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Original / *Síndrome metabólico*

Reduction of serum advanced glycation end-products with a low calorie Mediterranean diet

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Abstract

Dietary intake of advanced glycation end-products (AGEs) increases circulating and tissue levels of these substances, contributing to a state of increased oxidative stress and inflammation. A low dietary AGE intervention has been shown to reduce body AGE content. Mediterranean diets (MD) are theoretically considered low in AGEs, but the specific effects of a MD on AGEs serum levels has not been tested.

Methodology: forty-seven overweight and obese premenopausal women underwent a three-month calorie restriction treatment (20 kcal / kg initial weight) with a Mediterranean-type diet that excluded wine intake. The adherence to the MD was assessed before and at the end of treatment using an on-line questionnaire, which scores from 0 to 14 (minimal to maximal adherence). Body composition, insulin resistance, lipoproteins and carboxymethyl-lisine (CML) serum levels were measured at both time periods. Serum CML was assessed through ELISA (enzyme-linked immunosorbent assay). Compliance to calorie restriction was assessed according to weight loss (< or > 5 % initial weight).

Results: mean body weight, body fat, waist circumference, total cholesterol, triglycerides and serum CML fell significantly, together with an increase in the Mediterranean score, although none of the patients reached the highest score. Significant changes in CML and insulin resistance were observed in 17 women classified as compliant to caloric restriction, but not in the 27 participants who were considered adherent to the MD (according to improvement of the Mediterranean Score).

Conclusions: CML serum levels can be reduced through calorie restricted - Mediterranean-type diet. We could not reach a high enough MD score, so we cannot

REDUCCIÓN SÉRICA DE PRODUCTOS FINALES DE GLICACIÓN AVANZADA SÉRICOS CON UNA DIETA BAJA EN CALORÍAS TIPO MEDITERRÁNEO

Resumen

La ingesta dietaria de productos finales de glicación avanzada (AGEs) aumenta los niveles séricos y tisulares de estas sustancias, lo que contribuye a un estado de mayor estrés oxidativo e inflamación. Una intervención dietaria con bajo contenido de AGEs ha demostrado reducir el contenido de AGEs en el cuerpo. La dieta mediterránea (DM) se considera teóricamente baja en AGEs, pero los efectos específicos de este tipo de intervención en los niveles séricos de AGEs no ha sido probado.

Metodología: cuarenta y siete mujeres premenopáusicas con sobrepeso u obesidad se sometieron a tres meses de restricción calórica (20 kcal por kg de peso corporal inicial) con una dieta de tipo mediterráneo que excluía la ingesta de vino. La adherencia a la DM se evaluó al comienzo y al final del tratamiento utilizando una encuesta *on-line*, con puntuaciones de 0 a 14 (mínima a máxima adherencia a la DM). La composición corporal, la resistencia a la insulina, los niveles séricos de lipoproteínas y carboximetil-lisina (CML) se midieron en ambos períodos. El CML sérico se evaluó mediante ELISA (ensayo inmunoenzimático). La adherencia a la restricción calórica se evaluó de acuerdo con la pérdida de peso (< o > 5% del peso inicial).

Resultados: la media de peso corporal, grasa corporal, circunferencia de la cintura, colesterol total, triglicéridos y CML sérica disminuyeron significativamente, junto con un aumento en el puntaje de adherencia a la DM, aunque ninguno de los pacientes alcanzó la máxima puntuación. Hubo cambios significativos en los niveles de CML y de resistencia a la insulina en 17 mujeres clasificadas como adherentes a la restricción calórica, pero no en las 27 participantes que fueron consideradas adherentes a la DM (de acuerdo con la mejoría en el puntaje de la encuesta).

Conclusiones: los niveles séricos de CML disminuyeron tras la restricción calórica con una dieta tipo mediterránea. Dado que no se pudo alcanzar la puntuación máxima

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conclude whether the MD itself has an additive effect to caloric restriction.

Abbreviations

MD: Mediterranean Diet.
AGEs: Advanced Glycation End-products.
CML: Carboxymethyl-lisine.
ELISA: Enzyme-linked immunosorbent assay.

