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Research article

Enhancement the antioxidative and immunomodulatory functions of mesenchymal stem cells by tetrandrine

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ABSTRACT

In this study, mesenchymal stem cells (MSCs) were primed with Tetrandrine (TET) having anti-inflammatory and immunomodulatory effects to examine the effects of this molecule on the antioxidative potential of MSCs as well as their modulatory effects on activated peripheral blood mononuclear cells (PBMCs). The viability of primed MSCs was detected using MTT assay and Trypan blue staining. Moreover, flow cytometry technique was applied to evaluate cell cycle distribution and immunophenotype of MSCs. The production of superoxide dismutase 3 (SOD3), malondialdehyde (MDA), kynurenine, TGF-β, and IFN-γ were also measured by spectrophotometry to assess the alteration of antioxidative and immunomodulatory potential of MSCs. Then, TET-primed MSCs were cocultured with PBMCs. The MTT assay was used to measure the proliferation of PBMCs. Cell cycle progression of PBMCs and frequency of regulatory T cells were evaluated using Flow cytometry. ELISA assay was also applied to determine the concentrations of TGF-β and IFN-γ after coculturing. According to our data, TET enhanced the secretion of SOD3 and kynurenine from MSCs, while the production of IFN-γ was reduced. No changes were observed in the viability, proliferation, and immunophenotype of MSCs after priming with TET. Moreover, the proliferation and frequency of PBMCs in the S and G2/M phases of cell cycle reduced after co-culturing with TET-primed MSCs. The concentration of TGF-β was increased in the supernatant of PBMCs, but the level of IFN-γ was reduced.

Our data suggested this priming method as a novel strategy for increasing the antioxidative and immunomodulatory activity of MSCs.

1. Introduction

Today, the use of Mesenchymal stem cells (MSCs) which have the ability to self-renew, differentiate into another cell lineage, and modulate the immune system is considered one of the promising treatment strategies for different diseases, especially inflammatory and immune-related diseases [1–3]. MSCs can be harvested from many tissues although adipose tissue which have large quantities in the body and can be isolated with less invasive procedures, is the most popular source for obtaining high numbers of MSCs. There are different mechanisms by which MSCs regulate the functions of immune cells such as cell-cell interactions and secretion of immune

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modulators [4]. Additionally, extracellular vesicles (EVs) derived from MSCs can regulate different functions of immune cells by their surface molecules and transferring bioactive molecules such as lipids, proteins, cytokines, coding and noncoding RNAs, growth factors, and metabolites [5].

The most important modulators of MSCs are prostaglandin E2 (PGE2), TGF-β, IL-10, and indoleamine 2,3-dioxygenase (IDO) [6]. The mentioned immune-suppressive factors modify the phenotypic profile of immune cells, especially T helper (Th) cells, which orchestrate the progression of auto-immune diseases. Moreover, these modulators enhance the regulatory T (Treg) cell population, while suppress the mitotic cell cycle as well as the cytokine production of inflammatory Th cells (Th1 and Th17) [7].

Despite the favorable therapeutic potency of MSCs obtained from preclinical investigations, the outcomes of clinical studies have often been contradictory and exhibited limited efficacy following MSC therapy [8]. So far, different reasons have been presented to explain the limited functions of MSCs, including the unfriendly host microenvironment and the insufficient local inflammatory cytokines for all injected MSCs changing the phenotype and immunomodulatory function of MSCs [9]. Additionally, the origin (biological niche) of isolated MSCs as well as the conditions of donors (age, disease, and etc …), isolation method, and culture condition such as O2 tension can affect the phenotype of these cells and make heterogeneous MSCs [10,11]**.** Besides, the accumulation of reactive oxygen species (ROS) in injured tissue or during invitro culturing of MSCs, enhances malondialdehyde (MDA) and lipid peroxidation and subsequently diminishes the proliferation of these cells and their ability to inhibit T cells [12–14]. So far, various strategies have been established to overcome the limited capability and functional heterogeneity of MSCs, such as in vitro priming of MSCs with different biochemical and biological molecules [11,15]. There are several priming approaches including; priming with inflammatory cytokines, priming with different culture conditions and hypoxia, and priming with pharmacological drugs and biomolecules [16].

To date, many studies have reported the improvement of stem cell potentials following priming with different cytokines and hormones. However, using these agents has major drawbacks including high cost of large-scale production, the appearance of toxic effects, and unfavorable upregulation of HLA class I and II following cytokine priming that evoke alloreactive T cells in the host [1]. Therefore, it seems necessary to find more affordable and safe agents with therapeutic abilities. Recently, MSC priming with herbal components which are highly available and exert therapeutic potential is considered as a suitable alternative [17].

Tetrandrine (TET), as a bis-benzyl-isoquinoline alkaloid found in the phytochemical content of *Stephania tetrandra* and exerts different antioxidative and anti-inflammatory effects [18]. Moreover, different studies have declared the inhibitory effects of TET on the proliferation and differentiation of inflammatory Th cells (Th1, Th2, and Th17) [19,20]. Immunomodulatory effects of TET on MSCs have also been reported by a study, where priming of MSCs with TET enhanced the secretion of prostaglandin E2 (PGE2) and co-culturing of these cells with activated macrophages reduced the production of TNF-α by macrophages [21]. In spite of studies reported the effects of herbal components on the proliferation and differentiation of MSCs, the alteration in immunomodulatory properties of MSCs after priming with these components is rarely evaluated. Hence, we aimed to evaluate the efficacy of TET on modulatory function of MSCs by the measurement of TGF-β, IFN-γ, kynurenine (as an indicator of IDO activity), SOD3, and MDA levels. Also, we examined for the first time, the proliferation, differentiation, and cytokine production from T cells, after co-culturing with TET-primed MSCs.

