Original Article

Therapeutic Effects of Tretinoin and Caffeine-Treated Bone Marrow-Derived Mesenchymal Stem Cell on Immunological Features of Ulcerative Colitis: An Animal Model Study

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Abstract

Background: Ulcerative colitis (UC) is one of the inflammatory gastrointestinal diseases. It causes irritation, inflammation, and ulcers in the digestive tract. UC is distinguished clinically by abdominal and rectal pain and intestinal secretion abnormalities. Mesenchymal stem cell (MSC) therapy could be the underlying treatment for UC. This study aimed to compare the results of MSC therapy with tretinoin and caffeine in an animal model.

Materials and Methods: Sixty male BALB/c mice were randomly divided into six equal groups. Five groups were exposed to acetic acid-induced colitis, and one healthy negative control group was designed. The positive control group was UC-induced mouse model with no treatment. Besides, treatment groups were MSCs ($n = 2\times10^6$) that received tretinoin and caffeine. The treatment group was given mesalazine orally. The decision to begin treatment was taken after monitoring the symptoms of the UC.

Results: MSCs, tretinoin, and caffeine-treated MSCs significantly decrease inflammatory cytokines (interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α) and inflammatory mediators (myeloperoxidase (MPO) and nitric oxide (NO)) compared with the positive control group. However, the alleviated effects of tretinoin-treated MSCs significantly were more than those of MSCs and caffeine-treated MSCs.

Conclusion: MSC therapy is an effective option for UC and can prevent disease progression. The results represented a high developmental rate and simple cell application of MSC therapy in UC patients. Also, MSC therapy's ability for immunomodulation is strengthened by drugs that improve their microenvironment by binding to their receptors.

Keywords: Caffeine, inflammatory bowel disease, mesenchymal stem cells, tretinoin, ulcerative colitis

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Introduction

Inflammatory bowel disease (IBD) refers to various inflammatory gastrointestinal pathologies with unclear etiologies. Ulcerative colitis (UC) and Crohn's disease (CD) are two main IBD conditions characterized by increased inflammatory mediators such as prostaglandin E2, leukotriene B4, interleukin (IL)-6, and IL-1[1] and recurrent inflammatory assaults to mucosa or submucosa.[2] Following the release of inflammatory mediators and enzymes, the intestinal wall is damaged, and ulcers, bleeding, and diarrhea appear. Reducing inflammation is one of the main objectives of UC treatment.[3] Curative medical treatment for UC is not currently found, and evidence shows that more than 50% of patients may not respond to conventional therapies.[4] Corticosteroids are one of the therapeutic agents for reversing inflammation[5,6] Long-term side

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effects of corticosteroids are cataracts, osteoporosis, adrenal insufficiency, and cutaneous manifestations such as acne and hyperglycemia. Furthermore, corticosteroids may augment the susceptibility to infections, especially when used concomitantly with other immunosuppressive agents.[7,8] Comprehensive investigations are underway to manage the disease and prevent its occurrence and to utilize cell therapy and other alternative remedies to mitigate the harmful effects of inflammation.[9,10] MSC are being utilized for immune system modulation and treating immune-mediated disorders, owing to their unique properties, particularly their immunomodulatory potential.[11] Animal models of autoimmune disorders have been utilized to investigate the therapeutic potential of mesenchymal cells.[12] Over the past decade, the therapeutic or pre-therapeutic efficacy of specific agents that modulate or inhibit these mediators has been investigated in various forms of experimental colitis that simulate human IBD. Appropriate selection of these agents can help comprehend the underlying pathophysiological mechanisms and facilitate the development of novel hypotheses.[13] Tretinoin has been recognized for its antioxidative ability to remove chain reactions. The active metabolite of vitamin A is all-trans retinoic acid or tretinoin, which exhibits antioxidant, immune-stimulating, and anticancer activities.[14] Due to their augmented immunomodulatory potential, MSCs with tretinoin demonstrated enhanced immunomodulatory activity, inducing them a promising therapeutic option for inflammatory and autoimmune disorders.[15] Caffeine is a stimulant and promotes wakefulness. Also, caffeine exerts an inhibitory effect on phosphodiesterase.[16] Adenosine receptors are present on the surface of various types of cells, such as neuronal, immunological, and mesenchymal stem cells (MSCs). A study indicated that anti-inflammatory and immune system-modulating effects could be observed even at doses commonly associated with chronic tea consumption.[17] This study aimed to assess the therapeutic effects of MSCs that have undergone treatment with tretinoin and caffeine on the immunological manifestation of UC in a mouse model.

