

Peer Review File

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Reviewer A

Authors provide an educational piece on developing rabbit models for urethral stricture disease - recommend major revisions.

introduction - several other techniques of urethral stricture were described, please also include some basic information on success rates, etc

Reply: Thanks for your kind suggestion. We have modified our text as advised.

Changes in the text: Page 4, line 63-73, such as the following.

Further, electrocoagulation by video-urethroscopy has a failure rate of >40% because of epithelial cell regeneration (9, 10).

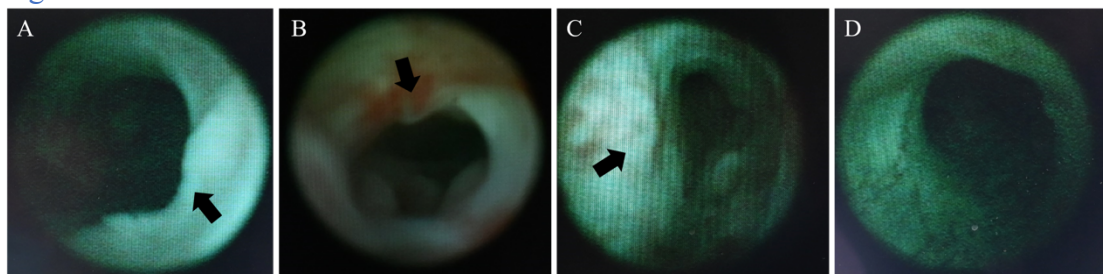
figure 2 - please make this figure 1 and reference it in the methods section

Reply: Thanks for point out this issue. We have made some improvement.

Changes in the text: Page 5, line 94-101.

figure 4 - please include arrows to the stricture site

Reply: We thank this suggestion. Arrows marking the stricture site have been added in Figure 4.



histologic evaluation - please specifically state whether the observed findings are similar to what is seen in human urethral stricture disease

Reply: Thanks for your kind advice. The content related to this has been added.

Changes in the text: Page 12-13 , line 255-256.

These features are similar to those observed in human urethral stricture disease.

are future studies planned to evaluate the differences between group 1 and group 3 or to evaluate stricture length?

Reply: Yes, we will conduct a more in-depth study in the future. Thanks for your comment.

please further described the one rabbit that died from urethral atresia - how was this diagnosed, when did it occur, how are these animals monitor, can you track urine output, can you track hydronephrosis

Reply: It was a pity that we heard the news from the breeder. We performed an autopsy on the rabbit and found urethral atresia, as well as an immensely large bladder with hydronephrosis. It occurred at fourth day after the surgery. These animals were monitor by the breeder. We didn't track the urine output, which may be an interesting issue. Thank you very much for your constructive comment.

Changes in the text: Page 8, line 152.

one rabbit died due to urethral atresia at 4 days after the procedure.

line 282 - Ventral half-circumferential electrocoagulation is recommended, and the need for whole-circumferential electrocoagulation can be adjusted according to experimental needs - please expand on how whole circumference can be adjusted to experimental needs compared to ventral

Reply: We appreciate the reviewer for pointing this important issue. Firstly, because the surgeons in our team are very experienced in urethral surgery and have a good understanding of urethral strictures, they know what level damage may cause ideal stricture. However, for newbies, this may need to be tried. Secondly, it is not absolute whether half- or whole- circumferential electrocoagulation. In this article, the half-circumferential electrocoagulation method didn't achieve a 100% successful rate. Although the whole-circumferential electrocoagulation method with a 100% successful rate, a rabbit died due to excessive urethral stricture (urethral atresia). If 3/4 circumferential electrocoagulation can achieve the desired outcomes with no animal died, we think that your team can take the 3/4 method. In a word, don't stick to data in this article, and you can choose the suitable electrocoagulation range according to the actual situation.

Changes in the text: Page 13, line 273-275.

Alternatively, whole-circumferential electrocoagulation can be considered according to experimental needs, as it could lead to excessive urethral stricture or even urethral atresia and death of the animal.

At last, thank you for your positive response to our work and the kind and valuable advice.

Reviewer B

The authors present 3 novel techniques for induction of urethral strictures in rabbit models. I will break my review up to major and minor considerations.

Major:

- In the intro section, the authors did not mention use of chemical agents such as bleomycin as another minimally invasive option for the induction of urethral strictures in animal models

Reply: Thanks for your kind suggestion. We have added the relevant content.

