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Comparative effects of glucose-lysine *versus* glucose-methionine Maillard reaction products consumption: *in vitro* and *in vivo* calcium availability

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The influence of glucose-lysine and glucose-methionine Maillard reaction products (MRPs) on calcium availability was studied in rats and in Caco-2 cells. Equimolar glucose/lysine and glucose/methionine mixtures (40% moisture) were heated (150°C, 30 or 90 min) to prepare samples (GL30, GL90, GM30, and GM90, respectively). For 21 days, the rats were fed the AIN-93G diet (control group) or diets containing separately 3% of the heated mixtures (GL30, GL90, GM30, and GM90 groups, respectively). In the last week of the trial, a calcium balance was performed. On day 21, the animals were sacrificed and their livers and femurs removed for analysis of calcium levels. The GL30 and GM30 samples and the corresponding raw mixtures were used for Caco-2 cells experiments. Fecal excretion of calcium decreased and urinary elimination increased in the GM30 and GM90 groups. In accordance, increased calcium transport in Caco-2 cells was found in the presence of the GM30 sample, compared with the raw sample. Bone calcium concentration was lower among the animals consuming MRP diets, compared with the control group. The possible long-term effects of MRP intake on calcium deposition in the bone should be further studied to ascertain the implications on related diseases.

Keywords: Bone / Caco-2 cells / Calcium bioavailability / Maillard reaction products

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1 Introduction

During the heat treatment of foods, or even during storage, free amino groups of amino acids, peptides, and proteins react with carbonyl groups from reducing sugars to form condensation compounds. These products, after chain reactions, lead to volatile compounds and complex colored products influencing the odor, taste, and typical color of processed food. The development of this reaction, known as Maillard reaction (MR), has been applied by the industry to enhance food attraction or introducing natural additives to the food [1]. Despite these positive technological aspects, the MR can lead to decreases in the nutritive value of foods, mainly because of the damage to protein quality caused by the destruction of amino acids or their bioavailability, especially in the case of lysine [2, 3]. Nevertheless, the nutritive consequences can not be standardized because they vary depending on the reactants, the heating temperature and duration, pH, water activity, *etc.* [4].

Rendleman [5] showed the bindings of calcium to soluble and insoluble melanoidins from glucose-glycine, fructose-glycine, and glucose-glutamic acid. According to O'Brien and Morrissey [6], using potentiometric titration, calcium and magnesium have a lower affinity for browning products than that observed for copper or iron. In this sense, our results [7] using MR products (MRPs) from glucose-lysine and glucose-methionine model systems heated for different times show that the presence of these products within solutions of minerals at intestinal pH and osmolality does not modify calcium or magnesium solubility. However, other studies performed in our research group reveal changes in calcium especiation in the presence of a heated casein-glucose-fructose mixture [8].

The *in vitro* chelating activity of MRPs suggests that mineral complexation could affect the bioavailability. Amadori products, premelanoidins, and some soluble low-molecular-weight melanoidins could form soluble complexes with calcium in the intestinal lumen and so improve its absorption and/or modify its normal metabolism. On the contrary, advanced MRPs would decrease calcium absorption [9]. Few data are available concerning the effects of

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Abbreviations: ANOVA, analysis of variane; **GL**, glucose-lysine; **GM**, glucose-methionine; **MRP**, Maillard reaction product

MRP on calcium availability *in vivo*, and these mainly refer to digestibility assays. Some studies report no modification in calcium utilization caused by the presence of MRP from glucose-glycine [10] and glucose-sodium monoglutamate in the diet [11] or improvement with xilose-lysine MRPs, attributed to the effects of the intestinal microflora [12]. Feeding rats a diet containing heated casein-glucose-fructose mixtures did not modify calcium digestibility, although urinary excretion of the mineral increased [8]. Increases in calcium digestibility were observed after consumption of a sterilized infant formula, but calcium retention was decreased due to lower intake [13]. In the other hand, recent studies using the human adenocarcinoma Caco-2 cell line [14, 15], have shown that the presence of heated casein-glucose-fructose mixtures increases the transport of soluble calcium [8].

