Author’s response to reviews

Title: Fasudil improves survival and promotes skeletal muscle development in a mouse model of spinal muscular atrophy

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Author’s response to reviews: see over
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Dear Dr. Lin Lee
Senior Editor, *BMC Medicine*

We thank you for your review of our manuscript entitled: “Fasudil improves survival and promotes skeletal muscle development in a mouse model of spinal muscular atrophy”. We are pleased to see that all of the reviewers are positive about the work. We hereby submit the revised version where we have addressed all of the concerns raised by the reviewers.

**REVIEWER #1**

**Comment #1:** The experiments are properly done and very convincing. The results are strongly discussed. There are no minor or major points, which need to be addressed.

We thank the reviewer for her positive feedback on our work.

**REVIEWER #2**

**Comment #1:** On page 4 the authors reference the differences between the two SMN genes citing an article by Lorson et al, 1999. It would be appropriate to also reference an article from the Burghes group (Monani et al, 1999) that documented precisely how many differences there are between the two genes. It would also be appropriate to cite the article by Coovret et al, 1997 later in the paragraph when referencing SMN2 copy number and disease severity.

The references mentioned above have been added to the specified text.

**Comment #2:** Figure 1 – The legend suggests 5 cohorts of mice whereas only three curves are apparent on the graph. It appears that the remaining two curves do not show up on the graph because they are masked by the Smn2B/+ (vehicle) curve. If so, it would be useful to indicate this in the legend.

Indeed, the WT and Smn2B/+ (Fasudil) curves overlapped with the Smn2B/+ (vehicle curve), seeing that all groups display 100% survival. We have reformatted Figure 1A to enable the visualization of all three curves.

**Comment #3:** Figure 1 – It is indicated that a treated animal perished because of dystocia. Does this mean that treated Smn2B/- mice are able to breed despite their severely compromised body size.

Indeed, to our own surprise, surviving Fasudil-treated Smn2B/- females are capable of reproducing. The dystocia-linked death may however point to the breeding limitations of these mice that still exhibit a neuromuscular/SMA phenotype. We have addressed this point in the Results Section, page 12, as follows: “Additionally, despite their initial compromised body size and neuromuscular function, surviving Fasudil-treated Smn2B/- females are able to reproduce, as exemplified by a female that was euthanized because of dystocia (Figure 1A). The dystocia-linked death, however, highlights the breeding limitations in these aging Fasudil-treated mice that still exhibit an SMA neuromuscular phenotype.”
Comment #4: The authors reference an article by Boyer in the text but missed including it in the bibliography section.

We have modified the original reference to the submitted work by Boyer et al. as follows: “JGB, unpublished data”. Furthermore, due to his initial characterization of the misregulated myogenin expression in SMA skeletal muscle, we have included JGB as a contributing author.

Comment #5: Figure 3 – The myofibers of treated animals are more than twice as large as those of untreated animals. Not surprisingly, the corresponding endplates are also larger. Considering that muscle mass contributes the bulk of an animal’s weight, it is puzzling that the treated animals did not differ in size or weight from their untreated littermates. Can the authors comment.

The increase in myofiber size in the absence of weight gain and increased strength is indeed surprising. However, various studies have previously reported similar observations, which we have addressed in the Discussion section, page 17: “Intriguingly, the dramatic increase in skeletal muscle myofiber size of Fasudil-treated Smn2B/- mice is not accompanied by changes in weight or strength, when compared to vehicle treated Smn2B/- mice. Previous studies have reported this phenomenon, providing a variety of potential explanations. In cases of sarcoplasmic hypertrophy, the non-contractile myofiber components expand while muscular strength remains unchanged (Kraemer et al, 2006). Further, the characterization of a postnatal myogenin knockout mouse model revealed normal skeletal muscle size albeit with a 30% weight loss compared to control littermates (Knapp et al, 2006). The authors suggest that this phenotype is caused by a slower growth rate and perturbed energy homeostasis. Finally, Rehfeldt et al. showed that mice homozygous for the Compact myostatin mutation (C/C) display muscular hyperplasia and increased muscle weight but with a reduction in overall body weight (Rehfeldt et al, 2005). The authors also identify a reduction in the number of capillaries per muscle in the C/C mice, subsequently impacting oxidative metabolism. Interestingly, recent work in the severe SMA mouse model demonstrated a significant decrease in the capillary bed density within skeletal muscle (Somers et al, 2011). Thus, the findings mentioned above highlight the fact that an increase in muscle size and or weight does not necessarily positively correlate with an increase in body weight. Regardless, the restoration of myofiber growth and skeletal muscle development by Fasudil, in the absence of weight gain, appears to be sufficient to providing therapeutic benefits to the Smn2B/- mice.”

Comment #6: On page 17, Bowerman and colleagues state that, “…given sufficient time, Fasudil administration allows for the improved development of NMJs in Smn2B/- mice.” What might be the mechanism underlying this improved development? Could the authors comment or speculate?

In this context we are using the word development to refer to the maturation of the neuromuscular junction. The text has now been modified for clarity to now read “We therefore suggest that although there was no initial improvement in the morphological aspects on pre-synaptic pathology, given sufficient time, Fasudil administration allows for the improved maturation of NMJs in Smn2B/- mice.” The improved NMJ maturation is evidenced by the increase in the complexity of the motor endplate, as quantified in Figure 7. We have also discussed the possible underlying mechanisms in the Discussion section, page 21: “Given the tight correlation between endplate maturation and neuromuscular activity (reviewed in Sanes et al, 1999), Fasudil may indirectly improve NMJ transmission, subsequently ameliorating motor endplate maturation. Alternatively, considering the crucial role of the actin cytoskeleton in the redistribution of AChRs during postsynaptic remodeling (Cartaud et al, 2011; Dobbin’s et al, 2006), Fasudil’s modulation of actin dynamics could directly restore normal AChR clustering. Clearly, the understanding and identification of Fasudil’s influence on NMJ maturation in SMA mice requires further investigation. Nevertheless, our work highlights the applicability of the compartmental degeneration hypothesis to SMA pathogenesis and the potential of therapies aimed at preventing synaptic degeneration.”
Comment #7: On page 30, bibliographic information for reference #21 is not complete.

This manuscript is still in press. We have made the appropriate adjustments to this reference (now #23) according to BMC Medicine’s guidelines.

We trust that you will find our revisions acceptable and look forward to a formal acceptance of the manuscript.

Sincerely,

Rashmi Kothary, Ph.D.
Senior Scientist