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Accelerated wound healing after topical application of hyaluronic acid cotton to hemorrhoidectomy wounds in a rat model

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Purpose: Anal wounds following hemorrhoidectomy can lead to severe pain and postoperative bleeding, impacting patient recovery and quality of life. Hyaluronic acid (HA) stimulates tissue regeneration and wound healing by accelerating cell migration and proliferation. This study aimed to investigate the differences in wound healing rate and completeness of recovery of perianal wounds topically treated with HA-soaked cotton in a murine model.
Methods: Forty-eight 8-week-old Sprague-Dawley rats with perianal wounds created using a biopsy punch were divided into 2 groups: simple dressing with gauze (control) and topical HA-soaked cotton. A single application of HA-soaked cotton was administered after surgery. Wound healing rate and completeness of recovery were evaluated by measuring the healed area and conducting histological analyses.
Results: The HA-cotton group exhibited a shorter complete wound healing duration compared to the control group (13.9 days *vs*. 16.4 days, P = 0.031). Differences in wound healing area between the 2 groups were greatest on postoperative day 2 (51.6% *vs*. 28.8%, P < 0.001). The HA-cotton group exhibited fewer cases of granulation tissue (2 *vs*. 5) or redness (0 vs. 3) upon complete wound healing. Histologically, the HA-cotton group showed accelerated reepithelialization, rapid shift to lymphocyte-dominant inflammation, enhanced fibroblast proliferation, and increased collagen deposition compared to the control group.
Conclusion: Herein, topical application of HA-soaked cotton on perianal wounds in rats resulted in accelerated wound healing, particularly in the initial stages, and improved completeness of recovery, underscoring the potential of the topical application of HA-soaked cotton on hemorrhoidectomy wounds in human patients to improve wound healing.
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Key Words: Hemorrhoidectomy, Hyaluronic acid cotton, Rat model, Wounds and injuries

INTRODUCTION

Hemorrhoids are a prevalent anorectal disorder characterized by the enlargement and distal displacement of normal anal cushions [1]. Typical symptoms of hemorrhoids include rectal bleeding, a perianal mass postdefecation, pain, mucus discharge,

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and pruritus [2]. Hemorrhoids are classified based on their location and extent of prolapse. Internal hemorrhoids originate from the internal hemorrhoidal plexus above the dentate line, while external hemorrhoids originate from the external hemorrhoidal plexus below the dentate line [3]. Treatment strategies differ depending on symptom severity. Conservative therapy, such as medication, is typically employed for patients with grade 1 or 2 hemorrhoids [4]. For patients with grade 3 and 4 hemorrhoids [5], hemorrhoidectomy is the most common surgical approach, despite its postoperative complications including pain and bleeding [6]. Delays in surgical wound healing after hemorrhoidectomy can lead to postoperative pain and an increased risk of postoperative hemorrhage. One study reported that 65% of patients experienced moderate or severe pain after hemorrhoidectomy, which notably delayed hospital discharge [7]. Postoperative hemorrhage is a rare but severe complication, with an incidence rate of approximately 0.9%–10% for delayed hemorrhage following surgery [8]. Previous studies have associated delayed postoperative bleeding with various factors, including the surgical procedure, infection, excessive straining during defecation, and the number of hemorrhoidal piles [9]. Therefore, efficient anal wound healing can mitigate associated symptoms, such as pain and hemorrhage, and reduce the likelihood of postoperative complications, ultimately facilitating faster recovery and reducing the time required to return to normal activities.