2. Material & methods

2.1. MSC culture

Adipose-derived MSCs at passages 2 were obtained from the cell bank of stem cell & regenerative medicine center of Kerman University of medical science (Kerman, Iran) and then cultured in 25 cm^2 tissue flask containing DMEM high glucose (Gibco, USA), 10 % heat-inactivated fetal bovine serum (FBS) (Gibco, USA), and 100 U/mL penicillin plus 100 μg/mL streptomycin (Gibco, USA) under controlled condition (at 5 % CO2, 95 % humidity, and 37 °C).

2.2. Viability and cell cycle assay of MSCs primed with TET

TET was purchased from Sigma Aldrich Company (CAS Number: 518-34-3, purity >98 %, USA). According to previous studies [20,21], three doses of TET were selected (5, 10, and 50 μM) for priming of MSCs, then, the viability rate and cell cycle phase distribution of MSCs were assessed. In brief, after passage three, when MSCs covered 80 % of the surface of a 25 cm^2 tissue flask, the cells were trypsinized (Sigma, USA), and 5×10^5 cells were seeded into a 96-well plate. After 24 h, floating cells were washed with DMEM and attached cells were cultured in the absence (non-primed group) or presence of different doses of TET for 48 h. One group of MSCs also treated with 2 % dimethyl sulfoxide (DMSO) as a control which is expected to be toxic for cells. For the MTT assay, as a viability test, MTT dye (20 μl) (Sigma, USA) was added to a final volume of 200 μl culture medium per well, and after 3 h incubation time at 37 °C, the culture fluid was removed. Then, the formazan crystals were dissolved by adding 100 μl DMSO (Merck, Germany) to each well, and the absorbance of the colored solution was detected at 570 nm by the ELISA reader. At the next step, to confirm the MTT results, the viability and cell cycle phase distribution of MSCs were reassessed with one selected dose of TET. For the trypan blue assay, 1×10^5 MSCs were stained with Trypan blue solution and the percentage of viability was calculated for each sample. Moreover, 2×10^6 MSCs were cultured in 6 well-plate and primed with TET as described above and used for DNA cell cycle analysis with propidium iodide (PI). The assay protocol was as follow: First, 5×10^5 cells were fixed with 70 % cold ethanol, and after pipetting and keeping the suspended cell on ice (for 15 min), the cells were centrifuged $400 \times g$ for 8 min and washed with phosphate-buffered saline (PBS) to remove ethanol. Then, the cell pellet was resuspended in 1 mL master mix solution containing 40 μg/mL PI and 10 μg/mL RNase A (Biolegend, USA) [22] to stain the DNA content of each cell. FlowJo software v10 was used for data acquisition and analysis.

2.3. Immunophenotyping of MSCs primed with TET

After priming of MSCs with 5 and 10 μM TET, the expression of some specific surface markers of MSCs was evaluated by flow cytometry. Briefly, the primed or non-primed MSCs were washed and resuspended in PBS. Then the cells were divided into different flow cytometry tubes (each tube containing 10^6 cell/100 μl) and each tube was stained with 5 μl of one of the fluorescently-labeled monoclonal antibodies (HLA-DR-PE, $CD29^-$ PE-Cy7, CD45-FITC, and CD90-APC, Biolegend, USA). All tubes were incubated at 4 °C for 30 min and then washed with 500 μl PBS (400×*g* for 5 min) to remove excess antibodies. The cells were resuspended in 250 μl PBS and were immediately assessed on BD FACS Calibur flow cytometer.

2.4. Antioxidative & immunoregulatory mediators of MSCs primed with TET

After 48 h incubation of MSCs with different doses of TET, the supernatant of each sample was collected. Afterward, the level of SOD3 was determined based on the protocol of Randox kit. The specific method established by Yagi, was applied to detect the levels of MDA with minor changes [23]. In summary, 100 μl of supernatant was added to 200 μl of a solution made by mixing 1.5 mL of trichloroacetic acid and 0.5 mL of thiobarbituric acid.

Then, the mixed samples were heated in a boiling water bath for 1 h. After cooling and centrifugation (10 min at 750×*g*), the absorbance of the separated pink phase was read at 532 nm. The standard curve of tetraethoxypropane was used to calculate the concentration of MDA [23]. To measure the levels of kynurenine which is an indicator of IDO activity, 100 μl of supernatant of each sample was added to 50 μl of 30 % trichloroacetic acid (Sigma, USA) and incubated at 56 °C for 20 min. Then 75 μl of the prepared solution was mixed with Ehrlich reagent (75 μl) and the absorbance of colored solution was read at 492 nm. The concentrations of IFN-γ and TGF-β were also detected by ELISA kit (Karmania Pars Gene, Kerman, Iran) according to the manufacturer's protocol.

