Author’s response to reviews

**Title:** Conditioning cortisol in humans: design and pilot study of a randomized controlled trial

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**Author’s response to reviews:**

Dear professor Guowei Li,

Please find enclosed our revision of the manuscript "Conditioning cortisol in humans: design and pilot study of a randomized controlled trial" (PAFS-D-18-00150). We would like to thank the reviewers and editor for the positive remarks and helpful comments. We have addressed all of their points to the best of our abilities. Attached you will find a point by point response to the comments, as well as the revised manuscript. All changes made in the manuscript are highlighted, and the manuscript and all figures were adjusted in accordance to the style of your journal. We hope that you will consider our revised manuscript suitable for publication in your journal.

Sincerely, on behalf of all co-authors,

Judith Tekampe, corresponding author

Point-by-point response to reviewers:

Reviewer #1:

I have no comments.
Thank you very much for reviewing our manuscript. We are delighted to hear that you are satisfied with the manuscript as is and recommended to accept it without further adjustments.

Reviewer #2:

This is an intriguing study, aimed at conditioning of cortisol responses in humans. As such, I can see numerous applications in both basic research (improved understanding of the HPA axis) and clinical research (stress-related conditions known to go together by HPA axis dysregulation). The paper is well-crafted, and the design is logical. The pilot study is of great interest for this endeavor, as it indicates that the study is feasible. I have some questions that the authors could address in a revised version of their manuscript:

Thank you very much for your compliments about our study and the questions you raised and valuable suggestions you provided for improving our manuscript.

1. The background section is brief and succinct, but I think it would be important to outline in a few sentences what the basic and clinical research applications of being able to condition cortisol responses could be. If the authors think that the background section is not an ideal place for that, then this should be picked up in the discussion.

Thank you for this suggestion. We agree that the possible applications of successful cortisol conditioning for clinical practice and further research could be described in more detail. We have addressed the possible clinical applications of conditioning cortisol more directly in the Background section (lines 85 to 88) and added a brief paragraph with concrete suggestions for clinical applications and further research in the Discussion section of the manuscript (lines 401 to 409). As at this point, it is still unclear whether cortisol can be conditioned in humans and possibilities for clinical applications and further research are thus rather speculative, we opted to place the more detailed description in the Discussion section rather than the Background.

Background (lines 85 to 88): “Successful conditioning of cortisol might thus not only provide new insights into the central regulation of the HPA axis, it might eventually also provide new ways to address dysregulation of the HPA axis in clinical settings, where it may become a valuable addition to existing treatment options for stress-related disorders.”

Discussion (lines 401 to 409): “If cortisol could successfully be conditioned, this would be of conceptual relevance, showing that Hypothalamic Pituitary Adrenal axis regulation can be influenced by associative learning processes. It would also provide opportunities for future research and clinical applications. As cortisol is a key stress-regulatory parameter and HPA axis dysregulation might play a role in stress-related disorders, cortisol conditioning could be investigated experimentally in patient populations, possibly using a stress-inducing challenge relevant and appropriate for the specific group (e.g. exposure to a phobic stimulus). Not only could this provide further insight into mechanisms underlying stress-related disorders, it may also identify cortisol conditioning as a valuable addition to existing cognitive-behavioral treatments of stress-related disorders.”
2. Not all readers will be familiar with the substances that are being mentioned in the text (e.g. sumatriptan). This should be briefly explained.

More important, though, is that the rationale for selecting hydrocortisone should be mentioned in the background section. It does not necessarily follow from the literature summary that this is the ideal choice.

Thank you for pointing this out. To aid the readers’ understanding of the substances used as unconditioned stimuli in previous studies, we added brief explanations about sumatriptan (line 92), dexamethasone (line 93) and corticotrophin releasing hormone (line 97), focusing on their effects on cortisol.

Background (lines 91 to 100): One study has found conditioned cortisol decreases after conditioning with sumatriptan, which inhibits the release of cortisol [20]. However, another study, in which the glucocorticoid dexamethasone, which inhibits the release of cortisol, was used as UCS has led to inconclusive results, showing a statistically significant interaction effect across groups (conditioned vs. placebo control) and measurement (evocation vs. no evocation), but post-hoc test remained statistically non-significant [19]. A third study did not show significant increases in cortisol and noradrenaline after conditioning with corticotropin releasing hormone, which stimulates the release of adrenocorticotropic hormone and in turn cortisol, although a post-hoc analysis revealed that participants with above-median cortisol levels at baseline did show significantly increased cortisol production as a conditioned effect [21].

