Author's response to reviews

Title: Daily egg consumption in hyperlipidemic adults - Effects on endothelial function and cardiovascular risk

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Version: 2 Date: 8 March 2010

Author's response to reviews: see over
March 4, 2010

Editor, *Nutrition Journal*

Dear Sir or Madam:

My co-authors and I respectfully re-submit the research manuscript: “Effects of Sugar-Sweetened and Sugar-Free Cocoa on Endothelial Function: A Randomized, Controlled Trial” for your consideration.

We have fully addressed the reviewers’ comments (below) and hope you now find our manuscript suitable for publication.

My address, phone and fax numbers, and e-mail address are provided above. My colleagues and I thank you in advance for your time and attention to our work, and look forward to your response.

Sincerely,

David L Katz, MD, MPH, FACPM, FACP
“The main limitation of this study is the very small study population (only 37 subjects) which limits its effectiveness in detecting true change/improvement in the flow-mediated dilation (FMD). The authors provided a power calculation based on a 3.5% change in FMD between egg and egg substitute. Authors should give the reference to their assumption.”

According to a report of the International Brachial Artery Reactivity Task Force for endothelial function studies, a sample size of 20-30 participants is sufficient to see significant improvement in FMD in a crossover design study [1]. Based on this, we believe our sample was ample enough to effectively detect significant improvement in FMD.

The 3.5% change in FMD represents a threshold widely used to represent a clinically meaningful difference.

“Since the study population is very small it is crucial to provide data on the patients' basic characteristics in a table. The data should include sex, age, CAD risk factors, body mass index, concomitant medications (especially those which can impact FMD, such as statins, ACE/ARB, ASA, calcium channel blockers, diuretics, nitrates, vitamins, food supplements, etc.), some laboratory parameters which could impact endothelial function such as lipids, fasting glucose, electrolytes, CBC, etc. In addition, patient's blood pressure should be included. What was the Framingham 10-year risk of the subjects? Table 1 should be more comprehensive and also should include the above data.”

We have provided additional characteristics of the study participants in Table 1. As we indicated in the methods, we excluded participants who were using lipid-lowering medications, insulin or glucose sensitizing medications, vasoactive medications or nutriceuticals (antihypertensives, glucocorticoids, antineoplastic agents, psychoactive agents, or bronchodilators); high dose vitamin E or C; and/or fiber supplements. We did not assess electrolytes or CBC. Two participants started statin drugs in the sustained phase of the trial. These participants were excluded from the study (see figure 1).

“We have provided the baseline diameter of the brachial artery (see Table 2).

“Did the authors find any correlation between total- or LDL-cholesterol and FMD? Did the authors control their findings to concomitant medications and or other variables which could affect FMD (such as lipoproteins, age, sex, hypertension, etc.)? Some studies have found an association between LDL change and FMD (Shechter M, et al. Improvement in endothelium-dependent brachial artery flow-mediated vasodilation with low-density lipoprotein cholesterol levels < 100 mg/dl. Am J Cardiol 2000;86:1256-1259).”

In a regression analysis we controlled for demographic information, lipoproteins and blood pressure. Controlling for these variables had no influence on our outcome. As we mentioned earlier, our participants were not on any medications.
“Authors should specify in a table the exact composition of each acute/ -6-week breakfast sandwich (proteins, fats, sugar, etc). Did the subjects also consume any beverage? Tea? Coffee?”

*We gave our study participants the option of consuming their breakfast with either water or 4 oz skim milk. They did not consume tea or coffee during their treatment assignments.*

“Was FMD measured while the patients were fasting? Were the patients under the influence of any medication? Influence of cigarettes?”

*The study participants fasted overnight before undergoing endothelial function assessment. As we indicated in the methods, all of our participants were non-smokers. We excluded participants who were using lipid-lowering medications, insulin or glucose sensitizing medications, vasoactive medications or nutriceuticals (antihypertensives, glucocorticoids, antineoplastic agents, psychoactive agents, or bronchodilators); high dose vitamin E or C; and/or fiber supplements.*

“Was the BART performed after a single dose of breakfast (Mickey – not sure what u mean)? Since FMD is blunted after a high fat meal (mainly due to high triglycerides) for about 6 hours it is important to report on lipoproteins (including triglycerides).”

