Evaluating the Safety and Efficacy of MSCs-derived Exosomes for Treatment of Refractory Perianal Fistula in IBD patients; Clinical Trial Phase I

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Abstract

Background and objectives:

Exosomes therapy is a promising technique that can decrease the concern related to the fate of transplanted stem cells to the fistula location. The unique properties of mesenchymal stem cells (MSCs)-derived exosomes can improve fistula healing because of their high ability to regulate and modify immune responses. This project aims to examine the safety and efficacy of implementing MSCs-exosomes in treating refractory fistulas in inflammatory bowel disease (IBD) patients.

Methods:

The exosomes were isolated from umbilical cord-derived MSCs and characterized using flow cytometry, western blotting, and transmittance electron microscope (TEM). Five patients (three men and two women) with refractory perianal Crohn's disease with an average age of 35.4 years old were included. The patients did not match the exclusion criteria, and the presence of refractory fistulas was the main inclusion criteria. These patients have their exosomes injections in the operation room. The therapy responses of patients were assessed using physical examination, face-to-face interviews, computerized tomography (CT) scan, and magnetic resonance imaging (MRI) six months later.

Result:

The Following injection, patients reported a 70% improvement on average. Two patients claimed complete healing after exosome injections, while one patient reported no improvement and active discharge from the fistula site. In addition, five patients reported no adverse effects, both systemic or on the injection site.

Conclusion: Injection of exosomes extracted from MSCs shows total safety and a satisfactory therapeutic impact, as shown in this and other research, and could play a significant role in the treatment of gastrointestinal fistulas in the future.

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What is already known?

· Mesenchymal stem cell-derived *exosomes have manifested anti-inflammatory properties in various settings both in animal and human subjects and* crohn's disease as part of inflammatory bowel diseases is associated with preanal fistula in severely active cases.

What is new here?

· This phase I clinical trial on human subjects with crohn's disease and preanal fistula were treated with MSC-derived exosomes; the results of follow up by MRI imaging and examination have shown significant healing potency with no adverse complications.

How can this study help patient care?

· Knowing the complexity of preanal fistula in crohn's disease and its complicated treatments and the high rate of recurrence in these patients the treatment is of offer importance. Exosome therapy is a new rising method of treatment that can be a solution for many crohn's patients.

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Keywords: IBD; Exosome; Crohn; MSCs; Fistula

1. Introduction

Perianal complications are common presentations of Crohn's disease associated with significant morbidity and economic cost on health care systems worldwide [1]. The low efficacy of the drug and surgical-based therapeutic methods and risks of stem cell transplantation moved the attention toward implementing MSCs-derived exosomes to treat fistula.

The standard surgical interventions for managing Crohn's perianal fistula include abscess drainage and seton replacement as the primary approaches to the acute phase of perianal sepsis [2]. Similar to other surgical methods, fistulotomy and novel strategies, including flaps, ligation of intersphincteric fistula tract (LIFT), video-assisted fistula therapy (VAAFT), and fistula tract laser treatment (FiLaC®), are considered the alternative approaches for the management of Crohn's fistula [3-8]. In addition to surgical intervention, medical management for perianal fistula is common to control sepsis and improve mucosal healing. Antibiotics and anti-tumor necrosis factor (TNF-α) drugs, as the immunotherapy mediated treatment, are usually the first step in treating Crohn's perianal fistula [9, 10].

Despite the considerable improvements in surgical and medical treatments, the effectiveness of the current methods to cure Crohn's perianal fistula is estimated to be up to 50%, and the optimal treatment for Crohn's perianal complications remains unachievable [11]. The significant failure rate of the current surgical and medical treatments can be related to the multiple causes, including complex pathophysiology of Crohn's disease, severe tissue injuries after a complicated immune process in the Gastrointestinal (GI) tract, the possibility of bacterial overgrowth, infections, and the underlying inflammation as a significant barrier for the efficient mucosal healing [12]. Thus, the demand for introducing novel medical approaches such as stem

cell therapy and exosome therapy has been growing over the past years to achieve optimal Crohn's perianal fistula treatment.

