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MILK IMPROVED THE METABOLIC SYNDROME IN OBESE β RATS

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Abstract The response of adult spontaneously obese rats from the IIMb/Beta strain fed a high calcium skimmed milk diet (MHCa), high calcium from carbonate (HCa) and a normal AIN 93 diet during 45 days was evaluated. Body weight, food intake and fecal fat excretion were measured. At the end of the experiment rats were euthanized, abdominal fat pads and livers were excised and weighed. Blood and liver triacylglycerols, total cholesterol and fractions were quantified. Body weight increase and abdominal fat pads in the MHCa group were significantly lower than in the other two. Plasma triacylglycerols, total and LDL-cholesterol were diminished in the MHCa group. Fecal lipid excretion was increased in the adult MHCa group. Total liver lipids and triacylglycerols showed a significant diminution in the MHCa group. These results suggest that calcium and other bioactive compounds from milk, most probably present in whey fraction, and not calcium carbonate exerted an "anti-obesity" effect on these rats.

Key words: milk, calcium, spontaneously obese rats

Resumen ***La leche mejoró el síndrome metabólico en ratas obesas β.*** Se evaluaron los efectos de dietas con distintos niveles y fuente de calcio sobre parámetros relacionados con el síndrome metabólico en ratas adultas espontáneamente obesas de la línea IIMb/Beta. Se suministraron durante 45 días tres dietas: alto nivel de calcio proveniente de leche descremada (MHCa); alto calcio proveniente de carbonato (HCa) y como referencia AIN 93, normocálcica. Se midieron peso corporal, ingesta de alimento y excreción fecal de grasa. Los animales se sacrificaron y se extrajeron y pesaron los panículos adiposos abdominales y el hígado. Se determinaron triacilgliceroles, colesterol total y fracciones en plasma y en hígado. El aumento de peso corporal, los panículos adiposos abdominales y los valores plasmáticos de triacilgliceroles y de colesterol y fracciones fueron significativamente menores en el grupo MHCa. La excreción fecal de grasa resultó aumentada en el grupo MHCa. Los lípidos totales y los triacilgliceroles hepáticos mostraron una disminución significativa en el grupo MHCa. Los resultados evidencian efectos beneficiosos del calcio de la leche y no del suplemento mineral, sugiriendo que una acción sinérgica con otros compuestos bioactivos, probablemente presentes en el suero de la leche, produciría los efectos "antiobesidad" en estas ratas.

Palabras clave: leche, calcio, ratas espontáneamente obesas

The nutritional approach to prevent and treat obesity has traditionally focused on factors that influence energy balance, such as reduced energy intake or increased energy expenditure. The identification of additional dietary factors without caloric value but able to influence obesity is important. There is a growing body of scientific evidence that suggests that dairy and or calcium (Ca) consumption is beneficially related to weight management[1].

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In 2000, Zemel forwarded the hypothesis that Ca stimulates lipolysis and inhibits lipogenesis due to the increased production of parathyroid hormones and/or 1,25-$(OH)_2$ vitamin D3[2]. Three years later it was suggested that Ca is also involved in the modulation of energy metabolism, exerting an "anti-obesity" effect. These hypotheses were confirmed by experiments carried on in humans and in mice[3-6].

An inverse relationship between overweight, obesity and Ca intake has been reported from the analysis of the data from important observational studies in different countries: NHANES III[7], the Coronary Artery Risk Development in Young Adults (CARDIA) study[8], the Heritage study[9] and the National Nutrition and Health Survey (ENNyS, Argentina)[10].

TABLE 1.– *Composition of the experimental diets (g/100 g)*

	MHCa	AIN	HCa
Sodium caseinate (Protein: 88.3%)[a]	-----	14.00	14.00
Dehydrated skimmed milkb	42.40	-----	-----
Sucrose	10.00	10.00	10.00
Mineral mixture[c]	3.50	3.50	3.50
$CaCO_3$	------	-------	1.75
Vitamin mixture[d]	1.00	1.00	1.00
Microcrystalline cellulose	5.00	5.00	5.00
Choline bitartrate	0.25	0.25	0.25
Cystein	0.18	0.18	0.18
Lipids[e]	4.00	4.00	4.00
Corn starch to complete 100g	33.70	62.10	60.30
Energy (Kcal/100g)	380.50	381.10	374.00
Energy (MJ/100g)	1.59	1.59	1.59

