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Evaluation of Inhibitory Effect of Abatacept (CTLA4-ig) and Conditioned Medium of Mesenchymal Stem Cell in an Acetic Acid-induced Mouse Model of Acute Colitis

Manizhe Faghih[1,2], Mona Moshiri[1,2], Fatemeh Ahmadzadeh[1,2], Maryam Ghasemi[2,3], Saeid Abediankenari[1,2*]

1. Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.

2. Immunogenetics Research Center, Mazandaran University of Medical Sciences, Sari, Iran.

3. Department of Pathology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.

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ABSTRACT

An individual with a genetic predisposition to inflammatory bowel disease (IBD) can experience inflammatory responses leading to conditions such as Crohn's disease (CD) or Ulcerative colitis (UC). Currently, stem cell therapies, particularly those utilizing mesenchymal stem cells (MSCs), are gaining attention due to their immunomodulatory properties, as demonstrated in clinical trials. Consequently, we decided to investigate the effects of mesenchymal stem cells-conditioned medium (MSC-CM) and Abatacept in an experimental model of acute colitis. MSC-CM was extracted from female BALB/C mice and stored for future use. Acute colitis was induced in BALB/C mice through the intrarectal administration of 100 µL of 4% acetic acid. Following this procedure, CM and Abatacept were administered intraperitoneally. Throughout the study, various parameters were monitored, including changes in body weight, bleeding, stool consistency, disease activity index (DAI), mortality rate, as well as the weight and length of the colon. Histopathological analyses were also conducted, along with monitoring changes in the levels of IL-10 and IFN-γ. The data collected are presented as mean ± SD and were analyzed using One-Way ANOVA. According to the results of the study, CM with and without Abatacept significantly reduced weight loss and bleeding as well as improved fecal consistency and DAI. Macroscopic examination of the colon showed that after infusion, colon length was reduced and histopathological analysis showed a decrease in mucosal changes. The secretion of IL-10 was increased while the IFN-γ level was reduced. Research indicates that the immunomodulatory properties of MSC secretion can have positive effects. We propose a combination therapy with MSC, which we believe could lead to improved outcomes in the treatment of acute colitis.

Keywords: Colitis, acetic acid, Abatacept, mesenchymal stem cell

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*Corresponding Saeid Abediankenari
Address: Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
E-mail: abedianlab@yahoo.co.uk

Introduction

Crohn's disease and ulcerative colitis are among the chronic diseases related to the immune system, the etiology of which is not completely known and are among the IBD diseases (1, 2). Ulcerative colitis is characterized by inflammation, damage to the intestine, and destruction of the lining and submucosa of the large intestine (3). Research in this area suggests that changes in gastrointestinal immune system tolerance may be responsible for this disease (4). Although the pathogenesis of IBD is still unclear, genetic and environmental factors such as smoking, non-steroidal anti-inflammatory drugs, and stress can play a role (5, 6). The infiltration of neutrophils and macrophages into the mucosal tissue of the large intestine is indeed a key characteristic of ulcerative colitis. These activated neutrophils produce reactive oxygen species, including superoxide ions, hydroxyl radicals, and hydrogen peroxide. These substances can affect the expression of inflammatory cytokine genes and enzymes involved in inflammatory responses, leading to the destruction of the intestinal structure. This can result in wounds, bleeding, and diarrhea (1, 7). Various chemicals are used to induce colitis, including Dextran sulfate sodium (DSS), 2,4,6-Trinitrobenzene sulfonic acid (TNBS), Oxazolone, indomethacin, and acetic acid. Injecting diluted acetic acid into the rectum is a by-product of induction of mucosal epithelial damage that mimics some of the features of ulcerative colitis. In this model, the injury is associated with epithelial necrosis and edema that penetrates the intestinal mucosa based on the concentration and duration of exposure to acetic acid. The advantages of this method are lower cost and easier induction of the disease (8-11). The importance of the CD28 stimulus signal in T cell function has made it a suitable target for drugs to modulate T effector and Treg function. Initial attempts to find a soluble CD28 protein that blocks the stimulus signal were ineffective for its ligand due to the low affinity of CD28, but in contrast to that, the soluble CTLA-4, which binds to the Ig constant by binding to CD80, CD86, and block the binding of these ligands has been very effective (12, 13). Both animal studies and clinical trials have demonstrated the effectiveness of Abatacept (CTLA4-Ig) in treating conditions such as rheumatoid arthritis, type 1 diabetes, spondyloarthropathies, lupus, and in kidney transplantation scenarios (12, 13). Mesenchymal stem cells (MSCs), known for their immune-regulating and inhibitory properties, are seen as a promising treatment option for inflammatory diseases. These cells can be activated in the target region by IFN-γ, TNF-α, and IL-1B. Subsequently, immunosuppressive factors such as indoleamine 2, 3 dioxygenase, PGE2, and NO trigger a shift towards M2 polarization and the induction of DC (14-16). A key function of these cells is to maintain equilibrium between Treg and Th1 / Th17 cells. They also aid in tissue repair and secrete regulatory factors like IL-4, IL-10, IL-11, IL-13, and TGF-B. The final outcome is influenced by the appropriate cell in the target area and the administration method, which includes factors like route, concentration, injection protocol, and pre-treatment with chemokine or cytokine (17-19). Some studies have used the secretome of MSCs instead of the whole cell to overcome the cell's low homing ability and enhance the therapeutic effect. This approach has shown better capability in reducing the Disease Activity Index (DAI), nitrite levels, and histological score. It also decreases inflammatory cytokines, improves the epithelial barrier, and shifts M1 to M2 with reduced levels of CXCL9, MCP-1, and iNOS but increased expression of CCL1, IL-10 (20). Given the role of abatacept (CTLA4-Ig) and mesenchymal stem cells (MSCs) in treating autoimmune and autoinflammatory diseases, they are anticipated to significantly improve the symptoms of