Introduction

Since Keys et. al. described a lower cardiovascular risk in certain Mediterranean countries[1], attributing the lesser mortality mainly to the ratio of monounsaturated to saturated fatty acids[2], numerous evidence has gathered respect to the beneficial cardiovascular effect of the Mediterranean Diet (MD). These original observations have been confirmed by epidemiological studies[3] as well as large primary prevention trials such as the PREDIMED[4]. It is worth mentioning that MD implies several food patterns and other behavioral and cultural traditions[5,6] and was declared as Intangible Cultural Heritage of Humanity by UNESCO. The traditional Chilean diet can be considered as a Mediterranean-type diet[7], but in the last 20 years a variety of processed foods described as "the western diet" have invaded Chilean kitchens, in association with an increased prevalence of obesity and secondary comorbidities, mainly diabetes and cancer[8], threatening young women in particular[9].

A great deal of research has aimed to study the role of specific nutrients contained in the MD and what are the mechanisms involved in disease prevention. Antioxidant vitamins, resveratrol and red wine polyphenols have been emphasized, due to their known antioxidant effects[10,11] and improvement of vascular reactivity[12].

High dietary intake of advanced glycation end-products (AGEs) has been associated with metabolic derangements, oxidative stress and inflammation[13], which can be successfully managed by simple changes in cooking methods[14]. Overall, the low AGE diet is similar to the MD, but less restrictive in the sense that all kind of meats and animal products are allowed as long as they are prepared under conditions of high humidity and lower heat[15,16]. MD involves patterns of food consumption rather than specific foods and recipes. These dietary patterns can be assessed through scores that rate weekly consumption of specific food groups, mainly non-refined cereals (whole grain bread and pasta, brown rice, etc), fruit, vegetables, legumes, potatoes, fish, meat and meat products, poultry, full fat dairy products (like cheese, yoghurt, milk), as well as olive oil and alcohol intake[17], according to the Mediterranean Pyramid[18].

Although traditional Chilean cooking used to be healthy, we have previously demonstrated that among adult Chileans, especially the younger ones dietary recalls show elevated ingestion of AGEs[19], contributing to the obesity epidemics.

The aim of the current study was to analyze the effects of a low calorie dietary intervention, following MD food recommendations, in young premenopausal overweight or obese women. We specifically assessed the effect of the intervention on body composition, metabolic variables, and serum levels of carboxymethyllisine (CML).

Methodology

Fifty premenopausal women aged between 25 and 45 years with a BMI between 25 and 32 kg/m^2 (overweight or obese) and interested in losing weight were initially screened at our Clinical Research Center (CEDINTA). Exclusion criteria were: diabetes mellitus, hypothyroidism, history of cancer, liver or kidney failure, or pregnancy. The study protocol followed ethical guidelines included in the Declaration of Helsinki and was approved by our local Ethics Committee. Each participant signed an informed consent before beginning the study (registered at www. clinicaltrials.gov number NCT01508091).

At baseline and after a 3-month period of caloric restriction using a Mediterranean-type diet, each participant filled-in an on-line dietary recall called *Aliméntate Sano - Fitbook* (*Healthy Feeding - Fitbook*) designed by Chilean investigators[20], which estimates adherence to a Mediterranean-type diet, through 14 items. Intake of vegetables (except potatoes), fruits, legumes, seeds and dried fruits, whole grain cereals, lean meats, seafood, low fat and fermented dairy products, vegetable oils (olive, canola or avocado), and moderate wine intake at meal times are classified as healthy (higher scores), while fatty or processed meats, high fat or non-fermented milk, sugar and excessive wine or no wine ingestion are considered non-healthy. Each item is scored as 0, 0.5 or 1, considering habitual intake and whether its quality is considered as beneficial or unhealthy. Final scores range from 0 (lack of adherence to MD) to 14 (maximal adherence).

In addition, each participant underwent anthropometric measurements (height, weight, waist and hip circumferences) and body composition measurements (total, bone, fat and lean body mass) using a Lunar Encore double beam densitometer (DEXA), at baseline and at completion of the study.

