Caffeine alters the effects of bone marrow-derived mesenchymal stem cells on neutrophils

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Conflict of interest

None declared

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Abstract

Background. It has been shown that mesenchymal stem cells (MSCs) express all four adenosine receptors' subtypes, and stimulation of these receptors plays an active role in bone marrow-derived mesenchymal stem cell proliferation and differentiation. The interaction between MSCs and immunocytes, such as neutrophils, has been investigated in some recent studies.

Objectives. This study was carried out to investigate the effects of caffeine as an adenosine antagonist on the effects of bone marrow-derived MSCs on neutrophils.

Material and methods. Mesenchymal stem cells were isolated from the bone marrow of rats and pulsed with different concentrations of caffeine (0.1, 0.5 and 1 mM) at different times (24, 48 and 72 h). Mesenchymal stem cells were co-cultured with neutrophils for 4 h and the functions of neutrophils were evaluated.

Results. The findings showed that MSCs pulsed with caffeine at low to moderate concentrations preserved the neutral red uptake by neutrophils and established the MSCs' ability to protect neutrophils from apoptosis. Mesenchymal stem cells treated with caffeine increased the phagocytosis of neutrophils and simultaneously diminished the production of potentially harmful reactive oxygen substances, more profound than MSCs without treatment. Nevertheless, a high concentration of caffeine could interfere with some aspects of the crosstalk between MSCs and neutrophils.

Conclusions. These findings may offer new insight into the potential mechanisms underlying the immunomodulatory effects of caffeine.

Key words: neutrophil, bone marrow-mesenchymal stem cell, caffeine

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Introduction

Caffeine (1, 3, 7-trimethylxanthine) is a natural product and a member of the methylxanthine family of drugs, which can be found in coffee, tea, soft drinks, chocolate, kola nuts, and certain medicines.^{1,2} Caffeine possesses various effects on different body systems, including endocrine, cardiovascular, respiratory, urinary, gastrointestinal metabolism, immunity, and especially the central nervous system.^{3,4} In fact, caffeine is the world's most widely and legally consumed psychoactive drug.³ Caffeine is structurally similar to adenosine. Indeed, it is capable of binding to adenosine receptors without activating them, suggesting that caffeine is a competitive inhibitor of adenosine.^{5,6} It also acts as a competitive, non-selective phosphodiesterase inhibitor and, therefore, raises intracellular cyclic adenosine monophosphate.⁶

Mesenchymal stem cells are plastic-adherent, fibroblast-like, and multipotent non-hematopoietic progenitor cells that differentiate into various mesenchymal lineages, including bone and cartilage.⁷ Mesenchymal stem cells also showed a potent immunoregulatory activity, which can be a worthwhile strategy for the treatment of autoimmune diseases and graft-versus-host disease.^{8–10} It has been observed that the MSCs' functions are under the control of a large number of signaling systems.^{11–13} Interestingly, it has been shown that MSCs express all four adenosine receptors' subtypes, and stimulation of these receptors plays an active role in bone marrow-derived mesenchymal stem cell proliferation and differentiation.¹⁴

The interaction between MSCs and immunocytes, such as neutrophils, has been investigated in some recent studies.^{15,16} Nonetheless, there is no information about the role of caffeine on the crosstalk between MSCs and neutrophils. The current survey was set out to investigate the effects of caffeine on the crosstalk between bone marrow-derived MSCs and neutrophils in rats.

Material and methods

Dextran was bought from Fresenius Kabi (Verona, Italy). Fetal calf serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO/Life Technologies Inc. (Gaithersburg, MD). Moreover, caffeine, nitro blue tetrazolium (NBT), natural red (NR), dioxin, dimethyl sulfoxide (DMSO), tetradecanoyl phorbol acetate (TPA), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT), and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO). In addition, May-Grunwald-Giemsa stain was ordered from Merck (Darmstadt, Germany).

Isolation and proliferation of mesenchymal stem cells

Mesenchymal stem cells were isolated as described elsewhere.¹⁷ In brief, bone marrow was aspirated from

the tibias and femurs of deeply anesthetized Wistar rats. After 2 washings, the cells were plated in 75-cm² tissue-culture flasks with concentrations of 0.3 to 0.4×10^6 cells/cm² in the DMEM medium, supplemented with 15% fetal calf serum. The cells were incubated in a humidified 5% CO₂ at 37°C. Four days after primary culture initiation, non-adherent cells were removed and adherent cells were fed every other day. Mesenchymal stem cells were removed by trypsin/EDTA when the cultures reached 80% confluence. The cells were counted and passed in 1:3 ratios (about 1.5×10^6 cells/75-cm² flask). Cell passage was done up to subculture 3. Then, MSCs were incubated with different concentrations of caffeine (0.1, 0.5 and 1 mM) at different times (24, 48 and 72 h). Afterwards, the medium was aspirated and cells were washed three times with PBS.

