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A mouse model for chronic pancreatitis via bile duct TNBS infusion.

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TITLE:

A mouse model for chronic pancreatitis via bile duct TNBS infusion

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KEYWORDS:
chronic pancreatitis, mouse model, bile duct infusion, TNBS

SUMMARY:
Chronic pancreatitis (CP) is a disease characterized by inflammation and fibrosis of the pancreas, often associated with intractable abdominal pain. This article focuses on refining the technique to generate a mouse model of CP via bile duct infusion with 2,4,6 -trinitrobenzene sulfonic acid (TNBS).

ABSTRACT:
Chronic pancreatitis (CP) is a complex disease involving pancreatic inflammation and fibrosis, glandular atrophy, abdominal pain and other symptoms. Several rodent models have been developed to study CP, of which the bile duct 2,4,6 -trinitrobenzene sulfonic acid (TNBS) infusion model replicates the features of neuropathic pain seen in CP. However, bile duct drug infusion in mice is technically challenging. This protocol demonstrates the procedure of bile duct TNBS infusion for the generation of the CP mouse model. TNBS was infused into the pancreas through the ampulla of Vater in the duodenum. This protocol optimized drug volume, surgical procedures, and drug handling during the procedure. TNBS-treated mice showed features of CP as reflected by bodyweight and pancreas weight reductions, changes in pain-associated behaviors, and abnormal pancreatic morphology. With the improvements to the protocol, mortality associated with TNBS injection was minimal. This procedure is not only critical in generating pancreatic disease models but is also useful in local pancreatic drug delivery.

INTRODUCTION:

Chronic pancreatitis (CP) is a chronic inflammatory disease characterized by the atrophy of the pancreas, fibrosis, abdominal pain, and eventual loss of both exocrine and endocrine functions[1]. Current medical and surgical treatments are not curative but are undertaken to relieve symptoms that are the consequence of the disease: refractory abdominal pain, endocrine and exocrine dysfunction. Therefore, more effective treatments are urgently needed[2]. Animal models provide an essential tool for developing a better understanding of the disease and investigating potential therapeutics[3]. Multiple mouse models for CP have been developed, of which cerulein and/or alcohol models are commonly used. Cerulein, an oligopeptide stimulating pancreatic secretion, has been shown to reproducibly induce a CP model featuring pancreatic atrophy, fibrosis, among others[4]. Another common model uses serial injections of L-arginine, which produces exocrine insufficiency similar to that observed in human patients[5]. CP can also be induced by complete or partial pancreatic duct ligation, as well as pancreatic duct hypertension[6,7]. Despite the variety of animal models available for CP, none of these models effectively reproduces the abdominal pain experienced by CP patients[8].

Previous studies showed that local pancreatic injection of 2,4,6 -trinitrobenzene sulfonic acid (TNBS) replicates the persistent pain experienced by CP patients[9-11]. TNBS-treated mice demonstrated abdominal hypersensitivity and increased pain-related behaviors as well as a "generalized hypersensitivity" to painful stimuli, a phenomenon that has been observed in CP patients[10]. In addition to accurately mimicking CP pain, the TNBS model replicates other pathological features of the human condition such as fibrosis, mononuclear cell infiltration, and replacement of acinar cells with fatty tissue[10,12]. However, TNBS infusion via bile duct is a technically challenging procedure in mice that may cause death. To our knowledge, there is no visual protocol to show how bile duct infusion is performed. In this article, we demonstrate the procedure of the bile duction infusion of TNBS to generate a CP mouse model. This procedure will help generate valuable animal models for the study of CP and other pancreatic diseases and can be used to infuse other materials (e.g., virus, cells) into the pancreas[13].

PROTOCOL:

All procedures were conducted with the approval of the Institutional Animal Care and Use Committees at the Medical University of South Carolina and the Ralph H. Johnson Medical Center. C57BL/6J male mice between 8-10 weeks of age were used in this study. Mice were housed under a standard 12 light/ 12 dark cycle with ad libitum access to chow and water.

1. Preparation of TNBS solution for injection

1.1. Prepare 10% ethanol in 0.9% saline. Dissolve stock TNBS (see **Table of Materials**) in 10% ethanol to a final concentration of 7.5 mM by adding 7.5 µL of TNBS into 1 mL of 10% ethanol.

CAUTION: TNBS presents a chemical hazard. Prepare the solution inside a fume hood and use personal protective equipment such as gloves, goggles, and a lab coat to avoid direct contact with TNBS.

