Author’s response to reviews

Title: THE EFFECT OF LOW LEVEL LASER IRRADIATION ON OXIDATIVE STRESS, MUSCLE DAMAGE AND FUNCTION FOLLOWING NEUROMUSCULAR ELECTRICAL STIMULATION. A DOUBLE BLIND, CROSS-OVER TRIAL

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Version: 2 Date: 23 Jul 2019

Author’s response to reviews:

Dear Editor,

Dear Reviewers,

Thank you very much for the thorough analysis of our manuscript, for valuable and helpful comments and for giving us the opportunity to revise our submission. We hope our replies and explanations, as well as the amendments to the manuscript (highlighted in yellow) will be satisfactory. The report and the manuscript improved thanks to your support.

In the following, please find our answers to your comments.

Reviewers’ comments:

Angus Lindsay (Reviewer 1):

Major comments:

1. There is concern over the length of time between the crossover. The referenced paper (Mackey et al. 2008) shows muscle damage following isometric contraction significantly elevates CK activity up to seven days post-exercise. While the authors have stated that eight days is a long enough washout period based on their own preliminary studies, the current study design did not
measure MVC or CK activity beyond 96 hours and it looks like there are differences between interventions at baseline even though it is a crossover design. Therefore, the eight-day duration between bouts may not allow full recovery and probably affected the response of the second bout to injury.

Response:

Please see in the table below CK and MVC values in the whole group (n=24), without dividing it into LLLT and sham-LLLT interventions (part I intervention and part II intervention, as provided in Figure 1):

<table>
<thead>
<tr>
<th>Study period</th>
<th>CK [x (SE)]</th>
<th>MVC [x (SE)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (intervention part I)</td>
<td>244.9 (55.5)</td>
<td>258.95 (18.54)</td>
</tr>
<tr>
<td>II (intervention part II)</td>
<td>194.9 (33.5)</td>
<td>264.29 (21.61)</td>
</tr>
</tbody>
</table>

After analysing individual data (of each participant), the differences between sham-LLLT and LLLT interventions (although they were not statistically significant), as shown in Figure 4 (previous version of the manuscript; (Figure 5AB in the revised manuscript), were rather due to slightly higher levels of CK in some of the participants at the beginning of the study (and in part I rather than in part II of the study- after eight days of washout, whereas only higher CK values in part II of the study would allow to conclude that the time of washout might not be sufficient). Therefore, we cannot assume that the washout period was insufficient to prevent the carryover effect. Nonetheless, we are aware that we were not able to fully control the activity of the participants before the study and between study periods (part I and part II). They were instructed to avoid any intense physical activities before the experiments (NMES sessions). However, we were not able to eliminate all the factors that could affect the parameters studied.

The duration of the washout needed for full recovery may depend, among other issues, on the intensity of physical activity. Our participants, who were moderately active, and they responded to a single NMES session with a moderate increase in CK activity compared to other authors' studies (which we described in the manuscript, lines 3376-3498). Mackey et al. [ref. 8 in the manuscript] found a significantly elevated CK activity up to seven days post-exercise, but they administered a 30 min. muscle stimulation in untrained men. On the other hand, Zorn et al. [36] observed only a minor elevation in circulating CK activity following electrical stimulation of both quadriceps muscles in trained participants. Therefore, we believe that our decision as to the duration of the washout period was rightly made, based on our pilot study.

Finally, we intended to introduce a sufficient washout period in our study but not to separate the study periods (bouts) with a too long break in order to avoid alterations in the characteristics of the subjects (e.g. health status or confounding factors that we could not control [references 21, 22, 24 in our manuscript]).
2. The statistical analyses have been conducted on absolute values. Given the MVC between the two groups at baseline looks to be large (~240 vs 280 Nm) and indicative of a group effect, it would be interesting to know whether the same statistical differences provided are also evident when compared as a % change from baseline in all parameters.

Response:

Similarly to the CK response (please see our reply to comment 1), the differences might be influenced by the differences in the activities of the participants between the study periods. The students were asked not to change their lifestyle during the experiment and to refrain from intense physical activity during the study course (lines 124-125 in the original submission). Nonetheless, this is true that one can never exclude the role of known and unknown confounders from the experiment. Also, the characteristics of both NMES, and MVC measurement procedures, based on the participants’ self-reported pain threshold and motivation, are prone to some variations, even in the same subjects. For more clarity, we have addressed these issues and supplemented the ‘Strengths and limitations of the study’ section (lines 42930-4321 in the revised submission) with further elaboration:

Furthermore, the characteristics of both NMES, and MVC values, dependent on the participants’ perceived pain threshold and their motivation, are therefore prone to some variations, even in the same subjects.