*We did not assess lipoprotein values after a single dose administration of treatment. However, in the sustained phased, we assessed and reported the influence of our treatment on lipoprotein.*

“How were the subjects randomized? Was the FMD reader blinded to the breakfast type?”

*We randomize as is typically recommended in a cross-over design trials. As we indicated in the methods (in the study design) our participants were first randomized to receive one of the two treatment sequence of egg and sausage/cheese. They were then randomized to one of the two treatment sequences of egg and egg substitute. Randomization was conducted by the data manager using a SAS (SAS version 9.1; SAS Institute, Cary, NC) algorithm. As we also indicated, this was a single blind trial with investigators blinded to treatments.*

“The BART protocol should be described in detail. Was a lower- or upper-cuff used? For how many minutes was the cuff inflated? Was the reading synchronized with ECG? Did authors measure nitroglycerin-induced vasodilation? What was the reproducibility of the test in the authors' lab?”

*We have described the BART in detail (see methods). Nitroglycerine was not administered. The reproducibility was 0.9. We have indicated this in the manuscript.*

“There are many variables which could potentially impact FMD, including carbohydrates etc. in the food. Authors should measure carbohydrate index of the food under which FMD was measured (Talia L, et al. The acute effect of various glycemic index dietary carbohydrates on endothelial function in normoglycemic subjects. J Am Coll Cardiol 2009;53:2283-2287)?”

*Unfortunately, we do not have data to measure carbohydrate index under which flow-mediated dilation was measured.*

“Page 9, 5st line of the Discussion: "while the subjects demonstrated impaired endothelial function at baseline...". Authors should define "normal" and "impaired" endothelial function and should give a reference.”
We have defined normal flow-mediated dilation and have provided reference for that.

“Did subjects undergo blood withdrawal on the same day as the BART? When exactly in relation to the FMD?”

Yes, our participants underwent blood withdrawal on the same day as the BART.

“What was the Framingham 10-year risk of the subjects?”

The mean Framingham 10-year risk for our study participants was 6.6%. We have reported these data in table 2.

“Page 5, 2nd paragraph: I did not see Figure 1.”

Figure 1 is the study flow diagram. We have included this figure in the paper.

“The Discussion section is too long and should be shortened.” We have shortened the discussion as suggested.

Reviewer 2

“The rationale for conducting the study is sound. However, a statement/paragraph should be added relating endothelial function, specifically FMD, to coronary outcomes and cardiovascular disease. See Yeboah J. Circulation, 2009.”

We have added a paragraph relating endothelial function and cardiovascular disease (see introduction).

“What was the rationale for conducting the acute ingestion trial and what results were you hoping to see following acute ingestion? How long post-ingestion were vascular and blood measurements made? How does the composition of the meals and timing of vascular/blood measurements compare to previous studies investigating the acute effects of meal ingestion?”

The rational for the acute phase was to show detrimental effects after ingestion of sausage plus cheese and no harmful effects after ingestion of eggs. Endothelial function assessment was done two hours after ingestion of the treatment assignments. Because EF is a dynamic response, both acute and ‘chronic’ indices are of interest. The breakfast administered is closely related to provocations used by Vogel and colleagues[2, 3].

“Prior to randomization, were subjects matched on pertinent variables (i.e., age, sex, BMI, cholesterol, etc.) to control for potential baseline differences?”

As indicated in the manuscript, this was a cross-over design trial. Participants were randomized to one of two sequence permutations. In a cross-over design trial, it is not necessary to randomize participants based on any pertinent variable since each participant serves as his/her control.

