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Efficacy of umbilical cord-derived mesenchymal stem cells and exosomes in conjunction with standard IBD drug on immune responses in an IBD mouse model

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Abstract

Background Inflammatory bowel disease (IBD) is a persistent inflammation of the digestive system, and Mesenchymal Stem Cells (MSCs) and their exosomes have demonstrated potential as treatments for this condition. The objective of this research was to examine the possible effectiveness of intraperitoneal injection of umbilical cord-MSCs (UC-MSCs) and their exosomes through a two-time injection regimen in a mouse model.

Method In this study, an animal model of a specific type of IBD in C57BL/6 mice, induced by dextran sulfate sodium (DSS), was utilized. The mice were treated with MSCs, exosomes, Mesalazine, and a combination of them. Upon sacrificing the mice, colon and spleen tissues were isolated to assess the changes in the mice's weight, colon length, spleen weight, and colitis' pathological symptoms. IL-10 and IL-17 levels were measured, and Treg and Th17 cell percentages were determined as well. Furthermore, colon tissue was stained to investigate histopathological changes.

Results In the groups that received MSCs, there was a significant reduction in the disease activity index and their combinations with exosomes and Mesalazine compared to the colitis group. Colon length increased in all groups except the exosome group. Histological measures were notably reduced in the MSC groups and their combinations. Significant increases in the IL-10 level of colon tissue and the proportion of Treg present in the spleen were observed

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in the groups receiving MSC and combination treatment. Furthermore, these groups showed a notable reduction in the percentage of spleen Th17 cells. However, IL17A decreased non-significantly in all groups.

Conclusion The results showed that intraperitoneal injection of UC-MSCs and their combination with exosome and Mesalazine in a murine colitis model improved the disease's symptoms. Therefore, MSCs and their combination with exosomes can be a promising therapeutic approach along with other common drugs for IBD, but exosomes alone could not significantly reduce the symptoms of colitis.

Keywords Mesenchymal stem cells, Exosome, Inflammatory bowel disease

Background

Inflammatory bowel disease (IBD) comprises two distinct aggressive inflammatory disorders known as ulcerative colitis (UC) and Crohn's disease (CD) [1]. Inflammation is a defining feature of CD, which can manifest in any part of the digestive system ranging from the mouth to the anus. The small intestine and upper region of the large intestine are more commonly affected. On the other hand, UC is a medical condition that leads to inflammation, ulcers, and swelling in the colon and rectum. Important complications associated with these conditions include diarrhea, weight loss, abdominal pain, bloody stools, and an elevated risk of colon cancer in the advanced stages of the disease [2]. IBD is mainly exacerbated by the lack of balance in immune response. IBD primarily arises from an inappropriate immune response in individuals who are genetically susceptible to pathogens. Its defining features include abnormal mucosal immune response and dysfunction in the integrity of the intestinal barrier [3]. IBD subtypes are increasingly prevalent worldwide, impacting patients' quality of life. Currently, there is no known cure for IBD. Existing treatments for inflammatory bowel disease focus on symptom management and enhancing the overall quality of life for those affected. These therapies often include the use of aminosalicylates, biologics/immunosuppressants, and corticosteroids. It is important to reduce the advancement of the disease and alleviate symptoms. However, new and secure treatment methods are urgently required to address the symptoms of UC without adverse effects to meet the unmet needs of patients [4].

MSCs are a kind of multipotent stem cells that possess the ability to regenerate and transform into different cell types. MSCs can be sourced from different origins, such as umbilical cord, adipose tissue, and bone marrow [5]. Evidence indicates that MSCs alleviate immune responses via interacting with natural killer cells (NK), T lymphocytes, B lymphocytes, and releasing immune regulatory factors [6]. The use of MSCs transplantation has become a hopeful method for alleviating IBD symptoms. MSCs have been discovered to possess immunomodulatory attributes, enabling them to regulate immune responses, diminish inflammation, and facilitate tissue regeneration [7]. Research has suggested that the beneficial effects of MSCs on inflammatory bowel disease (IBD) are attributed to their paracrine signaling mechanism. However, stem cell transplantation may have some drawbacks including the possibility of rejection by the body, high costs, and the potential risk of turning into cancerous cells. As a result, exosomes released by MSCs, known as MSCs-Exo, have gained significant notice as a potential alternative [8]. Previous research showed that exosomes are a great candidate for MSCs to carry out their immunomodulatory activity [9, 10].

Exosomes, produced by every type of cell, are a form of extracellular vesicles (EVs) with sizes typically falling within the range of 30 to 130 nanometers (nm). Encased in a phospholipid bilayer, they transport various cargoes like proteins, DNA, miRNA, and etc. Exosomes are essential for enabling cellular communication and the transfer of information between cells [11, 12]. Exosomes markers and other bioactive cargoes are reflected to their cell origin [13]. Research indicates that exosomes derived from mesenchymal stem cells (MSC-Exo) play a critical role in immune regulation [14]. MSC-Exo reduces the immune response by interacting with immune effector cells, carrying various anti-inflammatory substances and inhibiting inflammatory cytokines [12]. Recent studies have demonstrated that MSC-Exo can effectively reduce mouse colonic inflammation by reducing inflammatory responses in a manner dependent on macrophages [15]. Heidari et al. have shown that exosome-derived adipose MSCs are capable of reducing the levels of IL-12, TNF-α, IFN-γ, and IL-17, also clinical symptoms in colitis mice. Additionally, these exosomes growth the levels of IL-10, IL-4, and TGF-β [16]. Due to different properties in MSC-Exo such as non-oncogenicity, tissue-specific homing, and behaving therapeutic effects of MSC, they are usable as cell-free therapy in inflammatory diseases [17].

5-ASA (5-aminosalicylic acid), also recognized as Mesalazine, is still considered the initial approach for IBD, particularly effective in mild to moderate cases of ulcerative colitis [18]. Sulfasalazine, which is a prodrug of 5-ASA that is broken down in the colon by bacteria, releasing 5-ASA, which exerts its anti-inflammatory effects locally. Mesalazine has demonstrated efficacy in reducing inflammation and maintaining remission in individuals with UC [19]. That works by inhibiting the

production of inflammatory mediators, reducing mucosal inflammation, and promoting healing of the intestinal lining. While Mesalazine and sulfasalazine are effective in mild to moderate UC, more severe cases or those with inadequate response may require additional therapies such as corticosteroids, immunomodulators, or biologic agents [20].

In this research, our goal was to investigate the ways in which human umbilical cords-MSCs (UCMSCs) impact the immune system and their derived exosomes, both individually and in combination with each other and with Mesalazine, as a possible main therapy for inflammatory bowel disease. Our focus was on evaluating their impact on regulating anti-inflammatory and inflammatory cytokines and cells, also assessing the clinical symptoms of IBD using a mouse model. Additionally, another aim of our research was to investigate the possibility of a synergistic effect resulting from the combined application of these treatments, as well as their potential interaction with the standard IBD medication, Mesalazine. We aimed to determine if their effects could be enhanced when used together, potentially leading to improved outcomes in IBD treatment.