As an advanced regenerative medicine method, stem cell therapy acts through the suppression of inflammation to improve the healing process of Crohn's perianal fistula [13]. Early studies have introduced the application of stem cells as an effective method for managing Crohn's perianal fistula [14, 15]. Despite the immunomodulatory effects of stem cells and their significant efficacy for the treatment of perianal fistula, several safety concerns, including unwanted differentiation of transplanted stem cells, their possible malignant transformations, tumorigenicity, and adverse immunologic effects, have been raised about the administration of MSCs[16].

Early studies have shown that the immunomodulatory properties of stem cells are attributed to their extracellular secretions, such as extracellular vesicles and exosomes [17, 18]. Exosomes are enclosed lipid bilayer structures carrying bioactive and signaling molecules, including proteins, lipids, and nucleic acids between cells [19-21]. These bilayer lipid vesicles can be isolated from various body fluids, including blood, urine, saliva, and milk [22]. The small size (30- to 150-nm) vesicles can pass cell membrane and are highly biocompatible to warrant effective contents [23]. The role of exosomes in the gastrointestinal tract inflammatory disorders, such as intestinal mucosal inflammation accompanied by Crohn's disease, and ulcerative colitis, is related to numerous signaling pathways and molecules, including Zonula occurrence-1 (ZO-1), annexin-1 (ANXA1), Interleukin-6 (IL-6), TNF-α, extracellular regulated kinases (ERK) pathway, and tumor necrosis factor receptor 2 /nuclear factor kappa B (TNFR2/NF-κβ) pathway [24-28]. Based on the role of the exosomes in the inflammatory pathways of GI tract diseases and the immunomodulatory properties of the stem cell-derived exosomes, they might be safe and effective treatments for Crohn's perianal fistula. However, there is no previous study to assess the safety and effectiveness of applying stem cell-derived exosomes as a novel treatment for Crohn's perianal fistula patients.

This study aimed to evaluate the safety of administration of umbilical cord MSC-derived exosomes for treating patients with complex perianal fistula associated with Crohn's disease in phase I clinical trial. The isolated MSCs were characterized and induced to generate exosomes. The exosomes were isolated, purified, injected into petients, and monitored for six months (graphical abstract).

1. Materials and Methods

1.1 Human umbilical cords MSCs isolation and characterization

Human umbilical cords were obtained via ethical code" IR.TUMS.IKHC.REC.1400.184" based on research protocols from Imam Hospital. The cords were transferred in Phosphate buffer saline (PBS) supplemented with 100 U penicillin/ streptomycin (Pen/Strep; Gibco). The sample, after removal of blood vessels, was cut into 2–6 mm^3 pieces, washed with PBS, and treated with 2 mg/ml of type IV collagenase for two hours at 37 °C. Then, the sample was washed with PBS: consecutively the sample was filtered with a 70 μm nylon mesh to remove particles and aggregations. The isolated cells were suspended in D-MEM F12 culture medium (Gibco) supplemented with Pen/Strep, and 10 % fetal bovine serum (FBS; Gibco), then seeded in the flasks. The cells were placed in an incubator set at 37 °C containing 5 % CO2 at 90 % humidity. The exosomes were extracted in passages 2 and 3. The adipogenic and osteogenic differentiation capacities of MSCs were investigated in terms of the previous study.

1.2 Isolating and Characterizing Exosomes

MSCs in passage 3 were cultured in T175 flasks with D-MEM F12 culture medium (Gibco) supplemented with Pen/Strep , and 10 % exosome depleted FBS(Gibco for 48 h at 37 °C under 5% CO2. The conditioned media of MSCs (CM) were collected and centrifuged for 10 min at $400\times g$ to eliminate suspended cells, 30 min at $2500\times g$ to remove debris and apoptotic bodies, and then ultracentrifuged for 120 min at $100000\times g$ (Beckman, USA). The supernatants were collected and further ultracentrifuged for 120 min at $100000\times g$. Then the pellets containing exosomes were dissolved in PBS.

