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X-ray Visualization of Intraductal Ethanol-based Ablative Treatment for Prevention of Breast Cancer in Rat Models

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Abstract

There are still a limited number of primary interventions for prevention of breast cancer. For women at high risk of developing breast cancer, the most effective intervention is prophylactic mastectomy. This is a drastic surgical procedure in which the mammary epithelial cells that can give rise to breast cancer are completely removed along with the surrounding tissue. The goal of this protocol is to demonstrate feasibility of a minimally invasive intraductal procedure which could become a new primary intervention for breast cancer prevention. This local procedure would preferentially ablate mammary epithelial cells before they are able to become malignant. Intraductal methods to deliver solutions directly to these epithelial cells in rodent models of breast cancer has been developed by us, as well as others. The rat mammary gland consists of a single ductal tree and has a simpler and more linear architecture compared to the human breast. However, chemically induced rat models of breast cancer offer valuable tools for proof-of-concept studies of new preventive interventions and scalability from mouse models to humans. Here, we describe a procedure for intraductal delivery into the rat mammary ductal tree of an ethanol-based ablative solution containing tantalum oxide nanoparticles as X-ray contrast agent and ethyl cellulose as gelling agent. Delivery of aqueous reagents (e.g., cytotoxic compounds, siRNAs, AdCre) by intraductal injection has been described previously in mouse and rat models. This protocol description will emphasize methodological changes and steps that pertain uniquely to delivering an ablative solution, formulation consideration to minimize local and systemic side effects of the ablative solution, and X-ray imaging for *in vivo* assessment of ductal tree filling. Fluoroscopy and

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microCT techniques enable to determine success of ablative solution delivery and extent of ductal tree filling thanks to compatibility with tantalum-containing contrast agent.

SUMMARY:

A procedure for the delivery of a chemical ablative solution to the rat mammary ductal tree for image-guided preventive treatment of breast cancer is described. Mammary epithelial cells can be targeted with minimal collateral tissue damage through cannulation directly into the nipple opening and intraductal infusion of 70% ethanol-based ablative solution.

Keywords

ductal tree; intraductal; mammary gland; ductography; chemical ablation; image-guided procedure

INTRODUCTION:

For women in the USA[1], breast cancer (BC) continues to be most diagnosed cancer type and causes more deaths than any other cancer type, except lung cancer. Projections for 2022 estimate that 51,400 women will be diagnosed with carcinoma *in situ* and 287,850 with invasive carcinoma, and that 43,600 women will die from BC [1]. Despite the prevalence and mortality associated with BC, there are few options available for primary prevention and translational research on novel interventions for primary prevention is not prioritized by federal agencies[2]. Prophylactic mastectomy is the most effective intervention for primary prevention. However, this procedure is only recommended for high-risk individuals because it is a major surgery with life-changing consequences [3]. This surgery involves complete removal of the mammary epithelial cells from which carcinogenesis develops as well as the normal surrounding tissue. Individuals are often dissuaded from using this procedure as their first option of primary intervention due to the negative impact of physical, psychological, and social stress. For these reasons, even some high-risk individuals opt to not undergo this procedure and choose instead watchful waiting or similar surveillance strategies [3]. In our previous work, delivery of 70% ethanol (EtOH) directly into the ductal tree of mouse models was effective at chemically ablating mammary epithelial cells with limited damage to adjacent normal tissue and at preventing breast tumor formation [4]. EtOH is used in multiple clinical applications as either an ablative agent for local treatment of some cancers or sclerosing agent for local treatment of arteriovenous swelling and malformations [5–14]. The low toxicity and safety profile of EtOH is well established, as in some procedures up to 50 mL of 95% EtOH can be administered per session [5,10].

Complete removal of mammary epithelial cells from which BC develops is the most crucial component of both prophylactic mastectomy and local delivery of an ablative solution. Therefore, confirmation of complete ductal tree filling is necessary to guarantee that the ablative solution has come in direct contact with all mammary epithelial cells. Delivering a solution within the ductal tree(s) and its visualization by image-guided fluoroscopy or ductography are possible through clinical procedures that already exist [15–17]. Thus, it will be feasible to readily implement and evaluate this procedure in clinical trials. A key step in establishing the efficacy and translational feasibility of intraductal (ID) ablation as a

new intervention for primary prevention will be to demonstrate the feasibility of this X-ray visualization approach in animal models of increasing size and complexity of their ductal tree architecture [4,18,19]. Here, we describe a protocol which scales up this ablative procedure from mouse [20] to rat models. While mouse and rat ductal trees have a similar linear structure and branching pattern, the rat ductal tree is proportionally larger and is surrounded by a much denser stroma. We have implemented a method in our laboratory to successfully inject every mammary gland in a rat over a series of weekly sessions with an ablative solution containing a contrast agent. Session spacing is necessary to ensure the animals minimal side effects of EtOH (Figure 1 and Figure 2). The procedure involves injection of the ablative solution directly into the nipple opening of an isoflurane-anesthetized rat with a 33-gauge needle. Some key improvements of our procedure include the use of extended anti-inflammatory treatment, injection of higher volumes per ductal tree than suggested [21], and gastight syringes for liquid and gases. The duration of treatment with 5 mg/kg of carprofen, an NSAID, from 48 hrs before to one week after ID injections is comparable to anti-inflammatory protocol used for sclerosing therapy of venous malformation in the clinic. Treatment is performed on patients under systemic anesthesia followed by 2 d of anti-inflammatory medications such as NSAIDs. Anti-inflammatory treatment may be extended for a few more days to reduce local inflammation and any potential pain [13]. As in mice [20], intraperitoneal injection of a 5% sucrose solution mitigates short-term effect of alcohol intoxication in rats. Rats can be injected with up to 1 mL of 70% EtOH (up to 4 ducts; 0.2 g/dL of EtOH content in blood) in a single session when administered with this sucrose solution; animals fully recover within 4 h after ID injections. We perform sequential sessions to allow enough recovery time when injecting more than 4 glands and/or higher EtOH concentrations. Alcohol intoxication in women will be much less likely as ID injection of all ductal trees in both breasts, assuming 16 main ducts [16,17]and 2 mL per duct [22,23], with 70% EtOH would result in less than 0.1 g/dL of EtOH content in blood and may cause mild impairment.

