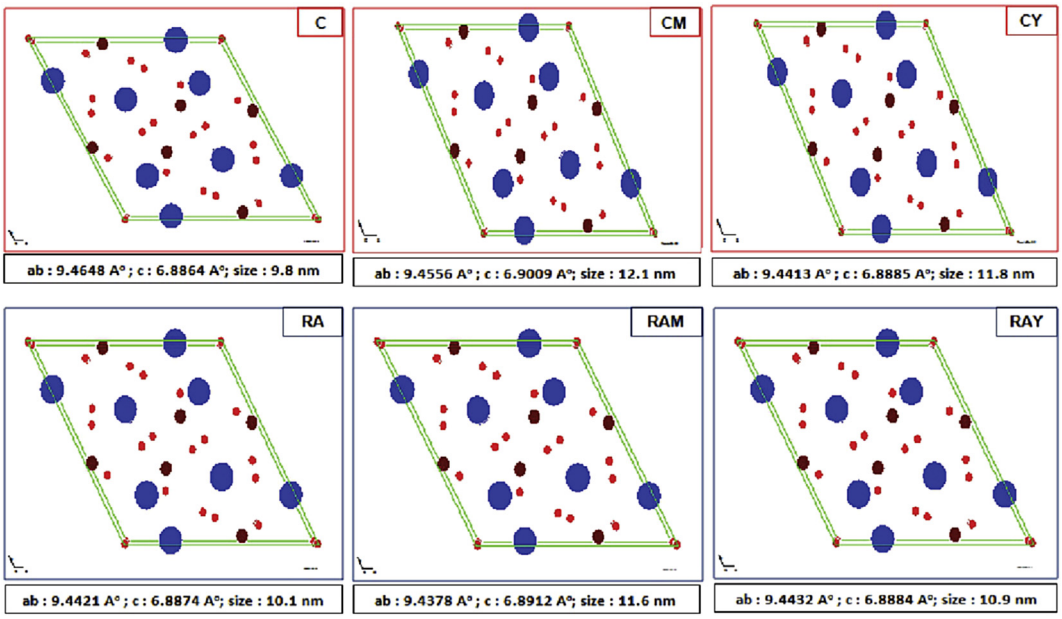

Figure 1 Hydroxyapatite crystal and lattice parameters of femur from CFA-induced RhA in rats. The size of crystal was increased in RhA groups with or without treatment and all control treatment group compared to control group. Blue circle, calcium; red circle, oxygen; and brown circle, phosphorus. C = control (untreated) group; CFA = complete Freund's adjuvant; CM = group receiving CSN1S2 protein from goat milk; CY = group receiving CSN1S2 protein from goat yoghurt; RA = RhA group; RAM = RhA group receiving CSN1S2 from goat milk; RAY = RhA group receiving CSN1S2 from goat yoghurt; RhA = rheumatoid arthritis. [a]: x axis; [b]: y axis; [c]: z axis

showed that there is hypermineralization and higher calcium levels in hydroxyapatite crystal structures from osteoporosis patients.[30,31] This phenomenon encouraged investigation to discover the ideal mineral composition of food aimed to inhibit osteoporosis and osteoporosis-related disease.

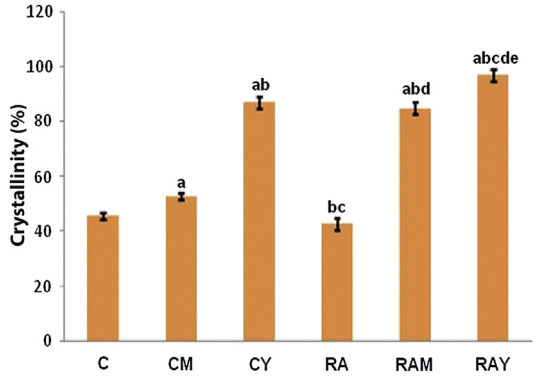

Figure 2 The percentage of femoral-bone crystallinity of CFA-induced RhA in rats. Treatment with the CSN1S2 protein from goat milk and increased crystallinity, which is significantly different in the RhA group as compared to the control group. C = control (untreated) group; CFA = complete Freund's adjuvant; CM = group receiving CSN1S2 protein from goat milk; CY = group receiving CSN1S2 protein from goat yoghurt; RA = RhA group; RAM = RhA group receiving CSN1S2 from goat milk; RAY = RhA group receiving CSN1S2 from goat yoghurt; RhA = rheumatoid arthritis. [a]: $p < 0.05$ in comparison with control group; [b]: $p < 0.05$ in comparison with CM group; [c]: $p < 0.05$ in comparison with CY group; [d]: $p < 0.05$ in comparison with RA group; [e]: $p < 0.05$ in comparison with RAM group.

