Video Article

Transplantation of Adipose Tissue-Derived Stem Cell Sheet to Reduce Leakage After Partial Colectomy in A Rat Model

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Abstract

Anastomotic leakage is a disastrous complication after colorectal surgery. Although current methods for leakage prevention have different levels of clinical efficacy, they are until now imperfect solutions. Stem cell therapy using ASC sheets could provide a solution to this problem. ASCs are considered as promising candidates for promoting tissue healing because of their trophic and immunomodulatory properties. Here, we provide methods to produce high-density ASC sheets, that are transplanted onto a colorectal anastomosis in a rat model to reduce the leakage. ASCs formed cell sheets in thermo-responsive culture dishes that could be easily detached. On the day of the transplantation, a partial colectomy with a 5-suture colorectal anastomosis was performed. Animals were immediately transplanted with 1 ASC sheet per rat. ASC sheets adhered spontaneously to the anastomosis without any glue, suture, or any biomaterial. Animal groups were sacrificed 3 and 7 days postoperatively. Compared to transplanted animals, the incidence of anastomotic abscesses and leakage was higher in control animals. In our model, the transplantation of ASC sheets after colorectal anastomosis was successful and associated with a lower leakage rate.

Video Link

The video component of this article can be found at https://www.jove.com/video/57213/

Introduction

Partial colectomy with a primary anastomosis is a commonly performed surgery that can be done for many diseases affecting the colon such as colorectal cancer, Crohn's disease and diverticulitis[1,2]. The most dreaded complication after colorectal anastomosis is anastomotic leakage[3]. Although several risk factors associated with anastomotic leaks have been identified, solutions for preventing leakage remain unkown[4,5].

Adipose tissue-derived stromal cells (ASCs) are associated with anti-inflammatory and trophic properties[6,7], which makes these cells promising candidates for regenerative therapies[8]. The effectiveness of ASCs to promote tissue healing was shown in various tissues such as cardiac muscle, skin, and oesophagus[9,10,11,12,13]. However, there are few reports on the use of ASCs to promote intestinal healing. Local transplantation of ASCs to experimental colorectal anastomoses via ASC-coated biosutures or serosal injections of ASCs showed either no improvement in healing[14] or did not prevent anastomotic leakage despite a more favourable anastomotic healing[15].

Local transplantation of ASCs in suspension or combined with biomaterials might be associated with insufficient cell retention or an inadequate distribution of transplanted cells[11]. Cell sheet technology[16,17] offers an innovative method of ASC delivery[18,19]. Therefore, in a previous study, a novel approach was proposed in which ASCs organized in a cell sheet could be applied on experimental colorectal anastomosis[20]. This study demonstrated that ASC sheet transplantation is successful in reducing colorectal anastomosis leakage after partial colectomy in a rat model. This article reports ASC sheet preparation and surgical transplantation technique.

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Protocol

Subcutaneous abdominal adipose tissue was obtained from human donors with approval of the Medical Ethical Committee (#MEC-2014-092), Erasmus MC University Medical Center, Rotterdam, The Netherlands. All animal experiments were approved by the Ethical Committee of Animal Experimentation, Erasmus MC University Medical Center, Rotterdam, The Netherlands (133-14-01).