[a]Provided by Molfino SA, Rafaela, Argentina; [b]Provided by La Sibila , Entre Ríos, Argentina
[c]Identical to AIN-93 mineral mixture; [d]Identical to AIN-93 vitamin mixture; [e]Commercial sunflower oil

Several rodent models have been used to investigate the interactions among increased dairy and/or Ca intake, weight gain or loss as well as fat accumulation. The transgenic aP2-*agouti* mice, that over-expresses the agouti gene in adipocytes was used to evaluate the effects of Ca or dairy on body weight (BW) and composition[1, 2, 12]. Papakonstantinou et al. demonstrated that Wistar rats fed a high Ca-high protein diet had smaller BW gain than control[13]. Other researches evaluated long term effects of high versus low Ca diets on BW and body fat on growing Sprague-Dawley rats, achieving inconsistent results[14]. A study by Parra et al. showed that Ca intake was able to reduce BW and body fat gain in wild 72 type C57BL/J6J male mice fed a high fat diet[15].

In contrast, several experiments by Zhang and Tordoff involving both C57BL/J6J mice and Sprague-Dawley rats fed normal or high energy density diets showed no effects of Ca on energy intake, BW or body fat content[16].

The objective of this study was to asses the effects of high Ca diets from dehydrated skimmed milk or calcium carbonate ($CaCO_3$) on adult IIMb/Beta rats. This line of rats was obtained by a high degree of inbreeding and upward selection of BW, it has been internationally recognized as a murine model for the study of obesity and diabetes[17, 18]. The IIMb/Beta line has proved to be a useful model for the evaluation of ingredients from functional foods for the treatment of obesity and co morbidities since it develops spontaneous peri-pubertal hypertriacylglycerolemic obesity and progressive glucose intolerance that evolves towards type 2 diabetes at adult age, when fed with a standard commercial rat chow or the AIN' 93 (American Institute of Nutrition Rodent diets recommendations) formula for experimental rodents[19, 20].

The particular characteristics of this strain led us to examine whether a high level of calcium from milk or from mineral supplement affected BW, fat distribution, blood and liver parameters related with the metabolic syndrome.

Materials and Methods

Male adult IIMb/Beta rats (180 days old) raised in the Biology Department of the School of Medicine of the National University of Rosario, were housed in individual cages at controlled room temperature (21 ± 1 °C) with 55 ± 10% humidity under 12-h light/dark cycles for 45 days. Throughout the experimental period, rats were allowed water and food *ad libitum*. They were maintained in keeping with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the protocol was approved by the Bioethics Committee of the National University of Rosario.

Initial body weights of 21 adult rats were measured (media ± SD: 369.0 g ± 20.0 g).

Animals were randomly divided into three groups of seven animals each. Food intake was measured 3 times a week and BW once a week. Food efficiency (g/g) was calculated according to the following equation:

Food conversion efficiency = BW increase (g) /Food intake (g) (1)

Feces were collected during the last four days of the experiment.

At the end of the experiment (45 days) fasting blood samples were collected under anesthesia (ketamine hydrochloride - 0.1 mg/100 g BW - and acetopromazine maleate - 0.1 mg/100 g BW). Rats were then killed by CO_2; abdominal fat pads (retroperitoneal and epidydimal) and livers were excised, rinsed in physiological solution, dried with filter paper and weighed[21].

Relative fat depots and liver weights were calculated as:

= Organ weight/Total body weight x 100 (2)

The livers were stored at -18° C until analyses.

The experimental diets (Table 1) were prepared according to AIN'93[22]. Diets were isocaloric and isolipidic. The mineral mix prepared and employed according to AIN`93 provided 0.5g Ca (from $CaCO_3$) per 100 g of diet in the normal AIN diet. The

TABLE 2.– *Body weight (BW) increase, food intake (g), food efficiency and fecal fat excretion (g /100g) in IIMb/Beta rats**

Diets	MHCa	AIN	HCa
BW increase	8.9 ± 6.3[a]	57.9 ± 10.8[b]	49.5 ± 9.2[b]
Total food intake	636.8 ± 29.6[a]	769.2 ± 30.4[b]	782.1 ± 158.0[b]
Food conversion efficiency	1.4 ± 1.0[a]	7.3 ± 1.4[b]	5.9 ± 1.1[b]
Faecal lipid excretion	1.9 ± 1.0[a]	0.8 ± 0.2[b]	0.9 ± 0.2[b]

** Data are means ± SD; n= 7.*
Means with no common superscript letters within a row are significantly different (p < 0.05).

