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Original Article

Altered immune responses in mice after receiving nicotine-pulsed mesenchymal stem cell-conditioned medium

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Abstract

Introduction*:* Previous investigations have documented that nicotine-pulsed mesenchymal stem cells (MSCs) can induce an anti-inflammatory phenotype in some immune cells in vitro. This study aimed to assess the effects of nicotine-pulsed MSCS in the function of immune cells, macrophages, and lymphocytes of mice receiving these cells.

Materials and methods: Bone marrow-derived MSCs (1.5×106) were seeded in a T75flask and incubated with 0, .1, .5, or 1 µM nicotine until the cells reached 90% confluency. Afterwards, immunophenotyping change, vitality, concentration of TGF-β, IL-10, and IDO levels of the MSC-conditioned medium were examined. Correspondent to in vitro results, the C57BL/6 mice intravenously received 400 µL of the conditioned medium of MSCs (CM), conditioned medium of nicotine (.5 µM)-pulsed MSCs (CMN), or medium. After 12 h, the lymphocytes, neutrophils, and peritoneal macrophages of the mice were isolated and their function was evaluated ex vivo.

Results: The least effective dose concentration of nicotine that led to an anti-inflammatory environment by the MSC-conditioned medium was 0.5 µM. Nicotine at this concentration prompted a higher level of TGF-β, IDO concentration in the conditioned medium. However, this concentration did not affect the MScs' markers expressions or MScs' vitality. T lymphocytes isolated from the mice receiving CMN showed a significant decrease in proliferation rate. The ratio of the IFN-γ gene expression to IL-4 gene expression in splenocytes was significantly reduced in the mice receiving CMN compared to the mice receiving CM. The neutral red uptake, respiratory burst, and nitric oxide production of the peritoneal macrophage only decreased in the mice treated with CMN. These factors also decreased in neutrophils isolated from mice receiving CM or CMN. However, these decreases were more prominent in the mice treated with CMN.

Conclusion: Treatment of MSCs by nicotine may be a promising strategy to enhance the immunomodulatory properties of these cells.

Keywords: Nicotine, Mesenchymal stem cells, Immunomodulation

1. Introduction

The discovery of non-hematopoietic, multipotent, fibroblast-like stem cells known as mesenchymal stem cells (MSCs) has revolutionized regenerative medicine and immune regulation [1, 2]. MSCs reside in the bone marrow and other mesenchymal tissues such as umbilical cord blood and dermal, peri-endothelial, and adipose tissues [1, 3]. MSCs are noted for playing an essential role in modulating the immune system via a close relationship with immune cells and secreting a wide variety of soluble immunoregulatory factors [4-7]. Nowadays, cell therapy with MSCs or using the conditioned media of these cells is considered a promising strategy to control and treat a variety of diseases associated with tissue damage and unbridled inflammation such as SARS-CoV-2 and autoimmune diseases [8-11].

The most important reason for the tendency to use MSCs is their immunoregulatory property along with their relatively easy isolation and rapid growth[11]. Furthermore, the benefits of immunosuppressive properties and the low immunogenicity of MSCs contribute to a lessened immune response induced by the implantation of allogeneic MSCs in comparison with other cells [4]. The use of the conditioned medium of allogeneic MSCs, given its cell-free nature as well as reduction of the risks associated with cell transfer (especially the transmission of some latent viruses), can be widely commercialized[12, 13]. However, due to some factors, particularly the inadequacy of reducing inflammation and properly regulating immune responses, the use of MSCs or their conditioned media has been limited[5]. The immunoregulatory function of MSCs can be affected by a broad spectrum of their surface nuclear receptors such as the receptors of TOLL-like, adenosine, calcitriol, retinoic acid, and steroid nuclear receptors [1,5,14-16]. Therefore, interventions such as the use of agonists or antagonists of these receptors may be effective in altering the immunomodulatory function of MSCs.

Second to caffeine, nicotine is the most widely used pharmacological substance consumed by a wide range of people. Nicotine alkaloid is one of the main stimulants of cholinergic nicotine receptors. Both choline acetyltransferase and acetylcholinesterase enzymes and also acetylcholine are expressed on in MSC population. Moreover, MSCs represent the nicotinic acetylcholine receptor subunits α3, α5, β2, β4, and α7 [17, 18]. Nicotine alkaloid is a well-known prototypic ligand for the nicotinic parasympathetic receptor. This alkaloid is found in high levels in tobacco leaves and in small amounts in other plants such as the Solanaceae family [14]. Of note, unlike cigarettes, nicotine itself has beneficial immune-regulating effects on some autoimmune disorders such as rheumatoid arthritis and the animal model of multiple sclerosis [19, 20]. Stimulation of MSCs with the nicotinic receptor agonist like nicotine promotes immediate and transient expansion in intracellular Ca^{2+} concentration
[18]. Recent studies have demonstrated that nicotine treatment, in addition to altering the proliferation and differentiation of MSCs, causes profound changes in the function of neutrophils directly adjacent to them or neutrophils treated with the conditioned medium produced by these MSCs[14, 21].Therefore, in vitro stimulation of nicotine receptors appears to alter the interaction of stem cells with adjacent immunocytes such as neutrophils. However, there is no information about the probable altered immune responses in a recipient after receiving a nicotine-pulsed MSC-conditioned medium compared to the

conditioned medium of un-pulsed MSCs. Thus, the present study was conducted to assess the effects of nicotine- pulsed MSCS in the function of neutrophils, macrophages, and lymphocytes of C57bl/6 mice receiving these cells.

