Research Square

Preprints are preliminary reports that have not undergone peer review. They should not be considered conclusive, used to inform clinical practice, or referenced by the media as validated information.

Preclinical investigation of expanded human adipose–derived stem cell dosage and timing for improved defecation function in immunodeficient mice

Ryota Mori (tamori0613@gmail.com)
Osaka University https://orcid.org/0000-0001-6119-2032
Norikatsu Miyoshi
Osaka University https://orcid.org/0000-0003-1113-8884
Shiki Fujino
Osaka University
Tsunekazu Mizushima
Osaka university
Ryohei Yukimoto
Osaka University
Takayuki Ogino
Osaka university
Hidekazu Takahashi
Osaka university
Mamoru Uemura
Osaka university
Yuichiro Doki
Osaka university
Hidetoshi Eguchi
Osaka university

Research Article

Keywords: Adipose derived stem cell, Anal sphincter injury, Fecal incontinence, LARS, Defecation function

Posted Date: July 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1850863/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background

Among treatment options for fecal incontinence, none are curative. Adipose-derived stem cells (ADSCs) have emerged as promising therapeutic agents, but most preclinical studies of their effectiveness for anal function have used autologous or allogeneic ADSCs. Here, we investigated the effectiveness, timing of administration, and required dosage of human (h)ADSCs for clinical application.

Methods

A 10-mm balloon catheter was used to induce anal sphincter injury in immunodeficient mice in three groups: ADSC (hADSCs injected after injury), phosphate-buffered saline (PBS; injected after injury), and control (uninjured). The effects of different timing and number of hADSCs administered was compared among groups using defecation status and pathological evaluation.

Results

In terms of defecation status, groups receiving $\geq 1 \times 10^4$ hADSCs immediately after injection showed improvement. Pathological images showed that compared with the PBS group, the thinnest part of the sphincter was thicker for animals that received $\geq 1 \times 10^4$ hADSCs, and fibrosis of the sphincter was notable in those treated with 1×10^3 hADSCs or PBS. Furthermore, although administration of hADSCs at 30 days following injury yielded no pathological signs of improvement, defecation status was improved.

Conclusions

hADSC administration in a mouse model of anal sphincter injury was effective. Injection of $\geq 1 \times 10^4$ hADSCs was necessary to improve defecation status, an effect detected in both the acute and chronic phases.

Background

Fecal incontinence is a common condition defined as the uncontrolled passage of feces or gas for at least one month and has a negative impact on quality of life.(1) Various factors are involved in its initiation, but the number of patients who develop the condition after surgery has increased. Chemoradiation and surgical techniques have improved remarkably in treating rectal cancer, and more patients are undergoing anus-preserving surgery, such as low anterior resection (LAR) or intersphincteric resection (ISR).(2) Although the advantage of these procedures is preservation of the anus, many patients develop severe bowel dysfunction, resulting in incontinence for flatus and feces, urgency, and frequent

bowel movements.(3) These symptoms are defined as LAR syndrome (LARS) and have a negative impact on quality of life.(4, 5) The various treatment options for LARS include medication, surgery, sacral neuromodulation, and biofeedback, but they relieve symptoms only and are not curative.

Cell transplantation recently has become a focus in the field of regenerative therapy because of the self-renewal capacity and differential potential of stem cells. Rapid advances in this field over the past two decades have suggested the possibility of curing previously incurable diseases.(6) In particular, adipose-derived stem cells (ADSCs) are promising therapeutic agents because of their ease of collection and expansion, low immunogenicity, and high ability to differentiate into multiple lineages, including muscle cells.(7) These cells also have paracrine functions and are associated with immunomodulation, cytokine secretion, and angiogenesis.(8, 9)

Initially, autologous cell transplantation was introduced into clinical practice because of safety and ethical concerns. The autologous approach, however, carries some disadvantages, including time-consuming culture methods and difficulty maintaining uniformity. Considering these problems, suitable methods need to be investigated for introducing appropriate cells into clinical treatment. Allogeneic transplantation offers one potential candidate approach.

The European Commission has approved allogeneic, expanded human (h)ADSCs (darvadstrocel; Takeda Pharmaceutical, Tokyo, Japan) for treatment of refractory complex perianal fistulas in patients with Crohn's disease.(10) Clinical trials for hADSC administration to patients with anorectal fistulae unrelated to Crohn's disease are currently underway.(11)

Results from animal models of defecation disorder point to improvements in anal function following ADSC administration, but some limitations need to be addressed before clinical application. First, in these studies, ADSCs were administered immediately after anorectal injury, which does not closely reflect chronic defecation dysfunction in clinical practice. Second, defecation function was measured only by anal pressure, yet studies show no correlation between anorectal pressure and defecation function,(12–14) and we have previously reported that defecation status correlates with defecation function.(15) Third, no one has reported on the optimal number of ADSCs to be administered. Finally, most studies used autologous or allogeneic ADSCs, and no evaluation of the effectiveness of hADSCs has been published.

Rats typically have been used as a model of defecation disorder.(16–18) Mice may have been overlooked because of their small size, which makes it difficult to create an anal sphincter injury model with uniform injury using conventional methods, such as cutting or partial removal of the sphincter muscle. Immunodeficient rats are rare and expensive for research use, however. For this reason, we established a mouse model of anal sphincter injury with dysfunction of defecation, using a balloon catheter to create the injury.(15) With this mouse model, we could easily conduct experiments using immunodeficient animals, allowing for studies of hADSCs instead of autologous or allogeneic transplantation, leading to more clinically relevant findings.

In this study, we investigated whether hADSCs improve defecation function, the dose of hADSCs required, and whether hADSCs improve defecation function both acutely and in the long term after injury, better reflecting the clinical situation.

Methods

Ethics

This study was approved by the Institutional Review Board (Permit number: 19388) and Animal Research Committee (Permit number: 02-031-002) and was conducted in accordance with the protocols approved by the Animal Care and Use Committee of Osaka University.

Animals

Immunodeficient mice (NOD/SCID) were obtained from Nippon Clare (Tokyo, Japan). All animals were bred in temperature- and humidity-controlled rooms with free access to food and water. For these studies, we used 6- to 8-week-old female mice.

hADSCs and cell culturing

We used two types of expanded hADSCs for this study. The cells and the medium (R:stem) were provided by ROHTO Pharmaceutical Co., Ltd. (Osaka, Japan). Normal hADSCs of LONZA (L-ADSCs; PT-5006, Basel, Switzerland) were cultured in R:stem at 37°C in a 5% CO_2 incubator, and the medium was changed every 4 days.(19) Accutase (Nacalai Tesque, Kyoto, Japan) was used for cell passaging. Normal hADSCs from ROHTO (R-ADSCs) were provided in a frozen state and used after being thawed and then washed with phosphate-buffered saline (PBS).

Anal sphincter injury mouse model and treatment protocol

A balloon catheter (10 mm diameter) was used to induce anal sphincter injury on the first day only, as we have previously reported, under general anesthesia using a mixture of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg).(15) The balloon dilation time was set at 2 min and performed twice. The ADSC group received 1000 µl of hADSCs dissolved in PBS immediately after injury at four perianal sites, and the PBS group received 1000 µl PBS as an acute injury comparator; for assessing the effect of hADSCs on chronic defecation disorders, both the balloon catheter and PBS groups received hADSCs at one month after injury. For the hADSCs intervention groups, 1×10^7 hADSCs were administered, but in a series of studies to identify the optimal dosage, different cell counts were applied (1×10^3, 1×10^4,1×10^5, 1×10^6, and 1×10^7). The series of procedures is shown in **Figure 1**.