Considering the above issue, as well as the Reviewer's comments, we attempted to analyse MVC data starting from an average baseline MVC value (all baseline values from part 1 and part 2 of the study - with no allocation to interventions) and standard deviation. Then, MVC results in subsequent time points after NMES were analysed separately in LLLT and sham-LLLT interventions (normalized on the average baseline MVC). As in original analyses (Figure 3 in the original and in the revised manuscript), only time main effect was seen (without significant differences between interventions; Figure 4 B of the revised manuscript). The same applies for percent changes in MVC, in comparison to baseline (Figure 4 A in the revised manuscript).

3. The statistical analyses conducted on the repeated measures is questionable. Post-hoc analyses are only to be used when a main effect is observed. T-tests can be used if an interaction is found. Because a two-way ANOVA was conducted and no time, group or interaction effect was measured, then I am unsure where the authors have got their statistical values from. For example, for TAC and TAC-UA in Table 3, no time, intervention or interaction effect is found. Therefore, post-hoc analyses cannot be conducted. So where has the statistical values presented in the table come from?

Response:

We agree with the Reviewer. Accordingly, we have revised Table 3 (now it is Table 4) and the description of the results (lines 2598-2654, 2843-2865), and presented only significant main effects.
4. The inference that LLLT had an effect on several indices of muscle damage, redox status and inflammation is based on statistics that were not conducted correctly. Furthermore, the authors make several inferred statements about the possible protective effect of LLLT before stating that no difference existed between groups. It is suggested the authors re-evaluate their discussion based on statistical analyses. For example, there was no intervention effect for changes in MDA concentration, yet the authors have detailed the possible benefit of LLLT on lipid peroxidation.

Response:

As suggested by the Reviewer, we have revised our discussion based on statistical analyses (lines 3743-3810; 4021-4056).

5. The authors need to consider the design or at least comment on the design with regard to the repeated bout effect. Because a single bout of exercise can provide protection from a subsequent bout of the same exercise (normally eccentric contractions), it is possible the crossover design here did not account for this effect. The authors might consider presenting or analysing data from the group as a single parallel design and/or as percent changes from baseline for each group.

Response:

We considered presenting data from two groups as a single parallel design. However, after analysing data of MVC and CK in parallel subgroups (in both part I and part II of the intervention), we gave up this idea, because their interpretation does not change, and it does not bring anything new other than what already results from crossover design.

Also, we have analysed changes in CK in the whole group in part I and part II of our study (without dividing on LLLT and sham-LLLT), as well as in group A (LLLT in part I vs. sham-LLLT in part II) and group B (sham-LLLT in part I vs. LLLT in part II of the study), separately. Any significant main effects were observed. However, we have to admit, that taking into account slightly higher (as seen above in the table placed in the response to comment 1) mean values of CK in the whole group in part I than in part II of the intervention (although differences between part I and part II were not statistically significant in any time-point), the repeated bout effect cannot be excluded. It has been stated in the revised manuscript (lines 3421-3432).

The repeated bout effect may be a serious potential confounder. It can be considered as a case of a period effect, which is among methodological problems specific for the crossover trials [references 22, 24, 25 in our manuscript]. We are address this issue in more detail in the answer to the Reviewer’s minor comment 6. We cannot definitely exclude the period effect from our study. Therefore we have enhanced the “Strengths and limitations of the study” section with the following statement (lines 4321-4345):

The study addressed a short, single intervention in healthy, stable participants. The subjects were already familiar with the NMES intervention and we provided an eight day the interval between study periods. Nonetheless, we cannot definitely exclude period effects, problems specific in
crossover designs, such as the learning effect or the repeated bout effect, as potential confounding factors.

6. The article requires grammatical consideration throughout for better readability.

Response:

We have advanced the paper accordingly.

Minor comments

1. The authors should consider plotting the torque tracings of the NMES intervention.

Response:

We thank the Reviewer for this suggestion. However, we have already added three figures to the manuscript, presenting the results of the study (relative changes in MVC, Figure 4 A and B, as well as relative changes in CK, Figure 5 B in the revised submission), and the text is also long (above 6.000 words of the manuscript), plus four tables, seven figures, some of them large, and references). The considered figure would add to the description of the procedure, nonetheless we hope that we have already provided sufficient information as to the intensity of NMES (lines 234-236; 303-307). The provided EECs and the description of the manoeuvre (such as pain threshold as the maximum for muscle contraction stimulation and the number of contractions) is in our view satisfactory to demonstrate the procedure. We hope the Reviewer will find our explanation satisfactory and acceptable.