2.5. Proliferation and cell cycle assay of activated T cells after Co-culture with TET-primed MSCs

For this step, MSCs were cultured in three 25 cm^2 tissue flasks, in the presence of 5, and 10 μM TET or in the absence of TET as a non-primed group for 48 h. Then, the proliferation of MSCs was inhibited with mitomycin c (10 μg/mL; Sigma, USA). The MSCs were trypsinized, washed, and reseeded in a 48-well plate (50 × 10^3 cell/well). After 24 h, peripheral blood mononuclear cells (PBMCs) were isolated from the blood of 5 healthy volunteers, and the MSCs were co-cultured with PBMCs (MSCs: PBMCs ratio, 1:10) for 72 h. Anti-CD3 and anti-CD28 antibodies (0.157 μg/mL and 0.085 μg/mL respectively; Biolegend, USA) were used to stimulate the T cell population in PBMCs. At the end of the co-culture period, the supernatant of each well was collected to measure the proliferation of stimulated PBMCs by MTT and cell cycle assay as described above.

2.6. Cytokine assay

The supernatants of co-cultured samples were collected after 72 h to detect the levels of secreted IFN-γ and TGF-β. The ELISA kit (Karmania Pars Gene, Kerman, Iran) was used to detect the level of cytokines. The test procedure was based on the provided protocol by Karmania Pars Gene company.

2.7. Flow cytometry assay of $CD4^+$ $CD25^+$ FOXP3+ Treg cells

To evaluate the frequency of $CD4^+$ $CD25^+$ FOXP3+ Treg cells, the MSCs and PBMCs were cocultured in a 6-well plate and the test procedure was similar to section 3-5 in the methods part. In the next step, the PBMCs were isolated from each well separately and washed with PBS. Then, 10^6 cells were resuspended in 100 μl of PBS-FBS (2 %) solution and anti-human antibody including CD4-FITC (Cat.NO:317408; Biolegend, USA), and CD25-APC (Cat.NO:302610; Biolegend, USA) was added to the cells and incubated at room temperature for 15 min. After the washing step, the cells were resuspended in 1 mL of 1X Fix/Perm buffer (Cat.NO:421403; Biolegend, USA) and incubated at room temperature for 15 min. Again, PBMCS were washed with 1 mL of 1X Perm buffer and FoxP3-PE Anti-human antibody (Cat.NO:320108; BioLegend, USA) was added to samples and incubated 30 min at 4 °C in the dark. Afterward, all samples were washed with 1X Perm buffer and resuspended in 500 μl of PBS. Finally, the percentage of $CD4^+$ $CD25^+$ FOXP3+ Treg cells population was determined on BD FACS Calibur flow cytometer.

2.8. Statistical analysis

After data collection, the Shapiro–Wilk test was applied to examine the normal distribution of variables. Then, One-way analysis of variance (ANOVA) or Student's t-test (or Kruskal–Wallis or Mann-Whitney test as nonparametric equivalent respectively) were selected to find out any statistically significant differences between the mean of three or two independent groups respectively. Additionally, after the rejection of the null hypothesis of equal means by the ANOVA test, the comparison between pairs of group means was assessed by Tuckey's post hoc test. GraphPad Prism 8.0.2. was selected to analyze data. Results were depicted as the mean ± SEM (standard error of the mean). p-values <0.05 were regarded as significant.

3. Results

3.1. TET priming maintains the viability and cell cycle progression of MSCs

To determine the viability, MSCs were primed with or without different doses of TET (5, 10, and 50 μM), and a toxic dose of DMSO (2 %), and all groups were compared with non-primed MSCs.

The MTT data indicated that while high concentration of TET (50 μM) and DMSO reduced the viability of MSCs (Fig. 1a, $p < 0.0001$), lower concentrations of TET (5, and 10 μM) showed no adverse effect on viability (Fig. 1a). The result of Trypan blue staining also showed a similar percentage of viability in non-primed and primed MSCs (5, and 10 μM, Fig. 1b). To confirm the results of MTT and Trypan blue staining, higher TET concentration (10 μM) was selected and cell cycle assay was performed in primed cells. The obtained data depicted that the accumulation of MSCs in G0/G1, S, and G2/M phases of the cell cycle was not different between primed and non-primed MSCs (Fig. 1c–and d), which confirmed the MTT results. Hence, doses 5, and 10 μM of TET were selected for other experiments in our study.

3.2. TET priming preserves the immunophenotype of MSCs

In this study, the effect of priming with 5 and 10 μM TET was examined on the expression of several specific markers of MSCs (HLA-DR and CD45 as negative markers; CD90 and CD29 as positive markers) (Fig. 2). Based on our flow cytometry data, primed MSCs had a positive expression for CD90 and CD29 (Fig. 2a) while had a negative expression for CD45 and HLA-DR (Fig. 2a). Moreover, no statistically significant difference was observed between the expression of markers in primed MSCs in comparison with non-primed MSCs (Fig. 2b).