Changes in the text: Page 3-4, line 59-62, 71-73.

Rabbit and dog models of US could be constructed by open surgery, electrocoagulation with or without endoscopy, electro-resection using a pediatric

resectoscope, the blast method with a special instrument, and administration of specific drugs, among others (7).

Finally, drug-induced models are too effort-intensive because medicines such as bleomycin need to be injected into the urethral submucosal tissue every other day for 6 weeks (12).

- What exactly is the purpose? In the intro, the authors discuss the disadvantages of open surgery to induce urethral stricture, however their techniques all employ open surgery, therefore I don't seem to understand the benefit, nor do I understand the novelty of the technique

Reply: We appreciate the reviewer for pointing this important issue. The purpose is to compare three surgical procedures and determine the appropriate one for the generation of urethral stricture (US) models in rabbits. Then, about the difference between the current open surgery and our procedures, we tried to answer as follows.

First of all, the current open surgery technically constructed a model of urethral defect, but not a real urethral stricture [1-3]. It could not reflect the true process of the US, lacking many typical histologic features of it.

Secondly, due to the above disadvantages, the urethral defect model is not suitable used for following experiments such as medicine treatment.

Thirdly, because part of the urethra is excised, it may cause severe wounds and a high incidence of urethral fistula formation.

Comparatively, we constructed a model with the histologic features of the US, which is suitably used for the following treatment experiments. Less bleeding and damage may occur due to the electrocoagulation technique.

We wish this reply could satisfy you and receive your support.

Reference:

[1] Zhong H, Shen Y, Zhao D, et al. Cell-Seeded Acellular Artery for Reconstruction of Long Urethral Defects in a Canine Model. *Stem Cells Int.* 2021,4;2021:8854479.

[2] De Filippo RE, Kornitzer BS, Yoo JJ, et al. Penile urethra replacement with autologous cell-seeded tubularized collagen matrices. *J Tissue Eng Regen Med.* 2015,9(3):257-64.

[3] Wang Y, Wang G, Hou X, et al. Urethral Tissue Reconstruction Using the Acellular Dermal Matrix Patch Modified with Collagen-Binding VEGF in Beagle Urethral Injury Models. *Biomed Res Int.* 2021,15;2021:5502740.

- RUG – Significant stricture was defined as urethral lumen decreasing by more than 50% of the sham group. The sham group is not an appropriate control to define the stricture. In order to accurately describe reduction in size, a pre-op RUG should have been performed as well, and those two images should have been compared

Reply: Thanks for your comment. You are right, and some scholars have done the same [1]. Yet, firstly, the pre-op RUG might make some injury to the urethra and glans, because we need to place a catheter and attach it round the glans site during the RUG process. Secondly, as reported, a positive correlation was found between the cross-sectional area at the maximum applied pressure and the age and the weight of the animals [2]. Time between pre- and post-operation was about 1 month, which mean a relatively long period of time for growing rabbits. At 4 weeks after the procedure,

these rabbits were about 4kg. Therefore, from two above points, we set up the sham group referring to the literature [3], and carefully selected rabbits of the same age in this study, and the weights of the rabbits were comparable between these groups.

Reference:

[1] Scott KA, Li G, Manwaring J, et al. Liquid buccal mucosa graft endoscopic urethroplasty: a validation animal study. *World J Urol.* 2020,38(9):2139-45.

[2] Andersen HL, Duch BU, Nielsen JB, et al. An experimental model for stricture studies in the anterior urethra of the male rabbit. *Urol Res.* 2003,31(6):363-7.

[3] Kurt O, Yesildag E, Yazici CM, et al. Effect of Tadalafil on Prevention of Urethral Stricture After Urethral Injury: An Experimental Study. *Urology.* 2016,91:243 e1-6.

- There was no comparison to other already established techniques – how can you determine that these novel techniques are in some way better?

Reply: We first want to thank you for your constructive reviews of our manuscript and say that we found your comments valuable. The model of urethral defect is commonly used instead of the urethral stricture, especially in the field of urethral tissue engineering. The urethral stricture model we described here are relatively simple to constructed and highly repeatable. We admit that we didn't directly compare our techniques with these already established. The main purpose of this article was to compare these three surgical procedures. However, we plan to make more explorations in the future studies. Thanks very much again.

