Comparing the calcium bioavailability from two types of nano-sized enriched milk using in-vivo assay

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Arezoo Erfanian [a,*], Babak Rasti [a], Yazid Manap [b]

[a] Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia
[b] Halal Products Research Institute, Universiti Putra Malaysia, 43400 Selangor, Malaysia

ABSTRACT

Calcium bioavailability from two types of enriched (calcium citrate and calcium carbonate) milks homogenized to a nano-sized particle distribution has been studied among 48 female Sprauge-dawley rats. Skim milk powder was enriched with some essential nutrients (Inulin, DHA & EPA, vitamins B_6, K_1, and D_3) as enhancers of calcium bioavailability according to recommended dietary allowances of the West European and North American. Ovariectomized and ovariectomized-osteoporosis rats were used as a menopause and menopause-osteoporosis model, respectively. Although, nano-sized enriched milk powders had the greatest calcium bioavailability among the groups, but bioavailability of nano-sized calcium carbonate-enriched-milk was significantly ($P < 0.05$) better than nano-sized calcium citrate-enriched-milk. Moreover, the trends were similar for bone calcium, strength and morphology. Therefore, based on the current results the calcium carbonate nano-sized enriched milk could be an effective enriched milk powder in ovariectomized-osteoporosis and ovariectomized rats as a model of menopause-osteoporosis and menopause women.

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1. Introduction

Calcium as an essential mineral is involved in several physiological processes such as bone development. A number of studies have shown that calcium is important for fracture prevention and bone density, especially among elderly women. In 2001, the National Academy of Sciences of the United States reported that adequate calcium intake is critical not only to achieve optimal peak bone mass, but also to modify the rate of bone loss associated with ageing. Gradual loss of bone with aging is normal; however, it may be accelerated by factors such as menopause, serious health conditions or their treatment, and lifestyle factors such as inadequate diet, lack of exercise, smoking, or excessive alcohol consumption (World Health Organization, 2004). Menopause is generally a normal biological and physiological event that occurs in women at an average age of 51. Hormone deficiency, especially in the elderly, can have negative results on body calcium balance, increasing bone loss. According to National Osteoporosis Foundation (NOF) (2002), estrogen hormones in women influence deposition of calcium into bones. Osteoporosis is generally thought as a "woman's disease"

because the prevalence of osteoporosis and the rate of fractures are much higher in postmenopausal women than in elderly men (Cawthon, 2011). Osteoporosis as a silent disease causes bones to become more penetrable. WHO considers osteoporosis as the second striking health care issue worldwide (WHO/FAO., 2003). In the year of 2000 alone, there were an estimated 9 million osteoporotic fractures worldwide, resulting in a loss of 5.8 million disability adjusted life-years (Johnell & Kanis, 2006). It is projected that approximately half of all hip fractures in the world occur in Asia. Currently, there is an increasing incidence of hip fractures in the developed cities in Asia. This is due to socio-economic development in many Asian countries and rapid ageing of the Asian population. One out of four hip fractures, occur in Asia and Latin America. This number of hip fractures will increase to 1 in 2 by 2050. Osteoporotic fractures are claimed to affect 50% of women aged over 50 years. One of the most important modifiable factors in the development and maintenance of bone mass is nutrition. Adequate nutrition plays a major role in the prevention and treatment of osteoporosis (Tucker et al., 2002).

On the other hand, osteoporosis and bone loss cannot be prevent by calcium intake alone, especially during menopause and aging (Heaney, Bilezikian, Holick, Nieves, & Weaver, 2006). To meet calcium recommendations, the bioavailability of calcium is an important factor to consider beyond simply the calcium content

of foods (Kwaka, Leeb, & Leeb, 2012). Bioavailability depends on several factors such as solubility and permeability through intestine. Solubility and permeability increase with decreasing particle size. Nanoparticles are specific dispersions or solid particles in the range of 10–1000 nm. Due to their small sizes, nanoparticles have various physiochemical properties in comparison with their respective bulk compounds (Sanguansri & Augustin, 2006). Physio-chemical properties involve changes in compound strength, optical properties (colour), the surface-to-volume ratio (solubility and reactivity), and conductivity of the nanoparticles (Garti, 2005). Solubility increases with decreasing particle size. Such a change arises because of the presence of an electrical charge on the particle, which is predominant in small particles (Behera, Sahoo, & Patil, 2010). Nanonization techniques are used to improve dissolution rates of mineral into the biological environment, in order to improve the oral bioavailability (Hetal, Bindesh, & Sneha, 2010). Nano delivery system can modify the distribution of the nutrients in the body, therefore, they can enhance nutrients' bioavailability (Whitesides & Grzybowski, 2002). According to previous study Mohanty, Dilnawaz, Mohanty, and Sahoo (2010), the most important factors to increase bioavailability is particle size reduction. Furthermore, bioavailabilities of calcium salts are dependent on the quality of the supplement's formula. Unsuitable formulated compounds do not disintegrate when in contact with gastric secretions, therefore, their absorption decreases (Heaney, 1991).