Materials and Methods

Animals

Sixty male bagg albino (BALB/c) mice, with an age range of 6–8 weeks and a weight of 20–30 g, were procured from the experimental animal care facility of the Pasteur Institute of Iran in Tehran City. The experimental animals were housed in plastic cages that were furnished with wood shavings and placed in a climate-controlled room maintained at a temperature of 25°C, for 12 hours. A total of 50 mice were prepared to serve as *in vivo* models of UC.

Colitis induction and experimental groups

Colitis was induced by the instructions provided in a previous study.[18] Before the induction of colitis, mice were subjected to a 36-hour fast. Under light sedation with ether, a pediatric-sized catheter with a 1-cm tip was introduced into the colon. Next, an intracolonic enema was utilized to administer a 500 µl solution of diluted acetic acid (4%) into the colon. Furthermore, 500 µl of phosphate-buffered saline (PBS) was administered intercolonially in the control group. The animals were maintained in a vertical, head-down position for one minute to prevent leakage of the acetic acid solution. The fifty mice with colitis were randomly divided into five groups, including a positive control group, a group treated with non-treated MSCs, a group treated with MSCs–tretinoin, a group treated with MSCs–caffeine, and a group treated with mesalazine. Each group consisted of ten mice. The colitis control group received intraperitoneal (IP) administration of PBS. IP treated cell groups twice with a one-weak interval of 2×10^6 cells each. The animals were euthanized on the 15th day after MSC implantation, and their colon tissues were extracted for analysis.

Isolation and proliferation of MSCs

As previously mentioned, the bone marrow-derived MSCs were separated based on their capacity to stick to the culture plates. The sacrificed BALB/c mice had their bone marrow drained from their tibias and femurs. The cells were grown in T25 culture flasks with Dulbecco's modified eagle medium (DMEM) supplemented with 15% FBS in a humidified incubator with 5% CO2 at 37°C after two rounds of washing. The adherent cells were fed twice weekly on the fourth day, while the non-adherent cells were carefully discarded. The MSCs were separated by trypsin or ethylenediamine tetraacetic acid at 80% confluence.[16]

Treatment of MSCs with tretinoin and caffeine

The MSCs were incubated independently with one mM caffeine[16] and one µM tretinoin[15] for 24 hours. Following the removal of the medium, the cells were washed three times with PBS, after which they were cultured for an additional 24 hours in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were subsequently harvested and prepared for injection.

Disease activity index (DAI)

The fecal occult blood and consistency were assessed daily, and the disease activity index (DAI) was determined by summing the parameter scores as outlined in Table 1.[18]

Preparation of colonic homogenate

The distal colonic tissue was incised, sectioned into fragments, and washed with phosphate-buffered saline. Subsequently, an equivalent tissue volume was homogenized in a 5 ml ice-cold physiological saline solution, followed by centrifugation at 10,000 ×g for ten minutes at 4°C.[18]

Assessment of myeloperoxidase (MPO)

The enzymatic activity of MPO in colonic homogenates has predominantly been utilized as a biochemical indicator

Table 1: Disease activity index (DAI)

Stool blood	Stool consistency	Score
Normal	Normal	0
Red	Soft	1
Dark red	Very soft	2
Black	Diarrhea	3

for granulocyte infiltration, particularly neutrophil infiltration, into gastrointestinal tissues. Briefly, 110 µL of tetramethylbenzidine (TMB) solution (2.9 mM TMB in 14.5% DMSO + 150 mM sodium phosphate buffer at pH 5.4) was added to 10 µL of the homogenized material. Subsequently, the absorbance was promptly quantified at 450 nm (regarding 620 nm) utilizing a microplate reader. Following this, the samples were subjected to an incubation period of 15 minutes at 37°C. The chemical reaction was terminated by adding 50 µl of 2M sulfuric acid (H2SO4), and the resultant absorbance was quantified at 450 nm using a spectrophotometer. 2.5 and 25 mU/mL concentrations of horseradish peroxidase (HRP) were employed for the assay, and 10 µl of the enzyme was added to the reaction mixture. Subsequently, the difference between the absorbance and the HRP standard curve was utilized to estimate MPO activity, with the results being displayed in milliunits per milliliter (mU/ml).[18]

