Author’s response to reviews

Title: Vitamin C and E supplementation does not affect heat shock proteins or endogenous antioxidants in trained skeletal muscles during 12 weeks of strength training

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Thanks to both reviewers for the excellent comments and questions which we think have improved the manuscript.

Reviewer reports:

Dianjianyi Sun (Reviewer 1): Results from this randomized trial showed that vitamin C and E supplementation did not affect antioxidant enzymes after strength training for 10 weeks, as well as the heat shock proteins in the mid of strength training for an acute exercise experiment. Here are some of my comments:

1. As readers are not quite familiar with all the outcome biomarkers, could the authors draw a figure indicating the mechanisms underlying the impact of endurance training on acute stress responses or long-term training adaptations.
We don’t think a figure is a good idea in an original paper. However, we have done some minor changes that might help the reader understand the impact of these proteins.

2. A table of participant characteristics was needed for either the long-term training adaptations experiment (10-weeks) or the acute stress responses experiment (at the 5th week).

Details about participant characteristics is found under Participants in the methods section. We will add more info about the 15 subjects volunteering for the acute experiment under Acute exercise session. Additionally, we have added information about strength (1RM) in the knee extensors in the revised manuscript.

3. More details should be illustrated in results part.

Unfortunately, some results were left out in the results part, but have now been added. Additionally, results on muscle strength are added to the revised manuscript.

4. What is the clinical application or the importance of public health for this study?

This study is related to sports nutrition and exercise adaptations. As indicated in the introduction antioxidant supplementation might affect the proteins investigated in this study. Negative effect in the adaptation in these proteins would, at least in theory, affect redox status, fatigue and restitution from exercise.
5. What are the other confounding effects?

There is always a chance that our results can be explained by confounders. Especially when it comes to studies involving humans.

The goal of the present study was to investigate the possible interaction of antioxidant supplementation on HSPs and antioxidant enzymes. We designed a RCT, where we controlled the training and diet (by dietary registrations) of the participants. Additionally, the groups were homogeneous when it comes to training experience, age, BMI, and adherence to training. We believe that this have minimized the chance of confounders. However, we acknowledge that controlling for all factors is impossible.

6. Why not control for the baseline characteristics (eg. Age, gender, BMI, energy intake, and etc.) in the statistical analysis?

As some proteins might be affected by (high) age (HSP; Morton et al., Sports Med, 2009; mnSOD: Lamberti et al., Mech Ageing Dev, 2007), the participants included in this study was quite similar regarding age and BMI. Furthermore, these proteins seem to increase in response to strength training in both sexes (HSP: Gjøvaag & Dahl, EJAP, 2006. mnSOD: Higashida, AJP, 2011; Higuchi, J Gerontol, 1985). We are unaware of any studies investigating if energy intake would have any effect on these variables. However, the energy intake of the participants did not vary a lot between participants (Paulsen et al., J Physiol, 2014). We cannot see the rationale for baseline control of these variables.

7. Like the limitations mentioned in the previous paper of "Vitamin C and E supplementation alters protein signaling after a strength training session, but not muscle growth during 10 weeks of training", the limitation in the current study were not fully addressed.

The paper you are referring to does not address limitations. However, our study published in BMC sports Science, Medicine, and Rehabilitation (Paulsen et al., 2014) discusses several limitations. The limitations discussed in the present study is mainly linked to what we see as a
limitation in our present study and not in general. We choose not to mention the same limitations as it might be repetitive. However, we have now added a reference to the study discussing limitations in the revised manuscript.

8. In Figure 4b, 4d, and 4f, the X-axis showed only "mid" and "post", why in the figure legend of "αB-crystallin pre-mid-post (B)" and "HSP70 pre-mid-post (F)"? Where is the "pre-"?

Thank you for noticing this. This is an error and removed from the revised manuscript.

9. Table 1 in the current study can be deleted or moved to the supplementary table.

If the editor agrees that table 1 can be a supplemental data it will be removed and moved to supplemental data.