As a control group, we used mice anesthetized following the same procedure as for animals with induced anorectal injury. After procedures, anesthesia was reversed using atipamezole (1 mg/kg).

Flow cytometric analysis

The expression of surface proteins by the hADSCs was examined using flow cytometry. Relative fluorescence intensities were measured using a BD FACS Aria II (BD Biosciences, CA, USA), with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-human CD90 (328107; BioLegend, San Diego, CA, USA), PE/Cyanine (Cy)7-conjugated anti-human CD34 (343515; BioLegend), allophycocyanin (APC)-conjugated anti-human CD73 (344005; BioLegend), APC/Cy7-conjugated anti-human CD45 (368515; BioLegend), and brilliant violet (BV) 421-conjugated anti-human CD105 (323219; BioLegend). As isotype controls for these antibodies, the following antibodies were used: FITC-conjugated anti-mouse IgG1, k isotype Ctrl (555748; BD Biosciences), PE/Cy7-conjugated anti-mouse IgG1, k isotype Ctrl (557872; BD Biosciences), APC-conjugated anti-mouse IgG1, k isotype Ctrl (554681; BD Biosciences), APC/Cy7-conjugated anti-mouse IgG1, k isotype Ctrl (557873; BD Biosciences), and BV421-conjugated anti-mouse IgG1, k isotype Ctrl (562438; BD Biosciences). 7-aminoactinomycin D (420403; BioLegend) was used to exclude any dead and damaged cells. Data were analyzed using the FlowJo 10.6.1 software program (FlowJo LLC, Ashland, OR, USA).

Evaluation of defecation function

Defecation function was evaluated by defecation status rather than by anorectal pressure. Fecal weight per stool for 24 h was normally measured on day 0 (before anal sphincter injury) and on days 1, 8, 15, 22, and 29 after anal sphincter injury. In the experiments that involved administering hADSCs at one month after injury (day 31), defecation status was measured on day 31 (before hADSC administration) and on days 38, 45, 52, and 59.

Histological examination

The rectum, anal sphincter, and surrounding connective tissue were resected under anesthesia for subsequent examination and fixed in 10% formalin, with care taken not to introduce deformities. After dehydration with an ethanol concentration series, samples were embedded in paraffin and the paraffin blocks sectioned at 3–5 μm onto slides. Sections were stained with hematoxylin and eosin or Masson's trichrome stain for detection of collagen deposits (TRM-1; Cosmo Bio, Tokyo, Japan).

Immunohistochemistry

For immunohistochemistry, paraffin blocks were deparaffinized and sectioned at 3 μm onto slides. Each slide was boiled for 15 min and then immersed in methanol-hydrogen peroxide solution for 25 min at room temperature to quench endogenous peroxidase. Slides then were blocked with blocking serum for 30 min at room temperature and incubated overnight with primary antibody, followed by a 30-min incubation with secondary antibody at room temperature. To counterstain DNA, slides were mounted with ProLong® Glass Antifade Mountant with NucBlue® Stain (P36983; Thermo Fisher Scientific, Waltham, MA, USA). The following primary antibodies were used for immunofluorescence studies: rabbit anti-HLA antibody (1:200, ab52922; Abcam, Cambridge, MA, USA), rabbit anti-Ki67 antibody (1:400, ab15580; Abcam, Cambridge, MA, USA), rabbit anti-CD206 antibody (1:200, 18704-1-AP; Proteintech Group Inc, Rosemont, IL, USA), and rat anti-F4/80 antibody (1:1000, MCA497RT; Bio-Rad Laboratories Inc, Hercules,

CA, USA). The following secondary antibodies were used: anti-rabbit IgG (1:1000, A11008; Alexa Fluor 488, Thermo Fisher Scientific, Waltham, MA, USA), and anti-rat IgG (1:400, 4418; Alexa Fluor 647, Cell Signaling Technology, MD, USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stain was performed using the MEBSTAIN Apoptosis TUNEL Kit Direct (8445; MBL, Aichi, Japan), following the manufacturer's instructions.

Imaging measurements and analyses

All images were captured with BIOREVO BZ-X710 (Keyence, Tokyo, Japan). Measurements of cross-sectional areas of the rectal lumen and anal sphincter and thickness of the anal sphincter were made using image-processing software (ImageJ, National Institutes of Health, Bethesda, MD, USA). The number of cells stained with fluorescent antibodies was automatically counted with a BR-Z analyzer (KEYENCE).

Statistical analysis

All statistical analyses were performed using JMP pro14.0.0 (SAS Institute Inc, Cary, NC, USA), and all data are presented as means ± standard error of the mean (SEM). Continuous variables were compared with the Mann–Whitney U test and student's t test. A p value lower than 0.05 was considered statistically significant.

Results

R-ADSCs and L-ADSCs had the same cell surface phenotype

Two different hADSCs, R-ADSCs and L-ADSCs, were analyzed by flow cytometry to assess for any differences in properties. Both types were positive for CD73, CD90, and CD105, which are considered positive markers for hADSCs, and negative for CD45 and CD34, which are considered negative markers (**Figure 2**).

hADSCs healed damaged anal sphincter muscles and promoted the recovery of defecation function in a mouse model of anal sphincter injury

NOD/SCID mice were divided into four groups (n = 4 per group): no anal sphincter injury (controls), injured animals administered 1×10^7 R-ADSCs, injured animals administered 1×10^7 L-ADSCs, and injured animals administered only PBS. Fecal weight per stool was measured on day 0 (before anal sphincter injury) and on days 1, 8, 15, 22, and 29 after anal sphincter injury in each group to evaluate defecation status. Then, on day 29, the mice were sacrificed and their anuses removed along with the surrounding connective tissue for pathological evaluation. A scheme of the experimental schedule is shown in **Figure 3A**.

Defecation status in both groups treated with hADSCs improved from day 8, and differences from the control group disappeared from day 22; however, the PBS group showed no improvement (**Figure 3B**). There was no difference in body weight among the four groups up to one month (**Figure 3C**). Because the

L-ADSC and R-ADSC groups did not differ, only R-ADSCs were used as the ADSC group in subsequent experiments.

Histological imaging showed that the injured anus was noticeably deformed, with fibrosis and tearing in the PBS compared with the ADSC group (**Figure 3D, E**). When the pathological images were analyzed, the PBS group had less sphincter area in cross section and less thickness in the thinnest part of the sphincter compared with the ADSC and control groups. The ADSC and control groups did not differ (**Figure 3F**).

hADSCs differentiated into muscle and surrounding connective tissue, showed increased M2 macrophages, promoted cell division, and inhibited cell necrosis

HLA expression in the ADSC groups was analyzed by immunohistochemistry. HLA-positive cells were observed in the muscle tissue and surrounding connective tissue in the ADSC group (**Figure 4A**), suggesting the potential of ADSCs to differentiate in the injured anal sphincter muscle and the surrounding connective tissue.