The current study developed two enriched milk homogenized to a nano-sized particle distribution (nano-sized enriched milks). Enrichment was consisted of calcium citrate/calcium carbonate and incorporating some enhancers for bioavailability of calcium (*i.e.* fat and water soluble vitamins including B_6, K_1, and D_3, inulin, and docosahexaenoic acid (DHA) as well as eicosapentaenoic acid (EPA)). The main purpose of this research was to study the influences of preparation condition on the calcium bioavailability from the designed enriched milks in ovariectomized-osteoporosis and ovariectomized rats. For this purpose, feces calcium, serum calcium, femur calcium content, femur breaking strength (maximum load) and femur structure morphology were measured.

2. Materials and methods

2.1. Milk enrichment

In this study, the enriched milks contained calcium citrate/calcium carbonate, vitamins K_1, D3, B_6, DHA & EPA (1:1), and inulin based on recommended dietary allowance of the Western European and North American (WHO, 2001). All of the above mentioned compounds were food grade and were provided by the Finuco (Sankyo Seifun Co, Ltd, Chugoku/Okayama, Japan). Skim milk was purchased from Fonterra (Kuala Lumpur, Malaysia). Milk enrichment was done based on the previous study (Erfanian, Mirhosseini, Abd-Manap, Rasti, & Hair Bejo, 2014).

2.2. Optimization of preparation condition of nano-sized enriched milks

To prepare the samples, enriched milks were dispersed in deionized water (20% w/v) (Erfanian et al., 2015). Briefly, coarse samples were obtained by mixing at 1000 rpm for 5 min (Silverson L4R, Buckinghamshire, UK). To produce fine calcium citrate/calcium carbonate nano enriched milks, different homogenization condition (200–400 bar pressure and 3–5 cycle) were used by a high-pressure homogenizer (APV, Crawley, UK). Polydispersity index (PDI) and mean particle size (MPS) of enriched milks were measured by dynamic light-scattering analysis (Malvern ZEN

1600, Worcester, UK). The experiment of each diluted sample was done in triplicate. In this study, the effect of two factors namely, x_1 (cycle), and x_2 (pressure), on dependent variables, Y_1 (PDI), and Y_2 (MPS (nm)) was studied using response surface methodology (RSM). Fourteen treatments including six center points were created based on a central composite design (CCD), to optimize the preparation condition of nano-sized enriched milks prepared by high-pressure homogenization. The quadratic, interaction, and main effects of factors on the responses were simultaneously studied. To identify the optimum level of preparation condition parameters leading to minimum PDI and minimum particle size, numeral and graphical optimization were carried out (version 16, Minitab Inc., State College, PA, USA) (Rasti, Jinap, Mozafari, & Abd-Manap, 2014). Numeral optimization to predict the exact optimum level of independent variables, leading to the desirable response goals was done by response optimizer (Minitab v. 16). The adequacy and validation of RSM model was evaluated using the *t*-test.

2.3. Transmission electron microscopy

An energy filtered transmission electron microscope (EFTEM; LEO 912 AB Omega, LEO Electron Optics, Oberkochen, Germany) was used to examine the nanoparticle morphology and structure. Reproducibility of the EFTEM images was assured by taking at least four pictures of each sample.

2.4. In-vivo study

This experiment was accepted by Animal Care and Use Committee of Faculty of Veterinary Medicine of Universiti Putra Malaysia (UPM, Selangor, Malaysia). 48 female Sprauge-Dawley rats aged 7-week-old were purchased by veterinary hospital, Universiti Putra Malaysia (Serdang, Selangor, Malaysia). The animals were housed at $22 \pm 2\,°C$, a relative humidity of $50 \pm 5\%$ and a 12 h/12 h day/night cycle. Each animal was embed in a plastic circular metabolic cage. Distilled water and pelleted rat feed (Gold coin animal feed, Kuala Lumpur, Malaysia) was provided *ad-libitum* for acclimatization (7 days). Ovariectomy was performed via surgical removal of the ovaries, which is a well-represented approach to mimic the menopausal condition in rats. The animals were anesthetized with a combination (2:1 v/v) of ketamine (80 mg/kg) and xylazine (10 mg/kg), intraperitoneally (Shirke, Jadhav, & Jagtap, 2008). Sham surgery was done according to the Bimonte-Nelson et al. (2003) procedure. After seven days, they were randomly distributed into three main groups (Sham, ovariectomized and ovariectomized-osteoporosis). All groups included eight rats each and kept for a duration of eight weeks. Sham and ovariectomized rats as first and second groups assigned to the treatment diets. Low-calcium diet fed to the third group which was ovariectomized rats for six weeks. The reason for such treatment was to induce osteoporosis in the rats (ovariectomized-osteoporosis rats). Then, they were fed with experimental diet for eight weeks the same as ovariectomized and sham rats by gavage (5 ml) twice daily. To assure all groups have been fed with similar treatment diets, the other two groups (ovariectomized and sham rats) were maintained on basal diet during that six weeks period.

2.5. Sample collection

Following overnight fasting, the rats were sacrificed under 2:1 (v/v) mixture of xylazine (25 mg/kg) and ketamine (10 mg/kg) anaesthesia (Ken et al., 2000). 5 mL blood was withdrawn by cardiac puncture and then immediately centrifuged at 5000 rpm (2850g) for eight min at 4 °C (Hettich® EBA 20 centrifuge, Andreas

Hettich GmbH & Co. KG, Tuttlingen, Germany) to discrete serum that was kept at −20 °C for further analysis. Femurs were removed, cleaned of adhering tissues, dried and saved for femur strength measurement, calcium determination, and bone structure morphology (Erfanian et al., 2014). For each individual rat, feces were collected in the last six days of the experiment. After that, the feces were finely grounded, air dried, and maintained at 4 °C until further analysis. The experiments (serum calcium, bone calcium, feces calcium, and bone morphology) were carried out in triplicate.

