Stem Cells: A Promising Alternative for the Treatment of Anal Fistulas

Pablo Piccinini[1], Mateo Santillán[2], Leonardo Ugartemendia[3], Augusto Carrie[2], Nicolás Avellaneda[1,2]

Department of Surgery, CEMIC. Buenos Aires, Argentina

1 Surgeon, Oral Pathology Unit.
2 Surgeon, General Surgery Service.
3 Specialist in Pharmaceutical Biotechnology Biochemistry Biotechnology (UBA). Eternity Biomedicine EterCell Laboratory.

ABSTRACT
We present two patients with trans-sphincteric anal fistulas in whom laser ablation treatment was combined with the application of lyophilized adipose-derived stem cells. The surgical technique and the method of obtaining and expanding the autologous stem cells obtained are described. Accelerated healing and a low level of postoperative pain were achieved. We propose the need to continue with this line of research.

Keywords: Anal fistula; Mesenchymal stem cells; Adipose-derived stem cells

INTRODUCTION

In the last decade, advances and discoveries in the field of biomedicine have led to numerous alternatives for the treatment of anal fistulas. These alternatives aim to improve patients' quality of life by reducing postoperative pain, treatment time, recurrence, and incontinence rates.[1] Pluripotent stem cells have been the subject of recent research, particularly for difficult-to-heal wounds and other medical disciplines.[2] Mesenchymal stem cells (MSC) are derived from various tissues, including bone marrow and adipose tissue (ADSC). There are two main ADSC techniques: lyophilized ADSC and autotransplantation of stem cells derived from centrifugally-fractionated fresh adipose tissue. The application of stem cells on scar tissue is based on the regeneration and migration of the microvasculature by their angiogenic action, both by differentiation into endothelial tissue and by secretion of proangiogenic factors such as VEGF-A4. Stem cells promote healing by encouraging the activity of keratinocytes and fibroblasts. Additionally, immunomodulation creates the ideal environment for healing. The objective of this work is to present two patients with cryptoglandular transsphincteric anal fistulas in whom the technique of lyophilized ADSC injection was used as a complement to standard laser treatment and to analyze its results.

CASES

Two patients with transsphincteric fistula at the level of the mid-anal canal, treated with laser photocoagulation and complementary administration of stem cells at a university hospital in Buenos Aires, Argentina, were retrospectively analyzed.

Patient 1: An 82-year-old female patient with a medical history marked by severe smoking, chronic obstructive pulmonary disease (COPD), and hemorrhoidectomy 25 years before, accompanied by postoperative stenosis and subsequent anoplasty, presented at our institution with a recurrent perianal abscess with spontaneous drainage. A 360° endorectal ultrasound revealed a solitary fistulous tract in the mid-anal canal. It had external and internal openings at seven o'clock. The patient's sphincter exhibited normal tonicity and did not demonstrate symptoms of fecal incontinence.

Patient 2: A 52-year-old male patient with a history of obesity and a herniated intervertebral disc presented at the clinic with a complaint of spontaneous purulent discharge. A 360° endoanal ultrasound was performed, which disclosed a single-tract transsphincteric fistula at the level of the middle anal canal, with internal and external openings at 6 o'clock. The patient had not received any prior treatment. The sphincter was found to be normotonic, with no symptoms of fecal incontinence.

Informed consent forms were signed by both patients, meticulously detailing the nature of the procedure and the potential associated risks. Postoperatively, patients were evaluated at 7 and 21 days following their surgical intervention, and thereafter, at 2, 4, and 12 months.
The method for obtaining mesenchymal cells is detailed below.

Adipose Tissue Harvest

The starting material collected consisted of 3 cc of adipose tissue and 90 cc of whole blood with anticoagulant. Adipose tissue samples were obtained by abdominoplasty, and the blood by venous puncture in tubes with sodium citrate. Samples from the abdomen were taken under local anesthesia by two surgeons specially trained in this technique. The donor site was infiltrated with 5 ml of 2% lidocaine and 5 ml of 0.2% bupivacaine with epinephrine for three minutes, following which the samples were obtained using a cold scalpel. Hemostasis of the cruciate bed was performed with an electrocautery. The samples were subsequently collected in sterile falcon-type tubes with a medium prepared for this purpose containing 1% antibiotics (penicillin and streptomycin).