Ki67 and TUNEL staining to assess the effect of hADSCs on cell division and apoptosis showed no difference between the PBS and control groups in the fraction of cells undergoing cell division, but hADSC treatment promoted cell division (**Figure 4B, C**). The percentage of cells undergoing apoptosis did not differ between the ADSC and control groups, but a higher percentage of apoptotic cells was found in the PBS group compared with the control group (**Figure 5A, B**). To examine the effect of hADSCs on the content of M2 macrophages, which affect wound healing, we performed double immunostaining using F4/80, a marker for macrophages, and CD206, a marker for M2 macrophages. As shown in **Figure 5C**, the ratio of M2 macrophages to total cell count was significantly higher in the ADSC group compared with the PBS and control groups. In addition, the ratio of M2 to all macrophages was significantly higher in the ADSC and control groups than in the PBS group (**Figure 5C**). These results indicate that M1 macrophages normally increase when tissue damage occurs, but that administration of hADSCs increased macrophage content in the surrounding connective tissue, especially of M2 macrophages.

More than 1×10^4 hADSCs were required to improve defecation function in a mouse model of anal sphincter injury

Next, we examined how many hADSCs would be needed for a positive effect. For this purpose, we used four doses of hADSCs (1×10^6, 1×10^5,1×10^4, and 1×10^3) administered after anal sphincter injury in NOD/SCID mice (n = 4 per dosage group). As shown in **Figure 6A**, groups receiving $\geq 1 \times 10^4$ hADSCs showed improvement in defecation status from day 21. In a comparison of these dosage groups with the three groups in the interventional experiments, body weight did not differ among the seven groups during one month (**Figure 6B**). In a comparison of dosage groups with the PBS animals, pathological images showed thinning and fibrosis of the sphincter muscle with the 1×10^3 hADSC treatment as well as in the PBS group (**Figure 6C**). Analysis of pathological images showed that the thinnest part of the sphincter was thicker in the group that received $\geq 1 \times 10^4$ hADSCs compared with the PBS group (**Figure 6D**). In

addition, the sphincter cross-sectional area was greater in animals that received 1×10^7 hADSCs compared with those administered 1×10^3 hADSCs or PBS only (**Figure 6D**). Similar to the results described in the previous section, however, the seven groups did not differ in length of the thickest part of the sphincter or in rectal lumen area (**Figure 6D**). Considering these findings, 1×10^4 hADSCs was set as the minimum dosage for improved defecation function in these animals.

hADSCs improved defecation function even if fibrosis was present in the anal sphincter

Finally, for examining whether administering hADSCs after injury chronicity affects defecation function, NOD/SCID mice were divided into three groups (n = 4 per group): no anal sphincter injury (controls), administration of 1×10^7 hADSCs at 30 days after injury, and administration of PBS 30 days after injury. Fecal weight per stool was measured on day 30 (before hADSC administration) and on days 38, 45, 52, and 59 after injury in each group to evaluate defecation status. On day 59, mice were sacrificed and their anuses removed along with the surrounding connective tissue for pathological evaluation. A scheme of the experimental schedule is given in **Figure 7A**. As shown in **Figure 7B**, animals with damaged sphincter muscles did not exhibit defecation status recovery at 30 days after anal injury. The three groups did not differ in body weight during this period (**Figure 7C**). Although no pathological improvement was observed after hADSC treatment at post-injury day 30, defecation function was improved at treatment day 14 with hADSCs (**Figure 7D–F**).

Discussion

Colorectal cancer is common globally, with rectal cancer accounting for one-third of cases.(20) Unfortunately, 80%–90% of patients who undergo LAR for rectal cancer experience a change in bowel habits after surgery, and the prevalence of severe LARS has not altered with time.(21, 22) LARS is said to be caused by direct injury to the anal sphincter muscle and damage to the nerves innervating the rectum and anus.(23) In this study, we demonstrated that ADSCs may represent an effective treatment candidate for LARS.

ADSCs can differentiate into various cell types, including bone, chondrocytes, muscle, adipocytes, neurons, and hepatocytes, and promote tissue regeneration and cell survival through paracrine secretion.(24, 25) We demonstrated in a mouse model of anal sphincter injury that ADSCs can differentiate into muscle tissue and surrounding connective tissue, activate cell division, and suppress apoptosis. Furthermore, in hADSC-treated animals, M2 macrophages were observed in large numbers in the connective tissue around the anal sphincter. This result is similar to previously reported findings and suggests that ADSCs also suppress the excessive immune response and contribute to the process of tissue healing.(26)

In terms of defecation function, the current results show that administration of 1×10^4 or more hADSCs immediately after injury improved defecation function. However, in terms of pathological results, 1 × 10[illegible] hADSCs were needed to yield benefit. Furthermore, in the chronic phase, hADSC administration at one

month after the injury did not yield positive results on pathology, but defecation function was improved. These findings indicate that recovery of the anal sphincter from injury is not the only factor that improves defecation function and that hADSCs may also have a positive effect on the nervous system.

In another study, using magnetic resonance imaging, we measured the volume of defecation-related muscle in patients with severe LARS and found an average volume of 36.5 ml.(27) The perianal area in the mice used in the current work was about 20.0 μl. Because the current findings highlighted 1×10^4 ADSCs as the minimum for defecation function recovery in mice, the extrapolated amount for humans would be about 2×10^7 ADSCs.

This study has several limitations. First, anal pressure was not measured in assessing the function of anal sphincter muscle. However, as noted, studies suggest no correlation between anal pressure and the function of anal sphincter muscle in clinical practice, and defecation status is likely more important for patients. Here, we took weight per piece of stool as a reflection of defecation function. Second, our mouse model of anal sphincter injury does not perfectly replicate the real-world clinical condition of LARS. Finally, ADSCs have not been evaluated for effects on the nervous system in a mouse model of anal sphincter injury.

Conclusions

Administration of expanded hADSCs in a mouse model of anal sphincter injury yielded improvement in defecation status and repair of anal sphincter muscles. In addition, a dosage $\geq 1 \times 10^4$ hADSCs was necessary to improve defecation status, which was effective in both the acute and chronic phases.

Abbreviations

hADSCs
Human adipose-derived stem cells
PBS
Phosphate-buffered saline
LAR
Low anterior resection
ISR
intersphincteric resection
LARS
Low anterior resection syndrome
L-ADSCs
Human adipose-derived stem cells of LONZA
R-ADSCs Human adipose-derived stem cells of ROHTO

Declarations

Ethics approval and consent to participate: This study was approved by the Institutional Review Board (Permit number: 19388) and Animal Research Committee (Permit number: 02-031-002) and was conducted in accordance with the protocols approved by the Animal Care and Use Committee of Osaka University. Informed Consent is not applicable.

Consent for publication: Not applicable.

Availability of data and materials: Not applicable.

Competing interests: This study was supported by ROHTO Pharmaceutical Co., Ltd., through a research grant and provision of two types of adipose-derived stem cells and culture medium.

Funding: Not applicable.

Authors' contributions: RM and RY collected the data; RM drafted the manuscript; SF, NM, and TM helped to finalize the manuscript. TO, HT, MU, YD, and HE proofread the content, and HE gave final approval of the article. All authors have read and approved the final manuscript.

Acknowledgements: The authors thank San Francisco Edit for English language editing.

