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Administration of caseinomacropeptide-enriched extract to mice enhances the calcium content of femur in a low-calcium diet

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ABSTRACT

Caseinomacropeptide (CMP) is the 64 C-terminal amino acid residue of κ-casein, formed by chymosin cleavage during cheese manufacture. This study examined the effects of oral administration of a CMP-enriched extract (CMPEE), obtained from a local dairy plant, on the Ca content of mouse femurs. Animals received low (0.1%, w/v), normal (0.5%, w/v) or high (1.2%, w/v) Ca diet for 3 or 8 weeks and CMPEE diluted (1:10) in their drinking water. No significant differences in Ca content were observed in faeces, kidney, urine or blood serum compared with control animals. The oral administration of CMP to mice significantly enhanced the Ca content in femur under a low-Ca diet model, especially during the period of full body development (3 weeks), in which case a significant 12% Ca increase was observed. These findings pave the way for further studies aimed at supplementing infant food with industrially-obtained CMP-enriched extract for enhanced bone health.

1. Introduction

Fermented dairy products have been traditionally perceived by consumers as good for health and their market has been constantly diversifying in variety of products and increasing in global sales worldwide (Nagpal et al., 2012). Among health benefits ascribed to these products are positive impact on lactose intolerance, weight control, prevention or management of different types of diarrhoea, inflammatory bowel disease, cancer and gut immunity, among others (Adolfsson, Meydani, & Russell, 2004; Lampe, 2011; McKinley, 2005). In terms of the components of fermented dairy products that justify their functionality, bioactive peptides, released from milk proteins during fermentation by proteolytic lactic acid bacteria or by enzymatic cleavage, are particularly relevant (Griffiths & Tellez, 2013). For these peptides, several beneficial physiological activities have been described as well, such as weight management, enhanced immunity and heart, digestive, dental and bone health (Korhonen, 2009). Bioactive peptides might be also produced by breakdown of milk proteins during gastrointestinal digestion (Furlund et al., 2013).

Another source of functional peptides might be food technology. Much effort has been invested in the last two decades in technologies for production, separation, fractioning and isolation of specific dairy proteins or peptides. However, their large-scale manufacture and commercial use remains limited and under development (Korhonen & Pihlanto, 2007). Milk and dairy products have been historically considered important for bone health due to their high bioavailable calcium contents (Caroli, Poli, Ricotta, Banfi, & Cocchi, 2011; Heaney, 2000). In addition to calcium, whey protein has been regarded as a bone protective ingredient (Takada et al., 1997). Caseinomacropeptide (CMP) or glycomacropeptide is the 64 C-terminal amino acid residue of κ-casein, formed by cleavage of this protein by chymosin (or pepsin) during the manufacture of cheese and released into the whey (Thoma-Worringer, Sørensen, & López-Fandiño, 2006). Reported biological activities of bovine glycomacropeptide include ability to bind cholera toxin and *Escherichia coli* enterotoxins, inhibition of bacterial and viral adhesion, suppression of gastric secretions, promotion of bifidobacterial growth and modulation of immune responses (Brody, 2000). In relation to mineral transport, CMP

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administration was reported to enhance zinc absorption in infant monkeys (Kelleher, Chatterton, Nielsen, & Lönnerdal, 2003), to exert inhibitory activity against enamel demineralisation and to promote tooth enamel remineralisation (Aimutis, 2004), and to inhibit bone loss in ovariectomised rats by increasing calcium bioavailability (Neeser et al., 2000).

