Assessing the effects of severe heat treatment of milk on calcium bioavailability: In vitro and in vivo studies

I. Seiquer,¹ C. Delgado-Andrade, A. Haro, and M. P. Navarro

Instituto de Nutrición (IFNA), Estación Experimental del Zaidín (CSIC), 18100 Armilla, Granada, Spain

ABSTRACT

Thermal processing of milk is a common practice. As milk is the main source of dietary calcium, this study aimed to assess the effects of overheating milk on calcium availability. Thus, thermally damaged milk (overheated, OH, milk; 3 cycles of sterilization at 116°C, 16 min) was compared with UHT milk (150°C, 6 s) in 2 types of assays: in vitro and in vivo (rats). In addition, the greater Maillard reaction rate associated with thermal treatment in OH milk was confirmed by determining specific (furosine) and unspecific markers (CieLab color). A negative effect on calcium solubility was observed after in vitro digestion of OH milk compared with UHT milk. Feeding rats the diet containing OH milk as the protein source led to significantly lower values of apparent calcium absorption and retention than those found among animals fed the UHT milk diet. Whereas reducing the absorption appears to result mainly from the decreased food intake, the negative effect on retention seems to be due to factors derived from milk thermal damage, such as the formation of Maillard reaction products. It was concluded that milk-processing conditions warrant special attention to prevent impaired dietary calcium utilization. This may be especially important in situations where milk and dairy products are the main dietary components, such as in early infancy.

Key words: calcium availability, Maillard reaction, milk thermal treatment

INTRODUCTION

Milk is a widely consumed beverage in Western countries; around 90 L/person is consumed annually in Europe and North America (International Dairy Federation, 2007). At present, only 2% of the milk produced in developed countries is consumed in its raw form, with the remaining volume submitted to processing techniques involving heat treatment (MAPA, 2006).

Heat treatment of milk has been a common practice since 1860, when Pasteur initially used it for preservation purposes (O'Connell and Fox, 2002).

In addition to destroying microorganisms and extending the shelf life of milk, heating processes may also produce modifications that affect the sensory and nutritional quality of the product (Burton, 1984). It is well documented that heat treatment of milk causes significant damage to proteins, and may reduce their nutritional quality and affect their functional properties (Delgado et al., 1992; alKanhal et al., 2001). Other effects such as folate losses (Forssén et al., 2000) or the isomerization of certain fatty acids (Mattila-Sandolm and Saarela, 2003) and sugars (Cattaneo et al., 2008) have also been described as a consequence of thermal processes applied to milk. As well as contributing protein, milk is a rich source of minerals, especially calcium; milk contributes 40% of total dietary calcium and is the major dietary source of this mineral (Seiquer et al., 2006). In vitro studies have shown that heat treatment reduces calcium solubility in goat's milk (de la Fuente et al., 1999) and in the presence of casein-sugar mixtures (Seiquer et al., 2001), but little information is available on the effects of thermal processes applied to milk on calcium absorption and metabolism at the cellular level or in in vivo assays.

According to Pellegrino et al. (1995), 2 groups of reactions may take place during heat treatment: type I, denaturation, degradation, and inactivation of heat-labile compounds, and type II, formation of new substances. Among these, Maillard reaction products (**MRP**) are very likely to be formed during heating of milk, due to its high protein and sugar content. It is known that the Maillard reaction (**MR**) in milk reduces its nutritive value even in the early stages, because lactose blocks the amino group of lysine to form the Amadori product lactulosyllysine (Erbersdobler, 1986). Besides the changes they provoke in protein availability, MRP may behave as chelating agents for metal cations, forming soluble and insoluble complexes that can affect mineral bioavailability (Friedman, 1996). The effects of MRP on calcium bioavailability have been studied using model systems from sugar-amino acids (Delgado-Andrade et al., 2005, 2006) or sugar-proteins (Seiquer et al., 2001)

Received May 25, 2010.

Accepted August 31, 2010.

¹Corresponding author: isabel.seiquer@eez.csic.es

and in browned diets (Mesías et al., 2009). However, the effects of MRP derived from milk-overheat treatment on calcium metabolism have been scarcely studied.

Although UHT milk is the type most consumed around the world, milk is frequently submitted to overheating for direct consumption or for baking. Thus, the aim of this work was to study the effects of overheating milk on calcium bioavailability. For this purpose, 2 types of assay were carried out: studies of calcium solubility after *in vitro* digestion, and calcium balance assays in rats. In addition, the MR rate associated with thermal treatment was evaluated by determining specific (furosine) and unspecific markers (CieLab color).

MATERIALS AND METHODS

All of the chemical products and solvents for all analyses were of the highest grade available and acquired from Sigma-Aldrich (St. Louis, MO) and Merck (Darmstadt, Germany). Furosine was obtained from Neosystem Laboratories (Strasbourg, France).

Samples

Semi-skim milk samples were provided by a local milk factory (Granada, Spain). One sample was subjected to direct ultra-high-temperature treatment (UHT milk), in which sterilization was achieved by heating to 150°C for 6 s. The other sample was subjected to a traditional in-bottle sterilization (116°C, 16 min), repeated 3 times, hereafter termed overheated milk (OH).

Samples were supplied to the investigators immediately after processing by the manufacturer and kept in their commercial packaging at 20 to 25°C for no longer than 2 wk. Within this time, samples were (1) lyophilized (TDS-3, FTS Systems Inc., Stone Ridge, NY) and stored at -20°C until they were used for MR marker determination and for biological experiments, and (2) used fresh in the *in vitro* digestion process.