2.6. Experimental analysis

To evaluate of biomechanical function of femurs, femur strength was measured using an Instron apparatus (5 kN model 3365, Norwood, MA, USA). Values for maximum load (N) were provided by the test results (Mohanty et al., 2010). Automatic blood chemical analyzer with Roche testing kits (Roche COBAS MIRA PLUS, Basel, Switzerland) was used to determine serum calcium. Atomic absorption spectrophotometer (Thermo Scientific, S Series, MA, USA) was measured total calcium concentration in the diet, bone and feces (Erfanian et al., 2014). Three micrographs were taken by scanning electron microscopy (SEM) at (×100) magnification (LEO 1455VPSEM, London, UK) (Erfanian et al., 2015). Relative bioavailability and apparent calcium absorption values were calculated based on procedures explained previously by Erfanian et al. (2014).

2.7. Statistical analysis

Minitab Software (Version 16, Minitab Inc., State College, PA, USA) was used to carry out the data analysis. To generate all treatments, a completely randomized design was employed. In each experiment, one-way ANOVA was used to compare between and within groups receiving similar diets. Tukey's multiple-range tests were applied to determine significant ($p < 0.05$) differences among treatment groups.

3. Results

3.1. Response surface analysis

As shown in Table 1, the arrangement of CCD was in such a way that allows the development of the appropriate empirical equations. Randomized experiments were carried out in order to reduce the effects of unexplained variability in the actual responses caused by extraneous parameters. In order to assess the repeatability of the method, the center point was repeated six times. Interaction, quadratic, and linear (main) effects of independent factors were assessed for the response variables (Y_i) by RSM. Table 2 presented two high-pressure variables regression coefficients, along with the corresponding R^2, lack of fit test, p-values, F-ratios and their significant probability of each parameter. The final reduced models fitted for MPS and PDI demonstrated high coefficients of determination (0.995 and 0.965) and (0.953 and 0.965) for nano-sized calcium carbonate-enriched-milk and nano-sized calcium citrate-enriched-milk, respectively. To describe the variation of responses as the function of the main preparation parameters, the satisfactory adjustment of the reduced response surface models were employed. PDI and MPS of nano-sized calcium carbonate-enriched-milk and nano-sized calcium citrate-enriched-milk were significantly ($p = 0.00$) influenced by pressure and cycle (Table 2).

In fact, PDI and MPS of nano-sized calcium carbonate-enriched-milk were significantly ($p = 0.00$) specified as a function of linear, quadratic and interaction impacts of both high-pressure homoge-

Table 1
Comparison between experimental and predicted values based on the final reduced models.

Run[1]	MPS[a]			PDI[a]		
	Y_0	Y_i	$Y_0 - Y_i$	Y_0	Y_i	$Y_0 - Y_i$
1	0.437	0.449	−0.012	0.494	0.497	−0.003
2	0.300	0.300	0.000	0.402	0.410	−0.008
3	0.322	0.300	0.022	0.409	0.410	−0.001
4	0.229	0.242	−0.013	0.420	0.422	−0.002
5	0.291	0.301	−0.010	0.510	0.510	0.000
6	0.300	0.005	0.295	0.421	0.410	0.011
7	0.338	0.011	0.327	0.290	0.291	−0.001
8	0.300	0.005	0.295	0.410	0.410	0.000
9	0.279	0.011	0.268	0.460	0.461	−0.001
10	0.300	0.005	0.295	0.410	0.410	0.000
11	0.452	0.011	0.441	0.360	0.359	0.001
12	0.318	0.008	0.310	0.494	0.491	0.003
13	0.300	0.005	0.295	0.410	0.410	0.000
14	0.281	0.008	0.273	0.408	0.408	0.000
Run[2]	MPS[a]			PDI[a]		
	Y_0	Y_i	$Y_0 - Y_i$	Y_0	Y_i	$Y_0 - Y_i$
1	0.497	0.498	−0.001	0.514	0.518	−0.004
2	0.350	0.341	0.009	0.452	0.457	−0.005
3	0.327	0.341	−0.014	0.439	0.457	−0.018
4	0.259	0.276	−0.017	0.480	0.471	−0.462
5	0.341	0.352	−0.011	0.560	0.553	0.007
6	0.326	0.341	−0.356	0.491	0.457	0.034
7	0.368	0.363	−0.358	0.320	0.326	−0.006
8	0.344	0.341	0.003	0.450	0.457	−0.007
9	0.341	0.324	0.017	0.480	0.490	−0.010
10	0.360	0.341	0.019	0.440	0.457	−0.017
11	0.483	0.488	−0.005	0.370	0.363	0.007
12	0.369	0.359	0.010	0.544	0.547	−0.003
13	0.340	0.341	−0.001	0.470	0.457	0.013
14	0.319	0.317	0.002	0.468	0.468	0.000

MPS: mean particle size; PDI: polydispersity index; Y_0: experimental value; Y_i: predicted value; $Y_0 - Y_i$: residue.
[1] Nano-sized calcium carbonate-enriched-milk.
[2] Nano-sized calcium citrate-enriched-milk.
[a] No significant difference ($p > 0.05$) between Y_0 and Y_i.

nization variables (Table 2). In this study, both MPS and PDI were reversely proportional to the main effect of the cycle and pressure (Table 2).