We also agree that our motivation to choose hydrocortisone as an unconditioned stimulus could be explained in the Background, rather than the Methods section of our Manuscript. Therefore we transferred the relevant information (line 107 to 112) to the Background section.

Background (line 107 to 112): “Hydrocortisone was chosen as unconditioned stimulus as it is the pharmacological equivalent of cortisol. In the central nervous system, hydrocortisone binds to mineralocorticoid as well as glucocorticoid receptors, while dexamethasone, which was previously used to condition cortisol [19], binds to glucocorticoid receptors only. Also, previous studies using hydrocortisone to manipulate cortisol levels showed promising results with important implications for the treatment of stress related disorders [28-30].“

3. I am not sure whether it is correct to conclude that previous studies have "shown inconsistencies" (page 4) and leave it at that. It becomes clear from the literature summary that these studies had very different aims and rationales. It is thus not necessarily surprising that the findings are "inconsistent". It would probably more to the point if the authors would state that the studies/findings are not comparable.

Thank you for bringing this up. We believe that the studies mentioned are comparable to a certain extent, as they all used a classical conditioning procedure aimed to influence cortisol levels. In the case of Petrakova (2017) and Sabbioni (1997) conditioning cortisol levels was the explicit aim of the study, whereas Benedetti (2003) used conditioning of cortisol and growth hormone levels to illustrate the differential effects of conditioning procedures and verbal suggestions on subjective symptoms (pain) and hormone release. However, we concur with the
reviewer that the mentioned studies are not 1-to-1 comparable as, for example, different unconditioned stimuli were used, which affect cortisol release in different directions and possibly target different regulatory mechanisms. We have acknowledged this in the Background section (lines 100 to 104).

Background (lines 100 to 104): “As the studies addressing conditioning of cortisol so far have all used different unconditioned stimuli, which affect cortisol release in different directions and possibly also via different regulatory mechanisms, it is difficult to compare the obtained results. In general, these studies provide indications that conditioning of cortisol might be possible, without allowing a clear conclusion at this time.”

4. The background section should briefly introduce the reason why the response to stress was examined. It becomes clear later on, but the reader is somewhat surprised at the end of the background section about this part of the design.

Thank you for bringing this to our attention. We agree that the objective of this study to investigate possible effects of cortisol conditioning under stress could be pointed out more clearly to the reader early on in the Background section. Therefore we added an introduction of this objective (lines 82 to 85).

Background (lines 82 to 85): “As the effects of interventions targeting cortisol emerge predominantly under stressful conditions, it would be of interest to investigate conditioning of cortisol not only under basal conditions, but also in response to stress.”

5. Since only women are going to be examined, are the authors going to test them in a specific part of the menstrual cycle? If not, why not? Also, are they going to incorporate BMI and/or smoking as covariates?

Thank you for raising this important question. We chose not to schedule the experimental sessions in a specific part of the menstrual cycle as there are large inter individual differences in the pattern of the menstrual cycles. Also, the influence of menstrual cycle phase on cortisol and other outcome parameters of this study is not clear and the ideal phase for measuring each of the parameters may differ (Strahler et al. 2017). However, menstrual cycle phase of the participants is recorded using the self-reported date of the first day of the last menstruation. Therefore, we will be able to determine if - in spite of randomization - there are differences between the two groups in their menstrual cycle phase during the experiment. Furthermore, other variables known to affect cortisol levels are assessed, including smoking behavior and BMI, so they can be used as covariates. Also, the participants are instructed to refrain from smoking in the two hours before each study appointment. We have mentioned these variables in the Methods section (lines 245 to 248) and acknowledged the fact that menstrual cycle phase will thus not actively be controlled for in the Discussion section (lines 395 to 397).

Methods (lines 245 to 248): “During the screening, demographic characteristics of the participants and variables known to affect cortisol levels (such as menstrual cycle phase, BMI and smoking and perceived stress) are assessed using self-report questionnaires [e.g. Perceived Stress Scale, PSS, 51].”
Discussion (lines 395 to 397): “Menstrual cycle phase, a factor known to affect cortisol responses to stress, is noted, but the experimental sessions are not be scheduled in a specific phase of the menstrual cycle.”

6. I can see that the authors chose to use a 100mg dose of hydrocortisone, because this dose has been shown to lead to marked cortisol increases in other studies. However, do they need to take into account body weight, and if not, why not? What were the reasons deciding against body weight-adjusted doses?

