Original Article

Mesenchymal Stromal Cell Therapy Improves Refractory Perianal Fistula in Crohn's Disease: Case Series Clinical Interventional Study

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Abstract

Objective: Perianal fistulas in Crohn's disease (CD) are the main challenges in inflammatory bowel diseases (IBDs). Some of the fistulas are refractory to any therapeutic strategy. The aim of this study was to evaluate the therapeutic effects of mesenchymal stromal cells (MSCs) as a novel promising modality for the treatment of fistulizing CD.

Materials and Methods: This case series clinical interventional study was conducted from 2014 to 2017 at Shariati Hospital, an IBD referral center in Tehran, Iran. Refractory adult patients with CD who had draining perianal fistulas were enrolled in this study. All patients were examined by a colorectal surgeon and the fistula imaging studies were performed by pelvic magnetic resonance imaging (MRI). After autologous bone marrow (BM) aspiration and MSCs isolation, the cells were cultured and passaged under current good manufacturing practice (cGMP) conditions. Four intra-fistula injections of cells, each containing 40×10^6 MSCs suspended in fibrin glue, were administered by an expert surgeon every 4 weeks. Procedure safety, feasibility and closure of the perianal fistulas at week 24 were assessed. Clinical examination and MRI findings were considered as the primary end points.

Results: In total, 5 patients (2 males and 3 females) were enrolled in this study. No adverse events were observed during the six-month follow-up in these patients. Both the Crohn's Disease Activity Index (CDAI) and Perianal Disease Activity Index (PDAI) scores decreased in all patients after cell injections and one patient achieved complete remission with closure of fistulas, discontinuation of fistula discharge, and closure of the external opening.

Conclusion: Local injection of MSCs combined with fibrin glue is potentially a safe and effective therapeutic approach for complex perianal fistulas in patients with CD.

Keywords: Cell Therapy, Crohn's Disease, Mesenchymal Stromal Cells, Perianal Fistulas

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Introduction

Crohn's disease (CD) is a chronic inflammatory condition characterized by transmural involvement of the gastrointestinal tract and fistula formation. Patients may suffer from fissures, canal stenosis and fistulas with or without abscess (1, 2). Perianal fistulas are one of the most disabling complications and a source of morbidity for CD patients. The cumulative incidence of perianal fistulas in patients with CD ranges from 18 to 43% (3, 4).

Understanding the pathogenesis of CD has revealed that unresolved chronic inflammation triggers the recruitment of activated immune cells including lymphocytes and macrophages over time. Production of tumour necrosis factor alpha (TNF-α), interleukin-12 (IL-12), IL-17 and IL-23 inflammatory cytokines in addition to impairments in healing mechanisms can cause epithelial defects and fistula formation (5-7). Adversely, diagnosis and optimal management of perianal fistulizing CD is challenging, as many patients do not respond to approved and available medical therapies that include administration of antibiotics, immunomodulators, and biological agents (8). Patients with perianal fistulas unresponsive to conventional medical or biological treatments should undergo surgical therapy. However, only one third of patients with complex perianal fistula achieve durable remission with either medical or surgical treatments (9). These limitations have encouraged considerable attention in investigating new alternative treatment options for perianal fistulizing CD.

Recently, stem cell therapy has become a highly promising approach to address important challenges in CD patients in general (10, 11). Several studies suggest that mesenchymal stromal cell (MSC) therapy could be an option to improve CD and Crohn's fistula (12, 13). MSCs are multipotent adult stem cells derived from various tissues such as bone marrow (BM), adipose tissue, umbilical cord, and placenta. MSCs are involved in anti-inflammatory events and tissue repair through their immunomodulatory, anti-fibrotic, and pro-angiogenic properties. MSCs suppress the proliferation of CD4+ and CD8+ T lymphocytes and natural killer cells (NK), stimulate the proliferation of regulatory T cells, and suppress immunoglobulin production through secretion of various bioactive molecules and cytokines (14, 15). MSC therapy has shown encouraging results in the treatment of refractory fistulizing CD. Clinical applications of MSCs have demonstrated their efficacy and safety as a promising alternative for the currently available treatments of perianal fistulas in CD. Moreover, local administration of autologous MSCs have shown significantly higher healing rate (HR) compared to the allogenic MSCs (16, 17).