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inflammatory bowel diseases like ulcerative colitis. Therefore, this study aims to investigate their role in modulating the immune system and alleviating disease symptoms.

Materials and methods

Animals

BALB /C female mice weighing about 18-25 g aged 6 to 8 weeks were obtained from the laboratory animal care center of Mazandaran University of Medical Sciences, Sari, Iran. They were kept in cages for two weeks before the start of the experiment and had adequate access to water and food. All stages of the experiment were conducted in accordance with the rules of the ethics committee (Code of Ethics: IR. MAZUMS. REC. 1400. 9137).

Isolation and characterization of MSCs

For this experiment, BALB /C mice aged between 6 to 8 weeks were humanely euthanized by cervical dislocation. The surface of the animal's body was sterilized using 70% ethanol. The femur and tibia were surgically extracted and placed on ice in a DMEM culture medium with penicillin /streptomycin, after the removal of muscle and soft tissue. The container with the bones and culture medium was transferred to a sterile environment. Both ends of the bones were cut off and the bone marrow was extracted using a syringe and needle 22, and then placed in the HBSS buffer. Cells were cultured at a density of 25 * 10^6 in each well of a 6-well plate in DMEM medium containing 15% FBS and penicillin /streptomycin. As mesenchymal stem cells adhere to plastic, the culture medium was gently replaced three hours post-culture, and for a period of 72 hours, the medium was refreshed every 8 hours. For 2 weeks, the medium was replaced every 3 days and at the end of this period, the first passage was performed. To confirm the identity of the isolated cells, these cells underwent immune phenotyping by flow cytometry for the markers CD105, CD44, CD45, and CD34. The isolated cells were identified by two positive markers (CD105 and CD44) and two negative markers (CD45 and CD34).

Preparation of mesenchymal stem conditioned medium

MSC cells, which had undergone 3-4 passages, were cultured to 80% confluence in a 6-well plate. After 24 hours, the medium was discarded from the cell culture well and the well was rinsed twice with PBS. Subsequently, 2 ml of DMEM medium without FBS was added to the well. After 48 hours, cell supernatants were gathered and centrifuged at 3000 rpm for 10 minutes. The supernatant was then stored at -70 C for future steps. The protein concentration in the conditioned medium isolated from MSC cells was measured using the Bradford standard method, employing the Bradford Protein Assay Kit (Bio basic INC, Cat. No: SK3041). The total protein concentration was found to be 154 µg/mL.

Inhibition of stimulation signal

Human CTLA4-ig fusion protein was purchased from Bristol-Myers Squibb Company, each vial contained 250 mg of abatacept. We injected intraperitoneally at a dose of 1000 µg/L and 500 µg/L (20 mg/kg).

Acute colitis in BALB / C mouse model and experimental design

For the induction of ulcerative colitis, a 100 µL dose of 4% acetic acid was administered via intrarectal injection. The mice were fasted for 24 hours prior to the procedure, with only water provided. Three days

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post-colitis induction, the first group of mice received an intrarectal injection of PBS. The second group was administered 4% acetic acid intrarectally. The third group received an intraperitoneal injection of Abatacept or CTLA4-ig at a dose of 1000 µg/mL on day 2, followed by a half dose (500 µg/mL) two days later on day 4/6. The fourth and fifth groups, in addition to the abatacept regimen, were intraperitoneally injected with 500 µL of CM-MSCs at protein concentrations of 50 µg/mL and 75 µg/mL respectively, on the same day. The final group received only an intraperitoneal injection of 500 µL of CM-MSCs at a protein concentration of 75 µg/mL.