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Pablo Piccinini ORCID 0000-0001-6866-942X, Mateo Santillán ORCID 0000-0002-9663-8601, Leonardo Ugartemendia ORCID 0009-0002-0945-1302, Augusto Carrie ORCID 0000-0003-4226-7240, Nicolás Avellaneda ORCID 0000-0002-6802-7125

Isolation and Culture of Stem Cells

The adipose tissue samples obtained were washed with Phosphate Buffered Saline (PBS) solution, which was supplemented with 1% antibiotics (100 µU/ml Penicillin and 100 µg/ml Streptomycin) until the residual blood was completely removed. Subsequently, the samples underwent a series of enzyme-based treatments, beginning with collagenase solution, which was applied with constant homogenization. The cell suspension was subsequently purified with 100 µm filters. The content was next brought to volume with Phosphate Buffered Saline (PBS) solution, which was supplemented with 1% bovine serum albumin (BSA), and then subjected to centrifugation. The oily and aqueous phases present in the tube were then discarded by overturning. The cell pellet obtained at this point is referred to as the Stromal Vascular Fraction (SVF), which is primarily composed of ADSCs, endothelial cells, fibroblasts, macrophages, pericytes, and pre-adipocytes.
The SVF was re-suspended in a culture medium containing concentrated and pure growth factors obtained from the patient's platelet concentrate and 1% PE. Antibiotics were not used in the primary culture since no antibiotics were observed and microbiological absence was noted. After 24 h, the cells began to adhere and spread on the surface of the culture bottle, reaching CFU formation at 48 h. After 72 h, the cells were examined under an inverted microscope with phase contrast, rinsed with phosphate-buffered saline (PBS) and culture medium, and fresh growth factors were added. Concentrated, pure growth factors are obtained after gently centrifuging citrate-anticoagulated whole blood for 15 min. Samples are frozen at -20°C for 2 h. After three cycles of cryolysis and incubation, they are centrifuged at 3000 RPM for 30 min.

Passaging of Stem Cells

The first passage, consisting of the lifting of cells and their subsequent transfer to a larger surface was performed when the cells reached a confluence greater than 80% in the T75 bottle.
After 3 days, when they had reached a confluence greater than 80%, the same procedure was repeated to perform the final expansion. Aliquots of the different cell suspensions were taken for counting and viability reading by the trypan blue exclusion method at the successive passages.

Cell Count

The trypan blue exclusion method was employed to count the cells. The SVF was counted, and then each time the cells were passaged (Table 1).
Daily observation of the cultures under an inverted phase contrast microscope permitted the documentation of permitted the documentation the time of attachment of the mesenchymal cells, the formation of CFU, and their characteristic fibroblastoid shape (spindle-shaped, elongated). This method also ensured the absence of microbiological contamination in the cultures.

Immunophenotypic analysis

The labeling of cells was achieved through the use of specific antibodies, which were incubated with a panel of monoclonal studies conjugated with CD90 (FITC), CD105 (PE), CD34 (PerCP CY-5.5), CD73 (APC), HLA-DR (V450), CD45 (V500), and isotype control antibodies. The acquisition of the samples was conducted using a Becton Dickinson 3-laser FacsCanto II flow cytometer, and the subsequent analysis was performed with Infinicyt software, version 1.7i. In both cases, the results obtained exceeded 98% for ADSCs (Figs. 1 and 2).

Table 1. Características del cultivo de células madre mesenquimales.

Cell count	Day 0: 400,000 in Stromal Vascular Fraction Day 12: 4.5 x 10^6 x 10^6 mesenchymal cells Day 21: 120 x 10^6 x 10^6 mesenchymal cells
Seeding cell density	δci: 5.3 x 10^3 mesenchymal cells/cm². δc 1st culture: 8.57 x 10^3 mesenchymal cells/cm². δc 2nd culture: 2.38 x 10^3 mesenchymal cells/cm².
Cell concentration	[ĉ]i: 4 x 10^4 cells/ml [ĉ] 1st culture: 1 x 10^5 x 10^5 cells/ml [ĉ] 2nd culture: 2.77 x 10^4 x 10^4 cells/ml
Maximum specific growth rate (µmax)	µmax (primary culture): 0.0144 µmax (1st passage): 0.0198 µmax (2nd passage): 0.0224

