Acute dairy milk ingestion does not improve nitric oxide-dependent vasodilation in the cutaneous microcirculation

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Abstract

In epidemiological studies, chronic dairy milk consumption is associated with improved vascular health and reduced age-related increases in blood pressure. Although milk protein supplementation augments conduit artery flow-mediated dilation, whether or not acute dairy milk intake may improve microvascular function remains unclear. We hypothesised that dairy milk would increase direct measurement of endothelial nitric oxide (NO)-dependent cutaneous vasodilation in response to local skin heating. Eleven men and women (61 (SEM 2) years) ingested two or four servings (473 and 946 ml) of 1 % dairy milk or a rice beverage on each of 4 separate study days. In a subset of five subjects, an additional protocol was completed after 473 ml of water ingestion. Once a stable blood flow occurred, 15 mM-N^G-nitro-L-arginine methyl ester was perfused (intradermal microdialysis) to quantify NO-dependent vasodilation. Red-blood-cell flux (RBF) was measured by laser-Doppler flowmetry, and cutaneous vascular conductance (CVC = RBF/mean arterial pressure) was calculated and normalised to maximum (%CVC$_{max}$; 28 mM-sodium nitroprusside). Full expression of cutaneous vasodilation was not different among dairy milk, rice beverage and water, and there was no effect of serving size on the total vasodilatory response. Contrary to our hypothesis, NO-dependent vasodilation was lower for dairy milk than rice beverage (D: 49 (SEM 5), R: 55 (SEM 5) %CVC$_{max}$; $P < 0.01$). Acute dairy milk ingestion does not augment NO-dependent vasodilation in the cutaneous microcirculation compared with a rice beverage control.

Key words: Dairy milk: Insulin: Microcirculation: Nitric oxide: Vasodilation

CVD is the leading cause of mortality in the USA and is responsible for approximately 17 % of national healthcare expenditures[1]. As such, effective non-pharmacological prevention strategies and early identification of modifiable lifestyle risk factors (e.g. dietary habits) are essential to limit the growing burden of CVD. Bioactive micronutrients and macronutrients in particular may have a preventive and protective effect against disease. Chronic dairy milk consumption is associated with attenuated age-related increases in blood pressure and improved cardiovascular outcomes. Increased dairy milk intake has positive effects on blood pressure[2–6] and measures of conduit-vessel function including pulse-wave velocity[7], and arterial stiffness[8]. However, little is known about the mechanisms by which dairy milk consumption may improve vessel function specifically at the level of the microcirculation.

The putative mechanisms mediating improvements in vascular function induced by dairy milk intake are probably complex and may involve the synergistic effects of milk proteins and elemental components. *In vitro* data indicate that bioactive peptides derived from the two primary milk proteins, whey and casein, exhibit angiotensin-converting enzyme (ACE) inhibitor properties[9–12] and direct antioxidant and radical scavenging activity[13–15]. In humans, chronic consumption of these peptides (1–10 weeks) decreases measures of systemic inflammation (IL-6, monocyte chemoattractant protein 1 and TNFα)[16–18], and the mineral composition in dairy milk (Ca, K and Mg) moderately reduces blood pressure[19,20]. The common vascular signalling pathway that links each of these purported mechanisms (angiotensin II inhibition, antioxidant properties, anti-inflammatory, etc.) is through increasing nitric oxide (NO) bioavailability. NO is a potent vasoprotective agent produced by the vascular endothelium and is essential for vessel health and function. Reduced NO bioavailability is prevalent in all cases of cardiovascular dysfunction, and precedes the onset of clinically detectable CVD[21–23]. Work conducted in our laboratory and in those of others has demonstrated a reduced NO-dependent vasodilation during local heating in healthy middle-aged[24] and older adults[25]. Further, the NO component of the local heating response provides a quantitative assessment of the magnitude of improvements in vascular function when examining the effects of interventions[26,27]. Therefore, we would expect our cohort of adults (55–75 years) to have age-related deficits in NO-dependent vasodilation that may be improved by the proposed dietary intervention.