The production of MSC- derived exosomes were measured by Bradford colorimetric assay (BCA) from 100 ml of CM. Size distribution of MSC- derived exosomes were determined by dynamic light scattering (DLS). Moreover, to determine the expression of CD9, CD63 and CD81 with flow cytometry and western blot, FITC mouse anti-CD63 (BD Pharmingen) and PE mouse anti-CD81 (BD Pharmingen) were used. Furthermore, the morphology and size of isolated exosomes were assessed using TEM in terms of the previous study.

1.3 Patients Characteristics

Allowance from the Imam Khomeini Hospital Complex – Tehran University of Medical Sciences ethical committee was obtained for an open-label, phase I prospective clinical trial to study 5 patients (IR.TUMS.IKHC.REC.1400.As Imam Khomeini hospital is a tertiary referral hospital, all patients who were enrolled in our study had refractory disease. Patients from 18 to 65 years with perianal Crohn's disease who were previously treated and had unhealed cryptoglandular fistulas were eligible for this study. All patients were treated for at least six months with 10 mg/kg intravenous infliximab and seton placement. Patients were excluded if they had anti-TNF α treatment from less than six months ago, surgical interventions, had a history of malignancies or immunodeficiency diseases or pregnant patients.

After obtaining an informed consent, the patients underwent a general examination and serologic evaluations, including complete blood count and electrolytes. Patients underwent an examination under anesthesia,the external orifice was located and the fistula tract was identified.

Conventional multiplanar, multisequence pelvic MRI for perianal fistula detection and characterization was performed before using MSCs-derived exosomes (baseline). The fistulas were classified according to the Park classification scheme. The architecture of the fistula and its cavity, extension, and T2 hyperintensity were assessed. Additionally the length of the hyperintense T2 tract was measured as quantitative marker of fistula activity.

1.4 Exosomes Administration Program

The procedure was undertaken in the operation room, and the patients were NPO 6 hours prior to the operation with intravenous sedation and oxygen supplement with a mask, placed in a lithotomy position. The external fistula opening was inspected, the tract was palpated, and using an Eisenhammer Retractor, the internal orifice was seen. The tract was irrigated with saline several times using a small catheter to clear the pus and fecal material. After irrigation, a flexible fistula probe was inserted into the tract. Using the probe as a guide, we injected 5 mL of exosome into the tissue surrounding the tract. The injection depth was about 2-3 mm of the soft tissue and sphincters of the anus. After the injection, the tract probe was extracted, the patients were observed in the operating room recovery for 3 hours, and vital signs were monitored. The patients were then transferred to the surgery ward and were observed for 48 hours. The patients were again examined under sedation in the operation room for further evaluation six months after injection.

1.5 Evaluating Safety

The main goal of this study was to assess the safety of using MSCs-derived exosomes for the treatment of IBD's perianal fistula. Patients were observed for 48 hours postoperatively to investigate acute adverse effects before discharge from the hospital. The patients were observed for ventricular tachycardia, myocardial infarction (MI), stroke, shortness of breath, coughing, and wheezing as possible severe adverse effects of administering exosome in the first 48 hours postoperatively. Additionally, monitoring the allergic symptoms, including hot flushes, ureteric, and skin rash, was performed in the first 48 hours. Blood tests, including complete blood count, , liver function tests, Erythrocyte sedimentation rate, creatinine, and blood urea nitrogen were checked every 6 hours. The patients who had no signs of adverse reaction and normal blood tests results were discharged after 48 hours. Patients were scheduled for weekly office visits and inspected for adverse effects, and blood tests were also taken for follow-up. Gastrointestinal outcomes such as nausea, vomiting, diarrhea, and abdominal cramping pains were observed monthly for six months.

1.6 Evaluating Efficacy

The first evaluation was performed around the first month postoperatively and every four weeks until the sixth postoperative month. At each evaluation, a detailed clinical examination was performed to assess for any adverse reaction or complication related to the MSCs-derived exosomes and assess the internal and external orifices of the fistula tract for healing. Clinical healing was defined as healed (no cessation of drainage with reepithelization of the external orifice), improved (decreased drainage), and no change. Short–term adverse outcomes were events occurring within four weeks of injection; long-term adverse outcomes were those occurring four weeks to 6 months following injection.