Maillard Reaction Markers

Furosine determination of the milk samples was performed by HPLC following the method described by Delgado-Andrade et al. (2007): 50 mg of the sample was hydrolyzed with 4 mL of 7.95 M HCl at 110°C for 23 h, after which, the usual procedure was followed. Furosine was quantified by the external standard method. The calibration curve was built from a stock solution (1 mg of furosine/mL) in the range 10 to 0.05 mg/L.

The HPLC system consisted of a series 200 pump, a series 225 autosampler, a series 200 UV-visible detector, a series 200 Peltier column oven, and TotalChrom

Workstation software v. 6.3.1 for data acquisition and treatment (PerkinElmer, Madrid, Spain).

The color determination of the different samples was carried out with a Chroma Meter CR-400 optical sensor (Konica Minolta Sensing Inc., Osaka, Japan) according to the CIELAB scale (CIE Colorimetric Committee, 1974). The system provides the values of 3 color components, named according to Kelly and Judd (1976): L^* (black-white component, luminosity), and the chromaticness coordinates, a^* (+red to -green component) and b^* (+yellow to -blue component). Samples were placed in a 34-mm optical glass cell and illuminated with D65 artificial daylight (10° standard angle) according to conditions provided by manufacturer. The yellowness index (YI) was calculated from the equation: $YI = 142.86 b^*/L^*$ and used as a color measurement related to browning intensity (Francis and Clydesdale, 1975). Each color value reported was the mean of 3 determinations at 22 to 24°C.

In Vitro Digestion of the Samples

Milk samples were subjected to successive *in vitro* gastric and intestinal digestion, as described previously (Mesías et al., 2009). Briefly, samples (10 mL) were digested with pepsin-HCl (pH 2) in a shaking water bath (37°C, 110 oscillations/min, 2 h) to simulate gastric digestion. For the intestinal digestion, a mixture of pancreatin and bile salts was added and the samples (pH 7.5) were incubated at 37°C at 110 oscillations/min for 2 h. After digestion, the digestive enzymes were inactivated by heat treatment for 4 min at 100°C in a polyethyleneglycol bath. The samples were then cooled by immersion in an ice bath and centrifuged at $3,200 \times g$ for 60 min at 4°C (CS-6R centrifuge, Beckman Instruments, Fullerton, CA) to separate the soluble (bioaccessible) and nonsoluble fractions. The supernatants were carefully separated and the percentages of soluble and insoluble calcium were calculated from the initial calcium content in the milk samples.

Diet Preparation

Diets were prepared so that milk was the main source of food in the diet, thus providing all protein and some fat and carbohydrates. The dietary protein level was adjusted to 10% (NRC, 1995) and, according to milk protein content, it was calculated that 29.43 g of milk (DM) was needed to provide 10 g of protein. The addition of 29.43 g of milk/100 g of diet also provided 17.01 g of carbohydrates and 4.73 g of fat; adequate levels of carbohydrates and fat according to requirements were achieved by adding the remaining ingredients. Soy oil

Table 1. Nutrient content of experimental diets¹

Item	UHT	OH
Moisture (%)	7.86 ± 0.65	8.14 ± 0.34
Protein (g/kg)	96.0 ± 5.02	95.2 ± 2.10
Fat (kg)	77.01 ± 4.42	73.59 ± 2.88
Calcium (g/kg)	6.20 ± 0.90	6.22 ± 0.42
Phosphorus (g/kg)	3.63 ± 0.21	3.24 ± 0.09
Magnesium (g/kg)	0.52 ± 0.01	0.51 ± 0.01
Iron (mg/kg)	49.05 ± 0.89	47.63 ± 0.53
Copper (mg/kg)	5.63 ± 0.10	5.21 ± 0.31
Zinc (mg/kg)	29.68 ± 0.48	28.54 ± 0.16

¹Data are means ± SE. No significant differences were found between samples ($P < 0.05$). Diets: UHT milk was sterilized by heating to 150°C for 6 s; OH (overheated) milk was subjected to a traditional in-bottle sterilization (116°C, 16 min) repeated 3 times.

was used as the fat source to meet the requirement for linoleic acid (NRC, 1995).

Semisynthetic diets were prepared with the following composition (on DM basis): 40% starch (Central Ibérica de Drogas, S.A., Madrid, Spain), 19.80% sugar (Confisa, S.A., Madrid, Spain), 2.80% soy oil (Carbonell, Córdoba, Spain), 5% cellulose (Central Ibérica de Drogas, S.A.), 0.2% DL-methionine (Merck), 2% mineral mixture (Merck), and 1% vitamin mixture (Roche, Basel, Switzerland). The only component that varied in the diets was that UHT and OH (29.43%) samples were used as a source of protein and partial source of carbohydrates and fat (diets UHT and OH, respectively). Diets were individually analyzed and no significant differences in nutrient content were found between them. Table 1 provides moisture and nutrient contents of the diets.

Biological Assay

Twenty-four weanling Wistar rats weighing 41.4 ± 0.5 g (mean ± SE) were housed individually in metabolic cages in an environmentally controlled room that was kept at 20 to 22°C and 55 to 70% humidity with a 12-h light–dark cycle. The rats were randomly distributed into 3 groups of 8 rats, and each group was assigned to 1 of the dietary treatments, designated UHT, UHT-paired, and OH. The animals in the UHT and OH groups had ad libitum access to their diets. With the aim of avoiding any influence of food intake among dietary treatments, a group of animals was fed the UHT diet at the same intake level as the OH group (the UHT-paired group), beginning the assay 1 d afterward to enable the pair-feeding. All animals had free access to demineralized water (Milli-Q Ultrapure Water System, Millipore Corp., Bedford, MA).