Methods

MSCs preparation and isolation

After filling out the informed consent letter, human umbilical cords (H-UC) were obtained from Imam Reza hospital from full-term pregnancy mothers. H-UC was transferred by transfer media containing 200 ml PBS (Biosera, france), 1% Pen/Strep (Gibco, USA), 1% diluted Betadine, 1% Amphotericin B, and 2% FBS (Biowest, South America) into GMP clean room. After removing blood vessels, the dissection of Wharton´s jelly, rinsed with PBS, and centrifuged at a speed of 1500–2000 RPM for 5 min. Then, the collagenase dispase enzyme (Boehringer Mannheim GmbH, Germany) was applied and placed for 60 min in a 37°C incubator. Next, H-UC pieces were centrifuged at 1500−200 RPM for 5 min; then trypsin was added and left to incubate for 30 min at 37°C. Finally, PBS was added before centrifugation at 1500–2000 RPM for 5 min. H-UC slices were cultured into α-MEM media with 20% FBS and 1% Pen/Strep in an incubator with 5% CO2 at 37°C. After a while, the MSCs were budding from Wharton's jelly and the slice were discarded. After reaching 90% confluency, cells were sub cultured.

Characterization and identification of MSCs

To evaluate the expression of MSCs markers anti-CD45, Anti-CD34, anti-CD90, and anti-CD105 antibodies were applied (BIO-RAD, USA). The Flow Jo software (version 10.5) was used to analyse the data of flow cytometry. To evaluate the capacity of MSCs to transform into different cell types, Oil Red-O and Alizarin Red (BIO-IDEA, Iran) staining techniques were utilized. Briefly, 3×10^5cells were placed into 6 well plates. To differentiate into adipocyte cell lineage, the adipocyte differential medium was applied into two wells, and one well was used as a control. The media was changed every three days. After 18–21 days Oil Red-O staining was conducted. To differentiate into osteocytes, the same amount of MSCs was cultured in an osteocyte differential medium for 18–21 days. After mineralization, Alizarin Red staining was done. Then, the result was observed with an optical microscope. The MSCs morphology was determined under the light microscope.

MSC-Exo preparation and isolation

For exosome isolation, the third passage of MSCs was maintained in a serum-free medium for 24 h at 37°C in a 5% CO2 incubator. Then, the supernatant was harvested and centrifuged at 3000 rpm to discard cell debris. The rest of the protocol was performed according to the EXOCIB kit (Cib biotech, Iran) protocol. After extraction, the MSC-Exo concentration was measured by BCA assay based on color intensity (Pars Tous Biotechnology, Iran).

Characterization and identification of MSCs-Exo

To assess the expression of CD9 and CD81, a western blot analysis was conducted. The size range of exosomes was determined using dynamic light scattering (DLS) analysis. The morphology of exosomes was examined through transmission electron microscopy (TEM). For TEM, exosomes were placed on a formvar-coated grid and treated with 2% paraformaldehyde. Subsequently, the exosomes were negatively stained with 2% uranyl acetate. The stained exosomes were then washed in double-distilled water before capturing images.

Animal study

The 45 male C57BL/6 mice, aged 9 weeks, were acquired from Mashhad University Medical Science. For two weeks, all mice were maintained in a controlled environment with unrestricted access to food and water. This study has been reported in line with the ARRIVE guidelines 2.0.

Ethics approval and consent to participate

This study was performed following the Declaration of Helsinki Ethical Principles and approved by the Ethics Committee of the Mashhad University of Medical Sciences, Mashhad, Iran (IR. MUMS. RES.1401.133).

(1) Title of the approved project: Evaluation of the effectiveness of umbilical cord mesenchymal stem cells along with the synergistic effect of the

Exosomes derived from them on the immune response of an IBD mouse model.
(2) Name of the institutional approval committee: the Ethics Committee of the Mashhad University of Medical Sciences, Mashhad, Iran.
(3) Approval number: IR. MUMS. RES.1401.133.
(4) Date of approval: 06/08/2022.

Study design and colitis induction

The 45 male C57BL/6 mice were randomly assigned to nine groups, with each group inclusive of 5 mice. Except for the first group (normal group), in the other 8 groups, colitis was induced by the following method: for inducing acute colitis, mice received 5% DSS (Cayman, Germany) in drinking water within the 7 days and they drank regular water for the remaining 3 days.

Mice were grouped as follows (Fig. 1):

1. The normal group: A group of healthy mice were given access with unrestricted availability of food and water and did not receive any treatment.
2. The DSS group (colitis group): A group of mice were given access to water that contained 5% DSS for a period of 7 days, after which they were allowed to drink regular water for the remaining 3 days. This group did not receive any other treatment as well.
3. The Mesalazine group: A group of colitis mice receiving 100 mg/kg Mesalazine (Cayman, Germany) from day 3 to day 10 every day by gavaging.
4. The Exo group: A group of colitis mice receiving150 µg Exo in 200 µl PBS by intraperitoneal (IP) injection on days 3 and 7.
5. The Exo + Mesalazine group: A group of colitis mice receiving the same amount of Exo on days 3 and 7, and besides they received 100 mg/kg Mesalasin everyday by gavaging.
6. The MSCs group: A group of colitis mice receiving 1×10^6 cells in 200 µl PBS intraperitoneally on days 3 and 7.
7. The MSCs + Mesalazine group: A group of colitis mice receiving the same amount of cells and Mesalazine with previous doses, routes, and on the same days.
8. The Exo + MSCs group: A group of colitis mice receiving the same amount of cells and Exo on days 3 and 7 intraperitoneally with 6 h interval.
9. The Exo + MSCs + Mesalazine group: A group of colitis mice receiving the same amount of cells, Exo, and Mesalazine with previous doses, routes, and on the same days.

The alteration in body weight changes, bleeding severity, stool consistency, and rectum prolapse were monitored on a daily basis and they achieved daily disease activity index (DAI, Table 1) using alterations in these clinical signs.

Colon and spleen evaluation using microscopic and macroscopic scoring

On the 10th day, the mice were euthanized by injecting 10 mg/kg xylazine (Merck, Germany) and70 mg/kg Ketamine (Merck, Germany) and cervical dislocation. Colon tissue was harvested from the rectum region to the ileocecal valve. After washing and removing stool with PBS, the colon and the spleen weight and length were