1.7 Statistical Analysis

In this study, we used IBM® SPSS® statistics 25 for statistical analysis. We used mean and standard deviation to present scale variables, and nominal variables were presented using frequency. Statistically, the significant difference was determined as *P-value* <0.05.

2. Results

2.1 Characterization of MSCs and MSCs-derived exosomes

Adipogenic and osteogenic differentiation capacity of umbilical cord-derived MSCs was confirmed with specific staining, Oil red O for adipogenic and Alizarin Red and ALP for osteogenic capacity (Fig 1A). Exosomes extracted from the human umbilical cord MSCs were visualized by TEM. Accordingly, the results showed a spherical shape with a diameter ranging from around 30–140 nm (Fig1B).

Western blot and Flow cytometry tests exhibited high expression of CD63, CD81, and CD9 (Fig1 C & D), consistent with the results of previous studies on exosome size distributions. DLS results also revealed that exosomes were less than 100 nm in diameter (Fig1E), which is consistent with the results of previous studies on exosome size distributions.

2.2 Study Population

All five patients enrolled in the trial had at least six months of follow-up and were included in the analysis. All five patients had Crohn's perianal fistula. The mean age was 35.4 years (range, 31-47 years), and we had three male and two female patients. The median duration of diseases at the time of study enrolment was 8.0 years (range, 2-15 years). The fistulas had been present for a median of 24 months (range, 6-36 months). Four patients had received treatment with infliximab for the median duration of 2 years (range, 1- 3 years) until six months before the intervention. Three patients had prior surgery for abscess drainage and seton placement (table.1). baseline MRI results showed that three patients had a single intersphincteric fistula tract. one patient had two fistula tracts(transsphincteric and intersphincteric) and one patient had four intersphincteric tracts.

Besides, three patients had perianal skin inflammation around the fistula; all three complained of severe burning, especially during defecation. One patient had significant fibrotic tissue around the external orifice and difficulty during defecation.

2.3 Effects of Mesenchymal Stem Cells Derived Exosomes administration

2.3.1 Safety of treatment:

No short-term or long-term adverse events occurred in patients after administration of MSCs-derived exosomes. Additionally, the no patient did not complain about adverse outcomes during follow-up evaluations. The lab tests showed no abnormality after injection and during follow-up, and no leukocytosis or impairment of liver function tests was observed.

2.3.2 Efficacy of treatment:

At the 6-month follow-up, response to treatment was observed in 4 patients (80%); one patient with severe fibrosis around the fistula tract and perianal region had no remarkable outcome with no change in drainage and reepithelialization. The fistula was completely resolved in 2 patients with intersphincteric fistula. The patient with transsphincteric fistula had significant drainage reduction and cessation of external office reepithelialization. The patient with four fistula tracts had significant improvement in symptoms, one of the tracts improved spontaneously, and two tracts had no sign of reepithelization or inflammation; therefore, a simple fistulotomy was performed (fig. 2). In all three patients with severe skin inflammation, there was no sign of skin inflammation after one month, and the state was the same after six months. The patients had no burning fell or pain during monthly visits. The MRI confirmed fistula treatment in 2 patients (fig. 3), also complete healing of 3 out of 4 tracts in one patient with no sign of inflammation. 2 patients had persistent tracts in MRI (one patient had clinical improvement).

3. Discussion

Perianal complications are the most common type of IBD fistula, which involve 20% of patients with Crohn's disease [29, 30]. Despite the significant improvement in medical treatments, the success rate of the current treatments is estimated at up 50%, and unfortunately, more than half of the cases are not curable [31]. Therefore, scientists seek advanced therapeutic methods implementing stem cells and stem cells derivatives to assist this group of patients involved in such inflammatory disorders.