The animals were fed the different diets over 28 d. In the last week of the experimental period (d 21–28), the calcium balance was performed. The complete as-

say involved a preliminary 21-d period during which solid food intake and BW changes were monitored, followed by a 7-d period in which feces and urine from each animal were collected daily and stored as a 1-wk pool. The feces were weighed, lyophilized, and then homogenized. Urine was collected in 0.5% HCl (vol/vol), filtered (Whatman Filter Paper No. 40, ashless, Whatman, UK), and diluted to an appropriate volume. To control for possible environmental contamination during urine and feces collection, blank cages were manipulated in the same way as those used for the animals. On d 28, after an overnight fast, animals were anesthetized with sodium pentobarbital (5 mg/100 g of BW; Abbott Laboratories, Granada, Spain) and terminal exsanguination was performed by cannulation of the carotid artery. Blood was drawn to obtain serum, and the liver, right kidney, right femur, small intestine, and cecal contents were removed, weighed, and frozen at –20°C until calcium analysis. All management and experimental procedures carried out in this study were done in strict accordance with current European regulations (86/609 E.E.C.) regarding laboratory animals. The Bioethics Committee for Animal Experimentation at Estación Experimental del Zaidín (CSIC, Granada, Spain) approved the study protocol.

Analytical Techniques

Analyses were performed in triplicate. Calcium analyses in all samples were carried out with flame atomic absorption spectrometry in a Perkin-Elmer Analyst 700 spectrophotometer (Norwalk, CT). Prior to analysis, aliquots of the samples (milk, milk in vitro digests, diets for biological assay, and feces/urine from rats) were completely digested by the addition of concentrated HNO₃ and HClO₄, and by heating at high temperatures in a sand beaker (Block Digestor Selecta S-509, J.P. Selecta, Barcelona, Spain). The organs from rats were dry-ashed in a muffle furnace (model 366, Selecta) at 450°C, and the white ashes obtained were dissolved with HCl:HNO₃:H₂O (1:1:2; Suprapur, Merck). All samples were diluted with Milli-Q water to an appropriate volume for measurement.

Standard solutions were prepared from a stock Tritisol solution of calcium [CaCl₂ in 6.5% HCl (vol/vol) and 1,000 mg of Ca, Merck]. Lanthanum chloride (Merck) was added to samples and standards to reach a final concentration of 0.3%. Pools of feces, urine, and diet were used as an internal control to assess precision. The interassay coefficient of variation was 2.35% in feces, 1.95% in urine, and 2.02% in the diet. Milk powder (certified reference material CRM 063; Community Bureau of Reference, Brussels, Belgium) was used to quantify accuracy, yielding a value of $13.48 \pm$

0.04 mg/g (mean $\pm$ SD; certified value: 13.49 ± 0.10 mg/g). All glassware and polyethylene sample bottles were washed with 10 *N* nitric acid and Milli-Q water was used throughout the study.

Using the data obtained for the calcium intake (I), fecal excretion (F), and urinary excretion (U), the following indices were calculated: apparent absorption ($A = I - F$), fractional absorption or digestibility ($\%A/I = A / I \times 100$), apparent retention ($R = A - U$), absorption efficiency ($\%R/A = R/A \times 100$), and utilization efficiency or bioavailability ($\%R/I = R / I \times 100$).

The chemical composition of the diets was determined by official methods (AOAC, 1995). In particular, nitrogen was analyzed in a Leco model FP-2000 protein/nitrogen analyzer (Leco Instruments, Madrid, Spain) calibrated with EDTA (Dumas method), fat was determined by hydrochloric hydrolysis followed by extraction in a Soxhlet apparatus, and moisture and ash content were determined by gravimetric methods.

Statistical Analysis

Data from the in vitro and in vivo and assays were statistically tested by one-way ANOVA, followed by Duncan's multiple-range test to compare means that showed significant variation ($P < 0.05$). The relationships between the different variables were evaluated by computing the relevant correlation coefficient (Pearson linear correlation) at the $P < 0.05$ confidence level. Statgraphics Plus, version 5.1 (Statpoint Technologies Inc., Warrenton, VA), was used to carry out the comparisons.

RESULTS AND DISCUSSION

Maillard Reaction Markers

Among the number of chemical indicators that can be used to evaluate early changes induced during milk processing, furosine is one of the most representative as it derives from the conversion by acid hydrolysis of lactulosyllysine, the rather stable Amadori product formed through the condensation between lactose and lysine. Thus, determination of furosine has been extensively used by many authors to quantify the extent of the MR in milk and milk products (Pellegrino et al., 1995; Elliott et al., 2005; Mendoza et al., 2005; Cattaneo et al., 2008).

A significant increase in furosine content was observed in overheated milk with respect to UHT milk samples (Table 2), which confirms that MR proceeded more extensively in the OH sample. A wide range of furosine content has been described in heat-treated milk, varying from 50 to 500 mg/100 g of protein (Pellegrino et

Table 2. Maillard reaction markers (furosine and CIELAB colors) determined in samples of milk subjected to UHT treatment and overheating¹

Item	UHT	OH
Furosine (mg/100 g of protein)	201.7 ± 1.7^a	348.1 ± 6.69^b
CIELAB colors		
L^*	90.51 ± 0.03^a	79.15 ± 0.10^b
a^*	0.05 ± 0.00^a	6.44 ± 0.08^b
b^*	14.71 ± 0.04^a	23.81 ± 0.12^b
YI^2	23.22 ± 0.17^a	42.98 ± 0.67^b

^{a,b}Different letters within a row indicate significant differences between samples ($P < 0.05$).