The cutaneous circulation is an accessible and representative circulation for the *in vivo* study of mechanisms mediating vascular function and dysfunction in humans[28–30]. Deficits in cutaneous function are highly correlated with measures of vessel dysfunction in the coronary and renal circulations[31,32]. Moreover, altered cutaneous microvascular function is evident before long-term changes in blood pressure or presentation of clinical symptoms[33]. As such, the cutaneous vascular bed has utility for the *in vivo* examination of molecular mechanisms by which intervention strategies may affect vessel function in humans. The cutaneous circulation has been previously used to study microvascular responses to several nutritional interventions[34–38], including acute oral micronutrient ingestion[36].

Given the epidemiological evidence that increased dairy milk consumption reduces CVD risk across the lifespan[39,40] and the evidence that the mechanisms associated with this decrease converge on the NO pathway, the aim of this study was to determine the mechanistic effect of acute milk consumption on cutaneous microvascular function in healthy older adults (55–75 years). We hypothesised that acute dairy milk consumption (two and four servings) would increase NO-dependent vasodilation in a dose-dependent manner compared with a non-dairy rice beverage control.

Methods

Subjects

All protocols were approved by the Institutional Review Board at The Pennsylvania State University and complied with the guidelines of the Declaration of Helsinki. All participants voluntarily provided written and verbal consent before the experiment. Eleven subjects (61 (SEM 2) years; five men, six women) participated in the study. Before participation, subjects underwent a medical screening that included a twelve-lead electrocardiogram, fasting blood chemistry and physical examination. Subjects also completed a 24-h ambulatory blood pressure monitoring while enrolled in the study. Inclusion criteria required a daily dairy milk intake of less than two servings. Daily dairy milk intake was assessed with a modified FFQ specific to dairy milk consumption. Subjects had a 2-d wash-in period of no dairy milk consumption and abstained from alcoholic and caffeinated beverages for 12 h, vigorous physical activity for 24 h and food for 8 h before each experiment. All subjects were non-smokers, non-diabetic, non-obese (BMI < 30 kg/m^2) and were not on any prescription medications that may alter vascular function (e.g. statins, antidepressants, antihypertensives, etc.). Women taking hormone replacement therapy were excluded from the study.

Experimental protocol

On four separate visits, subjects ingested 473 ml (two servings) of dairy milk (1 % fat; Giant Eagle), 946 ml (four servings) of 1 % fat dairy milk, 473 ml of a rice beverage (Nature's Promise Original Enriched) or 946 ml of rice beverage with a minimum of 1 week between visits. Although consideration was given to other milk alternatives, we chose a rice beverage as the control

beverage given its similarity to dairy milk in nutrient and energy content, as well as the absence of additional ingredients (e.g. soya) that may affect vascular function. A subset (*n* 5) of subjects also ingested 473 ml of water on an additional visit. The water trial was included to serve as an iso-volumetric, fasted reference value and was not included in the statistical analysis. The treatment order was randomly assigned for each subject. The energy, macronutrient and micronutrient contents of each treatment are displayed in Table 1.

All experiments were performed in a thermoneutral environment with subjects in a semi-supine position. Using a sterile technique, one intradermal microdialysis fibre (10 mm, 30-kDa membrane limit, MD 2000; Bioanalytical Systems) was placed in the ventral forearm skin. Ice was applied to the forearm for 5 min to anesthetise the skin before fibre placement. A 25-G needle was inserted into the skin with entry and exit points 2–3 cm apart. The fibre was threaded through the needle, which was subsequently removed leaving the semipermeable portion of the fibre remaining under the skin. Red-blood-cell flux (RBF), an index of skin blood flow, was measured by a laser-Doppler flowmetry probe placed in a local heating unit (MoorLab, Temperature Monitor SH02; Moor Instruments) directly over the microdialysis membrane. Brachial artery blood pressure was recorded at 5-min intervals (Cardiocap; GE Healthcare) throughout the protocol.

Localised microdialysis pharmaceutical perfusates were dissolved in lactated Ringer's just before use, microfiltered (Acrodisc; Pall) and covered in foil to prevent light degradation. Before baseline data collection, the fibre was perfused (2 µl/min) with lactated Ringer's for 60–90 min to allow the skin to recover from any trauma caused by the insertion of the microdialysis fibre. Subjects ingested the selected milk treatment after 30 min of hyperaemia. This time point for consumption was chosen so that the local heating plateau would occur 60–90 min post treatment, which is the time period after milk peptide ingestion when peak intestinal concentrations of bioactive peptides are recovered[41].

