A novel animal model of long-term sustainable anal sphincter dysfunction

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ABSTRACT

BACKGROUND: Although intersphincteric resection (ISR) can avoid the need for permanent colostomy in patients with lower rectal cancer, it sometimes causes anal sphincter dysfunction, thus resulting in a life-long, debilitating disorder due to incontinence of solid and liquid stool. The development of regenerative medicine could improve this condition by regenerating impaired anal muscle. In order to prove this hypothesis, preliminary experiments in animals will be indispensable, however, an adequate animal model is currently lacking. The purpose of this study was to establish a novel animal model with long-term sustainable anal sphincter dysfunction.

MATERIALS AND METHODS: Twenty male Sprague-Dawley rats were allocated into sham operation (n=10) and anal sphincter resection (ASR) (n=10) groups. The ASR group underwent removal of the left half of both the internal and external anal sphincters. Both groups were evaluated for anal

function by measuring their resting pressure before surgery and on postoperative day (POD) 1, 7, 14 and 28.

RESULTS: The rats in the sham operation group recovered their anal pressure up to baseline on POD 7. The rats in the ASR group showed a significant decrease in anal pressure on POD 1 ($P< 0.0001$) compared with the baseline, and kept this low pressure until POD 28 ($P< 0.0001$). The defect of the anal sphincter muscle was confirmed histologically in the ASR group on POD 28.

CONCLUSION: The present novel model exhibits continuous anal sphincter dysfunction for at least one month and may contribute to further studies evaluating the efficacy of therapies such as regenerative medicine.

Key words: Anal sphincter dysfunction, regenerative medicine, intersphincteric resection, animal model, rectal cancer

INTRODUCTION

Sphincter-preserving surgery for low rectal cancer has been widely performed with fair local control and postoperative survival rates (1). For the last decade, intersphincteric resection (ISR), the "ultimate" anal-preserving surgery, has been proposed and undertaken for patients with lower rectal cancer, which formerly was a definite indication for abdominoperineal resection with permanent colostomy (2,3). Although ISR has become a common procedure and can avoid the need for permanent colostomy in patients with lower rectal cancer, postoperative anal sphincter dysfunction of varying degrees has become a major problem. One potential cause of such postoperative defecatory dysfunction is anal sphincter damage caused by surgery (2,4-7).

The function of the anal sphincter is thought to be voluntarily regulated by the external anal striated muscle, while the internal anal smooth muscle is thought to be responsible for the basal tone (8). Manometric studies have

revealed that the anal resting pressure and maximum squeeze pressure are both decreased following ISR, with a reduced anal sensation or a reduced physiological recto-anal inhibitory reflex (9,10). These sphincter dysfunctions may be caused by direct injury during surgery (11) or may be a result of denervation of the internal sphincter (12). Damage to the anal sphincter is one of the major causes of postoperative defecatory disorders following anal function-preserving surgery, including ISR, and the anal function may not improve with time (4). Anal sphincter dysfunction results in a life-long, debilitating disorder due to incontinence of solid and liquid stool, and may spoil the quality of life for affected patients (7,13).

The development of regenerative medicine might be able to improve this condition in the future (14). Clinical trials are needed to demonstrate the utility of such procedures, however, it is difficult to study the anal sphincter after surgery in human subjects. Previous studies have introduced animal models that can be used to study the anatomy and physiology of the anal canal, and to solve the mechanism of anal function(15-18). However, these

studies were based on the hypotheses that these models had complete fecal incontinence. Since then, several conventional anal dysfunction animal models were reported, such as the sphincterotomy model (19-22), cryoinjured anal sphincter model (23) and pudendal nerve transection model (19,20). However, an adequate animal experimental model has not yet been established that accurately shows long-term dysfunction of the low internal anal sphincter pressure. In the current study, we tried to establish a reliable and reproducible animal model that shows long-lasting anal sphincter dysfunction.

MATERIALS AND METHODS

Twenty male age-matched Sprague-Dawley rats (Charles River Laboratories Japan Inc. Kanagawa, Japan) weighing 250 to 330 g were selected and randomly allocated to two groups: a sham group (n=10) and an anal sphincter resection (ASR) group (n=10). The animals were kept in a temperature- and humidity-controlled environment with a 12-hour

light-dark cycle, and they were allowed free access to water and food at all times. All procedures were performed according to protocols approved by the Animal Care and Use Committee of Nagasaki University.