Advances in cell replacement therapies have resulted in development of advanced therapy medicinal products (ATMPs) for the treatment of inflammatory bowel disease (IBD) patients. The advantages of MSCs for tissue repair and regeneration make them promising tools for treatment of inflammatory diseases. Recently, three adipose tissue-derived (AD)-MSC-based cell therapy products received approval for treatment of fistula in CD. Cupistem®, which is the first approved autologous AD-MSCs product, was developed by Anterogen Company (South Korea) and approved by the South Korea Ministry of Food and Drug Safety (MFDS) in 2012. Alofisel (darvadstrocel), which was an allogeneic product co-developed by TiGenix (USA) and Takeda (UK) pharmaceutical Companies, was approved by the European Medicines Agency (EMA) in 2018, and finally, Ryoncil (remestemcel-L) was developed by Mesoblast Company and is currently in an ongoing phase 1/2 clinical trial (18). The aim of this study was to evaluate the safety, feasibility and efficacy of intrafistula injection of autologous BM-MSCs suspended in fibrin glue into perianal CD fistulas that were refractory to conventional medical therapy.

Materials and Methods

Mesenchymal stromal cells isolation, expansion and characterization

In this case series clinical interventional study, BM-MSCs were isolated and expanded according to our previous report (19). Briefly, after approval from the Ethics Committee of the research council of digestive disease research institute (DDRI) at Tehran University of Medical Sciences (FWA00001331), each patient signed an informed consent for participation in the study and underwent general physical examination and virus screening, followed by BM aspiration, approximately four weeks before the first cell injection. BM aspiration (100-150 ml) was performed under local anesthesia from the posterior iliac crests of the patients. BM was placed in aseptic blood collection bag and washed with phosphate-buffered saline (PBS, Gibco, USA). Mononuclear cells (MNCs) were isolated by layering them on top of a density gradient solution (Ficol-Hypaque, 1.077 g/ml, Lymphodex; Inno-Train, Kronberg im Taunus, Germany) and were washed twice with PBS buffer. Then, the resuspended MNCs were seeded at a density of 1×10^6 cells/cm^2 in Alpha Modified Eagle's Medium (α-MEM, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and 2 mM L-glutamine (Gibco, USA) in T175 tissue culture flasks. On day 4, the medium was refreshed and nonadherent cells were transferred to new T175 flasks that contained 15 ml fresh medium, in which they were allowed to culture for an additional four days. The medium was refreshed every four days. Once they reached 80% confluency, the MSCs were harvested using trypsin/EDTA (Gibco,

USA) and seeded at a density of 2×10^5 cells/cm^2 in the same condition as before. The cells were passaged 3-4 times consecutively to provide enough cells for administration. After reaching 2×10^8 cells, MSCs were harvested and cryopreserved in 10% dimethyl sulfide (Sigma Aldrich, USA) at -80°C until the time of transplantation.

Quality control evaluation of the MSCs was performed by assessments of cell morphology and viability, immune phenotyping, and cytogenetic analysis, mycoplasma contamination, endotoxin tests using LAL test kit (Lonza, Walkersville, MD, USA; http://www.lonza.com) and microbial contamination test using BACTEC instrument (BD BACTEC; BD Diagnostics, Franklin, NJ, USA; http://www.bd.com).

The expression patterns of MSC specific surface markers (CD90, CD73, CD105, CD44 and CD29) and absence of hematopoietic specific markers (CD34 and CD45) were evaluated by flow cytometry (BD FACSCalibur™ cytometer and FlowJo 7-6-1 software). Table S1 lists the antibodies used in this assessment (See Supplementary Online Information at www.celljournal.org).