References

1. Paquette IM, Varma MG, Kaiser AM, Steele SR, Rafferty JF. The American Society of Colon and Rectal Surgeons' Clinical Practice Guideline for the Treatment of Fecal Incontinence. Dis Colon Rectum. 2015;58(7):623-36.
2. Toiyama Y, Kusunoki M. Changes in surgical therapies for rectal cancer over the past 100 years: A review. Ann Gastroenterol Surg. 2020;4(4):331-42.
3. Koda K, Yamazaki M, Shuto K, Kosugi C, Mori M, Narushima K, et al. Etiology and management of low anterior resection syndrome based on the normal defecation mechanism. Surg Today. 2019;49(10):803-8.
4. Emmertsen KJ, Laurberg S. Low anterior resection syndrome score: development and validation of a symptom-based scoring system for bowel dysfunction after low anterior resection for rectal cancer. Ann Surg. 2012;255(5):922-8.
5. Camilleri-Brennan J, Steele RJ. Quality of life after treatment for rectal cancer. Br J Surg. 1998;85(8):1036-43.
6. Madl CM, Heilshorn SC, Blau HM. Bioengineering strategies to accelerate stem cell therapeutics. Nature. 2018;557(7705):335-42.
7. Miyoshi N, Fujino S, Takahashi Y, Yasui M, Ohue M, Mizushima T. Implantation of human adipose-derived stromal cells for the functional recovery of a murine heat-damaged muscle model. Surg Today. 2020;50(12):1699-706.

8. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood. 2007;110(10):3499-506.
9. Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008;103(11):1204-19.
10. Panés J, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, et al. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. Lancet. 2016;388(10051):1281-90.
11. Maciel Gutiérrez VM, Gutiérrez Guillen SG, Centeno Flores MW, Valenzuela Pérez JA, Abarca Rendón FM, Hernández García FS, et al. Safety of Allogeneic Adipose Tissue-Derived Mesenchymal Stem Cells for the Treatment of Complex Perianal Fistulas Not Associated With Crohn's Disease: A Phase I Clinical Trial. Dis Colon Rectum. 2021;64(3):328-34.
12. Zutshi M, Salcedo L, Hammel J, Hull T. Anal physiology testing in fecal incontinence: is it of any value? Int J Colorectal Dis. 2010;25(2):277-82.
13. Bharucha AE. Fecal incontinence. Gastroenterology. 2003;124(6):1672-85.
14. Rao SS. Diagnosis and management of fecal incontinence. American College of Gastroenterology Practice Parameters Committee. Am J Gastroenterol. 2004;99(8):1585-604.
15. Yukimoto R, Miyoshi N, Fujino S, Mori R, Ogino T, Takahashi H, et al. Usefulness of An Anal Sphincter Injury Mouse Model by Means of a Balloon Catheter and a New Method of Evaluating Anal Sphincter Function. Ann Gastroenterol Surg. 2022;6(2):282-7.
16. Yamaguchi I, Fujita F, Yamanouchi K, Mishima T, Kawahara D, Sakai Y, et al. A novel animal model of long-term sustainable anal sphincter dysfunction. J Surg Res. 2013;184(2):813-8.
17. Kuismanen K, Juntunen M, Narra Girish N, Tuominen H, Huhtala H, Nieminen K, et al. Functional Outcome of Human Adipose Stem Cell Injections in Rat Anal Sphincter Acute Injury Model. Stem Cells Transl Med. 2018;7(3):295-304.
18. Salcedo L, Penn M, Damaser M, Balog B, Zutshi M. Functional outcome after anal sphincter injury and treatment with mesenchymal stem cells. Stem Cells Transl Med. 2014;3(6):760-7.
19. Fujino S, Ito A, Ohue M, Yasui M, Mizushima T, Doki Y, et al. Phenotypic heterogeneity of 2D organoid reflects clinical tumor characteristics. Biochem Biophys Res Commun. 2019;513(2):332-9.
20. Glimelius B, Tiret E, Cervantes A, Arnold D. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2013;24 Suppl 6:vi81-8.
21. Bryant CL, Lunniss PJ, Knowles CH, Thaha MA, Chan CL. Anterior resection syndrome. Lancet Oncol. 2012;13(9):e403-8.
22. Pieniowski EHA, Palmer GJ, Juul T, Lagergren P, Johar A, Emmertsen KJ, et al. Low Anterior Resection Syndrome and Quality of Life After Sphincter-Sparing Rectal Cancer Surgery: A Long-term Longitudinal Follow-up. Dis Colon Rectum. 2019;62(1):14-20.
23. Croese AD, Lonie JM, Trollope AF, Vangaveti VN, Ho YH. A meta-analysis of the prevalence of Low Anterior Resection Syndrome and systematic review of risk factors. Int J Surg. 2018;56:234-41.

24. Gorecka A, Salemi S, Haralampieva D, Moalli F, Stroka D, Candinas D, et al. Autologous transplantation of adipose-derived stem cells improves functional recovery of skeletal muscle without direct participation in new myofiber formation. Stem Cell Res Ther. 2018;9(1):195.
25. Sowa Y, Imura T, Numajiri T, Nishino K, Fushiki S. Adipose-derived stem cells produce factors enhancing peripheral nerve regeneration: influence of age and anatomic site of origin. Stem Cells Dev. 2012;21(11):1852-62.
26. Pilny E, Smolarczyk R, Jarosz-Biej M, Hadyk A, Skorupa A, Ciszek M, et al. Human ADSC xenograft through IL-6 secretion activates M2 macrophages responsible for the repair of damaged muscle tissue. Stem Cell Res Ther. 2019;10(1):93.
27. Mori R, Uemura M, Tsuboyama T, Fujino S, Hata T, Ogino T, et al. The prediction of postoperative anorectal dysfunction after low anterior resection for lower rectal cancer by measuring the volume of defecation-related muscles. Surg Today. 2022;52(5):745-54.

Figures

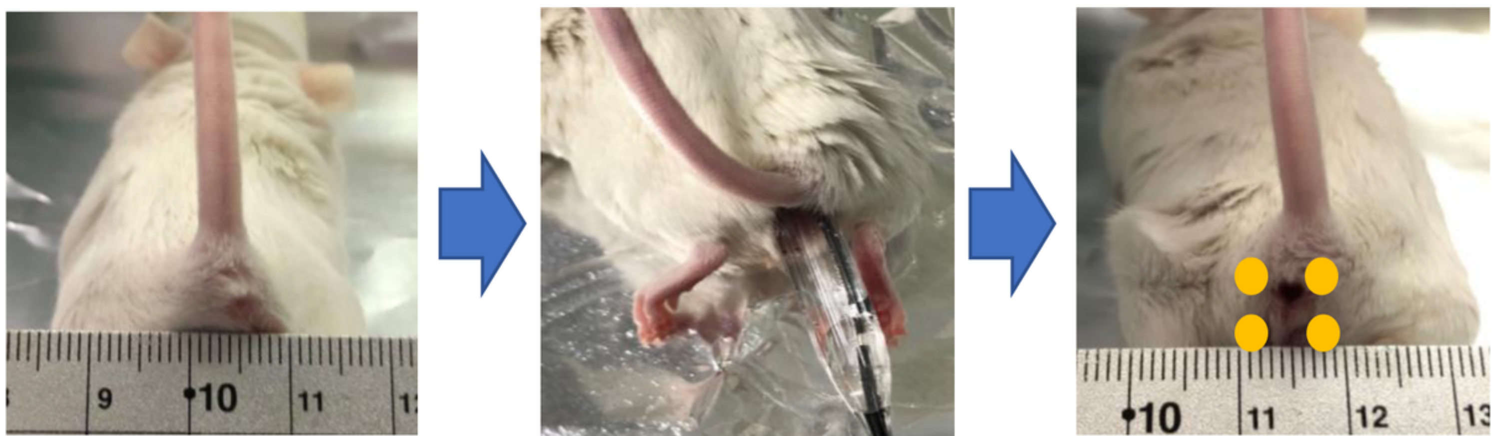


Figure 1

Images taken during and after anorectal injury induced with a balloon catheter in mice.