MSCs-derived products are promising therapeutic agents for treating inflammation-related disorders using their immunoregulatory effects. About two decades ago, it was shown that the application of MSCs for a female with Crohn's rectovaginal fistula, which was unresponsive to surgical and therapeutic treatment, was safe and successful [32]. Based on the different features of MSCs, including anti-inflammatory effects, immunomodulatory properties, proliferation, and differentiation ability, they might be effective in managing refractory IBD fistula. Early studies have shown that the MSCs-derived exosomes affect multiple signaling

pathways, including Zonula Occuldence-1 (ZO-1), annexin-1 (ANXA1), and IL6, TNF-α, ERK pathway, and TNFR2/NF-κβ pathway; to improve IBD's complications healing process. They significantly suppress the expression of inflammatory cytokines, such as IL-1β and IL-6, and upregulate the expression of anti-inflammatory cytokines, such as IL-10. Furthermore, extracellular vesicles reduce levels of cullin-1 and NEDDD8, which are related to neddylation as a post-translational pathway associated with IBD[33]. Additionally, exosomes decrease JAK1 and STAT1 phosphorylation in the colon, suggesting that these bilayer particles modulate immune responses by suppressing JAK1 and STAT1 [34].

Our study is the first to evaluate the safety and efficacy of using extracellular vesicles as a treatment for IBD perianal fistula. This study proves the safety of applying MSCs-derived exosomes for perianal Crohn's disease since we had no clinical symptoms or abnormal lab tests in any patients. The improvement of symptom rate of administrating MSCs-derived exosomes was 70%, and two patients had complete fistula obliteration. Skin inflammation was eliminated in all patients; it may be the reason for pain and burn alleviation. The patient who had fibrosis around the fistula did not benefit from treatment, and we hypothesize that exosomes cannot infiltrate fibrotic tissue and therefore are not a suitable treatment for chronic cases with significant fibrosis. Additionally, no patient complained of any adverse effects during follow-up, and no adverse outcomes occurred during examinations.

Prior to our study, similar studies evaluate the efficacy and safety of using MSCs as a novel treatment for IBD complications [14, 15]. As an example, Molendijk *et al.* demonstrated that the application of MSCs for the treatment of 21 patients with Crohn's perianal fistula was safe, and the success rate was 80%, which is approximately similar to the success rate of our study [35]. In another study, Lightner *et al.* have shown that using MSCs to manage Crohn's rectovaginal fistula was safe, and the efficacy of treatment was more than 70%, similar to our study [29]. Lightner *et* al. demonstrated that MSCs-coated plugs are as safe and effective as 80% in patients with rectovaginal Crohn's fistula [29]. Based on these studies and our results, the outcome of using MSCs-derived exosomes is similar to the application of MSCs, and it can be considered as a safe and effective novel management method for IBD's perianal fistula. The observed a 70% healing rate in patients from the application of MSCs-derived exosomes in our present study is comparable to patients treated with cultured autologous and allogeneic mesenchymal stem cells injection.

However, the outcomes are difficult to compare because different methods have been used to design studies. An issue that makes it difficult to compare the results of using exosomes with other studies is the number of perianal fistulae. Our study evaluates the efficacy of MSCs-derived exosomes for patients with complex perianal fistula. In the study with matrix-delivered autologous MSCs by Lightner *et al.,* patients could undergo the intervention if there was no concurrent fistula tract [29]. As in the study by Topal *et al.,* the complex perianal fistula was not considered an exclusion criterion in our study.

In addition, our study shows that the success rate of administrating MSCs-derived exosomes is similar and even more than using MSCs injection. However, there are several advantages for MSCs-derived exosomes in comparison with MSCs. Growing ethical and safety concerns about the application of MSCs are the main issues [16]. Concerns about the tumorigenicity of MSCs are rising. In detail, it is demonstrated that undifferentiated human embryonic stem cells (hESCs) can develop teratoma [36], and MSCs can differentiate into undesired tissues such as calcified tissues. Moreover, Kuriyan *et al.* revealed that the local microenvironment includes factors that induce undesirable differentiation of MSCs *in vivo* [37]. In addition to concerns about unwanted differentiation, MSCs may promote tumor growth and metastasis. After the injection of MSCs, they may migrate to the tumor site and suppress the anti-tumor immune response by their immunomodulatory effects [38]. In this study, we have illustrated that the application of exosomes for IBD's fistula does not lead to serious adverse outcomes in a 6-month follow-up. However, we cannot conclude that safety concerns about the application of MSCs are not related to exosomes certainly. It is necessary to perform studies with more participants and longer follow-ups to evaluate whether the safety concerns of MSCs and exosomes are similar.

