Original Article

Human Umbilical Cord Mesenchymal Stem Cells-Derived Exosomes Can Alleviate the Proctitis Model Through TLR4/NF-Kb Pathway

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Abstract

Background: Proctitis is a significant concern of inflammatory bowel diseases, especially ulcerative colitis. Exosomes are a new method for treating many diseases by their immunosuppressive and tissue-repairing potential. Here, we tried Mesenchymal stem cells (MSCs)-derived Exosomes for treating the proctitis model of rats.

Materials and Methods: Rats were assigned into four groups: sham, control group, rectal, and intraperitoneal exosome injection. The proctitis model was induced by rectal administration of 4% acetic acid. The exosome was derived from human MSCs isolated from human umbilical cords. After seven days, rectum samples were assessed for histopathological, IHC, and PCR analysis.

Results: The histopathologic scores, collagen deposition, and the expression of NF-κB, TLR4, TNFα, IL-6, and TGFβ were decreased in intraperitoneal exosome compared to controls. The result was not promising for the rectal administration of exosomes.

Conclusion: Exosomes can suppress the inflammatory response in the proctitis model and improve the rectum's healing process. Exosomes can inhabit the NF-κB/TLR4 pathway and downstream pro-inflammatory cytokines. This study implicates the therapeutic benefits of exomes in treating proctitis.

Keywords: Exosome, Mesenchymal stem cells, Proctitis, Inflammatory bowel disease

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Introduction

Proctitis, an inflammatory ulceration in the rectum, is rising from different etiology including inflammatory bowel disease (IBD), especially Ulcerative colitis, radiation, and microbial infection. Ulcerative colitis (UC) is classified depending on the area of involvement, which is called proctitis when the inflammation is limited to the rectum level. The prevalence of proctitis in UC patients has been reported to be about 30-60% (1). Approvable proctitis treatments are corticosteroids, 5-aminosalicylates, Thiopurines, and biological agents. Some patients will

eventually need a surgical procedure, which is invasive with multiple complications. In addition to the side effects of conventional therapeutic methods, it is not always helpful for the patients and keeps them in remission. Long-lasting active disease in patients is associated with decreasing quality of life and increased risk of colorectal cancer (2).

In recent decades, stem cell therapy has been developed for treating many diseases, including IBD (3). Tissue regeneration and immune response regulation features of stem cells make them a good candidate for treating IBD. Meanwhile, the therapeutic effect of MSC-exosome has been reported in DSS-induced colitis in mice (4).

Exosomes are small cell-derived vesicles of 30-150 nm secreted by various immune cells. Before exocytosis, exosomes reside in multi-vesicular bodies as intraluminal vesicles. Exosomes carry various molecular constitutions of their cell of origin, including protein, lipid, and nucleic acid (mRNA, microRNA) (5). They can be found in most bodily fluids, including blood, urine, saliva, amniotic fluid, breast milk, and most cell types (6). Exosomes were initially thought to serve as garbage bags for cells to eliminate unwanted constitutions. However, an increasing body of evidence has demonstrated that exosomes play an important role in cell-to-cell communications. In a recent study, it was indicated that the presence of Enterotoxigenic *Bacteroides fragilis* (ETBF) and its mechanism of inhibiting exosome-packaged miR-149-3p is closely linked to the occurrence of inflammatory bowel disease (IBD), colorectal cancer, and colitis-associated colorectal cancer (7). Therefore, exosome seems necessary for regulating inflammation and immune response in the intestine. Many functions have been related to the exosome, which the regulating of the TLR4/NF-κB pathway is one that can enumerate (8, 9).

Therefore, here, we aimed to treat the acetic acid-induced proctitis model in rats by mesenchymal stem-derived exosomes and evaluate the possible involvement of the TLR4/NF-κB pathway.