¹Diets: UHT milk was sterilized by heating to 150°C for 6 s; OH (overheated) milk was subjected to a traditional in-bottle sterilization (116°C, 16 min), repeated 3 times. Data are means $\pm$ SE.

²Yellowing index calculated as $142.86 \, b^*/L^*$.

al., 1995). Values observed in the present assay for UHT milk are higher than those reported in the literature, which are around 150 mg/100 mg of protein (Elliott et al., 2005; Mendoza et al., 2005), but similar to those described by Cattaneo et al. (2008) after heating milk at 150°C for 9 or 13 s (187 and 206 mg/100 mg of protein, respectively). According to Claeys et al. (2004), furosine values in UHT milk heated at the most intense parameters should not exceed 250 mg/100 g of protein. Furosine contents determined in OH milk are similar to those for direct UHT milk submitted to a second treatment at 148°C for 20 s to simulate overprocessing (351 mg/100 g of protein; Cattaneo et al., 2008).

The early stage of the MR results in a loss of nutritive value due to the blockage of lysine residues, which is well correlated with furosine content, but does not give rise to color or flavor changes. Storage or more intense heating give rise to the formation of brown components, which may be related to the changes in the visual color as measured by CIELAB color (Table 1; Pagliarini et al., 1990). The higher a^* and b^* values of OH milk compared with the UHT milk indicate the progression of the color to the red and the yellow zones, respectively, in accordance with the likely higher presence of MRP, although lipid oxidation and caramelization could also be partly responsible. Also, luminosity (L^*), the black-white component of the sample, significantly decreased in the OH milk, revealing a loss of lightness, probably due to the processing conditions applied during the thermal process. Measurement of luminosity by the L^* value has been associated to the presence of MRP in some foods (Aitameur et al., 2004; Delgado-Andrade et al., 2007), and in milk, it has also been used to estimate the stability of casein micelles (O'Sullivan et al., 2002; Smiddy et al., 2006). Casein micelles are colloidal casein associations able to scatter light that is responsible for the white color of milk; when casein micelles are disrupted, their ability to scat-

ter light decreases, which may be detected by a decline in the L^* value (O' Sullivan et al., 2002). Thus, in the OH milk, reductions of L^* could also be due to dissociations of casein micelles induced by heat, which in turn may be related with the formation of MRP through lysine-casein participation. Decreases in L^* values and increases in a^* and b^* values have been observed in nonfat dry milk during severe storage conditions (8 wk at 50°C), which has been related to the progress of the MR (Liu and Metzger, 2007). The YI proposed by Francis and Clydesdale (1975) is commonly employed as a color measurement related to browning intensity, but it also is a simple and reproducible index of commercial milk quality (Pagliarini et al., 1990). As expected, the more severe heat treatment in OH milk made this index increase significantly (Table 1), which confirms its suitability to characterize the MR development in milk.

Colors measured in the present assay were correlated with the furosine content of samples ($r = -0.9835$, 0.9831 , 0.9815 , and 0.9816 for L^* , a^* , b^* , and YI , respectively), thus confirming the usefulness of using color indices as indicators of heat damage in milk.

In Vitro Calcium Solubility

Results of calcium solubility after in vitro digestion of the milk samples are shown in Table 3. The first step to determining bioavailability is nutrient solubility in the intestinal tract, or bioaccessibility; Ca must be in a soluble form within the intestinal lumen before crossing the intestinal wall. Although they are minor constituents on a weight basis, minerals from casein micelles, mainly calcium and phosphate, play an essential role in maintaining micellar integrity (Smiddy et al., 2006). Heat treatment of milk alters the casein micelle structure producing the dissociation of κ , β , and α_s fractions and leads to a marked increase in the concentration of κ -casein in serum (Law, 1996). Because the κ fraction plays an essential role in maintaining the micelle structure by stabilizing and preventing α_s and β fractions from precipitating in the presence of Ca, it seems logical that severe heating of milk affects the ligands that maintain the calcium in solution. Other authors also have observed that heat treatment reduces calcium solubility in goat milk, and this reduction in solubility increases with the severity of heating (de la Fuente et al., 1999). Moreover, biologically active peptides released as casein phosphopeptides by enzymatic proteolysis during the gastrointestinal digestion of casein have a high calcium binding ability and enhance calcium absorption by inhibiting the luminal precipitation of calcium salts (Naito, 1990). After digestion most milk calcium is usually in soluble form, as was the case in UHT milk (Table 2) or in casein-glucose-fructose

Table 3. Total calcium content and soluble/insoluble fractions after in vitro digestion of samples of milk subjected to UHT treatment and overheating¹

Item	UHT	OH
Total content (mg/100 mL)	110.3 ± 1.8	109.9 ± 1.7
Soluble fraction (%)	63.2 ± 2.40 ^a	48.31 ± 2.46 ^b
Insoluble fraction (%)	37.7 ± 0.65 ^a	42.70 ± 2.21 ^b

^{a,b}Different letters within a row indicate significant differences between samples ($P < 0.05$).