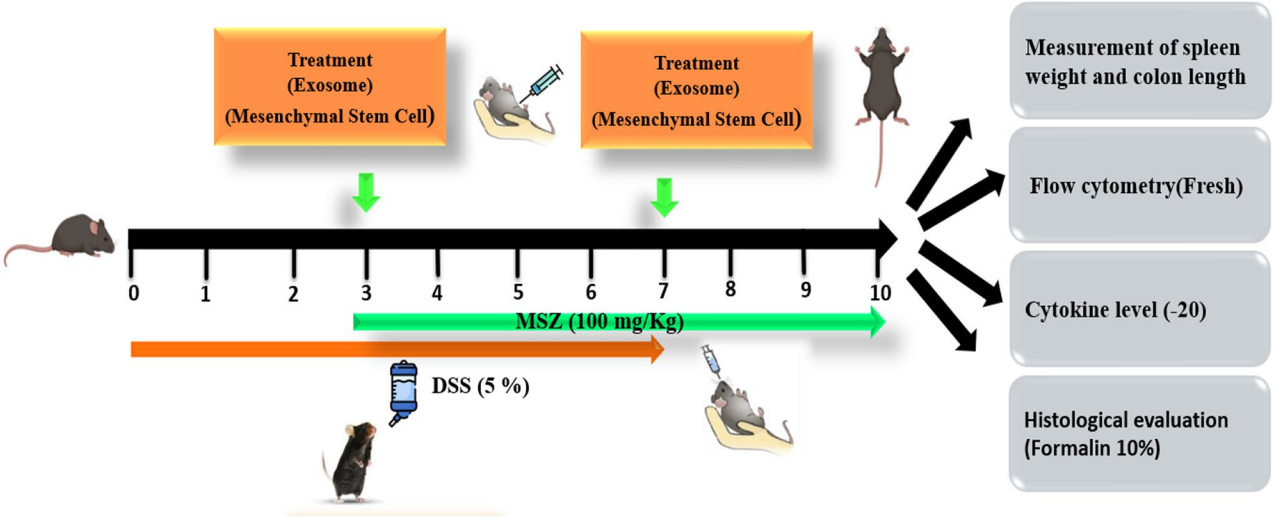

Fig. 1 A schematic illustrating the assessment of the therapeutic effectiveness of different interventions in DSS-induced colitis C57BL/6 mice. During the experiment, the mice were subjected to a 7-day exposure to 5% DSS. On days 3 and 7, different groups of mice were intraperitoneally injected with Exosomes, MSCs and any group that contains these substances. Mesalazine is also gavage from the third to the tenth day in related groups. Abbreviations: DSS; Dextran sulfate sodium, MSZ; Mesalazine

Table 1 Scoring system for disease activity index (DAI)

Disease Activity Index (DAI)				
Score	**Weight Lose**	**Stool Consistency**	**Rectal bleeding**	**Rectom Prolapse**
0	5% >	Normal	None	None
1	5%-10%	Soft	Red	Sign of prolapse
2	10%-15%	Very soft	Darck red	Clear prolapse
3	15% <	Diarrhea	Gross bleeding	Extensive prolapse

Table 2 Scoring system for histological changes

Histological changes				
Score	**inflammation**	**Mucosal damage**	**Crypt loss**	**Tissue pathology change**
0	None	None	None	None
1	Mild	Mucosa	1/3 crypt	1-25 %
2	Moderate	Submucosa	2/3 crypt	26—50 %
3	severe	Serosa and Muscle	All crypt	51-75 %
4			All crypts with epithelium	76-100 %

measured and the photo was taken. After macroscopic observations, the colon was segmented into small pieces, was immersed in 10% formalin to prepare for hematoxylin and eosin (H&E) staining (kianshimi, Iran) and another part was stored at -70 °C for cytokine evaluation. Histological scores were given to the intensity of inflammation, mucosal destruction, crypt loss, and pathological changes by a light microscope (Table 2).

Flow cytometry analysis of Th17 and Treg cells

As previously described [21]splenic cells were extracted. For stimulation of releasing intracellular cytokine, the cells were subjected to treatment with PMA/ionomycin (Biolegend, USA). Following a 6-hour incubation in a 5% CO2 and 37 °C, the cells were stained by anti-CD3, anti-CD4, anti-CD25, anti-FOXP3 (for Tregs), and were stained by anti-CD3, anti-CD4, and anti-IL17A antibodies (for Th17 cells) (Biolegend, USA). Labeled cells were analyzed with a FACSCalibur flow cytometer and FlowJo software.

Measuring anti-inflammatory and inflammatory cytokines level

For measuring IL-17 A and IL-10 levels, the homogenized colon tissue was prepared by a homogenizer device (Tissue lyser LT). The supernatant was collected for evaluating the expression of cytokines level and stored at -20 for ELISA assay (R&D Systems DuoSet® ELISA, china).

Statistical analysis

The data was analyzed using GraphPad Prism version 8. For evaluating group comparison one way -ANOVA and LSD test was performed. The results were reported as mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ was considered as significant difference.

Results

In vitro

Identification of MSC markers and differentiation potential

The UC-MSCs were isolated, and their positive and negative cell surface markers were analyzed using flow cytometry. As shown in Fig. 2a, the data indicated less than 5% expression of CD34 and CD45, confirming a negative profile for these markers, while CD90 and CD105 showed over 80% positivity. Differential staining was performed to evaluate the MSCs' differentiation potential into various cell lineages. Figure 2b and c illustrate the MSCs' successful differentiation into adipocytes and osteocytes, respectively, as confirmed by the staining results. Additionally, Fig. 2d shows the spindle-shaped morphology of the MSCs observed under a light microscope.

MSC-Exo characterization

Transmission electron microscopy (TEM) revealed oval-shaped exosomes with a bilayer membrane, ranging in size from 30 to 150 nm (Fig. 3a). Dynamic light scattering (DLS) analysis indicated an average exosome size of