The biological effects of inorganic compounds in processes associated with bone formation and turnover have also been described, leading to findings related to therapies for bone disease and degradation. An example of such therapy is the clinical use of strontium ranelate in patients with osteoporosis.[32] This study discovered several inorganic compounds in the CSN1S2 protein from goat milk and yoghurt, including phosphorus, sodium, calcium, iron, nickel, copper, and ytterbium. Moreover, the CSN1S2 protein from goat yoghurt has higher levels of phosphorus and lower levels of calcium and nickel relative to those observed in the CSN1S2 protein from goat milk. These minerals can be substituted or incorporated into hydroxyapatite crystals of the bone. Subsequently, the composition of minerals in hydroxyapatite crystals determines bone mesostructure and integrity.[33] This study demonstrated that the levels of calcium, chromium, iron, copper, zinc, nickel, and ytterbium were not significantly different between groups ($p > 0.05$). This result may be due to the substitution process that occurs when one of these minerals replaces a comparable atom due to the similarities in their radii and charges.[34] This finding indicates that CFA-induced RhA can cause osteoporosis, but possibly not as a result of changes in mineral concentration. We hypothesized that inflammation in RhA is a trigger of bone resorption. Our previous data found that CFA-induced RhA inflammation may not be interleukin-17 dependent, but rather dependent upon the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α), which could activate nuclear factor kappa-B signaling involved in inflammation and apoptosis in bone cells.[35–37]

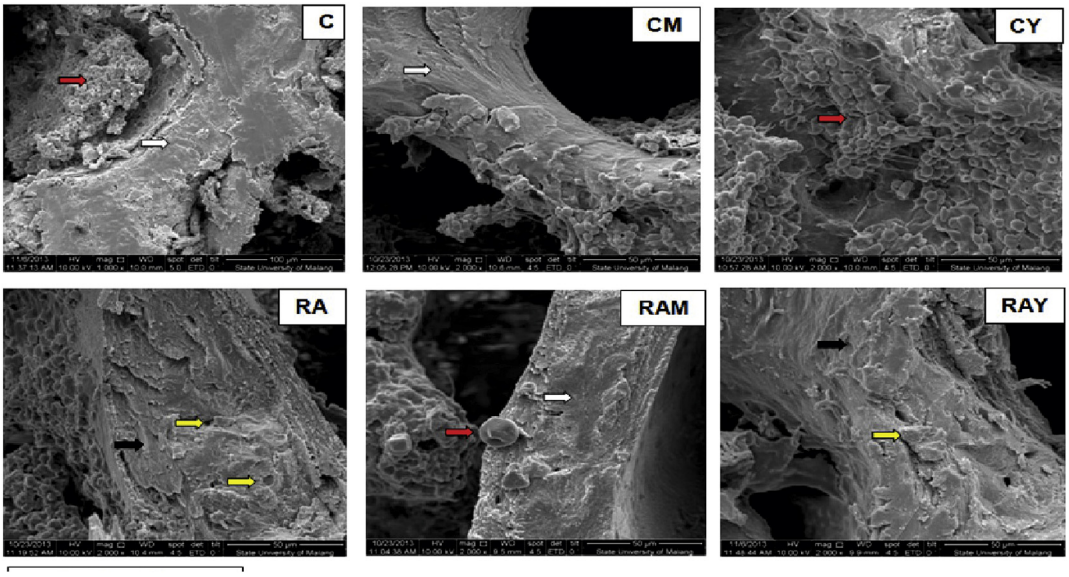

Figure 3 Micrograph illustrating the mesostructure of rat femurs. In normal rats, step-ladder patterns of collagen (white arrow) and granule formations (red arrow) covering the fibrillar collagen were observed. In RhA rats, there were irregular step-ladder patterns of collagen (black arrow) combined with resorption cavities and various holes (yellow arrow). The CSN1S2 protein from goat milk improved the collagen, as indicated by the reduction in holes and the arrangement of fibrillar collagen (step-ladder patterns or smooth surfaces) in RhA rats. In groups administered the CSN1S2 protein from goat yoghurt, the fibrillar collagen and irregular topography of the trabecular bone surface still existed in the RhA rats (SEM: 10.0 KV, magnification 2000×). C = control (untreated) group; CFA = complete Freund's adjuvant; CM = group receiving CSN1S2 protein from goat milk; CY = group receiving CSN1S2 protein from goat yoghurt; RA = RhA group; RAM = RhA group receiving CSN1S2 from goat milk; RAY = RhA group receiving CSN1S2 from goat yoghurt; RhA = rheumatoid arthritis; SEM = Scanning electron microscope.

The phosphorus level in the CY group was significantly lower as compared to the CM group ($p < 0.05$). This finding showed that the administration of yoghurt reduced the femoral bone phosphorus level in normal rats. The phosphorus level was significantly higher in the RAY group relative to that of the CY group ($p < 0.05$). The result indicated that inflammatory conditions elevated the accumulation of phosphorus absorbed from yoghurt, leading to the increase in femoral bone mineral content. Additionally, serum phosphorus levels also contributed to the femoral phosphorous level. Previous studies postulated that the elevation of phosphorus levels is related to tissue hypoxia and an increase in ATP degradation, resulting in the release of inorganic phosphorous from cells. Hypertrophy and hyperplasia creates a hypoxic environment in synovial joints.[38]

The composition of mineral elements determines the crystal size, crystal lattice parameters, and crystallinity. This study demonstrated that the crystal size was increased in the RA group and all treated groups compared to the control group. The crystallinity was not significantly different in the RA group as compared to the control group ($p > 0.05$). This finding indicated that CFA-induced RhA did not affect trabecular crystallinity. The treatment with the

Table 3 The characteristics of femoral trabecular bone in treated and control (untreated) groups.