¹Diets: UHT milk was sterilized by heating to 150°C for 6 s; OH (overheated) milk was subjected to a traditional in-bottle sterilization (116°C, 16 min), repeated 3 times. Data are means ± SE.

mixtures digested in the presence of calcium (Seiquer et al., 2001). However, heat induces dephosphorylation of casein and, as a result, the potential effect of casein phosphopeptides as mineral carriers is impaired (Meisel and Schlimmer, 1995). This could contribute to the significant increase of the insoluble calcium fraction observed after in vitro digestion of the OH milk (Table 2).

In addition to the effects of proteins disruption by heat, the effects of MRP on calcium solubility warrant consideration. Calcium appears to bind weakly to soluble and insoluble melanoidins (Rendleman, 1987), whereas reduced calcium binding ability has been attributed to low-molecular-weight MRP when compared with rates observed for other metals such as copper or iron (O'Brien and Morrissey, 1997). When minerals other than calcium are present, the formation of calcium-MRP chelates seems to be avoided, as has been shown in the presence of sugar-amino acid heated mixtures, where calcium solubility remain unmodified but copper, iron, or zinc solubility was significantly affected (Delgado-Andrade et al., 2004). However, after in vitro digestion of heated casein-sugar mixtures in a calcium solution, a significant increase in calcium insolubility has been observed compared with the unheated samples (Seiquer et al., 2001), which has been attributed to the formation of Ca-MRP insoluble complexes. In milk, calcium is present in a larger proportion than the rest of the divalent minerals, which probably allows for the formation of calcium-MRP complexes during the digestion process. As a result, Ca solubility should be negatively affected. The higher presence of MRP in the OH milk is supported by the furosine and CIELAB analyses results.

Biological Assay

Feeding rats the diet formulated with OH milk led to a minor voluntary food intake over the whole experimental period compared with the group fed the UHT diet (9.5 ± 0.6 and 11.0 ± 0.6 g/d, for the OH and

Figure 1. Body weight of rats fed the different diets (means $\pm$ SE, $n = 8$). Groups: UHT milk was sterilized by heating to 150°C for 6 s; OH (overheated) milk was subjected to a traditional in-bottle sterilization (116°C, 16 min), repeated 3 times; UHT-pair fed, group of rats fed the diet UHT at the same intake level as the OH group. ^{a,b}Different letters indicate significant differences between groups ($P < 0.05$).

UHT groups, respectively). As a consequence, the final weight of animals of the OH and UHT-pair-fed groups decreased by 16% compared with the UHT group (Figure 1). Experimental assays performed in rats fed diets containing small proportions of MRP-model systems show disparate results on food intake and weight gain. Thus, depending on the MRP composition, it has been observed that food intake does not vary (Furniss et al., 1989; Delgado-Andrade et al., 2006), tends to be reduced (Seiquer et al., 2001), or is negatively affected (O'Brien and Walker, 1988). However, according to our results, feeding a diet in which all of the protein sources are thermally damaged seems to lead to clear decreases in food intake and body weight gain, as was also observed by Sarriá et al. (2001) in rats fed bottle-sterilized infant formulas compared with powder formulas. Chemical changes occurred in the MR, such as protein alteration (e.g., loss of available lysine), and the formation of new compounds might affect food intake and BW gain. It has been described that increased lactulose, which results from lactose isomerization during milk heating (Sarriá et al. 2001), may decrease the flavor and smell acceptability of processed milk (Burton, 1988), which would directly affect food intake. Moreover, effects such as decreased gastric emptying and increased satiety have been attributed to lactulose (Washington et al., 1998). Thus, increased lactulose could have contributed to the negative effects on food intake and BW gain observed in the present assay among animals fed the OH diet.

As a consequence of the reduced food consumption, lower calcium intake was observed among animals

Table 4. Calcium balance (mg/d) in rats fed the different diets (d 21–28)¹

Calcium balance	UHT	UHT-pair fed	OH
Intake	76.9 $\pm$ 1.9 ^a	63.2 $\pm$ 0.8 ^b	62.1 $\pm$ 3.9 ^b
Fecal	41.1 $\pm$ 1.4 ^a	32.4 $\pm$ 1.7 ^b	35.3 $\pm$ 2.8 ^{ab}
Urinary	6.0 $\pm$ 0.5 ^a	4.8 $\pm$ 0.2 ^a	5.1 $\pm$ 0.5 ^a
Apparent absorption	35.9 $\pm$ 1.5 ^a	30.7 $\pm$ 1.5 ^b	26.7 $\pm$ 1.9 ^b
Apparent retention	29.8 $\pm$ 1.3 ^a	26.0 $\pm$ 1.5 ^{ab}	21.6 $\pm$ 1.8 ^b

^{a,b}Different letters within a row indicate significant differences between groups ($P < 0.05$).

¹Data are means $\pm$ SE of 8 animals. Groups: UHT milk was sterilized by heating to 150°C for 6 s; OH (overheated) milk was subjected to a traditional in-bottle sterilization (116°C, 16 min), repeated 3 times; UHT-pair fed, group of rats fed the diet UHT at the same intake level as the OH group.

belonging to OH and UHT-pair-fed groups compared with the UHT group (Table 4). The low calcium intake seemed to be the main factor behind the significantly lower calcium absorption in the indicated groups, although absorption efficiency or digestibility (%A/I) did not vary (Figure 2). That apparent calcium absorption did not differ among animals fed at the same intake level (UHT-pair-fed and OH groups) seems to indicate that consumption of overheated milk had no significant effect on the digestive utilization of calcium, except that resulting from the lower consumption of food compared with animals fed the UHT diet. However, although nonsignificant, a decrease of approximately 13% in apparent calcium absorption was observed in the OH group compared with the UHT-pair-fed group, which could be related to the lower solubility of calcium observed in the OH diet after the *in vitro* digestion process. Decreases in calcium apparent absorption as a result of lower food intake also have been observed in rats consuming bottle-sterilized infant formulas compared with powder formulas, although calcium digestibility was increased (Sarriá et al., 2001). According to the results of the present assay, feeding rats diets containing MRP does not usually cause changes in calcium digestibility, as seen with casein-sugar or glucose-lysine heated mixtures (Seiquer et al., 2001; Delgado-Andrade et al., 2006). In a recent study, Mesías et al. (2009) confirmed that the intake of MRP in humans does not induce significant variations in calcium absorption or digestibility.