Clinical activity index evaluation

During the study, the clinical signs of colitis including survival, body weight changes, stool consistency, bleeding severity, and the DAI were recorded (Table 1) (21). All mice were euthanized with Ether on day 8.

Table 1. Disease activity index (DAI) score

Weight loss (from baseline)	Stool consistency	Bleeding	Score
No weight loss or increase	Well-formed pellets	No blood by hemoccult	0
Weight loss of 1–5%	Soft Positive	hemoccult	1
Weight loss of 6–10%	Loose Visible	visible bleeding	2
Weight loss of 11–20%	Watery Gross	Gross bleeding	3
Weight loss of> 20%			

Colon macroscopic examination and Histological analysis

At the conclusion of the experiment, the intestine was extracted. The colon was then isolated from the small intestine and its length was measured. A small piece of colon tissue, about 1 to 2 mm, was separated and placed in a 10% formalin solution at room temperature. Depending on the size of the sample, it took between 6 to 24 hours for it to stabilize. Following this, Hematoxylin and eosin staining was carried out. Histological evaluation was then performed according to the criteria listed in Table 2, which included rchitectural changes, chronic inflammatory infiltrate, lamina propria neutrophils and eosinophils, neutrophils in the epithelium, crypt destruction, crypt abscess, and erosion or ulcers (22).

Cytokines assay

Cells numbering 5 * 10^5, which were previously isolated from the spleen of mice, were counted using a neobar slide and transferred to each well of a 24-well plate. These cells were then incubated for 72 hours, both in the presence and absence of PHA (23). Subsequently, the supernatant from the wells was collected and stored in a freezer at -70 °C. The cytokines IL-10 and IFN-γ were then measured in the supernatant using an ELISA kit (Raybiotec). Absorbance measurements were performed using an ELISA H1 Synergy BioTek reader at 480 nm.

Results

Characterization and differentiation potential of MSC

Mesenchymal stem cells isolated from bone marrow of mice showed typical immunophenotype of mouse mesenchymal stem cells, these results are shown in (Figure 1).

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Table 2. The original Geboes Score

Grade 0: Architectural changes	0.0 No abnormality 0.1 Mild abnormality 0.2 Mild/moderate diffuse or multifocal abnormalities 0.3 Severe diffuse or multifocal abnormalities
Grade 1: Chronic inflammatory infiltrate	1.0 No increase 1.1 Mild but unequivocal increase 1.2 Moderate increase 1.3 Marked increase
Grade 2A: Eosinophils in lamina propria	2A.0 No increase 2A.1 Mild but unequivocal increase 2A.2 Moderate increase 2A.3 Marked increase
Grade 2B: Neutrophils in lamina propria	2B.0 No increase 2B.1 Mild but unequivocal increase 2B.2 Moderate increase 2B.3 Marked increase
Grade 3: Neutrophils in epithelium	3.0 None 3.1 < 5% crypts involved 3.2 < 50% crypts involved 3.3 > 50% crypts involved
Grade 4: Crypt destruction	4.0 None 4.1 Probable: local excess of neutrophils in part of the crypts 4.2 Probable: marked attenuation 4.3 Unequivocal crypt destruction
Grade 5: Erosions and ulcerations	5.0 No erosion, ulceration or granulation tissue 5.1 Recovering epithelium + adjacent inflammation 5.2 Probable erosion: focally stripped 5.3 Unequivocal erosion 5.4 Ulcer or granulation tissue

