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Original Article

Attenuation of Differentiation of Mesenchymal Stem Cells Into Adipocytes by Co-administration of Calcium and All-trans Retinoic Acid

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Abstract

Background: Adipogenesis is affected by multiple factors, among which all-trans retinoic acid (ATRA) and Ca^{2+} are considered important factors.

Objectives: This study aimed to evaluate the effect of calcium, ATRA, and their underlying molecular mechanisms, alone and in combination, on adipocyte differentiation.

Methods: Human adipose-derived mesenchymal stem cells (hAD-MSCs) were differentiated into adipocytes and simultaneously exposed to 0.5 μM ATRA or 2.5 mM calcium, or both in combination for 14 days.

Results: Higher intracellular Ca^{2+} was observed in both Ca^{2+} and Ca^{2+} plus ATRA groups. Assessment of triglyceride content and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity indicated lower differentiation levels in all treatment groups than in the control group. Furthermore, the results demonstrated a higher expression of retinoic acid receptor alpha (RAR-α) and lower expression of peroxisome proliferator-activated receptor γ2 (PPARγ2) and glucose transferase-4 (GLUT4) in the treatment groups as compared with the control group. It is noteworthy that Ca^{2+} plus ATRA treatment caused more significant effects on gene expression levels ($P<0.05$).

Conclusion: In conclusion, combined treatment with Ca^{2+} and ATRA has a more pronounced inhibitory effect on adipocyte differentiation, indicating their cumulative effect.

Keywords: Retinoic acid, Calcium, Mesenchymal stem cell, Adipogenesis, GLUT4

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Background

Abdominal obesity occurs with an increase in both size and number of fat cells (1) and could therefore be the major risk factor for metabolic syndrome, heart diseases, stroke, and diabetes (2). Similar to bone marrow-derived mesenchymal stem cells (BM-MSCs), human adipose-derived mesenchymal stem cells (hAD-MSCs) are the pre-existing source of new fat cells, which play an important role in maintaining function and the mass of adult adipose tissue (3,4). These multipotent non-hematopoietic stem cells that are capable of differentiating into a variety of cell types could be found in many tissues such as adipose tissue. In addition, they can express cell surface markers such as CD105, CD73, and CD90 (5,6). To study the earliest regulation of adipocyte differentiation, in vitro differentiation of MSCs can be used as a model of adipogenesis (7). During this process, from pre-adipocytes to adipocytes, gene promoter activities are necessary, which are promoted by two important transcription factors including CCAAT/enhancer-binding protein α (C/EBPα) and nuclear receptor peroxisome proliferator-activated receptor γ_2 (PPARγ_2) (8). PPARγ_2 activation modulates the activity of AMPK and glucose uptake through glucose transferase-4 (GLUT4) and stimulates adipogenesis (9).

The adipogenesis process through adipocyte progenitors could also be affected by signaling molecules (10). These molecules alter the expression of numerous genes, thereby triggering or inhibiting the differentiation of MSCs into adipocytes (10).

One such important molecule is retinoic acid (RA), the active metabolite of vitamin A, with regulatory property in growth and development of multiple biological pathways (11). All-trans retinoic acid (ATRA) is the bioactive form with proven inhibitory effects on adipogenesis through its nuclear receptors (RARα, RARβ, and RARγ) and its ligand-binding inducible transcriptional factors that are known to be PPARβ/δ (12,13). Scientific evidence supports the key role of retinoic acid in the inhibition of adipogenesis in preliminary stages (14,15). The proliferative effects of RA were determined by regulating the calcium-signaling pathway (16).

Calreticulin is an endoplasmic protein that is bound to calcium in its inactive form and can be active via the other signals. However, the effects of calcium on adipogenesis and its hemostasis through calreticulin as Ca^{2+}-buffering chaperone are controversial. Some studies suggest the role of calcium in adipogenesis through a marked increase in PPARγ, the transcription factor that regulates gene expression necessary for the development of mature adipocytes (17). In contrast, the inhibitory effects of calcium on adipogenesis were also reported in the differentiated 3T3-L1 adipocytes, via downregulation of PPARγ and *C/EBPB* (18). There is also a study showing a dual effect of calcium, the inhibitory effect in early stages versus the stimulatory effect in later stages of differentiation (19). More importantly, the effects of calcium on retinoic acid signaling were also reported (20). Given that more complex pathways together influence the differentiation and adipose tissue formation, the effect of calcium on retinoic acid signaling in the adipogenesis process is still questionable due to the variability of consequences.