Most studies of the effects of calcium or CMP on bone health or composition has been performed in rat models (Juma, Sohn, & Arjmandi, 1999; Kim et al., 2009; Scholz-Ahrens et al., 2007; Zeni, Weisstaub, Di Gregorio, Ronanre de Ferrer, & de Portela, 2003). Mice models have been much less exploited for these purposes. However, more recently, Ohtani et al. (2009) reported that a black soybean snack containing 0.9% Ca was effective for the improvement of bone volume loss in ovariectomised C57BL/6J mouse model of osteoporosis. Pérez-Gallardo et al. (2009), also using C57BL/6J mice, studied the effects of calcium-enriched high-fat diet on calcium, magnesium and zinc deposition in the femur of mice. Finally, Lee et al. (2009) showed the beneficial effect of vitamin D2 and calcium-enhanced oak mushrooms on osteoporosis-like symptoms in the femur of ICR mice. The aim of this study was to examine the effects of the oral administration of a CMP-enriched extract (CMPEE) obtained in a dairy plant on the Ca content of the femur of mice fed a low, normal or high Ca diet. To the best of our knowledge this is the first study conducted in a mouse model to assess the effects of CMP on bone constitution.

2. Material and methods

2.1. Caseinomacropeptide-enriched extract

CMP-enriched extract (CMPEE) was obtained from sweet cheese-whey during the manufacture of Argentinean soft cheese. For cheese manufacture, the enzymatic action of commercial chymosin (Chy-Max plus, Chr. Hansen, Buenos Aires, Argentina) splits κ-casein into an insoluble peptide (para κ-casein, which remains part of the curd) and the soluble hydrophilic glycopeptide CMP.

The cheese-whey, containing CMP, had approximately 6% (w/v) of total solids, was submitted to nanofiltration (SepStream APV – FilmTec Membranes, Dow Chemical) for demineralisation and concentration to 20% (w/v) of total solids. Concentrated cheese whey was heated to 90 °C for 15 min at pH 5.60 to precipitate serum proteins, centrifuged (15,400 × *g*, 10 min, 55 °C). (GEA Westfalia Separator, Argentina, S.A.), refrigerated to 4 °C and ultrafiltered (polyethersulfone semi permeable membranes HFK-131 with nominal 10,000 Da cut-off) for lactose and salt removal. The ultrafiltration retentate was regarded as the CMPEE and had the following composition: total nitrogen, 1.5% (w/v); lactose, 8.0% (w/v). The concentration of CMP concentration in the extract was 2.6% (w/v), determined by reversed-phase high performance liquid chromatography (RP-HPLC) using Sigma C-7278 caseinomacropeptide (Sigma Chemical Co., St. Louis, MO, USA) as internal reference for quantification.

2.2. Animals and feeding procedures

Three week-old male BALB/c mice weighing approximately 7–8 g were obtained from the random-bred colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). Animals were maintained at the INLAIN animal facility. Feeding procedures with CMP started the same day mice were separated from their mothers (21 days old). Each experimental group (sampling point) consisted of 6–8 mice housed in groups of 3 or 4 in plastic cages and kept in a controlled environment (21 ± 2 °C and 55 ± 2% humidity), with a 12 h light/dark cycle. When required, animals were housed individually in mouse metabolic cages (Tecniplast, Italy). Mice were maintained and treated according to the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council (NIH). The animal assay was approved by the Ethical Committee for Animal Experimentation of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). The 3 R's principle (replacement, reduction and refinement) was taken into account for the experimental design.

Mice received ad libitum pelleted diets (AIN-76A rodent diets, Research Diets, Inc., New Jersey, U.S.A.). Diets contained 0.1% (w/w), 0.5% (w/w) or 1.2% (w/w) of Ca and were regarded as low, normal or high in Ca, respectively, according to the daily requirement of Ca in mice (Ritskes-Hoitinga, 2004). Mice were also offered, ad libitum, tap water (TW) (control groups) or CMP diluted (1 + 10) in TW (treated groups). CMP solutions were prepared fresh every day and offered in sterilised drinking bottles to avoid microbial contamination. Mice were weighed weekly.

Three-week old mice received low, normal or high Ca diets for 3 or 8 consecutive weeks, as well as TW (control group) or CMP solution (diluted 1 + 10) (treated group). The experiment was replicated twice (two independent assays). Two weeks before the end of the 8-week feeding period, one animal from each of the low and high Ca-diet groups was housed individually for 4 consecutive days in metabolic cages. Total faeces and urine were collected at days 2, 3 and 4 of housing. Material collected during the first day of housing was discarded. This procedure was repeated successively for 3 mice of each group.

