Dear Reviewer 2:

Thank you for your comments concerning our manuscript entitled “Melatonin inhibits apoptosis and oxidative stress of mouse Leydig cells via a SIRT1-dependent mechanism”. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction and supplement which we hope meet with approval. Revised portion are marked with blue (revised according to reviewers) in the paper. The main corrections in the paper and the response to you comments are as following:

The manuscript titled "Melatonin inhibits apoptosis and oxidative stress of mouse Leydig cells via a SIRT1-dependent mechanism" by Xu and colleagues is a well organized investigation that may have implications for the etiology and treatment of male infertility. Although well put together, the manuscript would benefit greatly from close English language editing to improve readability and grammar. That aside, I have a few suggestions for improvement:

1) MTT assay, Figure 1A and/or Materials and Methods: please indicate how statistics were determined, e.g., biological replicates versus replicate wells.

Response: For the question of biological replicates and replicate wells, we explained in materials and methods. The wells are repeated in triplicate and this experiment was performed three times independently. Thank you for your suggestion.

2) Line 80 indicates reductions in apoptosis to 70.2% and 82.3% in reference to Figure 2A. It is not intuitively clear from these images how these values were determined. Clarification would be helpful in the text, emphasizing that the reductions were 70.2% and 82.3% of the apoptotic rate of untreated cells. Also, for those unfamiliar with flow data, indicating which quadrants are considered apoptotic cells would be helpful. In fact, the flow plots might be better placed as a supplemental figure and leave the histogram of the apoptotic rate % as Figure 2A. Just a suggestion for simplification.

Response: Thank you for your suggestion. The suggestion made us realize that our
results analysis are not intuitive. These values (70.2% and 82.3%) really can't be seen intuitively in the results. Therefore, we replaced these values with intuitive expressions (significant decrease and P values). In addition, we explained the four quadrants and point out the quadrant of apoptosis in the revision.

3) Lines 102-103: "three" concentrations should be "five"

**Response:** Thank you very much for your correction. I have modified in the article.

Results section 2.6: should be referencing Figure 6 instead of 3 & 5

**Response:** This is the negligence in our writing. Thank you very much for your correction. We replaced Figure 3 & 5 with Figure 6 in result section 2.6.

4) Lines 146-147: these results indicate only partial reversal of oxidative stress when pretreating with the SIRT1 inhibitor. Therefore, one can only state that inhibition of oxidative stress in mouse Leydig cells is at least partially due to a SIRT1-dependent mechanism, although other mechanisms may also contribute.

**Response:** This comment is valuable and very helpful for improving our paper. We realized that this statement is not rigorous and we corrected it.

5) Finally, I have a bit of concern regarding the nature of the response and what appears to be a narrow therapeutic window in terms of dose and time. Can the authors speculate on why this is and how it might be relevant in an in vivo setting? This might be something to add to the Discussion.

**Response:** The production of free radicals can cause damage to biological macromolecules such as lipids, proteins, and nucleic acids, leading to cell damage. Melatonin can scavenge free radicals, protect against oxidation and inhibit lipid peroxidation to protect cell structures, prevent DNA damage, and reduce peroxide levels in the body.

For the problem of dose and time, we considered that there are two reasons. First, dose and time of melatonin depend on the degree of oxidative stress and apoptosis. If adding drug stimulation, we speculate that melatonin will play a protective role in advance and the concentration will increase. For example, melatonin treatment for 24 h relieves high glucose-induced apoptosis in Schwann cells (Tiong YL, 2019). Second, melatonin is often used in an effective range of concentration dose and time. The dose and time of
melatonin required by different individuals is also different, and the individual differences are large. It is necessary to try to determine the appropriate dose. Lower and higher concentrations of melatonin are not effective, which has been reported in many studies. For example, treatment of mouse granulosa cells with 0.1, 1, 10, and 100 μM melatonin revealed that the optimal concentration to inhibit palmitic acid-induced apoptosis was 10 μM rather than 0.1, 1 and 100 μM (Chen Z, 2019). In pig granulosa cells, melatonin with low concentration (0.1 μM) instead of high concentration (10 μM) could stimulate the synthesis of estradiol and make differentially expressed genes which associated with regulation of cell proliferation, cell cycle, and anti-apoptosis significantly enriched. We added discussion of this problem in the article (Liu Y, 2019).

For how it might be relevant in an in vivo setting, the difference between in vivo and in vitro is very large. In vivo experiment, melatonin often requires multiple injections, such as one week or longer. Studies have shown that melatonin injection for 7 days could inhibit apoptosis and oxidative stress of kidney and testis in mice. Melatonin injection for one month treatment may prevent formaldehyde-induced oxidative damage and apoptosis in rat testes. However, we did not study the effects in animals, which is a limitation of this article.

Thank you for your review.
Yours Sincerely