It is demonstrated that the efficacy of administrating extracellular vesicles depends on multiple factors. Previous studies have shown that the efficacy of MSC application for the treatment of Crohn's perianal

fistula is dependent on the dosage of locally injected stem cells. Based on these studies, the most popular dosage of MSCs for similar contexts is between 60×10^6 to 120×10^6 cells/ml for each patient [34, 39-41]. As the action mechanism of MSCs for the treatment of Crohn's perianal fistula is similar to the exosomes, it can be concluded that the dosage of injected exosomes affects the outcome of the intervention. In addition to the dosage of exosomes, the size of exosomes is a determinant factor for the efficacy of the treatment. To assure that the dosage of MSCs-derived exosomes is sufficient for the intervention, we measured MSCs-derived exosomes dosage using Bradford Colorimetric Assay at 570 nm. The results have shown that the MSCs-derived exosomes dosage was sufficient for the treatment.

Our current study has some important limitations. The first limitation of our study is the number of participants. Additionally, the patients were not selected blindly. The duration of follow-up was not as long as sufficient to rule out some adverse outcomes such as tumorigenicity of exosomes.

4. Conclusion

In conclusion, the application of MSCs-derived exosomes demonstrated great potential as a novel, safe treatment of perianal fistulas in IBD. The treatment has the potential to consider a much more effective alternative to current treatments for IBD's perianal fistula and is safer than using MSCs.

5. Declarations

Ethics statement

This study was approved by the Research Deputy and the Ethics Committee of the Tehran University of Medical Sciences (Reference number: IR.TUMS.IKHC.REC.1400.184) and conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and all subsequent revisions. A written informed consent form was obtained from all the participants

Consent for publication

All patients have signed informed consent forms.

Conflicts of interests

The authors declare no conflict of interests.

Data availability

Data available on request due to privacy/ethical restrictions.

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6. Authors' contributions

HN carried out flow cytometry and western blot studies and also participated in the MSCs and exosome isolation

AH, isolated and characterized MSCs and exosomes and prepared the samples for TEM and DLS assays.

MS conceived and designed the study and also participated in MSCs and exosome isolation.

RA coordinated the tasks and participated in drafting.

BB coordinated the tasks and participated in drafting.

MSF carried out surgical interventions

AK carried out surgical interventions

MR took part in anesthesia

MK interpreted MRI scans.

AK interpreted MRI scans.

FA conceived and designed the study and also contributed to patient follow-up.

SMA conceived and designed the study and also contributed to patient follow-up.

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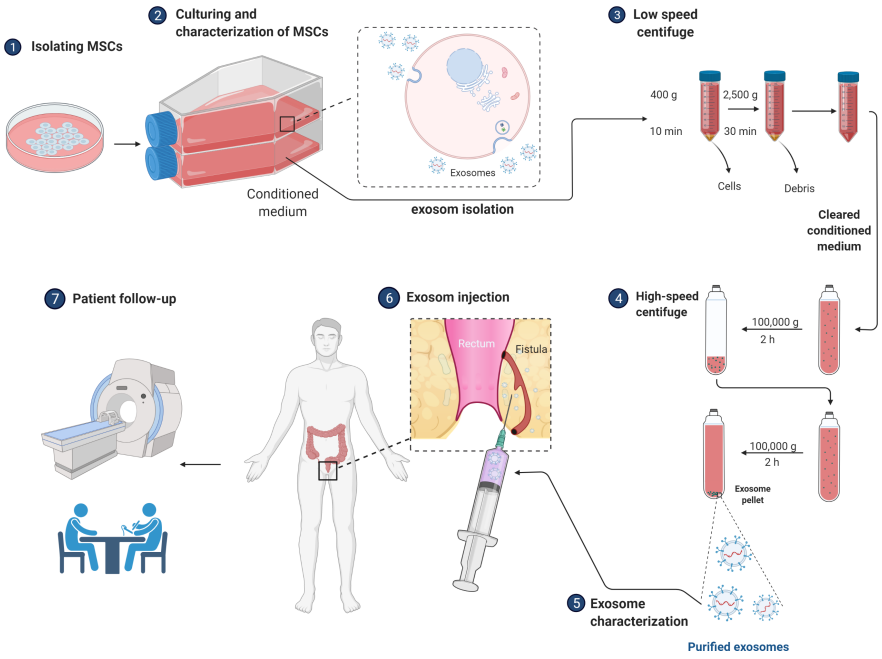
Table 1. The patient's detail before exosome therapy.