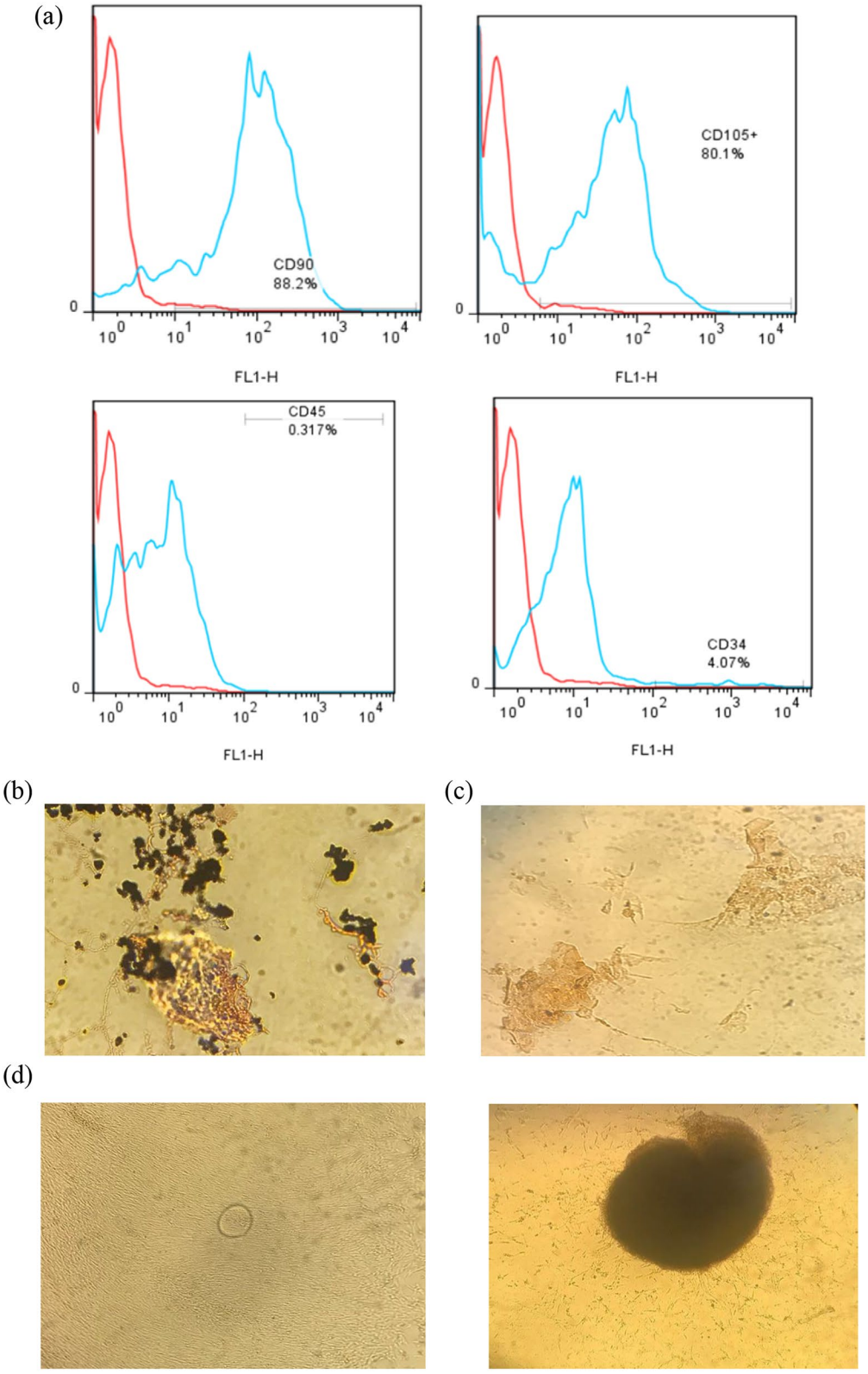

Fig. 2 Immunophenotyping characterization of UC-MSCs: (**a**) Flow cytometry analysis of MSC-positive markers (CD90 and CD105) and MSC-negative markers (CD45 and CD34) (Red line: unstained control, Blue line: a marker of interest). (**b**) Adipogenic differentiation of MSCs by oil Red-o staining. ×400, magnification. (**c**) Osteogenic differentiation of MSCs by Alizaren Red staining. ×400, magnification. (**d**) Phase-contrast micrographs of spindle-shaped MSCs that are budding from umbilical cord. ×100, magnification. Abbreviation: UC-MSCs; umbilical cord mesenchymal stem cells, MSCs; mesenchymal stem cells

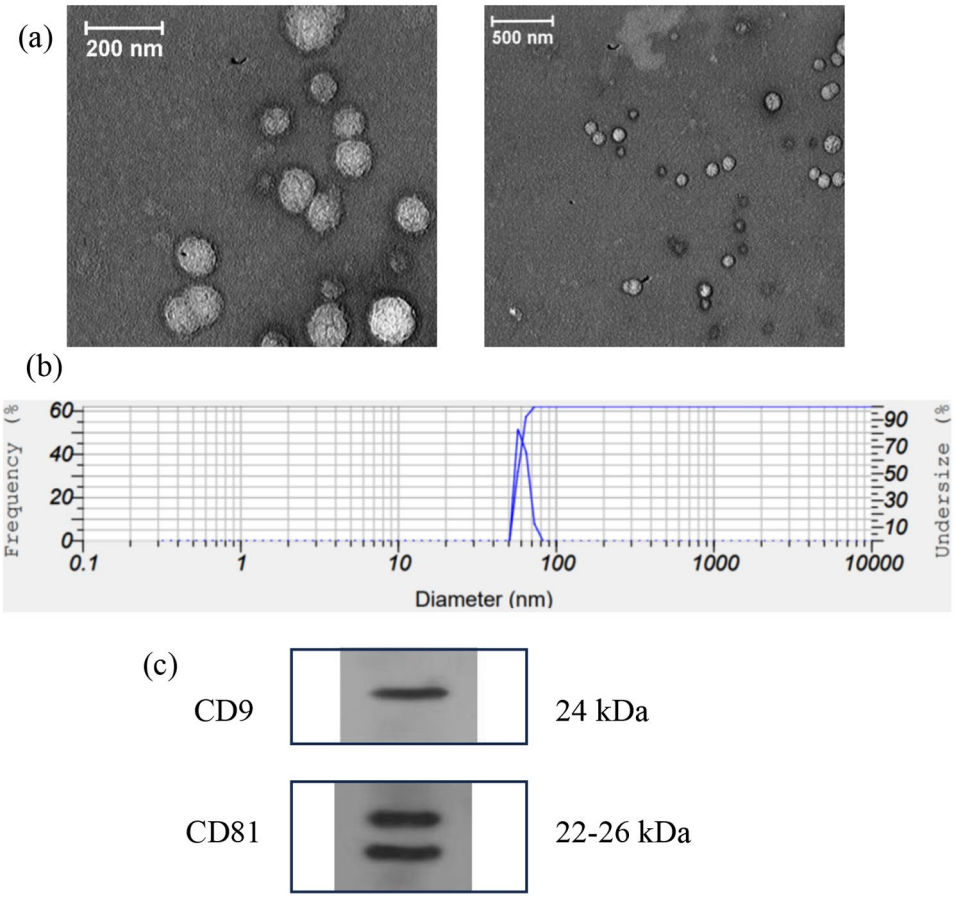

Fig. 3 Exosome characterization: (**a**) The oval-shaped exosome with dark membrane, which the size and the morphology was verified using TEM. (**b**) The confirmation size of exosomes in the solution with an average of 57 nm by DLS. (**c**) The western blot analysis of positive expression of CD9 and CD81 in exosomes. Full-length gels are presented in Supplementary Fig. 1. Abbreviations: TEM; transmission electron microscopy, DLS; dynamic light scattering

57 nm in solution (Fig. 3b). To confirm the presence of tetraspanin markers such as CD81 and CD9 on the exosome surface, western blot analysis was performed. The results demonstrated successful expression of both CD9 and CD81 (Fig. 3c). Full-length gels are provided in Supplementary Fig. 1.

In vivo

Evaluation of clinical complications

The therapeutic effects of MSCs, MSC-Exosomes, and their combination, alongside Mesalazine as the gold standard, were evaluated. Daily monitoring of the mice following colitis induction showed variations in stool consistency, weight loss, bleeding, and prolapse. Consequently, the Disease Activity Index (DAI) fluctuated throughout the observation period. DSS administration led to weight loss (Fig. 4a and b), which was prevented in most treatment groups. Notably, the combination therapy of Exosomes, MSCs, and Mesalazine significantly inhibited weight loss. As shown in Fig. 4c, d, and e, DSS treatment increased the DAI, while MSC administration significantly reduced it. Moreover, the combination therapy of Exosomes, MSCs, and Mesalazine resulted in a greater reduction in DAI compared to either treatment alone or the DSS group (Fig. 4d and e).

Macroscopic and microscopic effects of treatments on colon and spleen

Macroscopic changes in colon length and spleen weight, both associated with inflammation severity, were assessed. Figure 5a and b show that colon length reduction was prevented in all treatment groups except the exosome-only group. However, combination therapy significantly inhibited colon shortening. Spleen weight, an indicator of the severity of hemolysis in colitis, showed a marked increase in the DSS group (Fig. 5d). In contrast, MSC treatment and combination therapy significantly reduced spleen weight and hemolysis. Histological analysis (Fig. 6) revealed inflammation severity, mucosal damage, crypt loss, and tissue pathology using H&E staining. In the MSCs, Exosome, and Mesalazine combination therapy group, there was a significant reduction in mucosal damage, inflammation, crypt loss, and overall

