Original Article

Comparison of the Modulated Effects of Tretinoin and Calcitriol Treated Mesenchymal Stem Cell Supernatant on Macrophage Functions

Majdedin Ghalavand[1,2], Hadi Esmaeili Gouvarchin Ghaleh[2], Samira Mohammadi-Yeganeh[3,4*]

Abstract

Background: According to several studies, Calcitriol and Tretinoin can regulate differentiation and the growth of mesenchymal stem cells (MSCs). Nevertheless, the relationship between the supernatant of macrophage and mesenchymal stem cells is still under investigation. In the present work, a comparison is made between the modulated impacts of Calcitriol and Tretinoin treated mesenchymal stem cell supernatant on macrophage functions.

Materials and Methods: The isolation of mesenchymal stem cells was done using mouse bone marrow, and the various concentrations of Calcitriol (200 and 400 nM) and Tretinoin (25, 50, and 100 nM) were used to pulse MSCs for 48 h. Macrophages were then applied to co-culture the supernatant of MSCs for 4 hr. Consequently, macrophages were assessed for respiratory burst.

Results: Based on the obtained results, supernatant of bone marrow-derived MSCs pulsed with Calcitriol and Tretinoin can have the potential for decreasing the respiratory burst of macrophages considerably in comparison with the control group.

Conclusion: The anti-inflammatory M2 macrophage polarization can be accelerated using Calcitriol and Tretinoin by mesenchymal stem cells.

Keywords: Tretinoin, Calcitriol, Macrophage, Mesenchymal stem cell supernatant

1. Student Research Committee, Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
3. Medical Nanotechnology and Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding Author: Samira Mohammadi-Yeganeh, Medical Nanotechnology and Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran; Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Email:
s.mohammadiyeganeh@sbmu.ac.ir

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Introduction

The microenvironment encompassing stem cells is described as a stem cell niche consisting of an extracellular matrix, adhesive molecules, and adjacent cells. Both provide nutrients for the stem cells, and maintaining their properties of self-renewal and quiescence is the duty of the microenvironment (1). Stem cells are likely to increase or differentiate into different functional cells due to variations in the

microenvironment. Nevertheless, the exact differentiation mechanism is still under investigation (2). There have been two possibilities of activating signaling pathways inside stem cells due to cell contact and the combined actions of a group of cytokines (4). MSCs differentiated *in vitro* due to cytokine induction usually lack the functions of tissue cells. As highly plastic cells, macrophages provide numerous purposes, such as clearance of cellular debris, tissue development and homeostasis, removal of pathogens, and management of inflammatory responses (5). Within the M-CSF or GM-CSF-dependent differentiation of circulating monocytes, the postnatal growth of macrophages happens. Such cells are found in the bone marrow from myeloid-derived progenitor cells. The derived macrophage can encompass a wide range of phenotypic phases, directed via the formation of the cytokine milieu and the encompassing tissue niche (6). The macrophage activation is categorized into two sections despite its complexity. M1 classically activated macrophages or M2 alternatively activated macrophages. We selected two essential microenvironments in the present study, including Calcitriol and Tretinoin (7). As a group of synthetic and natural derivatives of vitamin A, retinoids are necessary for embryogenesis, vision, growth, and reproduction. Tretinoin (all-trans retinoic acid) is an active metabolite of retinoids by which a wide variety of biological processes, including cell proliferation, differentiation, and survival, are modulated. A particular group of nuclear receptors, the retinoic acid receptors (RARs), operating as ligand-inducible transcription agents, are involved in this process (8). The background of the theory of the vital role of vitamin A derivative in immunity returns to the early 20th century. Edward Mellanby and his co-worker Harry Green found the anti-infective impacts of vitamin A for the first time. Anti-neoplastic and immunomodulatory characteristics of Tretinoin were also stated. Tretinoin was clinically verified for treating cancers, including severe promyelocytic leukemia and Kaposi sarcoma, and inflammatory disorders, such as psoriasis and acne. Based on prior studies on Tretinoin, tissue destruction and inflammatory responses were alleviated (9). Furthermore, a group of autoimmune disorders, such as rheumatoid arthritis, type I diabetes, inflammatory bowel disease, and experimental autoimmune encephalomyelitis, were attenuated in animal samples. The profound impact of vitamin D on immunity was emphasized by the expression of vitamin D receptors (VDR) in immune cells (10). Nowadays, the essential function of vitamin D in improving both innate and adaptive immune systems is widely accepted. Intracellular vitamin D receptors (VDR) are responsible for a local immune influence of Vitamin D (11). Such receptors can be found in mesenchymal stem cells, monocytes/macrophages, B cells, T cells, dendritic cells (DCs), and natural killer cells (NK). A heterodimer is formed with retinoid X receptor (RXR) by vitamin D following the binding to its receptor VDR (a member of the nuclear receptor category) (12). Vitamin D Response Element (VDRE) is engaged, and activators and enzymes are recruited with histone acetylation activity by such complex. Accordingly, the targeted genes are regulated due to the structural variations in chromatin caused by such complex (13). On the other hand, phagocytosis is an important stage in the clearance of microbes. Surgical site infections, in particular perioperative, are associated with high morbidity and death. Macrophages provide the cellular host defense against infection or tissue harm. Inflammatory responses of macrophages include chemotaxis, phagocytosis, intracellular killing, and cytokine release (14 – 15). Numerous studies looked into the influence of anesthesia on macrophage phagocytosis and its mechanism. Researchers used RAW264.7 murine cells, peritoneal macrophages from mice, and THP-1 cells to study the effects of anesthesia agents (Isoflurane, Sevoflurane, and Propofol) on macrophage phagocytosis (16).