Fibrin glue preparation

Fibrin glue/gel is a two-component adhesive material composed of fibrinogen (sealant) and thrombin (catalyst), which is used to adhere tissues together and seal tissue defects. The fibrin glue was prepared from cord-blood-derived platelet rich plasma (PRP). The fibrinogen was precipitated from the PRP by ethanol precipitation at low temperature. The thrombin solution in the fibrin glue was obtained from diluted plasma through adjustment of the pH and centrifugation. Preparation of the fibrin glue was initiated (between 10 to 20 seconds) after mixing the fibrinogen and thrombin in the presence of $CaCl_2$ to convert prothrombin to thrombin. Fibrin Glue is commercially available from Royan Stem Cell Technology Co.

Patient criteria and treatment

This case series clinical interventional study was registered at www.clinicaltrials.gov. The registration number is: NCT01874015. Five patients (two males and three females) with refractory CD and active and persistent perianal fistulas were enrolled in this study.

Each patient signed a written informed consent prior to the study. Inclusion criteria consisted of: 18 years of age or older, confirmed diagnosis of CD for at least six months and presence of active and persistent refractory perianal fistula. Refractory fistula was defined as fistulas unresponsive to immunosuppressive therapy (e.g. azathioprine, 6-mercaptoputine and methotrexate) and/or anti-TNF therapy. The patients received 5-ASA, azathioprine, 6-mercaptoputine, methotrexate, or corticosteroids for at least eight weeks prior to their enrollment. Anti-TNF therapy must have been discontinued at least eight weeks before the enrollment. The exclusion criteria were the diagnosis of of fibrostenotic CD, any history of surgery four weeks prior to enrollment and previous history of any malignancies. Pelvic magnetic resonance imaging (MRI) was used to delineate the anatomy of the fistula track. The primary end point of the study was defined as closure of the treated perianal fistulas at week 24 post-transplantation, as assessed by both clinical examination and MRI. The secondary end points were reductions in Perianal Disease Activity Index (PDAI) and Crohn's Disease Activity Index (CDAI). Fistula closure was defined as the absence of discharge from the external orifice of the fistula after application of manual pressure along with re-epithelialization of the external orifice. Complete response was defined as closure of all fistulas and the absence of collections larger than 2 cm of the treated peri-anal fistulas as evaluated by MRI (9). Partial response was defined as a significant reduction of fistula discharge and a decrease in its diameter based on MRI imaging.

Cell transplantation

A total number of 4×10^7 MSCs were harvested and washed with PBS and re-suspended in sterile normal saline supplemented with 1% human serum albumin (HAS, Octapharma AG, Lachen, Switzerland, http://www.octapharma.com). A mixture of MSCs and fibrinogen, thrombin and calcium chloride (fibrin glue) was infused using a dual syringe injection system to fill both the peri-fistula and inside the fistula tract wall. The cells were injected every four weeks and four injections were performed per subject.

Injection procedure

After enrollment, all patients were examined by a colorectal surgeon. If a perianal abscess was detected, drainage was performed using seton placement and antibiotics were administered (oral ciprofloxacin and metronidazole) for two weeks before the first MSC injection. The injections were performed under local anesthesia and sterile conditions. The MSC suspension was mixed with fibrin glue and injected into the lumen and the walls of the fistula track. An average of 4×10^7 MSCs were transferred at each injection. The patients were monitored for possible adverse reactions such as fever for six hours after the procedure. CDAI (20) and PDAI (21) were calculated for each patient at baseline and at the end of the follow-up. The patients were examined three weeks after each injection, and the subsequent injections were performed every four weeks for up to four injections (Fig.1).

Ethical considerations

After receiving approval from the Ethics Committee of the research council of digestive disease research institute (DDRI) at Tehran University of Medical Sciences, the study conducted accordingly (FWA00001331).