3.2. Influence of homogenization condition on characteristics of calcium carbonate- and citrate-enriched-milks

Fig. 1(a–d) shows the particle size distribution of nano-sized calcium carbonate- and citrate-enriched-milks. Fig. 1(A–D) illustrates the transmission electron microscopy (TEM) figures of the enriched milks before and after using high-pressure homogenizer. As the Fig. 1 illustrates, the particle size significantly reduced in nano-sized enriched milk (Fig. 1b and d) compared with enriched milks (Fig. 1a and c). The results obtained from TEM supported the results of particle size analyzer.

3.3. Optimizing and validating of homogenization process

The multiple response optimizations leading to the minimum PDI and MPS of the nano-sized calcium carbonate- and citrate-enriched-milks were performed. The minimum MPS and PDI was predicted to be acquired with the combination of ∼300 bar pressure and 4 cycles. The minimum MPS and PDI were predicted to be obtained by the average pressure and cycle. The adequate fitness of the response equations was verified by this observation.

Table 2
Significance probability (p–values and F–ratio) of the independent variable effects in the reduced response surface models of nano-sized calcium carbonate-enriched and nano-sized calcium citrate-enriched-milks.

Variable			Main effects		Quadratic effects		Intteraction effects
			x_1	x_2	x_1^2	x_2^2	$x_1 x_2$
Nano-sized calcium carbonate-enriched-milk	PDI (Y_1)	P–value	0.00	0.00	0.00	–	0.00
		F–ratio	663.5	703.7	128.0	–	941.8
	MPS(Y_2)	p–value	0.00	0.00	–	0.00	0.00
		F–ratio	43.9	110.1	–	43.8	38.1
Nano-sized calcium citrate-enriched-milk	PDI (Y_1)	P–value	0.00	0.00	–	–	0.00
		F–ratio	148.23	83.27	–	–	124.93
	MPS(Y_2)	p–value	0.18	0.00	0.01	0.00	0.00
		F–ratio	2.21	711.98	15.99	679.85	52.19

PDI: polydispersity index; MPS: mean particle size; x_1: cycle; x_2: pressure.
Only the terms with statistical significance are included.

3.4. Influence of experimental diets on total serum calcium

Table 3 shows the serum calcium content of sham, ovariectomized and ovariectomized-osteoporosis rats. Based on the data, the amount of serum calcium of the rats fed with nano-sized calcium carbonate-enriched-milk was more than the rats fed with nano-sized calcium citrate-enriched-milk. It was observed that, although, the highest amounts of serum calcium were belonged to nano-sized calcium carbonate-enriched-milk groups; however, no significant ($p = 0.21$) difference was observed between the sham, ovariectomized and ovariectomized-osteoporosis groups and the experimental diets regardless of treatments.

3.5. Influence of experimental diets on femur calcium

One of the good indicators to estimate calcium bioavailability is measuring femur calcium content. Table 3 presents femur calcium content for experimental groups (sham, ovariectomized and ovariectomized-osteoporosis rats) fed with nano-sized enriched milks. It can be seen from the data in Table 3 that, the femur calcium content in ovariectomized rats was significantly ($p = 0.01$) higher than ovariectomized-osteoporosis rats fed with nano-sized calcium carbonate-enriched-milk, thus showing higher femoral density. In addition, the data in Table 3 provided that the amount of femur calcium in sham group had significantly ($p = 0.04$) rise compared to ovariectomized and ovariectomized-osteoporosis rats fed with nano-sized calcium carbonate-enriched-milk. The same results were seen in the experimental groups (sham, ovariectomized and ovariectomized-osteoporosis) fed with nano-sized calcium citrate-enriched-milk (Table 3). The results showed that the amount of femur calcium content of the rats which fed nano-sized calcium carbonate-enriched-milk had significant difference ($p = 0.00$) compared with the rats which fed nano-sized calcium citrate-enriched-milk (Table 3).

3.6. Influence of experimental diets on femurs' mechanical properties

Table 3 summarizes the data of mechanical properties in the sham, ovariectomized and ovariectomized-osteoporosis rats' femurs. It was realized that the maximum load of ovariectomized rats receiving nano-sized calcium carbonate-enriched-milk significantly ($p = 0.01$) enhanced compared with ovariectomized-osteoporosis rats (Table 3). Furthermore, the data in Table 3 illustrated that the amount of maximum load in sham group had significant ($p = 0.01$) rise compared with ovariectomized and ovariectomized-osteoporosis rats fed the same experimental diet. In addition, it was apparent from Table 3 that, the sham and ovariectomized rats fed a diet containing nano-sized calcium citrate-enriched-milk had similar maximum load with no signifi-

cant difference ($p = 0.09$). It was observed that, the maximum load of sham and ovariectomized rats receiving nano-sized calcium citrate-enriched-milk significantly ($p = 0.01$) enhanced compared with ovariectomized-osteoporosis rats. These results illustrated a significant rise ($p = 0.00$) in maximum load of the nano-sized calcium carbonate-enriched-milk compared with the nano-sized calcium citrate-enriched-milk among experimental groups (sham, ovariectomized and ovariectomized-osteoporosis). As a result, more force was needed to break the femurs of nano-sized calcium carbonate-enriched-milk groups in comparison with the other group (Table 3).