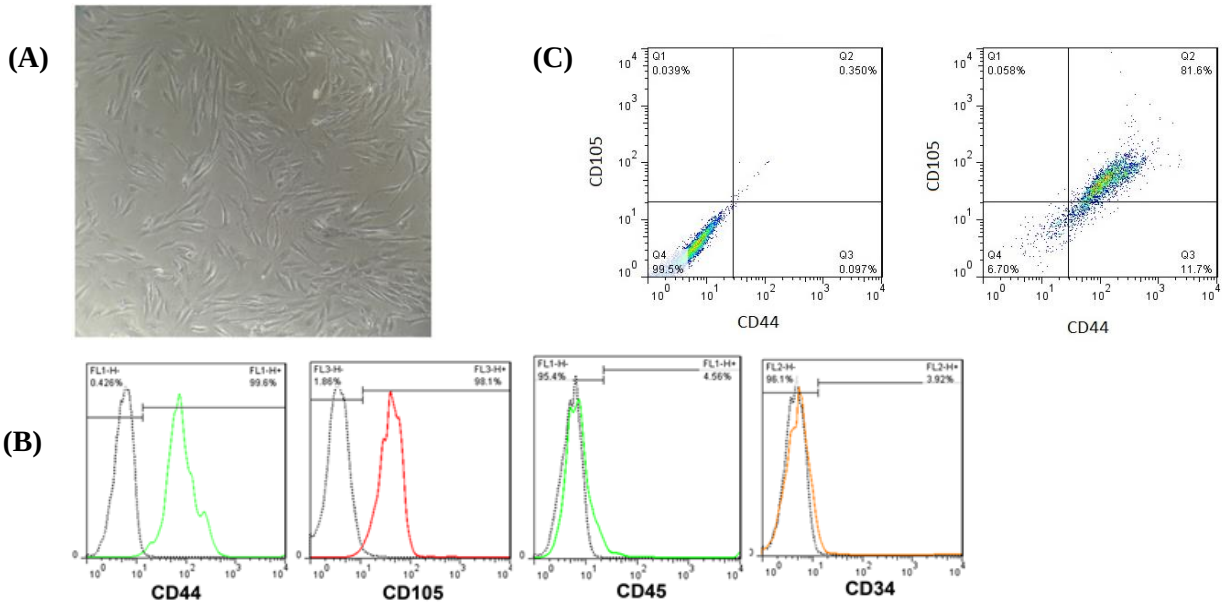

Fig. 1. Characterization of bone marrow mesenchymal stem cell. (a) Morphology of MSCs, (b) CD44, CD105, CD45 and CD34 markers are displayed as histograms and (c) CD44 and CD105 markers are displayed as Quadrants in addition to histograms.

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The effects of (MSC-CM or CTLA4-Ig) intraperitoneally injection on clinical symptoms

Mice exposed to acetic acid exhibited severe disease symptoms, including bloody diarrhea, progressive weight loss, increased mortality, and consequently, an elevated Disease Activity Index (DAI) as depicted in (Figure 2). While there was no significant difference observed in clinical symptoms, index, and survival among the treatment groups, the CM75 group showed a notable decrease compared to the acetic acid group ($P < 0.05$) (Figure 2).