1.2. Load 50 µL of 0.75% TNBS solution into an insulin syringe with a 31-gauge needle. Load 50 µL of 10% ethanol in saline into a syringe of the same size as vehicle control. Place the syringes on ice and protect them from light until needed.

2. Mouse preparation and surgery

2.1. Shave the hair from the abdominal surgical area.

2.2. Inject one pre-emptive dose of the analgesic (e.g., buprenorphine 0.1 mg/kg i.p.) before surgery.

2.3. Induce and maintain the mouse under general anesthesia with 1.5-2% isoflurane and 1 L/min of oxygen. Confirm the anesthetization by pinching the toes and observing the animal for a lack of reflex.

2.4. Place the mouse on a heated surgical pad during surgery. Apply veterinary ointment on each eye when the mouse is under anesthesia.

2.5. Disinfect the surgical site by wiping the surgical area 3x with 2% iodine, followed by 70% alcohol (**Table of Materials**).

2.6. Perform a laparotomy with micro scissors to generate a 0.5-1 cm incision.

2.7. Gently expose the duodenum and locate the common bile duct using cotton swabs (**Table of Materials**).

2.8. Place a straight micro hemo clip (**Table of Materials**) over the proximal common duct to prevent the flow of TNBS or vehicle solutions into the liver and the gallbladder (**Figure 1A**,**B**).

2.9. Gently expose the duodenum and insert the needle into the pancreatic duct through the papilla of Vater.

2.10. Once the needle is inside the duct, place a curved micro hemo clip (**Table of Materials**) over the duodenum surrounding the needle (**Figure 1A**) to secure the needle in place and prevent the injected solution from entering the duodenum.

2.11. Gradually infuse the solution (TNBS or vehicle) into the pancreatic duct over the course of one min.

NOTE: TNBS needs to be infused slowly over one min of time, and it is easy to control the infusion speed when the pancreas is perfused with an insulin syringe with a 5/16 inch and 31G needle. Keep the hand as stable as possible to avoid pricking the bile duct. If TNBS is successfully injected, yellow color may be visible inside the pancreas.

2.12. After infusion, carefully remove the micro clamp near the liver, and then remove the micro clamp holding the needle and the duodenum.

2.13. Carefully return the duodenum to its original position.

2.14. Leave 0.5 mL of warm sterile saline (36-37 °C) in the abdominal cavity before closure, to help the duodenum return to its original position and assist with the recovery of peristalsis.

2.15. Close the incision in the muscle layer using continuous suture with a 5-0 stitch. Close the skin using interrupted suture with a 4-0 stitch.

2.16. Place the cage containing mice on a heating pad to allow recovery from the anesthesia.

2.17. Confirm that the mice are warm and capable of spontaneous movement before returning them to the holding room.

2.18. Continue to provide an analgesic (e.g., buprenorphine 0.1 mg/kg i.p.) every 12 h and supplemental heat for 48 h post-surgery.

3. Monitoring mouse behavior

3.1. Remove sutures at day 7 post-surgery.

3.2. Monitor mouse health and behavior daily during the first-week post-surgery. Watch for signs of distress such as vocalizing, hunched back posture, or reduced locomotion. Measure the body weight every other day.

3.3. Use Von Frey monofilaments (VFFs) to measure abdominal mechanical hypersensitivity before, and 2, 3 weeks post-surgery as described[9,14].

3.3.1. Apply VFFs of different applied forces in the ascending order to the upper abdominal area 10x every 1-2 s. Consider the raising, retraction, or licking of the abdomen (withdrawal response) as a positive response.

3.3.2. Apply a stronger stimulus if a positive response is observed, and a weaker stimulus if a positive response is observed. The withdrawal threshold is the force at which the mouse responds 50% of the time.

4. Collection and histological analysis of pancreatic tissue

4.1. Sacrifice the mice under anesthesia by cervical dislocation, and carefully dissect the pancreas from the intestine and other organs.

4.2. Fix the pancreas in 10% paraformaldehyde for 24 h, embed in paraffin, cut tissue sections of 5 μm thickness, and place them on glass slides for staining.

4.3. Perform hematoxylin-eosin, and Masson's trichrome staining using standard methods as previously reported[4].

REPRESENTATIVE RESULTS

The bile duct infusion procedures were optimized to reduce mouse mortality associated with this procedure[10]. TNBS was first given in a total volume of 35 μL or 50 μL. Injection of TNBS in a volume of 50 μL could reach the whole pancreas and induce a more homogeneous disease phenotype (**Figure 1B**). In addition, injection of TNBS using an insulin syringe with 31G needle allowed for better control of infusion speed relative to regular syringes and needle sizes. Freshly prepared TNBS stored on ice and used within one hour of drug preparation also yielded a better outcome compared to TNBS prepared over one hour before use. With these improvements, the mortality of recipients was controlled and remained under 10%.