2.Materials and Methods

Enzyme-linked immunosorbent assay (ELISA) kits (Cat. Number# 900-TM98, 900-TM49, 900-M47 and 900-TM54) were obtained from PeproTech EC, Ltd. (London, UK). The RNX-Plus solution for RNA isolation was procured from Sinaclon (Tehran, Iran). SYBR Premix ex Taq II (Cat. Number#AGY1013N) and cDNA reverse transcription kits (Cat. Number#RR037A) were purchased from TAKARA (China). The monoclonal antibodies required for flow cytometry were purchased from abcam. Nicotine (Cat. Number#N3876) and other reagents were prepared from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Conditioned medium of MSC production and immunoregulatory assessment:

In brief, the femurs and tibias of male C57bl/6 mice were dissected and the marrow was flushed into a 15ml tube. The isolated cells were suspended in low-glucose Dulbecco's Modified Eagle's medium (DMEM, Cat. Number# D0819) and centrifuged at 1200 rpm for 5 min. The cells were seeded in T75flasks in low-glucose DMEM containing 15% fetal bovine serum (FBS). The cells were incubated in humidified 5% CO_2 at 37°C. The non-adherent cells of the flasks were discarded three days later. When adherent cells reached an about 90% confluence, the cells were isolated with trypsin/EDTA, counted, and passed at 1:3 ratios (about 1.5×10^{6} cells/75-cm^2 flask)[14].

MSCs at passage 3 were seeded in the T75flask and pulsed with different doses of nicotine (0, .1, .5, or 1 µM) until the cells reached 90% confluency. Afterwards, cells were rinsed twice and cultured in serum-free DMEM for 24 h. The conditioned media were collected, centrifuged at 12000g for 15 min, and filtered through a 0.22µm membrane. The average MSC count following the removal of the conditioned medium was 2.8×10^6 cells.

The secretion of IL-10 and TGF-β was monitored in the in aliquots from the MSC-conditioned medium by using commercial ELISA kits. The biological activity of indoleamine 2, 3-dioxygenase (IDO) was estimated as previously described. One part of 30% trichloroacetic acid was added with two parts of the conditioned medium, vortexed, and centrifuged for 5 min. Then, the supernatant was mixed with an equal volume of Ehrlich reagent (1mg p-dimethyl benzaldehyde in 5 µl glacial acetic acid) in a 96-well flat-bottomed plate, and the absorbance was recorded at 492 nm[22].

To monitor the effect of nicotine on the MSC immunophenotype, MSCs at passage 3 were isolated, washed, and stained with a fluorescently labeled monoclonal antibody against mouse CD45 (APC Anti-CD45 antibody [104] (ab210182)),CD44(FITC Anti-CD44 antibody [KM201] (ab25064)), and CD29 (PE/Cy7® Anti-Integrin beta 1 antibody [HMb1-1] (ab95622)) as described above(23). The stained MSCs were evaluated immediately on a FACS Calibur-flowcytometer (BD Biosciences, San Diego, CA) and Cyflogic software (CyFlo Ltd.). Moreover, the metabolic activity and vitality of MSCs were evaluated by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, as previously described[14].

Animal experiments:

Male C57bl/6mice aged 6-8 weeks were obtained from the Pasteur Institute of Iran. Animal procedures were set out in accordance with the Ethics Committee of the Faculty of Veterinary Medicine of Urmia University, Urmia, Iran. The mice were housed at constant temperature

(22±2˚C) with a 12-hour light/dark cycle and had access to water and food ad libitum. After the time required for the mice to adapt to the conditions, the C57BL/6 mice were randomly allocated to three equal groups (n=7) and intravenously received 400 µL of the conditioned medium of MScs (CM), conditioned medium of nicotine (.5 µM)-pulsed MSCs (CMN), or Dulbecco's Modified Eagles Medium alone. It is worth noting that the least effective dose concentration of nicotine that led to potent anti-inflammatory benefits in the MSCs was .5 µM and was, therefore, selected for *in vivo* investigation. After 12 h, the lymphocytes, neutrophils, and peritoneal macrophage of the mice were isolated under deep anesthesia, and their function was evaluated *ex vivo*.