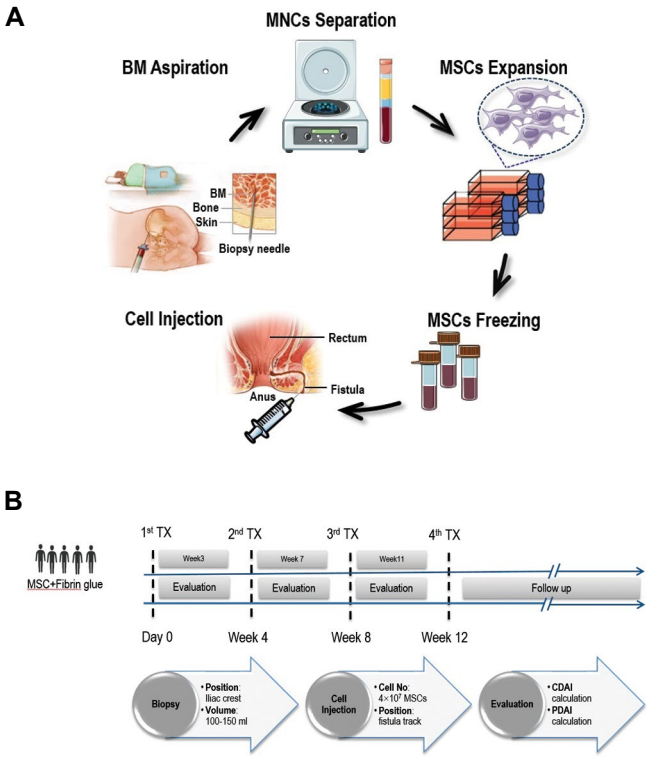

Fig.1: Steps of the injection of autologous BM-MSCs in CD patients. **A.** Isolation, expansion, characterization and intra-fistula injection of BM-MSCs in CD patients. **B.** The process time line of MSC therapy in CD patients. BM-MSCs; Bone marrow-derived mesenchymal stromal cells, CD; Crohn's disease, and MNCs; Mononuclear cells.

Statistical analysis

The student's t test for paired data was used to compare CDAI and PDAI before and after the treatment. The $P<0.05$ was considered as significant.

Results

Table 1 summarizes the patients' baseline characteristics.

Cell characteristics

MSCs from all patients were characterized in terms of cytogenetic integrity (karyotype analysis) identity (morphology), purity (surface marker expression patterns) and viability, according to the guidelines by the International Society for Cellular Therapy (ISCT) for MSCs. The isolated MSCs had a spindle-shaped morphology and expressed the general specific surface markers of MSCs including [CD90 (98.5%), CD73 (90.1%), CD105 (98.3%), CD44 (92%) and CD29 (96.4%)]. However, these cells did not express the hematopoietic stem cell markers [CD34 (1.59%), CD45 (0.06%) or CD11 (0.293%, (Fig.2A-F)]. Karyotype analysis of the expanded MSCs at passage 4 indicated normal karyotype without chromosomal aberrations (Fig.2F). Furthermore, MSCs from all CD patients met all other quality control criteria that included viability by more than 90% and absence of possible microbial contaminations before infusion. Table 2 lists the detailed results of the MNCs and MSC viability and cell count for all the patients.

Table 1: Baseline characteristics of the patients with Crohn's disease and refractory fistulas

Patient number	Age (Y)	Gender	Disease duration (Y)	Disease location	CDAI	PDAI	Previous surgery	Immunosuppressive	Biologics
1	48	F	15	Rectum left colon	169	5	No	Azathioprine	IFX
2	24	M	10	Ileum	99	7	No	Mesalazine	IFX
3	31	F	-	Rectum	166	6	No	Mesalazine	NA
4	31	F	4	Ileum	173	6	No	Azathioprine	NA
5	43	M	3	Ileum	167	9	No	Azathioprine	IFX

CDAI; Crohn's Disease Activity Index, PDAI; Perianal Disease Activity Index, NA; Not applicable, and IFX; Infliximab.