The purpose of this study was to analyze the effects of glucose-lysine *versus* heated glucose-methionine mixtures on *in vitro* and *in vivo* calcium availability. Two different heating times were chosen to compare the actions of earlier soluble Maillard products and those from the more advanced ones, containing insoluble products.

2 Materials and methods

2.1 Sample preparation

Glucose (Merck, Darmstadt, Germany), lysine, and methionine (Sigma Chemical, St. Louis, MO., USA) were used to prepare the samples. Equimolar mixtures of glucose-lysine-HCl (GL) or glucose-DL-methionine (GM) (both 40% moisture) were heated in open recipients in an oven (Selecta 2000210; Barcelona, Spain) at 1500°C for 30 or 90 min to obtain the GL30, GL90, GM30, and GM90 samples, respectively. This procedure tried to simulate a usual home cooking. After heating, the reaction was stopped by cooling in an ice bath and the products were then removed, frozen, lyophilized, and stored at 4°C as described by Delgado-Andrade *et al.* [16] until required for preparing the diets or to perform the Caco-2 cell assays. The samples were characterized for development of the brown color at 420 nm, pH values, and their effects on mineral solubility [7]. The free amino acid content in the samples was measured by high-performance liquid chromatography (HPLC), using the Water Pico-Tag method after derivatization with phenyl isothiocyanate without the hydrolysis step. The measured proportions of free residual lysine and methionine were 37.2%, 21.5%, 47.0%, and 30.4%, respectively, in the GL30, GL90, GM30, and GM90 samples from the amino acids present in the initial mixture.

2.2 Diet preparation

The AIN-93G purified diet for laboratory rodents [17] was used as control diet. Casein supplemented with cysteine is the protein source in this diet. The GL30, GL90, GM30, and GM90 samples were individually added to the AIN-93G diet to reach a final concentration of 3%. The diets were named GL30, GL90, GM30, and GM90, respectively. Individual analysis of the diets revealed no significant differences in nutrient content, as was to be expected since the diets were basically the AIN-93G diet. The mean ± SD nutrient content of the diets was: moisture (%) 8.0 ± 0.4, protein (g/kg) 181.7 ± 1.1, fat (g/kg) 69.4 ± 0.5, and calcium (g/kg) 4.8 ± 0.3.

2.3 Biological assays

Fifty-five weanling Wistar rats weighing 41.7 ± 0.4 (mean ± SE) were housed individually in metabolic cages in an environmentally controlled room which was kept at 20–22°C with a 12-h light-dark cycle and 55–70% humidity. The rats were randomly distributed into five groups of eleven rats and each group was assigned to one of the dietary treatments, designated control, GL30, GL90, GM30, and GM90 groups. All animals had *ad libitum* access to their diets and demineralized water (Milli-Q Ultrapure Water System; Millipore, Bedford, MA, USA). The animals were fed the different diets during 21 days. In the last week of the experimental period (days 14–21), the calcium balance was performed. The test involved a preliminary 14-day period during which solid food intake and body weight changes were monitored, followed by a 7-day period in which, moreover, feces and urine from each animal were collected daily and stored separately as a 1-week pool. The feces were weighed, lyophilized, and then homogenized. Urine was collected on 0.5% HCl (v/v), filtered (Whatman Filter Paper No. 40, ashless; Whatman, Maidstone, UK) and diluted to an appropriate volume. To control for possible environmental contamination during the collection of urine and feces, blank cages were manipulated in the same way as those used for the animals. On day 21, after an overnight fast, six animals of each group, since the rest of them were reserved for another kind of trials, were anesthetized with sodium pentobarbital (5 mg/100 g body weight) (Abbott Laboratories, Granada, Spain) and terminal exsanguination was performed by cannulation of the carotid artery. The liver and femur were removed, weighed, and frozen at –20°C until used for calcium analysis. All management and experimental procedures carried out in this study were done in strict accordance with the current European regulations (86/609 E.E.C.) regarding laboratory animals. The Committee of Bioethics for animal experimentation at our institution (UGR-CSIC) approved the study protocol.