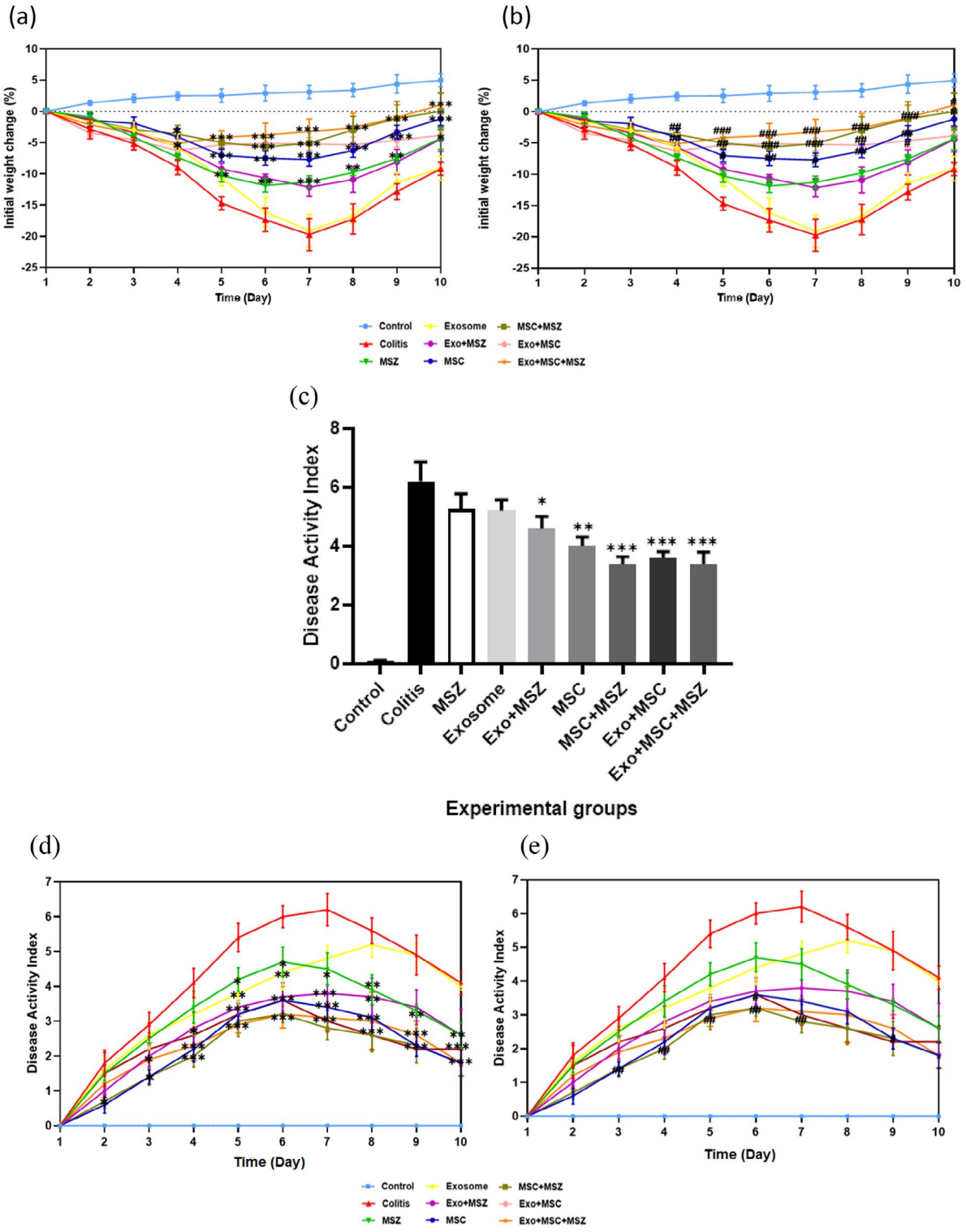

Fig. 4 Effects of different interventions on daily or/ and overal weight changes and disease activity index in DSS-induced colitis C57BL/6 mice. (5 mice in each experimental group). (**a**) Initial daily weight changes in comparison with colitis group. All data was presented as mean ± SD, (*:$p < 0.05$. **:$p < 0.01$. ***:$p < 0.001$). (**b**) Initial daily weight changes in comparison with Mesalaszine group (#:$p < 0.05$. ##: $p < 0.01$. ###: $p < 0.001$). (**c**) Overal DAI in comparison with mesalazine and colitis groups (*: $p < 0.05$. **: $p < 0.01$. *** in comparison with colitis group & $p < 0.001$. #:$p < 0.05$. ##: $p < 0.01$. ###: $p < 0.001$ in comparison with Mesalaszine group). (**d**) Daily DAI in comparison with colitis group (*:$p < 0.05$. **:$p < 0.01$. ***:$p < 0.001$((**e**) Daily DAI in comparison with Mesalazine group (#:$p < 0.05$. ##: $p < 0.01$. ###: $p < 0.001$). Abbreviations: DSS; Dextran sulfate sodium, DAI; Disease Activity Index, MSCs; mesenchymal stem cells, EXO: Exosome, MSZ: Mesalazine