Hyaluronic acid (HA) is an anionic, non-sulfated glycosaminoglycan found ubiquitously throughout connective, epithelial, and neural tissues. As a biopolymer and main constituent of the extracellular matrix [10], HA plays various roles including retaining moisture, maintaining extracellular spaces, regulating osmotic pressure, and providing synovial joint lubrication. Additionally, HA is widely used in cosmetics [11]. Research has shown that HA significantly contributes to cell proliferation and migration and plays a crucial role in wound healing by facilitating tissue regeneration, regulating inflammatory responses, and promoting neovascularization [12]. Consequently, several wound care products have been launched to harness the therapeutic potential of HA [13,14]. The extent to which a wound heals depends on its location in the body, and the time required for successful wound healing varies. A study involving patients who had undergone hemorrhoidectomy revealed that the duration for complete wound healing in patients susceptible to wound infection and open wounds resulting from defecation ranged from 3.1 to 6 weeks, which exceeded the healing time for other types of wounds [15]. However, few studies have investigated the effects of product application on wounds following hemorrhoidectomy. In a previous study on rats with perianal wounds, the wound healing time was compared between groups with HA-film dressing or HA-gel dressing and a group with simple disinfection. Results indicated an improved wound healing rate in HA-film groups compared to the simple disinfection group. The mean times to complete wound healing were 11.6, 11.9, and 13.8 days in the HA-film dressing, HA-gel dressing, and simple disinfection groups, respectively, indicating that the application of HA to wounds increased the rate of wound healing. Notably, the group treated with HA-gel dressing showed the largest increase in wound healing rate. The disparity in wound healing rates between the HA-film and HA-gel dressings suggests that the timing and location of HA application on wounds may influence the healing process. In addition, the thin film form of HA has the disadvantage of being difficult to handle because it can be easily torn, while the gel form of HA is relatively more manageable but tends to deviate from the application site [16].

In this study, a novel HA-soaked cotton ball was developed to address the limitations of thin films that are difficult to handle and to maintain prolonged contact time and broader coverage, thereby enhancing wound healing. The study aimed to assess the impact of HA-soaked cotton on perianal wound healing in a rat model.

METHODS

This study was approved by the Animal Experiment Ethics Committee at Seoul National University Bundang Hospital in Seongnam, Korea (No. BA2110-328/001-02).

Preparation of hyaluronic acid-soaked cotton

HA-soaked cotton was prepared following previously described methods [17]. Briefly, sodium hyaluronate with a molecular weight of 800–1,200 kDa (Hi-Aqua, Jinwoo Bio) and water were combined to form a HA paste containing 50%–95% water. After storing the HA paste in a refrigerator at 4 °C for 12 hours to restore water balance, the paste was placed in a spinning device and blended under 15–100 °C conditions. The resulting material was pressurized, discharged through a nozzle, and dried at 120 °C to yield HA fibers containing 5%–25% water. The HA fibers were then aggregated to produce HA-soaked cotton.

Animal experiments

The study involved 48 female 8-week-old Sprague-Dawley rats, weighing 180–200 g, housed under the following conditions: temperature, 21–22 °C; humidity, 40%–60%; and 12:12-hour light/dark cycle. Based on previous outcomes, a hypothesis that the time to gross complete healing of the experimental group would be shortened by at least 2.2 days compared to that of the control group was established. The 2-sided tests were used to determine statistical significance. Considering a standard deviation of 1.7 and 80% power, at least 26 rats were necessary allowing for a 10% dropout rate. Two rats from each group were sacrificed at 2, 7, 11, and 14 days after surgery, and a biopsy was performed to collect samples from the perianal wounds for histopathological examination. Rats with complete wound healing were euthanized on postoperative day (POD) 21 to conduct final evaluations and obtain histopathological samples. All surgical procedures were conducted under isoflurane anesthesia, with measures taken to minimize the suffering of the rats. Under anesthesia, an 8-mm biopsy punch was used to create a wound in the anal region of the rats. Thereafter, rats were divided into an experimental group (n = 16) and a control group (n = 16). In the experimental group, HA-soaked cotton (Jinwoo Bio) was applied once on the surgical wound area on the day of surgery to maintain uniformity with a previous study, whereas wounds in the control group were treated with only a simple dressing comprising gauze soaked in a saline solution (Fig. 1).