2.4 Cells

2.4.1 Cell culture

Caco-2 cells were purchased from the European Collection of Cell Culture (ECACC). All cell culture media and cell

culture grade chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Cells were grown in 75 cm^2 plastic flasks (Corning Costar, Cambridge, MA, USA) containing high-glucose Dulbecco's modified minimal essential medium (DMEM), with heat-inactivated fetal bovine serum (15%), $NaHCO_3$ (3.7 g/L), nonessential amino acids (1%), HEPES (15 mM), bovine insulin (0.1 IU/mL), and 1% antibiotic-antimycotic solution. The cells were maintained at 37°C in an incubator in an atmosphere of air/CO_2 (95 : 5) at 90% humidity, and the medium was changed every 2 days. The calcium content of the complete medium was 1.54 mM. Trypsinization and seeding of cells into bicameral chambers (Transwell, 24 mm diameter, 4.7 cm^2 area, 3 μm pore size; Costar) were performed and the development of functional tight junctions during cell differentiation in bicameral chambers was monitored by determining transepithelial electrical resistance (TEER) as described by Navarro *et al.* [18]. The monolayers used in this study exhibited adequate TEER values ranging from 500 to 650 Ω/cm^2.

2.4.2 Calcium transport

Twenty-one days after initial seeding, the calcium transport experiments were carried out. Spent culture medium was aspirated from the bicameral chambers and cells surfaces of the monolayer were washed three times with Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) at 37°C. Then, 2.5 mL of the transport solution was added to the basolateral chamber, and the test transport solutions were added to the apical chamber (1.5 mL). The transport solution contained 130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 7. Five different test transport solutions containing the same calcium concentration (3 mM) were added to the apical chamber. Calcium was added to DMEM from a 0.1 M stock solution prepared with $CaCO_3$ (Sigma Chemical) to achieve a final calcium concentration of 3 mM (DMEM-Ca). The rest of the test transport solutions were obtained by adding GL, GL30, GM, or GM30 samples to the DMEM-Ca in a concentration of 0.8 mg sample/mL, thus resulting the DMEM-GL, DMEM-GL30, DMEM-GM, and DMEM-GM30 test transport solutions, respectively. After adding the test transport solutions at 37°C to the apical chamber, the cell cultures were incubated at this same temperature in a humidified air : CO_2 atmosphere for 4 h. To calculate the calcium transported across the cell monolayer, the buffer from the basolateral chamber was removed and, to ensure complete collection, the wells were washed twice with deionized water. Cell viability after 4 h of exposure to the test transport solutions was assessed by trypan blue exclusion, and it was never <85%.

2.5 Analytical techniques

The liver and femur were dry-ashed in a muffle furnace (Selecta, Model 366, Barcelona, Spain) at 450°C and the white ashes obtained were dissolved with $HCl/HNO_3/H_2O$ (1 : 1 : 2) (Suprapur; Merck). Aliquots of the remaining samples were completely digested by the addition of concentrated HNO_3, $HClO_4$, and by heating at high temperatures in a sand beaker. All samples were diluted with Milli-Q water to an appropriate volume for measurement. Calcium analyses in all samples were carried out with flame atomic absorbance spectrometry (AAS) in a Perkin-Elmer Analyst 700 Spectrophotometer (Norwalk, CT, USA). Standard solutions were prepared from a stock Tritisol solution of calcium (Cl_2Ca in 6.5% HCl, 1000 mg 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 inter-assay coefficient of variation was 0.85% in feces, 0.95% in urine, and 2.07% 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 ± 0.04 mg/g (mean ± 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. The following indices were calculated using the data for calcium intake and fecal and urinary excretion obtained in the last week of the assay: percentage of calcium excreted in feces (fecal Ca/ingested Ca × 100); absorbed calcium (ingested Ca–fecal Ca); percentage of calcium excreted in urine (urinary Ca/absorbed Ca × 100); balance (absorbed Ca–urinary Ca).