Body weight loss is one of the characteristics of CP. Mice in the control group lost around 6% of their original body weight during the first 3 days after surgery, then gradually recovered (104.6% of original body weight at day 21) (**Figure 1C**). In contrast, mice receiving TNBS lost on average about 15% of their original body weight during the first 5 days and regained weight afterward (99.8% of original body weight at day 21) (**Figure 1C**). In addition, compared to controls, TNBS mice showed increased abdominal mechanical hypersensitivity at 2 and 3 weeks after TNBS injection (**Figure 1D**), which was likely associated with increased abdominal pain[10].

To confirm that the bile duct TNBS infusion effectively induced symptoms of pancreatic changes mimicking human CP, we collected pancreatic tissues from TNBS or vehicle-treated control mice at 3 weeks post-surgery. Both size, and weight per body weight of the pancreas were significantly reduced in TNBS mice compared with controls (**Figure 2A,B**), suggesting marked pancreatic atrophy consistent with the findings in humans with severe and long-term CP. In addition, the pancreas from the control mice appeared normal without obvious morphological changes, while TNBS mice showed vacuolization with massive loss of acinar cells replaced by fat cell infiltration and fibrosis (**Figure 2C**). These findings were consistent with reports from other studies[9,10].

FIGURE LEGENDS:

Figure 1: TNBS bile duct infusion for CP mice generation. (**A**) Illustration of bile duct injection. (**B**) Bile duct after injection of 50 μL of ink. (**C**) Averages of body weight change in mice receiving TNBS or vehicle. (**D**) Abdominal response threshold in TNBS and control mice at 3 weeks after infusion. Data were analyzed using available analysis software (e.g., GraphPad 8.2.1). Data are

presented as mean ± SEM. Differences between groups were analyzed using the Student's t-test, ** $p < 0.01$ was considered statistically significant.

Figure 2. Characterization of CP in TNBS-treated mice. (**A**) Micrographs of the pancreas from control (CTR) and TNBS mice. (**B**) The average pancreas weight divided by mouse bodyweight in CTR and TNBS mice. (**C**) Hematoxylin and eosin staining of pancreas sections of CTR and TNBS mice. Scale bar =100 μm. Data are presented as mean ± SEM. **, $p < 0.01$ by Student's t-test.

DISCUSSION:

Bile duct infusion of TNBS to induce chronic pancreatitis is technically challenging in mice, as up to 22.5% of mice can die within 3-4 days of drug infusion[10]. We refined the procedure based on previous studies and reduced early mouse mortality to <10%. For example, the increased drug volume (from 35 μL to 50μL) can ensure the drug reaches the whole pancreas. Using an insulin syringe and a smaller needle size (31G) reduces potential damage to the pancreatic duct and the leakage of bile into the gallbladder or the abdomen, which most likely would cause mouse death within the first several days after surgery. Clamping both ends of the bile duct can prevent TNBS from leaking to the gallbladder and the intestine, which may cause mortality. The hemo clips restrain TNBS within the injected pancreas, improving the efficacy while reducing damages to other tissues. In addition, TNBS is not stable at temperatures above 0 °C. Therefore, by using freshly prepared TNBS, CP induction achieved stable results.

This protocol effectively induces CP in male C57BL/6J mice at 8-12 weeks of ages and generates a model that mimics major symptoms of chronic pancreatitis, including bodyweight loss, pancreatic atrophy, fibrosis, and likely abdominal pain. Compared to other CP mouse models, the TNBS model is widely used to evaluate analgesic effects in addition to inflammation[10,15,16]. The other advantage of the TNBS model is that the drug is injected directly into the pancreas, which reduces damage to other organs that may interfere with the study[17]. This TNBS CP mouse model can be used to study pathogenesis as well as treatment options together with other chronic pancreatitis models.

One limitation of this study is that only mice between 8-10 weeks of age were used. Since mice at different ages may have difference pancreas sizes, which consequently affect TNBS-CP development. Therefore, whether mice at different ages/sizes should be given a different dose of TNBS needs to be tested. Nevertheless, this study demonstrated the procedures to successfully perform pancreatic duct infusion that may help with studies focused on pancreatic diseases.