Methods

Human umbilical cord-derived mesenchymal stem cells isolation and characterization: Umbilical cords were obtained from healthy infants of gestational age (39-40 weeks) delivered by cesarian section under sterile conditions in Imam Khomeini Hospital. It took place with the mothers' consent and in agreement with the Ethical protocol provided by the Ethical Committee of the Shahid Beheshti University of Medical Science. Umbilical cords were immediately put into Phosphate-buffered saline (PBS) supplemented with a cocktail of antibiotics (300 U/ml Penicillin, 300 µl/ml Streptomycin, 150 µg/ml Gentamycin, and one µg/ml fungizone; Sigma Aldrich, USA) and processed within 6-12h. Umbilical cords were washed twice with EBSS (Thermo Fisher Scientific, USA) containing 100 U/ml heparin. Subsequently, Umbilical veins were filled with Medium199 (M199) (Sigma, USA) enriched with 100U/ml heparin and an antibiotic cocktail. Then, Umbilical cords were incubated at 37ºC for 20 minutes. Afterward, Umbilical veins were washed by EBSS and gently massaged to extract suspension of endothelial and subendothelial cells. The acquired supernatant was centrifuged at 600 g for 10 min, and precipitates were resuspended in Dulbecco's Modified Eagles Medium (DMEM) (Sigma, USA) containing antibiotic cocktail (100U/ml penicillin and 100µg/ml Streptomycin) and 15% PBS. Then, cells were cultured in 25 cm^2 tissue flasks (Nunc A/S, Denmark) at 103 cells/cm2 concentration and incubated at 37ºC in a 5% CO_2 atmosphere. The medium was changed every three days to eliminate unattached cells for two weeks. Fibroblastoid cells were detached from the flasks with a solution of 0.05% Trypsin (Sigma-Aldrich, USA) and 0.02% EDTA (Sigma-Aldrich, USA). Harvested cells were dyed with Phycoerythrin (PE) or Fluorescein Isothiocyanate (FITC) conjugated antibodies: anti-CD29-PE, anti-CD73-FITC, and anti-CD105-PE. The non-specific antibody-anti Aspergillus niger glucose oxidase was added as a negative control to exclude non-specific cells. Subsequently, cells were incubated at 4ºC in the dark for 45 minutes. A flow cytometer (BD FACSort™ system, USA) was recruited to analyze the acquired data.

Exosome isolation and characterization: Umbilical cord-MSCs were cultured to 80% confluency and then cultured in a serum-free DMEM for 48 hours. The acquired supernatant was centrifuged at 300 g for 10

min, 2000 g for 10 min, 10,000 g for 30 min, and 100,000 g for 70 min to eliminate cell debris and large particles. Then, the participant was filtered through a 0.22 µm filter to exclude bigger vesicles. The obtained exosome pellets were washed with PBS and centrifuged at 100,000 g for 70 minutes to eliminate contaminating proteins, and the purified exosomes were stored at -70ºC. The characterization of MSC-derived exosomes was published in our previous study (10). The diameter of 30-150 nm and saucer-shaped nanovesicle, assessed by TEM, were in concordance with the exosome. The flow cytometry results confirmed the presence of CD63 and CD81 markers.

Animals and ethics: All procedures were performed according to NIH guidelines for Laboratory Animal Care and Welfare (NIH Publication No.85-23, revised in 1985) and Animal Care Committee (IACUC) and were approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences (Ethical number: IR.SBMU.MSP.REC.1400.360).

Twenty-four Wistar rats (weighing 250 ± 20g) were acquired from the Pasteur Institute of Iran (PII). During the study, rodents had unlimited access to fresh water and standard chewing pallets. Animals were randomly allocated into four groups:

- The control group
- The rectal administration of the exosome (rectal-exo) group
- The intraperitoneal administration of the exosome (IP-exo) group
- The sham group which received no treatment

Proctitis model and treatments: 4% acetic acid solution (Thermo Scientific™, US) was administered to induce the colitis model in the rectums of all rats except the sham group. All rodents were deprived of food 24 hours before the intervention but had access to fresh water. Rats were anesthetized with Ketamine (*IP*, 87mg/kg, Alfasan, the Netherlands) and Xylazine (IP, 13mg/kg, Alfasan, the Netherlands). Afterward, 2 ml of the acetic acid solution was infused intrarectally to induce proctitis in control and treated groups. All animals were kept in the Trendelenburg position for 30 seconds. to prevent fluid leakage. Next, the acetic acid was removed from the rectum. Treatments started one hour following colitis induction. 400 µg exosome was administered intraperitoneally in the IP-Exo group, and the rectal-Exo group received rectal administration of exosomes. The control group received intraperitoneal administration of PBS.