Subjects were instrumented with an intravenous catheter after fibre placement for the collection of blood samples. A fasted

Table 1. Micronutrient and macronutrient content of two and four servings of the 1 % fat dairy milk and rice beverage

	Per 2 dairy serving equivalents		Per 4 dairy serving equivalents	
	1 % milk	Rice beverage	1 % milk	Rice beverage
Serving size (ml)	473	473	946	946
Energy (kJ)	920	837	1841	1674
Energy (kcal)	220	200	440	400
Fat (g)	5	4	10	8
SFA (g)	3	0	6	0
Cholesterol (mg)	30	0	60	0
Protein (g)	16	0	32	0
Carbohydrates (g)	24	46	48	92
Sugars (g)	24	30	48	60
Fibre (g)	0	0	0	0
Na (mg)	260	180	520	360
Ca (mg)	600	600	1200	1200
K (mg)	740	0	1480	0

blood sample was taken before treatment administration. Blood samples were then taken every 30 min post treatment until the completion of the study. The blood samples were collected in EDTA-treated tubes, which were subsequently refrigerated and centrifuged. The plasma samples were stored at –80°C until future use. Plasma insulin concentrations were measured at baseline and at 90 min post ingestion using a commercially available ELISA (Mercodia) according to the manufacturer's instructions. Samples were analysed in duplicate with an average CV < 10 %.

Baseline measurements were collected for 20 min at a local skin temperature of 33°C. After a stable baseline period, the local skin temperature was increased to 42°C at a rate of 0·5°C every 5 s. Once a 10-min plateau was reached (approximately 40 min), the site was perfused with 15 mM N^G-nitro-L-arginine methyl ester (L-NAME; Calbiochem), a non-specific nitric oxide synthase (NOS) inhibitor, at a rate of 4 µl/min to quantify NO-dependent vasodilation[24,42,43]. After 10 min of stable RBF measurements (approximately 45 min), maximal vasodilation was induced by perfusing the fibre with 28 mM-sodium nitroprusside (USP) and increasing the local skin temperature to 43°C (30 min). Work conducted in our laboratory and in those of others has demonstrated that this protocol is highly specific to endothelial nitric oxide synthase (eNOS) production and allows the direct quantification of functional NO-dependent vasodilation in the cutaneous microcirculation[24,44,45].

Data acquisition and statistical analysis

Data were collected with Windaq (DATAQ Instruments) at a frequency of 40 Hz. Cutaneous vascular conductance (CVC) was calculated as RBF divided by mean arterial pressure. Data were normalised to a per cent of maximum CVC. CVC data were averaged over a stable 5-min period at baseline, the local heating plateau, the L-NAME plateau and maximum vasodilation. NO-dependent vasodilation was calculated as the difference between CVC at the local heating plateau and CVC at the post L-NAME plateau and expressed as a percentage of maximum. A three-way repeated-measures ANOVA was used to detect within-subject effects of dietary treatment and serving size on the phases of the local heating response. There was no main effect of serving size on functional vascular measures, and thus the data for the 473- and 946-ml servings were combined and a nested two-way repeated-measures ANOVA was performed to detect differences between dietary treatment on the parameters of the local heating response (version 9.4; SAS). Bonferroni *post hoc* corrections were performed to account for multiple comparisons when necessary. Significance was accepted using $\alpha = 0.05$. Unless otherwise indicated, all values are presented as means and standard errors.

Results

Subject characteristics are displayed in Table 2.

Fig. 1 depicts an original record of the response to skin local heating.

There were no differences in the local heating plateau between the dairy milk and rice beverage treatments (Fig. 2).

Table 2. Subject characteristics
(Mean values with their standard errors; *n* 11; 5 male, 6 female)

	Mean	SEM
Age (years)	61	2
BMI (kg/m^2)	26·1	0·6
DBP (mmHg)	74	2
SBP (mmHg)	125	5
HDL (mmol/l)	1·6	0·2
LDL (mmol/l)	3·2	0·3
Total cholesterol (mmol/l)	5·1	0·3
Fasting glucose (mmol/l)	5·3	0·2
HbA1c (%)	5·6	0·1