Neutrophil isolation and function assessment

Neutrophils were isolated from the heparinized cardiac puncture blood of deeply anesthetized C57bl/6 mice. Briefly, the samples were centrifuged, and the buffy coat was subjected to dextran sedimentation (1%, w/v) to remove the RBCs. Subsequently, the cells were centrifuged on a Ficoll–Hypaque density gradient. The mononuclear cell layer and plasma were removed. The remnant RBCs were removed via the ACK-RBC lysis buffer. The isolated neutrophils were washed twice and suspended in DMEM containing 10% heat-inactivated FBS [21]. The viability of the isolated neutrophils evaluated by trypan blue exclusion was never <95%.To monitor the vitality of neutrophils, a neutrophil suspension was added to each well of 96-well microplates (4×10^4/well) and pulsed with 20µl of the neutral red (NR) solution (3.3mg/ml) for 2 h at 37°C. The medium was carefully eliminated and the neutrophils were rinsed three times to delete un-endocytosed dye. The incorporated dye was then extracted by 200 µl of lysing buffer (1% acetic acid in 50% ethanol) and quantitated at 540 nm [21].

Neutrophils were also cultured with 1 mM of N-formylmethionyl-leucyl-phenylalanine (f-MLP) and .1% Nitroblue tetrazolium (NBT) dye. The unincorporated NBT was removed by washing in triplicate, and the reduced dye was solubilized in dioxane. The optical density was recorded at 520 nm. Moreover, neutrophils were incubated with 1 mM of f-MLP for 2 h, and nitric oxide production was monitored in the supernatant of the culture media [14].

Isolation of peritoneal macrophages and evaluation of their function:

5 ml of ice-cold PBS was injected into the peritoneal cavity of euthanized C57bl/6 mice. To dislodge the attached cells, the peritoneum of each mouse was gently massaged. The peritoneal fluid was collected and centrifuged at 1500 rpm for 10 min at 4˚C. To remove non-adherent cells, 1×10^6 cells are added to 48-well tissue culture plates and incubated for 2 h at 37˚C in a moist atmosphere of 5% CO_2. The non-adherent cells were remove by washing in triplicate with ice-cold PBS[6].

To assess the vitality of the macrophages, each well was pulsed with 40 µl of the NR solution (3.3 mg/ml) at 37°C. After 4 h, the medium was carefully removed and the cells were rinsed three times to remove the un-internalized dye. The incorporated dye was then solubilized by 400 µl of the lysing buffer (1% acetic acid in 50% ethanol) and the optical density at 540 nm was recorded[24].

To evaluate the respiratory burst of the macrophages, the cells were cultured for 20 min with .1% NBT and 100 ng/ml tetradecanoylphorbol acetate. The unused dyewas removed by washing in triplicate and the reduced NBT dye was solubilized in dioxin.The optical density was recorded at 520 nm[6, 25].

To monitor nitric oxide production by macrophages, the cells were pulsed with LPS (10 pg/mL).The potential of nitric

oxide production was monitored by the Griess method after 24 h[25].

Evaluation of splenic lymphocyte function

To evaluate the lymphocyte proliferation index, the spleens were aseptically removed, crushed, and elapsed through a filter with a size of 20 µL to prepare a single-cell suspension. The cells were incubated in 96-well flat-bottomed plates (1×10^5 cells/100 µl/well) and pulsed with phytohemagglutinin (PHA, 5 µg/ml) or the medium alone. After 4 h, each well was pulsed with 50 µl of the MTT solution (5mg/ml) for 4 h at 37°C. Then, 400µl of DMSO was added and shaken to solve the formazan crystals. The optical density at 550 nm was recorded. The results were reported as the ratio of the absorbance of stimulated splenocytes with PHA to OD550 of non-stimulated cells[6, 26].

Moreover, the splenocytes suspension (2×10^6/ml) was cultured in T75flasks and pulsed with phytohemagglutinin (PHA, 5 µg/ml) for 72 h. To monitor the expression of IFN-γ, and IL-4 total RNA from the splenocytes was extracted by using the RNX-Plus solution according to the manufacturer's guidelines and used to synthesize complementary DNA. PCR amplification was run in triplicate by an SYBR Green kit according to the manufacturer's guidelines[6].The reference gene was the HPRT gene. Forward and reverse primers for mRNA amplification are given in Table 1. The findings are presented as $2^{-\Delta\Delta Ct}$ (mean fold change).

Statistical Analysis

The normal distribution of the data was determined by the Shapiro-Wilk test. The findings were analyzed through one-way analysis of variance (ANOVA) and Tukey's *post hoc* test, expressed as means±SD. The level of significance was assumed at *P* values of <.05.

3. Results

In vitro results

Surface marker analysis demonstrated that third passage of MSCs or nicotine-pulsed MSCs were negative for CD45 expression but positive for CD44 and CD29 (Figure 1). More importantly, there was no statistical discrepancy between the MSCs or nicotine-primed MSCs in terms of expressing the pattern of the above marker (Figure 1).