Table 2: Quantity and viability of MNCs and MSCs

Patient	MNC count (viability)	1st MSC count (viability)	2nd MSC count (viability)	3rd MSC count (viability)	4th MSC count (viability)	Mean ± SD
1	2.1×10^9 (94%)	3.8×10^7 (95%)	4.2×10^7 (95%)	4.2×10^7 (95%)	4.0×10^7 (95%)	$4.05\times10^7 \pm 1.77\times10^6$
2	1.8×10^9 (100%)	4×10^7 (96%)	4.7×10^7 (95%)	4.5×10^7 (96%)	4.2×10^7 (97%)	$4.35\times10^7 \pm 2.87\times10^6$
3	1.4×10^9 (94%)	4×10^7 (96%)	3.8×10^7 (94%)	4×10^7 (93%)	3.6×10^7 (93%)	$3.85\times10^7 \pm 1.77\times10^6$
4	2.5×10^9 (96%)	4×10^7 (95%)	4×10^7 (93%)	3.4×10^7 (92%)	3.2×10^7 (95%)	$3.65\times10^7 \pm 3.81\times10^6$
5	1×10^9 (100%)	4×10^7 (92%)	4×10^7 (93%)	4.2×10^7 (97%)	4.5×10^7 (92.5%)	$4.17\times10^7 \pm 2.18\times10^6$

MNCs; Mononuclear cells and MSCs: Mesenchymal stromal cells

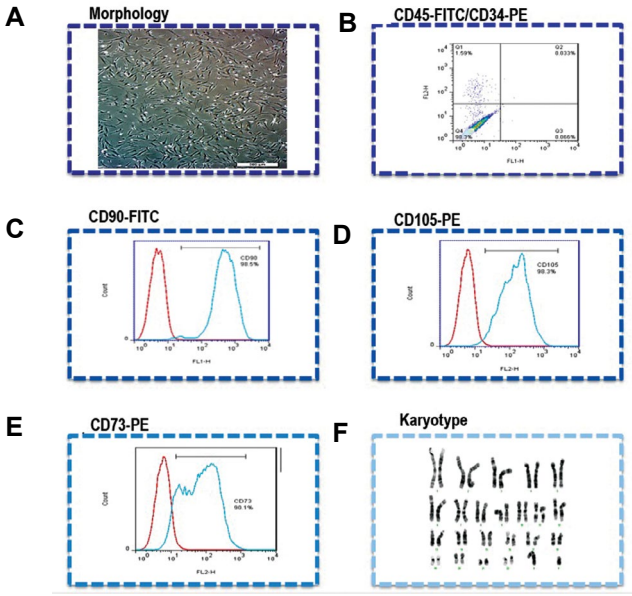

Fig.2: Detailed characterization of mesenchymal stromal cells (MSCs) from CD patients. **A.** Morphological examination revealed that MSCs were spindle-shaped and fibroblast-like cells in appearance. **B-E.** Immunophenotypic characterization of MSCs showed that these cells expressed general specific surface markers of MSCs [CD90 (98.5%), CD73 (90.1%), CD105 (98.3%), CD44 (92%) and CD29 (96.4%)], but did not express hematopoietic specific markers [CD34 (1.59%), CD45 (0.06%) and CD11 (0.293%)]. **F.** Normal diploid karyotype patterns of the isolated MSCs. MSCs; Mesenchymal stromal cells and CD; Crohn's disease.

Clinical assessment and follow-up

There were no adverse outcomes after the local injections and during the follow-up treatments in all five patients. One patient achieved complete remission during the six months of follow-up with fistula closure, cessation of fistula discharge, and closure of the external opening. A total number of three patients had partial responses with significant reductions of fistula discharge and decreased fistula diameters according to MRI imaging (Fig.3). One patient had no response to the treatment in terms of fistula closure, and a new perianal fistula track was observed during follow-up.