“What controls were implemented prior to endothelial function and blood testing (i.e., how many hours fasted, without caffeine, exercise, alcohol, time of day?)?”
Participants were required to fast overnight 8-10 hours prior to the scanning session. Early in the morning on the day of the scan, subjects underwent measurement of weight and blood pressure, then pre-prandial, BARS. Participants were asked to refrain from caffeine, exercise and alcohol.

“Please provide much more description regarding your endothelial function testing protocol. Where on the arm was the cuff placed? What was the occlusion pressure? What does “automate the brachial artery diameter measures” mean? What was the angle of insonation used to obtain accurate velocity measurements? Was an attempt made to calculate shear rate/shear stress as a stimulus inducing the FMD response? Why was FMD measured only at 60 sec post-occlusion compared to the true peak diameter (see Black MA et al. Hypertension, 2008)?”

We have provided a detailed description of the endothelial function testing protocol (see methods).

“Why were blood concentrations of lipids used as a baseline for both trials? Differences in blood concentrations may have differed over the course of 10 weeks had blood been drawn prior to each 6 week trial period. The same question for weight and BMI.”

The difficulty in analyzing the cross-over design is that there are not enough model degrees of freedom available to analyze the contrasts of interest while eliminating other parameters. Various authors[4, 5] have proposed various approaches to estimation of the treatment effect and its standard error, depending upon which of the baseline values are available.

“The contribution of nitric oxide to FMD differs depending on the technique utilized, particularly in regards to the use of upper arm vs. forearm occlusion (see Doshi SN et al. Clin Sci (Lond). 2001). This further necessitates further description of the technique utilized in the present study.”

We have provided detail of the technique used to assess the endothelial function (see methods).

“A paragraph needs to be included providing supporting evidence of the relationship between the endothelium, FMD, and cardiovascular disease. Without a discussion of this relationship(s), no connection can be made by the reader between endothelial function and cardiovascular complications.”

We have made this revision in the introduction section of the paper.

“The stated objective was “to assess the effects of daily egg consumption on endothelial function and serum lipids in hyperlipidemic adults”. However, this study also investigated the acute vascular and lipid effects of egg consumption. This should be stated as well.”

We have made this revision.

“Please include descriptive characteristics of subjects within the abstract (mean±SD for age, weight, BMI, serum cholesterol concentration, etc.).”

We have added descriptive characteristics of the study participants in the abstract.

“Please elaborate your Conclusions or combine the two sentences into one declarative sentence.”

We have combined the two sentences into one declarative sentence.

“Throughout the text please place the reference number before the period at the end of each sentence.”
We have made this correction throughout the manuscript.

“Page 5, paragraph 2: Triglycerides are listed twice within the same sentence. Also, it is stated “see figure 1.” No figures were included with the files I received. Should this be Table 1?”

We have removed one of the triglycerides from the sentence. Figure 1 is the study flow diagram. We have included this figure in the manuscript.

“Page 5, paragraph 3, 1st sentence: Please move this sentence to the end of paragraph 3 following the description of the acute phase meals.”

We have moved this sentence as suggested.

“What was the kilocalorie and macronutrient content of the meals used in the acute phase?”

We have provided this information in table 1.

“For the acute phase, three medium hardboiled eggs were consumed. However, only 2 eggs per day were consumed in the sustained phase. Please describe your rationale for this difference.”

The acute assessment is, by definition, limited to a single exposure and thus very dose-dependent; the sustained phase allows for detection of any effect that is cumulative over time. The 3 egg dosing was intended to avoid a misinterpretation due to a threshold effect.

“How long following the acute phase did subjects begin the 6 week trial?”

There was a 4-week washout period between the acute and the sustained phases (see methods & Figure 1).

“How was daily ingestion of eggs or egg substitute monitored?

Daily ingestion of treatment assignment was monitored through daily log. Participants were asked record their daily ingestion.

“Was the kilocalorie content of the two hardboiled eggs and ½ cup of egg substitute equal?”

No.

“What were the macronutrient compositions of the eggs, egg substitute, and breakfast sandwich? What was your rationale for choosing the breakfast sandwich as a comparison for the eggs in the acute phase?”