3.7. Influence of experimental diets on femur structure morphology

SEM images of the femurs are presented in Fig. 2. After 8 weeks of feeding with nano-sized calcium carbonate-enriched-milk, the SEM figures of the femurs' rat demonstrated that femur structure morphology was ameliorated in the ovariectomized group compared with the ovariectomized-osteoporosis group (Fig. 2E and H). A similar trend was observed for the rats which fed nano-sized calcium citrate-enriched-milk. As compared with nano-sized calcium citrate-enriched-milk, treatment with nano-sized calcium carbonate-enriched-milk represented better status of femurs among all experimental groups (sham, ovariectomized and ovariectomized-osteoporosis) (Fig. 2F and I). It was observed that canal openings and femur morphology had a great amelioration in nano-sized calcium carbonate-enriched-milk groups.

3.8. Influence of experimental diets on calcium absorption and bioavailability

Fig. 3a and b compares the data acquired from the analysis of calcium absorption and bioavailability. Under experimental conditions, the calcium absorption in ovariectomized rats which fed a diet containing nano-sized calcium carbonate-enriched-milk had a significant ($p = 0.02$) increase compared to ovariectomized-osteoporosis rats fed similar diet (Fig. 3a). Interestingly, it was observed that ovariectomized rats fed nano-sized calcium carbonate-enriched-milk had similar calcium absorption with sham group showing no significant difference ($p = 0.11$). Furthermore, as shown in Fig. 3a, the calcium absorption of nano-sized calcium citrate-enriched-milk had a significant ($p = 0.04$) increase in sham and ovariectomized rats compared with ovariectomized-osteoporosis rats, but there was no significant ($p = 0.18$) difference between sham and ovariectomized groups. In this study, significantly ($p = 0.02$) higher calcium absorption were found in the rats fed a diet containing nano-sized calcium carbonate-enriched-milk compared with the rats fed a diet containing nano-sized calcium citrate-enriched-milk among all experimental groups (sham,
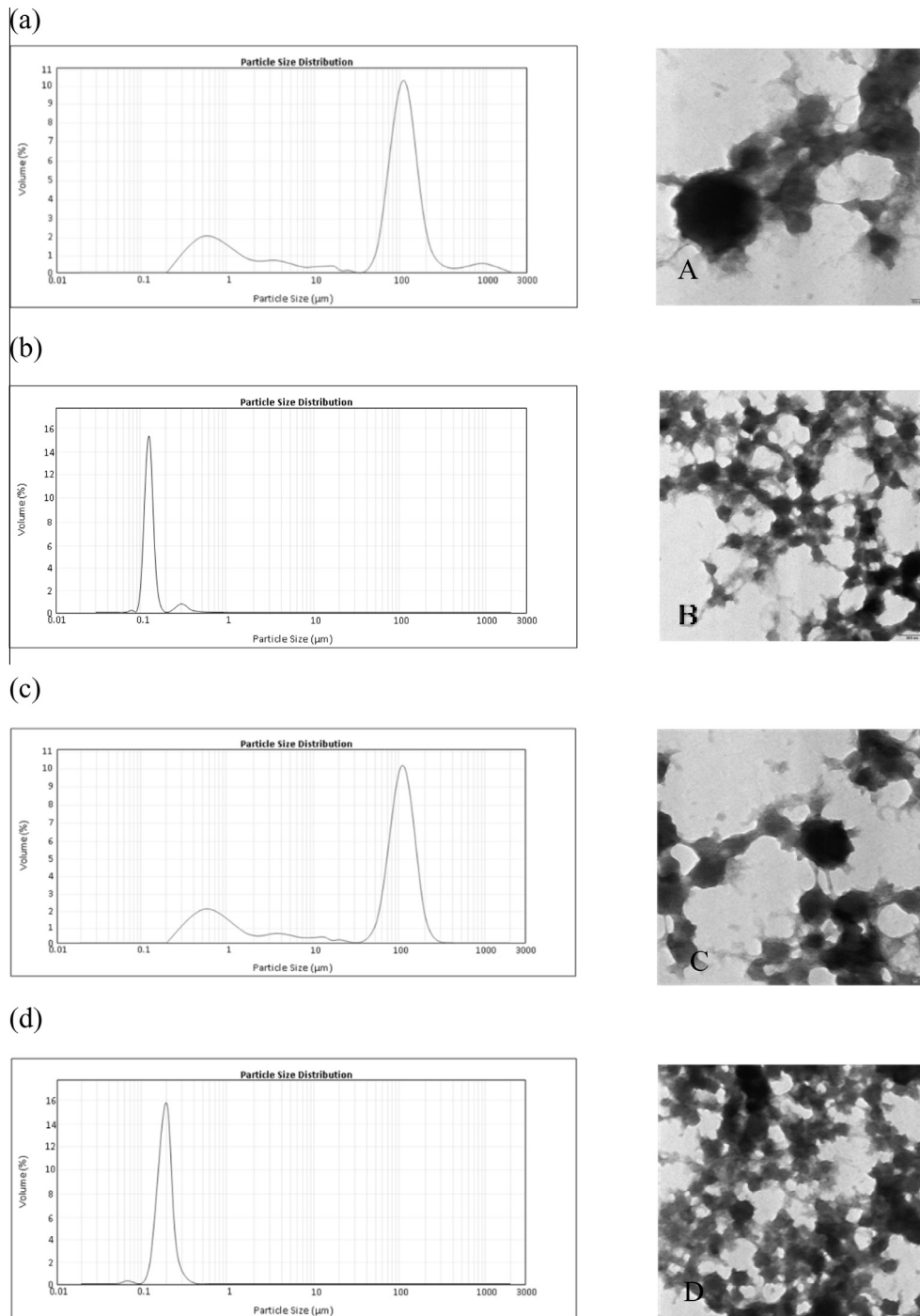
Fig. 1. Particle size distribution of (a) calcium carbonate-enriched-milk, (b) nano-sized calcium carbonate-enriched-milk, (c) calcium citrate-enriched-milk, and (d) nano-sized calcium citrate-enriched-milk. TEM (Transmission electron micrographs) of the milk particles, (A) calcium carbonate-enriched-milk, (B) nano-sized calcium carbonate-enriched-milk, (C) calcium citrate-enriched-milk, and (D) nano-sized calcium citrate-enriched-milk.