Marco Mensink, Ph.D., M.D. (Reviewer 2): In this manuscript Cumming et al described the effects of resistance exercise training, with or without vitC/E supplementation on the acute stress responses to exercise and adaptation to training with regards to NFkB pathway, HSP's and antioxidant enzymes. Data from this study with regard to hypertrophy related signaling and muscle strength/mass were already reported earlier (Paulsen, 2014).

The study and its analysis are of good quality.

The limited sample size and power of the study, in particular with regard to the acute stress response, makes interpretation of the data hard, and implication limited. This is acknowledged by the authors, but is a severe limitation. Data on the number of samples analysed for the different genes/proteins is missing, and should be added.
All figures include individual data points. This would show the reader how many samples are analyzed for each gene and protein. We have added info about the number of samples in the revised manuscript, in both the text and figure legends.

The results didn't reveal any acute exercise or training effect on the measured genes/proteins, except for IkBa in the placebo group at t=100. So any conclusions on a blunting effect of supplementation (as hypothesized) seems not warranted. This lack of exercise/training effects should be acknowledge in the conclusion, and carefully considered when drawing conclusions about supplementation (L283-286).

Thank you. This is a very good point. We have added a short discussion about this, and mentioned it in the conclusions in the revised manuscript.

Much of the pre-knowledge on HSP and antioxidant supplementation is derived from endurance exercise (training); Could the mode of exercise somehow be related to the lack of effect on HSP in the present study, besides the training status of the volunteers (L238-250).

This is a very good question. As several studies show increased HSP after strength training it could be that the total stimuli from the strength exercise overcomes a potential blunting effects from the antioxidant supplementation. Since endurance type exercises has less potential to increase levels of HSP, the detrimental effects of the supplementation might overcome the stimuli to stimulate HSP synthesis. However, we must acknowledge that we did not find any training effects on the HSPs (as pointed out over), which is a disadvantage in the present study.

However, we must point out that most studies that show blunting effects has done so at a mRNA level, and not at protein levels.

Comments

L73. What were the results of the endurance training supplementation trial (Cumming 2014b)

We did not find any effects of the supplementation in that study. The sentence is rewritten to point out this.
What was the training experience of the volunteers? How many years were the already active? Additional data on for example 1RM would be informative. This is relevant regarding the comments in the discussion on the role of training status.

The participants were active men and women with some strength training experience (at least 6mo). The participants trained 1-4 times per week before entering the study. We have rewritten this part so this is better highlighted.

Would love to see some information about the result of the program on strength and mass. Moreover presenting the results of the present manuscript at the background of results already published in the past (Paulsen 2014 a and c) would benefit the discussion. Sample size and power of the study seems large enough to detect effects of supplementation in other pathways (i.e. hypertrophy related signaling).

We have added the 1RM results for knee extension, and mentioned muscle mass (results from a previous published paper) in the introduction.

Was 1RM re-assessed during the intervention, and the training protocol adapted?

Yes, 1RM was tested pre-, mid (before the acute experiment) and post intervention. Since the participants trained with loads corresponding to 6 to 11RM the loads were adapted to fit this intensity. This means that the strength testing was only done to see changes in muscle strength.

I don't understand the information between brackets (6-10RM); does it mean 6 to repetitions? At which % of te 1RM?
The loads varied between weeks and exercises. So 6-10RM means that some weeks and exercises the participants trained with loads corresponding to 6RM and other 10RM. Details about the exercise program has been published previously (Paulsen et al., BMC, 2014).

6RM means (maximal) six repetitions. This means that the load need to be adjusted so the participants are not able to do any more repetitions.

10RM usually corresponds to 80% of 1RM, while 6RM about 90% of 1RM.

L105. How many exercises were performed?

Seven upper body exercises and six for lower body.

In general: Although data form the present study were published earlier, some details about the study design would benefit the present manuscript (L98).

More details and results is added to the revised manuscript.

L 111. What were the characteristics of these 15 volunteers, and how many per group?

Participant characteristics is added to the revised manuscript.

L119-133. I would suggest to describe the information on the supplements somewhat earlier in the method section, at least before the 'acute exercise session' section; as this makes it understandable why supplements were also provided during that session

We agree. Information about the supplementation is moved in the revised manuscript.