Neutrophil isolation and incubation with mesenchymal stem cells

Blood samples were isolated under anesthesia by cardiac puncture in sodium citrate. The blood was centrifuged and the buffy coat was subjected to dextran sedimentation (1% w/v), followed by centrifugation on a Ficoll-Hypaque density gradient. The plasma and the mononuclear cell layer were removed, and erythrocytes were eliminated using hypotonic lysis. The neutrophils were washed and suspended in DMEM.¹¹ Following this procedure, the purity of neutrophils was 95%.

For co-culture experiments, 2×10^6 neutrophils were added to each well of 24-well flat-bottomed plates, containing 2×10^5 MSCs and incubated for 4 h at 37°C in a moist atmosphere of 5% CO₂. Afterwards, the neutrophils were isolated and used for the next experiments.

Evaluation of neutrophils viability

The viability of neutrophils was assessed by the MTT assay, similar to the procedures described earlier.^{18,19} Briefly, 100 μ L of the neutrophil suspension (2×10^6 cells/ml) was added to each well of 96-well microplates and pulsed with 20 μ L of the MTT solution (5 mg/mL) for 4 h at 37°C. To dissolve the formazan crystals, 150 μ L DMSO was added to each well of 96-well microplates and the plates were shaken vigorously. At the end, a microplate reader (Dynatech, Denkendorf, Germany) was used to determine the optical density (OD) at 550 nm. In addition, the experiments were performed in triplicate sets.

Neutral red uptake

Briefly, 100 μ L of the neutrophil suspension (2×10^6 cell/mL) was added to each well of 96-well microplates and pulsed with 10 μ L of the NR solution (0.33%) for 2 h at 37°C. At the end of the incubation period, the medium was discarded and the neutrophils were twice washed in PBS.

The internalized NR was solubilized for 30-min incubation by mixing 100 μ L 10% acetic acid plus 40% ethanol solution. The optical density was measured at 550 nm.

Phagocytosis assay

This experiment was designed as previously described, with some modifications.²⁰ In brief, the cells were washed after stationary incubation of neutrophils with opsonized yeast at 37°C for 1 h, cytocentrifuged onto glass slides, and fixed in methanol. The slides were stained with May-Grunwald-Giemsa staining. Yeast ingestion was evaluated by light microscopy under oil immersion. Phagocytic activities of neutrophils were reported as percentage of neutrophils, internalized at least one yeast cell.

Respiratory burst

The NBT reduction assay was used to check the intracellular generation of reactive oxygen species (ROS) by neutrophils.²¹ In brief, neutrophils were incubated for 20 min with 100 ng/mL TPA and 0.1% NBT. The unused NBT was discarded through washing and the reduced dye was extracted in dioxin and quantitated at 520 nm.

Statistical analysis

The normal distribution of data was confirmed with the Kolmogorov-Smirnov test. Next, the results were analyzed by one-way ANOVA plus Dunnett's post-hoc test and presented as means $\pm$ SD. The minimal level of significance was reported at *p* values of less than 0.05.

Results

The MTT test showed that MSCs could significantly increase the viability of neutrophils (Fig. 1). Moreover, MSCs pulsed with 0.5 mM of caffeine for 72 h and MSCs treated with 1 mM of caffeine for 24, 48 and 72 h significantly diminished the survivability of neutrophils, compared to the control group (the MSCs, which were not pulsed with caffeine) (Fig. 1). These findings suggested that caffeine at high doses can decrease the protective role of MSCs on the viability of neutrophils, so that MSCs treated with 1 mM of caffeine for 72 h significantly decreased the viability of co-cultured neutrophils compared to neutrophils alone.

As exhibited in Fig. 2, the NR uptake by neutrophils did not show any significant difference between neutrophils alone and neutrophils co-cultured with MSCs without treatment, or the MSCs pulsed with caffeine at concentrations of 0.1 and 0.5 mM. However, MSCs treated with caffeine at a concentration of 1 mM significantly lowered the NR uptake by co-cultured neutrophils (Fig. 2).