2.3. Ca determination in faeces, kidney, urine, blood serum and femur

At the end of the feeding periods, animals were injected intraperitoneally with an anaesthetic cocktail containing 9 parts of ketamine (100 mg mL^{-1}), 9 parts of xylazine (20 mg mL^{-1}), 3 parts of acepromazine (10 mg mL^{-1}) and 79 parts of sterile saline solution. Blood was removed directly from the heart of anaesthetised animals, before they were sacrificed by cervical dislocation and opened for organ removal. Blood samples were allowed to stand at 37 °C for 30 min and centrifuged (5000 × *g*, 15 min, 5 °C). Blood serum was recovered and frozen stored (−70 °C). Kidneys were removed, washed in cold (5 °C) phosphate buffered saline (PBS) solution (pH 7.4) and fixed in 4% (w/v) formaldehyde buffered (pH 6.9) solution for 72 h at room temperature. Kidneys were dehydrated in a series of alcohol. Femurs were removed, cleaned of soft tissues and fixed in 4% (w/v) formaldehyde buffered (pH 6.9) solution for 96 h. Femurs were dehydrated in a series (1 h each) of alcohol of increasing concentration (50°, 70°, 80°, 96°, 100°). Collected faeces and dehydrated kidneys and femurs were dried at 101 ± 1 °C for 24 h and weighed. Faeces and kidneys were ashed in a muffle furnace until obtaining white and crystalline ashes, which were dissolved in 5–10 mL of 3.7% (w/v) HCl and diluted in 0.5% (w/v) lanthanum chloride. Urine and blood serum were diluted in 0.5% (w/v) or 0.2% (w/v), respectively, lanthanum chloride solution. Each dried and weighed femur was treated for 5 min in 1 mL 70% (w/v) HNO_3 and after 2 mL of 0.07% (w/v) HNO_3 were added and left overnight at 55 °C until complete dissolution. Bone solutions were diluted to 10 mL and Ca and Zn (when applicable) were quantified in lanthanum chloride-diluted samples by atomic absorption spectrophotometry (Atomic Absorption Spectrometer Perkin Elmer, model Aanalyst 300, calibration curve between 0.5 and 8 mg Ca L^{-1} (Ca or Zn standard Titrisol® Merck, Darmstadt, Germany).

2.4. Statistical analysis

A total of 156 mice were used in two independent assays (duplicates): 72 mice were used in the first assay (6 animals per group) and 84 animals were used in the second assay (7 animals per group). Each independent assay (duplicate) consisted of two feeding periods of 3 or 8 consecutive weeks, where animals received low, normal or high calcium diets and tap water or CMPEE. The statistical analysis was performed using R© software (2.12.2 version) (R Development Core Team, 2011). This non-parametric test has a lower power than the usual standard distribution-based ones. However, since normality cannot be assumed for this data, the usual tests are not applicable, since they would lead to erroneous conclusions. The test used in this case has no such normality requirement, and its conclusions are therefore valid. For each concentration of Ca in the diet (0.1, 0.5 or 1.2%, w/w) the mean content of Ca in femur (ppm) in the presence of CMP was compared with that obtained when only tap water (TW) was administered. Due to the sample size and the presence of outliers, a robust linear regression model (MM) and permutation tests were used. Permutation and robust tests provide an efficient approach to testing when the data do not conform to the distributional assumptions of the statistical method one wants to use (e.g., normality), when there are outliers, or when the sample size is not large enough to test normality (Legendre & Legendre, 1998). Values of Ca in faeces, urine, blood serum and kidneys and values of Zn in femur were analysed using one-way ANOVA, with the SPSS software (SPSS Inc., Chicago, IL, USA). The differences between means were detected by the Tukey's Multiple Range Test (SPSS, 1996). Data were considered significantly different when $p < 0.05$.