At a metabolic level, results showed a negative effect of OH diet consumption on apparent calcium retention (Table 4) compared with the UHT diet, although when calculated as a relative value from absorbed (%R/A) and ingested calcium (%R/I) (Figure 2), differences did not reach statistical significance. Contrary to results found for apparent absorption, the decrease in calcium retention cannot be attributed mainly to the minor

Figure 2. Biological indices in rats fed the different diets (means $\pm$ SE, $n = 8$). Groups: UHT milk was sterilized by heating to 150°C for 6 s; OH (overheated) milk was subjected to a traditional in-bottle sterilization (116°C, 16 min), repeated 3 times; UHT-pair fed, group of rats fed the diet UHT at the same intake level as the OH group. A = absorbed Ca; I = ingested Ca; R = retained Ca. No significant differences were found between groups ($P < 0.05$).

intake as no differences between UHT and UHT-pair-fed groups were observed. Thus, thermal damage and the presence of MRP seem to negatively affect calcium retention. Moreover, when statistical treatment (one-way ANOVA) was applied between groups at the same intake level (i.e., UHT-pair-fed and OH groups), calcium retention in the OH group clearly tended to decrease ($P = 0.09$ both for calcium balance and for bioavailability). Some *in vivo* experiments have shown that intake of diets containing MRP does not affect calcium bioavailability in rats (Delgado-Andrade et

al., 2006) or humans (Mesías et al., 2009). Conversely, feeding rats infant milk formula with a high rate of MR development (in bottle sterilized vs. powder) induces decreased calcium bioavailability (Sarriá et al., 2001). It should be noted that in the above assays of Delgado-Andrade et al. (2006) and Mesías et al. (2009), only a small proportion of the dietary protein was thermally damaged. Therefore, it may be hypothesized that when the entire dietary protein source is thermally damaged, as in diets based on overheated milk in which calcium is the major cation (Sarriá et al., 2001), Ca-MRP complexes are more likely to be formed and affect calcium metabolism. It has been confirmed that MRP formed in diverse food systems have different calcium-binding capacities as a result of important structural differences, which in turn may affect calcium bioavailability (Rendleman, 1987).

As expected, organs in animals fed the UHT-pair-fed and OH diets were smaller than those in animals fed the UHT diet, according to their lower BW (Table 5). Calcium content varied accordingly, and no differences were shown in the mineral concentration, except for the increase in the small intestine and the decrease in the liver observed in the OH group. It seems likely that some Ca complexes formed during OH milk digestion were able to be internalized into intestinal cells, but were not able to cross the cell monolayer and be absorbed. It is known that the amount of mineral internalized by intestinal cells cannot be considered totally available because many cells in the intestinal mucosa are desquamated and renewed daily (Ganong, 1993). Although previous studies have shown a negative effect of MRP intake on bone calcium (Delgado-Andrade et al., 2006), no such effects were observed after consump-

Table 5. Calcium concentration in organs of rats fed the different diets¹

Item	UHT	UHT-pair fed	OH
Liver weight, g	4.91 $\pm$ 0.18 ^a	4.09 $\pm$ 0.20 ^b	4.05 $\pm$ 0.16 ^b
Ca, mg	0.89 $\pm$ 0.09 ^a	0.92 $\pm$ 0.14 ^a	0.58 $\pm$ 0.02 ^b
Ca, mg/g	0.22 $\pm$ 0.04 ^a	0.23 $\pm$ 0.04 ^a	0.14 $\pm$ 0.01 ^b
Kidney weight, g	0.58 $\pm$ 0.02 ^a	0.53 $\pm$ 0.03 ^{ab}	0.50 $\pm$ 0.02 ^b
Ca, mg	0.06 $\pm$ 0.01 ^a	0.06 $\pm$ 0.01 ^a	0.05 $\pm$ 0.01 ^a
Ca, mg/g	0.10 $\pm$ 0.01 ^a	0.10 $\pm$ 0.01 ^a	0.10 $\pm$ 0.01 ^a
Femur weight, g	0.24 $\pm$ 0.01 ^a	0.22 $\pm$ 0.01 ^b	0.21 $\pm$ 0.01 ^{ab}
Ca, mg	46.0 $\pm$ 1.1 ^a	40.7 $\pm$ 0.8 ^b	41.2 $\pm$ 1.6 ^b
Ca, mg/g	191.8 $\pm$ 3.0 ^a	188.7 $\pm$ 2.5 ^a	193.0 $\pm$ 2.7 ^a
Ratio Ca:P	2.07 $\pm$ 0.03 ^a	2.04 $\pm$ 0.02 ^a	2.16 $\pm$ 0.04 ^b
Small intestine			
Ca, mg/g	0.17 $\pm$ 0.01 ^a	0.18 $\pm$ 0.01 ^a	0.28 $\pm$ 0.02 ^b
Cecum content weight, g	1.87 $\pm$ 0.08 ^a	1.70 $\pm$ 0.31 ^a	1.56 $\pm$ 0.18 ^a
Ca, mg/g	7.98 $\pm$ 1.00 ^a	7.65 $\pm$ 0.38 ^a	7.55 $\pm$ 0.73 ^a
Serum, mg/dL	11.5 $\pm$ 0.5 ^a	11.1 $\pm$ 0.3 ^a	12.3 $\pm$ 0.5 ^a

^{a,b}Different letters within a row indicate significant differences between groups ($P < 0.05$).