CDAI decreased to the mean of 118.4 points at the end of the treatment (154.8 to 36.4, P=0.004). There was also a significant decline in the PDAI after the treatment (6.6 to 4.6, P=0.04). Table 3 presents PDAI and CDAI scores before and after the intrafistula MSCs injections (Fig.4).

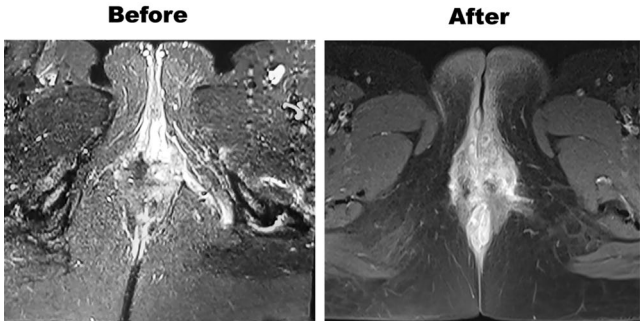

Fig.3: Short inversion recovery and T1-weighted, fat-suppressed (STIR T1W-FS) sequence with contrast injection of axial pelvic images before and after three weeks. Injection of mesenchymal stromal cells (MSCs) showed fistula tract closure on the left side at the 2 o'clock position of the anus. Decreased enhancement and shortening of the fistula tract with clinically reduced discharge was observed after treatment with MSCs.

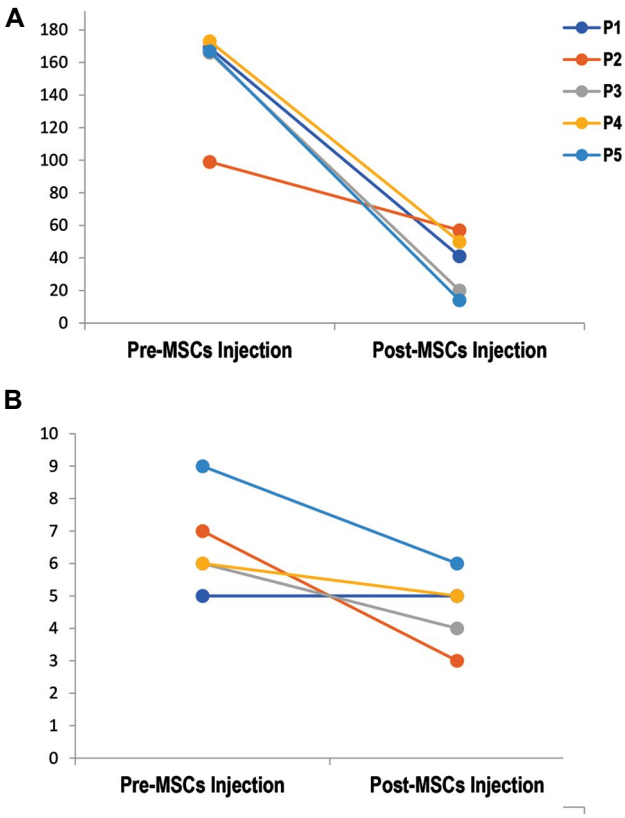

Fig.4: Perianal Disease Activity Index (PDAI) and Crohn's Disease Activity Index (CDAI) scores before and six months after intra-fistula mesenchymal stromal cells (MSCs) injections. **A.** CDAI scores before and after intra-fistula MSCs injection, **B.** PDAI scores before and after intra-fistula MSCs injection. Patients experienced clinical remission with the average of (CDAI<118.4) and (PDAI<4.6) scores by 6 months after local administration of MSCs.

Discussion

Using commercially available biological agents is the most frequent treatment strategy in 50% of IBD patients. However, long term therapy and many adverse event reports have caused serious limitations for this approach. Over the past few years, stem cell therapy has emerged as a promising option to treat soft-tissue injuries (22). Several studies have investigated the role of cell-based therapy with different types of stem cells, including HSCs, BM-MSCs and AD-MSCs from autologous or allogeneic sources for refractory CD. The results of such studies have successfully demonstrated the safety of this approach and the lack of serious adverse outcomes (23, 24).