Indicator	C	CM	CY	RA	RAM	RAY
Tb.Th (μm)	3.42 ± 1.03	2.00 ± 0.26*	1.46 ± 0.04*,**	1.90 ± 1.35	1.92 ± 0.72	3.09 ± 0.58**,***
Tb.Sp (μm)	1.26 ± 0.47	0.84 ± 0.00	1.24 ± 0.20**	0.82 ± 0.13***	0.84 ± 0.00***	0.94 ± 0.21
Tb.N (mm^{-1})	897.04 ± 331.97	1191.10 ± 12.66	823.62 ± 136.76**	1250.40 ± 207.13***	1167.3 ± 20.44***	1104.90 ± 247.18
BV/TV (%)	58.30 ± 12.70	40.50 ± 2.73*	50.80 ± 2.97**	48.85 ± 11.55	48.70 ± 5.80**	50.40 ± 4.16**

Data are presented as mean ± standard deviation.
BV/TV = trabecular volume; C = control (untreated) group; CFA = complete Freund's adjuvant; CM = group receiving CSN1S2 protein from goat milk; CY = group receiving CSN1S2 protein from goat yoghurt; RA = RhA group; RAM = RhA group receiving CSN1S2 from goat milk; RAY = RhA group receiving CSN1S2 from goat yoghurt; RhA = rheumatoid arthritis; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number.
* $p < 0.05$ in comparison with control group.
** $p < 0.05$ in comparison with CM group.
*** $p < 0.05$ in comparison with CY group.

CSN1S2 protein from goat milk and yoghurt significantly increased the crystallinity as compared with the control (untreated) or RA groups ($p < 0.05$). Our findings indicated that the CSN1S2 protein from goat milk and yoghurt inhibited the amorphous states of crystals in normal or RhA rats. The highest crystallinity was achieved by yoghurt treatment, which was confirmed by hypergranulation observed in the femur mesostructure. Osteoporosis is an amorphous process caused by an irregular degenerative condition, which is difficult to characterize in the scope of wide ranges of bone mineralization.[33] The growth of minerals in hydroxyapatite crystals occurs under specific orientations, where the c-axis of the crystal is approximately parallel to the length axis of the collagen fiber.[39]

Osteoporosis is characterized by a reduction in bone mass and micro-architectural deterioration of the bone, which increases bone fragility and fractures.[40] Trabecular bone strength is determined not only by the amount of composite material (mineral, protein, and water), but also the distribution of these materials (size, area, and structural properties). A number of properties, such as wall thickness, well-connected trabeculae, and plate-like trabeculae, confer better and stronger trabecular bone properties.[41–43] The trabecular region in the femur or tibia may be the optimal region for detecting such bone loss.[44] SEM of the femoral head of rats from the control (untreated) group showed step-ladder patterns of collagen with granule formations covering the fibrillar collagen. In RhA rats, there were irregular step-ladder patterns of collagen with resorption cavities and several hole sizes, but the levels of trabecular properties were not significantly different in the RA group as compared to the control group ($p > 0.05$). The CSN1S2 protein from goat milk repaired collagen structure in trabecular bone, as indicated by the reduction in holes and the rearrangement of fibrillar collagen into step-ladder patterns (smooth surfaces). In groups administered CSN1S2 protein from goat yoghurt, the fibrillar collagen, irregular topography of trabecular bone surface, and excessive granule formations remained. Excessive granule formation indicated excessive bone formation by osteoblasts. This may have been due to components of yoghurt, such as lactoferrin, angiogenin, and growth-factor binding proteins that contribute to the mitogenic activity of osteoblasts.[45,46]

Based on histomorphometry analysis, the CSN1S2 protein from goat milk was more effective than goat yoghurt in maintaining trabecular thickness in normal rats. The CSN1S2 protein from goat yoghurt increased trabecular number and volume, but induced trabecular spacing. The CSN1S2 protein from goat milk increased the trabecular volume in RhA rats, but reduced the trabecular thickness. Overall, this finding indicated that CFA-induced rheumatoid arthritis in rats induced femur osteoporosis and rearrangement of the trabecular wall. The decrease in trabecular thickness in the control group, but the increase observed in the RAY group, indicated that yoghurt induced trabecular bone turnover. The CSN1S2 protein from goat milk is more effective than that from goat yoghurt in repairing the crystallinity, collagen structure, and trabecular mesostructure.

In conclusion our study suggested that CFA-induced RhA in rats induced femoral bone osteoporosis. The CSN1S2 protein from goat milk was more effective than goat yoghurt at improving femoral bone crystallinity and mesostructure in RhA rats. Therefore, the CSN1S2 protein from goat milk is recommended as a source of bioactive peptides for anti-osteoporosis effects in RhA.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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