2. Line 62 - lack of desmin staining? Do you mean low protein levels of desmin following contraction?

Response:

Mackey et al. (2008) [8] showed a single fibre lacking staining for desmin (demonstrated loss in desmin immunoreactivity). This “lack of desmin immunoreactivity” is considered as immuno-histochemical evidence of muscle damage 48 hours after electrically stimulated isometric muscle contractions in humans. However, it cannot be explained as low protein levels of desmin only, since, as suggested in that paper, it may occur due to a reorganization in the myofibers or due to proteolytic digestion of the protein. Hence, we have changed “lack of desmin staining” to “loss in desmin immunoreactivity” (line 67).

3. Line 68 - why are oxidant/antioxidant status among top priorities?

Response:
Many authors attempt to explain the mechanisms underlying muscle damage induced by electrically evoked isometric contractions, but they remain speculative [ref. 6 in our manuscript]. It has been suggested that the mechanisms of muscle damage induced by eccentric voluntary contractions and by electrical stimulation are similar to some extent. Among many different factors, reactive oxygen species are known to be involved, directly or indirectly, in not only induction and progression of muscle damage, but also in recovery processes [6]. The important role of reactive oxygen species in muscle damage and function is confirmed in animal studies, in which inhibition of ROS production and diminishing oxidative damages (lipid peroxidation) resulted in an increase in maximal isometric force after repetitive electrically evoked contractions [Ryan et al., 2011]. Thus, measurements of oxidant/antioxidant status may be useful in the evaluation of the intensity of oxidative stress and its potential role in exercise-induced muscle damage and in muscle function.


4. Line 77 - what are vague findings? Subject number? Statistical analysis?

Response:

Our intention was to inform the readers that only one publication in peer-reviewed literature addressed this issue and that it was a pilot study. Nonetheless, we did not perform any critical appraisal of the methodological quality of this study. Thus, we have rewritten the sentence as follows:

As regards LLLT application for preventing or reducing delayed onset muscle fatigue and soreness following NMES, only a single, pilot, clinical study [20] was reported, with vague findings as to the association of LLLT and knee extensor torque following NMES.

Only a single, pilot, clinical study [20] addressed LLLT application for preventing or reducing muscle function following NMES. The authors reported that LLLT did not attenuate muscle fatigue evoked by NMES. Nonetheless, the study was performed in only five subjects, who received LLLT prior to a 3-minute session of NMES of the quadriceps muscle. Therefore, those findings need to be interpreted with caution.

5. Line 79 - no need to BOLD your objective

Response:

We have removed bold font.
6. Line 103 - what is the period effect?

Response:

We have addressed the period effect (specifically in lines 103 – 106 of the previous version of manuscript) as it is one of the aspects of crossover trial design critical to the potential risk of bias in the findings and interpretation. It is a potential phenomenon similar to the carryover effect, regarding the characteristics of participants in the subsequent periods (stages) of the study. The carryover effect is when the treatment effect from one period persists and has a residual effect into the subsequent period [ref. 21, 22, 24 in our manuscript]. The period effect, on the other hand, is likely in people with underlying medical conditions, whose characteristics could be instable throughout the study [24]. Also, if the intervention would influence the characteristics of the participants, the period effect needs to be considered [21, 22]. The learning effect is also assumed as an example of a period effect [Wellek S, Blettner M. Dtsch Arztebl Int 2012; 109:276-81].

In our study, the participants had already been familiar with both NMES and MVC measurements. The administered interventions did not have any prolonged effects. The treatment period was very short (a single intervention). For that reasons, in addition to the arguments already formulated in the paper (lines 103 - 106), we assumed that the period effect could not influence the study results and that the crossover design was appropriate in our study.

Indeed, as regards methodology, period effect is mentioned in only about 18-23% of the reports of cross-over trials [22, 25] and is often ignored in method quality analyses [24, 25].

7. Line 118 - abstract says 20-23 year olds.

Response:

These values in line 118 are given correctly (also in Table 1). The mistake occurred in the abstract. We have corrected it accordingly.

8. Line 179 - Did subjects complete a VAS after the squat as well?

Response:

At the end of the sentence (lines 180 – 181 in the previous version of manuscript) we stated ‘…and the assessment was repeated in the same manner’.