3. Results and discussion

Small animal models (especially rodents) are useful for the study of many functional activities of food ingredients as the first-step experiment towards human trials (Imaoka et al., 2004; Mañé Almero, 2007; Zhou & Gill, 2005). Animal studies, most of them carried out in rats, have shown positive effects of nondigestible oligosaccharides on mineral absorption and metabolism and bone composition and architecture (Scholz-Ahrens et al., 2007). However, no studies had been conducted to date to determine the effects of other food ingredients, such as CMP, on the mineral content of bones using a mouse model. Fig. 1 shows the RP-HPLC analyses of the CMP-enriched extract provided by the dairy industry. Comparing the profiles of the standard and the sample, it can be noted that the CMPEE is composed almost entirely of CMP. The glycosylated part of CMP eluted between minutes 4.5 and 5.7 whereas the two main peaks are the unglycosylated CMP genetic variants A (min 6.1) and B (min 6.9). It is also possible that other non-analysed components shown as minor peaks in the HPLC profile have contributed to some extent to the effects described below.

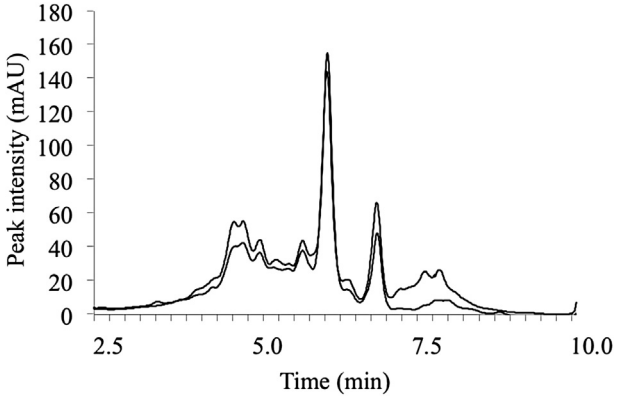

Fig. 1. RP-HPLC profile of CMP standard (0.4 mg mL^{-1} of CMP Sigma C-7278, Upper line before the highest peak and lower line after the highest peak) and the CMPEE (diluted 20 times in mobile phase, Lower line before the highest peak and upper line after the highest peak).

Mice received a low (0.1%), normal (0.5%) or high (1.2%) Ca diet and tap water (control groups) or CMP solution in their drinking bottles for 3 and 8 consecutive weeks and the Ca content of femur was studied by atomic absorption (Fig. 2). The content of Ca in the femur of mice that received diets containing 0.1% of Ca and CMP for 3 and 8 weeks (181.8 ± 17.1 and 211.9 ± 3.3 ppm, respectively) was significantly higher ($p = 0.020$ and 0.044, respectively) compared with control animals (161.8 ± 16.9 and 204.3 ± 10.5 ppm, respectively).

Fig. 3 shows the interaction between treatments (CMP versus TW) and feeding periods (3 versus 8 weeks). It can be observed that the effect of the oral administration of CMP was the same for both feeding periods: CMP increased the Ca concentration in femur compared with the administration of tap water only, the effect being of a stronger magnitude for the 3-week feeding period compared with the 8-week feeding period. There was no treatment–week interaction, since the two plotted lines presented positive slopes. This was confirmed statistically with $p = 0.2405$ (interaction not significant) when the MM-linear regression models with and without interaction were compared. Furthermore, a permutation test for the alternative hypothesis that CMP increases the amount of Ca in femur was performed, which was accepted since the test was statistically significant with $p = 0.044$ (8 weeks) and $p = 0.020$ (3 weeks). Then, the same analysis was performed for 0.5% and 1.2% Ca and again there was a parallel effect (i.e., no treatment–week interaction, with $p = 0.523$ and 0.336, respectively). The permutation test for the alternative hypothesis that CMP increases the amount of Ca in femur showed no differences for the 3-week feeding period (two sided $p = 0.387$ for 0.5% Ca and 0.502 for 1.2% Ca) nor for the 8-week feeding period (two sided $p = 0.814$ for 0.5% Ca and 0.432 for 1.2% Ca). It can be speculated that, in the presence of low Ca concentrations (low Ca diet), the CMP-enriched extract might have acted as a transporter for Ca deposition, as it was suggested that CMP might have a role in bone mineralisation (Brody, 2000) but, when Ca is highly available (normal or high Ca diets), it seems that bone mineralisation is normal and then the role of CMP is less evident. No differences in Ca content in blood serum, kidneys, faeces or urine were observed either due to CMP administration or in the Zn content of femur (Table 1).