X-ray imaging enables determination of how successful intraductal delivery is in each individual gland and if entire ductal tree is filled (Figures 1–3). Real-time fluoroscopy imaging in preparation for micro-CT scan and/or 3D reconstruction of DICOM file data can be used to assess the extent of solution delivery into the ductal tree and any leakage into the stroma. Use of fluoroscopy can help to limit overall radiation dose imposed on the animal. Fluoroscopy technique approximates more closely to the intended clinical application for image-guidance of this ablative treatment. We previously compared FDA-approved iodine-containing Isovue to tantalum oxide (TaO_x) nanoparticles in order to further refine the utility of our ablative solution [4,19]. We found that TaO_x was a superior micro-CT contrast agent for visualization of the initial filling of the ductal tree in mice than Isovue[4,19]. Here, we demonstrate that TaOx is a suitable contrast agent to visualize the initial filling of the rat ductal tree(Figures 2 and 3). Both in translational research and clinical practice applications, the gelling agent ethyl cellulose (EC) has been added to the EtOH solution to minimize diffusion from intended targeted regions [13,14,24–29]. We have observed in our studies that addition of up to 1.5% EC to EtOH-containing ablative solutions is compatible with TaO_x-based imaging (Figure 3). These as well as further refinements to the ablative solution may assist in ready translation of this image-guided procedure to the clinic.

PROTOCOL:

All described experiments were conducted under protocols approved by Institutional Animal Care and Use Committee at Michigan State University.

1. Extended anti-inflammatory treatment

1.1. Prepare sucralose gel cups as oral dosing of carprofen. Provide rats with this anti-inflammatory treatment from 2 d before receiving ID injection of 70% EtOH to 7 d after procedure.

1.2. Dilute in sterile PBS a working solution of carprofen for injection into the cup. From 50 mg/mL stock solution, prepare a diluted 2 mg/mL solution colored with 1% v/v sterile blue food dye and inject 500 μL into each cup. Addition of dye aids in visualization of complete mixing of the drug within the sucralose of the cup.

1.3. Follow manufacturer's recommendation to prepare cup for addition of carprofen. Unless otherwise indicated by vendor, warm the cup in a water bath at 60 °C for 15 min and dry off upon removal to reduce the risk of contamination.

1.3.1. Wipe-clean the lid of the sucralose cup with 70% EtOH in the area and introduce the needle of the syringe containing the carprofen working solution. Dispense the appropriate volume (500 μL) and cover the puncture with a sticker. Then

1.3.2. Shake the cup energetically for 15 s and then place that cup in vortex for additional 15 s. Visually assess the homogenous and complete mixing before storing for later use. Look for the presence of dark blue color.

NOTE: Allow cups to come to room temperature. Store cups at room temperature if desired but take care to pay attention to drug efficacy guidance from the manufacturer. Alternatively, store cups at 4 °C and use within a month. Dating the sticker is good practice for keeping track of the injection date without the risk of a pen or sharp marker puncturing the lid.

1.4. Just before use, wipe down the exterior of the cup with 70% EtOH. The lid should be removed before placing the cup into the animal cage. Cups must be replaced every other day or when empty. The level of gel should be checked daily to insure adequate dosing. One cup supplies carprofen for up to 2 rats for 1–2 d. Rats tend to eat one cup per day.

2. Preoperative preparation

NOTE: Ensure that animal preparation step precedes the ID injection procedure by 2–3 d.

2.1. Turn on isoflurane vaporizer (2–3% isoflurane, 1.5 L/min oxygen) to anesthetize the rat. Move animal to a nose cone on a warming pad. Apply eye lubricant to the rat and then position the animal to its back. Carefully monitor the animal's respiration to ensure that anesthetic plane is maintained at 1–3% of isoflurane.

NOTE: An electric razor may be used to remove excess fur before depilation. Extreme care must be taken not to damage any nipples with the razor. For this reason, you may skip this

step. Rats are more sensitive to depilatory cream than mice, so the removal of excess cream is very important. Avoid injecting an ethanol-containing solution into an area that already has an abrasion present from depilation. Some creams have added compounds such as aloe and lanolin that can help minimize likelihood of abrasions.

2.2. Use cotton-tip applicator to spread over-the-counter depilatory cream onto the nipple area. Use the applicator to rub the cream into the area for 10–30 s. Check that the fur is quickly loosened.

NOTE: Cream should remain on rat for the shortest possible interval and be removed completely to avoid burning the skin. Rats are even more sensitive than mice to this procedure.

2.3. After 10 –30 s of application, wet a gauze with warm water and use it to rinse cream and loosened fur from the animal. Perform at least three rinses of the area with fresh moistened gauze and dry with dry gauze after final rinse. Confirm good visibility and access to the area of the nipple from where fur is removed. Repeat depilatory procedure if necessary.

2.4. Place rat in a clean cage on a heating pad and allow it to recover. Check on the rat to make sure is fully recovered from anesthesia before bringing back to its permanent cage.

2.5. Place one carprofen-dosed (1 mg/cup) sucralose gel cup in the cage for anti-inflammatory treatment. Check gel consumption daily and replace with a fresh cup as appropriate. Do not leave cup for more than 2 d. Typically, cups will need to be replaced after 1 d.

3. Intraductal injection

3.1. TaO_x stock solution at 333.3 mM can be prepared as described[19] using sterile phosphate buffered saline (PBS). Warm solution if powder does not fully dissolve.