3.3. TET priming enhances the antioxidant & immunoregulatory mediators

The levels of SOD3 produced from primed MSCs were significantly higher ($p < 0.05$ for 10 μM TET and $p < 0.001$ for 5 μM TET) than that in the non-primed group (Fig. 3a). However, the levels of MDA were not different between groups (Fig. 3b). The results of IDO activity also revealed a remarkable enhancement of kynurenine in MSCs primed with 10 and 5 μM TET ($p < 0.01$ and $p < 0.0001$ respectively) compared to non-primed group (Fig. 3c). Moreover, 5 μM TET could upregulate the production of kynurenine more noticeable rather than 10 μM TET ($p < 0.0001$, Fig. 3c). Evaluation of cytokines also clarified a substantial decline in IFN-γ in the presence of lower dose of TET ($p < 0.05$, Fig. 3d), but not higher dose when compared to non-primed control MSCs. The concentrations of TGF-β were not different between MSC groups; however, an increment trend was observed in TET groups, especially at 5 μM

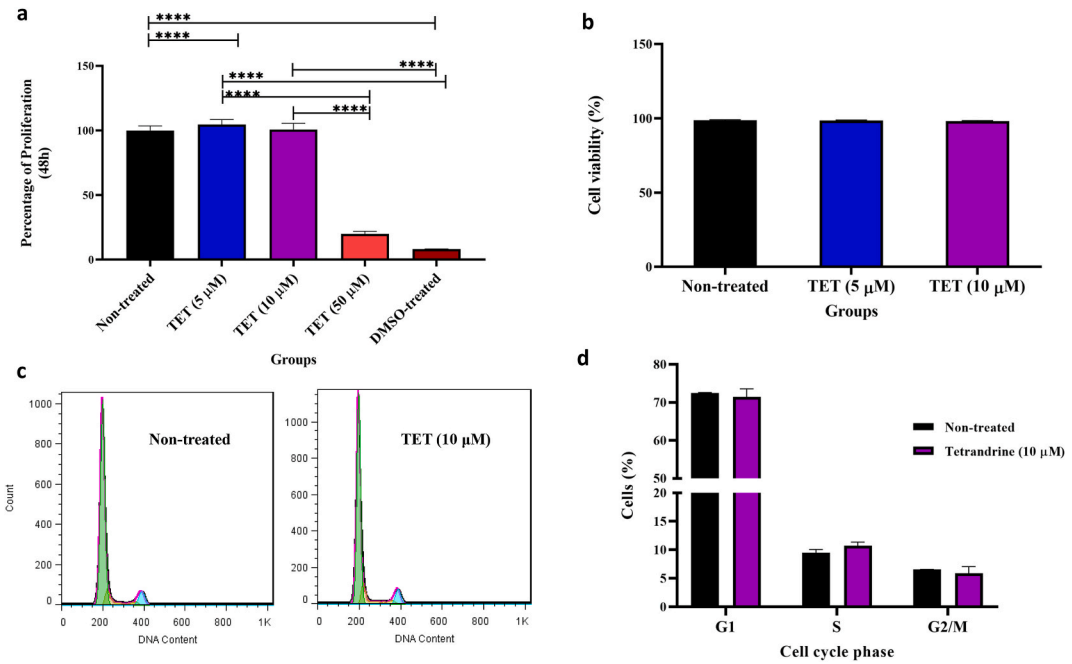

Fig. 1. The effect of TET on the viability and cell cycle progression of MSCs. The viability of MSCs was detected by the MTT test following priming with three different concentrations of TET (a, n = 5). Trypan blue staining (b, n = 5), and cell cycle assay (c and d, n = 3) were also applied to confirm the results of MTT for selected doses of TET. The graphs show the mean ± SEM. Significant differences are depicted with an asterisk (****$p < 0.0001$).