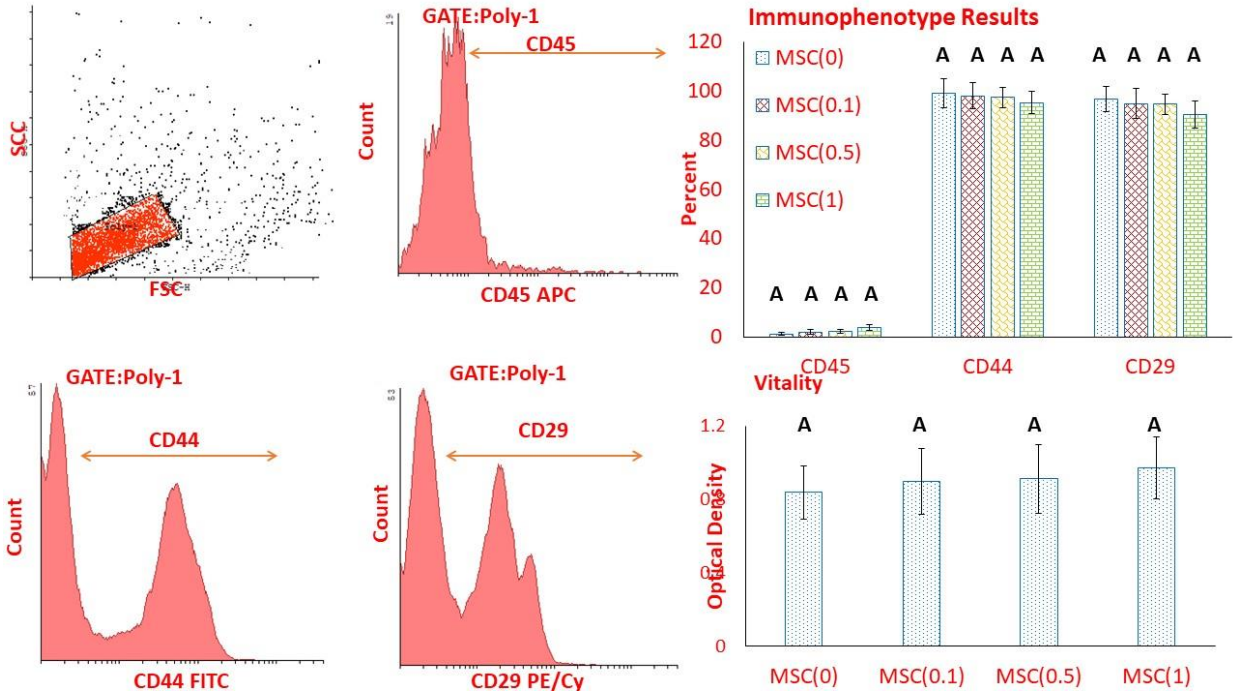

Figure 1. Analysis of surface marker and vitality of MSCs. MSCs at passage 3 were pulsed with different doses of nicotine (0, 0.1, 0.5, or 1 µM) until the cells reached 90% confluency asdetailed under Materials and Methods.CD45, CD44 and CD29 markers were used to prove the correct isolation of mesenchymal stem cells in the third passage. Since the CD45 marker is expressed on the surface of leukocytes, it was absent. On the other hand, there was no discrepancy in the expression of the CD45, CD44, and CD29 and vitality between the MSCs or nicotine-pulsed MSCs.The MTT assay is applied to evaluate mitochondrial metabolic activity as an indicator of cell vitality and functional ability. As shown in Figure 1, nicotine at all used concentrationscould not alter the proliferation rate of MSCs compared to MSCs without treatment Datawere presented as mean ± S.D. (Different letters indicate a significant difference at the level of $P<0.05$).

The MTT assay is applied to evaluate mitochondrial metabolic activity as an indicator of cell vitality and functional ability. As shown in Figure 1, nicotine at every used concentration could not alter the proliferation rate of MSCs compared to MSCs without treatment (Figure 1).

The secretion of immunoregulatory mediators by MSCs incubated with 0, .1, .5, or 1µM nicotine was monitored by determining TGF-β, IL-10, and IDO levels in MSC supernatants (Figure 2). MSCs Produced significantly higher levels of IDO and TGF-β levels when cultured with .5µM nicotine in comparison with the other groups (Figure 2). IL-10 production was significantly higher when MSCs were pulsed with .1µM and .5µM nicotine compared to 0µM and1µM nicotine (Figure 2). The production of TGF-β and IL-10 by MSCs was significantly reduced when MSCs were cultured with 1µM nicotine in comparison with the other groups. The concentration of IDO in the supernatant isolated from MSCs treated with 1µM nicotine reached the concentration produced by untreated MSCs (Figure 2).