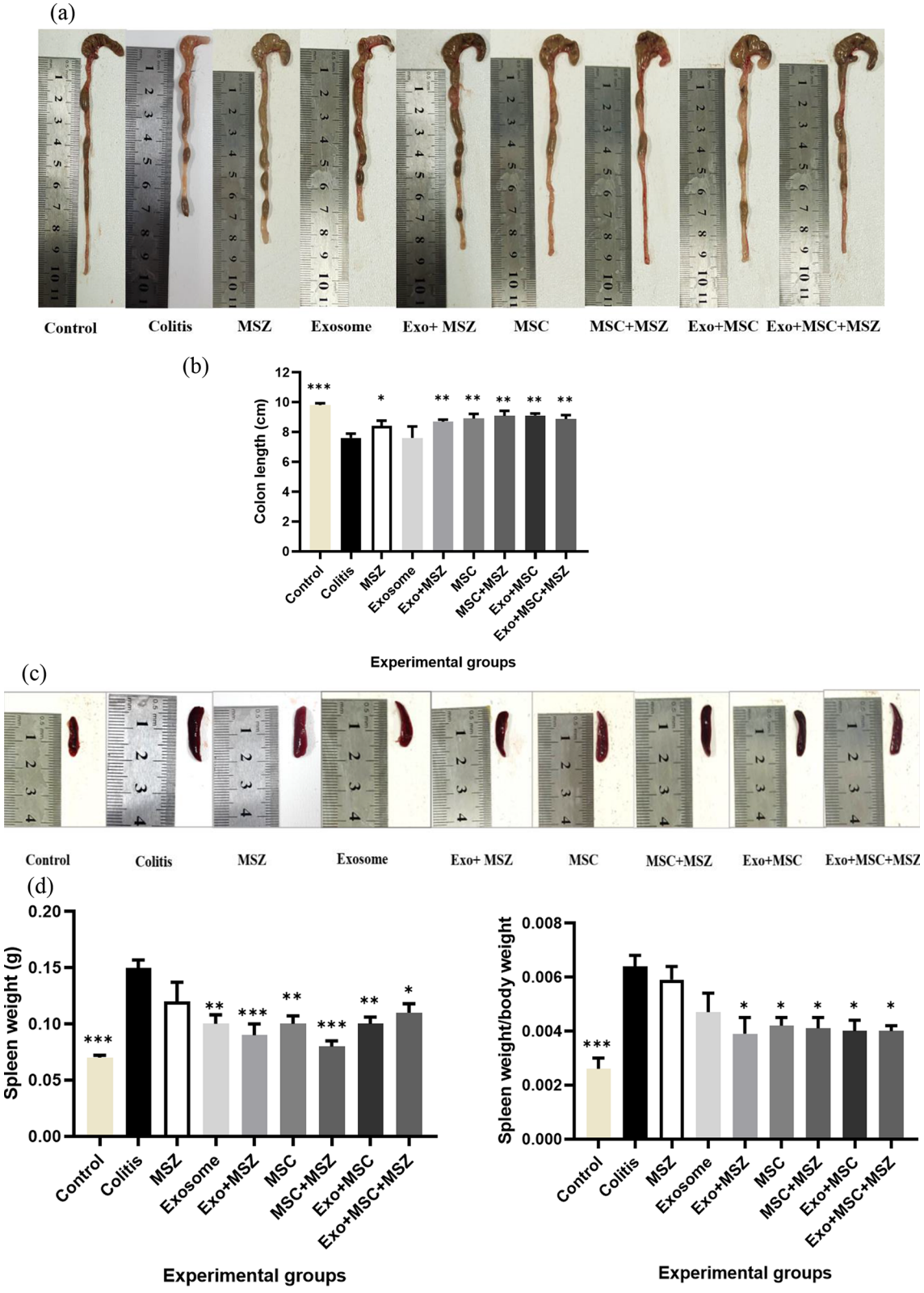

Fig. 5 The visible alterations in colon length and spleen weight subsequent to treatments administration during DSS-induced colitis. (**a**) Macroscopic changes in colon length in experimental groups during study. (**b**) Colon length alteration in experimental groups during study. (**c**) Macroscopic changes in spleen in experimental groups during study. (**d**) Spleen weight changes in comparison with colitis and mesalazine (*: $p < 0.05$. **: $p < 0.01$. *** in comparison with colitis group & $p < 0.001$. #: $p < 0.05$. ##: $p < 0.01$. ###: $p < 0.001$ in comparison with Mesalaszine group). Abbreviations: DSS; Dextran sulfate sodium, MSCs; mesenchymal stem cells, EXO: Exosome, MSZ: Mesalazine

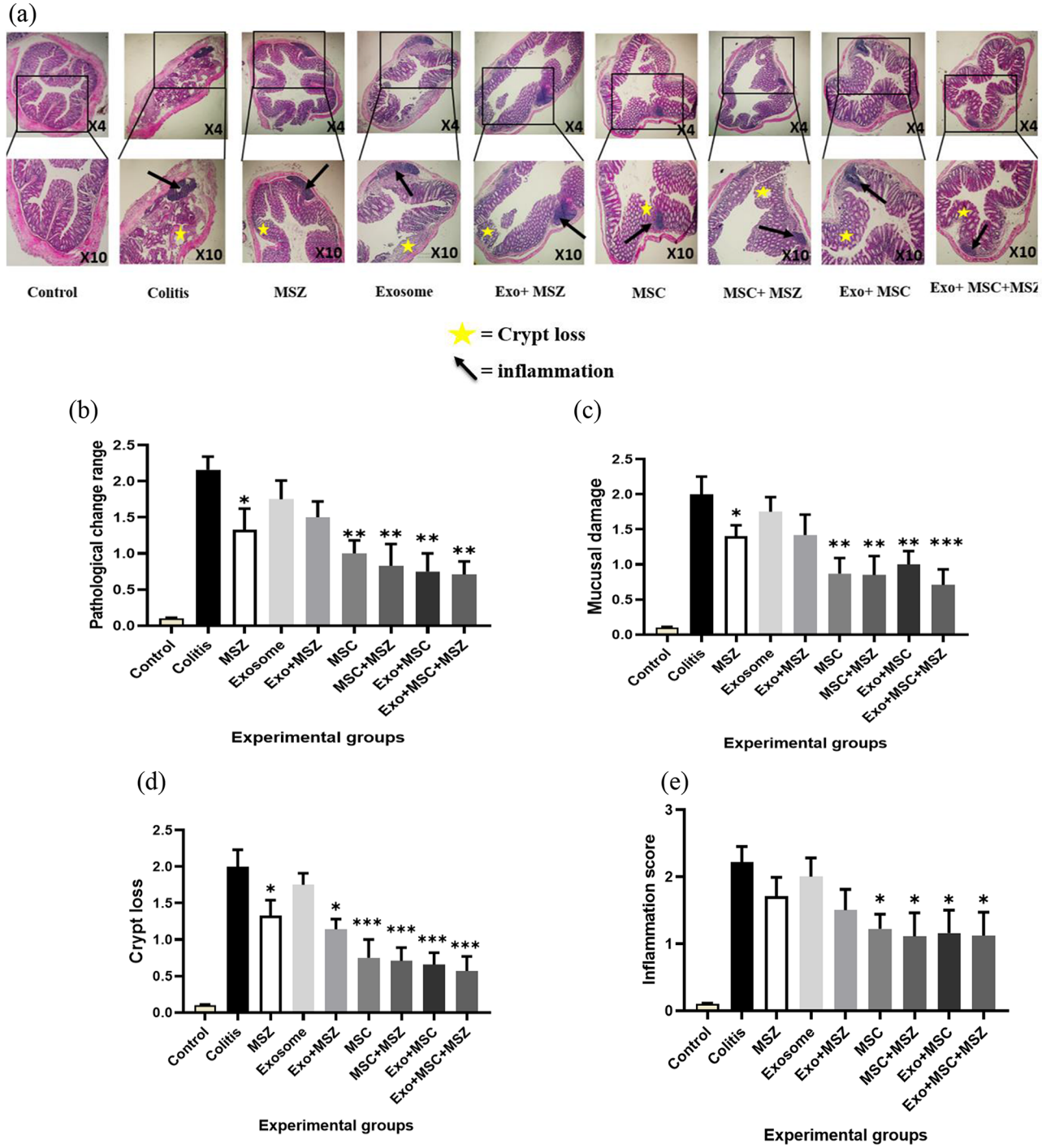

Fig. 6 Histological analysis in the mice' colons. (**a**) A typical histological graphs of mice' colons in all experimental groups. (**b**) Pathological change range, (**c**) Mucosal damage, (**d**) crypt loss, (**e**) and inflammation scores were defined by H&E staining. (*: $p < 0.05$. **: $p < 0.01$. *** in comparison with colitis group & $p < 0.001$. #: $p < 0.05$. ##: $p < 0.01$. ###: $p < 0.001$ in comparison with Mesalaszine group). Abbreviations: MSCs; mesenchymal stem cells, EXO: Exosome, MSZ: Mesalazine

histological scores compared to the DSS group (Fig. 6b, c, d, e).

Effects of treatments on Treg and Th17%

Treg and Th17 cells play crucial roles in regulating the immune response. We performed a flow cytometry analysis to evaluate the effects of different therapies on the proportions of Th17 and Treg cells in the spleens of colitis mice. As shown in Fig. 7b, the treatment groups, including MSCs, MSCs + Mesalazine, MSCs + Exo + Mesalazine, and MSCs + Exo, exhibited a significant increase in the proportion of Treg cells compared to the DSS group. In contrast, flow cytometry results for Th17 cells revealed a lower percentage of Th17 cells in all treatment groups compared to the colitis group, with combination therapy showing a particularly significant reduction (Fig. 7a).

Cytokines level in different groups

The data showed an increase in IL-10 levels across all treatment groups (Fig. 8a), with a significant rise observed in the MSCs, Exo + Mesalazine, and MSCs + Mesalazine groups. In contrast, IL-17 levels were reduced in all treatment groups compared to the DSS group, though this reduction was not statistically significant (Fig. 8b).