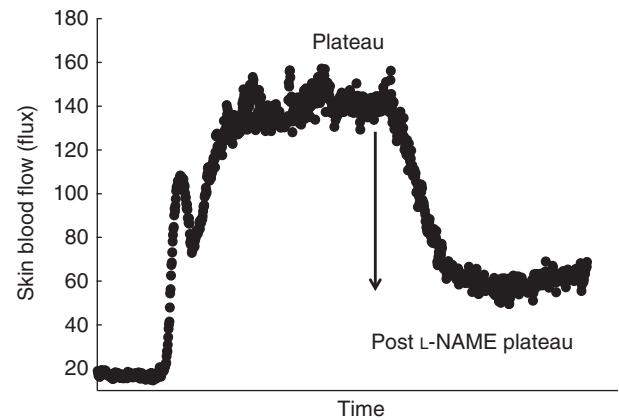DBP, diastolic blood pressure; SBP, systolic blood pressure; HbA1c, glycated Hb.

Fig. 1. Representative tracing of the local heating response in one subject. ↓, Decrease in skin blood flow with nitric oxide synthase inhibition. L-NAME, N^G-nitro-L-arginine methyl ester.

However, the %NO-dependent vasodilation was attenuated following dairy milk ingestion compared with the %NO-dependent vasodilation following rice beverage ingestion (R: 49 (SEM 5), D: 55 (SEM 5) %CVC$_{max}$; $P < 0.01$).

To determine whether the insulin responses contributed to the differences in NO-dependent vasodilation between milk treatments, plasma insulin concentrations were measured using the plasma samples from the 90-min time point, which coincided with the timing of NO quantification during the local heating protocol. The plasma insulin concentration following dairy milk consumption was lower compared with rice beverage consumption for both two and four servings (2D: 84 (SEM 10) pmol/l, 2R: 205 (SEM 20) pmol/l; $P < 0.001$, 4D: 161 (SEM 37) pmol/l, 4R: 311 (SEM 45) pmol/l; $P < 0.001$). The lower plasma insulin concentrations following the dairy milk treatments were associated with decreased NO-dependent vasodilation (Fig. 3).

Discussion

The principal finding of this study was that NO-dependent vasodilation was attenuated following acute dairy milk consumption compared with the rice beverage. Despite the local heating plateau being similar between dairy milk and rice beverage, when NO-dependent vasodilation was directly quantified it was reduced
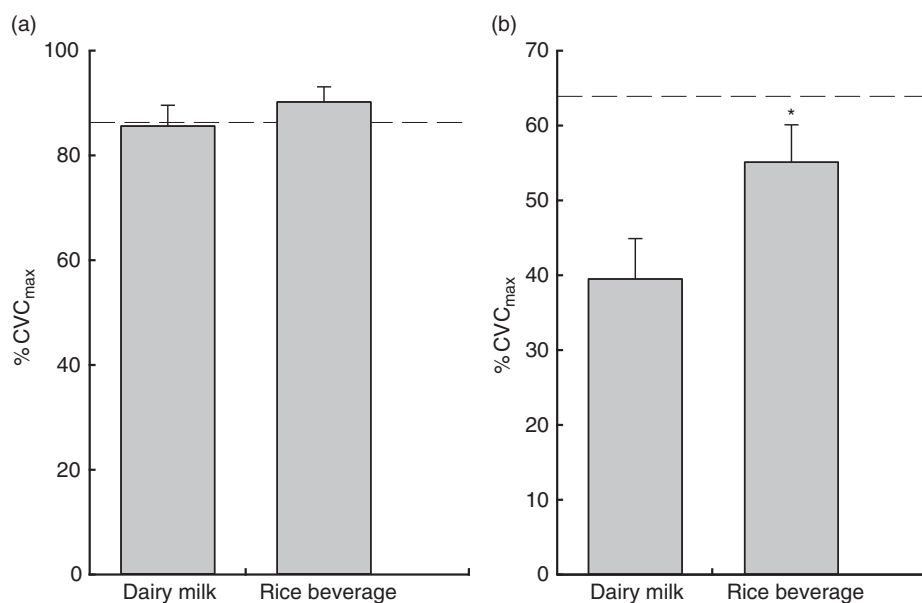
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Fig. 2. (a) Local heating plateau and (b) % nitric oxide (NO)-dependent dilation following dairy milk or rice beverage ingestion. * $P = 0.004$ difference *v.* dairy milk. – – –, Response in five subjects who completed a water (fasted) trial, included for reference. CVC, cutaneous vascular conductance.
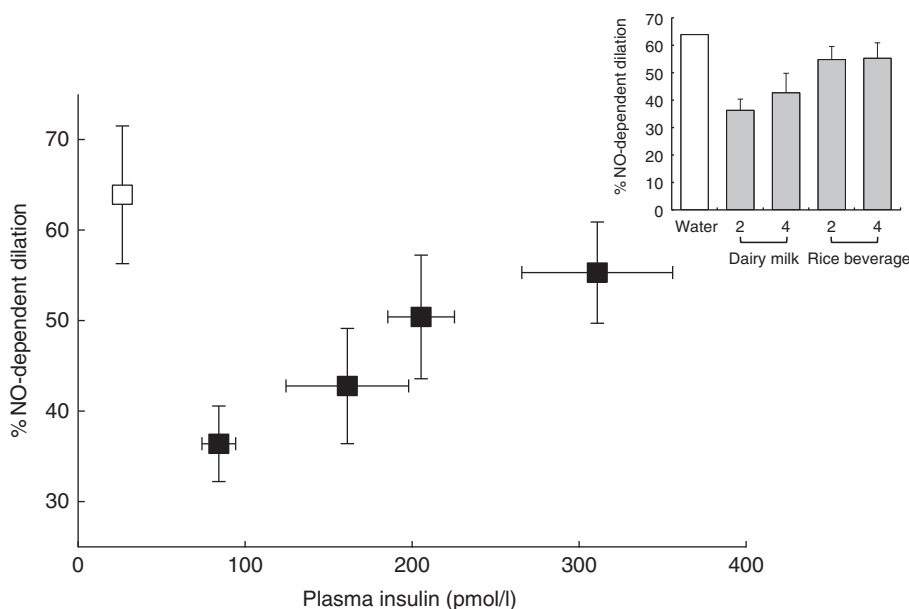