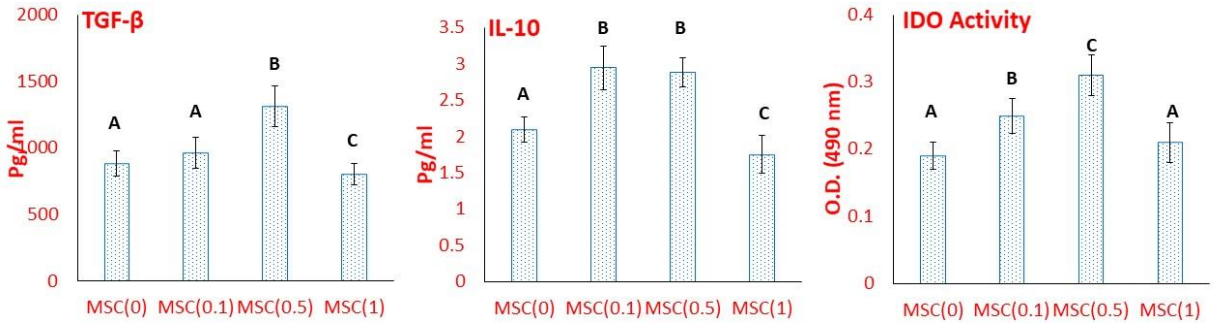

Figure 2. Assessment of the secretion of immunoregulatory mediators by MSCs. Passage 3 of MSCs were incubated with different doses of nicotine (0, 0.1, 0.5, or 1 µM)until the cells reached 90% confluency asdescribed under Materials and Methods. Resultswere reported as mean ± S.D. (Different letters indicate a significant difference at the level of P<0.05).

Overall, the most appropriate concentration of nicotine that created the best anti-inflammatory environment in mesenchymal stem cell secretions was .5 µM. Therefore, the conditioned medium of MSCs treated with .5 µM nicotine concentration was used for *in vivo* examination.

Ex vivo results

T lymphocytes isolated from the mice receiving CM or CMN showed a significant decrease in proliferation after stimulation by phytohemagglutinin. However, receiving CMN significantly lowered lymphocyte proliferation in splenocytes compared to the othergroups(Figure3).

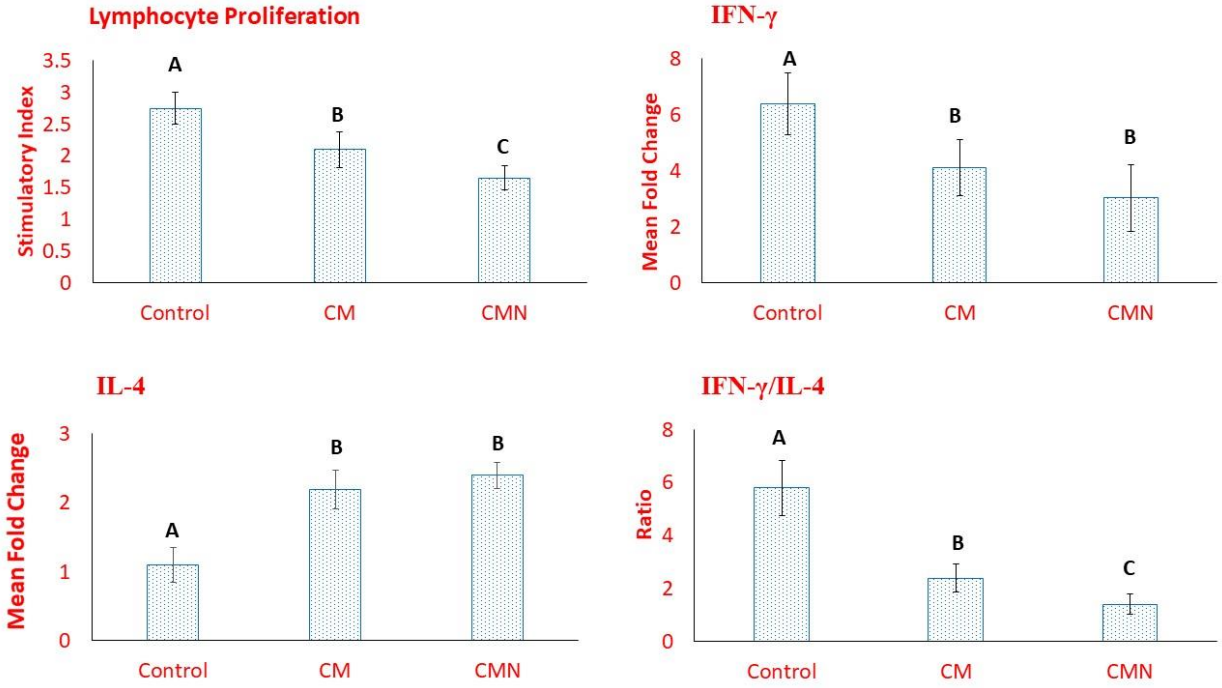

Figure 3. Evaluation of splenic lymphocyte function after co-culture withconditioned medium of MSCs (CM), conditioned medium of nicotine (0.5 µM)-pulsed MSCs (CMN). The findings were reported as mean ± S.D. (Different letters indicate a significant difference at the level of P<0.05).

As shown in Figure 3, intraperitoneal injection of CM or CMN caused a remarkable decrease in the mRNA expression of IFN-γ and a significant increase in IL-4in the splenocytes after stimulation by phytohemagglutinin. The results revealed that the highest decrease in the mRNA expression of IFN-γ occurred in

the group receiving the CMN compared to other groups. Figure 3 also demonstrates that there was no significant difference between the production potential of IL-4 between mice receiving the CMN or CM. More importantly, the ratio of IFN-γ gene expression to IL-4 gene expression in splenocytes was significantly reduced in the mice that received CMN compared to the mice that received CM (Figure 3).