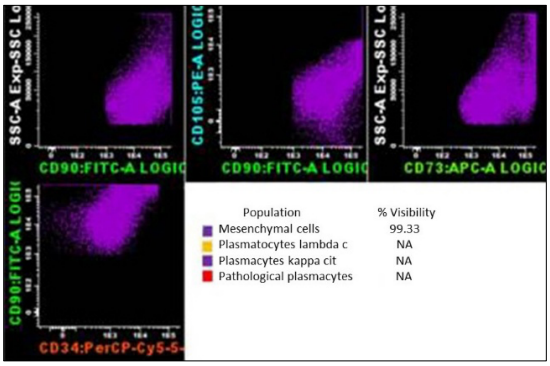

Figure 1. *Case 1. Adipose-derived stem cell culture. Mesenchymal cells: 99,33%.*CD73+, CD105+, CD90+.

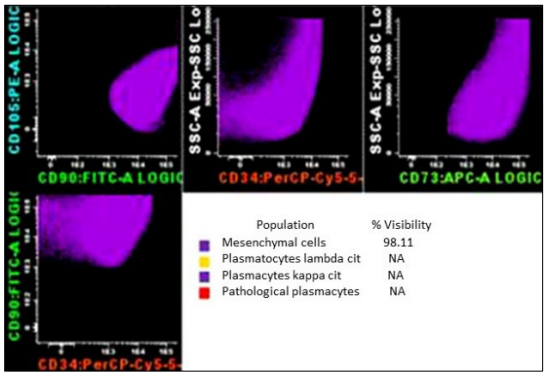

Figure 2. *Case 2. Adipose Tissue Stem Cell Culture. Mesenchymal Cells: 98,11%. CD73+, CD105+, CD90+.*

Surgical technique

The patient is positioned in the lithotomy position, and both pudendal nerves are infiltrated with 0.5% bupivacaine with epinephrine and 2% xylocaine. The fistulous tract is identified with . a probe. Photocoagulation is then performed using a 1470 diode laser at 7 watts of power in continuous mode with a circular optical fiber throughout the fistulous tract, starting the ablation from the internal opening towards the external opening. Following the completion of the fistulous tract treatment, stem cell injection is performed: The stem cells are injected in a total volume of 4 ml, divided into 0.5 ml for each of the four quadrants around the internal and external openings, and an additional 1.5 ml administered with a syringe and needle at the four cardinal points along the entire fistula tract. The total volume of stem cells injected is thus 6 ml (Fig. 3). The final volume of stem cells in the plasma-rich solution is 10 ml, with a concentration of 100 billion stem cells.

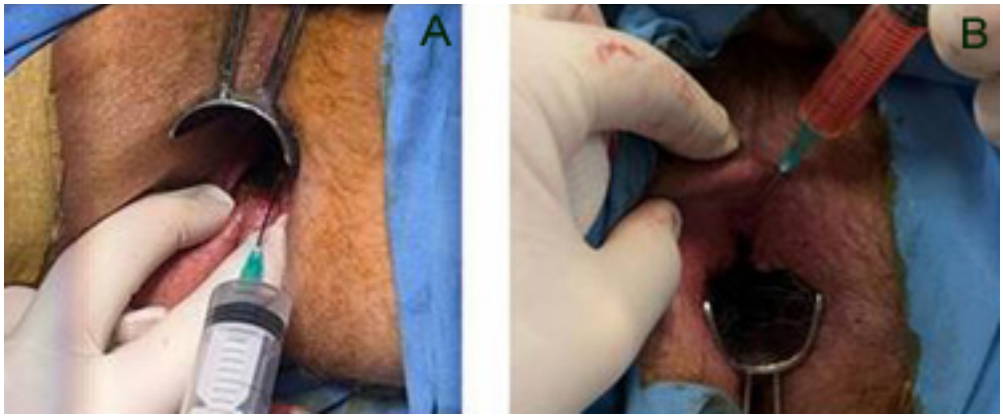

Figure 3. *After laser photocoagulation, lyophilized stem cells were injected into the fistulous tract of both patients.*

RESULTS

There were no complications during the follow-up period. Patient 1 required oral analgesia (etoricoxib with tramadol rescue) for seven days due to pain intensity of 3/10 on a visual analog scale. No other analgesic treatment was required. The wound was found to be completely healed on day 21.
Patient 2 reported pain of 2/10 on a visual analog scale and required oral analgesia (etoricoxib) without the use of rescue opioid. Wound healing was complete by day 25.
No postoperative antibiotic therapy was required in either case, and there was no recurrence or incontinence at the 12-month follow-up.