Experimental Procedures

Surgical procedure

The animals were anesthetized by inhalation of diethyl ether and by the intraperitoneal injection of pentobarbital sodium (32mg/kg). After anesthesia, the animals were placed in the prone position on an animal board, and the surgical site was shaved. An approximately 2 cm longitudinal incision was made on the left of the spine reaching the anal verge (Fig. 1A). For the ASR group, we performed sphincterectomy by removal of a left semicircle of both the internal anal sphincter (IAS) and external anal sphincter (EAS), including the subcutaneous fat tissue around the sphincters (Fig. 1B). The wound was sutured after the operation (Fig. 1C). For the sham group, a superficial dorsal incision (not involving the anal sphincter or

rectum) was performed, and the wound was sutured just after exfoliating the subcutaneous tissue.

Anal resting pressure measurement and analysis

The anorectal pressure was recorded by a catheter with a microminiature silicon strain gauge type sensor mounted at one end (CODMAN MICROSENSOR ICP Transducer, Codman, Raynham, USA), connected through a low profile connector (TEC-10D cable, Millar Instruments, Houston, USA), pressure control unit (TCB-500, Millar Instruments, Houston, USA) and digital data recording system (PowerLab 4/30, ADInstruments, Nagoya JPN). After pressure calibration, the rat anus was lubricated, and the tip of catheter was inserted 2 to 3 mm from the anal verge to be fixed and measure the pressure under anesthesia. After five minutes of stabilization, the measurement was started and recording was continued at least for three minutes. The anal pressure was recorded just before surgery as a baseline and on postoperative day (POD) 1, 7, 14 and 28.

We evaluated the mean pressures at each point and compared the sham group with the ASR group. On POD 28, the animals were euthanized with an overdose of inhaled anesthetic agent, and the rats were frozen to preserve the original anatomical position. The anatomical changes of the rectum and anal canal were examined histologically. They were dissected longitudinally along their dorsal and ventral aspects, fixed in 4% paraformaldehyde phosphate buffer solution (Wako Chemical Industries, Miyazaki, JPN), embedded in paraffin, sectioned (5 μ m) and stained with hematoxylin and eosin (H&E) stain.

Statistical Analyses

All data are presented as the means ± standard deviation. Overall comparisons between groups were made with the GraphPad Prism 5 software program (GraphPad Software. Inc., San Diego, USA). The statistical significance of differences was assessed by Student's t-test for comparisons of continuous variables between two groups and by a one-way

ANOVA for continuous variables for each group. A p-value < 0.05 was considered to be statistically significant.

RESULTS

Morbidities associated with the procedure

The preliminary experiments using over 20 rats were essential for developing the surgical technique. There were no lethal complications associated this procedure throughout the experiments. The minor complications were as follows: rectal mucosa damage with primary repair (3/34, 8.8%) and wound dehiscence (1/34, 3%).

Normal anal sphincter pressure waves

Before surgery, the anal pressure data showed spontaneous, consistent, rhythmic anal pressure contractions with multiple muscle fasciculations, producing multiple peaks in each pressure wave (Fig. 2A). The numeric values presented are the means of random sampling at each measurement

point.

Anal resting pressure analysis after sphincterectomy

On POD 1, there was a significant decrease in the mean resting pressure in both groups (ASR: 4.6±2.1 mmHg, sham: 11.9±7.4 mmHg) compared with the preoperative pressure (24.4±9.7 mmHg, P<0.001, 21.0±9.1 mmHg, P=0.03, respectively). On POD 7, the mean resting pressure of the sham group recovered to the baseline (16.9±6.7 mmHg). On the other hand, the mean resting pressure of the ASR group was kept at a low level compared to the baseline until the rats were euthanized on POD 28 (4.3±3.4 mmHg on POD 7, P<0.001, 5.6±2.6 mmHg on POD 14, P<0.001, 6.5±4.2 mmHg on POD 28, P<0.001) (Fig. 3A).

The whole changes of the anal pressure in both groups are shown in Figure 4. The anal pressure of the ASR group was significantly lower than that of the sham group at all points except before surgery.

Histology

The normal anal sphincter anatomy of the rat is shown in Figure 5A. The anal canal is 2-3 mm long, and is surrounded by the IAS and EAS. The IAS consists of smooth muscle cells, which are thickened at the distal part of the circular enteric muscular coat, and ends about 0.1 mm proximal to the anal orifice (15, Fig. 5A). Twenty-eight days after sphincterectomy, defect of both the IAS and EAS muscles were observed in the ASR group (Fig. 5B). H & E staining also showed that the site of sphinterectomy had been replaced by collagen fibers and fat tissue (Fig. 5C). No histological changes were observed in the sham group (picture not shown).