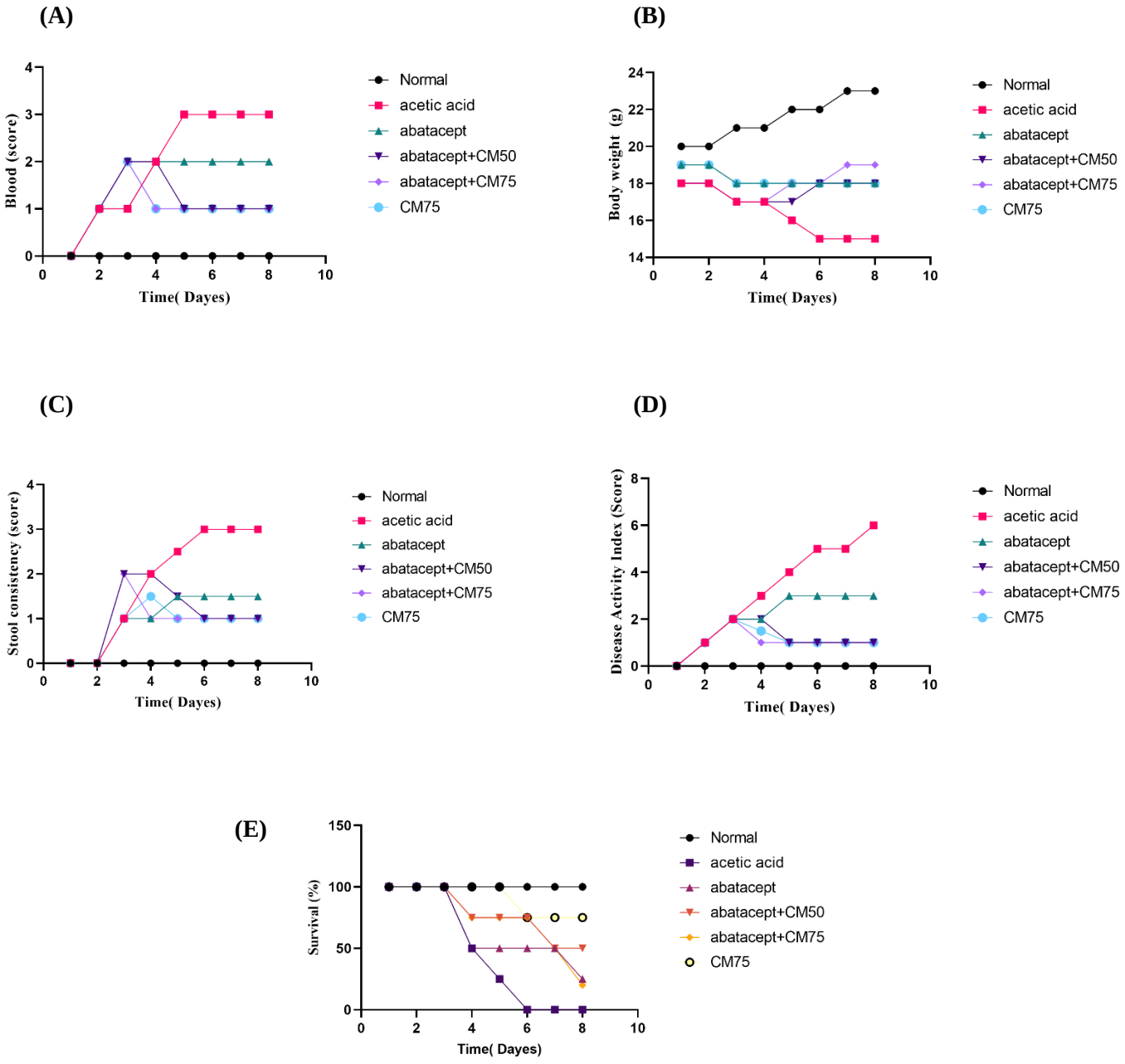

Fig. 2. The effects of MSC-CM injection on clinical symptoms. (a–e) show the clinical signs and survival of different groups. The number of mice in each group were 5. Level of significance was considered < 0.05(*p< 0.05) and the results are shown as mean ± SD. Analyses were done with Tukey's test and ANOVA. ANOVA, analysis of variance; DAI, disease activity index; MSC-CM, mesenchymal stem cell-conditioned medium.

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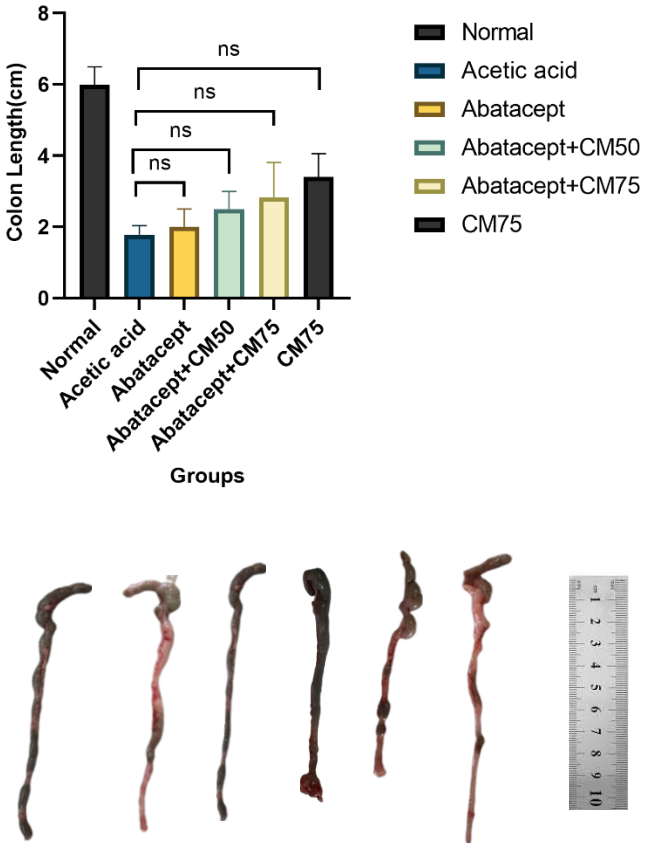
The effects of (MSC-CM or CTLA4-Ig) intraperitoneally injection on macroscopic features of colon

As depicted in (Figure 3), the length of the colon in the group treated with the conditioned medium (75 µg/mL) showed an increase compared to the other treatment groups. However, this increase was not statistically significant when compared to the acetic acid group ($p>0.05$).



CM75 Abatacept+CM75 Abatacept+CM50 Abatacept Acetic acid Normal

Fig. 3. The effects of MSC-CM injection on colon length (a) and (b) from right to left group 1-6. The number of mice in each group were 5. Level of significance was considered < 0.05 (*p < 0.05) and the results are shown as mean ± SD. Analyses were done with Tukey's test and ANOVA. ANOVA, analysis of variance.