Sample acquisition: All animals were euthanized seven days after the operation. For sample acquisition, rats were first anesthetized with Ketamine (87mg/kg, i.p) and Xylazine(13mg/kg). The abdomen was shaved using an electronic razor and cleaned with a povidone-iodine solution. Then, an abdominal incision was made, and the rectum was excised. After the macroscopic assessment, samples were cut longitudinally, irrigated with normal saline, and divided into two similar pieces; one piece of each sample was immediately put into liquid nitrogen at -70ºC and then kept at -80ºC for RNA extraction; the other piece was put into 4% formaldehyde solution for histopathological assessments. Rodents were euthanized with an i.p injection of 300 mg/kg Ketamine and 30mg/kg Xylazine.

Histopathological assessments: The rectum samples were kept in a 4% formaldehyde solution, embedded in paraffin wax, then cut into 4 µm slides by a microtome. Half of the samples in each group were dyed with hematoxylin and Eosin stain, and the other half were stained with Masson's trichrome staining per manufacturers' protocol. A single pathologist blinded to the study assessed all samples with a light microscope (Olympus, Japan). Pathological parameters were scored according to Table 1 (11).

Immunohistochemistry (IHC): The protein expression of IL-6 was assessed with Immunohistochemistry techniques. Tissue samples were kept in a 4% formaldehyde solution and subsequently fixed in paraffin blocks. Then, specimens were cut into 4 µm slides and deparaffinized with xylene solution. Afterward, samples were washed and rehydrated with gradient concentrations of Ethanol and normal saline solutions. Then, samples were incubated in 10% normal goat serum (ab7481, Abcam, Cambridge, United Kingdom) for 30 minutes at 37ºC to block non-specific antibody binding sites. Afterward, specimens were incubated in rabbit anti-IL-6 antibody (1:50, ab208113, Abcam, Cambridge, United Kingdom) solution at 4ºC for 24 hours and washed with PBS four times. Then, specimens were

Table 1: The histopathological scoring system.

Inflammatory severity	Inflammation extent	Tissue regeneration	Crypt damage	Tissue involvement
None	None	Complete regeneration and normal tissue	None	None
Slight	Mucosa	Almost complete	Basal 1/3 damage	1-25%
Moderate	Mucosa and Submucosa	Regeneration with crypt depletion	Basal 2/3 damage	26-50%
Severe	Transmural	Surface epithelium not intact	Only surface epithelium intact	51-75%
None	None	No tissue repair	The entire crypt and epithelium lost	76-100%

incubated in one hour of 1:1000 diluted goat anti-rabbit antibody (ab6721, Abcam, Cambridge, United Kingdom) solution. Samples were dyed with DAB (3,3'-diaminobenzidine) for developing signals, and subsequently, the nucleus was stained with hematoxylin. All samples were assessed by a light microscope (Olympus, Japan) and analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA, SCR_003070).

Real-time polymerase chain reaction (RT-PCR): Gene expression levels of TLR4, NF-κB, TNFα, TGFβ, IL-10, and SOD1 were determined using RT-PCR techniques. The precise method of rt-PCR was described in our previous research (12). In brief, the total RNA of samples was extracted with Trizol reagent (Sigma Aldrich, USA), and DNase1 was added to samples to eliminate possible DNA contaminations. Then, a commercial kit (Thermo Fisher Scientific, Lithuania) was consumed to generate cDNA. Reverse transcription was performed on cDNA in the thermocycler condition. The designed primers are mentioned in Table 2, and GAPDH was assigned as the housekeeping gene to normalize target gene expression. The relative threshold cycle (CT) was applied to measure gene expression levels and analyzed using the $2^{-\Delta Ct}$ method.

