# OPEN PEER REVIEW REPORT 1

**Name of journal:** Neural Regeneration Research
**Manuscript NO:** NRR-D-22-00293
**Title:** Metformin promotes angiogenesis and functional recovery in the aged mice after spinal cord injury by AMPK/eNOS pathway
**Reviewer's Name:** Úrzula Franco-Enzástiga
**Reviewer's country:** USA

## COMMENTS TO AUTHORS

This report describes a comprehensive series of experiments investigating the effects of metformin in mice undergoing spinal cord injury. The authors showed that metformin enhances the recovery of neurological functions, tissue repair and angiogenesis after spinal cord injury through the activation of AMPK/eNOS pathway.
Although this study will be an important contribution to the field, the authors could improve the paper by addressing issues related to statistics, experiments, results presentation and discussion. Specific major and minor points are detailed below.

Major points
1) The reviewer found a serious issue in the statistical analysis. The authors claimed that data were analyzed via one-way ANOVA followed by Student's t test for pairwise comparisons. As far as the reviewer knows, ANOVA cannot be followed by student t test. The authors will need to address that issue. The reviewer wonders if that is the reason why in Figure 5E there is a statistical difference even if the bars look very similar. Additionally, it would be better if the data are expressed as mean ± SEM instead of SD.
2) This paper must be re-written in an intelligible fashion. There are many mistakes in all the sections.
3) In figure 1, panels A and B: I recommend that, in addition to different colors, the representation of the different groups is made using different shapes (for instance triangles, circles and other shapes). The current representation in the paper does not allow to see the orange squares (18 months sham group), or wasn't it represented at all in the graphs? The authors need to clarify that.
4) Figure 1, panel C: This graph seems to be upside down. Generally, in pain journals, withdrawal threshold is expressed in grams, but the Y axis goes from 0 to 1.5 g, being ~1.5 g the withdrawal threshold of naïve or sham mice. Taking that into consideration, if the Y axis is the withdrawal threshold expressed in grams, as it is stated in this paper, then why is 1 g the withdrawal threshold of sham animals, while the injured animals have higher withdrawal threshold? This panel is confusing as it is represented. The authors need to clarify this. Additionally, in the results section, it was also stated that aged mice had higher threshold post SCI compared to young animals. If I am understanding the paper right, aren't injured aged animals more sensitive to tactile stimuli compared to young ones, and thus their withdrawal threshold should be lower compared to young animals?
5) Figure 2, panel C (sham 2 months) is not representative of the graph. Explain the reason why young sham animals have a higher level of Ki-67 immunofluorescence compared to old animals according to the picture shown.
6) Figure 2. In order to adjudge the changes in the levels of ANG-1, VEFG, BDNF and CTGF to SCI, the authors need to show the expression of mRNA in sham animals 2 months and 18 months and compared them with injured animals at both ages.
7) Provide discussion in regard to ANG-1, VEFG, BDNF and CTGF. They were not mentioned.
8) If metformin pathway provides vascular regeneration through AMPK/eNOS, it would be worth adding the microvascular 3D visualization parameters after metformin administration and Compound C.

9) Provide the rationale for the use of the selected doses of metformin and compound C. Provide details about the administration scheme. The reviewer wonders if the drugs were administered at the same time. On the other hand, compound C has been shown to induce apoptosis in the context of cancer. Thereafter, the authors need to provide rationale of the doses used. How many hours after metformin and compound C were BMS and tactile allodynia measured? Where these two evaluations performed in independent groups of animals?
10) Figure 4 shows the concentration of metformin in mM while the text (results and methods section) says μM.
11) The reviewer thinks that 8G is interesting because compound C seem to be changing the tissue arquitecture. The reviewer would like to see the histology of compound C alone in supplementary materials.
12) In the discussion section it is mentioned that there is a regression of angiogenesis after SCI. In the figure 6B, the quantification of vessels did not show that decrease. The authors should address that point.
Minor points
In key words:
1. remove the word "the" from the aged mice.
In the introduction (paragraph 3 and 4):
2. Martin-Montalvo et al., 2013 wrote that metformin has been used since 1960 for the treatment of type 2 diabetes. He did not write invented. Authors should correct that.
3. Remove the word meanwhile (Meanwhile, the fact that metformin treatment…).
4. The reviewer suggests the used of "additionally" or "moreover" instead of what's more.
5. In the phrase: its extensive biological effects have also manifested in central nervous system, such as Parkinson's disease. Change manifested for: shown
6. Mention in the text what does BMS stands for.
7. In the phrase: further attaching the importance of the potential therapeutic effect of metformin on SCI. The reviewer suggests the change of the word attaching to reinforcing.
8. Rewrite this sentence, it is confusing. Our present study may pave the way for working mechanism of therapeutic effect of metformin on the aged mice after SCI.
In the section materials and methods:
9. In the title: Construction of traumatic SCI model in mice and experimental design, delete the word construction.
10. Separate numbers from the units. For instance in this phrase: Metformin was diluted to achieve a final concentration of 20mg/ml. This is a common mistake that is repeated many times in the manuscript.
11. Indicate what does BMS stands for.
12. Provide detailed information regarding the electrophysiology experiments. Details about the transcranial stimulation pulse and how many pulses were averaged to obtain this trace are lacking. On the other hand, authors did not mention that they also measured MEP after 2 months (Methods). Authors should add that detail.
13. In the phrase: In brief, after anesthesia, induced electrodes were set up on the skull which represents the area of cortical motor center… Does induced means stimulating electrode? Additionally, change those words for: on the skull area corresponding to the motor area of cerebral cortex.
14. Rewrite this sentence: Transversely cryo-slices at 16 μm were obtained.
15. Does neck dislocation refer to cervical dislocation?
16. Report which endothelial cell medium was used.
17. Report what did the western blot lysis buffer contained. Which chemiluminescence kit did you use? Those details have to be reported.
18. qPCR number cycles, annealing temperatures and specific reagents used need to be added to the

methods section.

In the results section:

19. In the paragraph 1: Similarly, the area of sparing tissue was significantly less in aged spinal cord compared to those young SCI mice (Figure 1I). My comment: Figure 1I is of injured area, not spared area. Separate the number from the word (for instance separate 2months Sham) in figure 1.

20. In the phrase (paragraph 1): At the meantime, this method used here allows to quantitatively describe the difference between the young and aged spinal cord blood vessel network and provides more accurate quantifications data of the vascular networks. Remove at the meantime.

21. In the phrase (paragraph 1): 3D computational analysis of hierarchical image allows characterization. What does hierarchical mean?

22. Metformin promotes neurological functional recovery and tissue repair after in the aged mice after SCI. Remove the word after shown in italics.

23. Figure 6F: provide an explanation of sham animals with a value of almost 2 considering that in the other blots the same group has a value of 1. Were the values normalized?

24. In the discussion, authors could reorganize this statement: In our study, we found that upregulated AMPK/eNOS pathway in the presence of SCI and in SCMECs may be responsible for the metformin-based therapy for angiogenesis, as indicated by the Western blot results.