2.6 Statistical analysis

Data from the *in vivo* and *in vitro* assays were statistically tested by one-way analysis of variance (ANOVA), followed by the Duncan Test to compare means that showed significant variation ($P < 0.05$). The results concerning the calcium balance and calcium concentration in organs were also analyzed by two-way ANOVA to examine the effects of type of mixture (glucose-lysine or glucose-methionine), of the duration of the heat treatment (30 or 90 min) and of the interaction between mixture and duration time. Significance of the results was established at $P < 0.05$. Statgraphics Plus, Version 5.1, 2001 was used to carry out the comparisons.

3 Results and discussion

No significant differences were found in calcium intake during the last week of the assay among the animals given the MRP diets with respect to the control group, although in the GM30 group it tended to decrease and was lower than in the GL30 group (Table 1). According to the bibliography, this tendency may be due to the free methionine still present

Table 1. Calcium balance in rats fed the different diets (days 15–21)[a)]

Groups[b)]	Intake (mg/d)	F/I[c)] (%)	Absorbed (mg/d)	**U/A[c)] (%)**	Balance (mg/d)	Final weight (g)
C	63.33 ± 3.12^{ab}	38.23 ± 1.16^{a}	39.24 ± 2.31	6.75 ± 0.61^{a}	36.67 ± 2.31	141.78 ± 7.24^{a}
GL30	65.94 ± 3.35^{a}	34.35 ± 1.60^{a}	43.63 ± 2.99	6.99 ± 0.49^{a}	40.65 ± 2.90	143.50 ± 3.82^{a}
GL90	60.36 ± 3.31^{ab}	37.37 ± 2.22^{a}	38.23 ± 2.95	7.99 ± 0.96^{a}	35.36 ± 2.95	132.40 ± 7.22^{ab}
GM30	54.42 ± 2.61^{b}	26.85 ± 2.06^{b}	39.94 ± 2.51	11.88 ± 1.25^{b}	35.24 ± 2.42	116.25 ± 3.38^{b}
GM90	61.46 ± 3.17^{ab}	27.32 ± 2.74^{b}	44.66 ± 2.67	12.00 ± 1.22^{b}	39.49 ± 2.70	131.75 ± 6.36^{ab}
Two-way ANOVA						
Mixture[d)]	NS	$p = 0.000$	NS	$p = 0.000$	NS	$p = 0.015$
Heating time[e)]	NS	NS	NS	NS	NS	NS
Mixture × heating time[f)]	NS	NS	NS	NS	NS	$p = 0.020$

a) Different superscripts in the same column indicate significant differences ($p < 0.05$; one-way ANOVA and Duncan test). Values are mean ± SE of eleven animals.
b) C, group of rats fed the AIN-93G diet; GL30, group of rats fed the GL30 diet; GL90, group of rats fed the GL90 diet; GM30, group of rats fed the GM30 diet; GM90, group of rats fed the GM90 diet.
c) F, fecal calcium; I, calcium intake; U, urinary calcium; A, absorbed calcium.
d) Mixture, glucose-lysine or glucose-methionine.
e) Heating time, 30 or 90 min.
f) Interaction mixture × heating time

in the heated GM30 mixture, since this amino acid has been described as an appetite inhibitor and its excess has been related to lower body weights [19]. The nonsignificant difference in the calcium intake in these animals compared with the control group suggests that MRPs from the GM30 sample could have been able to counteract the negative effect of the free methionine ingested in this diet on appetite. However, the final body weight was significantly lower in this group.