Also at baseline and after the intervention, fasting blood samples were withdrawn for measurement of glucose, insulin, thyroid hormones and lipoproteins and an oral glucose tolerance test (OGTT), quantifying glucose and insulin levels 60, 90 and 120 min following a 75 g glucose challenge. Insulin resistance was evaluated by calculating the index of Matsuda[21] and the Homeostasis Model Assessment (HOMA-IR)[22]. Both serum glucose and insulin were measured by Roche kits.

Serum samples were tested for εN-carboxymethyllysine (CML) by enzyme-linked immunosorbent assay (ELISA), in a competitive assay, according to Makita et al[23] with some minor modifications. Briefly, 96-well ELISA plates (EIA/RIA plate, Costar, Cambridge, MA) were coated with 100uL/well of 3ug/mL CML-BSA (Cell Biolabs, USA) in coating buffer (0.1 M sodium bicarbonate, pH 9.6) overnight at 4°C. Wells were washed three times with 150ul washing buffer (PBS 0.05% Tween-20 and 1 mM NaN3), then blocked with 100ul blocking buffer (PBS 1% BSA) for 1h. After three rinses with washing buffer, 50ul of sample was added, followed by 50ul of anti-CML antibody 1:2000 (Ab3097, Abcam, USA) in blocking buffer and were incubated at room temperature for 2h with gentle agitation on a horizontal rotary shaker. Wells were then rinsed and the secondary antibody diluted 1:4000 in blocking buffer was then added to each well and the plates were incubated at 37°C for 1 h. After rinsing with washing buffer, 150ul pNPP substrate (Sigma, USA) was added to each well. Optical density at 405nm was determined by an ELISA reader (Start Fax 4200, Awardness technology) after 15 to 30 min. The intra-assay variation was ±2.4% and the inter-assay variation ±4.6%.

The intervention consisted in the prescription of a low calorie Mediterranean Diet (MD) (20 Kcal per kg initial body weight), with 5 daily meals, during 3 months. Distribution of macronutrients was 50 % carbohydrates, 25- 30 % lipids and 20 to 25 % protein (at least 1 g per kg initial body weight). One of the meals was provided by the research team, including dried fruits (175 Kcals/serving of 30 g), cereal bars (69 Kcals/serving of 20 g) or whole grain cookies (153 Kcals/serving of 3 cookies), to be ingested from one to three times per week, in order to improve compliance with dietary recommendations. Specific instructions were cutting out ingestion of pastry, sugared beverages, red meats, full fat dairy products, white bread, potatoes, rice and pasta, while stimulating intake of fish, poultry, vegetables and whole grain cereals in adequate portions. Patients were followed weekly by the research dietitian, to record weight changes and discuss meal alternatives when adherence was less than appropriate, reinforcing the selection of less caloric and more Mediterranean types of foods and ensuring adequate protein intake. No specific indications concerning cooking methods were advised. Patients were recommended to follow healthy lifestyles including increased physical activity, but this was not an active part of the intervention.

Compliance with the diet was not assessed by dietary recalls due to lack of precision and habitual underreporting[24,25], but instead according to changes in body weight defining compliant participants as those who achieved a weight reduction of > 5 % initial weight and non-compliant as those who did not achieve this goal. Adherence to the Mediterranean food selection was evaluated by the *Healthy Feeding – Fitbook* Score, considering both the total score and comparing baseline to final scores.

Results

Fifty premenopausal women were included in this study, aged 33 ± 5 years; three dropped out due to personal problems, so 47 were available for final analysis. Initial body mass index (BMI) ranged from 24.8 to 32.5 kg/m^2, while percentage of body fat fluctuated between 34 and 49 %. Table I depicts anthropometric and body composition measurements by DEXA at baseline and after the 3-months dietary intervention. Table II shows the changes in laboratory variables before and after the treatment period, highlighting a significant fall in CML, total cholesterol and triglyceride serum levels. Regarding parameters that define the metabolic syndrome at baseline, two participants had altered fasting glucose (fasting glucose ≥ 100 mg/dL), six were glucose intolerant (120 min serum glucose ≥ 140 mg/dL), nine were hypertriglyceridemic (serum TG > 150 mg/dL), blood pressure was elevated (> 135/85 mm Hg) in two participants and waist circumference was > 88 cm in 40. Nine participants had three or more criteria for diagnosis of the Metabolic Syndrome before the intervention and five at completion of the study.