DISCUSSION

The pelvic floor muscles help to regulate the defecatory process and maintain continence. These muscles include the IAS, EAS and the puborectalis muscle. The ISR technique has been used to extend the opportunity for sphincter preservation to patients with lower rectal cancer. The main concern with

regard to the ISR is the functional outcome. Patients may suffer from fecal incontinence due to partial or complete resection of the IAS. Fecal incontinence after ISR is mainly due to a decrease in resting pressure (2,4-8). Animal models which imitate the patient's state of anal sphincter dysfunction after ISR would be useful for exploring novel therapeutic approaches, including regenerative medicine. Several animal studies have been reported on anal sphincter dysfunction induced by surgical techniques (18-23). For instance, Salcedo, et al. (20) investigated two different models of anal sphincter dysfunction (i.e., sphincterotomy and bilateral pudendal nerve transection) in rodents. Although the resting pressure decreased significantly after each surgery, it recovered to the preoperative level in two weeks. The other reported animal models have also only kept the low anal pressure for a short period, which is not relevant to the human situation, because patients sometimes suffer from lifelong fecal incontinence after ISR. Consequently, the results of previous studies that were obtained from rodent models might not fully reflect the human situation. For this reason, a

model of long-term sustainable anal sphincter dysfunction has been desired. A major problem in rats is that the function can often recover over time, and rats can regain function without surgical repair (24). This might be attributed to the abundance of muscles around the coccyx. To solve this problem, we removed all of the tissue around the left half of the rectum and established a model with a low anal resting pressure, which continued to be low for at least one month. Furthermore, we believe that our model is suitable as an ISR model, in terms of the removal of sphincter muscles, which could contribute to evaluations of the state of clinical ISR.

Over 20 rats were required to establish this surgical technique. Initially, the IAS remained in the specimen after surgery in 12 rats. Therefore, we attempted to remove the distal edge of the IAS in order to induce anal sphincter dysfunction, and the skin incision was extended to the rectal mucosa beyond the anal verge. After the skin incision was extended, the surgical technique was established. On the other hand, we believe that extension of the skin incision also caused anal sphincter dysfunction in the

sham group. That is, a minute sphincterotomy was created that caused anal sphincter dysfunction in the short-term. To prevent this condition, a range of skin incisions must be repaired.

We removed not only the IAS, but also the EAS, in this model because these muscles were only a few millimeters in thickness and it was technically difficult to isolate. The EAS is usually preserved in the ISR technique in humans, so our model different in this respect. Therefore, a larger animal model would be needed to evaluate the true condition of ISR. However, in cases of advanced rectal cancer involving the EAS, it is necessary to also remove the EAS to perform an external sphincter resection (ESR). Our model is likely to mimic the conditions of ESR. From this point of view, our model should be useful for exploring novel therapeutic options for anal sphincter dysfunction.

We expect to use this model in future experiments to perform preclinical testing of pharmaceutical agents and regenerative treatment approaches, including stem cell therapy.

We also think that our model is applicable to studies to understand and evaluate treatments for anal sphincter dysfunction caused by other mechanisms, such as perineal laceration during childbirth and anal sphincter dysfunction associated with aging. These conditions are not caused by surgery, but may not recover during the natural course, as in ISR. Because our model exhibits anal sphincter dysfunction for at least one month, it may contribute to the identification of treatments for various types of anal sphincter dysfunction.

CONCLUSION

Our model is similar to the actual pathogenesis of anal sphincter dysfunction following very low rectal surgery in humans. We believe that this model may contribute to further studies of novel therapies for fecal incontinence, such as regenerative medicine.

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Figure legends

Figure 1: The surgical procedure for the anal sphincter resection (ASR). A) The incision line is made on the anal orifice (arrow). B) Removal of the sphincter muscles in the ASR group. The arrow shows the anal orifice. C) A cross-sectional view of the anal region. Dashed line shows resection line. The IAS and the EAS muscles are removed with paraproctium. R: rectum, T: testis, Co: Coccyx, IAS: internal anal sphincter, EAS; external anal sphincter. D) The anal orifice after suturing.

Figure 2: The anal sphincter pressure waves. A) Normal waves B) post-ASR (POD1), the anal pressure has decreased, and the rhythmic multiple fasciculation was unclear after surgery.

Figure 3: The sham operation group recovered the baseline anal pressure by POD7. The ASR group showed a significant decrease in anal pressure on POD1 ($p < 0.005$), and the low pressure continued until POD28 ($p < 0.001$).