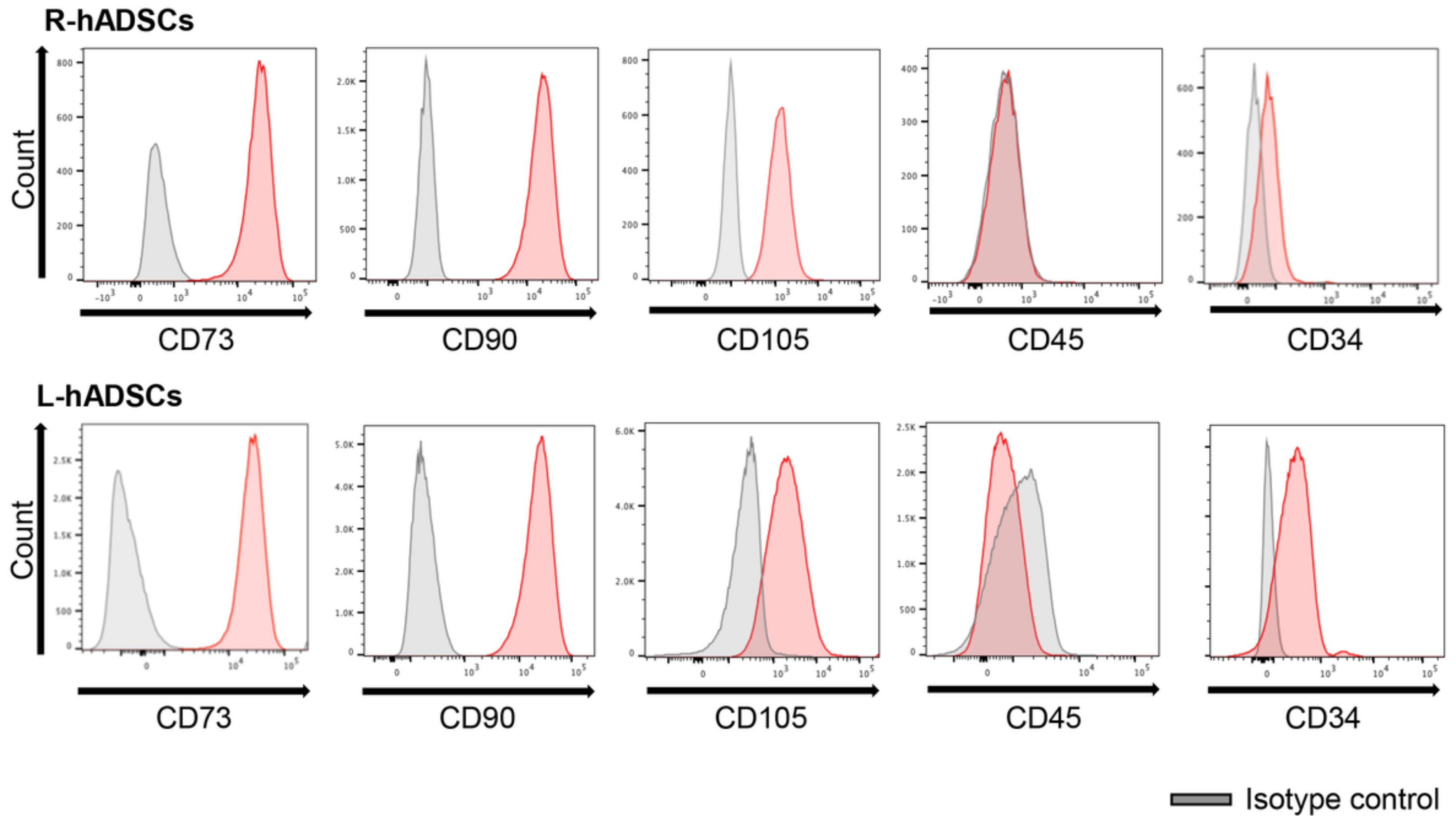


Figure 2

Surface marker analysis of hADSCs using representative positive and negative ADSC markers was performed using flow cytometry. CD73, CD90, and CD105 served as positive markers and CD45 and CD34 as negative markers. The results in the upper row were obtained from R-ADSCs, and those in the lower row were from L-ADSCs. Blue shading indicates isotype control.