Discussion

IBD is a chronic inflammatory disorder that affects the digestive tract, and its occurrence has been rising globally over time. Clinical improvement is frequently achieved with current IBD therapies. These treatments have noticeable side effects even though they work well. As of this now, IBD has no proven therapy. Since IBD is an autoimmune condition, one treatment strategy is to use immunosuppressive and anti-inflammatory medications to reduce the immune response. These therapies may, however, have a number of unfavourable side effects. Given the shortcomings of existing treatments, novel and creative therapeutic approaches are required. MSCs have become a potentially effective treatment for IBD, even in cases when traditional medications have failed to yield results [22, 23].

In this work, we examined the efficacy of intraperitoneally injecting UCMSCs, their exosomes, and mesalazine alone and in combination with each other's in the treatment of murine IBD model. Our findings revealed that UCMSCs and their exosomes are able to alleviate pathological, laboratory, and clinical colitis-related problems in DSS-Induced colitis. It was discovered that MSCs were more successful because, whether given either by themselves or in conjunction with exosomes and mesalazine, they dramatically decreased the symptoms of colitis. Exosomes alone did not, however, have a meaningful effect. When combined with exosomes and mesalazine, MSC treatment groups demonstrated a significant reduction in pathological damage, mucosal layer damage, crypt loss, and inflammation, as demonstrated by the results of H&E staining of pathology slides. In this regard, MSCs have been shown to promote the proliferation of IECs, leading to mucosal healing in colitis models. This effect is mediated, in part, by the production of TSG-6 by MSCs. TSG-6 is a multifunctional protein that has anti-inflammatory and tissue repair properties. Exosomes derived from MSCs also contain TSG-6, which suggests that they can contribute to the improvement of colitis by promoting mucosal healing [24].

Contradictions exist in this respect [15, 25], despite the fact that other preclinical and clinical investigations support the findings identical to those of our investigation.

For instance, intestinal fibrosis in a mouse model of chronic colitis was alleviated by intraperitoneal injection of umbilical cord mesenchymal stem cells in Choi et al.'s study [26] Similar to the outcomes of our investigation, Sun et al.'s study (2024) also showed that transplanting UCMSCs led to an increase in the mice's weight and a drop in the Disease Activity Index (DAI) in the animal phase. Additionally, in the human phase of the same investigation, patients in a 24-week follow-up showed clinical and paraclinical improvement after cell transplantation (17 Crohn's patients) [27]. Naturally, a comparative analysis of the treatment groups in our study demonstrated that stem cell and allogeneic injection as a combined treatment is more successful than exosome injection alone in improving the illness process. In contrast, exosomes outperformed MSCs in Jie et al.'s investigation [25]. Also, contrary to our results, some articles reported the effect of exosome alone [15, 16, 28].However, in line whit our results, the combination of MSCs, exosomes, and mesalazine has shown promising potential in the treatment of colitis [29–31]. This combination may have a synergistic effect in treating colitis.

The regenerative effects of MSCs and their paracrine signaling mechanisms, including the secretion of growth factors, cytokines, EVs, and exosomes, contribute to the improvement of tissue damage in the colon. These mechanisms promote tissue repair, angiogenesis, and the survival and regeneration of colon epithelial cells [32, 33].

Also, according to our test results, the treatment groups who received MSC either alone or in conjunction with mesalazine and exosomes had higher levels of cytokine IL-10 in the colon and a higher percentage of regulatory T cells (Tregs) in the spleen. When compared to the groups that received exosomes either alone or in conjunction with mesalazine, this increase was more notable. Due to their immunosuppressive characteristics Tregs can help control immune responses, lower inflammation, and preserve intestinal homeostasis. MSCs can control acquired immune cells, which can change the milieu to one that is Treg-rich. Paracrine substances like nitric

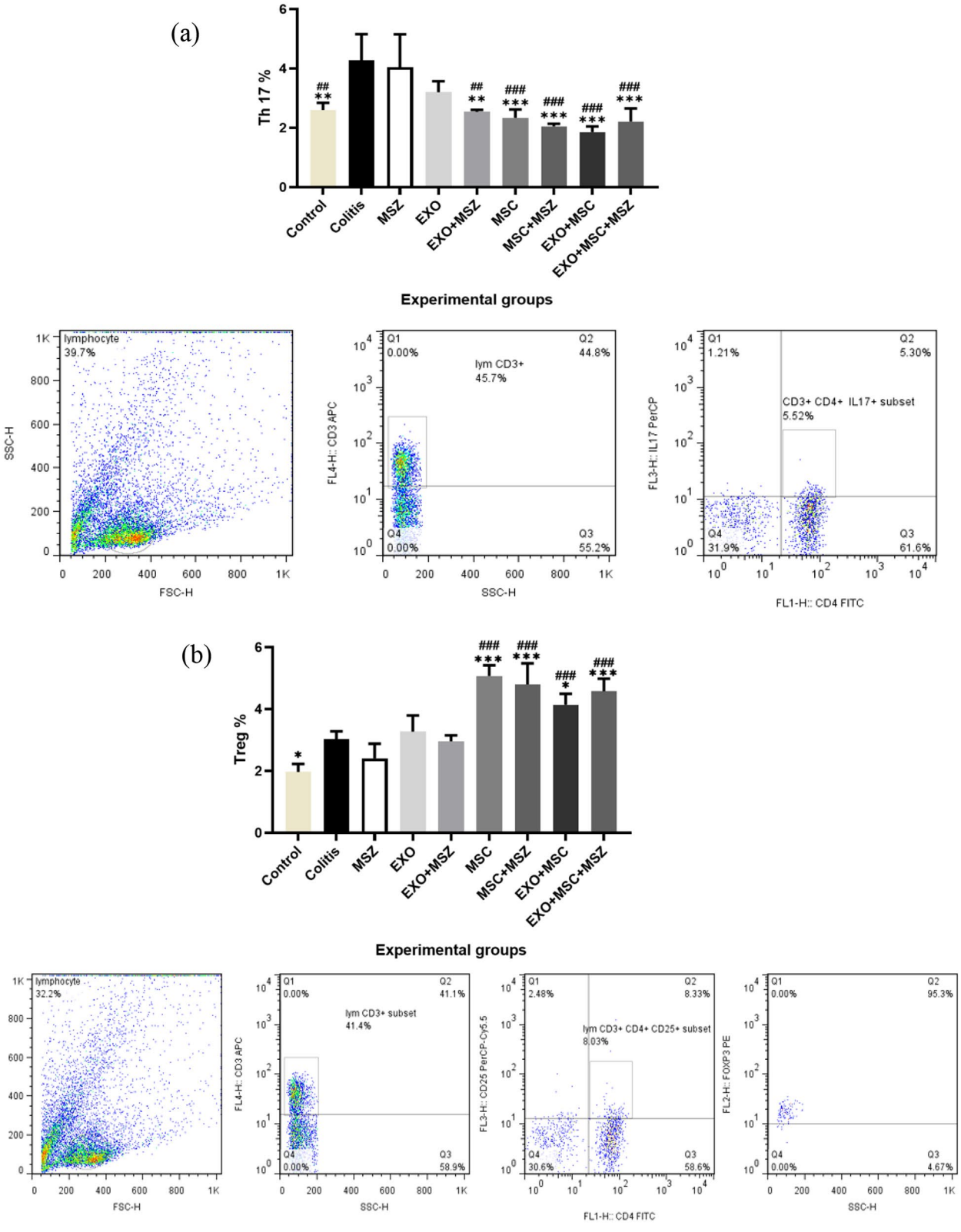

Fig. 7 T cell subset's analysing in the Mice' spleen. (**a**) Flow cytometry analysis of Th17 cells. (**b**) Flow cytometry analysis of Treg cells. (*: $p < 0.05$. **: $p < 0.01$. *** in comparison with colitis group & $p < 0.001$. #: $p < 0.05$. ##: $p < 0.01$. ###: $p < 0.001$ in comparison with Mesalaszine group). Abbreviations: MSCs; mesenchymal stem cells, EXO: Exosome, MSZ: Mesalazine