Table 2: Gene Primers. Abbreviations: Tumor necrosis factor alpha, TNFα; Nuclear factor kappa b, NF-Ƙb; Tissue Growth Factor β1: TGF-β1; Toll-like receptor 4: TLR4; Interleukin 10: IL 10; Super oxide dismutase: SOD.

Gene name	Primers (Forward and reverse)
TLR4	GACCTCAGCTTCAATGGTGTC
	TCAAGCCAAGAAATATGCCATC
NF-KB	TTCCCTGAAGTGGAGCTAGGA
	CATGTCGAGGAAGACACTGGA
TNFα	GAAAGCATGATCCGAGATGT
	CAGGAATGAGAAGAGGCTGA
TGF-β1	GCTAATGGTGGACCGCAACAAC
	CACTGCTTCCCGAATGTCTGAC
IL-10	GCAGACAAACAATACGC
	ACTTGCCCTCATCCC
SOD1	AGCTGCACCACAGCAAGCAC
	TCCACCACCCTTAGGGCTCA

Statistical analysis: We recruited GraphPad Prism (SCR_002798, version 9.4.1, San Diego, USA) to analyze the acquired data. Quantitative data are expressed as mean ± SD. One-way analysis of

Table 3: The histopathological score in sham, control, rectal-exo, and IP-exo groups. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control group.

	Inflammatory severity (0-3)	Inflammation extent (0-3)	Tissue regeneration (0-4)	Crypt damage (0-4)	Tissue involvement (1-4)	Total index (0-18)
Sham	0 (0-0)***	0 (0-0)***	0 (0-0)***	0 (0-0)***	1 (1-1)***	1 (1-1)***
Control	3 (3-3)	3 (3-3)	4 (3-4)	4 (3-4)	4 (3-4)	18 (16-18)
Rectal-Exo	2 (1-3)	2 (1-3)	2 (1-4)	2 (1-4)	2 (1-3)	11 (6-15)
IP-Exo	1 (0-1)*	1 (0-2)*	1 (1-3)	1 (0-3)*	1 (1-1)**	5 (3-6)*

Variance (ANOVA) was performed to compare the data, followed by Tukey's post hoc test for multiple compression. Histopathological scores were analyzed using the Kruskal–Wallis test. Significance was ascribed when probability values < 0.5.

Results

Histopathologic assessment: The histopathological scores of rectum samples are summarized in Table 3. While the rectal administration of exosome decreased the histopathological scores of rectum samples compared to the control groups, the difference between these two groups was insignificant (*P* for total index= 0.4799). Meanwhile, the IP administration of exosomes improved the pathological score significantly compared to controls (p-value for total index= 0.0243) (Figure 1).

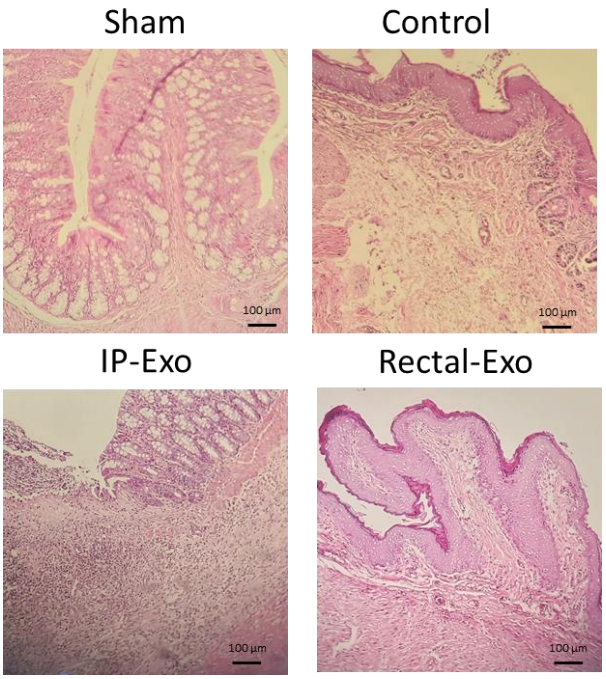

Figure 1. The H&E staining of the rectum samples.