3.2. Mix 3 parts of 333.3 mM TaO_x with 7 parts 100% EtOH for a final 70% EtOH 100 mM/ TaO_x solution. Optionally, add appropriate amount of 0.5–1.5% ethyl cellulose (EC) as gelling agents to maximize local retention of the ablative solution. Add 1% v/v blue food dye to the ablative solution for visual examination of delivery into the ductal tree during infusion.

3.3. Prepare a volume appropriate for experimental needs. Gland pairs 1 (cervical) and 6 (inguinal) can be filled with up to 100 μL of solution while all other pairs can be filled with up to 300 μL.

3.4. Anesthetize the rat as in preoperative preparation and move the rat to the nose cone once fully anesthetized. Apply eye lubricant before placing the animal on its back. Secure the rat beneath the stereoscope using tape near the nipples that will be injected, if desired. The weight of the rat is generally enough to keep it from moving substantially without taping.

3.5. Prepare nipples for injection by removing any dead skin that covers nipple opening with fine pointed forceps, if possible. Rats often have a plug protruding from the nipple opening that can prevent successful cannulation of the nipple if not removed.

NOTE: It is important to note that larger injection volumes of ablative solutions being used in rats may be more likely to result in superficial skin wounds near the injection site(s). For this reason, we have found that injecting every other nipple in a single session is less damaging and irritating to the animal than injecting adjacent nipples. Monitoring rats for any abrasions for 7 d post-injection helps to ensure no serious health effects from the animal scratching and introducing the possibility of infection from contamination with cage floor debris. Triple antibiotic ointment or washes with chlorhexidine solution can be used to treat any signs of injury infection that may occur (Table 1).

3.6. Use a 500 μL syringe with a 33-G needle to aspirate 101–301 μL of ablative solution. Aspirate an extra 1 μL of the solution for possible minor leakage when removing the cannulated needle.

NOTE: These are recommendation for volumes aimed at fully filling the ductal tree(s): up to 100 μL in cervical and inguinal glands, and up to 300 μL in the other glands. For other applications, it may be appropriate to use smaller or larger volumes.

3.7. Use tweezer to gently hold the nipple and cannulate the needle into the nipple opening. Gently continue inserting needle until the bevel is fully inside the nipple. To accommodate needle in the nipple, bring the nipple up towards the needle instead of pushing the needle down into the nipple. (Table 1). Take care to follow the path of the nipple opening.

NOTE: Rat nipples are generally much easier to manipulate as it has more fat surrounding the opening and cannulate successfully than those in mice.

3.8. Once the needle bevel is completely inserted, slowly infuse the solution at a constant rate of approximately 100 μL/min in rats. Abrupt changes in infusion rate can burst or damage the ductal tree. Wait for 30 s after end of infusion to remove the needle from cannulated tree with assistance with forceps; this ensures injected volume remains within the ductal tree (Figure 2) and reduces the likelihood of leakage.

3.9. Clean off any spilled solution with moistened gauze or an EtOH wipe to avoid extraneous contrast solution in images.

3.10. Inject intraperitoneally PBS containing 5% sucrose (10 mL/kg) to mitigate the effects of alcohol intoxication if ethanol is contained within ID injection solutions. This dose may be given at the beginning and the end of the procedure.

4. Micro-CT imaging

4.1. After all desired glands have been injected, move the animal swiftly to the micro-CT system and continue maintaining anesthesia using the incorporated isoflurane vaporizer.

4.2. Straighten the spine of the animal and tape each hind leg in an extended position to keep the leg bones of the animal further from the lower glands of interest in the scanned image.

4.3. Tape across the abdomen to minimize breathing artifact if scanning lower glands.

NOTE: Animals can be imaged with different scanning parameters (e.g., high resolution, longitudinal scans) if care is taken to determine an appropriate acceptable lifetime dose of radiation for rats and the cumulative dose does not exceed this level. Radiation exposure may be further reduced by acquiring fluoroscopy stills and videos without performing scans (Figure 2).

4.4. Perform TaO_x imaging of the rat ductal tree with good resolution and opportunity for repeated standard (2 min) acquisition scans using the following scan parameters: 90 kVp/88 μA; field of view (FOV), 72 mm; number of slices, 512; slice thickness, 72 μm; voxel resolution, 72 μm^3. High-resolution scans for longer time periods (4–14 min) can also be acquired in animals that will not be scanned longitudinally using the same parameters.

4.5. After data acquisition, carefully take rat away from anesthesia cone and place in new clean cage on a heating pad. Check on the rat to make sure is fully recovered from anesthesia before bringing back to its permanent cage. Place carprofen-containing sucralose cup and appropriately replace as described in step 2.5 to ensure animals continue to receive anti-inflammatory treatment for the next 7 d.

4.6. Process scanned images into quick renditions within the micro-CT software to better appreciate any contrast leaks, only partial filling or overfilling (Figure 2).

4.7. Proceed to next section to perform formal image processing publication or detailed analysis of scans if desired (Figure 3).

5. Image Analysis

5.1. Use specialized software packages to produce renderings of the filled ductal tree.

5.2. To segment the fat pad (unlike mice, the boundaries of this compartment are not as easily distinguishable from peritoneal cavity, femoral muscles, and skin due to similar Hounsfield units) within which the ductal tree of interest is contained, selecting “spline trace” option from the manual menu is the first step in creating a rendering. Spline trace the fat pad outline in every third slice.

5.3. Click the “propagate objects” option from the semi-automatic menu. This will propagate and connect all slices into a single segmented object of interest.

NOTE: Changing the threshold within the segmented region allows visualization of signal only within a certain range of Hounsfield units (HU); for other contrast agents or imaging paraments this range may need to be adjusted. A software package or Artificial Intelligence analysis may be used to make other measurements and images to show how much the ductal tree was filled.

5.4. Set HU values to low point of 300 and high point of 3,000 on the semi-automatic menu under the threshold volume tab. This allows for creation of a rendition only displaying the contrast (TaO_x) within the ductal tree.

5.5. Toggle “view button” to rendition as primary. This will change the display to only show the 3D rendition of the ductal tree.