Therefore, these controversial results led to our interest in assessing the effect of calcium, ATRA, and their underlying molecular mechanisms, alone and in combination, on adipocyte differentiation.

Materials and Methods

Isolation of Mesenchymal Stem Cells

MSCs were isolated from lipoaspirates of young females (n = 4, aged 25 to 35 years) according to our previous study (21). The protocol of this research was approved by the Ethics Committee of Hamadan University of Medical Sciences (Hamadan, Iran).

The lipoaspirate was washed several times with PBS (0.1 M, pH 7.45) containing 5% penicillin/streptomycin. Then, enzymatic digestion was performed by adding an equal volume of collagenase type I solution (1 mg/mL) in PBS (Thermo Fisher Scientific, USA) to lipoaspirate, followed by incubation and shaking at 37°C for 60 minutes in the presence of 5% CO_2. In the next step, the activity of collagenase was neutralized by adding an equal volume of cell culture medium containing DMEM-F12 and 10% fetal bovine serum. The pellet containing cells was prepared by centrifugation of this solution at 800 × g for 5 minutes. Finally, cells were transferred into T75 flask. The morphology of cells was evaluated using an inverted microscope (Nikon, Japan), and cells were sub-cultured until the fourth passage.

Immunophenotyping of Mesenchymal stem cells

The expression of MSC-specific cluster of differentiation (CD) markers including CD73, CD90, and CD44, and the lack of negative CD markers including CD45 and CD34 were determined by flow cytometry. For this purpose, at least 1×10^6 fourth-passage cells were harvested with 0.25% trypsin, washed with PBS, and fixed with 4% paraformaldehyde. After washing with PBS, cells were incubated for 30 minutes with fluorophore-conjugated antibodies (BD Biosciences, San Jose, USA) diluted in PBS/1% bovine serum albumin (BSA) (1:20). Finally, cells were analyzed by a flow cytometer (Sysmex-Partec, Germany). Rat IgG2b and rabbit polyclonal IgG were used as isotype controls (BD Biosciences, San Jose, USA) (22).

Differentiation and Treatment

After confirming the purity of isolated MSCs (fourth-passage) with 80-90% confluency, they were induced either in differentiation medium (control group) or in differentiation medium containing 0.5 µM ATRA (group 0.5AT), 2.5 mM Ca^{2+}(group 2.5Ca), or 0.5 µM ATRA + 2.5 mM Ca^{2+}(group 0.5AT-2.5Ca). The differentiation medium contained 10 µg/mL insulin, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 100 µM indomethacin (Sigma-Aldrich, UK). It must be noted that 2.5 mM calcium was added to the basal concentration of calcium (1.8 mM) that was determined by the manufacturer (Thermo Fisher Scientific, USA). All samples from four donors (n = 4) were evaluated in triplicate for each test and finally, the obtained data from all samples were pooled.

Cell Proliferation Assay

To determine any cytotoxic effects of the desired concentration of ATRA and Ca^{2+}, the cell viability assay was performed using MTS (3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H-tetrazolium) assay kit (Kiazist Life Sciences, Iran), according to the manufacturer's instructions. The assay was conducted on the third and fourteenth days after induction of differentiation.

After treatment, the MTS reagent (10 µL/well) was added to each well and the plate was incubated at 37°C under standard culture conditions for 4 hours. In the next step, formed formazan crystals were dissolved by a solving agent . Finally, the absorbance was read at 560 nm using a microplate reader. The proliferation rate was directly related to the absorbance value of each group.

Intracellular Calcium Mobilization

As we reported previously (23), cells were seeded in 96-well fluorometric plate and then treated with a calcium-sensitive probe, fluo-8, (Abcam, USA). The cells of different groups were incubated with flou-8 for 2 hours and finally, the signal intensity was captured (490-nm excitation and 525-nm emission) every 60 seconds for a total of 30 minutes by an HTS fluorometric plate reader (BioTek Instruments, Inc, USA).

Lipid Staining

Lipid droplets within cells were stained using Oil Red O staining method. After washing with PBS, fixation was performed by incubating the cells with 10% formalin for 10 minutes. Then, cells were rinsed in 60% isopropanol for 5 minutes. In the staining step, cells were covered with Oil Red O solution for another 5 minutes. Finally, cells were washed twice using deionized water and then counterstained with hematoxylin solution for 1 minute. After several washes with deionized water, cells were observed under a light microscope.