Evaluation of perianal wounds

Perianal wound images were captured using ImageJ software (National Institutes of Health) on PODs 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21. To estimate the wound healing rate, wound size on POD 0 was compared to the size on each subsequent POD to calculate the rate of reduction in wound size according to the formula:

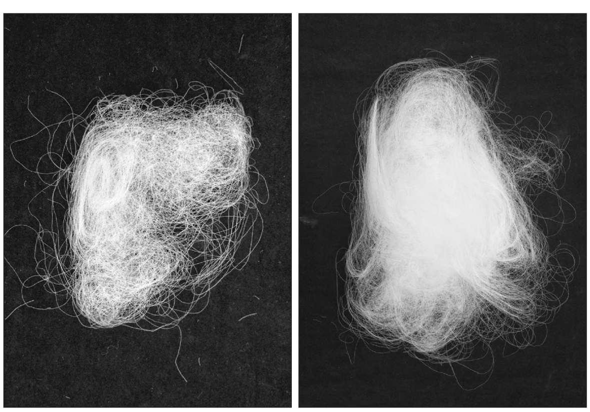

Fig. 1. Hyaluronic acid cotton used in the present study.

Equation of wound healing rate: $(1 - \text{day } \chi \text{ wound area} / \text{day } 0 \text{ wound area}) \times 100\ (\%)$, χ = *each POD (day when images were taken)*

The completeness of wound healing was evaluated based on the appearance of redness after reepithelialization and granuloma formation. Complete recovery was defined as any wound that reached 95% closure.

For histochemical analyses, the collected tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E and Masson's trichrome (MT). Reepithelialization, transition to lymphocyte-predominant inflammation, increased fibroblastic proliferation, and collagen deposition were evaluated.

Statistical analysis

Statistical analyses were conducted using the Wilcoxon rank-sum test because variables associated with the time (days) to complete recovery of the wound did not meet the assumption of normality. A 2-way repeated-measures analysis of variance was used to compare intergroup differences. All statistical analyses were conducted using R software (R version 3.5.1, The R Foundation).

RESULTS

This study initially included 48 rats, of which 32 and 16 rats were included in the statistical and histopathological analyses, respectively. Within the experimental group, 2 rats died because the stools did not pass out and remained in contact with the skin around the anus, causing wound infection and septic shock. Of the 32 rats, 14 from the experimental group and 16 from the control group were included in the final statistical analyses.

Gross evaluation of perianal wounds

The progression of wound recovery is shown in Fig. 2. The mean and median duration for complete healing were 15.2 and 14 days, respectively (Table 1). The mean time for complete

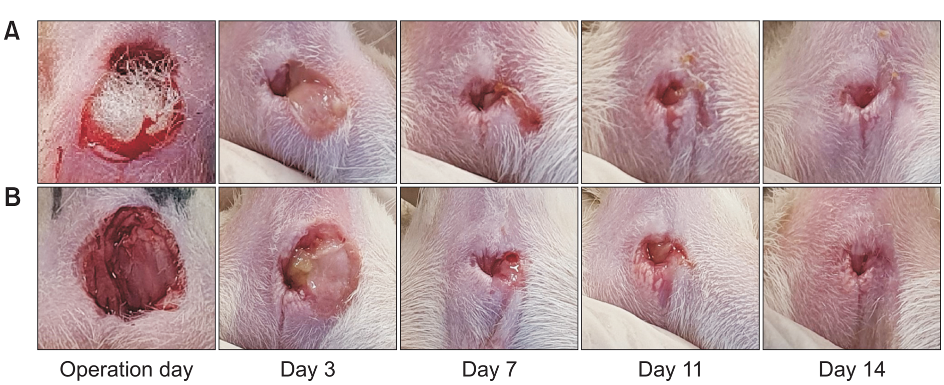

Fig. 2. Visualization of the wound recovery process. Gross appearance of a perianal wound at various stages of healing. (A) Hyaluronic acid cotton group. (B) Control group.