Masson's trichrome staining was performed to assess the collagen deposition in the rectum tissue (Figure 2). The mean collagen diameter was decreased in both rectal (P=0.0006) and IP (P<0.0001) administration of exosome compared to controls. However, it was lower in the IP-Exo group than the rectal-Exo group (P=0.0158). The difference between sham and IP-exo groups was insignificant (P= 0.8781).

Immunohistochemistry assessment: The expression levels of IL-6 in rectum tissue were compared among the groups (Figure 3). The results declared a significant increase in IL-6 expression in the control group compared to the sham ($P < 0.0001$). Although the IL-6 levels were decreased after rectal administration of exosome compared to the control (P = 0.0012), IL-6 levels in the rectal-Exo group were still significantly higher than in the sham group (P = 0.0004). The response to IP exosome has shown a noteworthy decrease in the rectum's IL-6 level compared to controls ($P < 0.0001$). Meanwhile, the Il-6 levels in the IP-Exo group were not significantly different from the sham group (P= 0.5011).

Inflammatory and anti-inflammatory markers gene expression: The result of rt-PCR showed increased levels of inflammatory markers, including NF-κB (P=0.0075), TLR4 (P=0.0136), TNFα (P=0.0025), and TGFβ (P=0.0337) in controls compared to the sham. TLR4 (P=0.0398), NF-κB (P=0.0354), TGFβ (P=0.0455), and TNFα (P = 0.0078) had a significant decline only in the IP exosome group compared to controls. In addition, IL-10, an anti-inflammatory marker, and SOD-1, an anti-oxidative enzyme, were increased significantly in the IP exosome group compared to all groups. The results are mentioned in Figure 4.

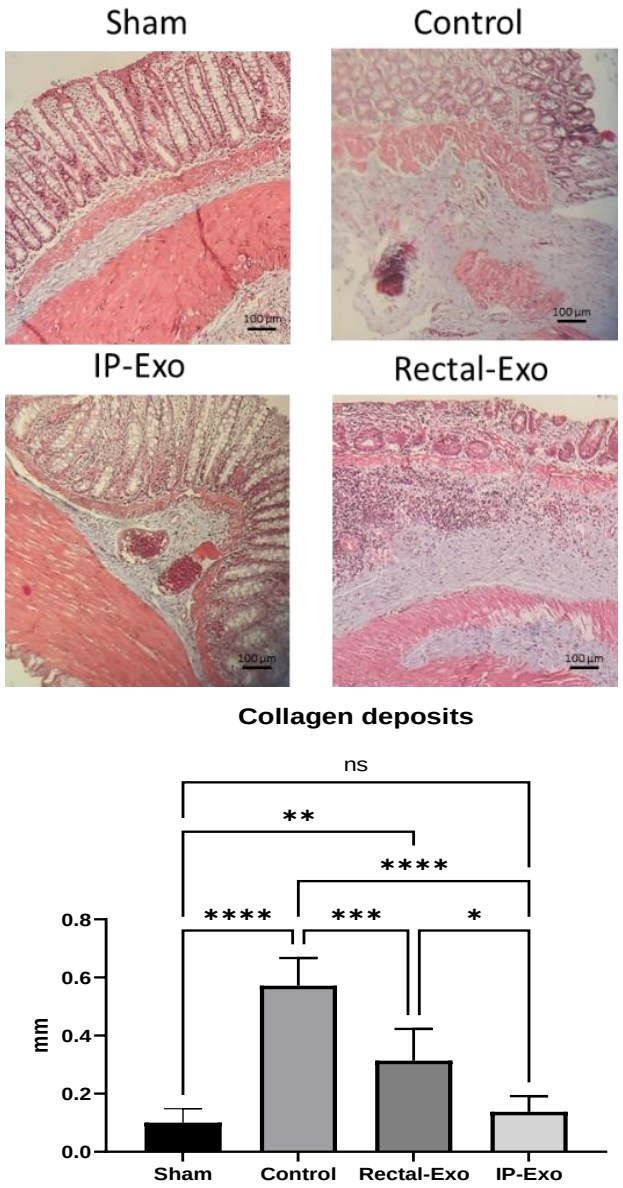

Figure 2. The trichrome staining of the rectum samples (a), and the diameter of collagen deposition (b). Bars represent means ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P* <0.0001 are statically significant.