¹Data are means $\pm$ SE of 8 animals. Groups: UHT milk was sterilized by heating to 150°C for 6 s; OH (overheated) milk was subjected to a traditional in-bottle sterilization (116°C, 16 min), repeated 3 times; UHT-pair fed, group of rats fed the diet UHT at the same intake level as the OH group.

tion of overheated milk. A reduction of the Ca/P ratio due to the decreased concentration of P was observed (our unpublished data).

CONCLUSIONS

In vitro and in vivo assays in the present study showed that thermal damage to milk may affect calcium availability. Calcium solubility was impaired after the in vitro digestion of OH milk compared with UHT milk, which could be the result of thermal damage to proteins and the increased presence of MRP, as supported by the analysis of MR markers. Consumption of a diet based on overheated milk led to significant reductions in apparent calcium absorption, mainly due to a decreased food intake compared with the UHT diet. A negative effect on calcium balance was also shown. We may conclude that processing conditions of milk warrant particular attention to prevent impaired dietary calcium utilization. This may be especially important in situations in which milk and dairy products are the main staples of the diet, such as in early infancy.

REFERENCES

- Aitameur, L., M. Zude, G. Trystram, and I. Birlouez-Aragon. 2004. Hydroxymethylfurfural: An indicative parameter of heat damage in cereal products. *Czech. J. Food Sci.* 22:99–101.
- alKhanal, H. A., A. A. al-Othman, and F. M. Hewedi. 2001. Changes in protein nutritional quality in fresh and combined ultra high temperature treated milk during storage. *Int. J. Food Sci. Nutr.* 52:509–514.
- AOAC. 1995. Official Methods of Analysis. 16th ed. Association of Official Analytical Chemists, Washington, DC.
- Burton, H. 1984. Reviews of the progress of dairy science: The bacteriological, chemical biochemical and physical changes that can occur in milk at temperatures of 100–150°C. *J. Dairy Res.* 51:341–363.
- Burton, H. 1988. Properties of UHT-processed milk. Pages 254–291 in *Ultra-High Temperature Processing of Milk and Milk Products*. H. Burton ed. Elsevier Science, London, UK.
- Cattaneo, S., F. Masoti, and L. Pellegrino. 2008. Effects of overprocessing on heat damage of UHT milk. *Eur. Food Res. Technol.* 226:1099–1106.
- CIE Colorimetric Committee. 1974. Technical notes: Working program on colour differences. *J. Op. Soc. Am.* 64:896–897.
- Claeys, W. L., C. Smout, A. M. Van Loey, and M. E. Hendrickx. 2004. From time temperature integrator kinetics to time temperature integrator tolerance levels: Heat-treated milk. *Biotechnol. Prog.* 20:1–12.
- de la Fuente, M. A., A. Olano, V. Casal, and M. Juárez. 1999. Effects of high pressure and heat treatment on the mineral balance of goats' milk. *J. Dairy Res.* 66:65–72.
- Delgado, T., N. Corzo, G. Santa-María, M. L. Jimeno, and A. Olano. 1992. Determination of furosine in milk samples by ion-pair reversed phase liquid chromatography. *Chromatographia* 33:374–376.
- Delgado-Andrade, C., I. Seiquer, and M. P. Navarro. 2005. Comparative effects of glucose-lysine versus glucose-methionine Maillard reaction products consumption: *in vitro* and *in vivo* calcium availability. *Mol. Nutr. Food Res.* 49:679–684.
- Delgado-Andrade, C., I. Seiquer, and M. P. Navarro. 2006. Changes in calcium absorption and subsequent tissue distribution induced by Maillard reaction products: *in vitro* and *in vivo* assays. *J. Sci. Food Agric.* 86:271–278.
- Delgado-Andrade, C., I. Seiquer, P. Navarro, and F. J. Morales. 2007. Maillard reaction products in diets usually consumed by adolescent population. *Mol. Nutr. Food Res.* 51:341–351.
- Delgado-Andrade, C., I. Seiquer, R. Nieto, and M. P. Navarro. 2004. Effects of heated glucose-lysine and glucose-methionine model-systems on mineral solubility. *Food Chem.* 87:329–337.
- Elliott, A. J., N. Datta, B. Amenu, and H. C. Deeth. 2005. Heat-induced and other chemical changes in commercial UHT milks. *J. Dairy Res.* 72:442–446.
- Erbersdobler, H. F. 1986. Twenty years of furosine—Better knowledge about the biological significance of Maillard reaction in food and nutrition. Pages 481–491 in *Amino-Carbonyl Reactions in Food and Biological Systems*. M. Fujimaki, M. Namiki, and H. Kato ed. Elsevier, Amsterdam, The Netherlands.
- Forssén, K. M., M. I. Jägerstad, K. Wigetz, and C. M. Witthöft. 2000. Folate and dairy products: A critical update. *J. Am. Coll. Nutr.* 19:100S–110S.
- Francis, F. J., and F. H. Clydesdale. 1975. Food colours. Pages 12–14 in *Food Colorimetry: Theory and Applications*. Avi Publishing Co., Westport, CT.
- Friedman, M. 1996. Food browning and its prevention: An overview. *J. Agric. Food Chem.* 44:631–653.
- Furniss, D. E., J. Vuichoid, P. A. Finot, and R. F. Hurrell. 1989. The effect of Maillard reaction products on Zn metabolism in the rat. *Br. J. Nutr.* 62:739–749.
- Ganong, W. F. 1993. Digestion and absorption. Pages 505–516 in *Review of Medical Physiology*. 14th ed. W. F. Ganong, ed. Appleton and Lange, San Francisco, CA.
- International Dairy Federation. 2007. IDF Bulletin 423/2007. www.fil-idf.org. IDF, Brussels, Belgium.
- Kelly, K., and D. Judd. 1976. Colour: Universal Language and Dictionary of Names. U.S. National Bureau of Standards, NBS Special Publication 440. Department of Commerce, Washington, DC.
- Law, A. J. R. 1996. Effects of heat treatment and acidification on the dissociation of bovine casein micelles. *J. Dairy Res.* 63:35–48.
- Liu, X., and L. E. Metzger. 2007. Application of fluorescence spectroscopy for monitoring changes in nonfat dry Milk during storage. *J. Dairy Sci.* 90:24–37.
- MAPA. 2006. Food in Spain. <http://www.mapa.es/es/alimentacion/pags/consumo/resumen.htm> Accessed May 10, 2010.
- Mattila-Sandholm, T., and M. Saarela. 2003. *Functional Dairy Products*. T. Mattila-Sandholm and M. Saarela, ed. CRC Press, New York, NY.
- Meisel, H., and E. Schlimmer. 1995. Casein-gebundener phosphor und der gehalt an freien aminosäuren in unterschiedlich wärmebehandelter Milch. *Kieler Milchw. Forsch.* 47:289–295.
- Mesías, M., I. Seiquer, and M. P. Navarro. 2009. Influence of diets rich in Maillard reaction products on calcium bioavailability. Assays in male adolescents and in Caco-2 cells. *J. Agric. Food Chem.* 57:9532–9538.
- Naito, H. 1990. Casein phosphopeptide: Intra-intestinal formation and significance in the bioavailability of calcium. Pages 207–220 in *Nutrition: Proteins and Amino Acids*. A. Yoshida, H. Naito, Y. Niiyama, and T. Suzuki, ed. Japan Scientific Societies Press, Tokyo, Japan.
- NRC. 1995. Nutrient requirements of the laboratory rat. Pages 11–79 in *Nutrient Requirements of Laboratory Animals*. National Academy Press, Washington, DC.
- O'Brien, J., and P. A. Morrissey. 1997. Metal ion complexation by products of the Maillard reaction. *Food Chem.* 58:17–27.
- O'Brien, J., and R. Walker. 1988. Toxicological effects of dietary Maillard reaction products in the rat. *Food Chem. Toxicol.* 26:775–783.
- O'Connell, J. E., and P. F. Fox. 2002. Heat stability of milk. Pages 1321–1326 in *Encyclopaedia of Dairy Sciences*. H. Roginski, J. W. Fuquay, and P. F. Fox, ed. Academic Press, London, UK.
- O'Sullivan, M. M., A. L. Kelly, and P. F. Fox. 2002. Influence of transglutaminase treatment on some physico-chemical properties of milk. *J. Dairy Res.* 69:433–442.
- Pagiarini, E., M. Vernile, and C. Peri. 1990. Kinetic study of color changes in milk due to heat. *J. Food Sci.* 55:1766–1767.