Table 1. Perianal wound recovery rates for hyaluronic acid cotton and control groups

Day	Recovery rate (%)			P-value
	Total (n = 30)	Treatment group (n = 14)	Control group (n = 16)	
2	39.4 ± 17.1	51.6 ± 7.9	28.8 ± 15.9	<0.001
4	59.5 ± 15.8	67.7 ± 5.3	52.4 ± 18.4	0.034
7	74.1 ± 13.1	78.7 ± 6.3	70.1 ± 16.1	0.120
9	83.3 ± 8.8	86.8 ± 3.5	80.3 ± 10.8	0.120
11	90.7 ± 7.8	93.7 ± 2.6	88.0 ± 9.9	0.047
14	93.5 ± 7.1	96.4 ± 3.1	90.9 ± 8.6	0.032
16	95.7 ± 6.1	97.7 ± 2.2	93.9 ± 7.7	0.227
18	97.8 ± 3.3	99.0 ± 1.8	96.8 ± 4.0	0.103
21	99.5 ± 1.3	99.9 ± 0.3	99.2 ± 1.7	0.403

Values are presented as mean ± standard deviation.

Table 2. Time to complete wound healing

Variable	Treatment group (n = 14)	Control group (n = 16)	P-value
Time (day)	13.9 ± 2.7	16.4 ± 3.4	0.031

Values are presented as mean ± standard deviation.

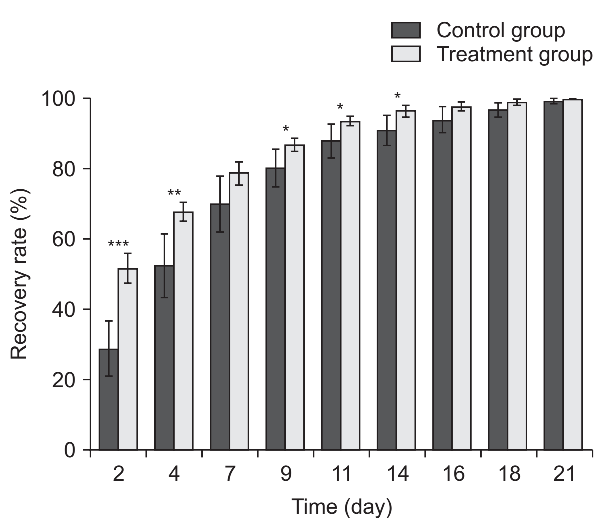

Fig. 3. Comparison of recovery rate between hyaluronic acid cotton and control groups up to postoperative day 21.

healing was 13.9 and 16.4 days in the HA-cotton and control groups, respectively, indicating a statistically significant difference (P = 0.031) (Table 2). A notable difference in the wound healing rate was the most substantial until POD 2 (51.6% in the experimental group *vs.* 28.8% in the control group, P < 0.001) (Fig. 3). On POD 21, granuloma formation and redness following complete wound recovery were compared. The experimental group showed lower instances of granuloma formation (2 cases, 14.3%) compared to the control group (5 cases, 31.3%), with no observed redness surrounding the wound

Table 3. Comparison of complete wound healing between hyaluronic acid cotton and control groups

Completeness of wound healing	Treatment group (n = 14)	Control group (n = 16)	P-value
Granulation tissue	2 (14.3)	5 (31.3)	0.399
Redness	0 (0)	3 (18.8)	0.228

Values are presented as number (%).

in the experimental group, in contrast to 3 cases (18.8%) in the control group (Table 3).