Histopathological findings

Histological evaluations were conducted using hematoxylin/ eosin staining. The group that received only Abatacept exhibited more severe chronic inflammatory infiltration and neutrophils in the epithelium compared to the group that received Abatacept combined with MSC-CM (50 and 75 µg/mL). However, this group was not significantly different from the group that received only MSC-CM at a concentration of 75 µg/ml in terms of chronic inflammatory infiltration and neutrophils in the lamina propria. In terms of crypt destruction, the group that received Abatacept combined with MSC-CM and the group that received CM alone performed better than the group that received only Abatacept (Figure 4a). The histological score revealed that the CM75 group had the lowest score compared to the acetic acid group ($p<0.0001$) (Figure 4b).

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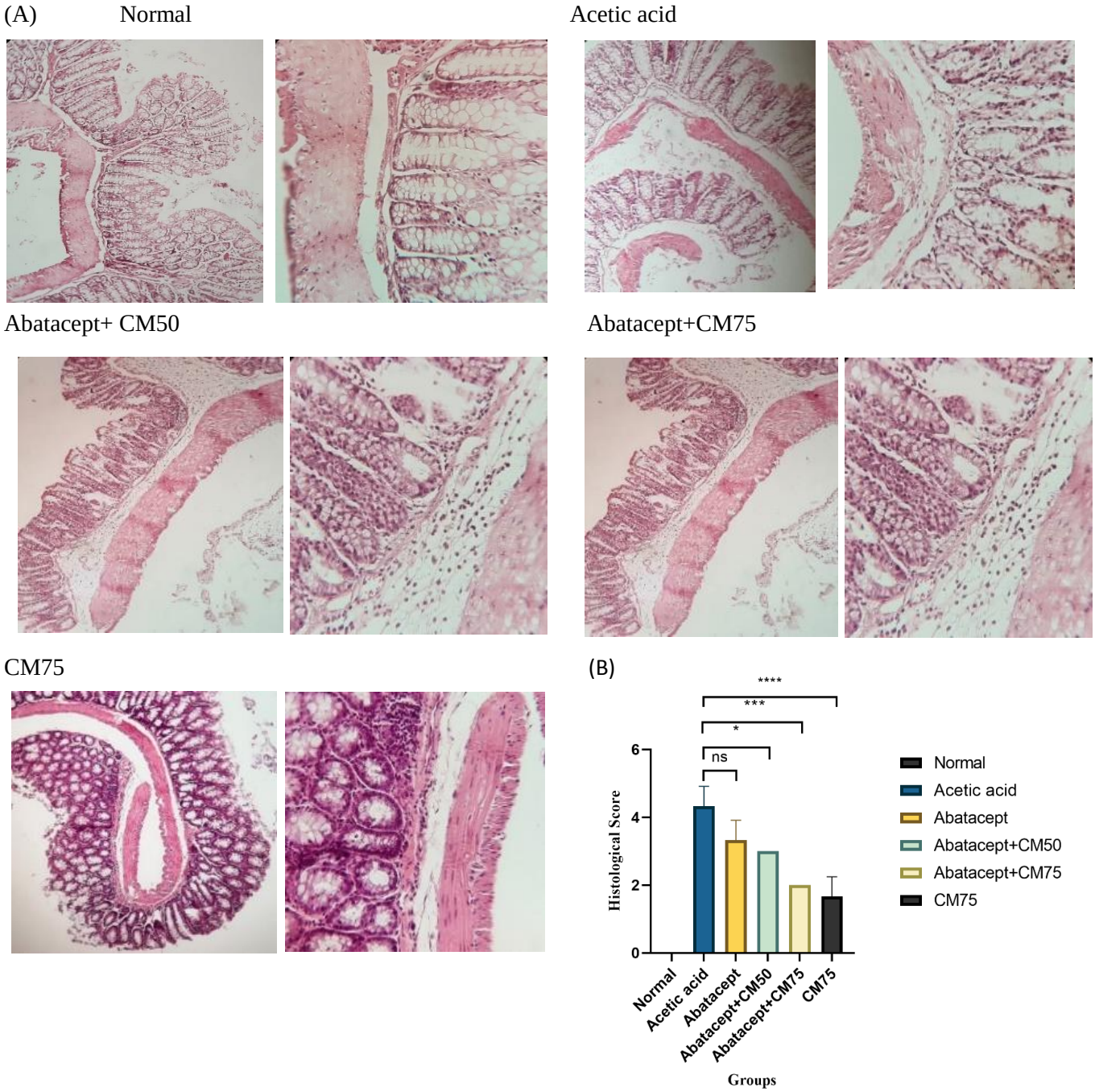

Fig. 4. Microscopic analysis of colon section (a) and histopathological score after H & E staining (b). In terms of crypt destruction, the group that received Abatacept with MSC-CM and CM alone was better than the group that received abatacept only. The significance level was determined less than 0.05, and the calculations were performed as mean ± SD, *p <0.05, ***p <0.001, ****p<0.0001. Analyses were done with Tukey's test and ANOVA. H&E, hematoxylin and eosin.