Apart from macrophages, exosomes also received a lot of attraction in the molecular mechanisms of pain and anesthesia. Exosomes are extracellular microvesicles involved in intercellular communication and capable of transporting cargo molecules such as medicines to nearby and far target areas. Recently, interest in the role of exosomes in a variety of pain conditions has increased. Their participation in pain processes has been shown through research on various chronic pain conditions. Additionally, animal and clinical research have

demonstrated the efficacy of exosome-based therapy in alleviating painful sensations with fewer side effects. The ability of exosomes to convey information and their existence in a variety of readily accessible body fluids implies that they may be used as novel non-invasive therapeutic methods in pain (17, 18). In this research, we compared the modulated impacts of Tretinoin and Calcitriol treated mesenchymal stem cell supernatant on the function of the macrophage cells.

Methods

MSCs isolation and proliferation: Mesenchymal stem cells(MSCs) were isolated based on a previously reported protocol with slight modifications (19). In brief, bone marrow was flushed out from Balb/c mice tibias and femurs under deep anesthesia. The cells were washed twice using Dulbecco's Modified Eagle Medium (DEMEM, Bio Idea, Tehran, Iran) media and centrifugation at 1200 rpm for 5 min. They were then plated in T-25 flasks in DMEM medium containing 15% Fetal Bovine Serum (FBS, Bio Idea, Tehran, Iran) and incubated at 37° C and 5% Co_2. 4 days after initiating primary culture, the culture media were collected, centrifuged, and cells were seeded in a T-25 flask. Cells were trypsinized with Trypsin/EDTA (Gibco, USA) at 70% confluence, counted, and passed at 1:3 ratios. The third generational passage cell suspension was collected. MSCs were then incubated for 48 hours with varying amounts of Calcitriol (200 and 400 nM) and Tretinoin (25, 50, and 100 nM). The MSC supernatant was then collected and used for further testing.

Peritoneal macrophage isolation and culture: Macrophage isolation was done from the peritoneal cavity of 6-8 weeks old from Balb/c mice. Harvested cells were cultured at a density of 1 million cells/ well in 24-well plates in RPMI (Bio Idea, Tehran, Iran) media containing 10% FBS. 4 hours after seeding, the non-adherent cell was removed by vigorous washing. The next day, washing was repeated to remove all non-adherent cells.

Macrophage co-cultured with MSC supernatant: Each well of a 96well microplate containing macrophages was co-cultured with 100 µl of MSC supernatant. The incubation was at 37 ° C in a humidified environment with 5% CO2.

Respiratory burst assessment: The Nitroblue tetrazolium (NBT) staining solution (0.2%w/v concentration) was prepared in phosphate-buffered saline (PBS). NBT powder was added to PBS (10 ml), incubated at 37°C for 30 min, and vortexed briefly. NBT reduction was utilized for measuring the intracellular generation of reactive oxygen species (ROS) (21-24). The incubation of the cells lasted for 30 min at 37 ° C, and NBT solution (1 mg/ml) was used for incubation for one hour at 37 ° C. In this experiment, the wasted NBT was rinsed and produced dye was detected at a wavelength of 520nm (19-21).

