Peer Review File

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Reviewer A

1. Please ensure that the full name of abbreviations is used when they occur for the first time in the manuscript.

We appreciate the reviewer's suggestion that we give the full name when using the abbreviation for the first time in the revision.

2. Please provide additional keywords for better indexing and search ability.

We agree the reviewer's suggestion that we had rewritten the keywords as follow. "Androgen, EPAC1, Erectile dysfunction, Penile, RhoA"

3. Please ensure that the images provided have a high resolution of over 300 dpi.

We provide images in high resolution >300dpi in the revised edition.

4. There are numerous typographical errors in the figure legends that need to be corrected.

We are grateful that the reviewer caught the errors and corrected them.

5. Please enlarge the font size of labels in the figures to improve readability.

We had enlarged the font size of labels in the figures in revision.

Reviewer B

Major

1.What post-hoc test was used to determine group differences following one-way ANOVA?

In order to test normality, the Shapiro-Wilk test was considered. If $p>0.05$, the data is normally distributed. The results showed that the characteristics of these statistical indicators are normally distributed by using the GraphPad Prism 7.0 Software normality procedure, respectively. Then, LSD-t test was used to determine group differences following one-way ANOVA.

2. In the introduction, the authors describe a study whereby expression of EPACs were

reduced in human airway smooth muscle cells following stimulation with testosterone. The present study demonstrates the opposite relationship in the corpus cavernosum following androgen deprivation. This disparity should be discussed.

The mechanism of down-regulation of EPAC in human airway smooth muscle cells stimulated by testosterone is unclear [19]. Meanwhile, in our experiment, the expression of EPAC was reduced in the penis tissue of castrated rats. This indicates that the relationship between EPACs and androgens in different species and tissues is complex and requires further research. We had added it in the discussion.

3. Several details regarding the animal procedures are missing from the methods section, such as anesthesia during the castrations, cavernosal injections, and ICP/MAP assessment, post-operative care following castration, the vehicle for testosterone, the administration of testosterone (presumably this was injected, but it is not specified).

We agree and provide a more detail information in the methods as follow.

After the rats were numbered 1-30, thirty 8-week-old Sprague-Dawley male rats were divided into six groups in a completely random manner(n=5): sham operation(sham), castrated, castrated + testosterone replacement (castrated + T), sham + EPAC1 over-expression lentivirus (sham + EPAC1), castrated + empty lentivirus vector (castrated + empty vector), and castrated + EPAC1. After the rats were anesthetized with 1% pentobarbital sodium (30 mg/kg) by intraperitoneal injection, bilateral testes and accessory glands were excised through scrotal incision to establish castrated rat model [3]. T replacement is given subcutaneous injection of testosterone propionate (3 mg/kg) every two days for 4 weeks. The other groups received equal amounts of vegetable oil (the vehicle for testosterone) in the same way. Four weeks after castration, a rubber band was used to ligate the penile root of the anesthetized rat. The lentivirus vectors carried the EPAC1 gene (1×10^8 TU/ml, 20 μl per rat, JiKai Gene Company, China) was injected into the middle of the corpus cavernosum of castrated+EPAC1 and sham+EPAC1 group rats by a micro syringe. At the same time, the same amount of empty lentivirus was injected into the penis of the castrated + empty vector group rats in the same way, while the rats in the other groups were injected with the same doses of physiological saline. After 3 minutes of injection, the rubber band tied to the penile root of the rat was removed [3.4]. The experimental rats were all raised in the Animal Experiment Center of Southwest Medical University. Room temperature was kept at 22-26 ℃, and the humidity at 40-60%. All rats were free to drink and eat. All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Southwestern Medical University and followed the Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

4. The NO data should not be presented in both Table 1 and Figure 1.

We agree and delete the NO data in Table 1 in revision.

5. The discussion is severely underdeveloped. The discussion is largely a restatement of the results section, which essentially reads as a list of individual group-group differences for each measure. There is limited discussion provided for how this work fits into the present body of literature or how this relates to other studies.

We agree and revised the discussion.

6. There is no clear concluding statement or paragraph.

We agreed and added this point in the last paragraph as follow.

This study found that low androgen status can inhibit erectile function by down-regulating the expression of EPAC1 in penile corpus cavernosum of castrated rats. The up-regulation of the expression of EPAC1 in penile cavernous tissue can significantly improve erectile function in castrated rats. Therefore, EPAC1 may be an important target for improving erectile function under low androgenic conditions, which provides an important direction for the clinical treatment of ED requiring maintenance of low androgen status.

Minor

1. Section 2.2 could be titled "Erectile function assessment" to enhance simplicity. The measurement of the ratio of ICPmax/MAP should be described in the body of the paragraph.

We agree with the reviewer's suggestion. Section 2.2 could be titled "Erectile function assessment" in revision.

Reviewer C

Major comments:

1. Were rats anesthetized before castration and ICP measurements?

We had added the methods of anesthesia in the first paragraph of section 2.1.