ovariectomized and ovariectomized-osteoporosis) (Fig. 3a). The same trends as absorption of calcium were found for the calcium bioavailability in the rats fed nano-sized calcium citrate-enriched-milk (Fig. 3b). The bioavailability of calcium had a positive significant ($p = 0.01$) effect in ovariectomized and sham rats compared with ovariectomized-osteoporosis rats. Moreover, after 8 weeks of feeding, the bioavailability of nano-sized calcium carbonate-enriched-milk was significantly ($p = 0.02$) higher in ovariectomized compared to ovariectomized-osteoporosis rats. Similar to the absorption of calcium, bioavailability of nano-sized
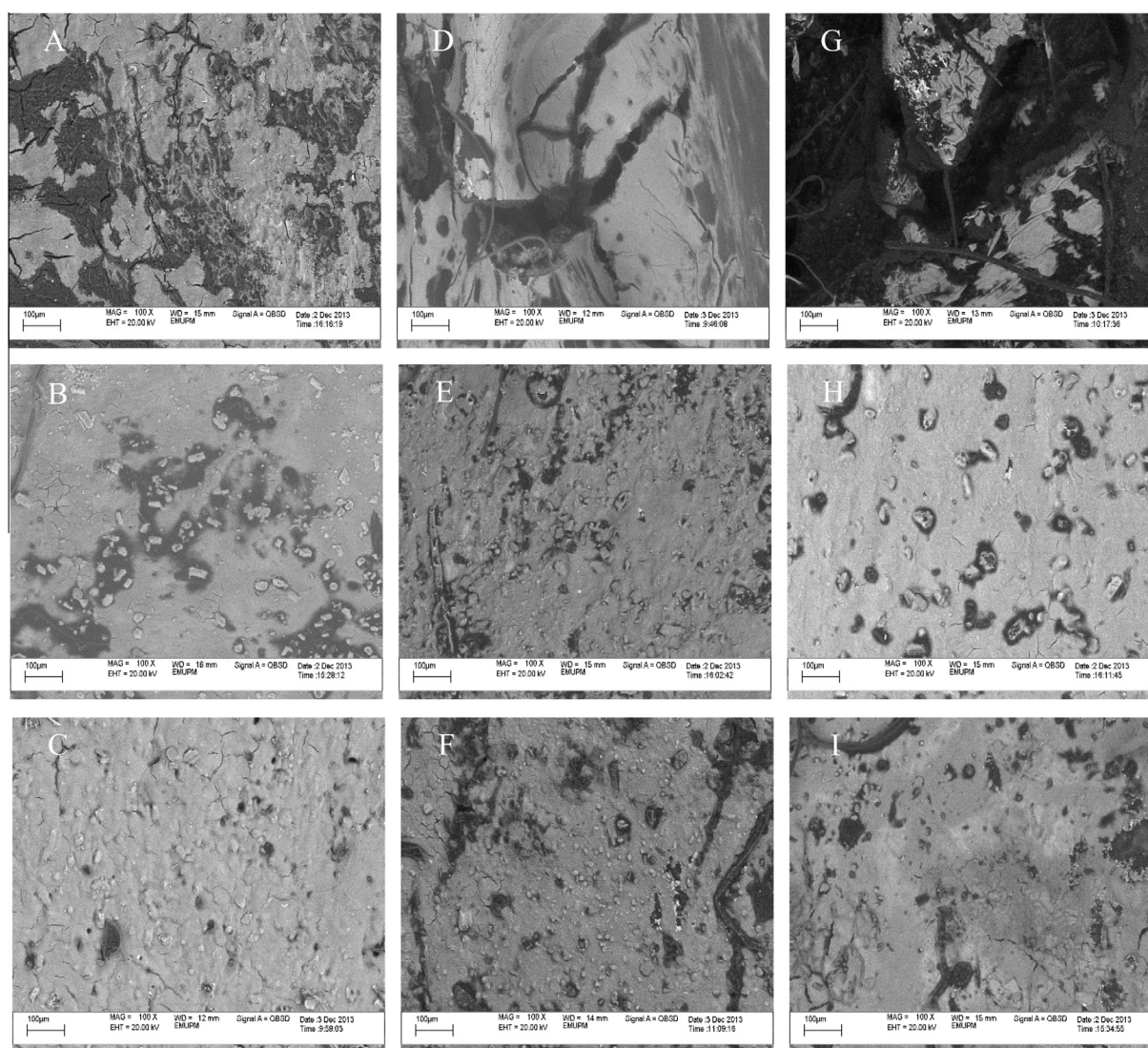
calcium carbonate-enriched-milk was significantly ($p = 0.02$) higher than nano-sized calcium citrate-enriched-milk within all experimental groups (sham, ovariectomized, and ovariectomized-osteoporosis) (Fig. 3b).