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**
Sex	Male	male	female	male	female
Age (years old)	35	47	40	24	31
Disease duration (year)	3	8	15	2	13
Number of fistula tracts	2	1	3	1	1
Type of fistula	perianal	perianal	perianal	perianal	perianal
Duration of fistula (month)	36	36	24	24	6
Δυρατιον οφ αντι-TNΦα τρεατμεντ	1	3	2	2	Never
Seton placement	yes	no	yes	Yes	No
Remission status	silent	silent	silent	Silent	Silent

Figure 3. MRI images of a patient with fistula before (A) and after 6-month (B) of exosome injection the tract has been resolved according to MRI images.



Graphical abstract

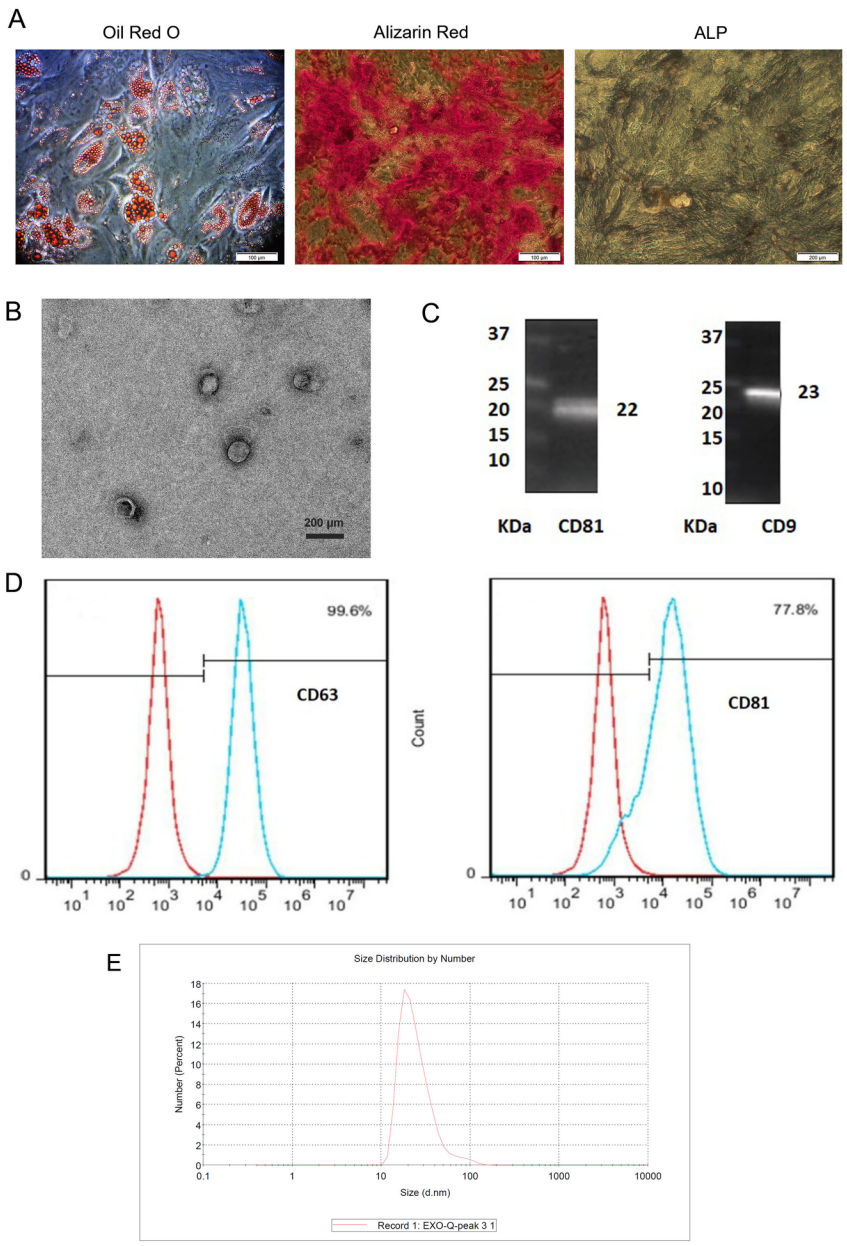