2. Serum levels of testosterone, a stress hormone, were measured after repeated ICP measurements. Were rats allowed to rest some time before blood collection, as measured testosterone values seem to have very small SDs, indicating very little fluctuation in testosterone levels.

The various electrical stimulation parameters including frequency, intensity and interval time have been reported[1-3]. This may be related to anesthetics, animal model and different measurement methods. Most research has suggested that the interval time should be at least 1 minute[4]. The interval of electrical stimulation was 3 minutes in this study. We collected the sample tissue 3 minutes after ICP determination. All samples of penile cavernous tissue were collected in this way.

References:

1.Long H, Jiang J, Xia J, Jiang R, et al. Icariin improves SHR erectile function via inhibiting eNOS uncoupling. *Andrologia*. 2018;50(9):e13084.

2.Zuo Z, Jiang J, Jiang R, Chen F, Liu J, Yang H, Cheng Y. Effect of periodontitis on erectile function and its possible mechanism. J Sex Med. 2011;8(9):2598-605.

3. Jiang J, He Y, and Jiang R. Ultrastructural changes of penile cavernous tissue in multiple sclerotic rats. J Sex Med 2009;6: 2206–2214.

4.Hox M, Mann-Gow T, Lund L, Zvara P. Cavernous Nerve Stimulation and Recording of Intracavernous Pressure in a Rat. J Vis Exp. 2018 Apr 23;(134). doi: 10.3791/56807.

3. Some of the techniques are not referenced. Please include references, unless they are new techniques not used before, in which case a detailed description should be provided.

We had added the references in the section 2.1.

4. Western blot: There is no need to include the calculation formula for P-Akt/Akt and P-eNOS/eNOS, as that is the standard way of presenting Western blot fata (such as EPAC/GAPDH).

We agree and delete the calculation formula in the revision.

5. What does the level of RhoA-GTP indicate?

RhoA-GTP is the active form of RhoA that performs physiological functions. RhoA is a GTP enzyme that, upon activation, can be converted from inactive RhoA-GDP to active RhoA-GTP, thereby activating downstream ROCK [1.2].

References:

1.Kim Jae-Gyu,Islam Rokibul,Cho Jung Y,et al.Regulation of RhoA GTPase and various transcription factors in the RhoA pathway.Journal of Cellular Physiology.2018;233 (9)：6381-6392.

2.Choi Eun-Kyoung,Kim Jae-Gyu,Kim Hee-Jun,et al.Regulation of RhoA GTPase and novel target proteins for ROCK.Small GTPases.2020;11 (2)：95-102.

6. Legend to Fig.1: (D) Is it a level or activity of NO?

It had been rexised as “The level of NO in each group”.

7. EPAC1 seems to be overexpressed in the penis of sham group after lentiviral injection over control levels. This was not commented in the manuscript. Would it be expected to affect downstream signaling?

After injection of the lentivirus carrying EPAC1, the sham+EPAC1 group showed a

significantly increase in EPAC1 levels compared to the sham group ($p<0.05$), while the activity of RhoA (RhoA-GTP) significantly decreased ($p<0.05$). However, there were no significant changes in p-AKT/AKT, p-eNOS/eNOS, NO levels, and the erectile function (ICPmax/MAP) of rats did not show any significant changes. It is suggested that the over-expression of EPAC1 did not significantly affect the erectile function of normal rats. We added it in the discussion.

8. Please replace "notably" with "significant" (if it is statistically different) in the Results section.

We agree the reviewer's suggestion that we had replaced "notably" with "significant" in revision.

9. What was the rationale for treatment with testosterone? The effect of testosterone replacement on the measured parameters including EPAC is only scarcely mentioned in the text in the Results section, and not at all discussed in the Discussion.

To investigate the effects of other factors other than testosterone (such as anesthesia, surgical emergencies, etc.) on experimental observation indicators, castrated rats were given testosterone replacement as a control group.

We had added the discussion on the data of the T replacement group.

10. Please do not repeat results in the Discussion.

We agree the reviewer's suggestion that we had rewritten the discussion.

11. Some references (such as ref 3, not related to aging) should be replaced with more pertinent ones.

Ref 3 is related to low androgen status and not related to aging.

Reviewer D

Interesting paper and well-designed study. It is unclear whether EPAC1 overexpression leads to improved erectile function. The authors did not test this, they found an increase of erection-promoting molecules only. They should revise their statement in the discussion. Also, the statement that testosterone replacement in prostate cancer patients cannot be done is not true. There is quite a lot of literature on this.

We agree the reviewer's view. However, although there are literature report that patients with prostate cancer may receive testosterone supplementation, current guidelines from the European Urology Association and the American Urology Association still consider androgen blockade as a classic treatment option for advanced prostate cancer. For a more accurate description, we had deleted "……and cannot be treated with testosterone supplementation in

prostatic cancer” in revision.