ACKNOWLEDGEMENTS:

This study was supported by the Department of Veterans Affairs (VA-ORD BLR&D Merit I01BX004536), and the National Institute of Health grants # 1R01DK105183, DK120394, and DK118529 to HW. We thank Dr. Hongju Wu for sharing technical experience.

DISCLOSURES:

All authors declare that they do not have conflict of interest.

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Figure 1

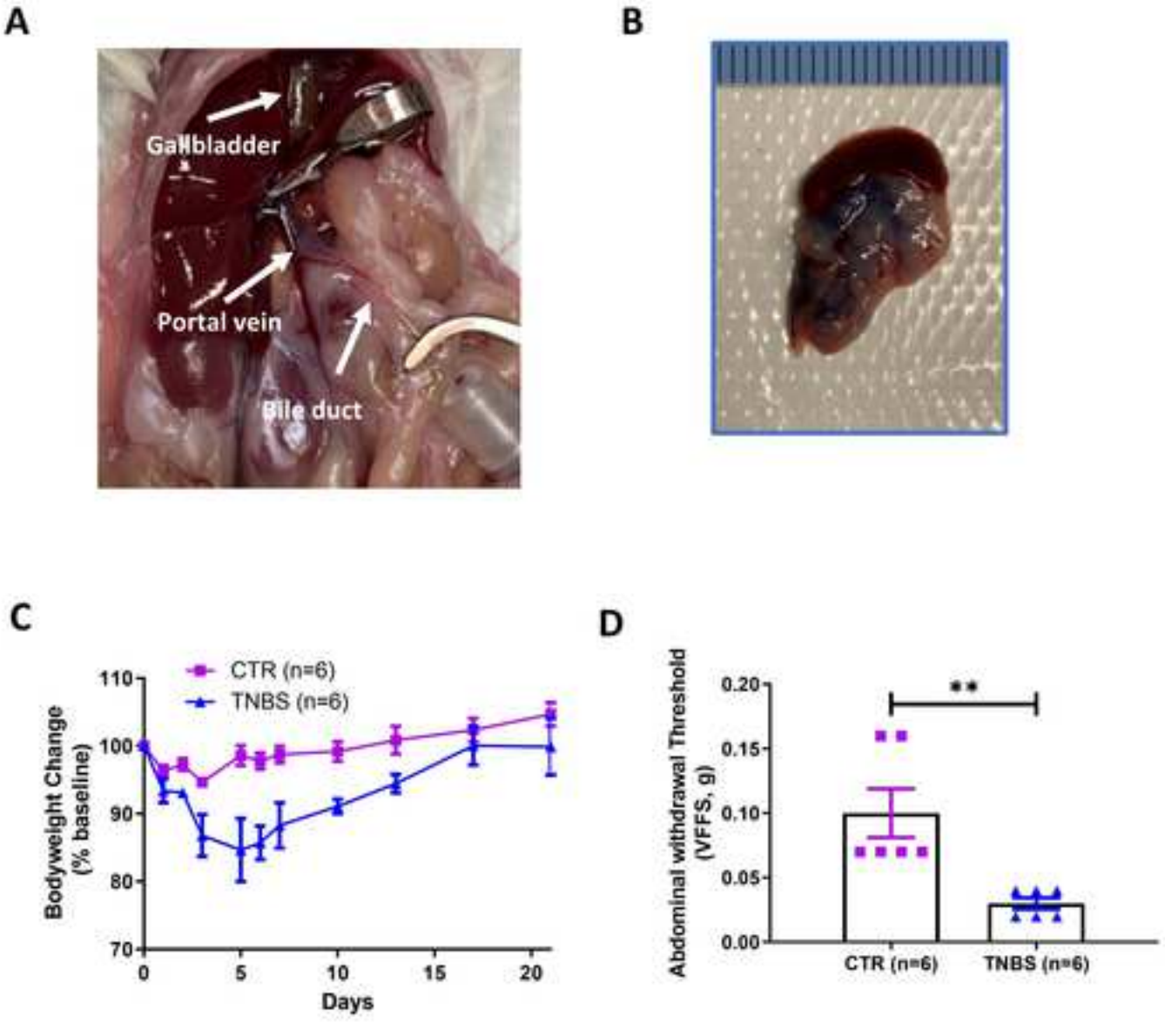

Figure 2

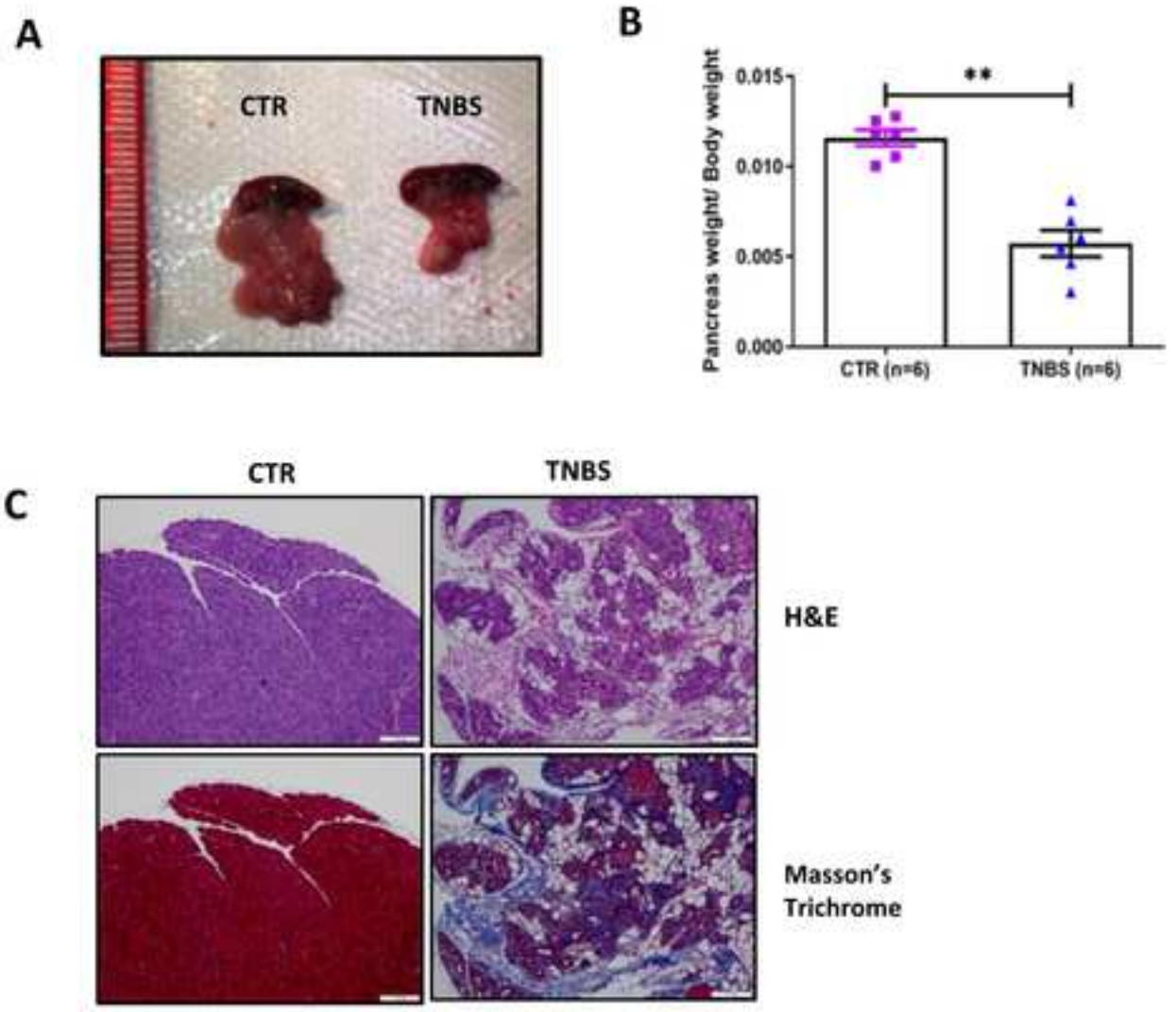

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_CP.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
10% Neutral buffered formalin v/v	Fisher Scientific	23426796	
Alcohol prep pads, sterile	Fisher Scientific	22-363-750	
Animal Anesthesia system	VetEquip, Inc.	901806	
Buprenorphine hydrochloride, injection	Par Sterile Products, LLC	NDC 42023-179-05	
Centrifuge tubes, 15 mL	Fisher Scientific	0553859A	
Ethanol, absolute (200 proof), molecular biology grade	Fisher Scientific	BP2818500	
Extra fine Micro Dissecting scissors 4” straight sharp	Roboz Surgical Instrument Co.	RS-5882	
Graefe forceps 4” extra delicate tip	Roboz Surgical Instrument Co.	RS-5136	
Heated pad	Amazon	B07HMKMBKM	
Hegar-Baumgartner Needle Holder 5.25”	Roboz Surgical Instrument Co.	RS-7850	
Insulin syringe with 31-gauge needle	BD	324909	
Iodine prep pads	Fisher Scientific	19-027048	
Isoflurane	Piramal Critical Care	NDC 66794-017-25	
Micro clip applying forceps 5.5”	Roboz Surgical Instrument Co.	RS-5410	
Micro clip, straight strong curved 1x6mm	Roboz Surgical Instrument Co.	RS-5433	
Micro clip, straight, 0.75mm clip width	Roboz Surgical Instrument Co.	RS-5420	
Picrylsulfonic acid solution, TNBS, 1M in H_2O	Millipore Sigma	92822-1ML	