1. Human ASCs Isolation and Culture

1. Prepare 3 mL of isolation medium for 1 g of adipose tissue. Mix low glucose Dulbecco's Modified Eagle's medium (LG-DMEM) with 10 g/L Bovine Serum Albumin (BSA) and 1 g/L collagenase type 1.
2. Dissect human subcutaneous abdominal adipose tissue (n = 8, all female, mean age 40 ± 9 years old) to small pieces (0.5 cm) using a sterile surgical blade size 10, Adson-Brown tissue forceps and Metzenbaum scissors in a sterile Biological SafetyCabinet.
3. Store the dissected tissue in a sterile glass media storage bottle. Weigh the total amount of dissected tissue and start the digestion with the prepared isolation medium (50 g of adipose tissue with 150 mL of isolation medium per bottle). The protocol can be paused here by storing the bottle with the dissected adipose tissue and isolation medium at 4 °C on a roller mixer overnight. Then prewarm the bottle the next day to room temperature 25 °C for 15 min before continuing with the next step.
NOTE: Subcutaneous abdominal adipose tissue was obtained as leftover material from donors undergoing breast reconstruction surgery with approval of the Erasmus MC Medical Ethical Committee (# MEC-200) and according to the Code of Conduct: "Proper Secondary Use of Human Tissue" (<http://www.federa.org>). Leftover adipose tissue was only used from donors who did not opt-out to secondary use.
4. Digest the dissected adipose tissue in a sterile glass media storage bottle in a shaker-incubator at 150 rpm and 37 °C for 1 h.
5. Divide the digested solution into 50 mL tubes and centrifuge the tubes for 10 min at 390 x g.
6. Prepare culture medium: LG-DMEM supplemented with 10% fetal bovine serum (FBS), 50 µg/mL gentamicin and 1.5 µg/mL ampothericin B.
7. Following the centrifugation, remove the supernatant and resuspend the cell pellet in 20 mL of culture medium (LG-DMEM supplemented with 10% fetal bovine serum (FBS), 50 µg/mL gentamicin and 1.5 µg/mL ampothericin B). Centrifuge the cells again for 5 min at 390 x g and remove the supernatant.
8. Resuspend the cell pellet with 10 mL of culture medium (LG-DMEM supplemented with 10% FBS, 50 µg/mL gentamicin and 1.5 µg/mL ampothericin B) and filter the cell suspension through a 100 µm filter.
9. Add 50 µL of 3% acetic acid/methylene blue solution (for red blood cell lysis) to 50 µL of cell suspension, mix the solution and use 10 µL to count the cells. Perform cell counting with a hemocytometer. Then plate the cells at a density of 40,000 cells/cm^2 in culture medium (LG-DMEM supplemented with 30% FBS, 50 µg/mL gentamicin and 1.5 µg/mL ampothericin B).
10. Incubate the cells at 37 °C in a humid atmosphere with 5% CO_2 for 24 h.
11. Following the incubation, wash the cells with warm (37 °C) phosphate buffered saline (PBS) to remove cell debris and replace the media with 10% FBS culture medium with freshly added ascorbic acid-2-phosphate (25 µg/mL) and human recombinant fibroblast growth factor 2 (FGF2, 1 ng/mL).
12. Subculture ASCs at 90% confluence using standard 0.25% trypsin EDTA solution (3 mL for a T175 flask). After 3-5 min, neutralize the 0.25% trypsin EDTA solution with 10 mL of culture medium (LG-DMEM supplemented with 10% FBS, 50 µg/mL gentamicin and 1.5 µg/mL ampothericin B).
NOTE: Flow cytometry analysis using common ASC surface markers and tri-lineage differentiation assays were performed previously and revealed that ASCs isolated using this protocol displayed ASC specific characteristics[21,22].
13. Wash the cells with 10 mL of culture medium (LG-DMEM supplemented with 10% FBS, 50 µg/mL gentamicin and 1.5 µg/mL ampothericin B) for a T175 flask and centrifuge the cells for 8 min at 250 x g. Remove the supernatant and resuspend the cells in 1 mL of culture medium. Count the cells with a hemocytometer.
14. Plate the cells in T175 culture flasks at a density of 8,000 cells/cm^2.
15. Freeze the remaining ASCs in liquid nitrogen with 10% dimethyl sulfoxide (DMSO) in culture medium before further use.

2. ASC Sheet Preparation

1. Pre-coat thermo-responsive culture dishes (3.5 cm diameter) with 1 mL of FBS, then place the dishes in an incubator at 37 °C for at least 30 min before cell seeding.
2. Trypsinize ASCs using standard 0.25% trypsin EDTA solution for 3-5 min (3 mL for a T175 culture flask). If there are any cell clumps after trypsinization, filter the cells through a 100 µm filter before counting.
3. Neutralize the trypsin solution with LG-DMEM supplemented with 10% FBS (10 mL for a T175 flask) and centrifuge the cell suspension for 8 min at 250 x g. Remove the supernatant and resuspend the cells in 1 mL of LG-DMEM supplemented with 10% FBS.
4. After coating the thermo-responsive culture dishes with FBS, move the dishes from the incubator to a warming plate at 37 °C and remove FBS from the dish.
5. Seed ASCs at 400,000 cells/cm^2 (in total, 3.52×10^6 cells diluted in 2 mL of culture medium per dish).
6. Carefully distribute the cells as evenly as possible by gently swinging the dish.
7. Culture ASC sheets for 48 h at 37 °C in a humid atmosphere with 5% CO_2.
NOTE: Try to minimize opening the door of the incubator after seeding ASCs to prevent temperature drops that may interfere with ASC sheet formation or cause premature detachment of formed ASC sheets.