Fig. 3. Plasma insulin response and corresponding % nitric oxide (NO)-dependent dilation 90 min following consumption of two or four servings of dairy milk or rice beverage. The inset depicts the %NO-dependent vasodilation for the dairy milk and rice beverage separated by dose (two servings: 473 ml and four servings: 946 ml). □, Response in five subjects who completed a water (fasted) trial, included for reference.

following dairy milk consumption. Plasma insulin concentrations were also lower following dairy milk ingestion and were associated with decreased NO-dependent vasodilation during local skin heating. Contrary to our hypothesis, these data suggest that acute dairy milk consumption does not augment NO-dependent vasodilation in the cutaneous microcirculation. It is important to note that although it appears that NO-dependent vasodilation following rice beverage consumption is unchanged compared with the fasted (water) trial and

NO-dependent vasodilation following dairy milk consumption is attenuated relative to the fasted state, we cannot determine how these data compare to a normal unfasted state based on our results.

The macronutrient content of the dairy milk and rice beverage differ substantially, which may explain differences observed in the present study. Although the dairy milk and rice beverage match closely in fat content, dairy milk has a lower carbohydrate content and thus a low glycaemic index

compared with the rice beverage[46], and was associated with a smaller insulin plasma response. Insulin induces vasodilation in the cutaneous microcirculation[47–49] through an NO-dependent mechanism[47]. In the present study, plasma insulin concentrations were lower following the dairy milk treatments relative to their respective iso-volumetric rice beverage treatments. Moreover, the lower plasma insulin concentrations following the dairy milk treatments were associated with reduced NO-dependent vasodilation. The low plasma insulin and high NO-dependent vasodilation following water ingestion may appear to conflict with the proposed role of insulin in augmenting NO-dependent vasodilation. However, we expect a low plasma insulin concentration following water ingestion because of the lack of macronutrients in water. At the same time, we expect a high NO-dependent vasodilation following water ingestion because of the absence of a postprandial hyperglycaemic response, which includes increases in oxidant species and thus reductions in NO bioavailability[50].