The assessment of macrophage viability: The viability of macrophages was assessed by the MTT test (21-23). First, macrophages and MSC were co-cultured, then 20 µl of the MTT solution ((4,5-Dimethyl thiazol -2-yl)-2,5- diphenyl tetrazolium bromide) was used to treat the cultures for four hours (37°C). Then, dimethyl sulfoxide (DMSO) was added, the mixture was shaken firmly to dissolve the formazan crystal and the optical density (OD) was measured at 560 nm. The trials were performed in triplicate (9).

Measurement of the level of Nitric Oxide production: Griess assay is the standard protocol for measurement of Nitric Oxide levels. A buffer with a ratio of 2:1was utilized for sample distillation. After that, 100 mL of each sample was poured into 96-well plates in three replications. The kit contained three copies of standard, and blank samples poured into 96-well plates. The samples were measured at a wavelength of 570 nm using an ELISA plate reader (20).

Reactive oxygen species (ROS) assay: Macrophage cells were cultured in a 96-well plate and then treated with MSCs and incubate at 37ºC for four h. carefully aspirate off the culture media and add 100 µl of Ready Assay Buffer (ROS assay Kit, TEB PAZHOUHAN

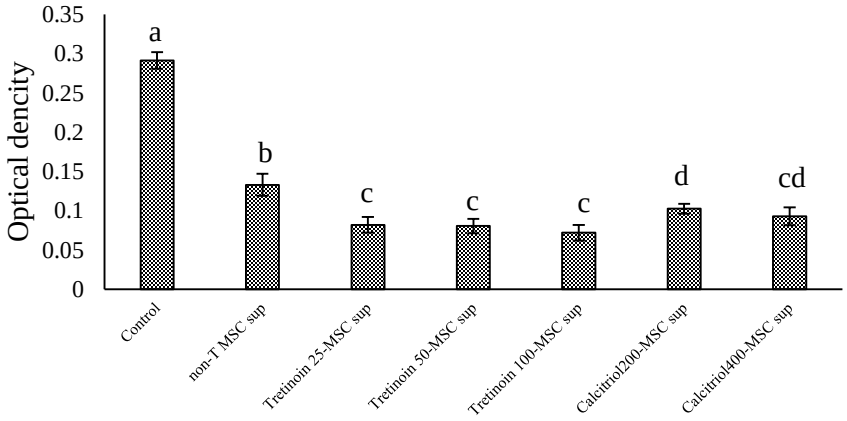

Figure 1. Evaluation of macrophage respiratory burst by Tretinoin and Calcitriol-treated MSCs. Significant statistical variations between groups are denoted by a separate superscript letter (P<0.05) in each index.

RAZI). After discarding Ready Assay Buffer from wells, 100 µl of DCF Staining Buffer was added to all wells except blank wells. Cover plate and incubate for 60 min at 37°C - protected from light.

After 60 min incubation, add 100 µl of R3 stimulator with desired concentration to designated positive control wells and incubate for an extra 20 minutes at 37°C - protected from light.

Then, aspirate DCF Staining Buffer and add 100 µl of Ready Assay Buffer. Measure the fluorescence intensity via an excitation wavelength between 480-500 nm and an emission wavelength between 510-550 nm (20-24).

Statistical analysis:

The one-way ANOVA approach followed by Dunnett's posthoc trial was used to analyze data, and the presentation of data was as means±SD. The statistical significance was defined as P-values of less than 0.05.