The phagocytic activity of neutrophils was significantly increased in the neutrophils co-cultured with the MSCs

Fig. 1. Effect of caffeine on modulation of the viability of the neutrophils by mesenchymal stem cells (MSCs). MSCs were isolated from bone marrow of rats and pulsed with different concentrations of caffeine (0 (control), 0.1, 0.5 and 1 mM) at different times (24, 48 and 72 h). Then mesenchymal stem cells co-cultured with neutrophils for 4 h. Greater optical density indicates higher levels of viability. The results showed that caffeine at least at higher doses could significantly decrease the protective role of MSCs on neutrophils apoptosis. Results were shown as mean $\pm$ S.D. Neut. – neutrophils alone, Control – neutrophils co-cultured with of MSCs alone (**p* < 0.01, ***p* < 0.001 vs neutrophils alone; # *p* < 0.01, ## *p* < 0.001 vs control)

Fig. 2. Modulation of Neutral red uptake by neutrophils. Greater optical density shows higher levels of Neutral red uptake by neutrophils. Results indicated that NR uptake by neutrophils didn't show any significant difference between neutrophils alone and neutrophils co-cultured with MSCs without treatment or MSCs pulsed with caffeine at concentrations of 0.1 and 0.5 mM. However, MSCs treated with caffeine at concentrations of 1 mM significantly decreased the NR uptake by co-cultured neutrophils. The values were presented as mean $\pm$ S.D. Neut. – neutrophils alone, Control – neutrophils co-cultured with of MSCs alone (**p* < 0.001 vs neutrophils alone or control)

pulsed with caffeine or the MSCs alone, compared to neutrophils without treatment (Fig. 3). The gained results also demonstrated that the phagocytic activity of co-cultured neutrophils and MSCs treated with caffeine was significantly more pronounced than phagocytosis observed by the co-cultured neutrophils and MSCs alone (Fig. 3).

Fig. 3. Modulation of phagocytic ability of opsonized yeast by neutrophils co-cultured with MSCs. Neutrophils were incubated with opsonized yeast at a ratio of 1:10 for 0.5 h at 37°C. Greater present value indicates higher levels of phagocytic ability. The phagocytosis activity of neutrophils was significantly increased following co-culture with of MSCs treated with at least 0.5 mM of caffeine for 24 h compared with control group. Moreover, MSCs treated with caffeine at least at concentration of 0.1 mM significantly increased the phagocytosis activity of neutrophils after 48 h and/or 72 h. Neut. – neutrophils alone, Control – neutrophils co-cultured with of MSCs alone (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ vs neutrophils alone # $p < 0.01$, ## $p < 0.001$ vs control)

Fig. 4. Evaluation of neutrophils respiratory burst after opsonized yeast ingestion by caffeine treated MSCs. Lesser optical density indicates lower levels of respiratory burst. Compared to control group, MSCs pulsed with 1 mM of caffeine for 24 h and of MSCs treated caffeine even at 0.1 mM for 48 h and/or 72 h significantly diminished the rate of respiratory burst of co-cultured neutrophils. Neut. – neutrophils alone, Control – neutrophils co-cultured with of MSCs alone (* $p < 0.01$, ** $p < 0.001$ vs neutrophils alone; # $p < 0.001$ vs control)

The NBT reduction assay was used to measure the reactive oxygen species (ROS) activity in neutrophils.²² The obtained findings expressed that the respiratory burst of neutrophils was significantly decreased in neutrophils co-cultured with the MSCs pulsed or without caffeine, compared to neutrophils without treatment (Fig. 4). The attained data also indicated that this reduction of the respiratory burst is more prominent in the neutrophils

co-cultured with caffeine (except caffeine at concentrations of 0.1 or 0.5 mM for 24 h) than that observed after co-culture of neutrophils and MSCs alone (Fig. 4).

Discussion

It has been revealed that MSCs interestingly produce adenosine and express adenosine receptors (A1R, A2AR, A2BR, and A3R), which clearly indicates that adenosine and adenosine receptors play an autocrine or paracrine role in the proliferation and differentiation of MSCs.^{14,23} Adenosine receptors are also differentially expressed in MSCs and involved in lineage-specific differentiation of MSCs. The A2B receptor is dominant in MSCs, and its expression and activity were transiently increased at the early stages of osteoblastic differentiation. During the later stages of osteoblastic differentiation, the expression of A2AR was increased.²³ On the other hand, differentiation of MSCs to adipocytes is associated with significant up-regulation in A1 and A2A receptors expression.²³ It has been known for a long time that the methylxanthine derivative, such as caffeine, can interfere with the adenosine/adenosine receptors biology.^{5,6}

Neutrophils are the most prominent cell type of the innate immune system and are predominant in host tissues during acute inflammatory processes.^{11,24} Neutrophils may also play an effective role in adaptive immunity.²⁴