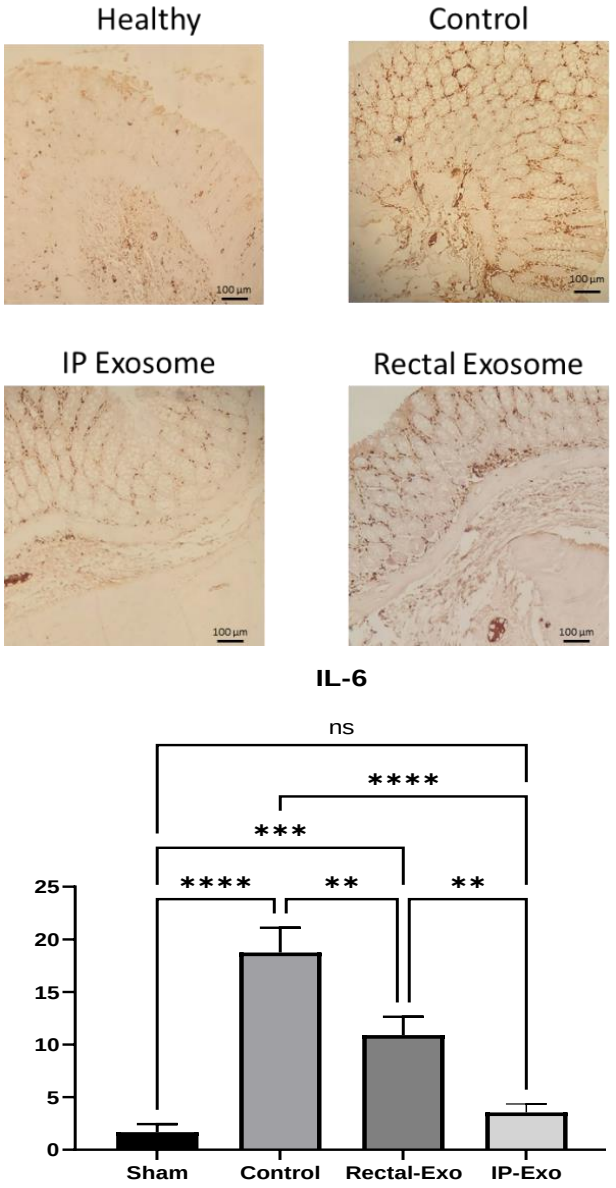

Figure 3. The IHC staining of IL-6 in the rectum (a) and the expression levels of IL-6 in the rectum (b). Bars represent means ± SD. ** *P*<0.01, *** *P*<0.001, and *****P* <0.0001 are statically significant.

Discussion

Our acetic acid-induced proctitis rats had shown ulceration, inflammation, necrosis, and neutrophil infiltration in their rectum. Previous studies on colorectal disease models administered exosomes by intraperitoneal or intravenous injection (13-17). We investigated the effect of local administration of exosomes to compare it with the effect of systemic or IP administration of exosomes on proctitis. The group with IP administration of umbilical cord-MSC-exosome showed dramatic responses to the treatment. The healing, anti-inflammatory, and anti-oxidative features of MSC-exosome showed a healing function in proctitis models. However, the rectal administration of the exosome was not as successful as IP administration in the histopathological and molecular tests. IP administration of exosomes seems more efficient due to systemic accessibility to immune cells and regulating cell-cell communication. However,