4. Discussion

In this study, the impact of two types of nano-sized enriched milks on calcium bioavailability were examined in ovariectomized and ovariectomized-osteoporosis rats. With respect to calcium, it is

Table 3

Serum calcium (mmol/L), Bone calcium content (mmol/g), and Maximum load (N) in sham, ovariectomized rats and ovariectomized-osteoporosis rats (Mean ± SD; $N = 8$).

Experiments	Serum calcium (mmol/L)		Femur calcium (mmol/g)		Maximum load (N)	
Groups/Diets	Nano-sized calcium carbonate-enriched-milk	Nano-sized calcium citrate-enriched-milk	Nano-sized calcium carbonate-enriched-milk	Nano-sized calcium citrate-enriched-milk	Nano-sized calcium carbonate-enriched-milk	Nano-sized calcium citrate-enriched-milk
Sham	3.25 ± 0.13[aA]	3.04 ± 0.19[aA]	6.60 ± 0.03[aA]	6.42 ± 0.04[bA]	101.15 ± 1.09[aA]	90.06 ± 1.21[bA]
OVX[1]	3.22 ± 0.16[aA]	3.05 ± 0.18[aA]	6.08 ± 0.01[aB]	5.56 ± 0.07[bB]	97.96 ± 1.20[aB]	87.37 ± 1.62[bA]
OVX-osteoporosis	3.20 ± 0.47[aA]	3.05 ± 0.16[aA]	3.82 ± 0.06[aC]	2.79 ± 0.08[bC]	74.83 ± 0.40[aC]	70.28 ± 0.21[bB]

Means for each sample sharing the same lowercase letter in each column is not significantly ($p > 0.05$) different.
Means for each sample sharing the same uppercase letter in each row is not significantly ($p > 0.05$) different.
$N = 8$: number of rats in each group.
[1] Ovariectomized.

Fig. 2. Bone SEM images of sham (A–C), ovariectomized (OVX) rats (D–F), and OVX-osteoporosis rats (G–I); rat feeding (A, D, and G), nano-sized calcium carbonate-enriched-milk (B, E, and H), and nano-sized calcium citrate-enriched-milk (C, F, and I).

estimated that only ∼50% of postmenopausal women consume dietary calcium adequate intake of per day. Therefore, it is necessary to persuade women to use the calcium-enriched foods (Breitman, Fonseca, Cheung, & Ward, 2003). Calcium supplements area reliable alternative for women who are not able to consume sufficient calcium from diet alone. When adequate amounts of calcium are not being obtained through the diet, supplementation should be considered. Park, Jeon, Ahn, and Kwak (2007) reported that the development of a highly absorptive calcium source in the intestine will be effective for bone metabolism, but absorption is only one part of the effectiveness of calcium. In (2000) Guéguen and Pointillart highlighted that calcium absorption in the intestines is only the first step in calcium bioavailability, and has to be followed by incorporation of absorbed calcium into the bone.

(a)

(b)
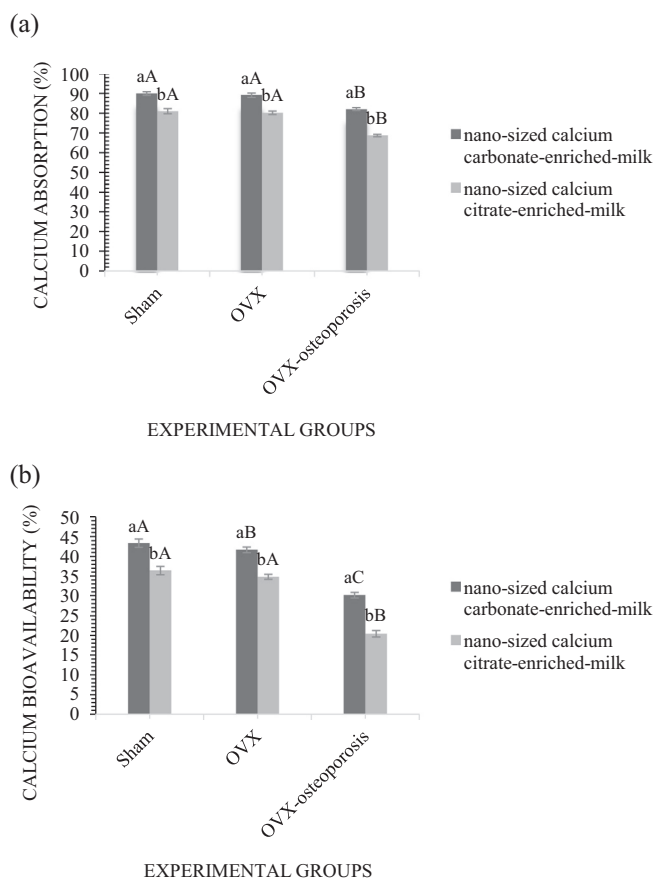
Fig. 3. (a) Absorption and (b) Bioavailability of calcium for sham, ovariectomized (OVX) rats, and OVX-osteoporosis rats. Different lower case letters shows significant difference ($p < 0.05$) between rats fed with different diets. Different upper case letters shows significant difference ($p < 0.05$) among rats fed with same diet.