Polypropylene Suture 4-0	Med-Vet International	MV-8683	
Polypropylene Suture 5-0	Med-Vet International	MV-8661	
Sodium chloride, 0.9% intravenous solution	VWR	2B1322Q	
Surgical drape, sterile	Med-Vet International	DR1826	
Tissue Cassette	Fisher Scientific	22-272416	
Von Frey filaments	Bioseb	EB2-VFF	

January 12, 2021

Journal of Visualized Experiments
Editorial office

Dear Editor and reviewers:

We appreciate your review of our manuscript entitled: “Generation of Chronic Pancreatitis Mouse Model via Bile Duct TNBS Infusion.” Thank you for your valuable comments. We have revised the manuscript based on your comments. Below are our point-to-point answers to your comments:

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
Answer: We have thoroughly proofread our manuscript, and all abbreviations are addressed at first use.

2. Please provide an email address for each author.
Answer:
Dr. Wenyu Gou: gou@musc.edu;
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3. Please refer to the instructions for authors and JoVE’s style guide to organize your manuscript in this order: Title, author names and affiliations (with email addresses)+corresponding author information; Summary; Abstract; Introduction; Protocol; Representative Results, Figure and Table Legends; Discussion; Acknowledgments; Disclosure; References; Figures (uploaded individually without legends); Tables (uploaded individually without legends); Table of Materials if applicable; Supplementary material
Answer: We have re-formatted the manuscript as requested.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.
Answer: This has been changed as suggested.

5. *Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).*
Answer: This has been revised.

6. Please introduce the information about the strain, age, and sex of the animals (line 160) to the beginning of the protocol and also specify details regarding housing, diet etc. If this information has been published, you can also cite that reference.
Answer: We have added this information in the revised manuscript.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Answer: We have added more details in the protocol so that the viewers can follow and replicate the protocol.

8. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
Answer: This has been changed.

9. As we are a methods journal, please add limitations of the technique to the Discussion.
Answer: Limitations of the technique have been added in the discussion.

10. Please include an Acknowledgements section after Discussion but before Disclosures, containing any acknowledgments and all funding sources for this work.
Answer: This has been added.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references and do not abbreviate journal names.
Answer: References have been re-formatted according to the Endnote style of JOVE.

Reviewers' comments:
Reviewer #1: Manuscript Summary: Excellent work. Congratulation
We appreciate the positive comments.

Reviewer #2:

Manuscript Summary:
We thank you for the comments: "*In the present manuscript, the authors demonstrate a valuable model for studying chronic pancreatitis, i.e. TNBS model. The protocol is easy to follow, yet needs to be improved in some aspects.*"

Major Concerns:
1. Intro: The authors state that "the TNBS model also replicates other pathological features of the human condition such as fibrosis, mononuclear cell infiltration, and replacement of acinar cells with fatty tissue". Please provide a supportive reference for this statement.
***Answer**:* As reported by Puig-Divi and colleagues, TNBS instillation into the pancreatic duct could result in chronic pancreatic disease. Morphological examination revealed changes mimicking features of chronic pancreatitis in humans, consisting of various degrees of periductal and lobular fibrosis, duct stenosis, patchy acute and chronic inflammatory cell infiltrates, and signs of gland atrophy in the rat model [1]. In addition, Cattaruzza demonstrated that TNBS infusion induced CP in mice, and the most striking feature was the massive loss of acinar cells and their replacement by fat and fibrosis as seen in human disease [2]. Both references are included in this revised manuscript.

2. Lin 69: define "ideal volume" already here and not as late as in the line 125.
Answer: We clarified in the protocol that TNBS was diluted into 50ul of volume with 10% of ethanol for injection in the revised manuscript.