REPRESENTATIVE RESULTS:

Each of the 12 mammary glands of a female rat contains a single ductal tree that opens at the nipple orifice. Despite the difference in size between mouse and rats, the developmental timing of mammary glands and the time that animals reach adulthood is very similar [30,31]. We briefly describe the key stages of mammary gland development in rats as representative of both rodent species. Terminal end buds (TEBs) are the highly proliferative structures at the tips of the elongating ductal tree that direct ductal branching [30,31]. The peak of proliferation and density of the TEBs occurs at 3–4 weeks of age during the elongating phase of the ductal tree during pubertal development [30]. By 9–10 weeks of age, there are few TEBs remaining as the ductal tree has grown to occupy the entire length of the fat pad [30]. After that, growth and expansion of the ductal tree proportional to that of the fat pad and of the animal [32]. Terminal ductal lobular units (TDLUs) in the human breast carry out a similar role to the TEBs in rodents. TDLUs are the main source for initiation of carcinogenesis and progression to BC [33,34]. We can inject up to 300 μL of 70% EtOH solution to fill the entire ductal tree of thoracic and abdominal mammary glands of 9-week-old Sprague-Dawley rat (Figures 1–3, ref [4,19]). Unlike mice [20], the nipples of cervical and inguinal glands of Sprague-Dawley rats are typically suitable for injection in more than 80% of animals, and up to 100 μL of 70% EtOH solution is required to fill the entire ductal tree (Figure 2). We routinely inject up to 10 mammary glands with the ablative solution under study. A typical experimental design consists of two independent weekly ID injection sessions, in which five alternating glands are infused with the ablative solution containing X-ray contrast agent and/or EC as gelling agent (Figure 2). For TaO_x-containing (50–200 mM) ablative solution, fluoroscopy and/or micro-CT scanning is performed after end of each session to determine and record the individual success of infusing each ductal tree with partial or full amount of infused solution (Figure 2). Immediate and longitudinal imaging after injection enables assessment of how changes in formulation, especially concentration of EC gelling agent, affects and limits outward diffusion of the ablative solution as a function of the injected volume (Figure 3). This imaging analysis provides cinformation to understand the optimal parameters to achieve maximal ablation with minimal collateral tissue damage.

DISCUSSION:

We have shown that ID delivery of 70% EtOH preferentially ablates the mammary epithelial cells with limited collateral damage to surrounding stroma and vasculature in mice [4]. Local ablation of the ductal tree is effective at preventing tumor formation in mouse models [4]. Here, we demonstrate that this ablative procedure can be scale up to rats. This is the next step in the path to translation of this ablative procedure as an alternative intervention to prophylactic mastectomy for primary prevention of breast cancer in high-risk individuals.

Addition of TaO_x nanoparticles as an X-ray contrast agent to the ablative solution allows to assess effectiveness of the solution at preventing tumor formation, as we can determine if procedure was successful or not a completely filling the ductal tree. Using fluoroscopy to visualize the injected mammary gland mirrors what will likely be done in clinic to guide this ID procedure. Image guidance of how much the solution has filled the ductal tree and when to stop infusion will be a key aspect of clinical implementation to ensure maximal filling of each ductal tree. Effectiveness of this ablative procedure requires that the infused solution makes direct contact with all epithelial cells to maximize rate of cell killing. Spare epithelial cells within one or more trees could eventually serve a source for BC development. Other groups reported ID delivery of viral particles (e.g., components of Cre/LoxP and/or Cas9/CRISPR systems), hormones and hormone antagonists (e.g., prolactin, fulvestrant), chemotherapeutic agents (e..g, cisplatin), siRNAs and/or antibodies or other targeting agents in mice [4,19,21,35–39 40–45], rats [21,33,46–48] and/or rabbits [18,49–53]. Successful cannulation of up to eight ductal trees per human breast for local delivery of chemotherapy has been reported in independent clinical studies [47,54,55]. Image guidance for infusion of these other solutions aimed at tumor prevention or geared towards local treatment would similarly maximize their effectiveness. We demonstrate here the scalability and refinement of this procedure from mouse to rat ductal tree. TaO_x nanoparticle in the murine [4,19] and rat (unpublished data) ductal tree provide high-resolution imaging that surpasses that FDA-approved iodine-containing X-ray contrast agents. Similarly, we are unaware of other ductal tree imaging approaches in mice [40,41] or other animal models [18] that can provide comparable resolution to TaO_x. Relevant for clinical translation is the fact that the gelling effect of EC in this intermediate size rat models is an enabling formulation refinement to minimize collateral tissue damage. As we continue to assess this ablative ID procedure for its ability to prevent BC, we will be able to determine more precisely from which glands BC develops through the added information provided by imaging after ID delivery in chemically induced and other rat models of BC. These data will determine the safety of this procedure and pinpoint any concerns or short-coming of whether partial or unsuccessful treated ductal trees might be more prone to develop BC in a high-risk woman.

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REFERENCES:

1. Siegel RL, Miller KD, Fuchs HE & Jemal A Cancer statistics, 2022. CA Cancer J Clin. 72 (1), 7–33, (2022). [PubMed: 35020204]
2. Wild CP The global cancer burden: necessity is the mother of prevention. Nat Rev Cancer. 19 (3), 123–124, (2019). [PubMed: 30683893]
3. Padamsee TJ, Wills CE, Yee LD & Paskett ED Decision making for breast cancer prevention among women at elevated risk. Breast Cancer Res. 19 (1), 34, (2017). [PubMed: 28340626]
4. Kenyon E et al. Ductal tree ablation by local delivery of ethanol prevents tumor formation in an aggressive mouse model of breast cancer. Breast Cancer Res. 21 (1), 129, (2019). [PubMed: 31779648]