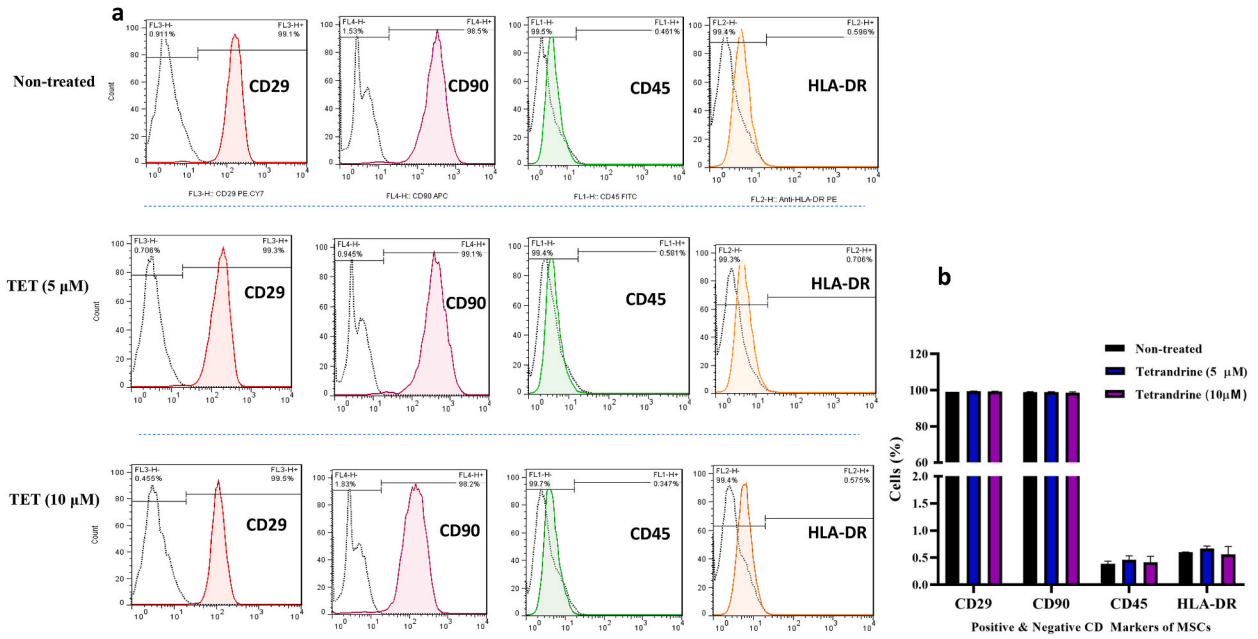

Fig. 2. The effect of TET on Immunophenotyping of MSCs. The expression of CD20, CD90, CD45, and HLA-DR was detected in non-primed and 5 and 10 μM TET-primed MSCs (a) after 48 h, and the results of all groups were compared with each other (b, n = 3). The graphs show the mean ± SEM.

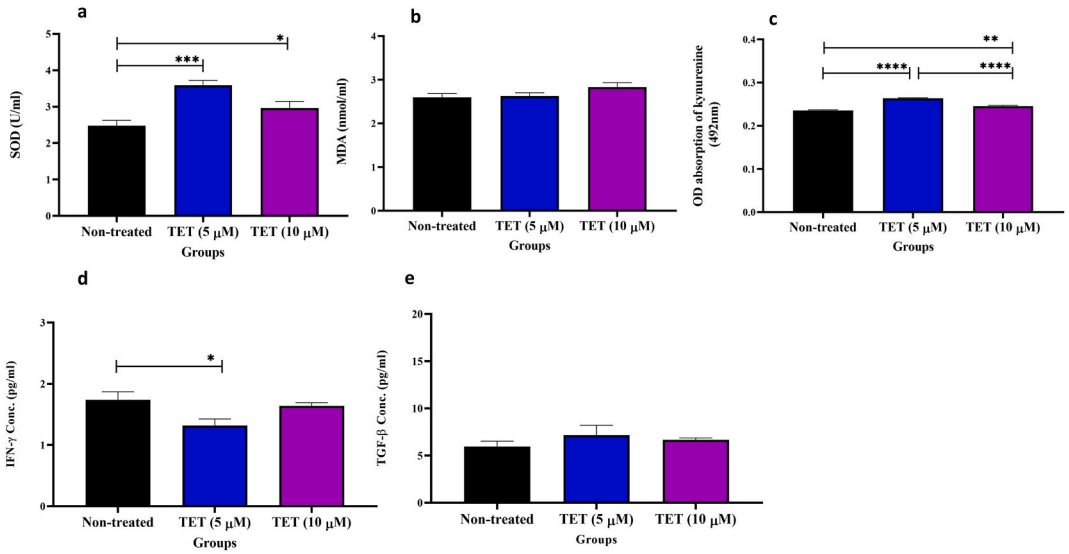

Fig. 3. The effect of TET on the production of Antioxidant & immunoregulatory mediators of MSCs. The levels of SOD3 (a), MDA (b), kynurenine (c), IFN-γ (d), and TGF-β (e) were detected in non-primed and TET-primed MSCs (5 and 10 μM) after 48 h (n = 5). The graphs show the mean ± SEM. Significant differences are depicted with asterisk (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

concentration (Fig. 3e).

3.4. TET-primed MSCs reduced the proliferation of activated T cells and the frequency of these cells in the S and G2/M phases of cell cycle

As shown in Fig. 4a, both group of MSCs primed with TET inhibited the proliferation of anti-CD3/CD28-stimulated T cells more significant ($p < 0.001$ for 10 μM TET group and $p < 0.0001$ for 5 μM TET group, Fig. 4a). To confirm the effect of TET on the reduction of PBMC proliferation, cell cycle distribution of these cells was also assessed after co-culture with 5 and 10 μM TET-primed MSCs. Analysis of the DNA content of PBMCs revealed the same percentages of PBMCs in G0/G1 in primed and non-primed MSCs groups