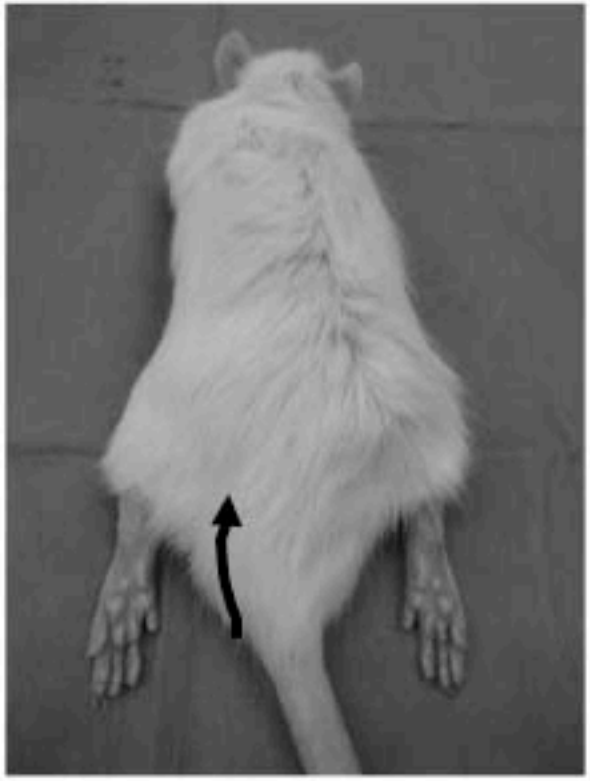
* $p < 0.05$ ** $p < 0.01$ (compared with the mean preoperative resting pressure).

Figure 4: A comparison of the mean anal pressure in the two groups. The postoperative pressure of the ASR group was always significantly lower than that of the sham group throughout the study * $p<0.05$ ** $p<0.01$ (compared with the sham group).

Figure 5: A) A vertical cross-sectional view of the anal region of a normal rat. IAS: internal anal sphincter. B) A cross-sectional view of the anal region of an ASR rat. A defect of the IAS is observed at the surgical site (circle). C) A vertical cross-sectional view of the anal region of an ASR rat. The IAS has been replaced by collagen fibers and fatty tissue.

Figure 1

A



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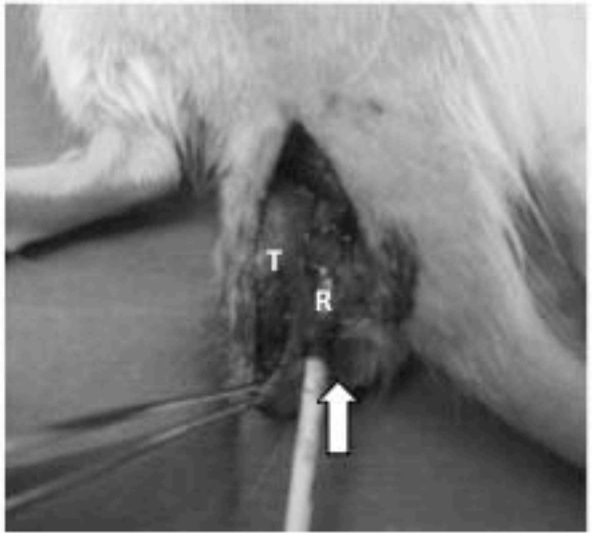

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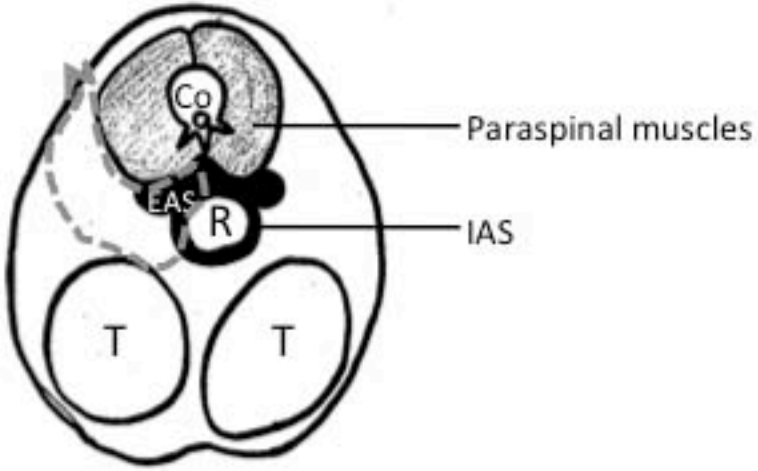