Reference:

[1] Zhong H, Shen Y, Zhao D, et al. Cell-Seeded Acellular Artery for Reconstruction of Long Urethral Defects in a Canine Model. *Stem Cells Int.* 2021,4;2021:8854479.

[2] Wang Y, Wang G, Hou X, et al. Urethral Tissue Reconstruction Using the Acellular Dermal Matrix Patch Modified with Collagen-Binding VEGF in Beagle Urethral Injury Models. *Biomed Res Int.* 2021,15;2021:5502740.

Minor:

- Title – title does not accurately convey the summary. Would say something along the lines of, “Three experimental procedures for the induction of urethral strictures in rabbit models....”

Reply: Thanks for your valuable suggestion. We modified the title as advised.

Changes in the text: Page 1, line 1-2.

Three new experimental models of anterior urethral stricture in rabbits

- Overall language and grammar were very poor throughout the paper

Reply: Thank you for your comments that help to improve our manuscript. The manuscript has been polished by an English language editing company.

Overall, the language in the paper makes it very difficult to follow and interpret the message being conveyed. In addition, the purpose of the study isn't quite clear initially and the design had multiple flaws.

Reply : We thank the reviewer for these very useful remarks. Thanks once more for your help on improving our manuscript. We hope our modifications to the manuscript meet your expectations

Reviewer C

The authors should be congratulated for proposing a novel surgical technique for creating urethral strictures (US) in a rabbit model, describing three different variations. They carefully describe their surgical methods -using standard instruments- and the evaluation of their outcomes -conducted by 3 methods (endoscopy, radiography and histology)-. I also would like to congratulate the authors for their outcomes in their attempt to create US, as they achieve a 100% rate in one of the arms, which seem extremely good.

While reading the manuscript, some concerns brought my attention, and I would like to address them.

Major comments:

- The sham group probably would be better with no intervention at all -not even dissecting the urethra-, as this could risk creating a stricture. Since the definition of US in this research is based on the lumen of the sham group, the authors should be certain that those rabbits have a complete normal urethra.

Reply: Thanks for your kind suggestion. Surgeons in our team are very experienced in both human and rabbit urethral surgery, so we are confident that there was no damage to the urethra in the sham group. A problem we considered was that the surgery itself might result in experimental deviation, so we did the intervention in the sham group. However, your comment is very valuable. We will consider the effect in future studies.

- Since the authors are comparing 3 methods for US development, the evaluation should be blinded. The urethrograms were performed by the same surgeons, which could lead to bias if they know the group of the technique used in each rabbit.

Reply: Thanks for your comment. These rabbits had no marks. Their information were noted and recorded individually which mean it only known by the observer (Zi-Wei Wei) who didn't participate in the procedures. The surgeons didn't deliberately try to remember the differences between rabbits, of course, we thought it was also a not easy thing. Therefore, we thought it was almost blinded to the surgeons. Thanks for your kind comment again.

- The same applies to the retrograde urethroscopy (should be blinded to the group of each rabbit). Also, the description of this procedure would include some information about how the authors evaluate the caliber of the stricture by endoscopy.

Reply: Thanks for your comment. Firstly, about the blind method, just the observer (Zi-Wei Wei) knew the information of each rabbit during the whole experimental process, but the surgeons didn't. Secondly, the endoscopy technique was used to help reconfirm the stricture condition, but not quantify the degree of stricture. It helps us know yes or not, according to the surgeons' clinical experience, and the condition of the urethral, such as surface color and smoothness and so on. I hope the above data and discussion have solved your doubts.

- Statistical analysis should be better described. All variables should be assessed for normal distribution, as this would affect the description -median and interquartile range- and also the statistical tests conducted. Also, the description of all measured urethras should be described -not only limiting to the “successful cases”-

Reply: Thanks for your comment. It is important that all variables are assessed for if normal distribution or not. We in fact conducted this procedure. We also have added all measured urethras information in the article as advised.

Changes in the text: Page 8, line 157-158.

The mean urethral diameters were 1.99 mm, 2.62 mm, and 0.88 mm in groups 1, 2, and 3, respectively.

- In the Results section, authors mentioned the total number of “successful cases” but, it is unclear if the authors independently compared the different evaluation methods. At would be possible that a rabbit would develop a stricture seen in the urethrography but not in the endoscopy, and this should be acknowledged. This aspect should be detailed.