Dairy milk proteins have a demonstrated positive benefit on vascular function in both animal and human models[16,51–54]. For example, milk peptides in hypertensive rat models show improvements in endothelial-dependent vasodilation, both *in vitro*[51] and following a 6-week supplementation[52], and eNOS expression following 5-d to 6-week supplementations[52,55]. In humans, vascular function, measured by flow-mediated vasodilation in the brachial artery[53,54], and reactive hyperaemia[16,53] are improved after 1–8 weeks of milk peptide ingestion. Reasons for the discrepancy between these findings and the present study include differences in the form of the milk proteins and the acute nature of the intervention. Most studies examining the effects of milk proteins on vascular function have used isolated milk protein hydrolysate[16,52–55] instead of fluid milk or other dairy products. Isolated milk peptides and dairy milk may differ in their resistance to peptidases during digestion and ability to transport across the intestinal wall in an active form. In addition, many of these studies have been chronic interventions (≥1 week)[16,52–54], which may be required to observe differences in vascular function. Ballard *et al.*[56] conducted a study examining acute low-fat milk consumption on vascular function and found that, unlike rice beverage ingestion, dairy milk ingestion maintained endothelial function by reducing the postprandial hyperglycaemia. Differences from the present study include measurement of conduit arterial function, quantified by brachial artery flow-mediated dilation, instead of microvascular function. Moreover, the study by Ballard *et al.* was conducted on individuals with metabolic syndrome, which may also explain the differences between the two studies.

It is important to note that in the present study the overall vasodilation response to local heating was not different between dairy milk and rice beverage treatments, or different compared with the fasted (water) trial. There is a great deal of redundancy in the mechanisms contributing to the cutaneous vasodilator response to local heat[57–60], and our data do not suggest that acute milk ingestion reduces vascular function. Rather, because there was no change of the local heating plateau, the acute exposure to higher insulin concentrations may have modulated the NO contribution to the total vasodilator response following rice beverage ingestion.

One of the strengths of the current study is that we directly quantified functional NO-dependent vasodilation using an eNOS-dependent stimulus[24]. In doing this, we were able to dissect out the amount of vasodilation due to NO with our specific dietary treatments. This is an initial first step in determining the direct acute effects of dairy milk on vascular function. The possibility remains that chronic dairy milk consumption improves endothelial function over a longer time course.

Limitations

Intestinal recovery of milk peptides occurs within 60–90 min of consumption[41], and acute milk consumption has been shown to alter conduit arterial function within 90 min post ingestion[56]. A chronic milk intervention may be appropriate to elucidate the effects of dairy milk intake on microvascular function, as it would allow for sufficient time for the milk peptides to act on the peripheral vasculature and would not be masked by an acute insulin response.

Because the proposed mechanisms by which dairy milk consumption may affect blood vessel function converge on the NO pathway, we focused primarily on NO-dependent mechanisms of microvascular function. However, it is possible that NO-independent mechanisms mediate improvements in vascular function observed with milk peptide consumption. Evidence for contribution from other mechanisms has been documented by increases in resistance vessel blood flow in response to reactive hyperaemia, a measure that is largely independent of NO, after milk peptide consumption[53]. *In vitro* casein-derived tripeptides induce endothelial-dependent dilation, an effect that is reduced with K^+ channel inhibition, NOS inhibition and bradykinin B2 receptor antagonists, suggesting additional roles for other vasodilatory substances including endothelium-derived hyperpolarising factor and bradykinin-mediated release of prostacyclin (PGI_2) in the beneficial vascular effects of milk peptides[61]. However, prostanoids do not contribute to the local heating response in the cutaneous circulation[62]; thus, other methods would be required to assess the potential role of PGI_2 in the vascular effects of dairy milk.

We chose to recruit healthy older individuals for this study because (1) our laboratory has previously shown a moderate age-related deficit in NO-dependent vasodilation in this population[24,25], and (2) this subject group represents a population that would most likely benefit from lifestyle modifications for the prevention of CVD. Other research in this area has focused on populations with overt CVD[63,64]. As such, it is possible that milk consumption has a more pronounced acute treatment effect on endothelial function in individuals with established vascular disease.

Summary

In summary, the local heating-induced vasodilatory response following dairy milk consumption was not different from the response following rice beverage consumption. Contrary to our hypothesis, NO-dependent vasodilation was decreased after dairy milk ingestion compared with rice beverage ingestion.

This finding may be associated with a lower acute insulin response following dairy milk intake. Although dairy milk consumption did not acutely increase NO bioavailability, chronic dairy milk consumption may improve or protect endothelial function given the potential ACE-inhibitory and antioxidant properties, as well as the long-term reductions in blood pressure previously observed[2–6].

Acknowledgements

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