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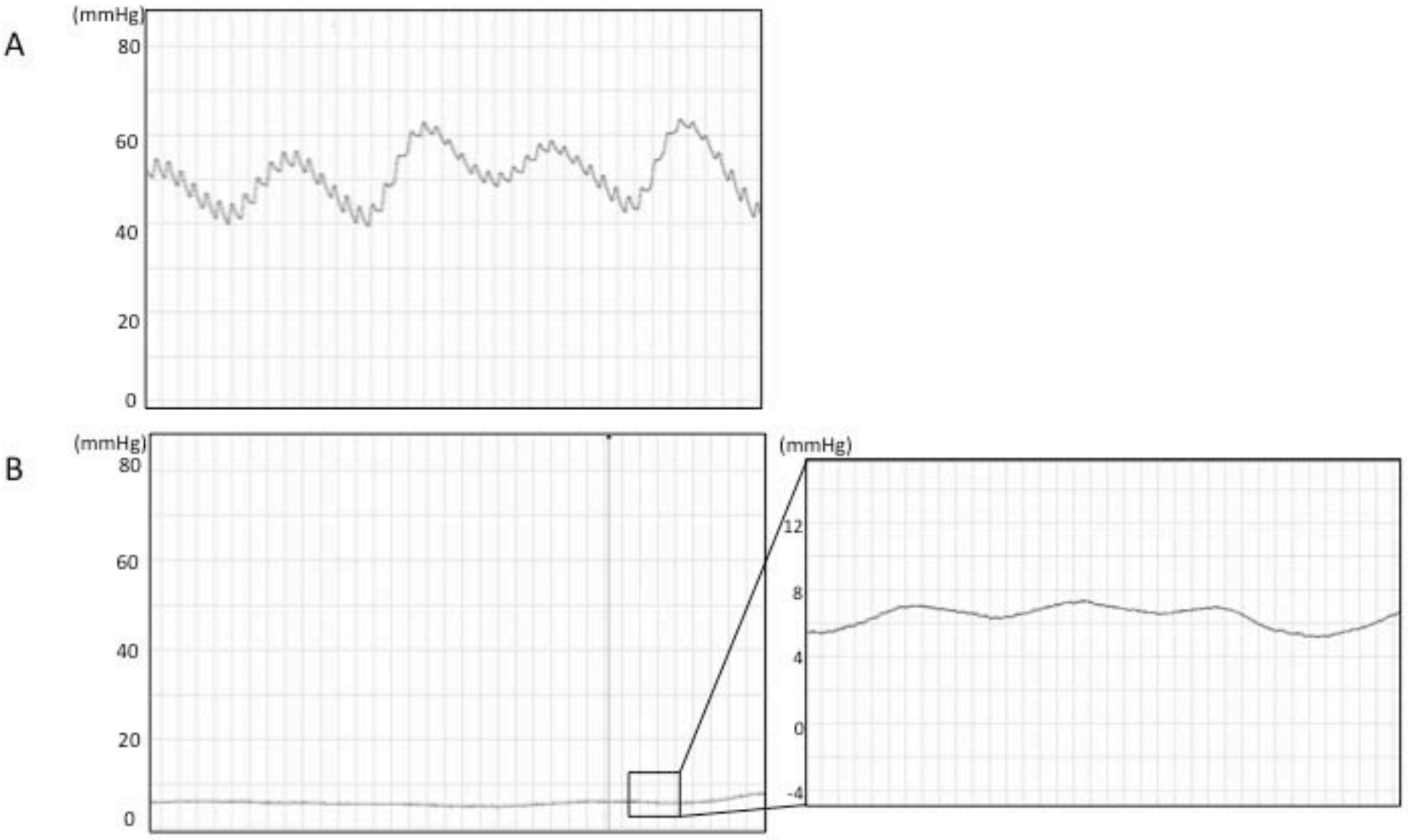