The lack of Ca deposition in kidney due to CMP administration could be regarded as a positive safety characteristic for a food ingredient (Burdock & Flamm, 1999). Having no previous information available as guideline, the 8-week feeding period was chosen in our study on the basis of previous reports. For example, Pérez-Gallardo et al. (2009) used an 8-week feeding period to study the retention of calcium, magnesium and zinc when a calcium-enriched high-fat diet was administered to mice. In another study (Lee et al., 2009), 4 weeks of administration of vitamin D2 and calcium-enhanced oak mushrooms to 4 week-old ICR mice was effective in overcoming the osteoporosis-like symptoms induced by a special diet in a bone mineralisation model. In those studies, the physiological parameters considered were bone histology and femur density and length, serum calcium levels, and the mRNA levels of active calcium transport genes. Then, the aforementioned studies allow determination of Ca content in the femur during short term (3 weeks) administration of CMP. This feeding period is also coincident with the period of full development in mice that goes from

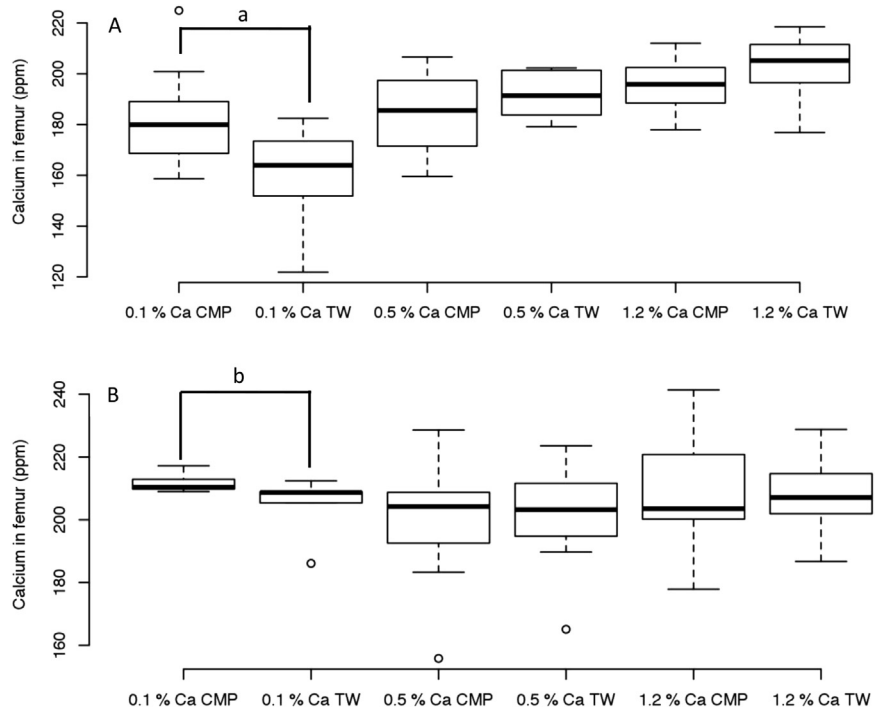

Fig. 2. Content of Ca (ppm) in the femur of mice that received rodent diet containing 0.1% (w/w), 0.5% (w/w) or 1.2% (w/w) of Ca for 3 (panel A) or 8 (panel B) weeks and tap water (TW) or CMP-enriched extract (CMP) in their drinking bottles. Due to the presence of outlier values and the sample size, a permutation test (considering all data) was performed to detect differences between groups. The letters a and b indicate differences ($p = 0.020$ and 0.044, for the feeding period of 3 and 8 weeks, respectively).