Histopathological evaluation

Representative H&E- and MT-stained images of the HA-cotton and control groups are shown in Fig. 4. On POD 2, both groups exhibited acute inflammation with neutrophil infiltration, venous congestion, and the initiation of granulation tissue formation. By POD 7, the experimental group showed recovery from acute inflammation and lymphocytic infiltration, while the control group displayed sustained acute inflammation and lymphocytic infiltration. On POD 11, both groups developed fibrosis. In the HA-cotton group, the area rebuilt with loose connective tissue was more prominent than that rebuilt with thick collagen deposition. However, fibrosis resulting from thick collagen deposition was observed. By POD 14, the HA-cotton group consistently demonstrated recovery of loose connective tissue, whereas the control group showed consistent fibrosis. A slight decrease in fibrosis was observed in the control group by POD 21 (complete wound recovery).

DISCUSSION

This murine study demonstrated that the application of HA-cotton balls to the perianal wounds of the HA-cotton group improved the wound healing rate. Histopathologically, the wound was more likely to return to normal connective tissue

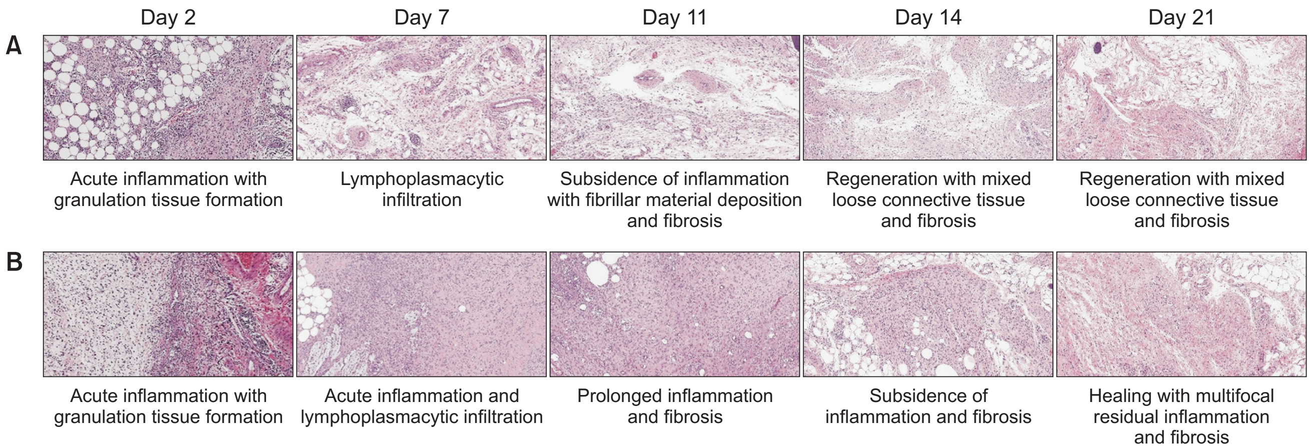

Fig. 4. Histopathologic findings (H&E, ×100). (A) Hyaluronic acid cotton apply group. (B) Control group.

than heal with fibrosis. In addition, no redness in granuloma formation was observed in fully recovered wounds. These results suggest that applying HA-soaked cotton to perianal wounds in patients undergoing hemorrhoidectomy expedites wound healing and may reduce pain and potential bleeding complications.