Nonetheless, for better clarity, we have rewritten it as follows:
Afterwards, standing with their feet spread shoulder-width apart, the subjects performed a squat slowly to 90° knee flexion and returned to the starting position. Then, the muscle soreness assessment was repeated in the same manner (lines 196-198).

9. Line 224 - These data would be better presented in a table.

Response:

We have placed these data in Table 3 in the revised manuscript.

10. Table 3 (GPx) - All the values seem to be consistent for this measurement except for 48 hours in the sham-LLLT group. Can this potentially be explained by something other than the intervention? It seems unusual that it suddenly drops and rebounds again 24 hours later.

Response:

We do not find any other explanation for this drop in GPx activity (and also in SOD activity) at 48 hours after NMES in sham-LLLT intervention, besides its relationship with excessive damage caused by free radicals, as confirmed by the simultaneous increase in plasma MDA as a marker of oxidative damage of lipids by free radicals. This issue has been explained in the discussion (lines 3643-3732 in the revised manuscript).

11. Line 256 - CRP did not change compared to baseline until 96 hours so the language "increased throughout" should be changed.

Response:

We agree with the Reviewer. The text has been changed accordingly (lines 2821-2832).

12. Line 312 - there is minimal evidence of secondary damage to muscle (see Warren et al. Minimal evidence for a secondary loss of strength after an acute muscle injury: a systematic review and meta-analysis)

Response:

In fact, Warren et al. reported minimal evidence to support a statistically significant secondary strength loss after muscle injury. However, they also found that “the mean immediate post-injury strength loss among the 223 studies varied substantially, and one might posit that greater initial injury would coincide with the appearance of a secondary strength loss”.

We did discuss (lines 309 – 316 of the previous version manuscript) the role of inflammatory cells and ROS in secondary muscle damage. In contrast, Warren et al., do not consider
secondary muscle damage. On the other hand, they discuss a scenario in which secondary injury occurs but a secondary strength loss does not, since the mechanisms of injury, and the recovery, are not homogenous, either temporally or spatially, within the muscle. The scenario of having a secondary injury without a secondary strength loss is analysed among practical and clinical implications of their review).

Finally, apart from Warren et al., secondary muscle damage and strength loss appear to be confirmed by our results, taking into account the relative changes in CK and MVC after NMES (Figures 4A, 4B and 5B in the revised manuscript).

13. Line 312 - "increased ROS production" - the authors never actually measured ROS production

Response:

In fact, we did not directly measure ROS production. However, the intensification of lipid damage caused by free radicals (confirmed by the increase in plasma MDA levels) may be due to increased production of ROS. Following this comment, we have added this explanation in the revised manuscript and we have changed the interpretation of our results in more cautiously (lines 3543-35960).

14. Line 315 - When was muscle fatigue measured in this study?

Response:

Please see our reply to comment 18. It also addresses comment 14.

15. Line 317 - Because no time or intervention effect was observed, the authors should limit the inference that LLLT might affect the antioxidant system.

Response:

We fully agree. We have removed this paragraph from the discussion section in the revised manuscript (lines 3743-3810).

16. Line 327 - can the authors provide a reference for this please.

Response:

The reference [Ribeiro et al, 2016] addresses the whole sentence. We hope it can remain unchanged.
17. Line 325 - 333 - This paragraph goes back and forth between satellite cells, muscle repair and ROS before circling back to the antioxidant defence system of this study. Can the authors please adjust the structure for readability please.

Response:

We have reorganised this paragraph for better readability (lines 3821-3932).

18. Line 357 and 375 - similar to above, muscle fatigue was not measured...or at least not presented.

Response:

The Reviewer is right. Indeed, we did not directly measure muscle fatigue. A more relevant parameter is myoelectric activity of particular muscles, as measured by sEMG. It is, however, useful to continuous monitoring of local muscle fatigue during performance of certain work [Cifrek et al., 2009]. Also, muscle fatigue is defined as a decrease in maximal force or power production in response to contractile activity [Gandevia, 2001]. Based on that, in our study, the decrease in MVC immediately after NMES may be considered as muscle fatigue. However, we used this term (muscle fatigue) also to describe changes in MVC in subsequent time-points. We admit that this was an over-interpretation. Describing the effect of LLLT on MVC, we have changed the expression ‘muscle fatigue’ to ‘recovery of muscle function’ throughout the revised manuscript.