- Pellegrino, E., I. De Noni, and P. Resmini. 1995. Coupling of lactulose and furosine indices for quality evaluation of sterilized milk. *Int. Dairy J.* 5:647–659.
- Mendoza, M. R., A. Olano, and M. Villamiel. 2005. Chemical indicators of heat treatment in fortified and special milks. *J. Agric. Food Chem.* 53:2995–2999.
- Rendleman, J. A. 1987. Complexation of calcium by the melanoidin and its role in determining bioavailability. *J. Food Sci.* 52:1699–1705.
- Sarriá, B., R. López-Fandiño, and M. P. Vaquero. 2001. Does processing of a powder or in-bottle-sterilized liquid infant formula affect calcium bioavailability? *Nutrition* 17:326–331.
- Seiquer, I., T. Aspe, P. Vaquero, and M. P. Navarro. 2001. Effects of heat treatment of casein in the presence of reducing sugars on calcium bioavailability: *in vitro* and *in vivo* assays. *J. Agric. Food Chem.* 49:1049–1055.
- Seiquer, I., M. López-Frías, M. Muñoz-Hoyos, G. Galdó, C. Delgado-Andrade, M. Mesías, and M. P. Navarro. 2006. Dietary calcium utilization among a group of spanish boys aged 11–14 years on their usual diets. *J. Physiol. Biochem.* 62:9–16.
- Smiddy, M. A., J. E. Martin, A. L. Kelly, C. G. de Kruif, and T. Huppertz. 2006. Stability of casein micelles cross-linked by transglutaminase. *J. Dairy Sci.* 89:1906–1914.
- Washington, N., M. Harris, A. Mussellwhite, and R. C. Spiller. 1998. Modifications of lactulose-induces diarrhea by psyllium: Effects on motility and fermentation. *Am. J. Clin. Nutr.* 67:317–321.