Delayed wound healing following hemorrhoidectomy increases the risk of postoperative pain and hemorrhage. Hemorrhoidectomy is primarily indicated for grade 4 hemorrhoids, associated symptoms such as anemia due to hemorrhage, presence of internal/external hemorrhoids with clinically significant symptoms, and failed nonoperative management [18]. Hemorrhoidectomy, which involves the surgical removal of the hemorrhoidal complex, surrounding connective tissues, and partially stapled mucosal defects, is highly effective but causes severe pain [19]. A meta-analysis of randomized controlled trials comparing the use of energy-based devices and conservative methods in hemorrhoidectomy revealed reduced postoperative pain within the first 24 hours in the group that underwent hemorrhoidectomy using an energy-based device [20]. Stapled hemorrhoidopexy with a circular stapling device was used to treat patients with grade 3 or 4 internal hemorrhoids. This surgical intervention removes the rectal mucosa, blocks the arterial/venous flow, and restores residual tissue to its original location above the dentate line [21]. In a study comparing stapled hemorrhoidopexy and conservative hemorrhoidectomy, severe pain during PODs 1–14 and baseline pain intensity were reduced [22]. However, a separate study evaluating long-term outcomes at 6 months after surgery reported no significant differences in pain [15]. Despite advances in surgical techniques and devices, postoperative pain remains a major challenge that decreases quality of life and hinders patients' return to normal activities. In a study that evaluated postoperative pain using numeric pain scales ranging from no pain (0) to worst pain (10), most patients who underwent conservative hemorrhoidectomy reported worst pain (7 points) on POD 1, followed by 5 and 3 points on PODs 7 and 14, respectively [22]. In the present study, the HA-cotton group demonstrated a notable improvement in wound healing rate, with a substantial difference observed between PODs 0 and 2. These findings suggest an overall reduction in posthemorrhoidectomy anal pain following an increase in the wound healing rate, indicating that HA treatment may ameliorate pain by accelerating wound healing, particularly during the early postoperative period. Various factors contribute to postoperative pain including, persistent wound inflammation, microbial exposure, and excessive strain on the anal sphincter muscle [23]. In the histopathological analysis, the HA-cotton group demonstrated an improvement in inflammation on POD 7, whereas the control group maintained acute inflammation. This suggests that mitigating perianal inflammation may lead to pain reduction. In addition, the HA-cotton group exhibited a greater coverage of loose connective tissue on POD 11 compared to the control group, indicating the potential reduction in anal sphincter muscle strain and subsequent pain relief.

The incidence of postoperative hemorrhage was approximately 8%, with delayed postoperative hemorrhage occurring between PODs 4 and 18 [24]. A cross-analysis study reported no difference in the incidence of postoperative hemorrhage between patients with grade 2 to 4 hemorrhoids who underwent LigaSure hemorrhoidectomy (an energy-based device, Medtronic) *vs.* conservative hemorrhoidectomy [25]. However, another study revealed a higher incidence of delayed hemorrhage in male patients who underwent LigaSure hemorrhoidectomy than in those who underwent conservative hemorrhoidectomy [24]. In the same study, multivariate analysis of predictors for postoperative hemorrhage highlighted male sex, energy-based devices (LigaSure), and postoperative constipation as significant risk factors. In the present study, the application of HA-soaked cotton to perianal wounds accelerated the healing process, particularly during the initial stage. The observed improvement in acute inflammation and increased coverage of loose connective tissue in the histopathological results can potentially minimize the likelihood of wound bleeding due to inflammation and prevent open wounds resulting from excessive strain. In addition, a reduction in the wound surface area may decrease the likelihood of postoperative hemorrhage by minimizing the duration of blood vessel exposure to the outside environment.

According to a past study, the prevalence of fecal incontinence in Korea is around 15.5% [26]. The primary risk factors for fecal incontinence in women were childbirth (91%) and vaginal delivery leading to perineal injury or complications requiring forceps. In men, approximately half of the patients with fecal continence had undergone anal surgery, which was the sole confirmed risk factor in 59% of cases [27]. These results suggest that scarring that develops after anal surgery may lead to long-term deterioration of anal function. Wound healing is a dynamic process involving a series of physiological phenomena, including coagulation, formation of granulation tissue, reepithelialization, and extracellular matrix remodeling [28,29]. During the final stages, a newly formed extracellular matrix sometimes forms scars that lack the flexibility or strength of the original tissue. In certain instances, normal scars may be replaced by pathological fibrosis, leading to the development of hypertrophic scars and keloids [30]. In the histopathological analysis, the wound was replaced by loose connective tissue in the HA-cotton group, indicating progressive wound healing. In contrast, the control group showed fibrosis owing to thick collagen deposition. HA-soaked cotton may potentially ameliorate symptoms associated with anal dysfunction, such as fecal incontinence resulting from reduced flexibility and excessive strain on the anal muscle because the anal wound undergoes scarring through the fibrotic process.