5. Kuang M et al. Ethanol ablation of hepatocellular carcinoma Up to 5.0 cm by using a multipronged injection needle with high-dose strategy. Radiology. 253 (2), 552–561, (2009). [PubMed: 19709992]
6. Ansari D & Andersson R Radiofrequency ablation or percutaneous ethanol injection for the treatment of liver tumors. World J Gastroenterol. 18 (10), 1003–1008, (2012). [PubMed: 22416173]
7. Zhang WY, Li ZS & Jin ZD Endoscopic ultrasound-guided ethanol ablation therapy for tumors. World J Gastroenterol. 19 (22), 3397–3403, (2013). [PubMed: 23801831]
8. Chin M, Chen CL, Chang K, Lee J & Samarasena J Ethanol Ablation of a Peripheral Nerve Sheath Tumor Presenting as a Small Bowel Obstruction. ACG Case Rep J. 3 (1), 31–32, (2015). [PubMed: 26504873]
9. Gueng M-K, Chou Y-H, Tiu C-M, Chiou S-Y & Cheng Y-F Pseudoaneurysm of the Breast Treated with Percutaneous Ethanol Injection. Journal of Medical Ultrasound. 22 (2), 114–116.
10. Zhang J et al. Comparison between absolute ethanol and bleomycin for the treatment of venous malformation in children. Exp Ther Med. 6 (2), 305–309, (2013). [PubMed: 24137179]
11. Wohlgemuth WA et al. Ethanolgel sclerotherapy of venous malformations improves health-related quality-of-life in adults and children - results of a prospective study. Eur Radiol. 27 (6), 2482–2488, (2017). [PubMed: 27699470]
12. Steiner F, FitzJohn T & Tan ST Ethanol sclerotherapy for venous malformation. ANZ J Surg. 86 (10), 790–795, (2016). [PubMed: 25182969]
13. Sannier K et al. A new sclerosing agent in the treatment of venous malformations. Study on 23 cases. Interv Neuroradiol. 10 (2), 113–127, (2004).
14. Dompmartin A et al. Radio-opaque ethylcellulose-ethanol is a safe and efficient sclerosing agent for venous malformations. Eur Radiol. 21 (12), 2647–2656, (2011). [PubMed: 21822948]
15. Faguy K Ductography: When, How, and Why. Radiol Technol. 92 (5), 487M–503M, (2021).
16. Slawson SH & Johnson BA Ductography: how to and what if? Radiographics. 21 (1), 133–150, (2001). [PubMed: 11158649]
17. Sheiman LS & Levesque PH The In's and Out's of Ductography: A Comprehensive Review. Curr Probl Diagn Radiol. 45 (1), 61–70, (2016). [PubMed: 26163736]
18. Clark A, Bird NK & Brock A Intraductal Delivery to the Rabbit Mammary Gland. J Vis Exp. 10.3791/55209 (121), (2017).
19. Chakravarty S et al. Tantalum oxide nanoparticles as versatile contrast agents for X-ray computed tomography. Nanoscale. 12 (14), 7720–7734, (2020). [PubMed: 32211669]
20. Kenyon E et al. Intraductal Delivery and X-ray Visualization of Ethanol-Based Ablative Solution for Prevention and Local Treatment of Breast Cancer in Mouse Models. J Vis Exp. 10.3791/63457 (182), (2022).
21. Murata S et al. Ductal access for prevention and therapy of mammary tumors. Cancer Res. 66 (2), 638–645, (2006). [PubMed: 16423990]
22. King BL & Love SM The intraductal approach to the breast: raison d'etre. Breast Cancer Res. 8 (2), 206, (2006). [PubMed: 16677416]
23. Love SM & Barsky SH Anatomy of the nipple and breast ducts revisited. Cancer. 101 (9), 1947–1957, (2004). [PubMed: 15382093]
24. Lai YE, Morhard R, Ramanujam N & Nolan MW Minimally invasive ethyl cellulose ethanol ablation in domesticated cats with naturally occurring head and neck cancers: Six cats. Vet Comp Oncol. 19 (3), 492–500, (2021). [PubMed: 33583138]
25. Mueller JL et al. Optimizing ethyl cellulose-ethanol delivery towards enabling ablation of cervical dysplasia. Sci Rep. 11 (1), 16869, (2021). [PubMed: 34413378]
26. Nief C et al. Polymer-assisted intratumoral delivery of ethanol: Preclinical investigation of safety and efficacy in a murine breast cancer model. PLoS One. 16 (1), e0234535, (2021). [PubMed: 33507942]
27. Chelales E et al. Radiologic-pathologic analysis of increased ethanol localization and ablative extent achieved by ethyl cellulose. Sci Rep. 11 (1), 20700, (2021). [PubMed: 34667252]
28. Morhard R et al. Understanding Factors Governing Distribution Volume of Ethyl Cellulose-Ethanol to Optimize Ablative Therapy in the Liver. IEEE Trans Biomed Eng. 67 (8), 2337–2348, (2020). [PubMed: 31841399]