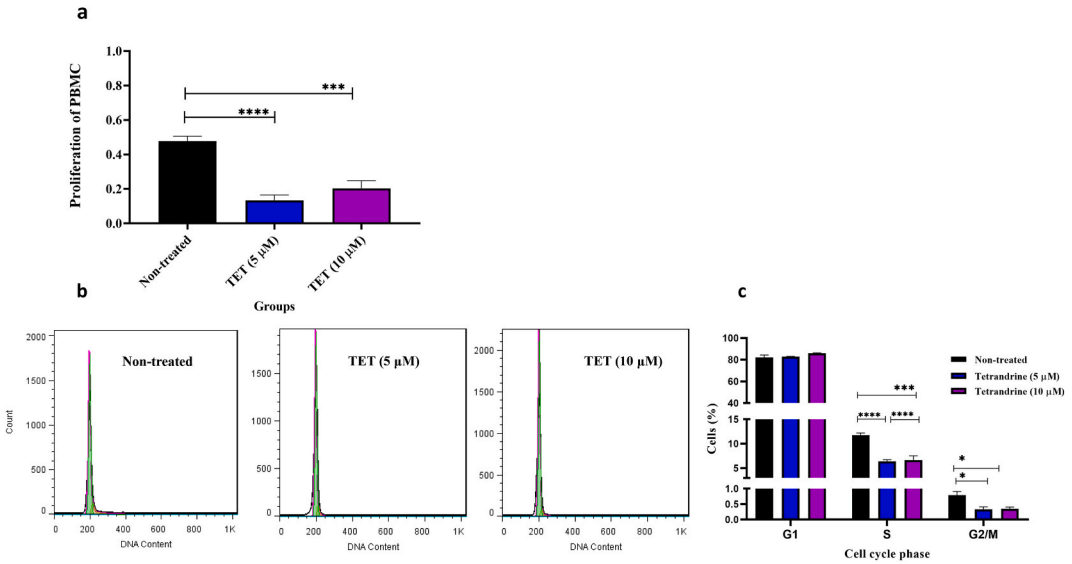

Fig. 4. The effect of TET-primed MSCs on the proliferation and cell cycle phase of Ab-activated T cells in PBMCs. The proliferation of activated T cells was detected by the MTT test following co-culture with TET-primed MSCs (a, n = 5). Cell cycle assay was also applied (b and c, n = 3) to confirm the results of MTT for T cells cocultured with MSCs which were primed with different doses of TET after 72 h. The graphs show the mean ± SEM. Significant differences are depicted with asterisk (*p < 0.05, ***p < 0.001, and ****p < 0.0001).

(Fig. 4b and c), whereas the entrance of PBMCs into S and G2/M phases of the cell cycle was arrested by MSCs primed with 5 μM TET (p < 0.0001 for S phase, p < 0.05 for G2/M phase) and 10 μM TET (p < 0.001 for S phase, p < 0.05 for G2/M phase) (Fig. 4b and c).

3.5. TET-primed MSCs modulate the cytokine production in the co-culture condition

The production of TGF-β and IFN-γ was detected in the supernatant of co-culture. The analysis of data displayed a considerable enhancement of TGF-β in both TET groups (p < 0.01, Fig. 5a) when compared with non-primed MSCs. Conversely, lower concentrations of IFN-γ were observed in both TET groups (p < 0.05, Fig. 5b) rather than the non-primed group.

3.6. TET-primed MSCs unaffected the percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells

To determine the source of TGF-β production, the population of $CD4^+$ $CD25^+$ FOXP3+ Treg cells was assessed. As depicted in Fig. 6, the co-culture of MSCs primed with 5 and 10 μM TET did not change the frequency of $CD4^+$ $CD25^+$ FOXP3+ Treg cells when compared with the non-primed MSCs group.

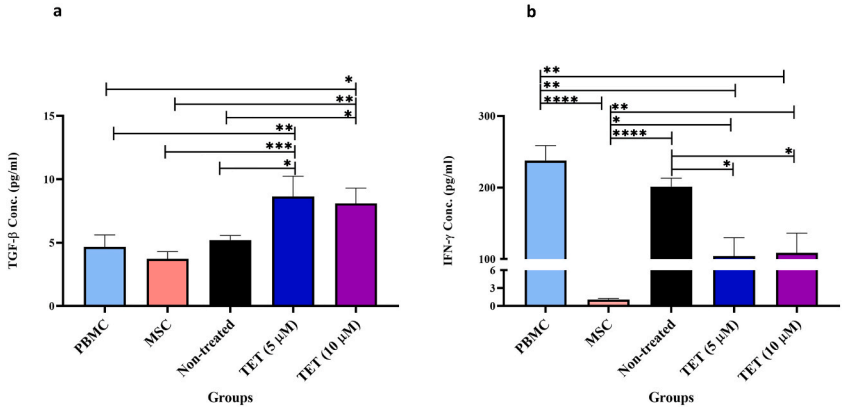

Fig. 5. The effect of TET-primed MSCs on cytokine production after co-culture with PBMCs. The levels of TGF-β (a) and IFN-γ (b) were detected by ELISA after co-culture of PBMC with non-primed and TET-primed MSCs. The graphs show the mean ± SEM. Significant differences are depicted with an asterisk (*p < 0.05,**p < 0.01, ***p < 0.001, and ****p < 0.0001).