According to Figure 4, receiving the untreated conditioned medium was not effective in altering the function of peritoneal macrophages (neutral red uptake, respiratory burst, and nitric oxide production). Nevertheless, the neutral red uptake, respiratory burst, and nitric oxide production of peritoneal macrophage significantly decreased in mice treated with CMN(Figure4).

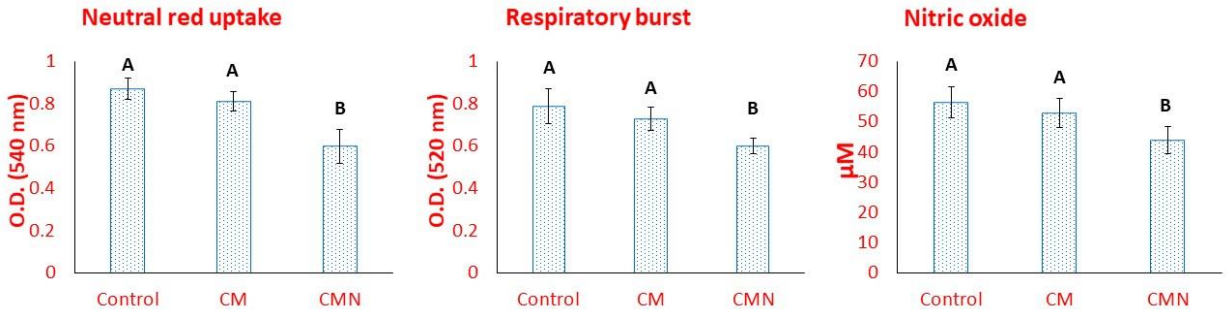

Figure 4. Assessment of macrophage functions after co-culture with conditioned medium of MSCs (CM) and conditioned medium of nicotine (0.5 µM)-pulsed MSCs (CMN) has been shown by Nutral Red uptake, Respiratory Burst ability and nitric oxide production test.Data were shown as mean ± S.D. (Different letters indicate a significant difference at the level of $P<0.05$).

As for the neutrophils, the results were somewhat different (Figure 5). The neutral red uptake, respiratory burst, and nitric oxide production of neutrophils declined in both mice receiving CM or CMN. However, these decreases were more prominent in the mice treated with CMN (Figure 5).

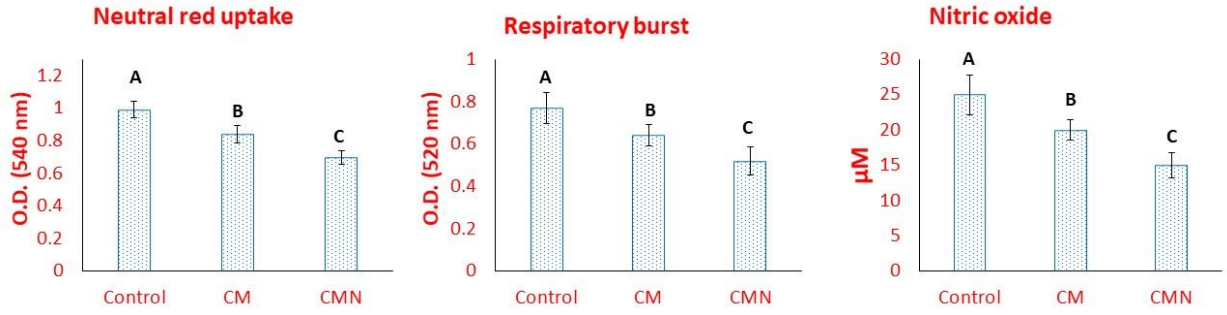

Figure 5. Evaluation of neutrophil functions after co-culture with conditioned medium of MSCs (CM), conditioned medium of nicotine (0.5 µM)-pulsed MSCs (CMN). Results were shown as mean ± S.D. (Different letters indicate a significant difference at the level of $P<0.05$).

4. Discussion

Cell therapy with MSCs has some limitations. For instance, the efficacy of implanted MSCs is highly dependent on environmental inflammatory conditions. It also carries the risk of transmitting retroviruses. This is while the conditioned media can be easily commercialized and turned into a drug-like product since they lack cells. Therefore, the use of conditioned environment and strengthening its immunomodulatory powers is a suitable

alternative to cell therapy which was used in this study [6, 27].

It is well-acknowledged that the cholinergic anti-inflammatory pathway through stimulation of α7 nicotinic acetylcholine receptor regulates immune responses[28]. Accordingly, nicotine can lower pro-inflammatory cytokine production, T cell activation, and antibody response[29]. MSCs can respond to nicotine as well as immune cells[14, 21], but the specific means whereby nicotine regulates the immunomodulatory benefits of MSCs need further elucidation. The present study was designed for this purpose.