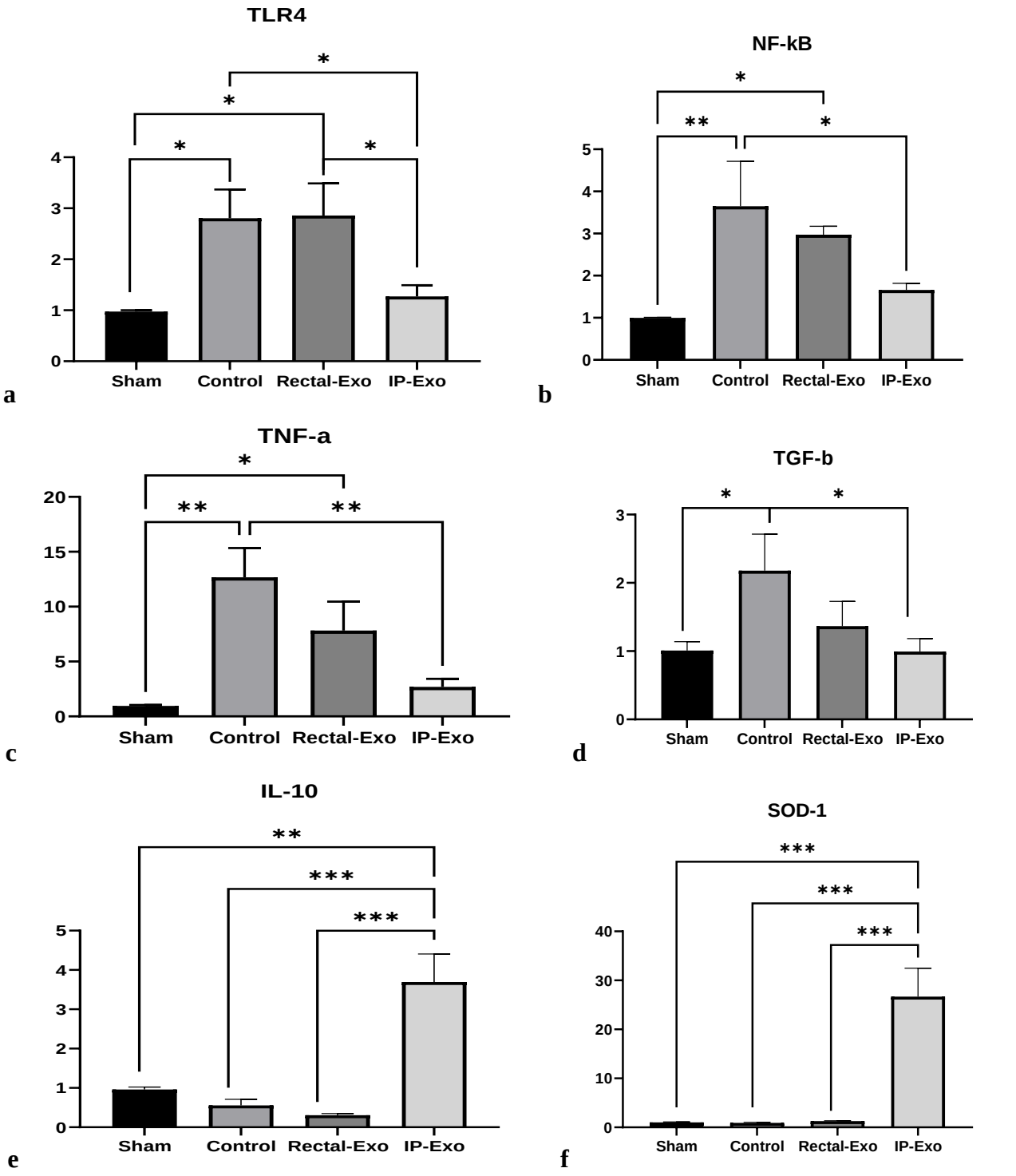

Figure 4. The IHC staining of IL-6 in the rectum (a) and the expression levels of IL-6 in the rectum (b). Bars represent means ± SD. ** *P*<0.01, *** *P*<0.001, and ****P <0.0001 are statically significant.

local administration of exosomes is limited to the immune responses in the rectum tissue and cannot inhibit systemic inflammation properly.

IL-6, a pro-inflammatory cytokine, has been shown to have an essential role in active and uncontrolled IBD, which can activate macrophage and

t cells' immune responses. Therefore, IL-6 can be considered a marker of active inflammation in the intestine (18). The results showed that exosome treatment decreased IL-6 levels in the rectum and reduced inflammation. In addition, the TGFβ, which increases in response to IL-6 stimulation (19), was decreased in our IP exosome group. The increased level of TGFβ has been shown in patients with IBD (20, 21), and it has been shown that TGFβ can increase collagen production and develop fibrosis in IBD (22). In addition to the TGFβ decrement, our treatment with exosomes also decreased collagen deposition, which can prevent fibrosis formation.