19. Line 376 - This statement is not corroborated by the statistical analyses. See comment in "major comments" section.

Response:

The statement “Also, our results suggest the beneficial effects of LLLT against oxidative stress and inflammation, induced by a single session of NMES, that probably result from preventive impact of LLLT on antioxidant enzymes”, was indirectly supported by our results, since significant time to intervention main effect was seen in case of CRP, as well as significant intervention effects were seen in case of antioxidant enzymes activities (i.e. GPx and SOD). Nonetheless, we did not observe any main effect of intervention in case of MDA, as the marker of lipid peroxidation. However, it should be mentioned that we measured only one parameter of free radicals-induced damages. Thus, the impact of LLLT on other markers (applying also for
free radicals-induced damages of proteins or DNA, not only lipids) should not be excluded. On the other hand, it has been suggested that the activity of antioxidant enzymes in blood also change in oxidative stress conditions [Finaud et al., 2006]. It is also indicated that exercise-induced changes in the activity of antioxidant enzymes may occur in two directions, i.e. increase in their activity resulting from adapting to moderately increased production of free radicals, as well as decrease in enzymatic activity in long-term or more intensive oxidative stress [Finaud et al., 2006].

According to some authors [Tauler et al., 2005; Trofin et al., 2014], the loss of antioxidant activity following physical exercise may be explained by an exercise-induced oxidative damage to proteins that modify catalytic activity of the enzymes. Therefore, the impairment of antioxidant system, observed in sham-LLLT intervention, 48 hours after a single session of NMES, might be associated with partial inactivation of enzymatic proteins, resulting from their allosteric or covalent modifications induced by reactive oxygen species. Thus, the decrease in antioxidant potential was probably reflected by accumulation of ROS generated (although it was not directly measured in our study) in response to exercise, which in turn resulted in progressive oxidative damages (as indicated by the increase in MDA, 48 hours post-NMES, seen in sham-LLLT intervention).

We have added the above explanations in the discussion section (lines 3643-37069). We have also adjusted the conclusions based on the changes in the markers measured directly and the reported significant main effects (lines 4454-4465).


John Dixon, PhD (Reviewer 2):

I thank the authors for submitting this manuscript which I enjoyed reading and which contains some very interesting data. The manuscript is generally written in a very clear and informative manner. However I have some comments, generally about clarity of results and inferences made.

ABSTRACT:

This was nicely written and clear. Perhaps you could clearly articulate the aim here.
Response:

Thank you for your suggestion. The aim has been articulated in the revised paper, accordingly.

BACKGROUND:

This is written in a very clear and logical manner. It summarises the rationale and the findings of previous studies that have examined the topic, and clarifies the gap in knowledge being addressed.

P 3 para 1 could the authors state briefly what the presumed underlying mechanism is by which LLLT produces potential benefits. My only other comments in this section would be purely stylistic; to change "objective" to "aim" (p3 l 75); to move the last paragraph/sentence (p3 L85) which sits somewhat in isolation, and place it in the previous paragraph with the aim/objective.

Response:

Thank you for this comment. We have added a brief description of presumed underlying mechanisms, by which LLLT produces potential benefits (lines 78-82).

We have changed the term "objective" to "aim" (p3 l 93) and moved the last paragraph/sentence (p3 L98-99) accordingly.

METHODS:

Again this is very clear and logical overall.

P 4 Participant section: Sample size - please could you just clarify what the required size actually was, and what parameters fed into that, where appropriate (e.g. power, alpha, detectable difference) for anyone such as myself used to more "traditional" estimations of sample size. The fig 1 contains 30 enrolled and 24 randomised.

Response:

For an assumed test power of 0.9, the minimal sample size was 23. This information has been added in the methods section, lines 137-138.

I am unclear on why SE is used for descriptive data (see below).

RESULTS/DISCUSSION
It is stated that results are presented as means and SEs. Variability for descriptive data should always be presented using SDs. Please could you amend that. A number of papers present the rationale for this such as: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3487226/

Response:

We are aware that SDs should be used to express variability and we did not use SE instead of SD to express variability of data. The use of SD is especially important in studies with parallel designs, when two different groups are compared compared [Barde MP, Barde PJ. What to use to express the variability of data: Standard deviation or standard error of mean? Perspect Clin Res. 2012;3(3):113-6. doi: 10.4103/2229-3485.100662].


As recommended [Barde, 2012], the use of SE should be limited to inferential statistics where the author explicitly wants to inform the reader about the precision of the study, and how well the sample truly represents the entire population. And that was our intention.