3. Partial Colectomy and Colorectal Anastomosis

1. Induce and maintain Male Wistar rats (weighing between 250-350 g) with 1 L/min of isoflurane (1.5-5%)/oxygen inhalation and inject 0.05 mg/kg buprenorphine intramuscularly.

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2. Once the animals are under general anesthesia, assess anesthetic depth using reflex testing. Apply vitamin A containing eye ointment to the eyes to prevent dryness. When anesthesia is considered sufficient for surgery, aseptically prepare the abdomen by shaving hairs and spraying the surgical area twice with 70% ethanol. Drape the abdomen with sterile paper drapes. Use an aseptic technique and maintain the sterile field during the whole surgical procedure.
3. Make a midline abdominal incision of 5 cm with a sterile surgical blade size 10 and extend the incision with Metzenbaum scissors. Identify and exteriorize the colon and pack the colon off from the remainder of the abdominal cavity with saline moistened gauzes. Identify the right, middle, and left colic arteries in the mesentery, bluntly dissect around each vessel using Halsted mosquito and ligate each individual vessel with non-absorbable braided silk 4/0.
4. Isolate the colonic segment between 1.0 cm aborally to the cecum and 0.5 cm above the caudal mesenteric artery. Transect through healthy colon using Metzenbaum scissors. After the resection of the colonic segment, bring the proximal and distal ends of the colon together by introducing two long cotton swabs trans-anally through the distal colon, and then into the proximal colon end.
5. Using a surgical microscope, create an insufficiently sutured end-to-end anastomosis by one-layer inverting suturing using 5 interrupted sutures (non-absorbable monofilament polyamide 8/0). Place the interrupted sutures through all layers of the colon wall and position the knots extraluminally.
6. Divide the rats randomly into control or ASC sheet group.

4. ASC Sheet Transplantation

1. Allow the culture dishes to cool down to room temperature 40 min before *in vivo* transplantation, to facilitate ASC sheet detachment.
   NOTE: After the detachment, ASC sheets shrink to approximately 2 cm in diameter. ASC sheet viability remains high for at least 3-6 h after the detachment[20].
2. Remove the culture medium and replace it with 1 mL of serum free LG-DMEM.
3. Gently grab the rims of the ASC sheet with atraumatic forceps and place the dish-side of the ASC sheet on top of the anastomotic line.
4. Carefully stretch out the ASC sheet approximately 0.25 cm above and below the anastomotic line using the atraumatic forceps and wrap the sheet around the colon. Lift the colon up to wrap the ASC sheet around the dorsal side.
   NOTE: ASC sheets adhere spontaneously to the anastomotic line, there is no need to use tissue glue or any other biomaterial. The control group does not receive any additional treatment.
5. Remove the cotton swabs and change the gloves and instruments. Replace the colon in the abdomen. Close the abdomen-linea alba with one layer of running sutures using absorbable braided polyglycolic acid 5/0. Suture the subcutis and the cutis together in one layer of running sutures using absorbable braided polyglycolic acid 5/0.