weaning (3 weeks of age) to adulthood (6 weeks of age) (Ritskes-Hoitinga, 2004). No significant differences in the body weight of animals were observed (Fig. 4) during the 3-week feeding period, nor after 8 weeks of oral administration of the product under study (data not shown). Similarly in terms of diet (low Ca diet), feeding period (4 weeks) and mouse age (4 weeks), Lee et al. (2009) observed the beneficial effect of administration of vitamin D2 and calcium-enhanced oak mushrooms on osteoporosis-like symptoms in a mouse model.

In relation to the capacity of CMP to affect mineral transport and absorption, Kelleher et al. (2003), reported that glycomacropeptide supplementation of infant formula positively affected zinc absorption, growth and nutritional status in infant rhesus monkeys. Regarding the possible future inclusion of CMP in food matrices, it is necessary to determine the physiologically active concentration, its stability to food processes, and the impact on the food structure. Few studies have been conducted up to now to determine the dose–response relationship with CMP administration. The physiologically active concentration of CMP is dependent on the desired bioactive effect (Thoma-Worringer et al., 2006). In vivo studies have

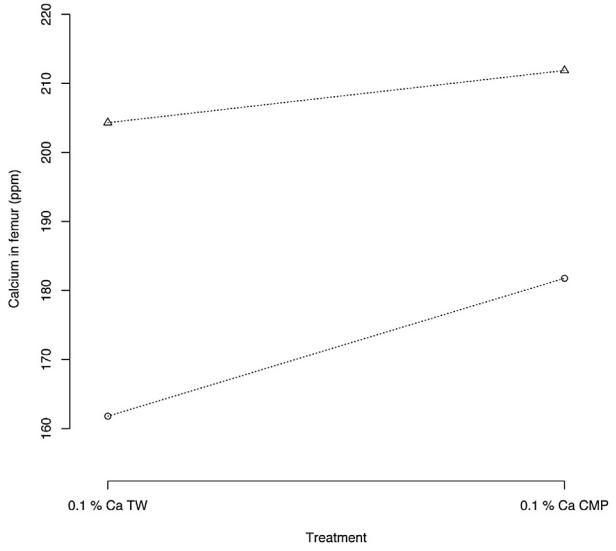

Fig. 3. Interaction plot between treatments (CMP or TW administration) and feeding periods (circles: 3 weeks, triangles: 8 weeks) for a rodent diet containing 0.1% Ca. The interaction between the factors treatments and weeks can be visualised by plotting the dependent variable (Ca content in femur) against treatments with a dashed line for each level of weeks. The parallelism between lines indicates that the effect of the treatment (CMP administration) was the same for 3 and 8 weeks ($p = 0.2405$ for the test of treatments–weeks interaction), but with a higher significance (line slope) for the 3-week feeding period, when the CMP administration is compared against TW administration.

Table 1
Calcium levels in faeces, urine, blood serum and kidneys and zinc levels in femur of mice that received rodent diet containing 0.1% (w/w) or 1.2% (w/w) of Ca either water or CMP for 8 weeks.[a]

Sample	Ca in diet			
	0.1% (w/w)		1.2% (w/w)	
	Tap water	CMP	Tap water	CMP
Ca content in samples				
Faeces (ppm)	9.08 ± 1.71^{a}	9.86 ± 1.22^{a}	83.56 ± 3.39^{b}	84.11 ± 2.98^{b}
Urine (mg L^{-1})	71.02 ± 2.41^{a}	69.39 ± 2.55^{a}	70.02 ± 4.93^{a}	66.44 ± 7.32^{a}
Blood serum (mg L^{-1})	12.48 ± 2.55^{a}	11.79 ± 4.60^{a}	36.12 ± 17.29^{b}	31.35 ± 17.51^{b}
Kidneys (ppm)	0.37 ± 0.07^{a}	0.34 ± 0.07^{a}	0.31 ± 0.06^{a}	0.34 ± 0.06^{a}
Zinc content in sample				
Femur (ppm)	0.19 ± 0.02^{a}	0.18 ± 0.02^{a}	0.20 ± 0.02^{a}	0.20 ± 0.01^{a}