In addition, as known CK values show great variability, and athletes with chronically low CK serum levels (low responders) have low variability when compared with those who have higher values (high responders) [Brancaccio P, Maffulli N, Limongelli FM. Creatine kinase monitoring in sport medicine. Br Med Bull. 2007;81-82:209-30]. Given the high variability of CK in population, in this particular case the use of SE may be better solution to visualize the results, while at the same time making it possible to assess the variability.

The use of SE in our study was prompted driven by the need to compare our results with corresponding studies, where SE was also presented, and also:


3. Antonialli FC, De Marchi T, Tomazoni SS, Vanin AA, dos Santos Grandinetti V, de Paiva PR, Pinto HD, Miranda EF, de Tarso Camillo de Carvalho P, Leal-Junior EC. Phototherapy in skeletal muscle performance and recovery after exercise: effect of


However, following the input from the Reviewer, in Table 4 (with biochemical parameters) we have placed range, to express variability (as also typically provided in other studies). That is why we have also placed data of CRP from Figure 5 (original submission) into Table 4 (revised manuscript).

In case of main parameters presented in Figures (MVC, CK), also following the comment of Reviewer 1, we have added figures with relative changes (percent changes in MVC and CK as compared to baseline values, Fig. 4A and 5B, respectively). Additionally, because of difference in baseline MVC between LLLT and sham-LLLT (although statistically nonsignificant), taking into account that it was the same group of participants, we calculated the changes in both interventions separately, but compared to mean baseline and SD from the whole group (LLLT and sham-LLLT). These changes have been expressed in 1/SD (Fig. 4B).

Results 1st para (L217): please clarify which phase is A and which is B.

Response:

This sentence is wrong. It should read “in the part I and part II of the study, respectively”. It has been corrected (line 235). Thank you for this remark.

Intensity/MVC (L216) : this para should be written more clearly. There is a mix of %MVC, and reference to Fig 3 which is in Nm. It does not look, as presented, that the sham "decrease in MVC…..seen throughout the recovery period" stated in the text, matches the data in Fig 3. Perhaps this is just a lack of clarity in the wording of "throughout", but it seems to increase between 0 and 24 hours.

Response:

We provided (only in the text) the values of EEC of the sixth and the last contractions as expressed in % MVC to present real intensity of NMES. These data was not presented in Figure 3. Figure 3 regards the changes in MVC [Nm] and it is described later in the text.
We stated that in the sham-LLLT intervention the decrease in MVC was seen throughout recovery period, because in each time-point post-NMES (i.e. at 0, 24, 48, 72 and 96 hours) MVC was significantly lower than at baseline (p<0.05). As suggested by the Reviewer, MVC (in absolute values) appears to increase between 0 and 24 hours (as shown in Figure 3). However, in sham-LLLT condition, the change at 24 h was not significant in comparison to 0 h. To be more precise, MVC decreased at 0 h, and it still remained lower throughout the recovery period, as compared to baseline. Nonetheless, we agree that this sentence might mislead the reader and have amended it. Following the Reviewer’s comment, we have re-written this part of the text (lines 237-238…). Also, as mentioned above It should be mentioned, that we have analysed relative changes in MVC (from baseline). In fact, as seen in Fig. 4A and 4B (added in revised manuscript), in sham-LLLT intervention, this parameter increased significantly increase in relative changes in MVC (Fig. 4A and 4B added in revised manuscript) was seen at 24, 48 and 72 hours after NMES (as compared to 0 h), and then, it significantly decreased at 96 hours (as compared to 48 and 72 hours).

Please could you clarify for the non-statistician or non-crossover expert, how/why there is a marked baseline between-group difference in MVC (fig 3) in a crossover study when it is the same subjects acting as their own control. Perhaps this just needs some minor rewording but it looks like the baseline difference between groups is bigger than any post-treatment group difference. If that is true, please clarify what is means.

Response:

Please see the explanation to comment 2 of Reviewer 1

Soreness (L230): change "insignificant" to "not statistically significant" if that is what is inferred.

Response:

Thank you for this remark. The text is corrected (line 24950).

DISCUSSION

To help the reader, I suggest start this section with a summary of the main findings. Currently it starts with a discussion of the level of stimulation.

Response:

Thank you for this point. We have improved the discussion section accordingly (2976-3021).

Broadly I think the textual results could be presented more clearly. It seems difficult for the clinical reader to assess and understand the results as written. Could this be improved?
Overall this reads well but my comments above re the Results need taking into consideration.

Response:

We have modified the Results taking into account your comments.

Once more, we would like to thank the Reviewers for their important comments and for their input to our report.