3. Line 94: "Gradually infuse the solution (TNBS or vehicle) into the pancreatic duct over the course of one minute." Please comment on how this gradual infusion is achieved already here not as late as in Line 127.
Answer: Gradual infusion can be achieved by injecting TNBS with an insulin syringe with a 31G needle. The needle has to be pushed extremely gently to make sure the solution is injected gradually. This has been updated in the manuscript.

4. Line 107: "Continue to provide analgesics and supplemental heat for another 48 hours post-surgery". Which analgesics are applied by the authors during this time period?
Answer: Buprenorphine (0.1 mg/kg i.p.) was given every 12 hours as analgesics for another 48 hours post-surgery.

5. Line 114: "Use Von Frey monofilaments to measure abdominal mechanical hypersensitivity before and 3 weeks post-surgery as described." Please describe the natural course of pain development in these animals and their survival curve.

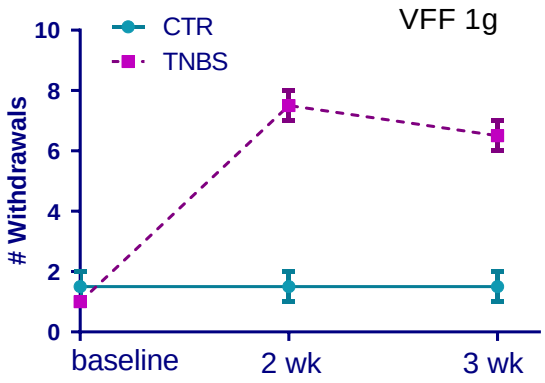

Fig.1 TNBS-induced CP increases pain behaviors. Mice abdominal mechanical sensitivity was measured by VFF method using applied forces of 1g in ascending order to the upper abdominal area for 10 times every 1-2s. A response was considered positive when the mice raised, retracted, or lick the abdomen (withdrawal response). The data are expressed as the average of the number of withdrawal responses per filament per experimental

Answer: In our study and the study by the Cattaruzz group [2], abdominal mechanical referred pain or sensitivity could be detected as early as 1 week after TNBS infusion and peaked at 2-3 weeks post TNBS infusion.

The mortality rate in our model is less than 10%, and deaths normally occurred within 48hrs after the surgery, mainly caused by surgery complications. Damage to the bile duct was the leading cause of mortality in our experience.

6. Line 116: I could not find any info on the abdominal sensitivity of these animals in the subsequent section.

Answer: Information on the abdominal sensitivity of animals has been added to protocol section 3.3.

7. Please provide a brief example of von Frey results as obtained from this model.

Answer: Figure 1D in the manuscript showed abdominal withdrawal threshold measured by the VFF method at 3 weeks after injection. In addition, Fig.1 above shows that TNBS-induced CP pain behaviors lasted for at least 3 weeks after TNBS infusion.

8. Please comment on the difficulties and caveats related to performing abdominal sensitivity testing via von Frey filaments in mice.

Answer: As pain cannot be directly measured in mice, stimulus-evoked methods such as abdominal sensitivity testing via von Frey filaments have been developed. The difficulties and caveats related to performing abdominal sensitivity testing were described by Deuis and colleagues [3]: "*Mice may respond to initial contact with a filament with a "touch-on" response, the manual von Frey method needs to be applied in the ascending order to the upper abdominal area. A touch-on reaction is more likely to occur if the filament is not applied perpendicularly, if the filament is not applied smoothly, or if the filament moves horizontally during the application, inducing scratching. Besides, rodents are intelligent and can learn that premature withdrawal will result in less human interaction and stimulation. Experimenters experienced in the technique can distinguish between "touch on" or false-positive responses, however, this can be difficult for inexperienced researchers and extensive training is usually required to produce high-quality data.*"

9. Von Frey testing is a form of evoked pain testing. Please comment on the suitability of the TNBS model for spontaneous pain testing.

Answer: This is a great point. CP patients may suffer from both stimulus-evoked pain and stimulus-independent or spontaneous pain [4]. Stimulus-evoked pain includes hyperalgesia or allodynia and can be measured in the TNBS mice using Von Frey testing. Spontaneous pain may include paroxysmal or continuous pain. The pain appears to be spontaneous, with no identifiable stimulus [3]. The distinction between these two types of pain is extremely difficult to distinguish. To the best of our knowledge, it is unclear if the TNBS mouse model can be used to recapitulate spontaneous pain based on current literature.

10. Please also briefly discuss the advantages of this model in comparison to multiple other CP models, as discussed in Reference 1.