The rationale for the sandwich was its relation to prior work; see response above.

“Please provide a reference for choosing blood flow within the first 15 sec after cuff deflation as an indicator of stimulus strength? How might have the results differed if the entire shear stress stimukus was accounted for? Refer to reviews by Pyke KE and Tschakovsky ME on this subject.”

We have provided a reference for choosing blood flow within the first 15 seconds after cuff deflation as an indicator of stimulus strength. Since we did not find any effect of SARM on the interpretation of our data,
other means of measuring SARM are unlikely to be of great utility. Note that this is a ‘supplemental,’ not a primary measure.

“We have provided reference for the VLDL calculation (see methods). Data we received from the lab does not contain VLDL values. This explains why we did not provide the results of this variable.

“Page 6, paragraph 3: What do you mean by saying Tchol, TRIG, and HDL were obtained by direct measurements? Please provide a reference for your calculation of VLDL and LDL? No mention of the VLDL results are provided.”

Blood pressure was determined with the use of the Datascope Accutorr Plus automatic digital blood pressure device with the participant supine after a 5-min period of rest. Both systolic and diastolic pressures were calculated as the mean value of 2 readings 5 min apart. All blood pressure measurements were obtained by one investigator.

“Was dietary pattern recorded the three days prior to vascular testing or at three arbitrary days during the 6 week trials? Were three day food records replicated prior to each measurement of endothelial function?”

The three-day food diary we collected was the dietary pattern prior to the participants’ endothelial function testing. We have removed the 3-day food diary data as suggested by reviewer 3.

“We have made this reference in this paragraph.

“Page 8, paragraph 1: Please refer readers to Table 1 within the 1st paragraph of page 8.”

We have included these data in Table 2.

“Do the tables represent data from 60 subjects or 56?”

There were 40 participants in this study.

“Significant differences in SARM were detected at baseline. Was an attempt made to correct for this?”

In all our analyses we compared the pre-post scores of an assignment to those of another and thereby control for any build in, systematic, pre/post changes.

“We have made this correction in the manuscript.

“The n for Table 3 should be 36 and not 40 as stated within the text.”

We have made this correction.
“Is a 1.0% improvement in FMD physiologically important/significant? What is the meaningfulness of a change of this magnitude? See Shimbo D et al. Atherosclerosis. 2007.”

**Generally not; the prevailing view in the literature suggests that a difference of roughly 3% is clinically meaningful [6]. However, since FMD is a continuous variable, there is no absolute threshold effect, and smaller variances are not entirely unimportant. The Shimbo paper did not convey a refutation of this view.**

“What was the total protein intake of the subjects? This data should be included in Table 3 as well.”

**We removed dietary data as suggested by reviewer 3.**

“Where are the VLDL results as mentioned in the Methods?”

**The VLDL data were used to determine LDL. The results of the LDL data are presented.**

“Page 9, paragraph 2, sentence 3: Who are you comparing the subjects in the present study with when saying “the subjects demonstrated impaired endothelial function at baseline? Sentence 4: This sentence is very confusing to me. Please re-word.”

**This is relative to a healthy endothelial function which is typically >10%.**

**We have re-worded this sentence.**

“Page 9, paragraph 3, sentence 4: Please provide references following ‘but is at odds with the reported literature.’ ”

**We have provided this reference.**

“Please provide a reference when discussing differences in gastric transit times.”

**We have provided a reference.**

“Page 10, paragraph 1, sentence 2: Several studies have examined FMD serially over time following meal ingestion (see Vogel, Plotnick, Marchesi, Ng, etc.). Page 10, paragraph 3: This paragraph is confusing to me. You begin by discussing the effects of egg consumption on HDL. However, no mention is made to your present results. In the next sentence, an attempt is made to explain a mechanism of why blood cholesterol does not increase with increasing dietary cholesterol intake. I am not seeing the connection here.”