Completeness of recovery was analyzed by measuring the redness and granulation tissue formation, yet no significant differences were observed owing to the small sample size. A specific sample size was established to investigate the differences in the duration of wound healing. Redness was reported in 19% of patients in the control group, whereas no redness was reported in the HA-cotton group. Granulation tissue formation was observed in 31% and 14% of the control and HA-cotton groups, respectively. This finding suggests that HA-soaked cotton may clinically reduce anal skin tags following hemorrhoidectomy or alleviate perianal discomfort and inflammation occurring 30 days postoperatively.

During this study, 2 rats in the HA-cotton group died 2 days postoperatively due to wound complications. Typically, HA-soaked cotton dissolves within a few hours and transforms into a gel upon contact with the wound. However, an investigation into the cause of death in the 2 rats revealed that their stool fibers became entangled with the HA-soaked cotton before it fully dissolved and transformed into a gel. Consequently, the rats experienced difficulty in defecating, leading to the accumulation of stool around the wound. This resulted in wound infection, which triggered the progression of anal wound inflammation and systemic inflammation, ultimately leading to septic shock and death. The likelihood of encountering such an issue is low for human patients as they are less likely to defecate on the day of surgery, and the HA-soaked cotton can be removed if necessary. However, this issue should be considered for clinical application, and caution should be exercised regarding the possible side effects.

The present study had some limitations. First, this was a preclinical study that investigated the efficacy of HA-soaked cotton on perianal wound healing in a rat model. Consequently, the physiological function and healing rate of the rat anus differs from those of humans. HA-soaked cotton has limitations in clinical settings, based only on the findings of this study. Furthermore, it remains uncertain whether the acceleration of anal wound healing in humans is directly related to pain reduction at the surgical site, mitigation of potential bleeding complications, and preservation of long-term anal function as anticipated. Further human studies are necessary to evaluate the application of HA-soaked cotton to wounds following hemorrhoidectomy. Second, only a single application of HA-soaked cotton was administered following surgery. Therefore, we could not determine whether there were any significant disparities in the wound healing rate between the group receiving multiple applications of HA-soaked cotton on perianal wounds and the group receiving a single application of HA-soaked cotton. Nevertheless, our findings indicated that one application initially improved wound healing and that a prolonged contact time along with a broader coverage area accelerated the healing process. Third, the evaluation of the wound healing area based solely on image analysis lacked objectivity. To address this limitation, histopathological analyses were performed, revealing qualitative differences in the extent of wound healing. However, considering the small sample size, a quantitative analysis could not be performed.

This rodent study demonstrated that the topical application of HA-soaked cotton to perianal wounds accelerated wound healing, especially in the initial stage. In addition, rapid improvement in acute inflammation was observed histologically, and wound healing was achieved by the replacement of loose connective tissue. Based on these findings, we expect that HA-soaked cotton may reduce the severity and duration of posthemorrhoidectomy pain. Moreover, quick wound recovery can reduce the likelihood of posthemorrhoidectomy hemorrhage. Minimizing the progression to fibrosis during wound recovery may sustain the flexibility of the anal wounds following surgery, thereby reducing the risk of long-term anal dysfunction. Consequently, the application of HA-soaked cotton to wounds following hemorrhoidectomy can shorten the recovery period and expedite the return to routine activities by reducing postoperative acute pain and the possibility of side effects, while preserving long-term anal sphincter muscle function and ensuring a better quality of life. Further clinical studies are necessary to elaborate on the effects of HA-soaked cotton, as demonstrated in preclinical studies, on patients undergoing this novel procedure.

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Conflict of Interest

Duck-Woo Kim is the Editor-in-Chief of *Annals of Surgical Treatment and Research*. He was not involved in the review process of this article. The authors declare that the research was conducted without any other commercial or financial relationships, including potential conflicts of interest with Jinwoo Bio Co., Ltd.

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