The Mediterranean Diet Score at baseline averaged 4.98 ± 2, oscillating between 1 and 10. After the dietary intervention it improved significantly, reaching 8.17 ± 1.6 (p < 0.01) ranging from 4.5 – 11 points, but none reached 14 points. The change in this score was significantly correlated with the fall of cholesterol levels (Spearman's rho = - 0.3, p =0.045) but was not associated with changes in CML or triglyceride concentrations. Figure 1 depicts non significant changes in CML serum levels according to improvement (more than 2 points) or no improvement in the Mediterranean Score (less than 2). The analysis was repeated considering a higher improvement of the score (> or < 4 points), again observing a comparable unsignificant decrease of serum CML in both groups (data not shown).

Table I
Anthropometric data before and after treatment

	Baseline (n = 47)	After diet (n = 47)	P
Weight (kg)	72.2 ± 6.9	69.2 ± 6.8	**< 0.001**
Height (kg)	160.3 ± 6.0	160.3 ± 6.0	
BMI (kg/mt²)	28.1 ± 2.0	26.9 ± 2.2	**< 0.001**
Waist (cm)	94.5 ± 6.1	90.0 ± 6.5	**< 0.001**
Hip (cm)	104.5 ± 5.3	102.5 ± 5.2	**< 0.001**
Total body fat (kg)	30.1 ± 4.5	28.2 ± 4.7	**< 0.001**
Trunk fat (kg)	15.8 ± 2.7	14.5 ± 2.8	**< 0.001**
Fat mass index (kg/mt²)	11.7 ± 1.7	11. 0 ± 1.8	**< 0.001**
Total lean mass (k)	39.2 ± 3.7	38.6 ± 3.7	**0.002**
Bone mineral content (kg)	2.4 ± 0.2	2.4 ± 0.2	0.477

Table II
Laboratory variables before and after treatment

|	Baseline (n = 47)	After diet (n = 47)	P
Carboxymethyl-lisine (ug/ml)	0.6 (0.1 – 1.3)	0.4 (0.1 - 1.4)	**0.0001**
Fasting serum glucose (mg/dl)	86.9 ± 1.3	89.2 ± 1.3	0.188
120 Min serum glucose (mg/dl)	99 (38-202)	106 (33 – 176)	0.475
Fasting serum insulin (u/dl)	8.6 (1.8 – 30.2)	7.6 (1.7 -51.5)	0.844
120 Min serum insulin (u/dl)	56.5 (4.6 – 252.6)	48.6 (4.1 – 203.9)	0.247
HOMA-IR	1.7 (0.4 – 6.4)	1.6 (0.3 - 12.8)	0.589
Total cholesterol (mg/dl)	184 ± 37	173 ± 32	**0.010**
HDL cholesterol (mg/dl)	58 (36 – 79)	52 (34 - 91)	0.233
Triglycerydes (mg/dl)	96 (49 – 409)	87 (42 – 299)	**0.021**
Serum creatinine (g/dl)	0.72 ± 0.12	0.73 ± 0.09	0.511
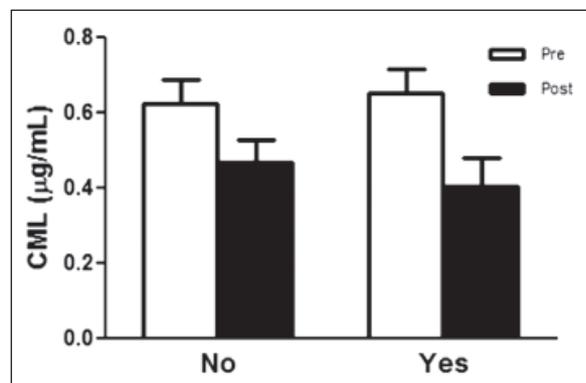
Fig. 1.–CML serum levels according to improvement of the mediterranean score.
Non significant serum levels CML (mean + SD) before (white) and after (black) dietary treatment, according to changes of the Mediterranean Score (NO : < 2 points improvement after the intervention, YES : > 2 points improvement after the intervention).
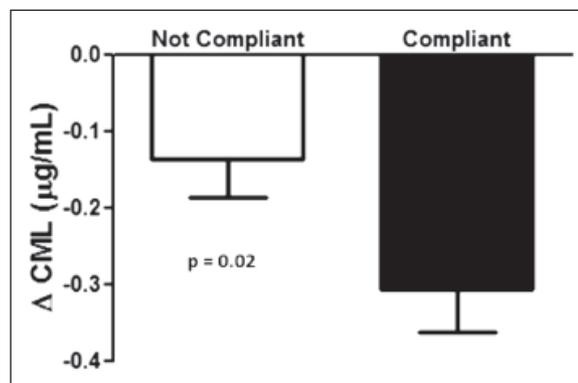