L132. Did subjects use NSAID’s or was this prohibited during the study? (as for other medication)
In general, the participants were not allowed to take any form of medication or supplementation that could affect training adaptations. This includes NSAIDS. We have added this to the revised manuscript under “supplementation”

L136. Where the pre and post training biopsies taken in the fasted state? Or after breakfast, with or without supplements?

The biopsies taken pre and post training intervention was not taken in a fasted state. As this would be ideal, we were not able to organize this within the time schedule we had. However, the participants took the pre and post biopsy at the same time of the day, and they were told to have the same meal before the biopsy. Additionally, the participants were not allowed to train in the days before the biopsy.

The pre biopsy was taken without supplementation, while the post biopsy was taken when the participants received supplements.

The baseline muscle biopsy samples during the acute session were taken postprandial (2h after the breakfast). Is this comparable to the pre and post training sample as is relevant for the data presented in figure 4 (pre-mid-post)

The time between breakfast and the biopsy, and what the participants had for breakfast was different between the two pre-biopsies. The goal of the acute (pre) biopsy was not to compare it to the pre intervention/training biopsy but to get insight in what happens during a training session. The standardizing of the breakfast in the acute study was because we measured protein synthesis and signaling. Since these analyses are sensitive to protein ingestion we tried to minimize any confounding factors.

L139. How and where were the three samples taken in the acute study? Same or different incision? Proximal or distal, and distance?

Biopsies was taken from the left leg. One insertion was made for the pre sample(s) (two samples was taken for the pre due to the protein synthesis analyses), and another insertion was made for
the post samples. The sampling was done in different directions, one proximal and one distal. Info about this is now added to the revised manuscript.

L158. Details for HSP 27 are missing (->figure 4)

Thank you for noticing this! This is a major mistake. Information about the method is added to the revised manuscript.

How many muscle biopsy samples could be analysed for the different groups/ timepoints? Was a sample available for each volunteer at each occasion? Indicate those numbers in the text/figure legends

No, some samples were not available for all analyses, e.g ELISA. Information about ELISA is added to the revised manuscript.

Figure 1. I suggest to add a heading indicating the protein measured (IkBa). Like is done for the other figures. And indicate the significant effect at t=100 for placebo group

Heading indicating protein is added to figure 1 (2). We choose to don’t add signs to indicate difference between pre, as this might be confusing. However, we will add it to the revised figures as this help readers that just skim trough the article and look at the figures.

Figure 2 and 3: why not combine? As they contain same level of data (gene expression in acute study)

There is no reason why we should not combine these figures. A combined figure is added to the revised manuscript.
Figure 4: representative immunoblots for HSP 27 are missing; please indicate that P-value relates to difference between groups (and not over time).

HSP27 is analysed with ELISA (info added to the revised manuscript). So no immunoblots are available.

Information about the P-value is added to the revised manuscript.

L 211. How many subjects were in each analysis?

Information about how many participants in each analysis is added to the revised manuscript and indicated in each figure.

L 213. Also describe the results presented in Figure 5 in the main text

Thank you, and sorry. This is again a major mistake by us. The results are presented in the revised manuscript.

L236/237. How many biopsies were available?. This relates to earlier remarks I made. As the authors acknowledge the limited sample size and power, I would like to see numbers throughout the manuscript.

Info is added to the figure legends.

L245. What is the authors' definition of 'moderately'
We see that this word is confusing. We changed it to recreationally strength trained. Recreationally trained is defined as someone who had trained for 1-4 sessions per week the last six months.

L258. Can an estimation be given what power would have been needed according to the authors to draw more solid conclusions?

The initial power calculations was done for lean mass. There are no previous studies investigating antioxidant supplementation on HSP adaptations in humans. However, based on the results in the present study, we would need 499 participants in each group to find significant differences between groups for αB-crystallin, over 42000 for HSP70. That means that the observed differences (if any) would not have any significant importance. However, as discussed, the story might have been different if we found training effects.

(calculations done by: http://clincalc.com/stats/samplesize.aspx)