Determination of nitric oxide (NO) concentration in the colonic homogenate

The presence of NO in colonic tissues was assessed utilizing the Griess reagent, a commonly employed colorimetric technique for quantifying NO content. In brief, 50 µl of homogenized colonic tissue was mixed with 50 µl of Griess reagent (comprising 0.1% sulfanilamide, 3% phosphoric acid, and 0.1% naphthyl ethylenediamine) and allowed to incubate in the dark at 25°C for 10 minutes. The absorbance was subsequently measured at 540 nm using a microplate reader, and the nitrite level was estimated using a standard curve.[18]

Assessment of inflammatory cytokines in colonic homogenate

Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1), and IL-6 levels were quantified in colon samples using a commercially available enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's guidelines (Karmania Pars Gene, Iran).

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences version 23 software. In the case of nonparametric variables, such as the severity of the disease, which were assessed with discontinuous ranks, the Kruskal–Wallis test was utilized to evaluate differences between the groups. Also, the Mann–Whitney U-test with the Bonferroni correction was conducted subsequently. For continuous data, the one-way analysis of variance (ANOVA) and Dunnett's *post hoc* test were performed after verifying their normal distribution using the Kolmogorov–Smirnov test. The results were presented as means and standard deviation (SD), and statistical significance was determined at a significance level of $P < 0.05$.

Results

DAI

The study results indicated that normal stools were observed in all mice in the negative control group. Conversely, the positive control group displayed the most unfavorable stool consistency. Non-treated MSCs, MSCs–caffeine, and MSCs–tretinoin groups were significantly improved compared with the positive control group. There was no significant statistical difference between the MSCs–caffeine and MSCs–tretinoin groups. As expected, mesalazine showed the most significant reduction in DAI compared with other treatment groups, as illustrated in Figure 1.

MPO assay

The study results indicate a significant difference in MPO enzyme activity among the examined groups. The MPO activity was the lowest in the negative control group and the highest in the positive control group. The groups treated with untreated MSCs and MSCs–tretinoin and caffeine exhibited intermediate MPO enzyme activity, which was significantly lower than the positive control group. There was a significant difference between the MSCs–tretinoin and caffeine groups. Additionally, the mesalazine group demonstrated notably lower MPO activity than the other treatment groups analyzed [Figure 2].

NO assay

The study's findings indicated that the positive control treatment exhibited the highest NO (µM) level. The group administered with MSCs–tretinoin showed a significant decrease in NO levels compared with the positive control group. In contrast, the caffeine-treated MSC therapy group and untreated MSC therapy group displayed almost similar NO levels, which were lower than those of the positive control

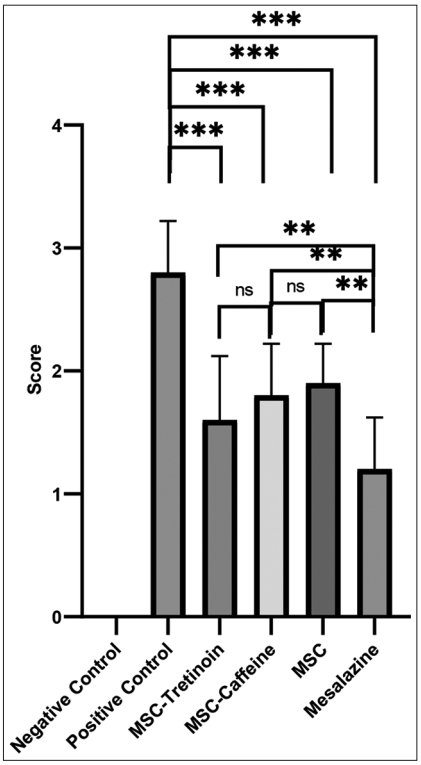

Figure 1: Disease activity index in the studied groups (* indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$)

group. Moreover, the group treated with mesalazine showed a lower NO level than the positive control group [Figure 3].

IL-1, IL-6, and TNF-α cytokine assay

The findings showed a statistically significant difference in the IL-1, IL-6, and TNF-α levels across the study groups. Compared to other groups, the negative control and positive control groups had the lowest and highest IL-1, IL-6, and TNF-α levels, respectively. IL-1, IL-6, and TNF-α levels decreased significantly in the MSC groups treated with tretinoin and caffeine and in the untreated MSC therapy group compared with the positive control group. Additionally, compared to the positive control group, mice receiving mesalazine displayed reduced IL-1, IL-6, and TNF-α levels [Figures 4-6].