5. Post-operative Evaluation and Follow-up

1. Immediately rehydrate the animals with a subcutaneous injection of 5 mL warm saline postoperatively. Place the animals under a heat lamp to maintain body temperature and monitor the vital signs until animals regain sufficient consciousness to maintain sternal recumbency.
   NOTE: Animals that had undergone surgery were not returned to the company of other animals until fully recovered. After recovery, allow free access to water and regular rat chow. For post-surgical pain relief, administer 0.05 mg/kg buprenorphine subcutaneously every 6-8 h for 3 days. No antibiotics were administered at any time in this study.
2. Obtain daily clinical evaluations of wellness and weight. In case of a very low wellness score or severe weight loss, animals should be euthanized by exsanguination via cardiac puncture while still under anesthesia.
3. On postoperative day 3 or day 7, anesthetize the rats again with 1 L/min of isoflurane (1.5-5%)/oxygen inhalation without buprenorphine, and perform a re-laparotomy with a U-shaped incision. Check the abdomen for the signs of peritonitis, stricture, fibrous adhesions, and the presence of abscesses. Score the severity of adhesions and abscess both in the abdomen and at the anastomotic area.
4. Determine the anastomotic bursting pressure by the insufflation of air in a closed segment of the colon including the anastomosis. Record the air pressure and the place of rupture when the first air leak as bursting pressure.
5. Remove the colon from each sacrificed animal and fix the cut segment of the colon -containing the colorectal anastomosis- with 4% buffered formaldehyde, following by paraffin embedding and cutting the colon into 4 µm thick sections.
6. Euthanize the animals while under anesthesia directly after collection of the anastomosis segment by exsanguination via cardiac puncture. Confirm animal death with the absence of breathing movement and a heartbeat.
7. Perform histochemical stainings such as Hematoxylin & Eosin and Picro Sirius red, and immunohistochemical stainings with antibodies against human mitochondria, rat endothelial cells (anti-CD34) and cells of the immune system (CD3+, CD163+).
8. Digitalize the slides for computerized analysis and compare the animal groups.

Representative Results

A flow chart of this study depicting both ASC sheet culture and the procedure of colon resection and anastomosis is shown in **Figure 1**. **Figure 2** shows ASC sheet microscopic morphology and the macroscopic appearance of the ASC sheet during and after the detachment. **Figure 3** shows the different steps of ASC sheet detachment and transplantation. **Figure 4** shows the presence of the ASC sheet at the anastomotic line and prevention from leakage 3 days postoperatively.

The follow-up period allowed for colorectal anastomosis evaluation. The different assessments are shown in the figures and table. Compared to control animals, the transplanted animal groups showed less frequent anastomotic dehiscence and leakage on postoperative day 3 and less frequent abscess formation on postoperative day 7. Compared to control animals, transplanted animals did not develop significant adhesions or stricture formation. **Table 1** shows the macroscopic findings at the end of the follow up periods in transplanted and non-transplanted animals.

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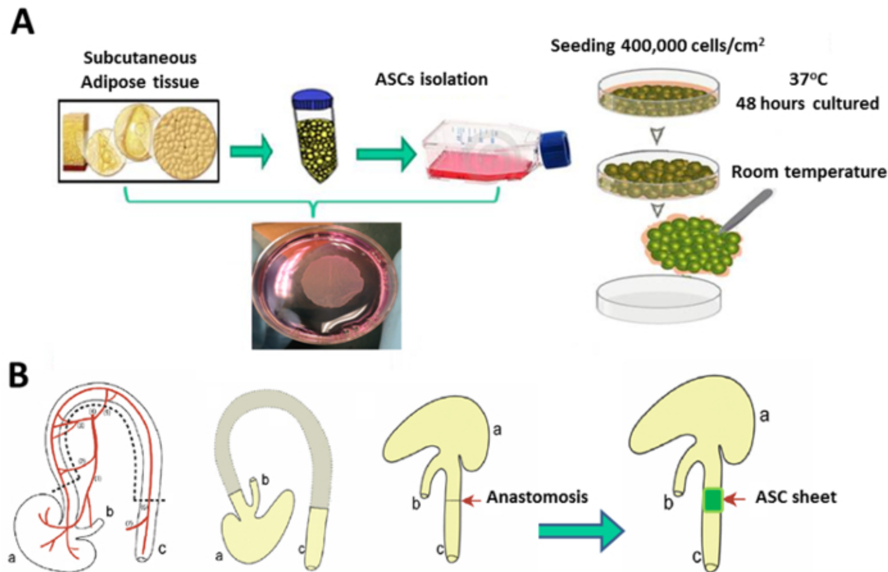

