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Reviewer Comments

This study aims to determine if Botulinum Toxin-A injection reduces fibrosis induced in spinal cord-injured animals through attenuation of the TGF-β1 pathway. The study aimed to measure the effect of Botulinum Toxin-A injection on expression of this pathway.

Abstract

Comment 1: The Early and Late groups need to explain in the Abstract, as this is published separately.

Reply 1: we have modified our text as advised (see Page 3, line 36-39).

Changes in the text 1:

The sentence was modified as following: *0.*9% saline was injected into the detrusor in the Sham and T10 transection groups simultaneously with the surgery, while 2U/rat botulinum toxin A was injected into the detrusor simultaneously with the surgery in the Early group and 4 weeks following the surgery in Late group.

The original sentence was as following: For bladder wall injection, 0.9% saline was injected into the Sham and T10 transection groups and 2U/rat botulinum toxin A was injected into the Early group simultaneously with the transection, while 2U/rat botulinum toxin A was injected into the Late group 4 weeks following the transection.

Comment 2: The conclusions say that Botulinum Toxin-A injection prevented fibrosis – this is an overstatement – it certainly reduced it.

Reply 2: we have modified our text as advised (see Page 3, line 36-39). *We also added a conclusion (see Page 3, line 50).*

Changes in the text 2:

The sentence was modified as following: Intradetrusor botulinum toxin A injection reduced bladder fibrosis in rats with spinal cord injury, which was more obviously in the Early group than in the Late group. The mechanisms might be mediated by suppression of transforming growth factor β1 expression.

The original sentence was as following: Intradetrusor botulinum toxin A injection prevented bladder fibrosis in rats with spinal cord injury, which might be mediated by suppression of transforming growth factor β1 expression.

Background

Comment 3: P5 You speak about measuring the effect of Botulinum Toxin-A injection on TGF-β1 expression. It is not clear whether you are talking about the effect on the TGF-β1 receptor or the cytokine itself. Could this be made clear in this section.

Reply 3: we are talking about the effect on the TGF-β1 cytokine.

Changes in the text 3:

The sentence was modified as following: Transforming growth factor β1 (TGF-β1) is

a key cytokine in promoting fibrosis.

The original sentence was as following: Transforming growth factor β1 (TGF-β1) plays a key role in promoting fibrosis.

Methods

Comment 4: P5 rat models Line 76 The concept of "early" and 'late' needs explanation here, as it is used throughout the study and they seem to comprise two of the four groups
What was the sham procedure

Reply 4:

1) We have modified our text as advised in **Comment 5** introducing the concept of "early" and "late" earlier. To do this, we integrated the two parts of "Rat models" and "Bladder wall injection" together and polished the language (see Page 6, line 80-89).

2) As advised, we added the explanation of sham procedure (see Page 6, line 80-81)

Changes in the text 4:

1) The Sham group underwent T10 sham operation, which opened the spinal canal and the dura mater, but did not damage the spinal cord and then closed the wound. The other 3 groups underwent T10 spinal cord transection. For intradetrusor injection, agents (totally 80 μl) were injected into 4 sites around the bladder base and 4 sites around the middle of the bladder between the serosal layer and the muscle layer via a midline abdominal incision using a syringe needle (30G). 0.9% saline was injected into the detrusor in the Sham and T10 transection groups simultaneously with the surgery, while 2U/rat BoNTA (Hengli®, Lanzhou Biological Products, China) was injected into the detrusor simultaneously with the surgery in the Early group and 4 weeks following the surgery in Late group. T10 spinal cord transection and intradetrusor injection were made under 2% isoflurane anesthesia.

2)The Sham group underwent T10 sham operation, which opened the spinal canal and the dura mater, but did not damage the spinal cord and then closed the wound.

Comment 5: P6 bladder wall injection, line 82 Important is the number of units of BoNTA injected (2U) and how this relates to the number than would be injected to patients – is it done on a basis of normalisation to body weight? Here the Early and Late concepts are introduced but it would be better earlier.