**We have addressed this (see discussion).**

**Reviewer 3**

This study addresses an interesting nutritional subject concluding that three eggs don’t have any acute detrimental effect on endothelial function; similarly, consuming two eggs daily for 6 weeks has no unfavourable effect on both endothelial function and lipidemic pattern in hyperlipidemic subjects. Furthermore, egg substitute had beneficial effects after 6 weeks and sausage/cheese breakfast sandwich had no acute unfavourable effects. Therefore, the take home message might be that hyperlipidemic subjects have probably not unfavourable consequences from a so frequently eating eggs. However, a number of methodological problems (or at least the way in which they are presented) heavily limit the strength of the
results of this study. Due to the nature of this study, probably the protocol should have been registered in a public trial registry.

“The authors affirm that literature is not univocal eggs consumption, cholesterol levels and CV risk and that, taken as a whole, we have very few evidences for a so strong indication that eggs consumption has detrimental health effects. However, they should also quote other recent researches that have shown how eggs consumption is associated with increased risk of diabetes (Djoussé L et al, Diabetes Care, 2009), with (#1/day) increased mortality and even more indiabetics (Djoussé L et al, AJCN, 2008) or in women (#2/day) with a significant correlation with cholesterol concentrations (Nakamura Y et al, AJCN, 2004) and other references.”

We have reported these other research findings in the discussion.

“The authors have to explain why they considered hyperlipidemic adults. This information appears abruptly in the last three words of the section Introduction. “

We have previously studied healthy, normolipidemic adults[7]; this study addresses whether eggs may be safely included in the diets of adults with overt cardiac risk factors with which dietary cholesterol has been associated.

“In the section Subjects and Methods it needs to be reported habitual alcohol intake and it should be clearly stated if subjects assumed other drugs (ASA, etc.). These informations may be reported in Table 1.”

Participants were asked to refrain from caffeine, exercise and alcohol. We excluded participants who were using lipid-lowering medications, insulin or glucose sensitizing medications, vasoactive medications or nutriceuticals (antihypertensives, glucocorticoids, antineoplastic agents, psychoactive agents, or bronchodilators); high dose vitamin E or C; and/or fiber supplements.

“Despite, the ultrasonographer (only one ultrasonographer ? Please specify) was “strictly blinded” the study design is to be considered randomized, open, crossover trial, not “single-blind” (page 5, line 7 from the bottom).”

Only the project coordinator who was interacting with and providing treatment to the participants was not blinded. All investigators were blinded to the treatment assignments which participants were receiving. Because the participants were aware of what they were receiving but the investigators were not, we believe this should be considered as single blind trial.

“It is not described how much time after eggs ingestion was measured the FMD (page 6, paragraph on endothelial function) in both studies (in fasting conditions in the chronic study ?), neither how much time was maintained inflated the sphygmomanometer cuff in order to occlude the brachial artery. The accuracy and reproducibility of FMD measurements for their laboratory is not reported. The authors utilize an old fashion system for FMD measurements that doesn’t allow to monitor continuously the brachial artery diameter after deflating the cuff, in consequence of it the 60 seconds post-cuff release value is not always the peak value coinciding with FMD. The “indicator of stimulus stress” (page 6, lines 6-7 from the bottom) it is not a clear procedure, despite it doesn’t seem necessary to be reported, it should at least be presented in a better and more comprehensive way, actually it seems not understandable and may be omitted from results and tables.”

We have provided details on how we assessed the endothelial function (see methods).
“A major limitation is also the fact (as the authors acknowledge) that FMD was measured only one time after eggs ingestion and not monitored for a prolonged time (i.e. 30’, 60’, 90’, 120’ or: 60’, 120’, 180’).”

**We have mentioned this in the limitation section of the paper.**

“Since hyperlipidemic subjects with an average age of 60yrs old were investigated a significant flaw of this study is the lacking of the endothelium-independent dilation measurements that is obtained after sublingual glyceryl-trinitrate administration (GTN).”