Mature neutrophils are normally found in the bloodstream and inflamed tissues, instead of bone marrow. Mesenchymal stem cells localized in the perivascular and periendothelial areas can directly crosstalk with neutrophils.^{25,26} It is necessary to notice that MSCs localized in the perivascular area, derived from various tissues, have shown a phenotype similar to that of the bone marrow-derived MSCs.²⁶ Certainly, the isolation and expansion of MSCs from the bone marrow are easier than isolating MSCs from other tissues. Similar to the present study, some former studies also used the bone marrow derived MSCs to investigate the interaction between MSCs and neutrophils.^{11,27}

Neutrophil homeostasis and turnover are highly regulated in the body. Circulating neutrophils have a short life span of 6-10 h, after which the cells undergo apoptosis.²⁸ It was shown that MSCs significantly protect neutrophils from apoptosis and increase the life span of these cells.^{25,29} The MTT assay is a rapid test for assessing cell viability.¹¹ The findings showed that caffeine at higher doses could significantly decrease the protective role of MSCs on neutrophils apoptosis. Previous studies indicated that MSCs diminish the mitochondrial pro-apoptotic protein, Bax in neutrophils through IL-6 secretion.²⁷ It is also known that adenosine produced by MSCs can stimulate the A2B receptors through autocrine or paracrine routes, and potently stimulates IL-6 secretion.²³ According to the antagonistic effects of caffeine on adenosine receptor, it is possible that

caffeine at higher doses may interfere with IL-6 secretion by MSCs. However, the precise mechanisms involved in these effects are yet to be clarified.

Neutral red can be ingested and accumulated in the lysosomes of neutrophils depending on the level of cell activation. The neutral red uptake by neutrophils depends on different factors connected with cell viability, activity, and cell membrane integrity.³⁰

Phagocytosis is an essential function of neutrophils, which participates in the uptake of pathogens, apoptotic bodies, and debris.³¹ The phagocytic activity of neutrophils was markedly increased in neutrophils co-cultured with the bone marrow-derived MSCs compared to neutrophils without MSCs. Moreover, it has been demonstrated that the caffeine treated bone marrow-derived MSCs may cause a significant increase in the phagocytic ability of neutrophils more profound than MSCs alone.

Reactive oxygen species (ROS) are one of the important factors involved in the elimination of invading microbes by neutrophils.²² In addition to encountering pathogens, different stimuli may induce the respiratory burst in neutrophils.²⁷ Nonetheless, when the production of ROS is excessive or inappropriate, ROS participate in severe host tissue damages and in different immunopathological conditions.³² In this survey, it was observed that MSCs could significantly reduce the ROS production by neutrophils. In this regard, the former data indicated that the supernatant of MSCs could inhibit the basal and f-MLP-stimulated production of ROS by neutrophils.²⁷ The obtained data also indicated that MSCs pulsed with caffeine could profoundly inhibit the ROS production by neutrophils more pronounced than MSCs without treatment. Of note, higher phagocytic activity without the production of potentially harmful ROS can help phagocytes reduce inflammation.

Our in vitro findings suggest that at least some of the effects of caffeine on the interaction between MSCs and neutrophils may be different between the low to moderate and high concentrations of caffeine. Previous works also confirmed that caffeine had dose-dependent effects on the osteogenic differentiation of MSCs: 0.1 mM caffeine significantly potentiated mineralization and alkaline phosphatase activity, and upregulated the osteogenic differentiation of MSCs. However, a concentration of caffeine greater than 0.3 mM diminished the osteogenic differentiation of MSCs.³ A number of in vitro and in vivo studies have indicated that caffeine could modulate both innate and acquired immune responses.^{6,33,34} Moreover, it has been demonstrated that the effects of caffeine may be partly related to the dose of caffeine.⁶ Interestingly, some evidence has suggested that caffeine, even at the concentrations that are relevant to normal human consumption, may possess anti-inflammatory and immunomodulatory effects.⁶ Based on the attained data, it has been proposed in this paper that some of the immunomodulatory and anti-inflammatory effects of caffeine may be due to the change in the interaction between MSCs and neutrophils.

Conclusions

The observations in this research suggest that bone marrow derived MSCs pulsed with caffeine at low (0.1 mM) to moderate (0.5 mM) concentrations preserve the basic activity of neutrophils and established the MSCs ability to protect neutrophils from apoptosis. Mesenchymal stem cells treated with caffeine increased the phagocytosis of neutrophils and simultaneously, diminished the production of reactive oxygen substances more profound than MSCs without treatment. Nevertheless, a high concentration of caffeine could interfere with some aspects of the crosstalk between MSCs and neutrophils. Overall, these findings may offer new insight into the potential mechanisms underlying the immunomodulatory and anti-inflammatory effects of caffeine.

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