Calcium in feces, expressed as a percentage of the mineral excreted from the intake (% F/I), decreased in the groups fed MRPs from GM mixture but not in the GL groups (Table 1). The statistical analysis showed significant differences due to the type of glucose-amino acid mixture, and so the effect observed in the fecal output might be attributed to the MRPs from GM system, since this action has not been observed in studies of methionine addition to the diet. Moreover, the chelating activity of some melanoidins on calcium has also been reported [20, 21]. Soluble and insoluble melanoidins can complex calcium with different complexation capabilities depending on the reactants involved [5]. In addition, soluble melanoidins may be absorbed [22] and some MRP-mineral complexes favor mineral availability due to neutralization of their charges, which facilitates transport through the cell membrane [23]. Accordingly, it may be suggested that some soluble MRP from the GM mixture with capacity to complex calcium under the intestinal conditions [7] or their intestinal degradation products might promote calcium absorption. This would explain the reduction of fecal excretion in the GM30 and GM90 groups.

The results found for calcium transport in Caco-2 cells are in agreement with the decrease in the fecal excretion of calcium observed in the GM groups *in vivo* (Fig. 1). The presence of the GM30 sample in the culture medium increased the calcium transport, compared with the raw mixture. Thus, connecting the *in vitro* and *in vivo* assays, it seems logical that a higher calcium transport across the intestinal monolayer leads to lower fecal excretion of the element. Certainly, some MRPs could be changed *in vivo* in the gastrointestinal tract of man and animals by the digestive process, and, even more importantly, by the action of the intestinal microflora. These mechanisms that could modify the structure and so affect the calcium-binding activity of original MRPs, would not have taken place in the *in vitro* cell system. Nevertheless, both *in vitro* and *in vivo* assays are in agreement suggesting a positive influence of MRPs from the GM mixture on fractional calcium absorption. This can be related to the investigations of Senior *et al.* [24] who described the favorable effects of some premelanoidins on calcium absorption. Despite of the modification in fecal excretion, the calcium absorbed, in absolute values, did not

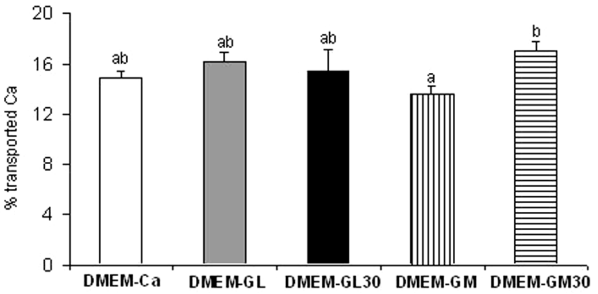

Figure 1. Calcium transport in Caco-2 cells after 4h of exposure to 3 mM Ca solutions. Data are mean ± SE of at least three wells from a representative experiments. Different letters indicate significant differences (one-way ANOVA and Duncan test, $P < 0.05$).

vary in all the animals with respect to the control group. However, the fraction absorbed increased in the animals consuming MRP from the heated GM mixtures.

Previous assays developed in our laboratory revealed no modification in calcium transport in Caco-2 cells in the presence of fructosil lysine [25]. In accordance with this, the calcium transport in the DMEM-GL30 test transport solution (Fig. 1) was unaffected and, despite the above-mentioned limitations of *in vitro* studies, results were consistent with the absence of variations observed in the efficiency of calcium absorption in the groups fed the GL diets. In the animals of the GM30 and GM90 groups the urinary excretion increased, a feature not manifested in the other groups (Table 1). Comparison of data for this parameter using multifactorial ANOVA showed an effect associated with the type of glucose-amino acid mixture ($P = 0.000$). In this respect, it may be suggested that free methionine these diets could be responsible for the increased elimination of calcium, since the intake of methionine-supplemented diets has been related to higher calcium contents in urine because of the action of filtered, and poorly reabsorbed, SO_4^{2-} ions in the kidney, which are able to bind calcium and provoke its elimination [26, 27].

The decrease in fecal excretion of calcium might counteract the increase in the urinary elimination in the above-mentioned groups, given that none of the animals in this assay presented quantitative changes in the calcium balance. No differences were found in calcium retention, either deriving from variations in the heating time or the glucose-amino acid mixture (Table 1).