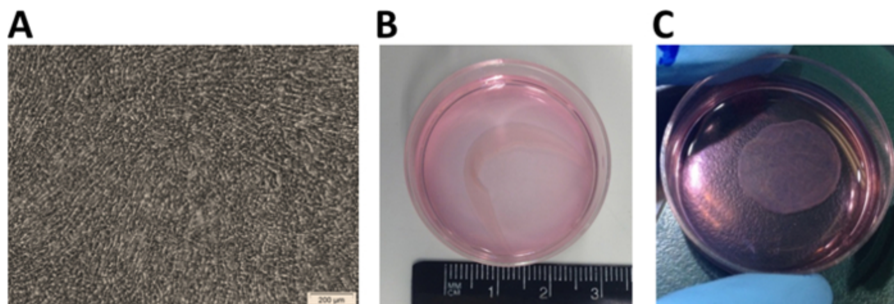

Figure 1: Study flow chart. A) ASCs were isolated and expanded before seeding onto thermo-responsive culture dishes. ASCs were seeded at a density of 400,000 cells/cm^2 and cultured for 48 h before the transplantation. On the day of transplantation, ASC sheet were detached by allowing the thermo-responsive culture dish to cool down to room temperature for 30 min. B) Scheme of surgical procedure; after ligation of supplying vessels a partial colectomy and colorectal anastomosis is performed. An ASC sheet was wrapped around the anastomosis in the ASC sheet group; a) caecum, b) terminal ileum, c) anus. The schematic overview of the surgical procedure was partly adapted with permission from Wu Z. *et al*.[24] Please click here to view a larger version of this figure.

Figure 2: ASC sheet before and after detachment. A) ASC sheet in thermo-responsive culture dishes after 48 h of culture 40x magnification. ASC sheets were obtained by culturing ASCs on commercial thermo-responsive culture dishes. B-C) ASC sheet during (B) and after detachment (C). When the thermo-responsive culture dishes were allowed to cool down to room temperature, the ASC sheet spontaneously detached from the dish surface as one intact ASC sheet. No enzymatic treatment was necessary. The size of the ASC sheet reduced after the detachment. Please click here to view a larger version of this figure.

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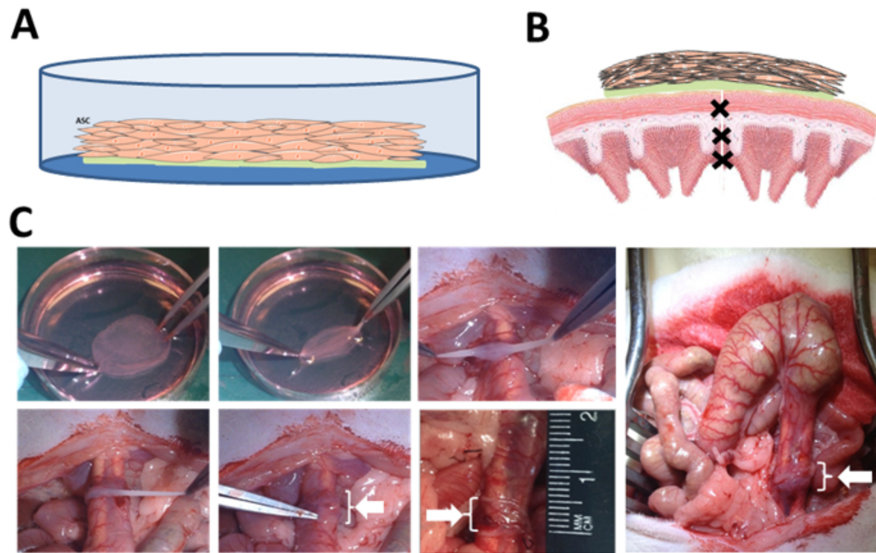

Figure 3: ASC sheet transplantation. A) Depiction of ASC sheet orientation in the thermo-responsive culture dish.[26] B) Orientation of ASC sheet after the transplantation. The dish side of the ASC sheet was placed on top of the serosal surface of the anastomotic line (anastomotic line is indicated with crosses). C) Intra-operative views of the different steps of transplantation. Two atraumatic forceps were used to lift up the ASC sheet and wrap it around the anastomotic line. White arrows indicate ASC sheet location. The images of transplantation were partly adapted with permission from Sukho, P., *et al.*[20] Please click here to view a larger version of this figure.