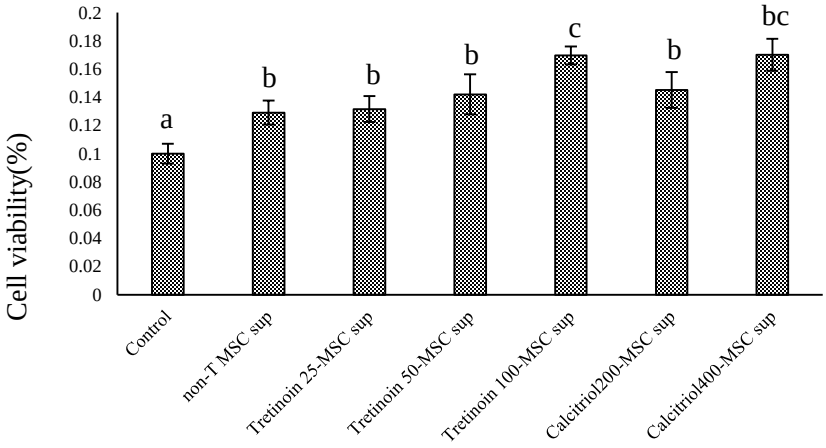

Figure 2. Effect of Tretinoin and Calcitriol on modulation of macrophage viability by MSCs. Significant statistical variations between groups are denoted by a separate superscript letter (P<0.05) in each index.

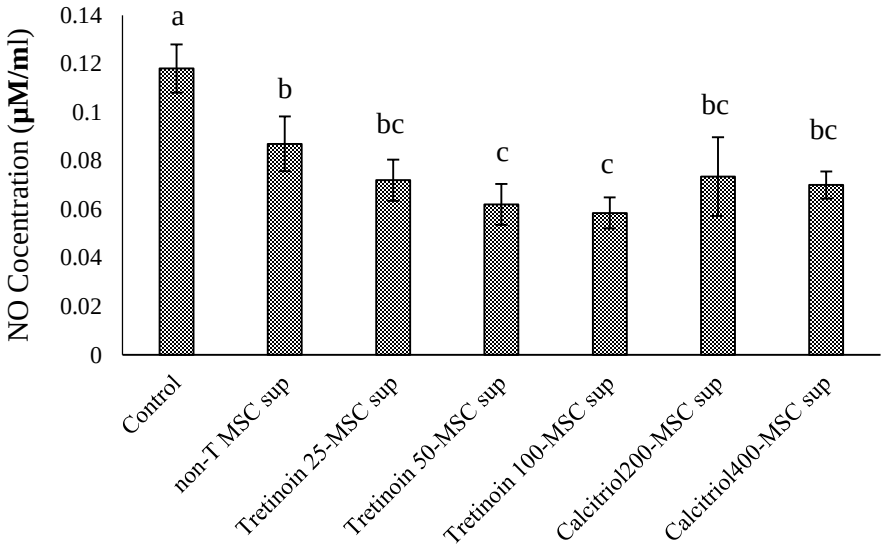

Figure 3. Effect of Tretinoin and Calcitriol on modulation of macrophage nitric oxide level by MSCs. Significant statistical variations between groups are denoted by a separate superscript letter (P<0.05) in each index.

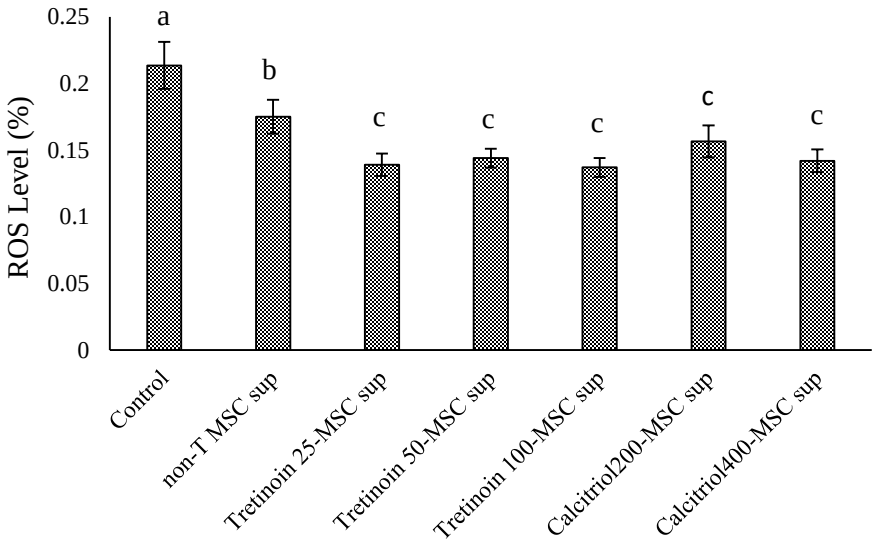

Figure 4. Effect of Tretinoin and Calcitriol on modulation of macrophage reactive oxygen species level by MSCs. Significant statistical variations between groups are denoted by a separate superscript letter (P<0.05) in each index.