The immunomodulatory effects of (MSC-CM or CTLA4-Ig) on cytokine production

The ELISA test revealed a significant decrease in the level of IFN-γ in the CM75 group compared to the acetic acid group ($p<0.05$). However, there was no significant increase in the level of IL-10 in the CM75 and Abatacept + CM75 groups (Figure 5).

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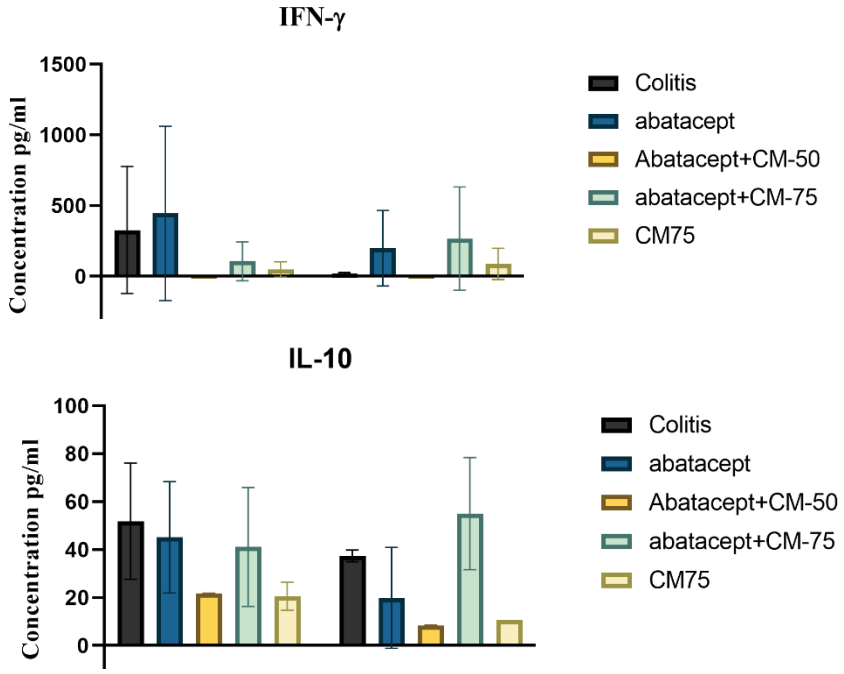

Fig. 5. Changes in cytokines level. MSC-CM injection reduced IFN-γ production and increased the levels of anti-inflammatory cytokine IL-10.

Discussion

Both the innate and adaptive immune systems play a role in the pathogenesis of IBD, with alterations in the epithelial barrier and an imbalance between these two systems leading to gut inflammation. A key characteristic of ulcerative colitis is the infiltration of neutrophils and macrophages into the mucosal tissue of the large intestine. These activated neutrophils produce reactive oxygen species, including superoxide ions, hydroxyl radicals, and hydrogen peroxide, which cause lipid peroxidation, increase mucosal and blood vessel permeability, enhance neutrophil entry into the mucosal tissue, and affect the expression of inflammatory cytokine genes and enzymes involved in inflammatory responses, leading to the destruction of the intestinal structure (1, 7). In addition to Th1 and Th2 immune responses, Th17 and Treg cells also play a role. Th1 cytokines such as IFN-γ are secreted at higher levels in Crohn's disease than in ulcerative colitis, and some studies have shown that ulcerative colitis is Th2-dependent, with large amounts of IL-5 and IL-13 cytokines being secreted. The anti-inflammatory role of Treg in IBD is disrupted in this disease, and TGF-B signaling is impaired. As a result, T effector cells in the lamina propria of patients do not respond to Treg activity, reducing the anti-inflammatory activity of Treg and increasing the pathogenesis of the disease as much as increasing T effector activity (24, 25). In a clinical trial study on ulcerative colitis, treatment with MSCs improved inflammation of the intestinal mucosa and deep and diffuse ulcers in 83% of patients (26). One of its main roles is to maintain the balance between Treg and Th1 / Th17, it also repairs tissue and secretes regulatory factors such as IL-4, IL-10, IL-11, IL-13 and TGF-B (16). In a 2011 study by Duijvestein et al., the effect of IFN-γ pre-treatment on the immunomodulatory properties of MSC in two models of colitis induced by DSS and TNBS was examined. In the mouse model, DSS-induced colitis reduced disease