29. Morhard R et al. Development of enhanced ethanol ablation as an alternative to surgery in treatment of superficial solid tumors. Sci Rep. 7 (1), 8750, (2017). [PubMed: 28821832]
30. Russo IH & Russo J Developmental stage of the rat mammary gland as determinant of its susceptibility to 7,12-dimethylbenz[a]anthracene. J Natl Cancer Inst. 61 (6), 1439–1449, (1978). [PubMed: 102856]
31. Paine IS & Lewis MT The Terminal End Bud: the Little Engine that Could. J Mammary Gland Biol Neoplasia. 22 (2), 93–108, (2017). [PubMed: 28168376]
32. Hinck L & Silberstein GB Key stages in mammary gland development: the mammary end bud as a motile organ. Breast Cancer Res. 7 (6), 245–251, (2005). [PubMed: 16280048]
33. Sivaraman L et al. Effect of selective ablation of proliferating mammary epithelial cells on MNU induced rat mammary tumorigenesis. Breast Cancer Res Treat. 73 (1), 75–83, (2002). [PubMed: 12083633]
34. Cardiff RD & Wellings SR The comparative pathology of human and mouse mammary glands. J Mammary Gland Biol Neoplasia. 4 (1), 105–122, (1999). [PubMed: 10219910]
35. Brock A et al. Silencing HoxA1 by intraductal injection of siRNA lipidoid nanoparticles prevents mammary tumor progression in mice. Sci Transl Med. 6 (217), 217ra212, (2014).
36. de Groot JS et al. Intraductal cisplatin treatment in a BRCA-associated breast cancer mouse model attenuates tumor development but leads to systemic tumors in aged female mice. Oncotarget. 8 (37), 60750–60763, (2017). [PubMed: 28977823]
37. Wang G et al. Intraductal fulvestrant for Therapy of ERalpha-positive Ductal Carcinoma in Situ (DCIS) of the breast- a Preclinical Study. Carcinogenesis. 10.1093/carcin/bgz084, (2019).
38. Yoshida T et al. Effective treatment of ductal carcinoma in situ with a HER-2- targeted alpha-particle emitting radionuclide in a preclinical model of human breast cancer. Oncotarget. 7 (22), 33306–33315, (2016). [PubMed: 27119227]
39. Chun YS et al. Intraductally administered pegylated liposomal doxorubicin reduces mammary stem cell function in the mammary gland but in the long term, induces malignant tumors. Breast Cancer Res Treat. 135 (1), 201–208, (2012). [PubMed: 22752247]
40. Markiewicz E et al. High resolution 3D MRI of mouse mammary glands with intraductal injection of contrast media. Magn Reson Imaging. 33 (1), 161–165, (2015). [PubMed: 25179139]
41. Markiewicz E et al. MRI ductography of contrast agent distribution and leakage in normal mouse mammary ducts and ducts with in situ cancer. Magn Reson Imaging. 40 48–52, (2017). [PubMed: 28366759]
42. Annunziato S et al. Comparative oncogenomics identifies combinations of driver genes and drug targets in BRCA1-mutated breast cancer. Nat Commun. 10 (1), 397, (2019). [PubMed: 30674894]
43. Rutkowski MR et al. Initiation of metastatic breast carcinoma by targeting of the ductal epithelium with adenovirus-cre: a novel transgenic mouse model of breast cancer. J Vis Exp. 10.3791/51171 (85), (2014).
44. Xiang D, Tao L & Li Z Modeling Breast Cancer via an Intraductal Injection of Cre-expressing Adenovirus into the Mouse Mammary Gland. J Vis Exp. 10.3791/59502 (148), (2019).
45. Barham W, Sherrill T, Connelly L, Blackwell TS & Yull FE Intraductal injection of LPS as a mouse model of mastitis: signaling visualized via an NF-kappaB reporter transgenic. J Vis Exp. 10.3791/4030 (67), e4030, (2012). [PubMed: 22971993]
46. Chun YS et al. Intraductal administration of a polymeric nanoparticle formulation of curcumin (NanoCurc) significantly attenuates incidence of mammary tumors in a rodent chemical carcinogenesis model: Implications for breast cancer chemoprevention in at-risk populations. Carcinogenesis. 33 (11), 2242–2249, (2012). [PubMed: 22831956]
47. Stearns V et al. Preclinical and clinical evaluation of intraductally administered agents in early breast cancer. Sci Transl Med. 3 (106), 106ra108, (2011).
48. Okugawa H et al. Effect of perductal paclitaxel exposure on the development of MNU-induced mammary carcinoma in female S-D rats. Breast Cancer Res Treat. 91 (1), 29–34, (2005). [PubMed: 15868429]
49. Falconer IR The distribution of 131 I- or 125 I-labelled prolactin in rabbit mammary tissue after intravenous or intraductal injection. J Endocrinol. 53 (3), 58–59, (1972). [PubMed: 5039234]

50. Fiddler TJ, Birkinshaw M & Falconer IR Effects of intraductal prolactin on some aspects of the ultrastructure and biochemistry of mammary tissue in the pseudopregnant rabbit. J Endocrinol. 49 (3), 459–469, (1971). [PubMed: 5104551]

51. Fiddler TJ & Falconer IR The effect of intraductal prolactin on protein and nucleic acid biosynthesis in the rabbit mammary gland. Biochem J. 115 (5), 58P–59P, (1969).

52. Bourne RA, Bryant JA & Falconer IR Stimulation of DNA synthesis by prolactin in rabbit mammary tissue. J Cell Sci. 14 (1), 105–111, (1974). [PubMed: 4856240]

53. Chadwick A Detection and Assay of Prolactin by the Local Lactogenic Response in the Rabbit. J Endocrinol. 27 253–263, (1963). [PubMed: 14079513]

54. Mahoney ME et al. Intraductal therapy of ductal carcinoma in situ: a presurgery study. Clin Breast Cancer. 13 (4), 280–286, (2013). [PubMed: 23664819]

55. Love SM et al. A feasibility study of the intraductal administration of chemotherapy. Cancer Prev Res (Phila). 6 (1), 51–58, (2013). [PubMed: 23169924]

