In vivo model of Hirschsprung-associated enterocolitis using benzalkonium chloride

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ABSTRACT

BACKGROUND Hirschsprung-associated enterocolitis (HAEC) is a life-threatening complication of Hirschsprung’s disease. Studies using animal models on the pathogenesis of HAEC are limited. Thus, this study aimed to establish a rat model of HAEC using topical application of 0.1% benzalkonium chloride (BAC) in the sigmoid colon.

METHODS 55 male Sprague Dawley rats aged 10−12 weeks old were separated into 11 groups. The control group (n = 5) was euthanized on day-7, and the other 10 groups (n = 5 in each group) treated with 0.1% BAC in the sigmoid colon for 15 min to induce Hirschsprung’s disease were euthanized on day-7, -10, -12, -14, -17, -19, -21, -23, -25, and -28. The sigmoid colon was excised, fixed in formalin, and sectioned for histological examinations with hematoxylin and eosin staining. The degree of HAEC was compared within all groups.

RESULTS Rats that were sacrificed on day-7 to -12 showed the 1st degree or early HAEC, which was most likely caused by BAC application. The 2nd degree of HAEC occurred in rats that were sacrificed on day-14 that showed a macrophage infiltration in the sigmoid colon, thus fulfilled the initial criteria for HAEC (p = 0.0025 versus control). The degree of enterocolitis increased with time, and the highest degree was found in rats that were sacrificed on day-28 (p<0.001 versus control).

CONCLUSIONS Topical application of 0.1% BAC for 15 min was successfully produced HAEC model in rats, which was occurred on day-14 after the application. This model provides a useful resource for further research on the pathogenesis of HAEC.

KEYWORDS animal model, colitis, ganglions, Hirschsprung’s disease

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normal innervation and various diseases related to that innervation. It has been demonstrated that the application of 0.1% benzalkonium chloride (BAC) for 15 min in the serosal surface of the bowel eventually leads to the narrow aganglionic segment of the bowel, possibly due to irreversible injury of the myenteric nerve cells, and also results in luminal dilation in the proximal untreated segments in the rats’ bowel and decompression of the treated segments, which mimics aganglionosis in Hirschsprung’s disease patients. In fact, BAC is an enteric neurotoxic agent that can be applied topically to the bowel and is a powerful cationic detergent that can destroy bacteria and mammalian tissues due to its ability to disrupt membrane cells. Transgenic animal models of Hirschsprung’s disease, which show similarities to the human condition, are also available although they have a short life span due to enterocolitis. Thus, further study on the chronic complication of Hirschsprung’s disease, such as HAEC, is almost impossible.

Previous studies have evaluated the functional and pathological aspects of the ENS in Hirschsprung’s disease, but no studies have evaluated the long-term complication, such as HAEC. Therefore, important questions such as the molecular mechanism, most involved enteric cells in the pathogenesis, and long-term cell-based therapy for HAEC remain unanswered. Hence, an in vivo model of HAEC, which is easy to breed and has a long enough life span, is needed.

This study aimed to establish an in vivo animal model of HAEC using BAC application by examining the histomorphological changes of the sigmoid colon of BAC-treated rats.

METHODS

This study was conducted in the Animal Laboratory of National Institute of Health Research and Development, Indonesian Ministry of Health, Indonesia and received its approval from the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No: 1069/UN2.F1/ETIK/2018).

Animals

A total of 55 adult male Sprague-Dawley rats at 10–12 weeks old were housed in polypropylene cages in a room maintained at 23 ± 2°C with a 12 hour-light/dark cycle. All rats included in this study were healthy (not having symptoms of anorexia, excessive saliva, and mucopurulent secretion of the eyes and nasal). The rats were then randomly divided into 11 groups. Each group consisted of five rats determined by Federer formula. In the treatment group, 0.1% BAC was applied for 15 min to the sigmoid colon. We also included the control with the application of saline to the sigmoid colon.

Surgical procedure

All animals underwent midline incision laparotomy under general ketamine hydroxychloride and xylazine anesthesia. The application of 0.1% BAC to the sigmoid colon was carried out according to research conducted by Yoneda et al. In brief, the sigmoid colon was identified and removed from the abdomen. In the sigmoid mesentery avascular area, a small opening was made, a 1 × 2 cm gauze was soaked in 0.1% BAC (Sigma-Aldrich, USA) and placed around 1 cm from the sigmoid colon, and a layer of plastic was put beneath the colon to prevent spreading of the solution (Figure 1a). Three drops of 0.1% BAC solution were placed on the gauze every 5 min to avoid dehydration of the bowel for a 15 min period before the gauze was removed and washed well with 0.9% normal saline. In the control group, rats were also treated similarly, but saline was applied instead of 0.1% BAC. After the procedure, the abdomen was closed, and rats were socially housed with a standard diet and tap water ad libitum.