Therefore, calcium bioavailability from different salts is determined by calcium incorporation in the bone.

In fact, several factors can influence mineral bioavailability in the diet. Using suitable composition and methodology for food enrichment is necessary when dietary calcium intake is inadequate or calcium bioavailability is low. Two of the commonly used calcium sources for food enrichment are calcium citrate and calcium carbonate. They are widely used in calcium enriched food supplements. Calcium carbonate has 40% and calcium citrate has 21% of elemental calcium. Thus, in the present study, they were selected as the preferred sources of calcium for enrichment. Both human and animal studies showed that there is a difference in calcium bioavailability among various calcium salts such as citrate and carbonate (Cocato, 2007; Sakhaee, Bhuket, Adams-Huet, & Rao, 1999). Heller, Greer, Haynes, Poindexter, and Pak (2000) conducted a randomized cross-over study on bioavailability of a single dose of supplements including both forms of calcium. They found the lower absorption of calcium citrate than calcium carbonate. Kenny et al. (2004) revealed that calcium citrate to be more effective in decreasing the markers of bone reabsorption. However, other study showed that different calcium salts have similar calcium bioavailability (Varnai et al., 2003). In (2001) Heaney et al. published a paper in which they observed no important distinction between calcium citrate and calcium carbonate. The present study showed that calcium carbonate showed higher absorption and bioavailability compared to calcium citrate in the nano-sized enriched milk. It might be related to the composition and nano-size reduction of enriched milks which affected the calcium

absorption. As mentioned above, previous researchers explained the positive effects of calcium absorption enhancers like inulin, omega-3 PUFA, vitamins B_6, K_1 and D_3 on intestinal mineral absorption in animals and humans (Nicole et al., 2008).

Furthermore, the processing of food ingredients into nano-sized particles can make them different from the ones existing naturally. Indeed, their enhanced bioavailability and absorption would result in higher internal exposure. Based on findings of Chaudhry et al. (2008) this alteration in properties of the nanoscale processed food might change the way through which the food ingredients breakdown in the gut and; consequently, the way through which they are treated in the gastrointestinal tract. FAO/WHO pointed that, the nano-particles not only trigger biological effects, but also interact with other nearby compounds (Food & Agriculture Organization of the United Nations (FAO) & World Health Organization (WHO), 2009). The nanoparticles also carry these substances into different biological tissues. The carrier ability of nanoparticles may affect the molecules absorption. Therefore, in order to improve the oral bioavailability, nanonization technique is used to enhance mineral dissolution rate into the biological environment (Hetal et al., 2010). Recent study by Mohanty et al. (2010) on the influence of delivery system and particle size reduction on bioavailability revealed that the most great factors to increase bioavailability and absorption is reduction of particle size. Some researchers mentioned that, these mechanisms might increase the solubility of the active ingredient, the rate of mass transfer, and the retention time or the absorption by direct uptake of the nano-particle (Porter & Charman, 2001). Hillyer and Albrecht (2001) assessed gastrointestinal uptake of nano-particles with reducing the droplet size. They have shown that smaller particles were absorbed easier and faster than bigger particles. In another major study, oral administration of milk enriched with nano-calcium to ovariectomized rats can change the calcium metabolism (Park, Ahn, & Kwak, 2008). Moreover, Jinno et al. (2006) reported that ovarian hormone deficiency resulted by bone loss is prevented by reduction of nutrient particle size specifically supplementation with nano-calcium. The results from this study on femur calcium and femur strength are in agreement with the findings of Jinno et al. (2006). Furthermore, the present results seem to be consistent with other researchers' observations which showed the importance of supplementation with nano-calcium to increase bone calcium metabolism in ovariectomized rats (Park et al., 2007). In addition, the results showed that nano-sized particles can be an effective solution for enhancing calcium absorption and bioavailability and preventing bone loss induced by ovariectomy.

5. Conclusion

In this study, the effects of particle size reduction on the calcium bioavailability was investigated in ovariectomized-osteoporosis and ovariectomized rats. The major finding was that bioavailability of calcium can be improved by reducing particle size. The present study found that the combination of composition (calcium carbonate/ calcium citrate, DHA & EPA, vitamins D_3, B_6, K_1, and inulin) and reducing particle size could help to increase the calcium bioavailability. In the present study, nano-sized calcium carbonate-enriched-milk was more effective than nano-sized calcium citrate-enriched-milk on bioavailability and absorption of calcium. In general, it seems that an effective way to prevent bone loss and fracture induced by osteoporosis and ovarian hormone deficiency could be particle size reduction. Thus, such enriched milk products are recommended for subjects at risk of bone loss (especially women over 50 years of age). The knowledge gained through the experiments, outlined in this study, will help

menopausal women to prevent and reduce bone loss. However, considerably more work need to be done to determine the mechanism of the system in human body.

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