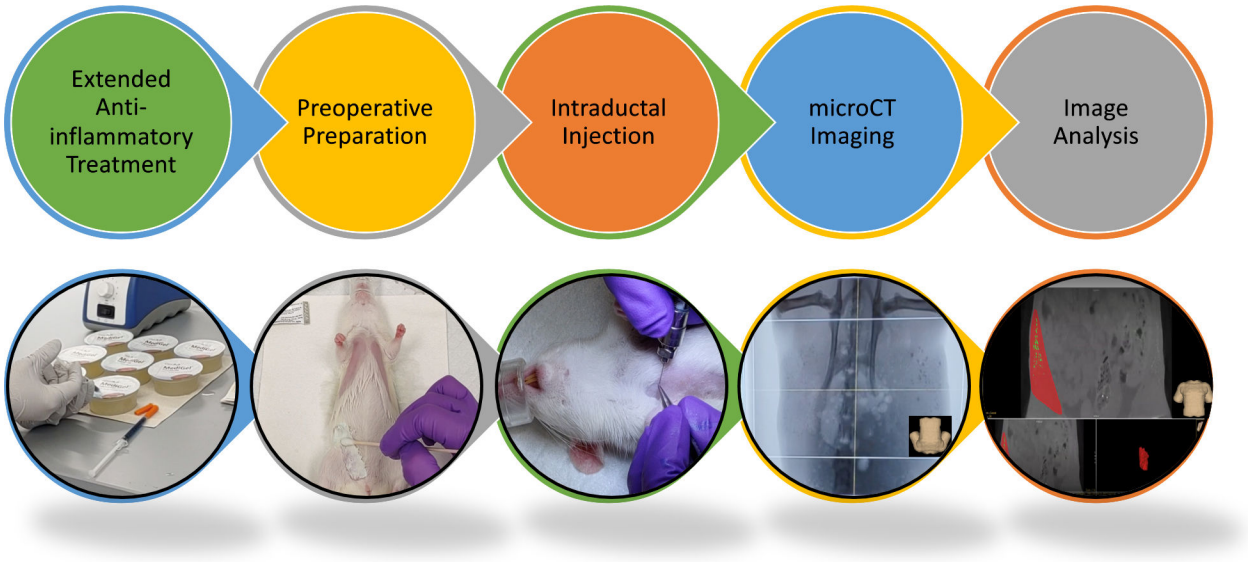

Figure 1. Workflow of intraductal procedure and image analysis.
Key steps of the ID procedure are highlighted. Please see video for more details.

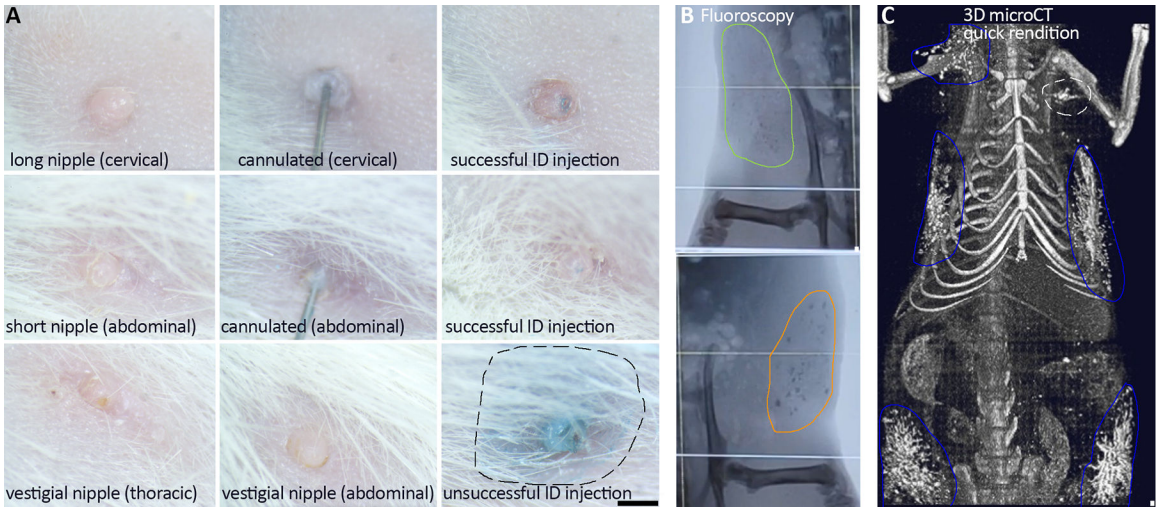

Figure 2. Successful cannulation and delivery of ablative solution to multiple mammary glands.

A) Typical presentation of nipple shapes in Sprague-Dawley rat strain. Long nipples are easier to cannulate than short nipples, whereas too short or vestigial nipples cannot be cannulated. Once cannulated, both long and short nipples can be infused with the solution and achieve similar success rates. Blue food dye in the injected solution may be used as *in vivo* evidence of ductal tree filling and delivery success (most apparent, dome formation, for an unsuccessful fat pad injection). Real-time fluoroscopy (**B**) and post-image acquisition 3D micro-CT rendition (**C**) provide *in vivo* evidence of delivery success and more quantitative assessment of the solution reaching the TEBs. **B)** Each abdominal mammary gland of the first pair (#4, #10) received ablative solution with 1% EC (orange outline) or without it (green outline) **C)** Successful delivery (blue outline) of ablative solution in right cervical (#7), second pair of thoracic (#3, #9) and first pair of abdominal (#4, #10) mammary glands, and unsuccessful injection (dashed white outline) in left thoracic (#1) gland. Scale bars correspond to 1 mm in images at different magnification.