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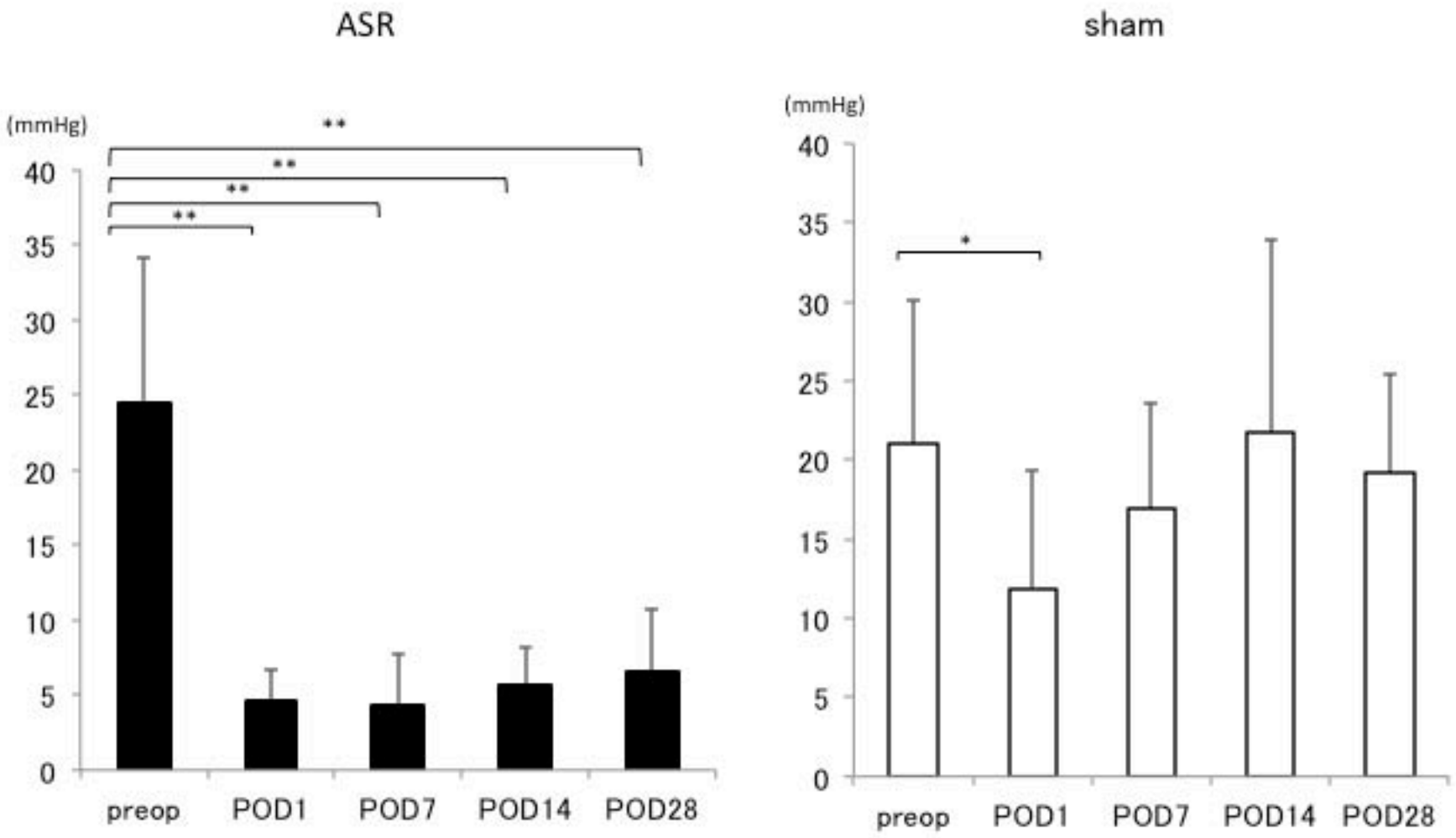
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Figure 4