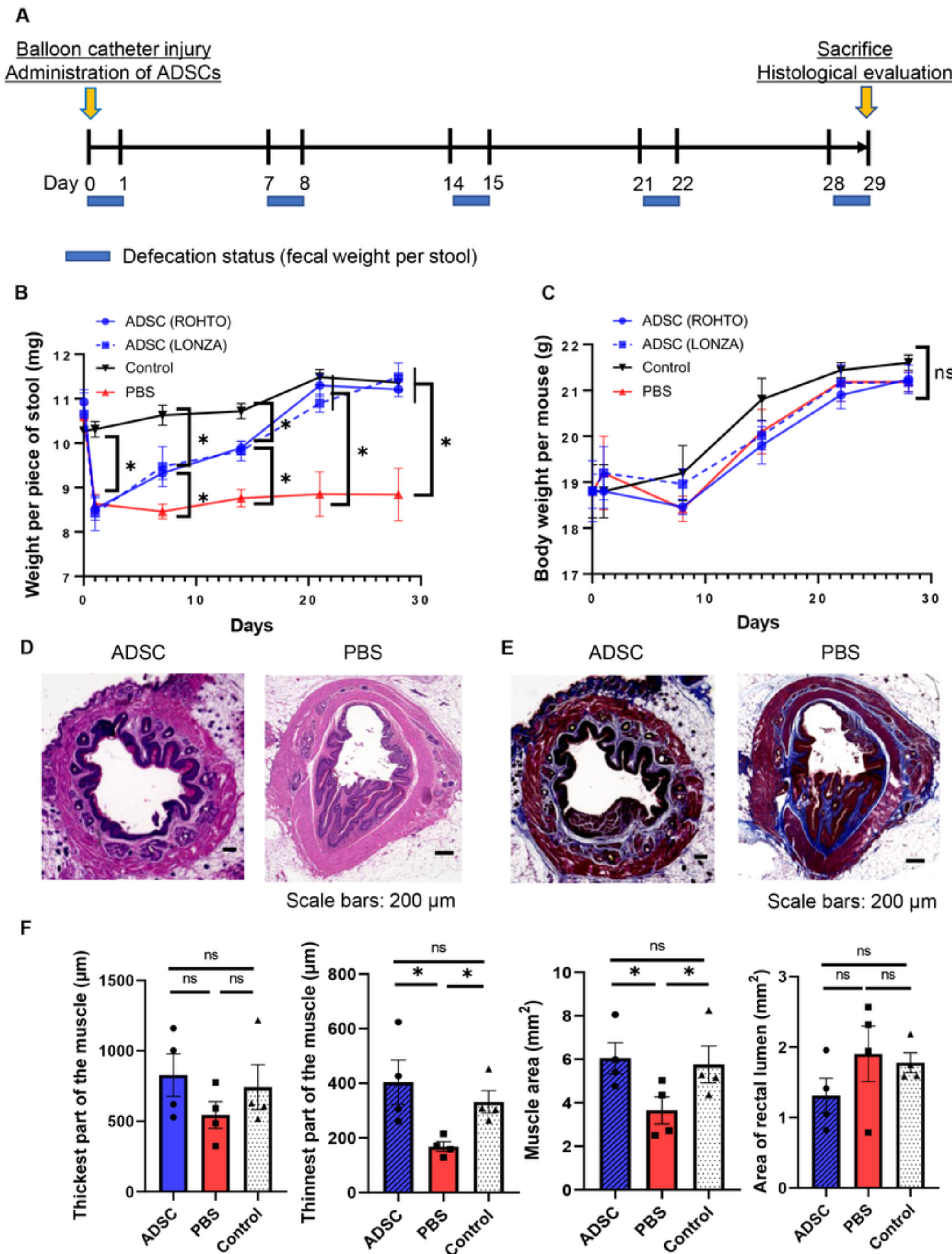
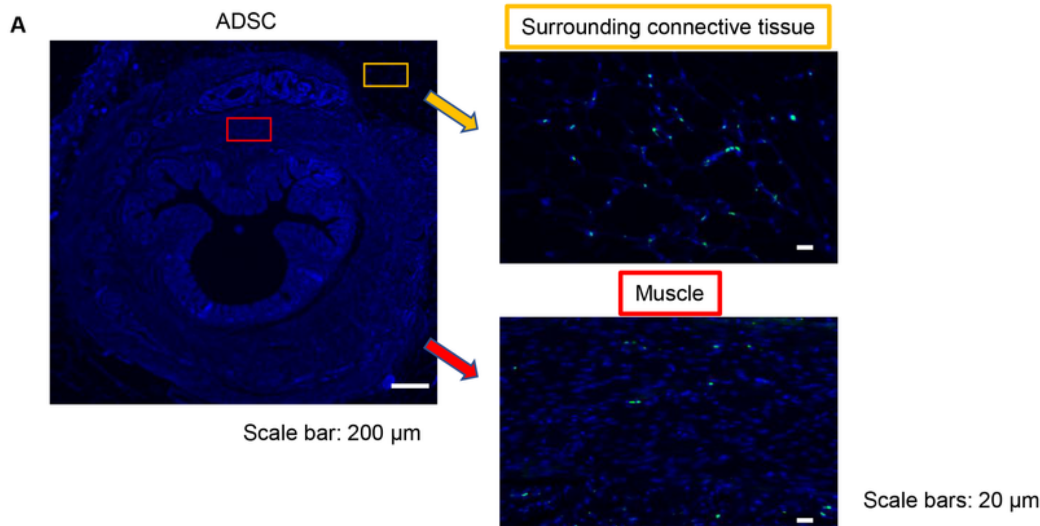
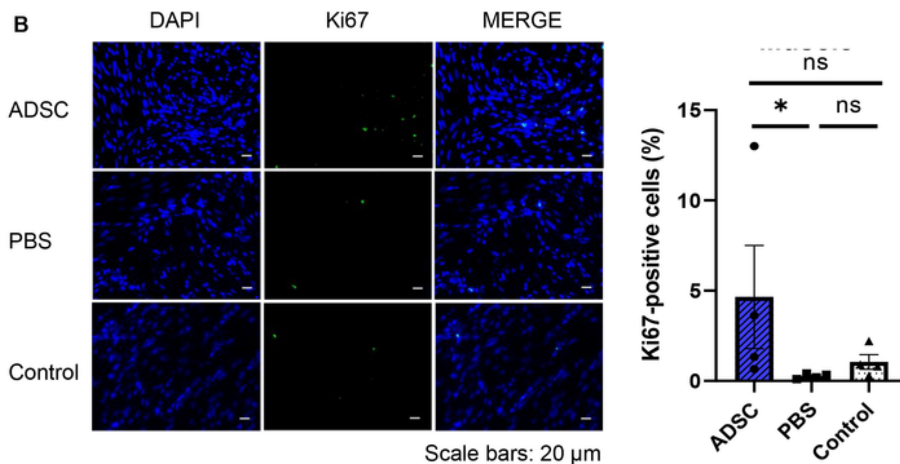


Figure 3

Examination of ADSC effectiveness with administration in the acute phase. ADSCs were administered in a mouse model of anal sphincter injury. (A) Experimental schedule. (B) Defecation status of the four groups. Each bar represents the mean ± SEM of quadruple measurements of weight per piece of stool (**P* < .05). (C) Body weight of the four groups. Each bar represents the mean ± SEM of quadruple measurements (**P* < .05). (D) Representative photomicrographs of histological sections of the anal

sphincter and surrounding connective tissue on day 29 with hematoxylin and eosin staining (R-ADSC and PBS groups). (E) Representative photomicrographs of histological sections of the anal sphincter on day 29 with TUNEL staining (R-ADSC and PBS groups). (F) Bar plot of pathological evaluation of the anal sphincter on day 29 (R-ADSC, PBS, and control groups). Cross-sectional area of the rectal lumen and anal sphincter and thickness of the anal sphincter were used as criteria for pathological evaluation. Each bar represents the mean ± SEM of quadruple measurements (**P* < .05).





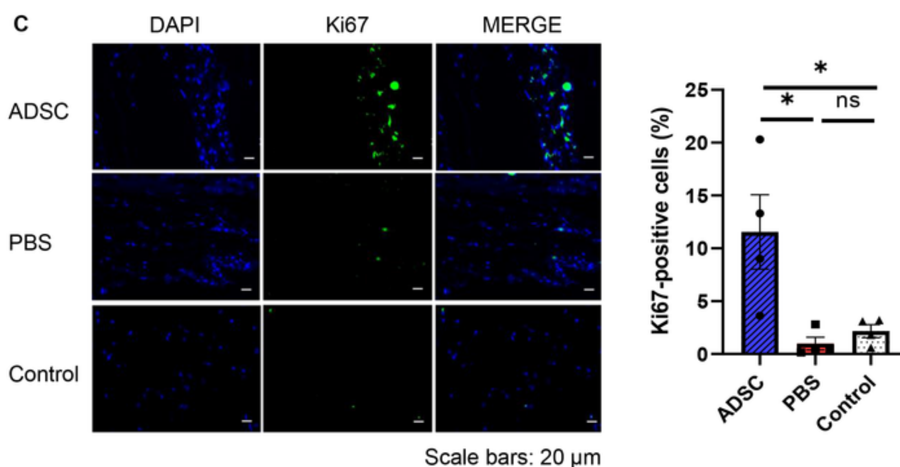


Figure 4

Evaluation of pathological images by fluorescent immunostaining to assess effects of hADSC administration. (A) Representative images of HLA expression (green) with nuclei staining (blue) in the ADSC and PBS groups. (B) Representative images of Ki67 expression (green) with nuclei staining (blue) in the anal sphincter and bar plot of expression rate in ADSC, PBS, and control groups (*$P < .05$). (C) Representative images of Ki67 expression (green) with nuclei staining (blue) in the surrounding connective tissue and bar plot of expression rate in the ADSC, PBS, and control groups (*$P < .05$).