Discussion

UC is a chronic inflammatory disease characterized by inflammation of the colon's mucous layers. Despite extensive research, the precise etiology of UC remains unknown. However, several factors have been hypothesized to contribute to the pathophysiology of the disease, including immunological factors, genetic factors, and environmental variables.[19] These factors may interact to produce a chronic inflammatory response in the colon, leading to the characteristic symptoms of UC. Understanding the underlying mechanisms of UC is essential for developing effective treatments and improving patient outcomes.[20-22] Despite a definitive cure for UC, patients have been subjected to various experimental treatments. These include amino salicylic acids, corticosteroids, thiopurines, and anti-TNF-α therapy. While the efficacy of these treatments remains unproven, they continue to be used to alleviate the symptoms and improve the quality of life of those suffering from UC. However, it is essential to mention that individual responses to these treatments can vary significantly, and careful monitoring by a healthcare professional is necessary.[23,24] Novel UC therapies aim to mitigate the disease's progression and maintain remission. One such emerging treatment modality is the use of MSC therapy.[25] MSCs have been found to possess remarkable abilities to differentiate into a variety of cell types and to produce cytokines and chemokines that play crucial roles in repairing damaged tissues and regulating the immune response in the gastrointestinal tract. These unique properties of MSCs have made them attractive targets for therapeutic interventions in diseases such as UC, where tissue damage and aberrant immune responses are hallmarks of the condition. In addition, MSCs have shown the potential to develop into both ectodermal and endodermal cells, further broadening their scope of application in regenerative medicine.[26] MSCs have been shown to have significant anti-inflammatory properties and can be a valuable option for treating UC due to their ability to reduce inflammation and tissue damage. In some studies, MSCs have been genetically modified to increase their therapeutic benefits in treating nephritis, ischemic lung injury, and arthritis. This suggests that gene-modified MSCs may offer even more significant potential for treating UC and other inflammatory diseases.[27,28]

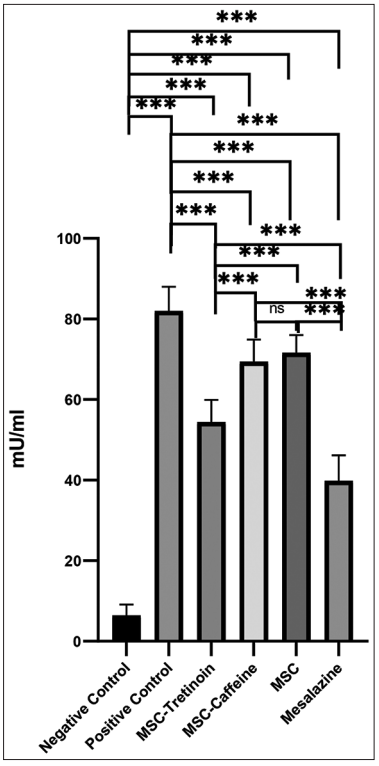

Figure 2: Comparison of the level of myeloperoxidase in the studied groups (* indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$)

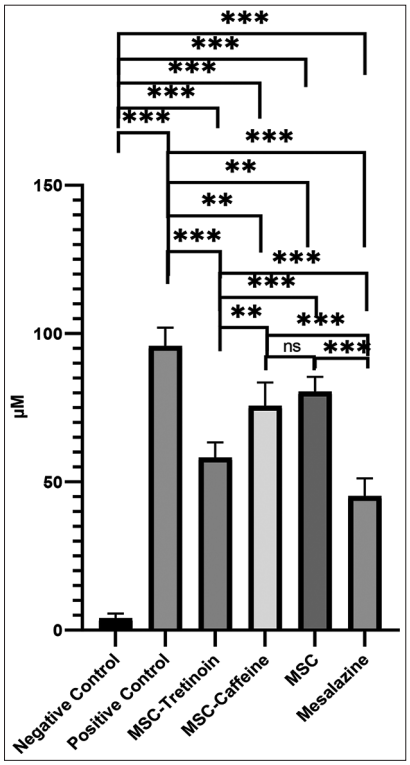

Figure 3: Comparison of the level of nitric oxide in the studied groups (* indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$)