[a] Values are means ± standard deviations; values in rows with different superscript letters are significantly different ($P < 0.05$). CMP was diluted 1 + 10 in tap water.

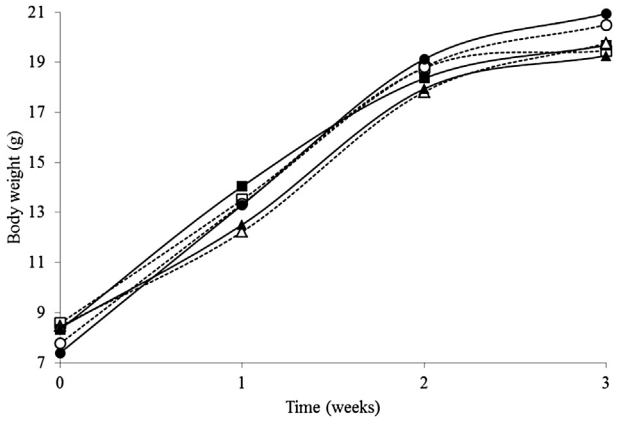

Fig. 4. Body weight of mice that received rodent diet containing 0.1% (w/w) (squares), 0.5% (w/w) (triangles) or 1.2% (w/w) (circles) of Ca for 3 weeks and tap water (dashed line-open symbol) or CMP-enriched extract (solid line-full symbol). No significant differences were detected among groups at weeks 1, 2 or 3 of feeding.

shown that 1 mg kg^{-1} CMP was antithrombotic in guinea-pigs and therefore probably corresponds to an active dose in humans (Chabance et al., 1995, 1998). A daily intake of 0.2 g of CMP was considered as physiological for the stimulation of pancreatic secretion in rats (Pedersen et al., 2000). In this study, in mice, the active dose of CMP for enhanced Ca deposition, in particular in growing individuals receiving a low Ca diet was approximately 13 mg day^{-1}. CMP is a phosphorylated sequence derived from milk casein (Thoma-Worringer et al., 2006). Phosphopeptides can form soluble organophosphate salts and may function as carriers for different minerals, especially Ca. Hence, they might exert an influence on absorption of Ca or other minerals in the intestine (Meisel, 1997). Therefore, in this study, the enhanced Ca content in the femur of mice fed a low Ca diet and CMP might be due to a better exploitation of the low amounts of circulating Ca in the presence of CMP. Nutritional deficiencies have always been a major consideration in paediatrics, especially in preterm infants (Shah & Shah, 2009; Suskind, 2009), in whom bone mineral deficiency of prematurity is caused by the lack of simultaneous availability of Ca and anorganic phosphate during rapid skeletal growth (Maas, Pohlandt, Mihatsch, & Franz, 2012). Thus, these results are encouraging for further animal and human studies that examine the possibility of supplementing infant food with CMP-enriched extracts for enhanced bone health if human trials can confirm the results observed in animals.

4. Conclusions

The oral administration of CMP to mice significantly enhanced the content of Ca in femur under a low-Ca diet model, especially during the period of full body development, where a significant 12% of Ca enhancement in bone was observed followed CMPEE administration. These findings pave the way for further animal and human studies aimed at supplementing infant food with industrially-obtained CMP-enriched extract for enhanced bone health when Ca intake in the diet is, perhaps, insufficient. From an industrial perspective, the results obtained in this study also suggest the possibility of obtaining a functional ingredient (CMP-enriched extract) from cheese whey.

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