TABLE 3.– *Plasma parameters in IIMb/Beta rats (mmol/l)**

Diets	MHCa	AIN	HCa
Glycemia	10.50 ± 2.70[a]	11.90 ± 1.40[a]	10.30 ± 0.60[a]
TAG	1.97 ± 0.39[a]	2.83 ± 0.44[b]	2.88 ± 0.44[b]
Total Chol	3.63 ± 0.31[a]	4.37 ± 0.61[b]	4.42 ± 0.60[b]
LDL-Chol	0.71 ± 0.39[a]	1.28 ± 0.43[b]	1.04 ± 0.54[b]
HDL-Chol	1.05 ± 0.08[a]	1.12 ± 0.12[a]	1.24 ± 0.13[a]

** Data are means ± SD; n= 7.*
Means with no common superscript letters within a row are significantly different (p < 0.05).

TABLE 4.– Post mortem *studies in IIMb/Beta rats**

Diets	MHCa	AIN	HCa
Abdominal fat pads relative weight	4.10 ± 0.69[a]	5.30 ± 1.00[b]	5.40 ± 1.03[b]
Liver relative weight	4.63 ± 0.28[a]	4.97 ± 0.29[a]	4.55 ± 0.43[a]
Total liver lipids (g/100g)	2.59 ± 0.79[a]	8.02 ± 3.72[b]	6.47 ± 1.97[b]
Liver TAG (g/100g)	1.49 ± 0.80[a]	2.74 ± 0.87[b]	2.80 ± 0.69[b]
Liver total Chol (mg/100g)	139.60 ± 67.80[a]	175.60 ± 39.20[a]	159.30 ± 31.10[a]

** Data are means ± SD; n= 7.*
Means with no common superscript letters within a row are significantly different (p < 0.05).

additional Ca in the high Ca diet (HCa) (Ca 1.2 g/100 g) was provided by $CaCO_3$ (Merck Chemicals, Millipore; Catalogue Nº 102067, Germany), whereas skimmed dehydrated milk provided by La Sibila, Nogoyá, Entre Rios, Argentina, was the additional Ca source in the Milk High Calcium (MHCa) diet (Ca 1.2 g /100 g).

Final BW, food efficiency and BW increase were obtained. Blood parameters related to obesity were evaluated at the beginning and at the end of the experiment; these included: glycemia, blood triacylglycerols (TAG), total cholesterol (Chol), LDL-Chol and HDL Chol with enzymatic spectrophotometrical methods using Wiener Laboratories kits (Wiener Laboratorios SAIC; Rosario, Argentina), (Biotraza, Model 7 Spectrophotometer, China).

Feces collected during the last four days of the experiment were dried in an oven (Dalvo Instrumentos Premium, SP 464; Santa Fe, Argentina) at 42º C during 12 h, ground to 0.5–1.0 mm mesh, and dried again at 105 ºC for 2 h. Dried fecal samples (1.5–2.0 g) were homogenized in a Potter-Elvejahn device (Fisher Scientific, W 10691, UK), and lipids were extracted with chloroform (pa ACS, CAS 67-66-3; Cecarelli, Argentina) / methanol (Absolute ACS, CAS 67-56-1; Cecarelli, Argentina) (2:1, v/v). Total lipids were determined gravimetrically (Ohaus, Adventurer scale, 210g x 0.1mg) after solvent evaporation.

Fecal relative fat excretion was calculated as = fecal fat/ fat intake x 100 (3).

Liver samples were homogenized in a Potter-Elvejahn homogenator and lipids were extracted with chloroform/methanol according to Folch[23]. Total lipids were determined gravimetrically after evaporation of the solvents.

Liver TAG and total Chol were determined with the same analytical procedures used for plasma.

Results are expressed as mean ± standard deviation (SD). Data were analyzed using one way analysis of variance (ANOVA), and the Bonferroni multiple comparisons test was performed when significant differences were observed. Statistical analyses were carried out using Graph Pad Prism 3.02 Version program (April 2000). A value of p below 0.05 ($p < 0.05$) was considered significant.

Results

Animals began the experiment with not significantly different body weights. Final BW increase was significantly lower in the MHCa group.

Food conversion efficiency was significantly lower in the MHCa group, in coincidence with its smaller BW increase (Table 2).

