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Mouse model of endoscopically ablated enteric nervous system

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ABSTRACT

Background: Current transgenic animal models of Hirschsprung disease are restricted by limited survival and need for special dietary care. We used small animal colonoscopy to produce chemically ablated enteric nervous system in the distal colon and rectum of normal mice.

Materials and methods: Adult C57BL/6 mice underwent colonoscopy with submucosal injection of 75–100 μL of saline ($n = 2$) or 0.002% ($n = 2$), 0.02% ($n = 15$), or 0.2% ($n = 2$) benzalkonium chloride (BAC). Each mouse received 1–3 injections in the distal colon and rectum. Mice were sacrificed on postprocedure day 7 or 28. Injection sites were analyzed histologically and with immunostaining for β-tubulin III.

Results: Submucosal injection of 0.02% BAC resulted in megacolon and obliteration of 82 ± 8.8% of myenteric ganglia at the injection site on postprocedure day 7 compared with normal colon. This effect was sustained until day 28. Injection of 0.002% BAC had little effect on the myenteric neuronal network at these time points. Multiple injections of 0.002% or 0.02% BAC (up to three injections per mouse) were well tolerated. Injection of 0.2% BAC caused acute toxicity or death.

Conclusions: A novel model of chemically ablated enteric nervous system in the mouse colon and rectum is introduced. This model can be valuable in evaluating targeted cell delivery therapies for Hirschsprung disease.

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1. Introduction

Hirschsprung disease is a congenital disorder with an incidence of 1 in 5000 live births and is characterized by lack of enteric ganglia, most commonly in the rectum and distal colon [1]. Current therapy consists of surgical removal of the affected segment, but cell-based therapies using autologous neural crest–derived cells are under investigation. Such studies require reliable animal models of enteric aganglionosis and typically use rodents homozygous for the lethal spotting mutation involving the endothelin-3 gene in mice [2] and endothelin receptor type B gene in rats [3]. However, these animal models are restricted by limited survival rate and life span as well as need for special dietary care. Surgical models of Hirschsprung disease use chemical ablation of the enteric nervous system (ENS) with benzalkonium chloride (BAC) [4,5]. These have been shown to produce reliable aganglionosis in experimental animals, but the technique

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requires laparotomy for serosal application of BAC to the intestinal segment of interest. Anorectal injection of BAC has been previously shown to be a suitable alternative, mitigating the need for laparotomy [6]. In the present study, we used miniature endoscopy to produce a more targeted model of chemically ablated ENS in the distal colon and rectum of normal mice.

2. Methods

2.1. *Miniature endoscopy*

Eight to 12-wk old male or female C57BL/6J mice were obtained from an institutionally maintained breeding colony (originally from Charles Rivers Laboratories, Wilmington, MA) and placed on a water-only diet overnight. Anesthesia was induced with 3% isoflurane then decreased to 1% for maintenance of anesthesia. Colonoscopy was then performed by insertion of a lubricated miniature 1.9-mm endoscope (Fig. 1A) in the mouse rectum (Karl Storz, Tuttlingen, Germany). Using a 30-gauge custom-made needle fitted with pulled and beveled capillary tubing (Fig. 1B), the ventral rectum was injected submucosally with 100 μL of either sterile saline ($n = 2$) or BAC at a concentration of 0.002% ($n = 2$), 0.02% ($n = 15$), or 0.2% ($n = 2$) diluted in sterile water. To facilitate later identification of the injection sites, a permanent carbon ink suspension (GI Supply, Camp Hill, PA) at a final concentration of 1.5% was used. Mice injected with BAC at the lower two concentrations received 2–3 injections at multiple longitudinal levels, each consisting of 100 μL. After the procedure, all mice were monitored for appropriate recovery from anesthesia and placed back in standard laboratory mouse cages with full access to water and chow and sacrificed 7 or 28 d after the endoscopic procedure.

2.2. *Histology and immunohistochemistry*

The isolated colon specimens were flushed with cold phosphate-buffered saline to remove luminal contents then filled with warm HistoGel (American MasterTech, Lodi, CA) and fixed overnight in 10% buffered formalin solution. Specimens were embedded in paraffin and sectioned at 3 μm. Hematoxylin and eosin staining and immunohistochemistry were performed per standard protocol. Heat-mediated antigen retrieval was performed in Citra buffer (Fisher Scientific, Pittsburgh, PA). Slides were stained with mouse anti–β tubulin III antibody (Abcam, Cambridge, MA) at 1:1000 dilution and conjugated with goat anti-mouse secondary antibody (Life Technologies, Grand Island, NY) at 1:200 dilution, both diluted in blocking solution consisting of 2% bovine serum albumin and 4% normal goat serum. 4′,6-Diamidino-2-phenylindole (Life Technologies) was used as a counterstain.

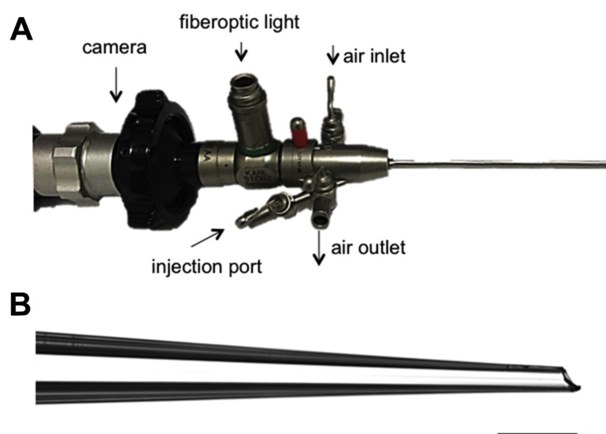

Fig. 1 – Endoscopy setup showing (A) the miniature endoscope and (B) a beveled glass needle used for injection into the submucosa of mouse colon. (Color version of figure is available online.)

2.3. *Data analysis*

Immunofluorescence and bright-field images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Ink-laden areas of the rectum were identified on bright-field images, and the perimeter was measured in micrometers. On the corresponding fluorescence images, the number of β-tubulin III positive ganglia was counted and normalized both to the perimeter and the number of ganglia per unit length of saline-injected mouse rectum. Statistical analysis was performed using the one-tailed Student *t*-test.

2.4. *Ethics statement*

All animal studies were approved by the animal research committee at UCLA. The UCLA facility is an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

3. Results

Endoscopic injections in the rectum produced a submucosal bulge (Fig. 2A), which confirmed successful cannulation of the submucosal layer. This was verified by histologic examination, which showed ink in the submucosal space between the muscularis mucosa and muscularis propria (Fig. 2B). There were no perforations related to endoscopy. The two mice injected with 0.2% BAC and one of 15 mice injected with 0.02% BAC died within 24 h of injection (mortality 100% and 6.7%, respectively). All other mice that were injected with either saline or BAC at 0.002% or 0.02% fully recovered from the endoscopic procedure, tolerated chow diet, and showed no signs of distress after the procedure and until the time of elective sacrifice on postprocedure day 7 or 28. On gross examination, saline- and 0.002% BAC-injected mice showed evidence of submucosal ink marking the injection sites but otherwise unremarkable colons on necropsy. However, mice injected with 0.02% BAC and sacrificed on postprocedure days 7 and 28 had megacolon extending to the proximal colon and cecum (Fig. 3).

Confirmatory anti–β tubulin III staining of specimens injected with 0.002% showed no aganglionosis at the injection site on day 7 (Fig. 4A and C) but moderately effective ablation of the ENS on day 28 with 47% ± 30.8% obliteration of native

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