Thank you for this interesting question. High inter-individual variability exists in the timing and extent of the uptake and distribution of orally administered substances. To actively control for volume of distribution, body-weight adjusted doses have been used in conditioning paradigms targeting immune and glycemic responses. However, all studies concerning cortisol conditioning to date have used fixed doses as a UCS. As body weight of the participants is noted during screening, we are able to examine its possible influence on conditioned responses.

7. The collection of sAA has its own intricacies. Some argue that salivary flow rate needs to be measured and controlled for (and some argue that this is not necessary). What is the authors’ stance on this question? Also, how exactly is saliva going to be collected (passive drool, chewing on salivettes, etc.)?

Thank you for bringing this up. We are indeed aware of the ongoing discussion about the ideal measurement procedures for salivary alpha amylase, especially concerning the additional measurement of salivary flow rate. We made a motivated choice for measurement procedures that we deemed most appropriate specifically for this study. In this study saliva is collected using salivettes with cotton swabs and the measurements of the main outcome parameter cortisol and secondary outcome parameter alpha-amylase are derived from the same samples. Salivettes are a well-documented and valid way to collect saliva samples for subsequent cortisol determination (Kudielka et al. 2012). Alpha-amylase is predominantly of interest in response to psycho-social stress exposure in this study. This particular response has previously been shown not to be affected by salivary flow rate (Rohleder et al. 2006). Moreover, as collecting saliva by means of salivettes (or absorbent materials in general) is much faster (Beltzer et al. 2010) and possibly less bothersome for the participants than collecting saliva by passive drool, we deemed the use of salivettes to be more suitable in our study protocol. We added more information about the exact sampling method in the Method section (lines 182 to 187).

Methods (lines 182 to 187): “Cortisol and alpha-amylase are assessed under basal conditions and as markers of the psychophysiological response to stress by means of saliva samples [41, 42] collected with salivettes (Sarstedt, Rommelsdorf, Germany). Participants will be instructed to place the cotton swab contained in the salivette tube in their mouth and move it through their mouth using their tongue for one minute. Participants are specifically instructed not to bite or chew on the cotton swab and not to touch it with their hands.”

8. More details are needed on the assessment of HR and EDA. Are the authors interested in SCR/SCL? Sampling rates?
Thank you for this suggestion. We agree that the measurement of Heart rate and skin conductance could be explained with more detail and have added the requested information in the Method section of the manuscript (lines 191 to 198).

Method (lines 191 to 198): “Heart rate and skin conductance level are assessed as a measure of autonomic activity, using a noninvasive Biopac© apparatus consisting of the MP150 Data Acquisition System and the ECG100C Electrocardiogram Amplifier and the GSR100C module with a sampling rate of 1000 per second. For heart rate recordings, electrodes are applied to the participants’ body employing a Lead-II configuration (one on the chest, one on the ribs; no ground is needed because of the simultaneous recording of skin conductance) and a high pass filter of 0.5 Hertz is used. For skin conductance two electrodes being applied to the skin of the participants’ non-dominant hand. Gain is set to 5μΩ/V and a low pass filter of 10 Hertz is used.”

9. Figure 2 was not incorporated in the review material. I am not sure how long the baseline/accommodation phase is at the beginning. Maybe I overlooked it, but it should be at least 30 minutes.

Thank you for pointing this out to us. We added Figure 2 to the materials submitted with the manuscript. We did not include a standardized accommodation period at the beginning of each appointment, but each appointment begins with a rather extensive explanation of the study procedures and questions about variables that possibly influence the study outcomes (smoking etc.). Furthermore, due to the repetitive nature of the conditioning protocol (same laboratory environment, same experimenter, same measurement procedures) and the extensive behavioral instructions to be observed by the participants before each of the appointments (which are also closely monitored), we believe that effects of novelty of the situation on the measurements taken after the first screening appointment are unlikely. Also, observing an accommodation period of 30 minutes before each of the seven study appointments would add extensively to the time investment requested of the participants, which is already substantial in this study. Moreover, the study protocol is of course the same for both the conditioned and the control group. Tainting of baseline measurements due to a lack of an accommodation period would therefore occur in both groups and not affect the outcome of this study. We have added a brief discussion about possible effects of novelty and anticipation in the Discussion section of the manuscript (lines 377 to 379).

Discussion (lines 377 to 379): “This highly repetitive nature of the study procedures have the additional advantage that all events occurring during the study appointments (with exception of the TSST in the last session) are highly predictable for the participants, and thereby minimize effects of novelty or anticipation.”