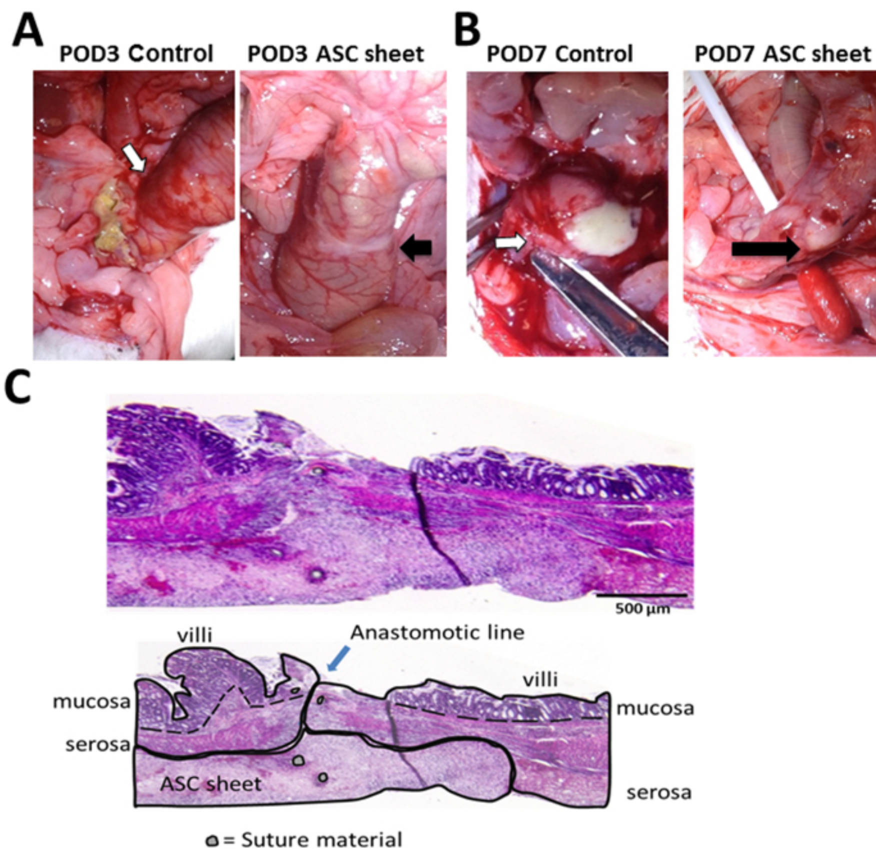

Figure 4: Colorectal anastomosis macroscopic and histological evaluation. A-B) Compared to the control group, macroscopic observation showed reduced leakage and abscess formation at post-operative day 3 (A) and 7 (B), respectively.White arrows point at anastomotic area and black arrows point at transplanted ASC sheets.C) Representative cross-sections of the colorectal anastomosis site in transplanted animals stained with H&E. The sheet structure could be identified at the anastomosis site up to 7 days postoperatively.The images were partly adapted with permission from Sukho, P., *et al.*[20] Please click here to view a larger version of this figure.

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	Post-operative day 3			Post-operative day 7		
	Control (%)	**ASC sheet (%)**	***p*-value**	**Control (%)**	**ASC sheet (%)**	***p*-value**
Peritonitis	1/14(7.1)	0/14(0)	NS	0/15(0)	0/14(0)	NS
Anastomotic disruption	10/14(71.4)	2/14(14.3)	0.002	3/15(20)	2/14(14.3)	NS
Stricture	2/14(14.3)	2/14(14.3)	NS	2/15(13)	2/14(14.3)	NS
Abscess at anastomosis	14/14(100)	12/14(85.7)	NS	10/15(66.7)	4/14(28.6)	0.04
Adhesion at anastomosis	14/14(100)	14/14(100)	NS	15/15(100)	14/14(100)	NS
Abscess elsewhere	11/14(78.5)	8/14(57.1)	NS	6/15(40)	4/14(28.6)	NS
Adhesion elsewhere	8/14(57.1)	3/14(21.42)	NS	9/15(60)	7/14(50)	NS

Table 1: Post-operative macroscopic findings.