Reply 5:

1) BoNTA injecting dose may be an important factor affecting the degree of bladder fibrosis in the study and we calculated the dose based on body weight. The explanation is in the Discussion: Line to Line (see Page 6, line 80-81).

2) As advised, we introduced the concept of "early" and "late" earlier (see Reply 4).

Comment 6: P6 bladder wall injection, line 88 the manual expression of the bladder regime changed after 14 days, why was this?

Reply 6: Because the bladder function changes over time, the manual expression of the bladder regime changed. According to previous experience and reports, complete urinary retention occurs in the early stage of spinal cord transection due to spinal shock, while detrusor overactivity and reflexing urinary incontinence usually appear 14 days after the surgery.

Comment 7: P6 cystometry, line 96 Can it be made clear that all animals has cystometry 8 weeks after the sham or T10 section (the latter ± BoNTA treatment)
Reply 7: We have modified our text as advised (see Page 6, line 94-95) and made some language polish.
Changes in the text 7: 8 weeks post spinalization, conscious cystometry was recorded following the measurement of body weight in all rats.

Comment 8: P6 cystometry, line 96 I am not clear what you mean by the 'rats were recovered from anesthesia – was this after the cystometry was done?
Reply 8: We have clarified our text as advised (see Page 6, line 98).
Changes in the text 8: Cystometric parameters was recorded according to previous report after two stable micturition cycles at an infusing speed of 0.08 ml/min with 0.9% saline after the rats recovered from anesthesia for 1h.

Comment 9: P6 cystometry, line 96 May be a 'non-voiding contraction' is a better term than 'DO'.
Reply 9: We have replaced "OD" with "non-voiding contraction" as advised for rat cystometry throughout the manuscript.

Comment 10: P7 histology If the proportion of collagen was measured on H&E stained sections what is the point of the Masson's (trichrome) stain?
Reply 10: We also doubted whether we should keep the Masson' staining. Actually, we performed Masson' staining of the bladder in all rats, but when we analyzed the collagen content quantitatively, we found that the results were greatly affected by subjective factors and were prone to false conclusions. However, Masson' staining made the presentation of bladder fibrosis more intuitively and it was a pity to delete the figures, so, we kept the figures.

Comment 11: P7 Western blot Is it the TGF-β receptors, or the protein itself?
Reply 11: It is the TGF-β1 protein in Western blot.
Changes in the text 11: We added "protein" in Page 7, Line 117. *The modified sentence is as following:* Evaluation of TGF-β1 protein expression with Western blot.

Results

Comment 12: P9 line 148 It might be better to say that bladder weight was intermediate between the sham and the T10 group in the two BoNTA-treated groups.
Reply 12: We have modified our text as advised (see Page 9, line 152-153).

Changes in the text 12: The bladder weight in the SCI rats was increased significantly compared with the Sham group (P<0.01), meanwhile, it was intermediate between the Sham group and the T10 transection group in the two BoNTA-treated groups (Table 1).

Comment 13: P9 line 155, etc Here also the cystometric activity was intermediate in the two BoNTA-treated groups.

Reply 13: We have modified our text as advised (see: Page 9, line 160-161; Page 10, line 170-171; Page 10, line 177-178).

Changes in the text 13:

1) The NVC frequency increased significantly in the SCI rats compared with the Sham group (*P*<0.05), meanwhile, it was intermediate between the Sham group and the T10 transection group in the two BoNTA-treated groups (*Table 2, Figure 1*). Notably, the NVC frequency decreased significantly in the Early group compared with the Late group (*P*<0.05) (*Table 2, Figure 1*).

2) The detrusor connective tissue percentage increased significantly in the SCI rats compared with the Sham group (P<0.05), meanwhile, it was intermediate between the Sham group and the T10 transection group in the two BoNTA-treated groups *(Figure 2)*. Notably, the detrusor connective tissue percentage was tended to decrease in the Early group compared with the Late group (P=0.133) *(Figure 2)*.

3) TGF-β1 protein expression increased significantly in the SCI rats compared with the Sham group (*P*<0.05), meanwhile, it was intermediate between the Sham group and the T10 transection group in the two BoNTA-treated groups (*Figure 3*). Notably, TGF-β1 protein decreased significantly in the Early group compared with the Late group (*P*<0.05) (*Figure 3*).