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progression, and in the TNBS-induced model, it reduced symptoms. Also, in the MSC + IFN-γ pre-treatment group, weight gain and survival of rats and decreased inflammatory cytokines in colon tissue were observed. In addition, increasing the ability to inhibit the Th1 response reduced tissue damage (27). In a 2012 study by Fan et al., the therapeutic effect of IL-1β-primed MSC in a model of induced colitis with DSS was examined. They showed that it modulates the balance of immune system cells in the spleen and mesenteric lymph nodes, thereby reducing M1 and increasing CD 206^{+} M2, Treg and Th2 were observed. On the other hand, by increasing the expression of CXCR4, it has increased its ability to migrate to the inflammatory region of the intestine (28). In our study, we also indicated that MSC-CM can reduce the level of IFN-γ production, although it leads to an increase in the level of IL-10 secretion, especially when combined with Abatacept (CTLA4-Ig). In IBD, the use of Abatacept has not been very effective in clinical trials. A balance between the T effector and Treg is essential for maintaining colonic homeostasis, although a decrease in Treg count and an increase in Th17 in the peripheral blood of IBD patients have been observed. The number of Treg also increased in the lamina propria and mesenteric lymph nodes, which is essential for inhibiting the onset of active inflammation. The treatment by Abatacept did not alter Foxp3+ Treg numbers in the colon tissue. It is not known whether it has inhibitory activity, but laboratory studies have suggested that it has no effect on Treg function. Hypotheses have been put forward that include: The stimulus signal is essential for naïve T, unlike the T memory and T effector (TEM), which are more present in the gut, because the CD28 pathway is not important in the pathogenesis of the disease. Most TCD8+ and TCD4+ cells do not express CD28 in the intestinal epithelium, and the OX40 / OX40L pathway, which is not targeted by this drug, is more significant in IBD inflammation. It also does not affect the number and function of Treg. Inflammatory mediators that activate leukocytes should also be considered, as blocking T activity alone is insufficient (29, 30). In a 2017 study by Pouya et al., they examined the effect of MSC-CM in a mouse model of acute colitis with DSS, and monitored changes in weight, bleeding, stool consistency, DAI, and colon length during the study. The results showed improvements in weight loss, bleeding, stool consistency, and DAI. Macroscopic examination of the colon also revealed a reduction in colon inflammation and mucosal damage (31). This study corroborated our findings that in the MSC-CM with Abatacept group and the MSC-CM alone group, the histological score and crypt destruction were reduced, and the colon length was increased. Daily monitoring of clinical symptoms indicated that intraperitoneal injection of MSC-CM with or without Abatacept led to the inhibition of body weight loss, reduction in bleeding, improvement in stool consistency, and ultimately a reduction in the DAI. Traditional therapies for IBD, such as 5-amino salicylic acid, steroids, and antimicrobial drugs, have many limitations and side effects. In the last two decades, biological therapies like the signaling antagonist IL-12-IL-23 with Ustekinumab, anti-IL23p19, and Anti-TNFα have revolutionized the treatment of IBD. However, all of these are still in the clinical trial phase and have not been finally confirmed. These drugs also have many side effects and can lead to treatment resistance and recovery (32-34). Due to the limitations of cell therapy, including improper replacement in tissue and changes in its proper function under different conditions, cell-free therapy can be more effective today. Based on the results, MSC-CM with Abatacept can be used in inflammatory and autoimmune diseases such as IBD, to decrease mucosal damage, macroscopic tissue changes, and local inflammation by reducing the secretion of inflammatory cytokines and increasing anti-inflammatory cytokines. However, this study shows that

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Abatacept alone was an insufficient treatment and required combination with MSC-CM for better effects in Colitis. Current treatments in acute colitis and preventing its progression to the chronic stage of the disease have not been effective, and since any chronic inflammation is initially in the acute stage of the disease, treatments to prevent the disease from becoming chronic can be treated earlier. Limitations of this study include not using different doses of Abatacept as well as higher doses of medium condition in combination with Abatacept, which can be more effective in improving mucosal damage and inflammation.

The objective of this study was to explore their potential in modulating the immune system and alleviating disease symptoms, with the ultimate goal of paving a new path in the treatment of ulcerative colitis. Given the roles of MSCs and Abatacept in treating autoimmune and autoinflammatory diseases, our findings suggest that they could play a significant role in improving symptoms of inflammatory bowel diseases such as ulcerative colitis. However, further experimental studies and clinical trials are needed to substantiate these findings.

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