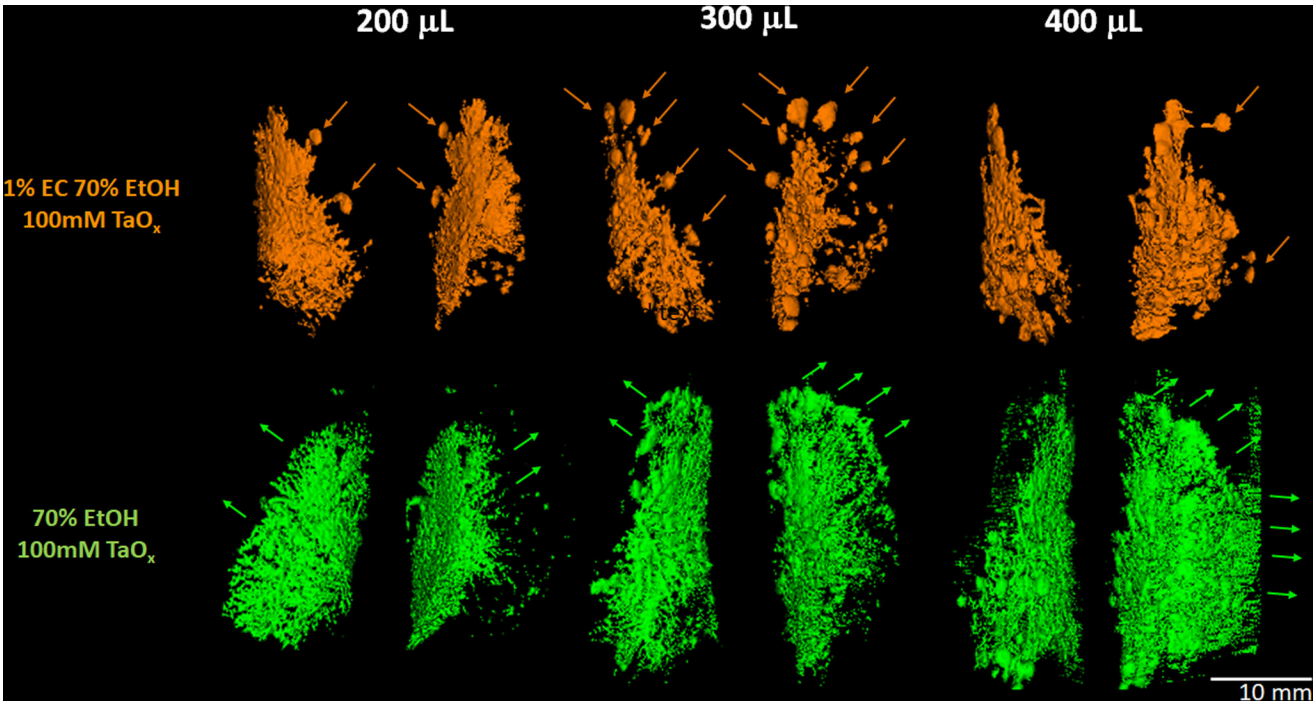

Figure 3. 3D reconstruction and assessment of ablative solution filling and diffusion.

70% EtOH/100 mM TaO_X nanoparticles with 1% EC (top) or without EC (bottom) were intraductally injected into the second abdominal mammary gland pair (#4 and #10) and immediately imaged by micro-CT. Each Sprague-Dawley rat received an increasing volume of either solution. Individual ductal trees were reconstructed using an image analysis software package (spline trace + propagate object + threshold rendition). With 1% EC, the solution can be seen reaching the terminal ends. As delivered volume is increased the number of TEBs filled is more apparent. Scale bar corresponds to 10 mm in all renditions.

Table 1.

Troubleshooting and helpful tips.

Issue	Appearance	Solution
Short nipple (Fig. 2)	Nipple has low profile – hard to grab	It is sometimes easier to hold the skin near the nipple and target the center of the nipple with the needle. The needle will likely dive under the skin. Pulling up slowly may reveal the nipple to be slightly over the tip of the needle and give room to grab and pull it the rest of the way onto the needle. Be very careful when diving below the skin about the angle of the needle. It is easy to inadvertently get a fat pad injection by stabbing at the wrong angle.
Fat pad injection (Fig. 2)	Swollen around nipple and possibly in nipple itself – easiest to see if color is added to injection solution	If nipple is swelling with first few ul injected, remove needle, and attempt to insert again with more care taken of angle. Begin injection again and watch for further swelling. If swelling continues, abandon attempt. It is very rare to successfully inject a nipple that has started out as a fat pad injection.
Wounds/ scabbing	Open wound or scabbing near injection site of EtOH solution	Rats are more likely than mice to develop wounds or scabbing near the injection area. If wounds are found, apply triple antibiotic ointment to open wounds but leave scabbed wounds alone. Applying ointment to scabs can increase likelihood animal will bother the scab and remove it. Check every 1–2 days until healed depending on severity of wound. Carprofen should be given until healed even if beyond normal window.
Inject alternating glands	N/A	Larger injection volumes in rats make it more likely to cause skin abrasions if injecting consecutive glands. For least likelihood of trauma to injection area, alternate glands injected within a single session (i.e. inject #1, 3, 4 and 6 rather than #1–4). Spacing between third (#3 and #9) and fourth (#4 and #10) gland pairs allows injection of both of these glands in one session.

Materials

Name	Company	Catalog Number	Comments
AnalyzeDirect v12.0	Caliper	n/a	For micro-CT image processing
Carprieve, Carprofen 50 mg/mL	Allivet	50647	For anti-inflammatory treatment
Ethyl cellulose	Acros Organics	9004-57-3	For intraductal injection
Evans blue	Sigma	E2129-50G	For injection visualization
Hot water bath	Toolots	Yidu_HH-S2	For preparing carprofen cups
MediGel Sucralose Cups	ClearH2O	74-02-5022	For delivery of carprofen
Model 1750 TTL, PTFE Luer Lock Syringe, 500µL	Hamilton	81220	For intraductal injection
Photoshop 2021	Adobe	n/a	For image processing
Quantum GX2 microCT Imaging System	Perkin Elmer	CLS149276	For micro-CT image acquisition
Metal Hub Needle, 33 gauge, custom (30° bevel angle, 0.4 in, point style 4)	Hamilton	7747-01	For intraductal injection
Stereo Microscope SZM Series	AmScope	SM-4TPZ-144	For intraductal injection
Sterile blue food dye	McCormick	930641	For injection visualization
Sterile phosphate buffered saline (PBS)	ThermoFisher	14190250	For solution preparation
Stickers	DOT Scientific	DOTSCI-C50	For preparing carprofen cups
Sucrose	Calbiochem	8550-5KG	For intraductal injection
Syringes	Fisher	14-826-79	For preparing carprofen cups
Vortex	VWR	10153-834	For preparing carprofen cups
Warming pump/pad(s)	Braintree Scientific	HTP-1500 120V; AP-R 26E	For intraductal injection/preoperative preparation