Tissue preparation

All rats were euthanized using an overdose of inhaled anesthetic (isoflurane) followed by cervical dislocation that was performed on day-7, -10, -12, -14, -17, -19, -21, -23, -25, and -28 after 0.1% BAC application due to crypt cell proliferation around 2–3 days, whereas rats in the control group were euthanized 7 days after the sham procedure. Sigmoid colon samples were immediately obtained, rinsed three times with saline solution, weighed, and kept in the 10% neutral buffered formalin pot until they were examined. Paraffin-embedded colonic tissue was sliced into 5-µm-thick longitudinal sections and mounted on glass slides. The BAC-treated and control sigmoid colon were stained with hematoxylin and eosin. The aganglionic cells per slice were recorded, and the degree of enterocolitis was scored according to Teitelbaum et al and Cheng et al by three independent researchers blinded to the experimental conditions. Five randomly selected fields from each specimen (n = 3 per experimental condition) were scored for evidence of sigmoid colon.
injury. The degree of HAEC was ranged as follows: 
0 = normal, no evidence of injury; 1 = crypt dilation and mucin retention; 2 = cryptitis and crypt abscess and macrophage and neutrophil infiltration; 3 = multiple crypt abscess; 4 = fibrinopurulent debris and mucosa ulceration; and 5 = necrosis and transluminal perforation as previously described.\(^{12,13}\)

### Statistical analysis

Data were expressed as the mean and standard deviation. A comparison among groups was performed using one-way analysis of variance with Tukey’s post-hoc multiple comparison. A \(p\)-value of <0.05 was considered statistically significant.

### RESULTS

All rats were successfully survived during the experimental period. The postoperative BAC-treated colonic segment is shown in Figure 1a. BAC application was successfully produced narrow, transitional, and dilation segments in all rats, mimicking the short segment of Hirschsprung’s disease in humans (Figure 1b). As shown in Figure 2, the control group demonstrated normal ganglion of the sigmoid colon (Figure 2a), whereas the BAC-treated groups revealed an aganglionosis short segment in the sigmoid colon (Figure 2b–d). We found that aganglionosis started to occur on day-7 after BAC application (Figure 2b), and the number of ganglia was decreased over time, such as on day-7 (Figure 2b), -14 (Figure 2c), and -28 (Figure 2d) after BAC application.

Isolated segments of the sigmoid colon treated with 0.1% BAC has also demonstrated the longer the rats was sacrificed, the higher the degree of HAEC was (Figure 3b–f and Figure 4). HAEC was not occurred in the control group (Figure 3a). The 1\(^{st}\) degree of HAEC characterized by the presence of Lieberkühn gland dilatation was appeared in rats that were sacrificed on day-7 to -12 (Figure 3b). The degree of HAEC was similar in rats having normal saline (control) and in rats having 0.1% BAC that were sacrificed on day-7 to -12 (\(p = 0.41\)) (Figure 4). The 2\(^{nd}\) degree of HAEC was shown by the infiltration of inflammatory cells in the isolated sigmoid colon crypts. The 2\(^{nd}\) degree of HAEC occurred in rats that were sacrificed on day-14 and -17 (Figure 3c) and was higher, compared with the control (\(p = 0.03\)) (Figure 4). Rats that were sacrificed on day-19

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**Figure 1.** Surgical procedure and macroscopic presentation of the 0.1% BAC application. (a) 0.1% BAC application in the sigmoid colon of rats. 1 × 2 cm gauze wrapped the sigmoid colon, with blue plastic layer beneath to prevent the spread of 0.1%.BAC to other tissues; (b) autopsy findings in rats at 1 week after 0.1% BAC application. Narrow (white arrow), transitional (yellow arrow), and dilated segment (red arrow) were shown 7 days after 0.1% BAC application, mimicking the anatomical condition of Hirschsprung’s disease. BAC=benzalkonium chloride

**Figure 2.** Representative photomicrographs of sections from sigmoid colon tissue stained by H&E (100× magnification). Ganglionic area in the control group (a), and aganglionic (blue arrow and circle) and ganglionic areas (black arrow) on day-7 post BAC application group (b), day-14 post BAC application group (c), and day-28 post BAC application group (d). BAC=benzalkonium chloride; H&E=hematoxylin and eosin
and day-21 experienced the 3rd degree of HAEC, indicated by the presence of polymorphonuclear infiltration in the crypts of the sigmoid colon. Meanwhile, the 4th–5th degree of HAEC was demonstrated in rats that were sacrificed on day-23 to -28, characterized by the presence of fibrinogen in the Lieberkühn gland, necrosis and desquamation of mucosal villi epithelial cells, and necrosis of the Lieberkühn gland. The degree of HAEC was higher in rats sacrificed on day-19 to -28, compared with the control and rats sacrificed on day-7 ($p = 0.01$) (Figure 3d–f and Figure 4).