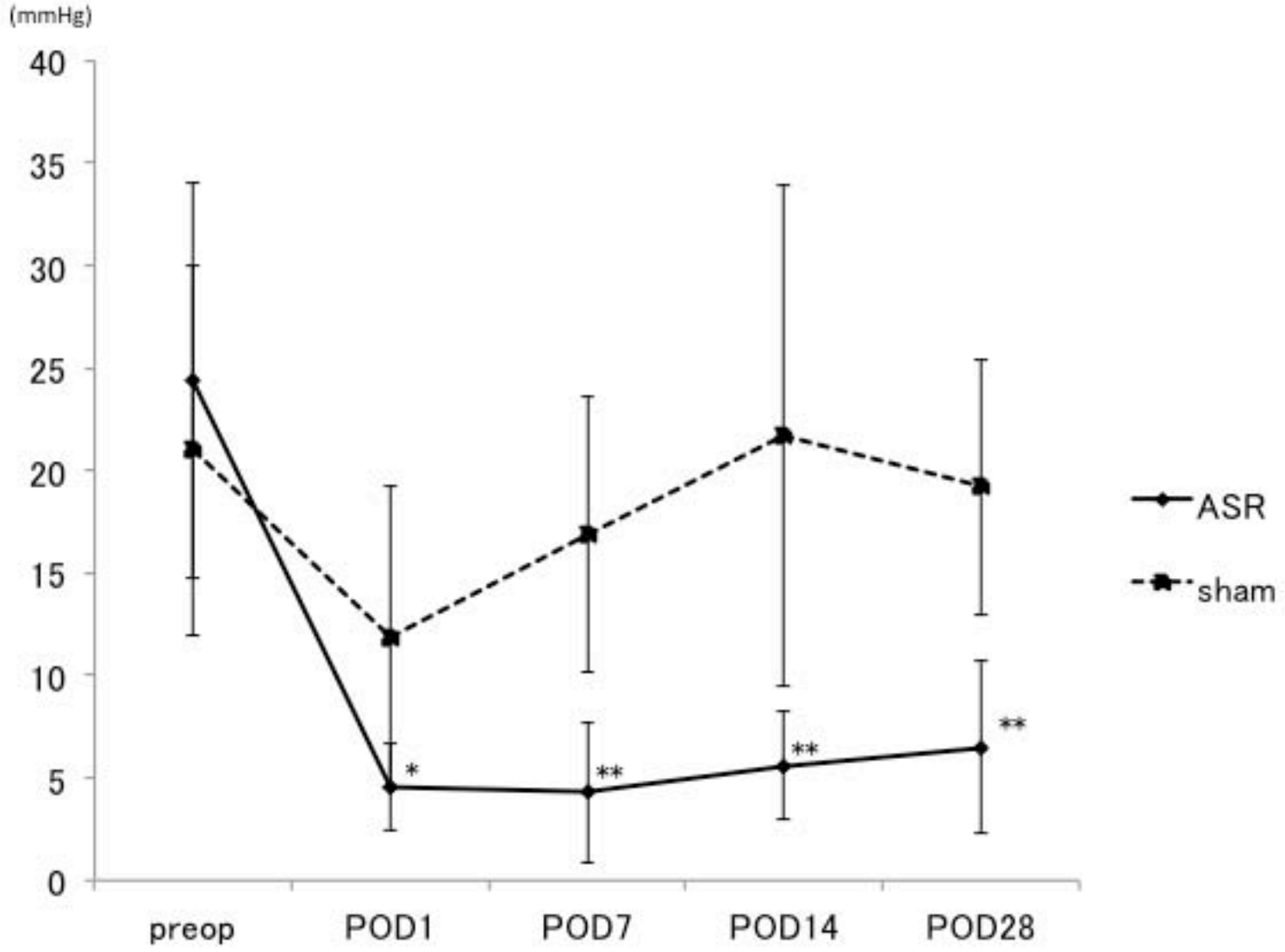
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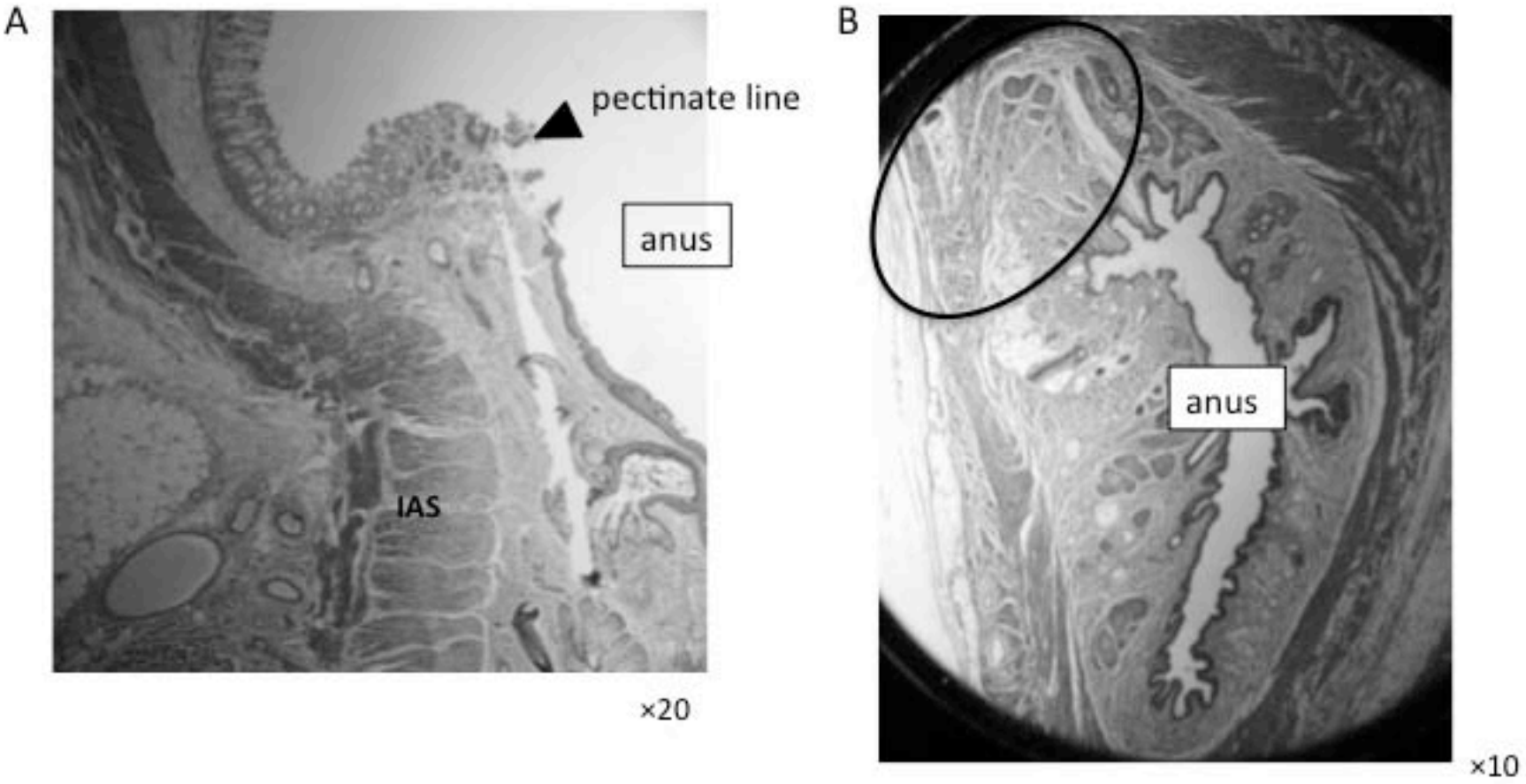
Figure 5