Reply: Thank you very much for raising this important question. You are right, only endoscopy could not exactly evaluate the stricture. Compare with urethrography, it mainly helps us know the condition of the urethral, such as surface color and smoothness and so on. We hope our reply meet your expectations. Thanks again.

- The authors stated in the Results section: “For these successful cases, the mean urethral diameters were 3.96mm, 4.37mm, and 2.64mm in groups 1, 2, and 3, respectively. The diameter in the sham group was measured at the same point with a mean of 4.2mm”. If the diameter in the group 2 is greater than in the sham group, how this could be considered a success case?

Reply: Thanks so much for your careful review. This was indeed an accidental mistake. The data shown in Figure 2D was correct in the initial manuscript. We have corrected it in the text.

Changes in the text: Page 8, line 158-161

In the cases in which US was successfully established, the mean urethral diameters (measured at the narrowest point of the strictures) were 1.57 mm, 1.8 mm, and 0.96 mm in groups 1, 2, and 3, respectively.

- A Limitations paragraph is missing in the Discussion section and should be included.

Reply: Thanks for your comment. We added it in the discussion section.

Changes in the text: Page 13-14, line 277-281

Despite the promising findings, it is necessary to apply these procedures and further observe this model in larger sample sizes to confirm our findings. Moreover, as scar formation caused by electrocautery may not have the same characteristics as strictures caused by urethritis or straddle trauma in humans, this animal model may not be suitable for all kinds of human anterior urethral strictures.

Minor concerns

- English language would need revision.

Reply: Thanks for your comment. We have improved many inappropriate expressions. Moreover, a native English language editor helped us revised the manuscript in detail.

- Discussion is too long.

Reply: Thanks for your comment. We have streamlined the discussion section.

- In the Discussion is stated: “Firstly, the urethral internal diameter of adult New Zealand male rabbits is 5-7 cm long”. This should be corrected.

Reply: Thanks for your comment. We have corrected it.

Changes in the text: Page 10, line 210-211

One of the main advantages is that the urethral internal diameter of adult New Zealand male rabbits is similar to that of a 1-year-old male infant

- Figure 1 is no relevant -all the information is mentioned in the text-. Also, the describe data would be better displayed in a table, instead a graph-.

Reply: Thanks for your kind suggestion. We added a table with more detailed information , which also included all measured urethras you mentioned above. Sorry for not deleting the graph, because some professors may prefer it. We hope you can understand !

Changes in the text: Table 1

Table 1. Data of study groups at the day of surgery and the 4th week after surgery.^{a,2}

Parameters ^{a,2}	Group 1 ^{a,2}	Group 2 ^{a,2}	P value ^{a,2}	Group 3 ^{a,2}	P value ^{b,2}	P value ^{c,2}	Group 4 ^{a,2}	P value ^{d,2}
Weight, kg ^{a,2}	3.23 ± 0.16 ^{a,2}	3.19 ± 0.13 ^{a,2}	0.55 ^{a,2}	3.24± 0.13 ^{a,2}	0.93 ^{a,2}	0.27 ^{a,2}	3.31 ± 0.19 ^{a,2}	0.61 ^{a,2}
Operative time, min ^{a,2}	18.58 ± 1.88 ^{a,2}	21.25 ± 3.52 ^{a,2}	0.02 ^{a,2}	22.08 ± 1.83 ^{a,2}	<0.001 ^{a,2}	0.13 ^{a,2}	15.25 ± 1.71 ^{a,2}	<0.001 ^{a,2}
Number of success, n (%) ^{a,2}	9 (75%) ^{a,2}	5 (41.67%) ^{a,2}	0.1 ^{a,2}	11 (91.67%) ^{a,2}	0.27 ^{a,2}	0.009 ^{a,2}	0 (0%) ^{a,2}	<0.001 ^{a,2}
Urethral diameter, mm ^{a,2}	1.99 _± 0.83 ^{a,2}	2.62 ± 0.76 ^{a,2}	0.07 ^{a,2}	0.88 ± 0.54 ^{a,2}	0.001 ^{a,2}	<0.001 ^{a,2}	4.23 ± 0.22 ^{a,2}	<0.001 ^{a,2}
Urethral diameter of success, mm ^{a,2}	1.57 ± 0.36 ^{a,2}	1.8± 0.28 ^{a,2}	0.18 ^{a,2}	0.96 ± 0.49 ^{a,2}	0.01 ^{a,2}	0.005 ^{a,2}	4.23 ± 0.22 ^{a,2}	<0.001 ^{a,2}

a-c. Statistical analysis was performed with[Mann-Whitney U tes] for each two experimental groups in measurement data, while Chi-Square Test in counting data. a. between groups 1 and 2; b. between groups 1 and 3; c. between groups 2 and 3.^{a,2}

d. Statistical analysis was performed with Kruskal-Wallis H test for four groups.^{a,2}

- Figure 5 is of no interest -all samples should be equal-.