Previous data have indicated that nicotine causes instant and temporary enchantment in the intracellular Ca^{2+} concentration and phosphorylation of the extracellular signal-regulated kinases 1 and 2 in the MSC population [17]. The intracellular increase in calcium changes the proliferation, cytoskeletal rearrangement, and differentiation of MSCs[30]. Our results showed that the immunophenotype markers of MSCs remained mostly similar among groups. Previous studies have examined the effect of nicotine on mesenchymal stem cell proliferation and differentiation. It has been realized that rat MSCs exposed to 25, 50, and 100μM nicotine experienced a dose-dependent reduction in COL2A1 and ACAN gene expression and chondrogenic induction after four weeks compared to untreated MSCs[31]. An *in vitro* study about human MSCs showed that nicotine at physiological concentrations (.1 to 1 μM) had minimal effects on chondrogenic induction, whereas 10 μM nicotine inhibited chondrogenic induction [32]. Shen et al. reported that human MSCs treated with 50–100 nM nicotine for seven days had a significant rise in the cell number; nevertheless, nicotine at 1 μM significantly reduced cell proliferation [33]. On the other hand, it has been observed that human umbilical cord blood cells pulsed with 3–9mM nicotine have dose-dependent increases in apoptosis [32, 34]. Significant reductions in proliferation have also been reported after the exposure of MSCs to a concentration of 0.1–10μM nicotine [32]. The data in our study showed that after 48 h of exposure, nicotine at all concentrations could not alter the proliferation rate of MSCs.

A recent *in vitro* study also suggested that nicotine can alter MSCs' immunoregulatory properties. Accordingly, the viability and phagocytic activity of neutrophils co-cultured with nicotine-primed MSCs significantly increased compared to untreated MSCs. Yet, MSCs primed with 1μM nicotine reduced the respiratory burst of co-cultured neutrophils, triggered by opsonized yeast [21]. CM-MSCs can be therapeutically administered because of their potent immunomodulatory properties, similarly to MSCs. Soluble factors such as indoleamine 2,3-dioxygenase (IDO), TGF-β, and IL-10[23, 35]. IDO production by MSCs promotes the discharge of tryptophan, which is an indispensable amino acid for lymphocyte proliferation [23]. TGF-β and IL-10 cytokines are among the most famous cytokines that maintain homeostasis during immune responses[36]. TGF-β and IL-10 also mediate T lymphocyte suppression by MSCs[23]. The results of this study indicated that MSCs pulsed with .5μM nicotine secreted significantly higher levels of IDO and TGF-β compared to other groups. The highest level of IL-10 was observed in the conditioned medium of MSCs pulsed with .1μM and .5μM nicotine. However, a concentration of 1μM of nicotine interfered with MSCs' regulatory functions.

It was also shown that 24 hours after nicotine injection, the expression level of TGF-β is greatly raised, which in turn causes the cells to polarize towards Treg. High values also rise with increase in nicotine concentration from .1mμ to 1mμ[37] Nicotine appears to play this role as an inhibitor of immune function and

inhibit the secretion of many cytokines. Many studies have shown that innate immune system receptors such as TLR2 and TLR4 and NOD receptors are significantly reduced in smokers. They are also reported to alter the secretion of cytokines IL6, IL8, TNF-α, IL10 and the chemokines CCL-2, CCL-5, CXCL9 and CXCL10[38].Nicotine α7 receptor appears to be expressed on TCD4 + lymphocytes and stimulation with nicotine in these receptors reduces T proliferation and activation of T lymphocytes. All of these events lower the secretion of IL-17, IL-17F, IL-21, and IL-22 cytokines. Increased polarization toward Th2 also increases IL-4 production. Decreased T.bet expression and increased GATA-3 expression suppress Th1 and Th17 more. T lymphocytes with α7 -/- marker are not affected by nicotine. There is a hypothesis that repeated administration of nicotine may also cause a state of unresponsiveness and tolerance in the immune system[39].Therefore, based on *in vitro* results, the least effective dose concentration of nicotine leading to an anti-inflammatory environment by the MSC-conditioned medium was .5 µM.

Then, for the first time, we compared the *in vivo* immunomodulatory effects of the conditioned medium isolated from nicotine-treated MSCs at a .5 µM concentration with the conditioned medium isolated from untreated MSCs. The suppressive effect of MSCs or their conditioned medium on lymphocyte proliferation in various models of autoimmune and inflammatory diseases has been reported in scientific sources[23]. Our results also indicated that T lymphocytes isolated from mice receiving CMN significantly lowered the proliferation index after stimulation by phytohemagglutinin compared to T lymphocytes isolated from mice receiving CM. Furthermore, the ratio of IFN-γ gene expression to IL-4 gene expression in splenocytes was significantly reduced in the mice receiving CMN compared to those receiving CM. Besides assessing the role of CMN on the proliferation of T lymphocytes, the production of IL-4 and IFN-γ by phytohemagglutinin-stimulated lymphocytes was assessed.IL-4 represents type II immune responses and IFN-γ represents type I responses. In brief, type I immunity is protective against most infections and for perpetuating auto-inflammatory and autoimmune responses, while type II responses protect against worm parasites and help eliminate cell-mediated inflammation[40]. Of course, if an immunopathological condition is caused by the polarization of one side of one of these types of immune responses, a change in the type of polarization will ameliorate the destructive effects of the disease. The polarization of immune responses to type II responses after treatment with MSCs or their conditioned medium has been shown in auto-immune and auto-inflammatory models [41, 42]. More importantly, our results demonstrated a more potent polarization of immune responses toward type II responses in mice receiving CMN compared to those receiving CM.