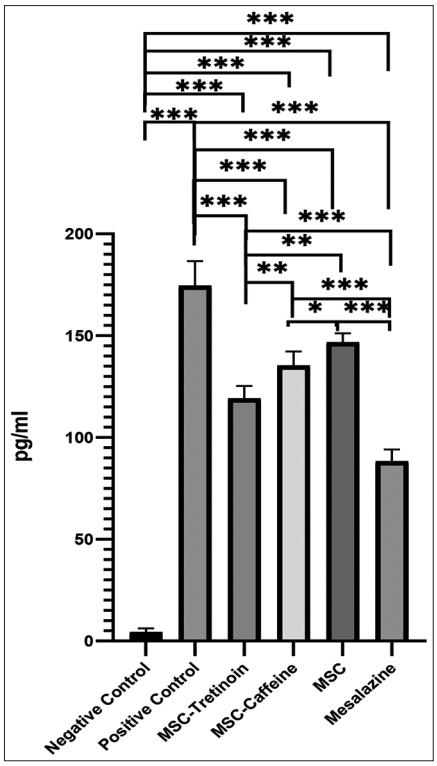

Figure 4: Comparison of cytokine IL-1 levels in the studied groups (* indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$)

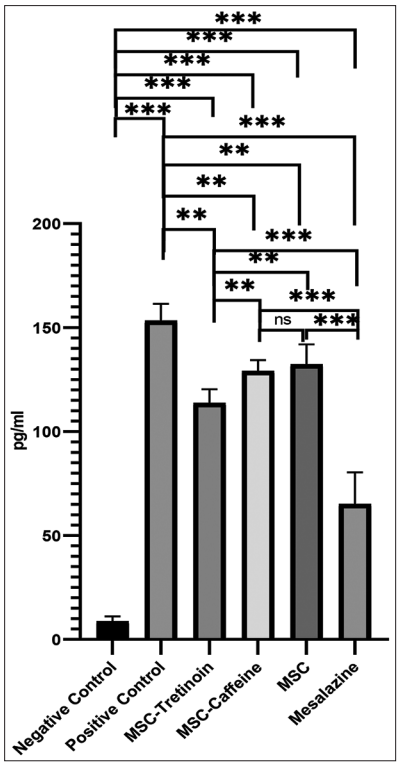

Figure 5: Comparison of cytokine IL-6 levels in the studied groups (* indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$)

The current study indicated that tretinoin and caffeine acted as microenvironments that enhanced the functionality of MSCs, thereby reducing disease activity criteria, as well as MPO and NO production, respectively. Furthermore, the treated MSCs were found to decrease the levels of inflammatory cytokines. The comparison of the two treatments revealed that tretinoin was more effective than caffeine. These findings demonstrate that MSCs treated with tretinoin and caffeine are promising therapeutic options for UC.

A recent study demonstrated that in patients suffering from UC, the monocyte populations that infiltrate the lamina propria and peripheral circulation at the onset of inflammation prompt the release of TNF-α upon activation of T cells. This pro-inflammatory cytokine plays a crucial role in exacerbating the Th17 response, thereby aggravating inflammation observed in UC.[29] In the current study, the therapeutic groups of MSCs were treated with tretinoin and caffeine, and the untreated MSC therapy groups exhibited downregulation of TNF-α. This reduction may enhance the anti-inflammatory effects of Th1 and Th17 responses.

Another study investigated the immunomodulatory properties of MSCs subjected to caffeine treatment in adjuvant-induced arthritis. The current study's results indicate that caffeine, when administered at a concentration of 0.5 mM or higher, has a remarkable anti-inflammatory effect on MSCs. At this concentration, caffeine can effectively reduce the levels of IFN-γ, IL-6, and IL-1 while elevating the levels of indoleamine 2, TGF-β, and IL-10 compared with other groups. Remarkably, these changes in cytokine expression occur without any substantial impact on the viability or markers of MSCs. Furthermore, the concomitant administration of MSC therapy and caffeine has been shown to enhance weight gain and mitigate illness severity to a greater extent than MSC therapy alone. Significantly, incorporating caffeine into MSCs led to a significant reduction in the serum levels of C-reactive protein, NO, MPO, and TNF-α, accompanied by a substantial increase in IL-10 levels. These findings suggest that the synergistic effects of MSC therapy and caffeine could hold great promise as a novel therapeutic strategy for various medical conditions.[30,31]

Based on the current study's results, the administration of caffeine-treated MSCs significantly reduced the levels of MPO, NO, and cytokines such as IL-1, IL-6, and TNF-α compared with animals with UC. These findings are consistent with previous research in this area.