Fig. 2.–Delta CML serum levels according to compliance to caloric restriction.
Changes in CML serum levels according to compliance with the intervention. Not Compliant (white bar): lost < 5 % body weight versus Compliant (black bar): Lost > 5 % body weight; p = 0.02.

At baseline, CML serum levels did not correlate with BMI, waist circumference or body fat. After 3 months, 17 participants were compliant (loss of more than 5% of initial body weight) and 30 were non-compliant. Table III depicts changes in laboratory variables comparing both groups. There were significantly higher reductions in CML and HOMA-IR among compliant participants. Changes in CML serum levels were significantly associated with changes in fasting glucose (Spearman rho = 0.3 p = 0.03), but not with changes in serum lipids, Mediterranean Score, HOMA-IR, BMI nor body fat.

Discussion

In the current study we observed that reducing caloric intake and stimulating preference for foods contained in Mediterranean type diets, except for wine, positively influenced body fat, serum CML and lipoproteins in overweighed to obese premenopausal women.

In this trial we recommended energy restriction while simultaneously suggesting Mediterranean foods (such as legumes, whole grain cereals, fish, fermented milk and olive oil), establishing criteria of compliance to these two components. Adherence to energy restriction was assessed by % weight loss, and adherence to the Mediterranean diet was verified by changes in a previously validated score. This type of energy restriction is different from the regular low fat diet traditionally recommended for the treatment of obesity, because of its higher content in fat, specifically monounsaturated fatty acids contained in olive oil, and also emphasis in certain groups of foods. Our results indicate that the Mediterranean Score increased significantly, but did not reach 13 or 14 as expected, so compliance in this respect was far from perfect. Actually, in spite of weekly sessions with the dietitian, the Mediterranean Scores obtained were comparable to that of the control group in the PREDI-MED study[4]. A possible explanation is that patients did not consume all the foods recommended in this type of diet due to its price, because in spite of local production, in Chile olive oil and fish are very expensive, thus less ingested by the general population.

Serum CML levels fell independently of changes in the *Healthy Feeding - Fitbook Score*. A potential explanation for this lack of association is that MD questionnaires do not take into account cooking methods and consumption of processed foods as specific dietary recalls designed to quantify AGEs intake[16].

Since compliance with the MD was less than expected, we could not evaluate its individual effects over the measured variables. In contrast, adherence to calorie restriction was assessed by changes in body weight, the best indicator of energy balance, more reliable than dietary recalls[26], and our results suggest that compliance with this intervention was responsible for the reduction of serum CML. Even though we did not specifically prescribe a low AGE diet by overseeing cooking methods, the low calorie diet necessarily implied reducing intake of pastry, processed and fried red fatty meats, cheese, and other greasy snacks while simultaneously decreasing portions of most meals except for vegetables, indirectly reducing intake of AGEs. Comparable results were found by Gugliucci et. al.[32] in a 2 month reduced calorie diet in overweight/obese Japanese men and women, observing a 7.2 % decrease of fluorescent serum AGEs. Other interventions have aimed to reduce circulating AGEs using dietary recommendations directed to reducing AGEs intake[16], observing weight maintenance[27] or weight loss[28], depending on associated calorie restriction. In our study we observed a 39 % decrease of serum CML in the whole sample, which was higher among compliant women (53 versus 30 %, p = 0.017).