DISCUSSION

Research has focused on the management of perianal fistulas due to the complexity of the pathology and the potential for complications, particularly incontinence. The advent of new technologies has expanded treatment options, allowing for a more tailored approach and improving efficacy in reducing pain, recurrence, and incontinence rates. Treatment options currently available include fistulotomy, fistulectomy, line placement, fibrin instillation, intersphincteric ligation of the fistulous tract, laser ablation, endoscopic ablation, and flap anoplasty. However, due to significant variability among techniques, lack of standardization, and the paucity of randomized trials, no single treatment has been shown to be superior over time.[7]
Regarding anal pathology, the use of adipose tissue stem cell expansion for the treatment of complex anal fistulas has been reported since 2009, especially in patients with inflammatory bowel disease.[8] A 2019 meta-analysis observed promising cure rates of complex fistulas in patients with and without Crohn's disease, noting the need for a large-scale, randomized, double-blind clinical trial to evaluate the true impact of this therapeutic.[9] A recent literature review by Zahra et al.[10] compared the efficacy of alternative treatments (diode laser, MSCs, ADSCs) with conventional treatment (fistulectomy and mucosal advancement flap) and found a better response to alternative treatments in patients with Crohn's disease and similar results in complex fistulae not associated with inflammatory bowel disease.[10]
The utilization of MSCs in various autoimmune diseases has led to the proposal of its application in the treatment of Crohn's disease.[11] This proposal is supported by evidence demonstrating its efficacy in reducing healing time and recurrence rate.[12] However, the literature lacks sufficient evidence to determine the non-inferiority and/or superiority of this treatment compared to other therapeutic modalities for the management of cryptoglandular fistulae.[13]
A recent meta-analysis by Wang et al.[13] evaluated 10 randomized controlled clinical trials using different amounts of MSCs. The analysis revealed that the incorporation of stem cells into the conventional treatment, which predominantly involved the use of fibrin glue, led to better short-term and long-term outcomes. In a randomized trial, Ascanelli et al14 observed an advantage in healing time when surgical treatment was combined with ADSC injection compared to surgical treatment alone. They also noted a significant difference in postoperative pain, time to healing, and time to return to work in favor of stem cell injection. However, abdominal bruising and pain were found in the first postoperative week in patients in whom liposuction was performed. Notably, no significant differences were observed in patient satisfaction between the two groups. Application of both cryopreserved and fresh adipose tissue-derived MSCs is based on the same principle of injecting stem cells into the damaged tissue. Compared to fresh tissue transplantation, the application of expanded MSCs is a higher cost procedure due to the need for a laboratory for cell line expansion. On the other hand, the necessity of liposuction necessitates adequate training and carries the risks associated with such a procedure.
In our group, we believe that larger volume injections and the presence of pro-inflammatory agents in fresh adipose tissue could lead to a greater inflammatory response and, therefore, less efficacy

and greater pain. In our opinion, a randomized clinical trial comparing lyophilized MSCs with fresh adipose tissue would be desirable.

In this study, we present our preliminary findings on the application of lyophilized ADSCs in combination with conventional laser therapy for the treatment of complex perianal fistulas. The simplicity and reproducibility of the surgical technique, and the postoperative healing time are encouraging.

Stem cell treatment has emerged as a new alternative for complex anal fistula. The risk of the treatment is low, as no major complications associated with the procedure have been reported.15 Its benefits could have an impact on the treatment of complex fistulas, which are the most demanding for the health system.

The heterogeneity of the published studies, the variation in surgical technique, and even the lack of differentiation between treatments with lyophilized or non-lyophilized stem cells, mean that the results cannot be considered conclusive. In most cases, this technique was used as an adjunct to standard treatment (laser, fibrin glue, curettage), which makes it difficult to analyze the results.

The need for prospective cohort studies comparing this technique with other therapeutic options is highlighted in order to provide a higher level of evidence of the benefits of its application.

CONCLUSION

We present two cases in which a currently available treatment for transsphincteric fistula was combined with the application of lyophilized adipose-derived stem cells. To reproduce our results, we publish the surgical technique used and the method of obtaining and expanding the autologous stem cells.

Our observations included accelerated healing and low postoperative pain. We propose the need to continue with this line of research.

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