MSCs have been extensively investigated and applied in regenerative medicine, tissue repair, and immunomodulation and are proposed to exert beneficial effects in fistulizing CD. Previous studies have shown the safety and effectiveness of a single injection of AD-MSCs in the treatment of fistulas in CD (25). The long-term evaluation of safety and efficacy of allogeneic BM-MSCs in CD patients has revealed that BM-MSC therapy is safe, as it resulted in successful fistula closure rates of 63, 100, and 43% after 4 years in three different MSC-based cohort studies (26). Moreover, a recent meta-

analysis study evaluated the efficacy and safety of local administration of MSCs for complex perianal fistula. The results have shown that application of MSCs alone or in combination with fibrin glue is effective with no serious adverse events.

Moreover, application of fibrin glue had a synergistic effect on the fistula closure (27). In this case series study we evaluated the safety and efficacy of intra-fistula injections of autologous BM-MSCs combined with fibrin glue for treating refractory fistulas in five CD patients. Our data indicated significant improvements in both CDAI and PDAI scores; however, only one patient showed complete closure and 60% of the subjects had a partial response. A randomized double-blinded placebo-controlled trial was conducted in 2016 to compare the impact of AD-MSCs and normal saline on fistula closure. Although the data showed significant improvement in PDAI scores, the CDAI scores did not notably differ between the two groups (28). In another study, patients with refractory CD had local injection of 2×10^6 autologous AD-MSCs plus fibrin glue or fibrin glue alone. The results showed more than 70% improvement in patients who received AD-MSCs plus fibrin glue compared to 16% in the fibrin glue-alone group ($P<0.001$) (29). Furthermore, a follow-up study was done by this research group for 49 patients for 42 months. Complete closure was seen in 12 of 21 patients who received autologous AD-MSCs plus fibrin glue and in 3 of 13 who received only fibrin glue (30). In 2013, Lee et al. (31) evaluated the effects of autologous AD-MSCs injection in 33 patients with fistulizing CD; almost 80% of the patients showed complete closure of the fistula tract after just a single injection and approximately 90% of them had no evidence of recurrence after 12 months. In another study, five out of six patients showed incomplete closure with 50% reduction in fistula drainage. Data from another randomized, double-blinded, placebo-controlled study that used allogeneic BM-MSCs in 21 patients with refractory perianal fistulizing CD, demonstrated that patients had healed perianal fistulas by week 12 after the BM-MSCs injection that persisted up to week 24, compared to those that received placebo. No significant adverse effects were observed (32).

Currently, the most common challenge reported for stem cell therapy in CD studies is patient recruitment. Furthermore, compared to biological agents, the only disadvantage for the cell-based therapeutic strategy is the invasiveness of local administration of MSCs. In addition, this procedure has no extra risk for an anal sphincter injury (25). Altogether, it seems that patients with refractory fistulizing CD might have benefited from BM-MSCs treatment. Nonetheless, short term follow-up and the low number of patients were the most notable limitations of this study. Larger sample size or repeated injections of MSCs may lead to more improved results in future studies.

Conclusion

We conclude that intra-fistula injection of BM-MSCs with fibrin glue is safe and effective in treating refractory fistulas in CD patients.

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Authors' Contributions

R.M., S.N., N.A., H.B., M.V., H.R.A., M.M., A.A.; Participated in study design, drafting the manuscript, proofreading, and final approval and overall supervision of the study. M.V., H.A.A., S.T., B.S., N.H.-K.; Participated in data collection and analysis, drafting, and editing the manuscript for submission. A.B., S.-E.H., N.J., S.M., V.A.; Performed MSC isolation, expansion and characterization. M.N., A.A., M.V., M.M.; Contributed in data interpretation and finalization of the data. All authors read and approved the final manuscript.

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