Discussion

Anastomotic leakage is the most serious adverse event following colon resection with a primary anastomosis. Optimal techniques to prevent anastomotic disruption and leakage are still lacking. Local application of an array of biomaterials has been conducted, with varying results[25,26,27]. The aim of cell therapies is to facilitate tissue repair by tissue replacement or the stimulation of local healing through paracrine secretion.

In this rat model, ASC sheet transplantation was technically successful. Clinical and histological evaluations demonstrated the effectiveness of the ASC sheet at reducing leakage after colon resection with primary anastomosis.

The transplantation of an ASC sheet to colorectal anastomoses is a novel approach in colorectal surgery. In this study, for the first time, a high-density ASC sheet was transplanted. The choice for ASCs delivered as a cell sheet was based on the previously reported advantages of cell sheet technology[28,29] over conventional cell transplantation techniques. In addition, the choice for ASCs was based on multiple studies that demonstrated their anti-inflammatory and trophic abilities, especially in cardiac muscle- and skin-healing[11,12,19,32].

ASC sheet preparation and detachment can be challenging. In some ASC donors, ASC sheets detached prematurely from the culture dish and folded, precluding their use. In this study, the exact cause for this problem in these specific donors was not identified. Since a high consumption of culture medium was notified in these donors, it was assumed that individual variations in proliferation rate may play an important role. Serum coating assisted in cell adhesion to the surface. Together with careful distribution of the cells in the culture dish and minimizing opening of the incubator door after seeding ASCs (preventing temperature drops), less folding of the ASC sheets occurred. In this way, ASC sheets from all donors were successfully transplanted, confirming the feasibility of the technique.

On the day of the transplantation, most ASC sheets spontaneously detached after allowing the thermo-responsive culture dish to cool down to room temperature. However, a limited number of sheets did not fully detach. Gentle shaking of the culture dish or soft flushing at the rim using a pipette eventually assisted in complete detachments.

For successful ASC sheet transplantation to colorectal anastomoses, several critical steps need to be addressed. First, excessive blood at the anastomosis site should be removed to ensure the contact between the ASC sheet and the serosal surface. Second, the dish side of the ASC sheet should be placed on the serosal surface of the anastomosis. It is postulated that the function of cell sheets after harvest is maintained due to the preservation of cell surface proteins and cell-to-cell junction proteins. Additionally, the ability of ASC sheets to spontaneously adhere to the serosal surface in a short time might be facilitated by the presence of deposited ECM that is produced during in vitro culture[19,20,21]. Third, the size of the ASC sheet and the colon diameter should be comparable to allow complete coverage of the anastomosis site. When larger surfaces need to be covered, the use of several sheets can be considered.

There are some limitations of this protocol for ASC sheet preparation and transplantation. When using thermo-responsive culture dishes to prepare the ASC sheet, the temperature should strictly be maintained at 37 °C during the entire process to prevent premature detachment. After the transplantation, the ASC sheet survival rate is unknown. Although previous experiments showed that ASCs were viable at 3 days postoperatively, mitochondrial activity of these ASCs was diminished at 7 days postoperatively[17]. The cell survival rate and dose effect of the transplantation are important questions that need to be addressed in future studies. Observer bias cannot be completely ruled out since the ASC sheet can be identified macroscopically and microscopically at post-operative evaluations. Although transplanted animals did not show more postoperative adhesion formation compared to control animals[17], the evaluation of the colorectal anastomosis can be hindered by the presence of these intra-abdominal adhesions.

An advantage of this application technique is that it is simple and easy to perform. With only a small amount of practice and using universal atraumatic forceps, intestinal surgeons should be able to wrap the ASC sheet around the anastomosis. In addition, surgery time is not greatly affected since ASC sheets immediately adhere to host tissues. Moreover, when using ASC sheets, no synthetic biomaterial remains in the body minimizing the risk of a foreign body reaction.

Transplanted ASCs organized in cell sheets demonstrated their ability to reduce colorectal anastomosis leakage. In view of these promising results, future studies should be conducted to evaluate long-term effects both for therapeutic and safety reasons. Besides that, several methods

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have been described to modify the therapeutic effects of ASC sheets such as inducing overexpression of vascular endothelial growth factor[30]. These methods may further enhance tissue repair using ASC sheets and should be considered in future studies.

Disclosures

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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