**DISCUSSION**

In the present study, we demonstrated that 15 min of 0.1% BAC application to isolate the segment of...
the sigmoid colon caused 100% of early aganglionosis on day-7, although only in a short segment. This is an important step because the short segment of aganglionosis occupies the highest frequency of Hirschsprung’s disease in clinical settings. This finding is similar to a study conducted by Yoneda et al that showed the BAC application could be employed to yield an animal model of Hirschsprung’s disease in which the myenteric plexus was selectively ablated. They also reported that 12 weeks after BAC application, the histological examination revealed a total disappearance of rectal ganglia and nerve fibers after 7 days of 0.1% BAC application for 15 min. There was a narrow segment at the site of BAC application accompanied by a transitional and dilated segment similar to that of patients with Hirschsprung’s disease. Garcia et al also reported that 1-week application of BAC in the isolated segment of the colon resulted in more than 90% loss of myenteric plexus. Furthermore, a previous study showed that between day-7 to -84, 0.1% BAC application for 30 min reduced the number of ganglia and ganglionic cells in the rat colon to the greatest extent.

In the present study, we demonstrated that the 1st degree of enterocolitis had occurred on day-7 after BAC application in the isolated segment of the sigmoid colon. The grade of enterocolitis continued to increase until the end of the present study (28 days after BAC application). Although the 1st degree of enterocolitis had occurred on day-7 to -12 after BAC application, this could not be considered as the onset of HAEC. The inflammatory process that occurred on day-7 to -12 was most likely due to previous exposure to BAC. BAC is a quaternary ammonium, which is the most commonly used preservative in antiglaucoma medications. It was shown that BAC was able to induce an inflammatory reaction when tissues were exposed to BAC. Cheng et al proved that in endothelin receptor B (Ednrb) transgenic mice, the 1st degree of HAEC was demonstrated by the presence of cryptic dilation, mucus retention, and inflammatory cell infiltration. In our present study, although the presence of dilated crypt and mucus retention indicated the 1st degree of enterocolitis, it was not followed by inflammatory cell infiltration. Therefore, we suggested that this was not the initial time of HAEC but a result of previous exposure to BAC. Conversely, the 2nd degree of HAEC, which occurred on day-14 (or 7 days after the occurrence of Hirschsprung’s disease), was the first occurrence of HAEC as the mononuclear cell and neutrophil infiltration were seen, indicating an inflammatory process. Furthermore, a previous study has shown that the elimination half-life of BAC is around 8 hours; thus, enterocolitis occurred on day-14 in the present study was caused by Hirschsprung’s disease. Our findings were in line with previous studies that showed HAEC would occur when Hirschsprung’s disease diagnosis was delayed for more than 7 days of life span. Garcia et al also reported that HAEC that occurred early in the present study (14 days after BAC application) is useful for further study of the pathogenesis of HAEC which is largely unknown, and its predisposing factors and specific prevention strategies have not been established. As previously stated, HAEC is characterized by a variety of clinical features. The histological assessment results of patients with HAEC showed the presence of cryptitis with a severe inflammatory process and neutrophil infiltration in the crypt.

Multiple genetic defects are closely related to the occurrence of Hirschsprung’s disease. The most commonly reported defects are genetic in the rearranged during transfection (Ret) and Ednrb, both of which have an important role in the development of ENS. Currently, there are two available transgenic animal models of Hirschsprung’s disease, namely Ret-/- and Ednrb-/- mice, but both transgenic animals have a high mortality rate. The Ednrb-/- mice have a mean survival time of 26.9 days; thus, making it impossible to study the pathogenesis of HAEC. In addition, our experimental animal model has a long-lasting colonic aganglionosis, providing a resource to study the long-term cell-based therapy for HAEC, molecular mechanisms, and enteric cells mostly involved in the pathogenesis of HAEC. This study has a limitation that the gauze wrap may incompletely envelop the small portion of the sigmoid colon, resulting in ganglia being exposed to BAC. Thus, we suggest using a similar gauze wrap size to cover the sigmoid colon for future studies.

In conclusion, we were able to create an experimental animal model of HAEC with enterocolitis that occurred early (14 days after BAC application) and was able to survive up to 28 days, providing data to further study the pathogenesis of HAEC. In addition, the BAC-induced sigmoid colon aganglionosis in this model is quite easy to perform.
Conflict of Interest
Vivian Soetikno is the editorial board member but was not involved in the review or decision process of the article.

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