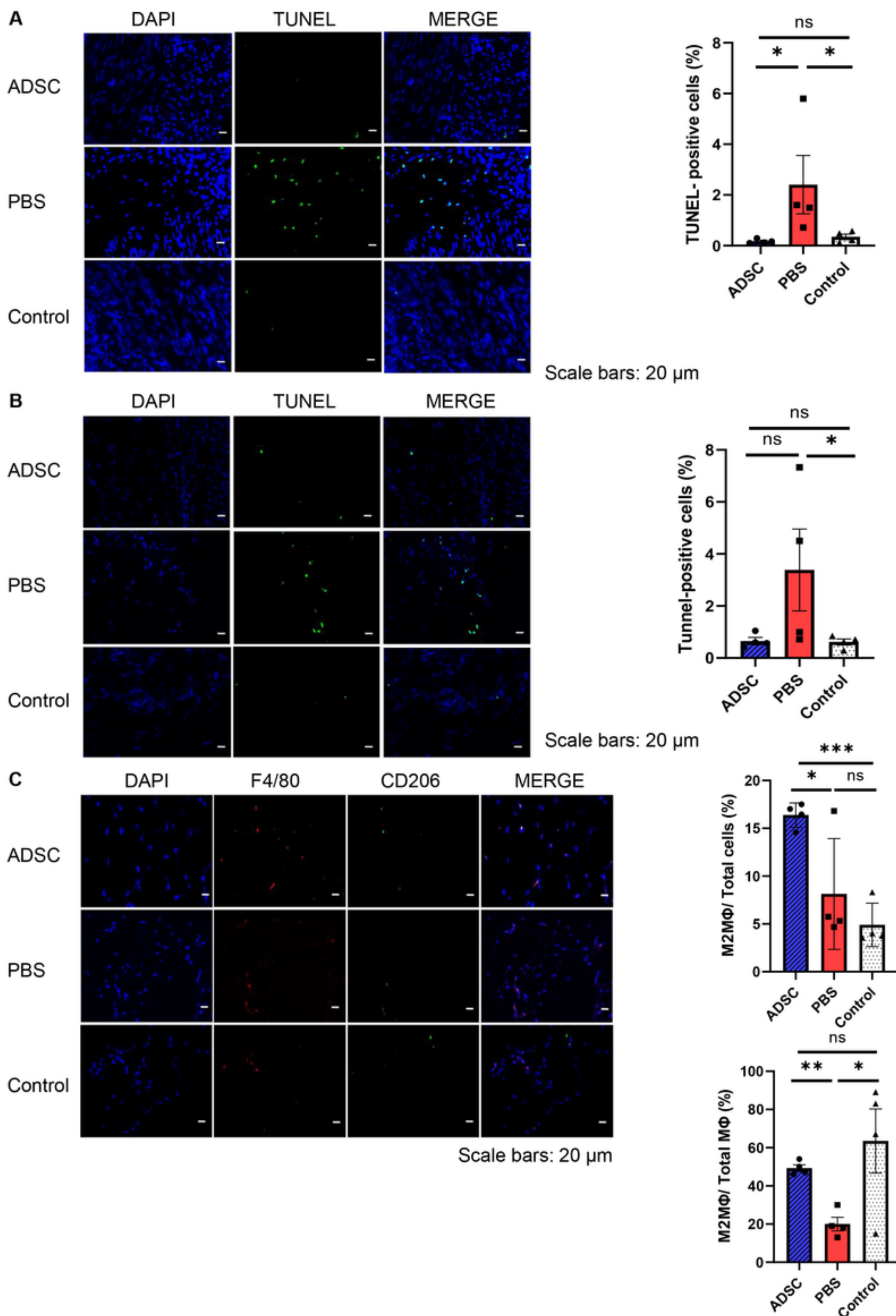


Figure 5

Evaluation of pathological images by fluorescence immunostaining to assess the effects of hADSC administration on day 29. (A) Representative images of TUNEL staining (green) with nuclei staining (blue) in anal sphincter and bar plot of positive rate on day 29 in ADSC, PBS, and control groups (**P* < .05). (B) Representative images of TUNEL staining (green) with nuclei staining (blue) in the surrounding connective tissue and bar plot of positive rate on day 29 in ADSC, PBS, and control groups (**P* < .05). (C)

Examining the effect of hADSCs on M2 macrophages in adipose tissue by double immunostaining using F4/80 (red), a marker for macrophages, and CD206 (green), a marker for M2 macrophages with nuclei staining (blue). Representative images of macrophages and a bar plot showing the percentage of M2 macrophages relative to all cells and the percentage of M2 macrophages relative to all macrophages on day 29.