To define the *in vivo* effect of CM or CMN on innate immunity responses, neutrophils and macrophages were isolated from the mice receiving CM or CMN. Neutral red is a cationic dye that is endocytosed depending on the activity level of the membrane part of neutrophil and macrophage cells and stored in the lysosomal fraction of these cells [14, 21]. In other words, when these cells are stimulated by environmental factors such as immune complexes, their rate of neutral dye uptake will increase [21]. The increased endocytotic activity of innate immune cells has been observed in immunopathological conditions[43]. To eliminate the invaders, neutrophils and macrophages own or generate a variety of cytotoxic mediators, including numerous proteases, reactive oxygen species (ROS), and reactive nitrogen substances (e.g., nitric oxide)[14, 21]. Chakkarwar (2011) reported that nicotine significantly reduced NO

production. The presence of a nicotine inhibitor such as Fenofibrate eliminates the effects of nicotine in inhibiting NO production. The presence of nicotine in the vicinity of cells reduces the effects of phagocytosis and respiratory explosion and causes immunosuppressive and anti-inflammatory effects[44] In the case of neutral test(NR), nicotine has been shown to be more effective than similar compounds and nicotine derivatives such as caffeine, niacinamide and nicotinic acid in most cell lines to reduce the rate of neutral cation depletion and cell inhibition[45]

In MTT test, no significant difference was observed between different groups treated with supernatant of mesenchymal stem cells with nicotine and without nicotine and also between control groups. The reason seems to lie in the concentration of nicotine. As stated, the effects of nicotine proliferation and survival are not observed at concentrations below 100 µm. In fact, cell viability remains intact at concentrations below 100 µm nicotine[46]. Some studies suggest that higher concentrations of nicotine are required to induce apoptosis and cell death in different cell lines. These studies show that concentrations higher than 6 µm are required to observe the effect of nicotine on cell growth and proliferation[47] and another interesting study indicated that concentrations around 1 µm can also increase growth and proliferation[48].

Nevertheless, in many immunopathological conditions, the exaggerated production of these mediators causes extensive tissue damage [21]. Reduced production of ROS and nitrogen mediators, especially by neutrophils, has been shown *in vitro* or *in vivo* by receiving stem cells or their conditioned medium[49]. The NBT reduction assay is performed to monitor ROS production after induction of respiratory burst [21]. The grease test is also a known test to evaluate the production capacity of nitric oxide[50].According to the results of this study, it seems that receiving conditioned medium from untreated MSCs does not change the *ex vivo* functions of peritoneal macrophages. The neutral red uptake, respiratory burst, and nitric oxide production of peritoneal macrophage were only lowered in the mice treated with CMN. However, the neutral red uptake, respiratory burst, and nitric oxide production of neutrophils were decreased in both mice receiving CM or CMN. However, these reductions were more prominent in the mice treated with CMN. All these data indicate a profound effect of nicotine in strengthening the immunomodulatory potential of the conditioned medium of MSCs.

Taken together, these findings indicated for the first time that the treatment of MSCs by nicotine can be a promising strategy to increase the immunomodulatory properties of these cells. Based on in vitro results, the least effective dose concentration of nicotine leading to an anti-inflammatory environment by the MSC-conditioned medium was .5 µM. Nicotine at this concentration prompted a higher level of TGF-β, IDO concentration in the conditioned medium compared to the other treatments. However, this concentration did not affect the MScs' markers expressions or MScs' vitality. Based on *ex vivo* results, the T lymphocytes isolated from the mice receiving nicotine (.5 µM)-pulsed MSCs (CMN) showed a significant decrease in proliferation after stimulation by phytohemagglutinin. Moreover, the ratio of the IFN-γ gene expression to IL-4 gene expression in splenocytes was significantly reduced in the mice receiving CMN compared to the mice receiving CM. The neutral red uptake, respiratory burst, and nitric oxide production of the peritoneal macrophage were only lowered in the mice treated with CMN. The neutral red uptake, respiratory burst, and nitric oxide production of neutrophils decreased in both mice receiving CM or CMN. However, these decreases were more prominent in

mice treated with CMN. These results can offer a novel insight into the potential mechanisms underlying the immunoregulatory effects of nicotine although these findings are preliminary and further research with another source of MSCs or *in vivo* models seems necessary.

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Ethical approval

Ethics code and its issuing reference should be mentioned.

Conflict of interest

The authors declare no conflict of interest.

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Genes	Forward primer	Reverse primer	Refrences
IL-4	5′-CACTTAGCTGTGACACACTTCTCGAGAGAC-3′	5′-CAAGAAGTTTTCCAACGTACTCTGGTTGGC-3′	(51)
IFN-γ	5′-TCAAGTGGCATAGATGTGGAAGAA-3′	5′-TGGCTCTGCAGGATTTTCATG-3′	(52)

Table 1. Primer pairs used for IL-4 and IFN-γ were shown in up table.