Reply: Thanks for your comment. This mainly aimed to provide more information about the wound statue at 4 weeks after the procedure. We wish this reply could satisfy you and receive your support.

- Figure 6 should detail the intervention group where the sample is from.

Reply: Thanks for your kind advice. Because their histology were the same, we pick up one typical picture from three intervention groups, but not three. In other word, the one picture represents all the three intervention groups. We modified it as advised.

Changes in the text: Page 19-20, line 424-425

The images for the experimental groups are of samples taken from group 3.

Besides all these comments, I find the author’s work highly interesting and valuable. I hope those comments would help the authors to enhance their manuscript and increase the chances for a future publication.

Reply: In all, thank you for your positive response to our work and these very kind and valuable suggestions. We hope the article now can better meet the high standards for publication.

Reviewer D

The authors should be commended for their attempts to move the field of experimental animal models concerning urethral stricture disease and surgery forward by introducing novel surgical techniques to construct a successful animal model imitating the human stricture disease.

The manuscript is well structured, and the described techniques and results could benefit other researchers in this field.

The authors should, however, additionally address the following issues.

Major:

1. you should mention in your manuscript that the scar formation caused by electrocautery may not have the same characteristics as strictures caused by urethritis or straddle trauma in humans or even some iatrogenic strictures caused by friction of catheter or resectoscopes. Considering that such animal models may not apply to all kinds of human anterior urethral strictures, they should be interpreted with caution.

Reply: Thank you, you are right. We have modified it as advised. Thanks for your kind comment again.

Changes in the text: Page 13-14, line 279-281

Moreover, as scar formation caused by electrocautery may not have the same characteristics as strictures caused by urethritis or straddle trauma in humans, this animal model may not be suitable for all kinds of human anterior urethral strictures.

2. your writing style and use of scientific language should be improved. Many spelling mistakes and sentences made no sense or gave the wrong meaning (for example, Line 92, " The urethra was placed in an 8 Fr transurethral catheter").

P.S.: I attached a PDF with some improvement suggestions.

Reply: Thanks so much for your careful review. Your kind suggestions are very valuable. We have polished the text as advised. Moreover, the manuscript has been edited by a native English editor.

3. the argument used to explain the difference in success rate between the dorsal and circumferential electrocoagulation also applies to the ventral semi-circumferential coagulation group. Could you clearly define the difference in the success rate between dorsal and ventral approaches since the corpus spongiosum surrounds both sides of the urethral mucosa (ventral and dorsal) to the same extent along the penile urethra?

Reply: We appreciate the reviewer for pointing this important issue. You are right. We think the additional suture of ventral incision may play another important role in the dorsal semi-circumferential coagulation group, as the dorsal group had 'dorsal coagulation + ventral suture' but the ventral group had only 'ventral coagulation'. However, the specific reason or mechanism still needs further study.

Changes in the text: Page 12, line 246.

However, the specific mechanism needs further study.

Minor:

1. You refer to the urethral diameter as cm, but you mean mm. Please correct it.

Reply: Thanks for your comment. We have corrected it.

Changes in the text: Page 10, line 210-211

One of the main advantages is that the urethral internal diameter of adult New Zealand male rabbits is similar to that of a 1-year-old male infant

2. please rephrase some sentences or words used which make no sense (see the attached pdf)

Reply: Your kind suggestions are very valuable. We have improved many inappropriate expressions and polished the text as advised. Moreover, a native English language editor helped us revised the manuscript in detail.

3. Figure 7 (Immunohistochemistry of the urethral) is very dark, and the differences in color among sham and experimental groups ist not easily visible. Could you increase the contrast and the brightness of these images?

Reply: Thank you for your advice. We have modified as advised.

Changes in the text: Figure 7