Western-type diets contain elevated quantities of AGEs[29], which can be absorbed in the digestive tract causing an increase of serum and tissue levels, with adverse consequences on health[13,27]. This study confirms that it is possible to reduce circulating levels of these substances by decreasing caloric intake globally. A low AGE diet has already been shown to reduce circulating AGE levels[14], and the effects of calorie restriction plus low AGEs intake have been recently demonstrated in

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Table III
Changes in laboratory variables comparing compliant versus non-compliant women*

	Compliant (n = 17)	Non-compliant (n= 30)	P
Delta carboxymethyl-lisine (ug/ml)	-0.35 (-0.6 - 0.1)	-0.10 (-0.8 - 0.2)	**0.020**
Delta fasting serum glucose (mg/dl)	-4.0 (-21 – 22)	4.3 (-24 – 23)	0.124
Delta fasting serum insulin (u/dl)	-1.0 (-6.7 - 6.2)	0.8 (-15.9 – 37)	0.068
Delta homa-ir	- 0.2 (-1.6 - 1.3	0.3 (-3.1 - 9.5)	**0.039**
Delta total cholesterol (mg/dl)	-171.2 ± 41.2	-175.3 ± 48.4	0.773
Delta hdl cholesterol (mg/dl)	-1.82 ± 9.0	-0.76 ± 8.6	0.692
Delta tryglycerides (mg/dl)	-23 (-110 – 86)	-6 (-95 – 90)	0.382

*Compliance to caloric restriction was classified according to loss of > 5 % body weight (compliant) or < 5 % loss (non-compliant).

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Mexican obese men[28]. This study adds information on the effects of a Mediterranean type of calorie restriction, without intentionally trying to lower AGEs intake in young overweight/obese Chilean women. In our earlier studies we have detected high AGEs intake (mainly in breakfast cereals, sweet snacks, powder milk, and melted cheese) especially among younger people, as the present sample[19]. We have measured the content of CML and other AGEs in the most frequently consumed foodstuffs (unpublished data), confirming that our progressively obese population is exposed to an elevated dietary intake of these glycotoxins. The Mediterranean diet protects from development of cardiovascular diseases and diabetes[30], and can be considered as a low-AGE diet[16]. Although MD is a form of low AGE diet, not every low AGE diet is Mediterranean. In the present intervention we made sure that patients consumed at least 1 g/kg protein, specially from animal origin such as eggs, dairy products, fish and white meats, to prevent from loss of lean body mass, so if these were cooked at high temperatures or be provided as processed foods (powder milk or canned sea products) their content in AGEs could be elevated. Respecting the MD component of our diet, it is noteworthy that adoption of MD in western countries is usually assessed through semiquantitative scores derived from dietary recalls or filled in by the patient as in this study, but these methods have been highly questioned recently[24,25]. Moreover, the Mediterranean Pyramid has been modified several times[31], and requires locally defined portions to assess food patterns, so the method we employed in this study although debatable, is very similar to other published Mediterranean Scores. Adherence to other kinds of low-calorie interventions can also lower the intake of glycotoxins and serum AGEs simply due to reduction in nutrient intake[32].

In conclusion, a significant reduction of CML serum levels was achieved by effectively reducing calorie intake while simultaneously favoring Mediterranean nutrients, without specifically indicating to lower ingestion of foods containing elevated amounts of AGEs. Dietary recalls are inadequate to track the precise alimentary selections of each patient, but according to the attained *Fitbook* scores, our patients did not follow all our indications, so we could not address the separate contribution of the Mediterranean Diet on AGEs serum levels.

Acknowledgements

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