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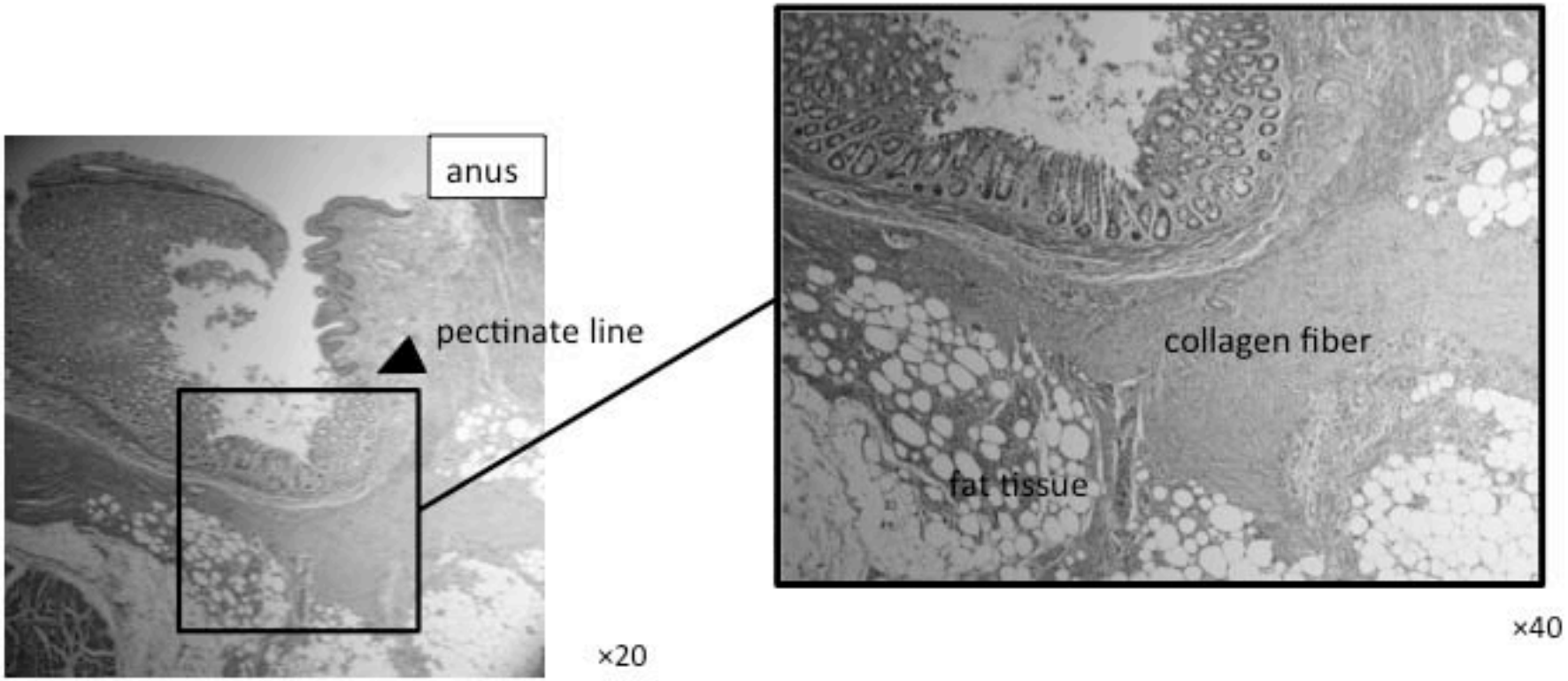
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