Treatment of proctitis rising from different etiology can be challenging for therapists. Especially proctitis in the IBD background, drug resistance, and relapse are common complaints. The long-term side effects prevent clinicians from using corticosteroids as maintenance therapy in IBD. Anti-TNF agents also have drawbacks; conditions such as heart failure, active infections, and cancer can be exacerbated and limit the use of biological agents (23). On the other hand, there is a risk of relapse by discontinuing the treatment. The annual risk of relapse is reported to be 36%, and after 16 months, nearly half of the patients have worsened their conditions (24). In addition, some patients become resistant to anti-TNF and require a higher dosage, probably due to anti-drug antibodies that inhibit the drug effect and elevate serum clearance (25, 26). Therefore, new treatments are desirable to keep the patient's condition in remission for longer.

As the consequence of proctitis is a significant concern in patients, we tried to assess the new method for treating the proctitis model. The potential of MSC and the MSC-derived exosome has been shown in treating IBD (27, 28, 4). Exosomes are cell-derived vesicles that contain cytokine, RNA, and DNA, which secret and alter cells' function. They are produced by merging multiple endosomal vesicles and acting by paracrine signaling to other cells. The transmitting information of exosomes can be various depending on their origin cells.

Recently, studies have shown that exosomes can be a regulatory key for TLRs (29). Exosomes, as the cell-to-cell messenger, can activate or inhibit the TLRs, depending on their nature. The previous study on cerebral ischemic injury showed that MSC-derived exosome miR-542-3p could inhabit TLR4, preventing the glial cell's inflammatory response (8). Another study showed that MSC Exosomal miR-146a could alleviate the inflammatory response and decrease IL-6, IL-1β, and TNFα in the diabetic retinopathy model by suppressing the TLR4/NF-κB pathway (9).

Toll-like receptors (TLR), the critical points of the innate immune system, proved to participate in a wide range of inflammatory diseases (30). TLRs showed vital roles in intestinal inflammations, and previous research showed that TLR4 is expressed in enterocytes and intestinal mucosa. By activating the NF-κB pathway, TLR4 initiates the inflammatory cytokines released from enterocytes such as TNFα, IL-1, IL-6, and IFNγ. TLR4-/- mice and MyD88-/- (a TLR4 downstream molecule) showed decreased acute inflammatory cells and epithelial proliferation in the UC model. The results indicated that the TLR4/NF-κB pathway is crucial for intestinal response to injury, bacterial infection, and developing UC (31). Also, Previous research on the ulcerative colitis model in mice showed that inhibiting the TLR4/NF-κB pathway by Xianglian Pill attenuated UC in mice (32). The meta-analysis of TLR4 polymorphism showed that there are types of TLR4 polymorphism that can increase the risk of developing IBD significantly (33). Our result also indicated that the exosome administration decreased TLR4/NF-κB expression and its downstream inflammatory cytokines, TNFα, TGFβ, and IL-6. Suppressing the TLR4/NF-κB expression after IP administration of exosome suggests that TLR4 can be one of the critical pathways in proctitis, which can be targeted by MSCs-derived exosome.

Future studies can focus on further elucidating the exact mechanism of exosomes through the TLR4/NF-κB pathway and their regulation of immune responses in the treatment of proctitis and inflammatory bowel diseases. Evaluating exosomes in TLR4 knockout models can provide valuable insights into the underlying molecular pathways involved.

Conclusion

In conclusion, our study investigated the effects of local administration of exosomes compared to IP administration on proctitis in rats induced by acetic acid. While the IP administration of umbilical cord

MSC exosomes showed dramatic responses in terms of healing, anti-inflammatory, and anti-oxidative features, the rectal administration of exosomes was less successful in histopathological and molecular tests. This finding suggests that the IP administration of exosomes is more efficient due to its systemic accessibility to immune cells and regulation of cell-cell communication.

Acknowledgment

None.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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