Answer: The following information has been included in the discussion: "TNBS-treated mice demonstrated abdominal hypersensitivity and increased pain-related behaviors as well as a "generalized hypersensitivity" to painful stimuli, a phenomenon that has been observed in CP patients [2]. In addition to accurately mimicking CP pain, the TNBS model also replicates other pathological features of the human condition such as fibrosis, mononuclear cell infiltration, and replacement of acinar cells with fatty tissue [1, 2]. Compared to other CP mouse models, the TNBS model is most commonly used to evaluate analgesic effects in addition to inflammation [2, 5, 6]. The other advantage of the TNBS model is that the drug is injected directly into the pancreas, which reduces damage to other organs that may interfere with the study [7].

Minor Concerns: none

Reviewer #3:

Manuscript Summary:

We appreciate the reviewer's comments: "The JoVE62080 manuscript (title: Generation of Chronic Pancreatitis Mouse Model via Bile Duct TNBS Infusion) present the step-by-step surgical method for chronic pancreatitis induction by TNBS. This is a rarely used method in the research field of chronic pancreatitis, but the authors highlighted the advantages of this model".

Minor Concerns:

1. Please write the Trinitrobenzene Sulfonic Acid to abstract and introduction as well, not just the abbreviation the TNBS.

Answer: This has been changed as suggested.

2. The use of % in case of solutions or reagents are not exact enough, the w/v% or w/w % are better. However, I recommend using M (mol/L) for the final concentration of TNBS, because the purchased stock solution of TNBS is also expressed in M, it is 1M.

Answer: We appreciate the comments. We have added that 0.75% TNBS = 0.0075 mol/L =

0.00855 g/mL in the revised manuscript.

3. The further thing is that the "Prepare 0.75% TNBS (Table of Materials) by dissolving in 10% ethanol and dilute to ideal volume using saline" sentence is not fully clear. First you prepare 0.75% TNBS in 10% ethanol, then you further dissolve it in saline or you dilute the stock TNBS in 10v/v% ethanol (in saline) solution to 0.75%? Please clarify that sentence
Answer: We have clarified how to prepare TNBS in the method section. Please also see response to Review 2, question #2.

4. The needle what is injected into the pancreatic duct is sharp and pricking. How can you prevent the perforation of pancreatic duct? Can an unsharpened needle be a better solution for this procedure?
Answer: That's a great point. However, if the needle is unsharpened, it is hard to inject into the bile duct through the papilla of Vater. The only way to avoid perforation of the pancreas duct is to keep the hand stable during infusion.

5. The authors mentioned that the 50 µL from TNBS solution can cause homogenous disease phenotype. However, the weight of mice can influence the required volume of TNBS. The size of pancreas in 20g mice or 30g mice are different. It is recommended to correct the TNBS volume with the weight of animal. Furthermore, the weight of animal should be written in the protocol
Answer: We agree that mouse body weights can vary based on the age. We proposed that TNBS volume may need to be adjusted based on body weight.

6. Please indicate the mouse strain in the beginning of the protocol
Answer: This information has been added as suggested.

References:

1. Puig-Diví, V., et al., *Induction of chronic pancreatic disease by trinitrobenzene sulfonic acid infusion into rat pancreatic ducts.* Pancreas, 1996. **13**(4): p. 417-24.
2. Cattaruzza, F., et al., *Transient receptor potential ankyrin 1 mediates chronic pancreatitis pain in mice.* Am J Physiol Gastrointest Liver Physiol, 2013. **304**(11): p. G1002-12.
3. Deuis, J.R., L.S. Dvorakova, and I. Vetter, *Methods Used to Evaluate Pain Behaviors in Rodents.* Front Mol Neurosci, 2017. **10**: p. 284.
4. Cruz-Almeida, Y. and R.B. Fillingim, *Can quantitative sensory testing move us closer to mechanism-based pain management?* Pain Med, 2014. **15**(1): p. 61-72.
5. Puig-Divi, V., et al., *Induction of chronic pancreatic disease by trinitrobenzene sulfonic acid infusion into rat pancreatic ducts.* Pancreas, 1996. **13**(4): p. 417-24.
6. Xu, G.Y., et al., *Enhanced excitability and suppression of A-type K+ current of pancreas-specific afferent neurons in a rat model of chronic pancreatitis.* Am J Physiol Gastrointest

Liver Physiol, 2006. **291**(3): p. G424-31.
7. Drewes, A.M., et al., *Pain in chronic pancreatitis: the role of neuropathic pain mechanisms.* Gut, 2008. **57**(11): p. 1616-27.