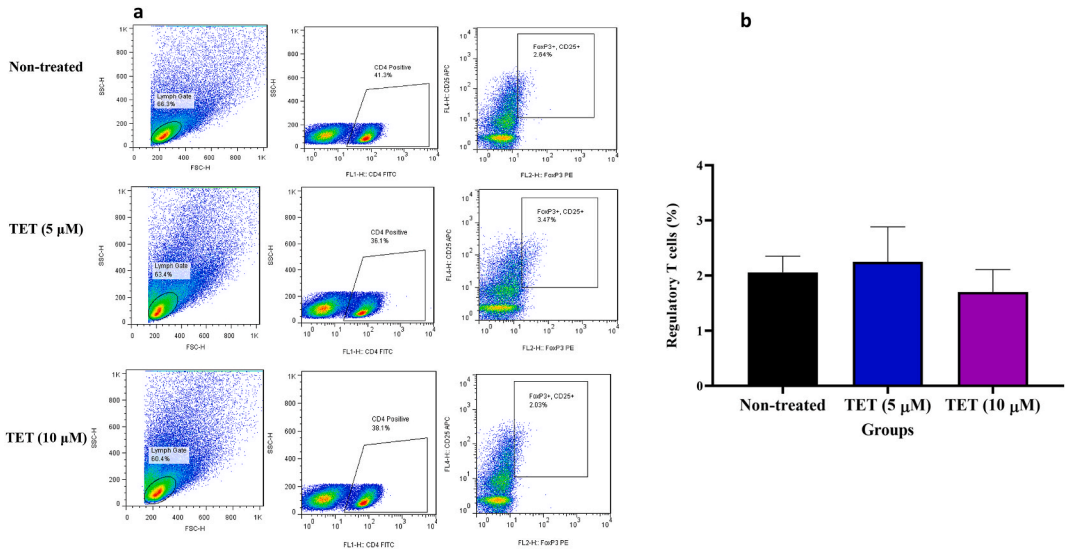

Fig. 6. The effect of TET-primed MSCs on the frequency of $CD4^+$ $CD25^+$ FOXP3+ Treg cells population. Flow cytometry assay was applied to detect the Treg cell percentage in PBMC cocultured with MSCs which were primed with both doses of TET (5 and 10 μM) after 72 h (a). Then, the effect of non-primed and both group of TET-primed MSCs was compared with each other (b, n = 3). The graphs show the mean ± SEM.

4. Discussion

As mentioned earlier, preserving or boosting the therapeutic potency of MSCs is one of the main issues to reach satisfactory outcomes in the clinical trials [15]. To achieve this goal, many recent experimental studies have been designed based on the invitro priming or invivo combination therapy of MSCs with various biomolecules, hormones, and inflammatory cytokines. Among different biomolecules, the use of herbal components which are highly available, low cost, and safe is a promising tool for MSC priming [24,25]. Hence, at this study we used TET, as a potent immunomodulatory herbal component for MSC priming. Our results showed no undesirable effects of TET on the viability, proliferation, and phenotypic pattern of MSCs. Moreover, the production of antioxidative and immunomodulatory factors of MSCs enhanced as a result of priming with TET. Consequently, the proliferation and function of activated T cells reduced after coculturing with TET-primed MSCs.

As previously explained, the cell culture process augments the rates of intracellular ROS and MDA due to the high O2 tension, lack of the anti-oxidant molecules, and pro-oxidant metal ions in the culture media [13,26]. According to our findings, high production of SOD3 was observed in primed MSCs, but no rise was detected in the MDA level. Similarly, Bao et al. observed that treatment of rat spinal cord astrocytes with TET restored the overproduction of ROS and MDA and increased the level of SOD in an oxygen-glucose-serum deprivation/reoxygenation (OGSD/R) rat model [27]. Also, Wang et al. found that TET decreases neurological damage after ischemia-reperfusion (I/R) injury through the reduction of nitric oxide (NO), MDA, and enhancement of glutathione peroxidase (GPx) levels [28]. These findings suggested that TET might protect MSCs from oxidative damage through the enhancement of SOD3.

Kynurenine is a metabolite of IDO activity, with inhibitory effects on the proliferation and function of T cells [7]. This is the first report to disclose the remarkable increase of kynurenine in MSCs following priming with TET. Although there has been no study about the effects of TET on the production of kynurenine, other alkaloids such as caffeine could increase IDO activity in MSCs [29]. So, it is very likely that one of the possible mechanisms by which kynurenine enhanced is higher production of SOD3 in TET group rather than that in non-primed group. In this context, Sah et al. showed that MSCs transduced with the SOD3 gene enhanced the expression of IDO, TGF-β, and IL-10 rather than those in non-transduced MSCs [30]. However, further studies are essential to clarify the exact mechanisms of TET on the kynurenine production pathway.

In 2017, Wu et al. reported that heterogeneous MSCs derived from adipose tissue show different cytokine secretion patterns. In addition, the frequency of MSCs that secret IFN-γ or TGF-β was affected by cytokine stimulation [31]. On the other hand, the balance of inflammatory and anti-inflammatory cytokines contributes to the differentiation of various type of immune cells especially T cells [32]. Hence, we assessed the effect of TET on the production of two important cytokines from MSCs, IFN-γ and TGF-β. IFN-γ plays a vital role for differentiation of inflammatory immune cells such as M1 macrophages, Th1, and cytotoxic T cell [33]. Conversely TGF-β reduces the differentiation of inflammatory cells and their cytokines and increases the population of immune suppressor cells such as Treg cells and M1 macrophages, and regulatory B (Breg) cells [34,35]. Our data showed a significant decrease in the levels of IFN-γ in MSCs primed with 5 μM tetrandrine and a slight increase in TGF-β level, although not statistically remarkable. In parallel with this study, Yang et al. found that TET could augment the immunomodulatory function of MSCs by affecting NF-Kβ/COX2 signaling pathway and enhancement of PGE2 [21]. The regulation of cytokine production by tetrandrine has also been pointed out in many in vitro and in vivo studies. For example, Gao et al. evaluated the anti-rheumatic effects of TET and indicated that this molecule reduced

the production of IL-1β, TNF-α, and IL-6 from LPS-stimulated macrophages by the inactivation of NF- Kβ signaling, and alleviated the inflammation in the cartilage of arthritic rats [36]. Another study also declared that TET sustained the tolerogenic state of DCs determined with overexpression of IL-10, lower production of IL-2, and as a result, enhanced the population of Treg [37].