Little is said in the bibliography concerning the calcium balance and MRP intake, and most published data are disparate. Sarriá *et al.* [13] reported a considerable decrease in calcium retention after feeding rats a liquid infant formula containing browning products; other authors have reported no variations in retention in animals fed MRPs from a heated glucose-glycine mixture [10] or in animals given a diet containing a heated mixture of casein-glucose-fructose [8].

After consumption of diets containing MRPs, an abnormal redistribution of calcium toward unspecific organs seemed to occur: the hepatic concentration (expressed as calcium concentration in liver ashes) increased in all groups, although not significantly in the GM30 group (Fig. 2). Some authors have shown the importance of hepatic tissue as an MRP target in the organism [11, 28]. The browning products consumed in this assay may bind calcium and somehow behave as calcium carriers to the liver.

Calcium concentration in bone ashes decreased in all animals of the trial compared with the control group (Fig. 2). Given that the quantity of ashes did not decrease in any group, these results seemed to be a specific effect on bone

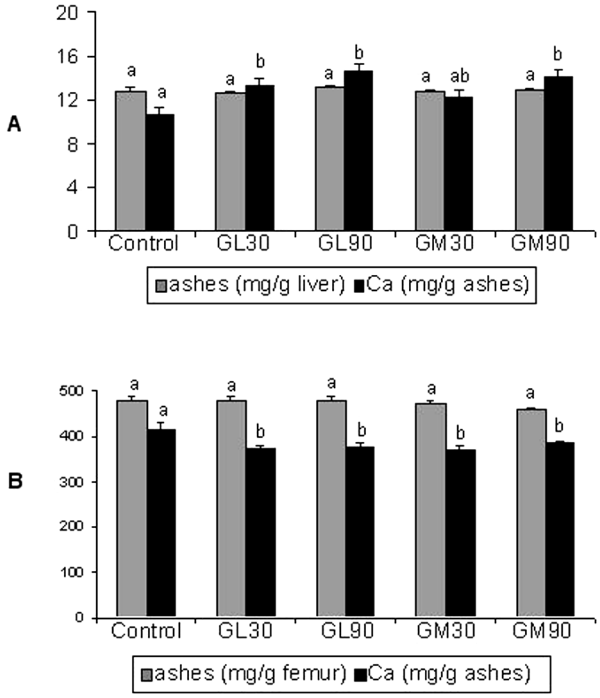

Figure 2. Ashes and calcium content in (A) liver and (B) femur. Data are mean ± SE of six animals. Different letters indicate significant differences (one-way ANOVA and Duncan test, $P < 0.05$). No significant differences or interactions were found in the two-way ANOVA test.

calcium. This decrease could be attributed to MRP intake, since subsequent assays performed in our laboratory have confirmed that the addition of free methionine and lysine to the diet in the same quantity as in the samples of this assay does not alter the calcium concentration in the bones of rats (unpublished data). In agreement with this, the literature describes reductions in bone strength and calcium content in bones in animals consuming diets including MRPs [29, 30]. Some authors have also observed a lower ash content in the bone [28], a result not found among the animals in our assay (Fig. 2).

In view of the results found in bones, the implication of consumed MPRs on *in vivo* glycation might be taken into account, since it has been suggested that the effects of some exogenous Maillard products could supplement the action of the endogenous Maillard products [22]. It has been found that advanced glycation end products may alter the bone protein matrix and modify calcium deposition [31].

4 Concluding remarks

In summary, the consumption of MR products from a heated GM mixture, compared to those from a heated GL mixture, induced a lower rate of fecal excretion of calcium and an increase of urinary calcium, probably due to the con-

sumption of associated free methionine. Neither the type of the heated mixture nor the duration of the heat treatment affected the calcium balance under our experimental conditions. However, calcium concentration in the femur clearly decreased among the animals consuming the MRP diets, in both amino acid-glucose mixtures and heating times. The deficit of bone calcium could be an indicator of abnormal bone mineralization. This might be taken into account in physiologic grown situations, in which calcium demands are higher, and in aging, when calcium absorption may be compromised and when the *in vivo* glycation of long-life proteins is significant.

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