Glycemia did not show differences between groups at the end of the experiment. Plasma TAG were significantly lower in the MHCa group than in both AIN and HCa groups.

A lower concentration in LDL-Chol was observed in the MHCa group, responsible for the lower levels in total Chol in this group. No significant differences were observed in HDL Chol values among groups (Table 3).

The lower BW increase registered in MHCa group was accompanied by significantly smaller abdominal fat depots relative weights. Liver relative weights did not differ among groups.

Total liver lipids and TAG were significantly lower in MHCa group. No differences were registered in liver total Chol (Table 4).

Discussion

The use of different nutrients, macro or micro, as functional dietary components for the prevention of obesity and associated morbidities has been recommended in recent years and strongly supported by international organizations involved in the promotion of human health.

The inbred line of rats IIMb/Beta is a suitable model for evaluating the effects of different treatments on the expression of its spontaneous obesity and co-morbidities. The spontaneous development of obesity and associated morbidities with the usual AIN'93 diet in this strain of rats makes unnecessary the use of high energy density diets to induce obesity. In contrast, most of the studies concerning calcium effects on parameters related to obesity report the use of high fat, high calorie diets designed to induce the pathology in laboratory rodents[24].

A significant diminution in BW increase and food intake during the experimental period was registered in the MHCa group in coincidence with results reported by others. Food conversion efficiency was significantly lower in MHCa group (Table 2). These results may be justified by the greater bioavailability of Ca from milk compared to Ca from carbonate in the HCa and in AIN diets[25].

Plasma parameters related to obesity as TAG, total Chol, LDL Chol showed a significant diminution in MHCa group compared to the others. The presence of milk in the diet apparently buffers the increase in blood Chol by diminishing the levels of LDL fraction in this group (Table 3). A high level in plasma TAG is a typical feature of the IIMb/Beta line of rats from puberty onwards. Hence this response to MHCa diet represents an important benefit.

The increased fecal fat excretion in the MHCa, can be considered as another contribution of milk –and dairy– to the improvement of obesity and its associated morbidities (Table 2). Fecal fat losses in the form of Ca salts of fatty acids represent an extra energy loss that is not present in the other diets. This effect, due to differences in the physicochemical form of dairy Ca or to cofactors in dairy products has been reported by others[26, 27].

Hepatic lipid content, particularly TAG, showed a diminution in MHCa group compared to the others (Table 4). These findings represent another goal in the study of IIMb/Beta obesity, including the possibility that liver lipid deposit might be modulated by the composition of the diet. An increased deposit of lipids, mainly TAG in liver is being considered as another feature frequently associated to obesity. Recently, a strong association between metabolic syndrome and non alcoholic fatty liver disease (NAFLD) was reported; approximately 30% of NAFLD cases concurrently presented the whole metabolic syndrome, whereas 90% presented at least one of its features[28].

There are studies that conclude that there is a greater enhancement in weight loss when dairy foods are consumed compared to Ca supplements, indicating that compounds present in dairy products also contribute to weight loss[29, 30]. Other studies, in a Chinese population[31] and also in rats and mice[16], demonstrated no effects of calcium mineral supplements on body weight or composition. The additional effect of dairy products could be related to the angiotensin converting enzyme inhibitory activity of dairy or to the rich concentration of branched amino acids and /or bioactive peptides present in dairy whey protein.

In conclusion, in these spontaneously obese rats, a high dietary Ca level from skimmed milk and not the mineral Ca supplement produced significant diminutions in BW increase and smaller fat depots as well as attenuation in plasma lipid levels, particularly TAG.

The fact that in the MHCa group total liver lipids, especially TAG, were significantly lower than in HCa and AIN, represents a new and interesting approach to the study of fatty liver as another component of the obesity syndrome developed by these rats.

Further studies are needed to explain the mechanisms involved in the increase of fecal fat losses induced by dairy Ca and its importance on BW control.

Moreover, the bioactive components of dairy products, most probably part of the milk whey fraction, that would play an additional role in the "antiobesity" effect remain to be elucidated.

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Conflict of interest: The authors declare that they have no conflict of interest.

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One of the first duties of the physician is to educate the masses not to take medicine.

Uno de los primeros deberes del médico es educar a las masas a no tomar remedios.

Sir William Osler (1849-1919)

En: *http://www.oslersymposia.org/about-Sir-William-Osler.html*