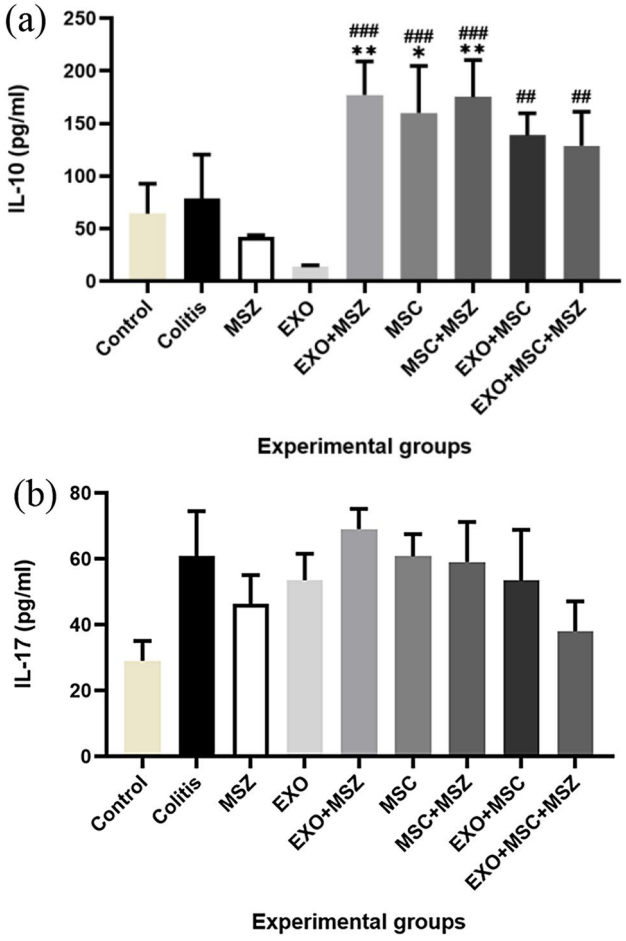

Fig. 8 The cytokine production levels of small intestine cells in experimental groups. (**a**) The IL-10 levels in experimental groups. (**b**) The IL-17 levels in experimental groups. (*: $p < 0.05$. **: $p < 0.01$. *** in comparison with colitis group & $p < 0.001$. #: $p < 0.05$. ##: $p < 0.01$. ###: $p < 0.001$ in comparison with Mesalaszine group). Abbreviations: MSCs; mesenchymal stem cells, EXO: Exosome, MSZ: Mesalazine

oxide (NO), HGF, PGE2, TGF-β, and indoleamine 2 and 3 dioxygenases (IDO) are secreted in order to achieve this [34, 35]. IL-10 is an anti-inflammatory cytokine that can further contribute to the suppression of inflammatory processes. The upregulation of Tregs and IL-10 production by MSCs contribute to the amelioration of colonic inflammation and reduction in inflammatory cytokines such as IFN-γ and IL-17 [36, 37].

Additionally, the tests showed a decrease in the percentage of Th17 cells in the spleen and a decrement in the amount of IL-17 in the colon in the treatment groups. This decrease in Th17% was more significant in the groups receiving MSC alone or in combination with exosomes and Mesalazine, or the group receiving exosomes and Mesalazine, compared to the groups receiving exosomes alone. Th17 cells and IL-17 are associated with pro-inflammatory responses, and their reduction suggests a dampening of the inflammatory processes in the treated groups. Furthermore, MSCs can inhibit Th17 and Th1 responses while promoting Treg and Th2 responses. This modulation of T-cell responses is achieved through various mechanisms, including the downregulation of IL-2 receptor expression and the inhibition of the IL-2 signalling pathway. MSCs also produce NO and IDO, which suppress T cell proliferation [38].

Evidence shows that exosomes-derived from MSCs have immunomodulatory properties. Exosomes can also directly provide functional mitochondria to intestinal epithelial cells, further enhancing their energy metabolism [39]. Moreover, the presence of miR-326 in MSC-derived exosomes has been shown to inhibit demyelination and alleviate IBD in a mouse model. This further highlights the potential of exosomes in modulating inflammatory processes and promoting tissue repair [40].

Conclusion

In summary, our findings suggest that MSCs and their derived exosomes could be utilized as a standalone treatment or as an adjunct therapy alongside standard drugs for IBD patients. Colon tissue repair and regeneration, promoting mucosal healing, and immunomodulation are some MSC's mechanisms to achieve this goal. The combination therapy may provide enhanced therapeutic benefits compared to using exosomes alone. More studies and experiments are required to confirm these results and determine the optimal treatment protocols for using MSCs and exosomes in IBD treatment.

Abbreviations

IBD	Inflammatory bowel disease
MSCs	Mesenchymal Stem Cells
UC-MSCs	Umbilical Cord-Derived Mesenchymal Stem Cells
DSS	dextran sulfate sodium
Treg	T regulatory cells
UC	ulcerative colitis
CD	Crohn's disease
NK	Natural killer cells
EVs	Extracellular vesicles
MSC-EXo	Exosomes derived from mesenchymal stem cells
5-ASA	5-aminosalicylic acid
H-UC	Human umbilical cord
DLS	dynamic light scattering
TEM	transmission electron microscopy
MSZ	Mesalazine
H&E	Hematoxylin and Eosin
DAI	Disease Activity Index
NO	nitric oxide
IDO	indoleamine 2 and 3 dioxygenases
TJs	tight junctions

Supplementary Information

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Supplementary Material 1

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Author contributions
The project was designed and overseen by Prof J.TA and M.kh who also approved the quality control of cells and exosomes before injection. They played a critical role in reviewing and revising the final manuscript. F.KH and SF.R were equally and mostly in charge of cellular and exosomal extraction, characterization, and culture. They also contributed to all animal processes, conducting laboratory tests, collecting and analyzing data, and drafting the initial version of the manuscript. Dr N.KF, E.N, MA.KH and M.H equally participated in animal handling, exosome extraction and characterization, cell cultures, and data analysis. SA-E, S.SN, and A.F contributed to the study design, manuscript revision, and final approval. All authors have read and given their approval for the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
This study was performed following the Declaration of Helsinki Ethical Principles and approved by the Ethics Committee of the Mashhad University of Medical Sciences, Mashhad, Iran (IR. MUMS. RES.1401.133). (1) Title of the approved project: Evaluation of the effectiveness of umbilical cord mesenchymal stem cells along with the synergistic effect of the Exosomes derived from them on the immune response of an IBD mouse model. (2) Name of the institutional approval committee: the Ethics Committee of the Mashhad University of Medical Sciences, Mashhad, Iran. (3) Approval number: IR. MUMS. RES.1401.133. (4) Date of approval: 06/08/2022. After filling out the informed consent letter, human umbilical cords (H-UC) were obtained from Imam Reza hospital from full-term pregnancy mothers.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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