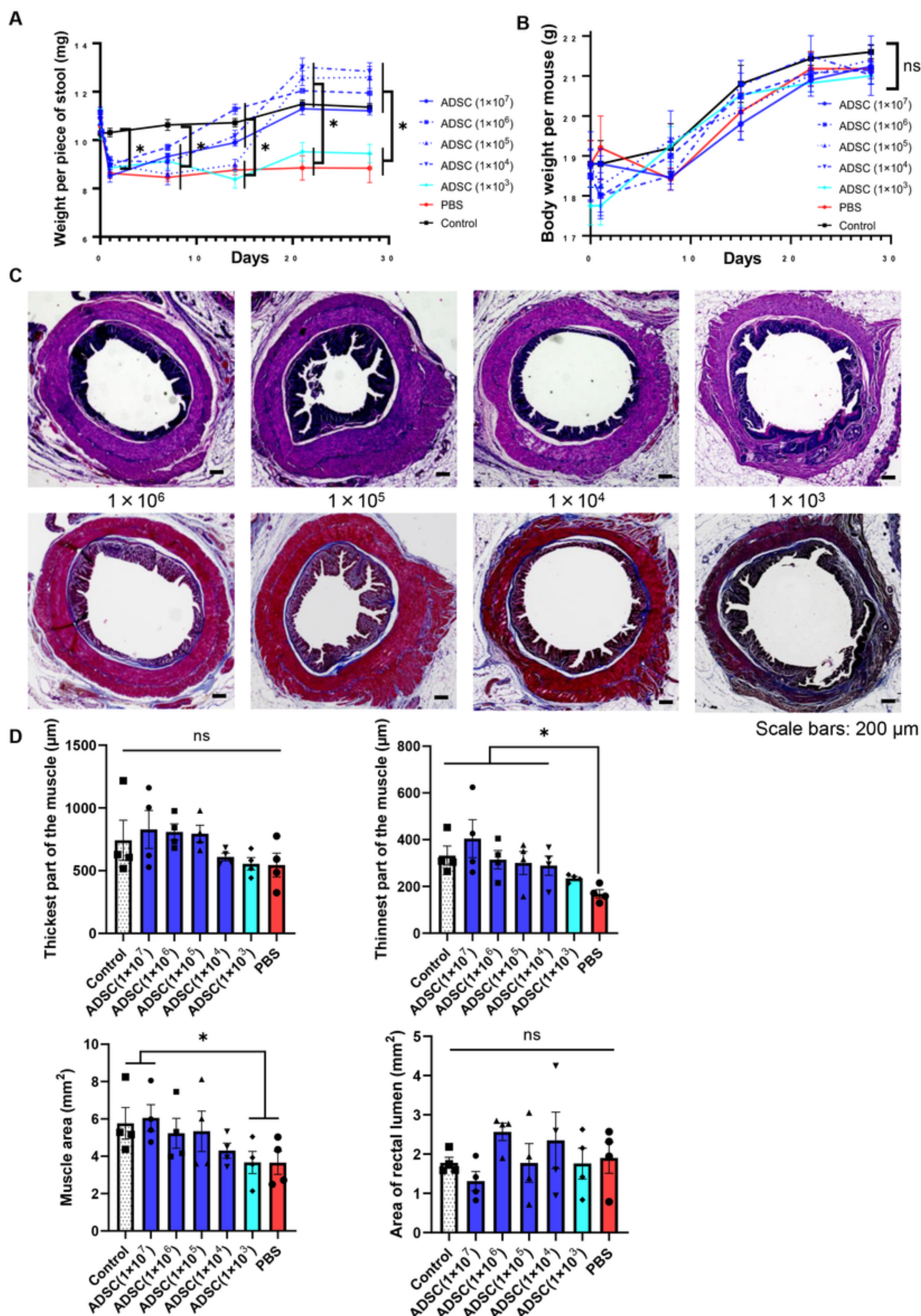


Figure 6

Examination of the number of effective doses of hADSCs. (A) Defecation status of the seven groups. Each bar represents the mean ± SEM of quadruple measurements of weight per piece of stool (*$P < .05$). (B) Body weight of the seven groups. Each bar represents the mean ± SEM of quadruple measurements (*$P < .05$). (C) Representative photomicrographs of histological sections of the anal sphincter on day 29 with hematoxylin and eosin staining and TUNEL staining (1×10^6, 1×10^5, 1×10^4, and 1×10^3 ADSCs). (D) Bar plot of pathological evaluation of the anal sphincter in the seven groups on day 29. Cross-sectional area of the rectal lumen and anal sphincter and thickness of the anal sphincter were used as criteria for pathological evaluation. Each bar represents the mean ± SEM of quadruple measurements (*$P < .05$).

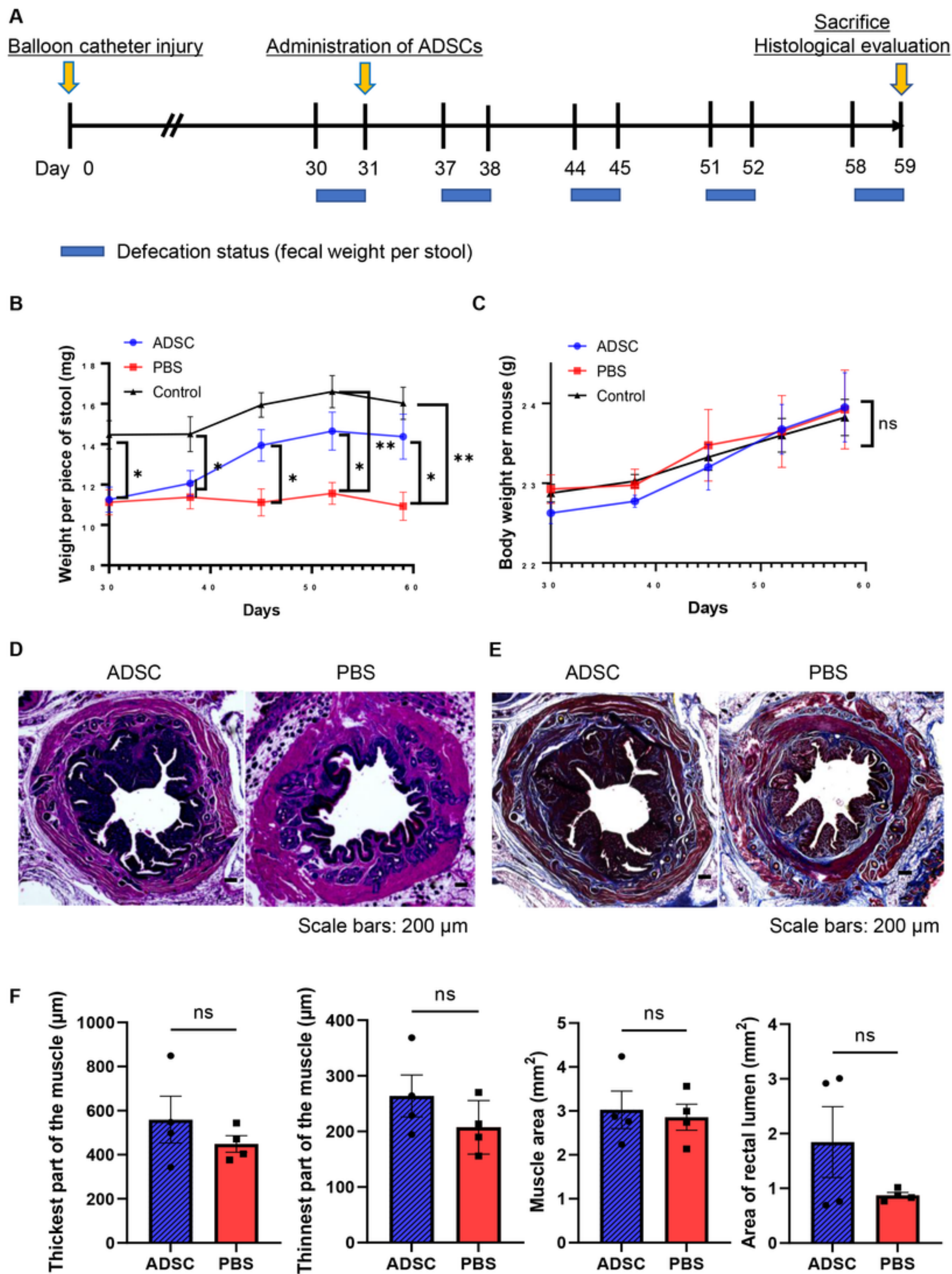


Figure 7

Examination of whether hADSCs are effective when administered in the chronic phase. NOD/SCID mice were divided into three groups (n = 4 per group): without anal sphincter injury (controls), and those administered 1×10^7 hADSCs or only PBS 30 days after anal sphincter injury. Fecal weight per stool was measured on day 30 (before hADSC administration) and on days 38, 45, 52, and 59 after injury to evaluate defecation status. On day 59, mice were sacrificed and their anuses removed along with the

surrounding connective tissue for pathological evaluation. (A) Experimental schedule. (B) Defecation status of the three groups. Each bar represents the mean ± SEM of quadruple measurements of weight per piece of stool (*$P < .05$, **$P < .01$). (C) Body weight of the three groups. Each bar represents the mean ± SEM of quadruple measurements (*$P < .05$). (D) Representative photomicrographs of histological sections of the anal sphincter and surrounding connective tissue on day 59 with hematoxylin and eosin staining (ADSC and PBS groups). (E) Representative photomicrographs of histological sections of the anal sphincter and surrounding connective tissue on day 59 with TUNEL staining (ADSC and PBS groups). (F) Bar plot of pathological evaluation of the anal sphincter on day 59 (ADSC and PBS groups). Cross-sectional area of the rectal lumen and anal sphincter and thickness of the anal sphincter were used as criteria for pathological evaluation. Each bar represents the mean ± SEM of quadruple measurements (*$P < .05$).