In the second part of our study, the TET was omitted from the culture media, to understand the durability of changes induced in MSCs and evaluate the potency of primed cells on inhibition of stimulated peripheral blood mononuclear cells (PBMCs) proliferation, in fact reflecting proliferation of T cells stimulated with anti-CD3 and anti-CD28 antibodies and also alteration of phenotypic features of immune cells. MTT data declared that TET-primed MSCs suppress the proliferation of activated PBMCs more intensively in comparison with non-primed MSCs. In line with the MTT results, cell cycle analysis also revealed that TET-primed MSCs arrested notably the entrance of PBMCs into the DNA synthesis (S) and mitotic (G2/M) phases. The results of the cytokine assay after coculture also showed that the secretion of IFN-γ was lower from PBMCs co-cultured with TET-primed MSCs, while the levels of TGF-β were higher in comparison with the control group. Additionally, after the comparison of released cytokines between groups, it can be assumed that the main producer cells for IFN-γ are T cells and for TGF-β are both MSCs and T cells, although the exact clarification of source of these cytokines is not possible in coculture condition. In conformity with our findings, Yang et al. identified TET as a suitable molecule for the primed of MSCs that reduced TNF-a secretion from mouse macrophages after co-culturing with TET-primed MSCs [21]. Based on the results obtained in the first part of our study, it seems that enhancement of IDO activity in TET-primed MSCs could arrest the proliferation of T lymphocytes via the reduction of tryptophan required for growth of PBMCs [38]. Another reason for diminished proliferation and IFN-γ secretion from PBMCs could be probably the increment of SOD3 in the TET-primed MSCs. As previously described, SOD3 could repress the activity of stimulated-T cells by blocking the ERK, p38, and NF-kB signaling pathways and inhibition of CD25 and CD69 expression which are necessary for the activation of T cells respectively [39].

In the second part, it also revealed that the levels of TGF-β increased in the supernatant of PBMCs co-cultured with the TET-primed group, whereas the number of $CD4^+$ $CD25^+$ FOXP3+ Treg cells was not statistically different between groups. As previously mentioned, TGF-β, is necessary for the differentiation of $CD4^+$ $CD25^+$ FOXP3+ Treg cells and we did not observe high production of TGF-β by TET-primed MSCs in the first part of the study. So, the same level of TGF-β produced by TET-primed or non-primed MSCs in the first part of our study may be the main reason for similar frequency of $CD4^+$ $CD25^+$ FOXP3+ Treg cells between groups. Also, to justify the higher levels of kynurenine, which is in favor of Treg production, in TET groups, we can refer to a study that demonstrated the different behavior of active and non-active T cells in response to the tryptophan reduction and kynurenine elevation in the environment; in fact, while anergy and apoptosis were observed in active $CD4^+$ T cells and $CD8^+$ T cells, resting $CD4^+$ T cells differentiate into $CD4^+$ $CD25^+$ FOXP3+ Treg cells [38] and as mentioned earlier, we activated T cells using specific antibodies. Moreover, to explain the enhancement of TGF-β, but not Treg cells in the co-culture of TET-primed groups rather than the control group, we can point out studies that introduced other type of cells producing TGF-β. For example, it is reported that kynurenine secreted from MSCs could enhance the differentiation of M2 macrophages secreting anti-inflammatory IL-10 and TGF-β [38,40]. Another study also reported that human MSCs through cell-cell contact, could increase the induction of $CD8^+$ Treg secreting TGF-β [41–43]. Therefore, the evaluation of other regulatory cells such as M2 macrophages, Breg, and $CD8^+$ Treg cells could be valuable for subsequent studies. In addition to invitro priming of MSCs with TET, invivo administration of these cells with TET could be considered as a new method of combination therapy to boost the therapeutic effects of MSCs.

To conclude, our data revealed that in vitro priming of MSCs with TET could increase the immunomodulatory and antioxidant properties of these cells without any adverse effects on cell viability and proliferation. Moreover, suppressed proliferation and modulated secretion of cytokines in activated T cells after co-culture with TET-primed MSCs confirmed the persistency of TET effects on the immunomodulatory properties of MSCs. Therefore, priming of MSCs with tetrandrine might enhance the therapeutic efficacy of these cells. However, additional investigations are recommended to clear the exact mechanisms and pathways used by tetrandrine to modulate MSC activities.

Data availability

All data generated or analyzed during this study are included in the article and supplementary materials.

CRediT authorship contribution statement

Shohreh Fadaghi: Writing – original draft, Methodology, Data curation. **Merat Mahmoodi:** Supervision, Conceptualization. **Ali Derakhshani:** Methodology, Data curation. **Farnaz Sedghy:** Formal analysis. **Mahdi Ranjkesh:** Methodology. **Ahmadreza Behzadi:** Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35667.

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