Comment 14: The pressure traces themselves look rather strange with oscillations above and below the base-line in the T10 and the two BoNTA-treated groups. Can you explain this – it looks as if the recording system is imposing its own filtration on the traces?

Did you measure compliance, this might be expected to decrease with more fibrosis

Reply 14:

1) Because the time spent for cystometry is different in rats, the size for the same time scale and pressure scale are different when we save the traces as TIFF. For visual beauty, we replaced the original scales with a fixed size scale. Of course, we carefully polished the picture.

2) Actually, we measured bladder compliance. However, there was no significant difference among the three groups with T10 spinal cord transection. 8 weeks may be too short to evaluate the changes of bladder compliance.

Comment 15: P9 line 161 What is 'bladder volume' referring to here?

Reply 15: We have added our explanation as advised (see: Page 6, line 100).

Changes in the text 15: Bladder volume was defined as the infusion volume when the fluid releases from urethra.

Comment 16: P10 Figure 2 Is it possible to add the individual data points to the bar chart in Part B, as the bars in the T10 and Late groups look similar. And also for the data in Figure 3 B,C.

Reply 16: We have added the individual data points to the bar chart in Figure 2B, Figure 3 B,C as suggested.

Discussion

Comment 17: P10 line 180 I would have thought that the improvement of bladder compliance would also reduce the risk of upper tract damage.

Reply 17: We have modified our text as advised (see: Page 10, line 187).

Changes in the text 17: The improvement of DO and bladder fibrosis could reduce detrusor pressure and increase bladder volume, thus improving urinary dysfunction, reducing the risk of hydronephrosis and protecting renal function in SCI patients.

Comment 18: P10 line 183 etc Here is a good discussion of why the BoNTA dose was used. Could this be referred to in the Methods

Reply 18: It is a good suggestion to explain the reasons for BoNTA dose earlier, however, the paragraph is too long after we integrated the two parts of "Rat models" and "Bladder wall injection" together as **Reply 4** in **Comment 4 (**see Page 6, line 80-89), so we think it may be better to put the explanation in Discussion.

Comment 19: P11 line 192 etc This paragraph deals with the important question of timing of BoNTA injections after spinal cord injury to reduced fibrosis development. The answer is not quite clear from this study, although there is a suggestion that early injection is better. So maybe a positive result about timing is overstated here as there is a suggestion that early injection may be better but there is no statistical evidence. How do time scales in a rat compare to the human condition? Also how might this study be improved to provide a mire definite answer about this important question?

Reply 19: We agree with the reviewer's suggestion and further research is needed to provide a definite answer for the benefit of early BoNTA injection. We added some explanation **(**see Page 11, line 207-211).

Changes in the text 19:

Neurogenic bladder fibrosis in patients with supraspinal cord injury is usually irreversible, and early BoNTA injection might prevent the fibrosis. However, we found that the bladder/body weight, DO frequency and bladder volume improved significantly in the Early group than in the Late group, while there was no significant difference between them in the bladder weight, DO amplitude and connective tissue percentage. Thus, this study showed early BoNTA injection had a tendency to prevent bladder fibrosis, but further research is needed to provide a definite answer.

Comment 20: P11 line 206 You are arguing that the replacement of smooth muscle with fibrosis will reduce detrusor contractile function, rather than a change to the physiological properties of detrusor. Have you tested this in vitro?

Reply 20: This is an interesting question that we need further exploration.

Conclusions

Comment 21: P12 line 225 You say "The results support the early usage of BoNTA to prevent bladder fibrosis in SCI patients…" I think this is a little strong, there is no statistical demonstration about this just a suggestive trend.

Reply 21: We agree with the reviewer's suggestion and modified the conclusion (see Page 12, line 231-232).

Changes in the text 21: The results suggest the usage of BoNTA to prevent bladder fibrosis in SCI patients with DO and demonstrate a mechanism of BoNTA action by downregulating TGF-β1 expression.