Previous studies have revealed that the immunoregulatory function of MSCs is influenced by caffeine, depending on the dosage administered. These findings suggest that caffeine may modulate the effect on the immunomodulatory properties of MSCs and warrant further investigation into the potential therapeutic implications of this interaction.[16] A recent study revealed a fascinating finding that suggests that the coculturing of macrophages with MSCs in the presence of caffeine can significantly enhance the polarization of macrophages toward the anti-inflammatory M2 phenotype, surpassing the effects of MSCs alone. The study accomplished this by enhancing the

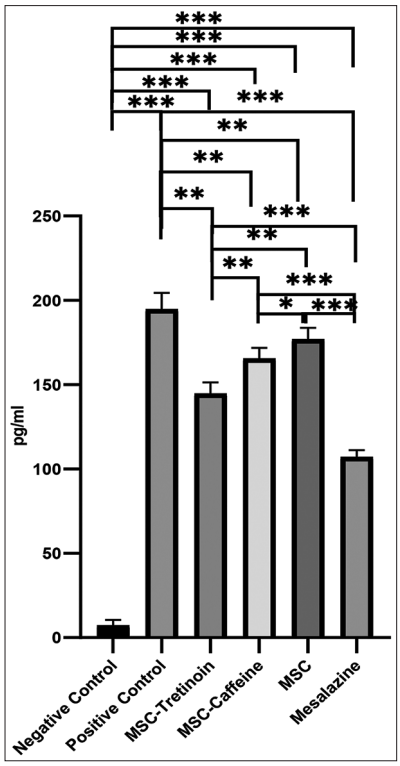

Figure 6: Comparison of cytokine TNF-α levels in the studied groups (* indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$)

phagocytic potential of cocultured macrophages and reducing their oxygen production.[31] MPO serum level is a pivotal biomarker for assessing oxidative and inflammatory stress in autoimmune conditions.[32] Numerous medical conditions have provided evidence of the harmful effects that NO can have on nervous tissue.[33] The findings proved that the administration of MSCs in conjunction with caffeine significantly reduced the serum levels of NO and MPO in mice when compared to the administration of MSCs alone. These observations suggest a potential therapeutic benefit of caffeine supplementation in enhancing the effectiveness of MSC therapy.[30] In particular, the present investigation revealed a marked reduction in the levels of MPO and NO, as well as a significant decrease in the levels of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α in animals with UC who received MSCs treated with tretinoin, as compared to the positive control group. A study was conducted to investigate the impact of tretinoin in inbred white mice after being challenged with sheep red blood cells (SRBCs). Results revealed that tretinoin had a significant effect on the immunity of the mice. In particular, while the cellular immunity decreased, the humoral immunity was enhanced following the SRBC-induced challenge. Furthermore, tretinoin has been found to significantly reduce the secretion of IL-17 and augment the synthesis of IL-10, in addition to lowering Nitro blue tetrazolium and lymphocyte proliferation. However, there was no significant alteration in the quantity of IFN-γ or the frequency of FoxP3 + Treg cells. Despite the growth of FoxP3 + Treg cells not being the sole factor contributing to the *in vivo* immunomodulatory effects of tretinoin, it is postulated that the observed immunosuppression from the pro-inflammatory cytokine IL-17 to the anti-inflammatory cytokine IL-10 may be the primary mechanism underlying these effects.[34]

Conclusion

The current study's findings indicate that the administration of MSCs treated with tretinoin and caffeine, along with the untreated MSC treatment group, led to a marked reduction in the MPO, NO, IL-1, IL-6, and TNF-α, in animals with UC. Also, tretinoin significantly enhances the immunomodulatory capacity of MSCs compared with caffeine, as evidenced by the results of MSCs treated with both compounds.

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Ethics approval and consent to participate

The study was approved by the Institutional Review Board of Baqiyatallah University of Medical Sciences (IR.BMSU.AEC.1400.009).

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Nil.

Conflicts of interest

There are no conflicts of interest.

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