Figure 1. Characterization of MSCs and MSC-derived exosomes. The results of the adipogenic and osteogenic capacity of UC-MSCs (A). The results of characterization of exosomes by TEM (B), western blotting (C), flow cytometry (D) and DLS (E).

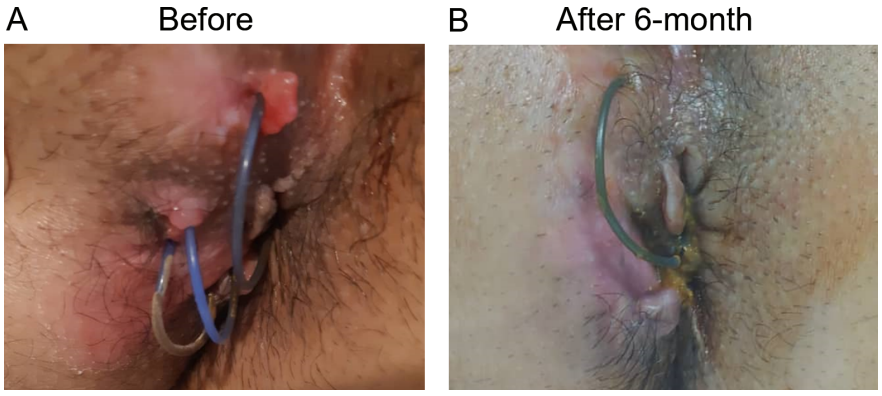


Figure 2. A) A case of crohn's disease with 3 perianal fistula tracts before exosome therapy. B) The same patient 6 months after treatment

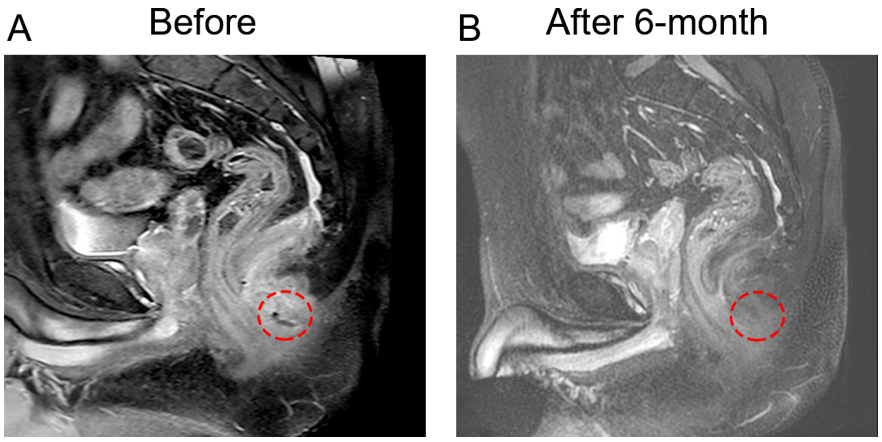


Figure 3. MRI images of a patient with fistula before (A) and after 6-month (B) of exosome injection the tract has been resolved according to MRI images.