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Correspondence

The Effects of AGEing on Diet

To the Editor-in-Chief:

We have read with interest the recent article by Cai et al.[1] However, we believe that the methods used do not permit the conclusion that advanced glycation end products (AGEs) are responsible for the biological effects on longevity observed by the authors.

The authors fed groups of mice either a calorie restricted (CR) diet, an autoclaved CR diet to generate AGEs (CR-high AGE), or a regular diet. The data in Table 1 of their article[1] show that both CR groups were fed a diet with the same caloric density as the regular diet (each 4 kcal/g), and Table 2 and Figure 2 (inset) show that both the CR- and CR-high AGE-fed animals consumed 40% less calories and food, respectively, than the animals fed the regular diet. These data establish that calorie restriction was achieved by a 40% reduction in food availability. On top of the reduced food availability, the CR-high AGE diet was autoclaved for 15 minutes at 120°C to generate AGEs. This practice is not commonly used or recommended for the preservation of animal diets because many vitamins and phytochemicals are heat labile. Thus, the CR-high AGE-fed animals received 40% less of a diet that was potentially already poor in vitamins and micronutrients. Because the adequate supply of essential vitamins, phytochemicals, and micronutrients was not ensured, it is possible that the observed effects of the CR-high AGE diet were not attributable to the presence of AGEs in this diet but rather to vitamin and/or micronutrient deficiency. It is known that thiamin, calcium pantothenate, vitamin B12, and vitamin D are heat labile. In light of the fact that heat-induced thiamin deficiency results in similar *in vivo* effects as observed by Cai and colleagues,[1] namely oxidative stress, inflammation, and AGE formation,[2,3] it may be likely that the observed effects of the CR-high AGE diet were not mediated by AGEs but by vitamin deficiencies.

A second methodological problem concerns the reported values for reduced and oxidized glutathione (GSH) in whole blood. The GSH concentration in whole blood is normally ~50 times higher than that of glutathione disulfide (GSSG). However, GSH oxidizes rapidly when exposed to air and precautions need to be taken to prevent GSH oxidation during sample preparation. The authors did not describe how GSH oxidation was prevented, and because the data in Figure 5B show GSH/GSSG ratios between 1.3 and 2, it is highly likely that a large part of GSH was oxidized to GSSG, probably during sample preparation. Thus, the data presented in Figure 5B are most likely meaningless.

One of the major obstacles to obtaining unequivocal data in AGE research concerns the heating of food to generate AGEs. Heating profoundly alters food structure, taste, and composition far beyond AGE formation. Heated food, although containing higher levels of AGEs than unheated food, cannot be used to single out AGEs as culprits for any observed biological effect.

In addition, AGEs are a diverse group of chemical modifications of free amino acids, peptides, and proteins, and their analysis is complex. Adequate characterization of the AGE modifications induced by heating, providing a balance of ingested and excreted AGEs, and controlling for other possible influences induced by heating, including vitamin analysis, are essential to allow proper interpretation of the results obtained. Ideally, dietary intervention studies with AGEs should be done by adding defined AGEs to the same dietary background, essentially as done by the authors in their 6-month study. It is not easily understandable why this has not also been done for the life-long calorie restriction study.

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T.B. is employed by the Nestlé Research Center, which is affiliated with the Nestlé Company.

Author's Reply:

In response to the questions from Buetler and Henle, we offer the following comments:

In their correspondence, Buetler and Henle question the role of the negative effects of heating on diet in our model system. We are well aware that certain vitamins in calorie-restricted or low-AGE diets are altered by heat and need to be supplemented in sterilized diets, especially when nutrient-restricted. For these reasons, the NIH-31 diet is prepared by the manufacturer to be autoclaved (according to the manufacturer's instruction; Harlan Teklad), and we have taken this into consideration, as cited in the article.[1] A review of the methodology used by the reports cited in their letter indicates major differences from ours. Our approximately two-fold increase in AGE levels above the standard method contrasts to the approximately nine-fold increase in such reports. We therefore feel that the expressed concerns about diets used in research in oxidant stress and/or aging, although of general relevance, are not applicable to our system.

In regards to the question about GSH determinations, this topic has been extensively covered in the literature, both by our group and by many others. Not only are we aware that it is critical to expeditiously handle blood specimens intended for measurements of markers of oxidant stress (OS), but it is routine practice to add inhibitors of oxidation immediately after blood collection, followed by freezing at −80°C until they are assayed. The commercial kits for many of these assays also provide inhibitors as standard practice.

In addition, Buetler and Henle comment on the lack of a significant difference in GSH levels between the low- and high-AGE diet groups. We are very familiar with the articles quoted in this letter because they are authored by respected scientists who are among our collaborators. They found a difference of ~1.5-fold in circulating GSH levels between the low- and high-AGE diets.[2,3] Thus, our data are clearly within the range published by others who are actively engaged in animal research in the area of AGEs and oxidant stress. Beyond the GSH depletion data, however, we supplied evidence in terms of several well-established measures of native oxidants, 8-isoprostanes in plasma as well as of tissue OS-responsive genes, as receptor for advanced glycation end products and $p66^{shc}$. Taken together, these data support a link of dietary AGEs to oxidant stress and injury.

There is a considerable body of literature that demonstrates that AGEs in the diet contribute to serum levels of AGEs, and that the body burden of AGEs contributes to cardiovascular and kidney disease, insulin resistance, and diabetes type I and II. Other studies have satisfied Koch's postulate, namely that injecting a single AGE-modified molecule induces, and inhibition of AGE formation or levels reduces or prevents, some or all of these conditions. We have always been careful to not single out AGEs as the culprit. However, because this is an important question, in this study we performed critical experiments by supplementing a low-AGE diet with a specific AGE, methylglyoxal. We found a progressive increase in circulating AGEs and oxidative stress in the current study. This confirmed the role of AGEs in the diet as a significant contributor to oxidant stress. Buetler and Henle do not understand "why this was not done for the life-long calorie restriction study." These experiments are very long and expensive. Nonetheless, these long-term studies are ongoing, and whereas the data at 2 years confirm the currently referenced article,[1] they will not be completed for another year.

Buetler and Henle deny that AGEs have negative biological effects. We would like to note that Buetler and colleagues have argued in similar letters to the editor that AGEs N-carboxy-methyl lysine neither bind nor activate RAGE despite a large number of high-quality, peer-reviewed full-length articles by several groups of independent investigators demonstrating that AGEs promote inflammation and oxidant stress via AGE receptors, including RAGE, or other mechanisms in several animal models including mice, rats, and humans.[3–16]

It is in fact difficult to understand the exact nature of the above concerns. We would like to raise the legitimate question of whether the views expressed above may be influenced by commercial interests seeking to cast doubts on findings illuminating the potentially serious effects of AGEs present in processed foods on the health of the general public. What Buetler and Henle neglect to add is that heating of diets is a universally applied, singularly simple, and inexpensive step that renders foods not only edible and transportable, but also invariably flavorful, an aspect easily exploitable and hugely profitable for the food industry. Our research published in *The American Journal of Pathology*[1] and numerous other animal and clinical reports by both ourselves and others[1,5–7,10,15,16] show that it is possible to decrease oxidants in the diet without sacrificing nutrients, calories, or taste. Being asked to rethink the current commercial approach may be challenging for the industry, but the health-related costs associated with denying these facts are already far greater.

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