**We will not directly refute this point, but simply note that in every study to date using GTN, all subjects dilate in response. Thus, it seems that the non-endothelial dependent vasodilatory response to nitrates is established as a universal phenomenon, and the inclusion of nitrates when they do no longer add to the interpretation of the data raises human subjects concerns which we confronted with our IRB. We concluded that we simply could not justify this element in the protocol.**

“About 3-d food diary (page 7), it is not reported how (software, etc…) data were evaluated. From Table 3, we know only data about calories, fat and carbohydrate intakes. The authors should add the data relative to protein and cholesterol intakes. These data are in fact crucial for the interpretation of the study results (protein and cholesterol intakes may influence the FMD), given the fact that cholesterol levels reduced in egg substitute treatment group. However, it is also to be noticed that probably food diary data are inaccurate. In fact, the total calories intake is frankly underreported since the corresponding intake for kg of BW is rather low (about 22 kcal) and body weight is reported stable. Probably it is better to delete the data concerning the 3-d food diary despite this fact underscores the study.”

**We have removed the 3-day food diary as suggested.**

“It is really strange that all lipidemic measurements reduced were reduced, significantly or not, whatever the treatment”

“Page 9, lines 10-11 “While….”: this sentence is not understandable in this context. Why is baseline FMD impaired ?”

**This is considered impaired relative to healthy endothelial function which is typically >10%. We have clarified this in the paper.**

“In general, the authors should report also more recent literature.”

**We have updated the literature throughout the manuscript.**

“Abstract, page 2, please specify that results are expressed in terms of “change” (after minus before)”

**We have now indicated our data a presented in the abstract.**

“Page 3, lines 4-6 from the bottom “.eggs are an….”: this paragraph is not necessary.”

**We have removed this paragraph as suggested.**

“Page 3, last sentence needs at least one reference.”

**We have added a reference to this sentence.**
“Page 6-7: methods for lipid profile determination are not reported. Please, add reference for Friedwald equation.”

We have added reference for Friedwald equation as suggested.

“Data are widely duplicated in tables and results section. It should have been useful to show a diagram flow to resume lines 1-6 at page 8.”

We have removed duplicate data in the tables and the results section. We have provided a flow diagram.

“In no part of the manuscript it is reported if data are expressed in mean +/- DS or SEM.”

We have indicated in the footnotes of the tables that data are presented as mean +/- SD.

“Please, report a column with ranges in Table 1, report also BMI, clinical characteristics, working activity, and so on (please retype this table).”

We have included the BMI and clinical characteristics in table 1. We did not working activity data.

“Please, report body weight and BMI with one decimal in Table 2.”

We have made this correction in table 3.

“Please, indicate mean +/- ? in tables”

We have indicated in the footnotes in all tables our data are presented.

“Page 7, line 3 from the bottom: is 3.5% in absolute value ?”

Yes, this is in absolute value.

“page 9, line 12: please replace “egg” with “Egg”.

We have made this correction.

“page 7, line 13: please replace “significance” with “significant”.

We have replaced “significance” with “significant” in line 13 as suggested.

“page 8, line 12 and 17: please delete “see”.

We have deleted “see”.

“page 10, line 4 from the bottom: please replace “responses” with “response”.

We have made this correction as suggested.

“page 20, Table 2: please replace “student” with “Student’s”

We have made this correction as suggested.
“page 20, Table 2: please replace “ttest” with “t-test”

We have replace “ttest” with “t-test” in Table 3.

“page 20, Table 2: please replace “obtain” with “obtained”

We have made this correction as suggested.

“Table 2 and Table 3, please homogenise data relative to “Change”

We do not understand what you mean to homogenize data relative to change.

“Table 2 and Table 3: please replace “Repeated Measures ANOVA” with “ANOVA for repeated measurements”

We have made this change as suggested.

“Table 3 caption please remove capital letters.”

We have made these changes as suggested.

“Table 3, probably it is better to suppress “Stimulus adjusted response measure”

We have made this change as suggested.

REFERENCES:


