Author’s response to reviews

Title: Blood pressure and endothelial function in healthy, pregnant women after acute and daily consumption of flavanol-rich chocolate: a pilot, randomized controlled trial

Authors:

Jaime A Mogollon (jaime-andres.mogollon.1@ulaval.ca)
Emmanuel Bujold (Emmanuel.Bujold@crchul.ulaval.ca)
Simone Lemieux (Simone.Lemieux@fsaa.ulaval.ca)
Claudine Blanchet (claudine.blanchet@crsfa.ulaval.ca)
Laurent Bazinet (Laurent.Bazinet@fsaa.ulaval.ca)
Charles Couillard (Charles.Couillard@fsaa.ulaval.ca)
Martin Noël (martin.noel@me.com)
Sylvie Dodin (sylvie.dodin@fmed.ulaval.ca)
Mélodie Bourdages (melodie.bourdages.1@ulaval.ca)

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Author’s response to reviews:

Institut des Nutraceutiques et des Aliments Fonctionnels (INAF)
Université Laval
Québec (Québec) Canada G1V 0A6

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Editor
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Sir/Madam:

Please find enclosed our manuscript entitled “Blood Pressure and Endothelial Function in Healthy, Pregnant Women after Acute and Daily Consumption of Flavanol-rich Chocolate: A Pilot, Randomized, Controlled Trial” which we are re-submitting for exclusive publication as an article in the Nutrition Journal.

First, we thank you and your reviewers for your rapid response. We are also grateful for the attention paid to our manuscript. The reviewers’ comments were very relevant and have significantly improved the text.

Point-by-point changes made in response to the reviewers’ comments

Audrey F. Saftlas (Referee 1)

1. “The statements in the abstract and in the text, which assert that no trials have examined the effects of chocolate in pregnant women, are no longer accurate. Findings from a chocolate trial among pregnant women in Italy were recently

Indeed, the recent study by Di Renzo et al. was published just after our manuscript was submitted to your journal. Their findings have been added in the second paragraph of the Introduction.

Changes made: “Finally, a non-placebo, controlled, non-blinded study by Di Renzo et al. [1] suggested that modest daily intake of high-cocoa content chocolate contributes to BP reduction during pregnancy.”

2. A major design flaw of the trial was the decision to measure plasma flavanols in blood samples collected following a 12-hour fast, which accounts for non-detectable levels of epicatechin in samples collected following 6- and 12-weeks of daily high-flavanol chocolate intake. As noted in the discussion, it is well known that epicatechin clearance from the plasma is rapid and most would be eliminated over the 12 hours following ingestion. Therefore, the study provides no direct evidence of high-flavanol exposure to test for an association of chronic high-flavanol chocolate intake (versus low-flavanol intake) with the study outcomes of blood pressure and flow-mediated dilation. a) This point needs to be made clear in the abstract, results, and discussion sections. Currently, the conclusion of the abstract states that the study results “demonstrate higher plasma concentration of epicatechin and theobromine in the intervention group”. This statement should be revised and clarified to indicate that increased levels of epicatechin could only be demonstrated with acute ingestion of flavanol-rich chocolate.

We agree that epicatechin levels in blood samples, collected after a 12-hour fast at weeks 6 and 12, account for non-detectable levels of epicatechin. Indeed, this important point was already mentioned in the Discussion, but we agree that it needs to be revised and clarified. The conclusion in the Abstract and Discussion has been modified accordingly.

Changes made: “...and demonstrate higher plasma epicatechin and theobromine concentrations in the intervention group after acute ingestion.

...its effectiveness in increasing blood theobromine and flavanol metabolite concentrations after acute ingestion.”

3. The experimental chocolate differed from the “placebo” chocolate only on the flavanol content while all other key components of chocolate (ie, theobromine, caffeine) were identical. These similarities should be emphasized in the methods section and a rationale for this choice of a placebo should be provided.

In this clinical trial, aiming to test the hypothesis that high-flavanol cocoa could improve FMD and BP in pregnant women, we decided to use placebo cocoa which differed from experimental cocoa only in its flavanol content. Indeed, flavanols and theobromine are the 2 major constituents of cocoa, and accumulating epidemiological and clinical data support cocoa’s enhancement of endothelium function via improvement of nitric oxide synthesis. To date, this
endothelium-protective action of cocoa has only been explained by the presence of flavanol compounds. As we stated in the Discussion, it has emerged recently that theobromine could have relevant effects on cardiovascular risk factors, such as FMD and BP, but only one clinical trial supports this hypothesis. It would be relevant, in a clinical trial, to verify whether theobromine accounts for or enhances the effects of flavanols. To test this hypothesis, cocoa placebo without theobromine and flavanols will be administered. As suggested, we emphasized similarities of our chocolate intervention and provided the rationale for our choice in the Recruitment section.

Changes made: “Therefore, to isolate the effects of flavanols, our chocolate placebo was identical to experimental chocolate in its content of all other nutrients except for flavanols (including theobromine and caffeine).”

4. Methods. Dose: What is the authors’ rationale for prescribing a total daily intake of 20-g of chocolate?

Very few clinical trials have explored the chronic effect of daily chocolate intake. In diabetic patients, 30-day, thrice-daily consumption of chocolate containing 371 mg of flavanols significantly improved baseline FMD [2]. Our cocoa provided 400 mg of total flavanols. High-flavanol cocoa was provided by the Barry Caillebot Company which has developed a specific process that preserves flavanols in chocolate produced from cocoa beans. All steps in chocolate production (fermentation, drying, roasting, and alkalinisation) have been optimised to preserve antioxidants as much as possible. Indeed, our 20-g chocolate provided 64 mg of catechin and epicatechin. Cocoa tested in a recent study by Di Renzo et al. only provided 12.6 mg of epicatechin and catechin [1].

Changes made: “…a total of 20-g daily providing 400 mg of total flavanols and 64 mg of epicatechin and catechin. This daily flavanol intake should be sufficient to exert beneficial effects. Indeed, in a similar study of non-pregnant women, chronic consumption of 373 mg flavanol was associated with significant outcomes on BP and endothelial function. Moreover, its caloric value was easily replaced by a snack of equivalent energy. Therefore, the balance between beneficial effects, caloric value and fat content was carefully considered.”

Table 1. Chocolate composition (20 g)

<table>
<thead>
<tr>
<th>Components</th>
<th>High-flavanol chocolate</th>
<th>Low-flavanol chocolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>102</td>
<td>102</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>1.35</td>
<td>1.35</td>
</tr>
<tr>
<td>Total flavanols (mg)</td>
<td>400 &lt;60</td>
<td></td>
</tr>
<tr>
<td>Total catechin + epicatechin (mg)</td>
<td>64 14</td>
<td></td>
</tr>
<tr>
<td>Caffeine (mg)</td>
<td>23.6</td>
<td>23.6</td>
</tr>
</tbody>
</table>
Theobromine (mg) 150 150

5. Methods. Timing of intake: Why were women followed in the first 24 weeks of pregnancy rather than later in pregnancy when blood pressures tend to rise from the nadir at 22-24 weeks? The choice of the gestational age at intervention and its potential impact on study findings should also be addressed in the discussion.

Actually, patients were enrolled at 12 weeks of gestation, but gestational duration at randomisation was 21.1 weeks. Therefore, the women were followed from approximately 21 to 33 weeks of gestation (for a total of 12 weeks of intervention).

Changes made:

Table 3. Baseline characteristics of 44 pregnant women by study arm

<table>
<thead>
<tr>
<th>Baseline characteristics High-flavanol chocolate (n=23)</th>
<th>Low-flavanol chocolate (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation duration at randomisation (weeks) 21.13 ± 1.10</td>
<td>21.10 ± 1.61</td>
</tr>
</tbody>
</table>

6. Results (1st paragraph and Consort diagram): The number of women who declined to participate (n=45) should also include women who had transportation problems (n=4) and those who cited personal reasons (n=33). The 5 women who had a miscarriage should be categorized with the other women who failed to meet inclusion criteria (n=27); and the breakdown of reasons for exclusion should be given. The study’s participation rate and response rate should also be provided. The authors should comment on how the low participation rate may have impacted their study.

We clarified the first paragraph (page 14) and CONSORT diagram (Figure 1). In an effort to simplify the flow diagram, we included a breakdown of reasons for not meeting the inclusion criteria and for declining to participate (Results section, page 14). Finally, 44 (33%) of women who met the inclusion criteria were randomised. We compared the characteristics of women who declined to participate with those of randomised women and did not see any significant difference between the 2 groups.

Changes made: “We approached 176 women, of whom 27 were excluded as they failed to meet the inclusion criteria: 12 were over 16 weeks pregnant, 6 were smokers, 5 had body mass index >30, 4 did not meet other criteria, and 5 had spontaneous abortions (n=5). One hundred women declined to participate for different reasons: 72 had logistical or personal problems (transportation, work, other children at home, too time-consuming), 18 did not return calls, and 10 refused to give a reason for withdrawing their participation. Finally, 44 (33%) of the 132 pregnant women who met the inclusion criteria and who agreed to participate were randomised.” “The baseline characteristics of women who declined to participate were not significantly different from those of randomised women (data not included).”

7. Results (p. 13, 2nd paragraph): “… significant increase in plasma theobromine concentrations was observed in both groups at 180 minutes and was slightly but
significantly more marked in the experimental group. Therefore, theobromine
concentrations served as a marker of chocolate compliance”

a) Theobromine is a well-established marker of chocolate intake, but it’s not clear
why there would be a greater increase in theobromine levels in the experimental
group when the theobromine content was identical in the high-flavanol and
low-flavanol chocolate bars.

The marginal but statistically significant increase in theobromine levels in the
experimental group compared to the placebo group could be attributed to
different rates of intestinal metabolism, which is influenced by diet. Indeed,
orally-administered theobromine is rapidly and almost completely absorbed by
the gastrointestinal tract [3]. At baseline, theobromine concentrations were also
slightly lower in the placebo group, but theobromine sources in diet measured by
FFQ in the last month preceding randomisation and at each follow-up visit were
not significantly different between the 2 groups.

Changes made: Discussion (page 21). "The marginal but statistically-significant
increase in theobromine levels in the experimental group at 180 minutes and 12 weeks post-randomisation could be attributed to
different rates of intestinal metabolism, which is influenced by diet. Indeed,
oraly-administered theobromine is rapidly and almost completely absorbed by
the gastrointestinal tract [3]. At baseline, theobromine concentrations were also
slightly lower in the placebo group, but its sources in diet, measured by FFQ in
the last month preceding randomisation and each follow-up visit, were not
significantly different between the 2 groups."

b) Table 5: Is the standard deviation for high-flavanol chocolate at 180 minutes
correct (i.e., 6.51 ± 0.08) correct? It is much lower than the standard deviations
shown for the other measures of theobromine.

We corrected the typographical error in Table 5 (page 39).

Changes made:
Table 5. Acute changes in methylxanthine concentrations after single-dose
chocolate intake
Low-flavanol
chocolate
(n=21) High-flavanol
chocolate
(n=23) P value of change
between treatments
Theobromine (µg/mL)
0 min (baseline) 0.35 ± 0.37 0.45 ± 0.43 -
180 min 5.47 ± 1.29 6.51 ± 0.80 0.0122
P value (180 vs. 0 min) <0.0001 <0.0001
8. Tables 4-5. The measurements taken at 60 minutes and 120 minutes after a single dose acute intake should be included in the tables. Methylxanthine concentrations were measured only at 180 minutes.

9. Minor Essential Revisions: Tables 4-10. Note in the table headings that the data reflect means with standard deviations.

Changes made: We added a footnote with central tendency and statistical dispersion measures in each table.

Andrea Tranquilli (Referee 2)

1. The Authors concentrate their attention mostly on the results in terms of flavanol action (the real difference in the two arms). It appears, conversely, and is scarcely addressed that there is a significant increase in theobromine concentrations in women with the most significant results. It should be evidenced by what mechanism flavanols may contribute to increase theobromine absorption.

To our knowledge, no data have provided evidence that flavanols contribute to increase theobromine absorption. As discussed in response to the first reviewer, the marginal but statistically significant elevation of theobromine levels in the experimental compared to the placebo group could be attributed to different rates of intestinal metabolism, which is influenced by diet.

2. It should be better emphasized how the effect observed may be due to both epicatechines and theobromine.

We addressed this hypothesis in the Discussion (page 23).

Changes made: “Although the beneficial outcome of chocolate on FMD and BP has been largely associated with flavanols, a recent clinical trial indicated that theobromine could be partly responsible for the BP-lowering action of chocolate [4]. Our results could have been masked by its effects, which would confirm the importance of carefully selecting an adequate placebo”.

Pablo Stiefe (Referee 3)

1. After a long introduction speaking almost exclusively of preeclampsia, the authors design a study based in two groups of healthy pregnant women, for finally hypothesize that the absence of effects on BP or FMD was attributable to their "healthy, normotensive female population, among whom only small changes in BP or FMD were to be expected”. Then, why did not they study preeclamptic women? As they recognized their results are not applicable to high risk pregnant patients. In the revised version of the manuscript this should be emphasized in the abstract.

We agree that our Introduction emphasized preeclampsia, as our pilot study was aimed at evaluating the feasibility of a large-scale trial among women at high risk of preeclampsia. Therefore, we first tested the feasibility of our methodology and procedures in healthy, pregnant women. In the Discussion (page 25), we recognized that our results may not be applicable to high-risk pregnant women.
2. It is stated that the participants were asked to avoid foods rich in polyphenol or theobromine, as tea, coffee, fruit juice and wine. But what about olive oil? It has been recently reported that olive oil polyphenols decrease blood pressure and improve flow-mediated dilatation as well as several markers of endothelial function in young hypertensive women (Am J Hypertension 2012 Dec;25(12):1299-1304). The authors must clarify if the studied subjects have consumed olive oil and in what quantity. Moreover, the previous mentioned reference must be added to the text and discussed together with their results.

We thank the reviewer for updated data on olive oil’s polyphenol effects on BP, FMD as well as several markers of endothelial function in young hypertensive women. Nevertheless, our randomised, double-blind clinical trial was specifically designed to verify the action of cocoa rich in flavanols. Flavanols are polyphenols not found in olive oil. We did not measure olive oil consumption in our study, but the 2 groups should have been randomised, as for olive oil consumption. Moreover, in the mentioned study, the authors used a specific kind of virgin olive oil that could have been less frequently available on the commercial market and, therefore, less likely to be consumed by our participants. In addition, analysis of polyphenol content in their study revealed that most phenols found were hydroxytyrosol, tyrosol and vanillic acid, which could have produced a different pathway of antioxidant action compared to the flavanols in our chocolate (epicatechin and catechin).

3. It is not clear for this referee why 176 women were assessed but only 44 were randomized. Why such an elevated number of women declined to participate (n=45) or cited personal reasons for not participate (n=33).

As suggested by the first reviewer, we clarified this point in the Methods section and in the flow chart.

4. P value must be added to tables 2 and 3, or if there are not any significant differences, it should be commented as a footnote.

We agree with the reviewer that P values should be added in Tables 2 and 3. Nevertheless, as suggested by CONSORT recommendations, P value should not be included to evaluate differences between groups. The authors of the CONSORT stated that such hypothesis testing could mislead researchers and readers. Rather, comparisons at baseline should be based on consideration of prognostic strength of the variables measured and the size of any chance imbalances that may have occurred [5].

5. Table 3: BMI was measured during pregnancy?
BMI was evaluated as a parameter of changes in randomisation time up to weeks 6 and 12. Therefore, BMI did not serve to evaluate nutritional condition of the pregnant women.

6. In tables 7 and 8 it seems to be some significant differences regarding diastolic blood pressure, of unclear clinical significance. The authors should interpret these differences and particularly the increase of diastolic blood
pressure in both groups, observed in table 8, since there are significant changes (even with p values of 0.0007). The enrollment seems to begin on the 21th week and the intervention lasted for 12 week. Therefore, should this change be related to the normal increase in blood pressure values from the second to the third trimester of pregnancy?

As suggested, these changes, which are statistically significant within groups but not between groups, were probably related to the physiological increase of BP observed from the second to the third trimester of pregnancy. We addressed this hypothesis in the Discussion (page 19).

Changes made: "In our study, DBP increased significantly from baseline to 12 weeks in the high-flavanol and low-flavanol chocolate groups, but the difference between groups was not significant. It is probable that this elevation was related to normal increases from the second to the third trimester of pregnancy."

7. Are all the data expressed as means ± SD? This was mentioned only in tables 2 and 3.

We added a footnote on central tendency and statistical dispersion measures in each table.

8. Non-paired T test is valid for comparison between groups. For changes in the same group the use of a paired T test should be considered.

We agree with this comment. Indeed, changes within groups were analysed by paired t test, and the differences between groups were evaluated by non-paired t test. We included this statement in the Sample size and Statistical sections (2nd paragraph, page 14).

Changes made: “Non-paired t tests were performed for planned comparisons between groups. Changes within groups were evaluated by paired t test.”

9. Did the investigators measure variability and reproducibility of the technique for measuring FMD, in their participant subjects?

Although we did not measure accuracy and reproducibility in our participants, FMD was performed under strictly-controlled conditions, as stated in the Methods section, and all women were scanned by the same individual at each visit. Accuracy and reproducibility were measured previously by other groups [6], and the coefficients of variation were 9.8%, 10.6%, 6.6%, and 9.2% at 4 to 6 h, 1 week, 1 month, and 3 months, respectively. The latter information has been added in the Methods section.

Changes made: “Accuracy and reproducibility were measured previously by other groups [6] and the coefficients of variation were 9.8%, 10.6%, 6.6%, and 9.2% at 4-6 hours, 1 week, 1 month, and 3 months, respectively.”

10. In page 7, first paragraph, it is stated "we enrolled non-smoking, normotensive... between the 7th and 12th weeks of gestation". However, in table 3 is mentioned that mean gestational age in both groups at enrolment was 21.13 and 21.10 weeks, respectively. It seems to be an error there; may you please clarify this point?
We agree that information regarding enrolment needs to be clarified. Indeed, 12 weeks corresponds to ultrasound time to confirm pregnancy. 20 to 22 weeks correspond to randomisation time. We clarified our statement in Table 3.

11. The key words “preeclampsia” and “hypertension” should simply be deleted since this is a study on normotensive pregnant.

As this clinical study involved normotensive, healthy women, we agree that the key words “preeclampsia” and “hypertension” could be deleted. Nevertheless, as our study is the first step in a large-scale trial evaluating these outcomes in a population of pregnant women at risk of preeclampsia, we prefer to retain these 2 informative key words.

Thank you for your consideration of our work. Please address all correspondence concerning this manuscript to me at Université Laval and feel free to communicate with me by e-mail (jaime-andres.mogollon.1@ulaval.ca).

Sincerely,

Andres Mogollon, M.Sc.
Institut des Nutraceutiques et des Aliments Fonctionnels (INAF)
Pavillon des services, Université Laval
2440, boul. Hochelaga, Local 2827
Québec (Québec) Canada G1V 0A6