Results

Respiratory burst assays: NBT assay showed that Tretinoin and Calcitriol-treated MSCs significantly diminished the rate of the respiratory bust of co-cultured macrophages in a non-dose-dependent manner compared to the control group (Figure 1).

Cell viability assay: Macrophage metabolic activity measured by MTT assay was used to evaluate the survivability of macrophages. Our findings indicated that, in a non-dose-dependent manner, the survival rate of co-cultured with Tretinoin and Calcitriol-treated MSCs, was significantly greater than that of macrophages co-cultured with non-treated MSCs (Figure 2).

Nitric Oxide assay: NO results revealed that NO

production decreased significantly in the Tretinoin and Calcitriol treated groups and a non-dose-dependent manner (Figure 3).

Reactive oxygen species (ROS) assay: Compared to the negative control group, ROS production decreased significantly in the Tretinoin and Calcitriol treated groups in the non-dose-dependent manner (Figure 4).

Discussion

Macrophages profoundly influence the expression and activation of immune responses and cancer development. They have a significant role in the host's defense and the pathophysiology of chronic and autoimmune conditions because they can eliminate pathogens and guide other immune cells (25). Furthermore, macrophages substantially influence the following stages of cellular functions and rehabilitation, including eliminating damaged tissue and the clearance of apoptotic cells. Flexible macrophages are likely to change their functional phenotype due to their environmental impact. (25-27). M1 macrophages or macrophages with classic activation have shown inflammatory and anti-microbial characteristics. It showed that M2 macrophages produce fewer pro-inflammatory cytokines, leading to their great potential for resolving inflammation through high phagocytic activities and trophic factor secretion (28). Owing to the interconnection of MSCs in tissue and bone marrow with macrophages, they may have a critical role in determining macrophages phenotype in microenvironments milieu (25-27). Calcitriol is likely capable of altering the interaction between MSC and macrophages as an environmental agent. Previous reports indicated that Calcitriol inhibits MSC proliferation, arrests the cell cycle, and accumulates MSCs in the G0/G1 phase without apoptosis induction (26-28). The expression of survivin was also dysregulated—furthermore, co-culture of MSCs with macrophages induced phagocytic activity of macrophages against Escherichia coli (27, 28). M2 macrophages usually have greater phagocytic as a characteristic. Reactive oxygen species (ROS) have considerable potential for removing invading microorganisms via phagocytes. ROS leads to critical host tissue injury and immuno- pathological states due to ROS's excessive or improper production. A diminished quantity of ROS is produced by M2 macrophages having excellent anti-inflammatory characteristics (29, 30). Other studies have reported a notable rise in uptake of neutral red and phagocytic activity of opsonized heat-killed baker's yeast could be created by the bone marrow-derived MSCs pulsed with Calcitriol. In addition, the Calcitriol treatment improved the viability of macrophages (17, 18, 29). Compared with the control group, a significant reduction was observed in the respiratory burst of co-cultured macrophages with Calcitriol-treated MSCs. According to the other studies, vitamin D3 treatment on MSCs potentiates the anti-inflammatory functions of MSCs-educated macrophages without any change in the microbicidal potential of macrophages after challenge with the opsonized pathogen (30). Hui Zha et al. showed that Anesthetics influence macrophage phagocytosis and the degree of receptor expression in macrophages. Isoflurane and sevoflurane, but not propofol, significantly reduced macrophage phagocytosis. They reported a reduction in macrophage phagocytosis in vitro in response to the volatile anesthesia Isoflurane and sevoflurane, but not in response to the intravenously administered propofol. They showed that sevoflurane also directly inhibited Rap1, a small GTPase necessary for phagocytosis (16).

Conclusion

Our data indicated that the supernatant of MSCs, isolated from bone marrow, pulsed with Tretinoin and Calcitriol might significantly reduce the respiratory burst of macrophages compared to the control group. Hence, immunomodulatory characteristics of Tretinoin and Calcitriol are evident since anti-inflammatory M2 macrophage polarization can be accelerated and potentiated by MSCs.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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