Triglyceride Assay

To confirm Oil Red O staining, the triglyceride (TG) content of cells was quantified. After discarding the medium, cells (5×10^5 cells) were trypsinized and washed with PBS. Triglyceride content was measured using a Glycerol Cell-Based Assay Kit (Cayman Chemical, USA). After sonication of the cells (10 cycles for 1 minute) and centrifugation at $8000\times g$ for 10 minutes at 4°C, the supernatant was collected to assess the TG content according to the manufacturer's instructions. For normalization, the protein content was determined by a commercial bicinchoninic acid (BCA) assay kit (Kiazist Life Sciences, Iran).

GAPDH Activity Assay

Cells were trypsinized, centrifuged at $14000\times g$, and washed with cold PBS. Then, the assay was conducted by addition of 100 µL of ice-cold GAPDH assay buffer according to GAPDH assay kit instructions (Abcam, USA) (24).

Real-time RT-PCR

To evaluate gene expression related to adipogenesis, real-time RT-PCR was performed. For this purpose, the cell medium was discarded and TRIzol reagent (Thermo Fisher Scientific, USA) was added to each dish. Then, the attached cells were scraped and transferred into an RNase-free microtube.

RNA extraction was carried out by adding chloroform, isopropanol, and ethanol, followed by several stages of centrifugation (according to the TRIzol extraction protocol). The quantity and quality of extracted RNA were assessed by NanoDrop apparatus (Onec UV-Vis Spectrophotometer Thermo Scientific).

Next, cDNA was synthesized by Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Primer3 software was used to design the forward and reverse primers (Table 1). The real-time gene expression level was monitored using qPCR master mix (Ampliqon, Denmark) and LightCycler® 96 System (Roche, Germany). Relative gene expressions (fold change) were calculated based on $2^{-\Delta\Delta Ct}$ method developed by Livak and Schmittgen (25,26), and data were normalized to RPII (RNA Polymerase II) expression as a reference gene.

Western Blotting

The protein expression levels of GLUT4 and PPARγ2 were analyzed by western blotting. To prepare the suitable amount of protein, cells were cultured in 60 mm dishes. After reaching the proper confluency, cells were lysed with RIPA buffer (Kiazist Life Sciences, Iran) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, USA). After the determination of protein content by the bicinchoninic acid method, 50 µg of total protein from each group was resolved by SDS-PAGE and transferred to nitrocellulose membrane. In the next step, the membrane was washed with TBST buffer (20 mM Tris.HCl, 500 mM NaCl, pH 7.4) and blocked by 3% BSA prepared in TBST buffer (20 mM Tris.HCl, 500 mM NaCl, 0.5% Tween 20, pH 7.4). Then, the membrane was incubated with goat polyclonal anti-GLUT4, anti-PPARγ2, and anti-cofilin antibody (Santa Cruz Biotechnology, USA) diluted in TBST buffer (anti-GLUT4, 1:1000, anti-PPARγ2, 1:500, and anti-cofilin, 1:500) at 4°C for 24 hours. Next, the membrane was washed three times with 0.5% TBST buffer, incubated with the HRP-conjugated donkey anti-goat secondary antibody (1:10 000 in TBST buffer) for 1 hour and washed again with the same buffer (three times).

Table 1. Primer Sequences

Gene name	Primer sequence	Product size	Ta	Accession No.
PPAR-γ2	F: CTATTGACCCAGAAAGCGAT R: CGTAATGTGGAGTAGAAATGC	210	54	NM_005037
Calreticulin	F: CTCTGGCAGGTCAAGTCT R: TTGTCTTCTTCCTCCTCCTTAA	170	54	NM_004343
GLUT4	F: AGGATCGGTTCTTTCATCTTCGC R: GTTCCCCATCTTCGGAGCCTA	98	59	NM_001042
RAR-α	F: CCTATGCTGGGTGGACTCT R: GGCACTATCTCTTCAGAACTGCT	131	57	NM_000964
RPII	F: GCACCATCAAGAGAGTCCAGT R: ATTTGATGCCACCCTCCGTCA	81	57	NM_000937

Finally, immunoreactive bands were obtained using an enzyme-linked chemiluminescence detection kit (Kiazist Life Sciences, Iran). In this study, cofilin was used as a reference protein for the normalization of GLUT4 and PPARγ2.

Statistical Analysis

All values are given as mean ± standard error of the mean (SEM), except for gene expression fold change that data are presented in mean ± standard deviation (SD). Data analysis was carried out using one-way ANOVA followed by post hoc Tukey test. $P < 0.05$ was deemed statistically significant. The plotting was performed by GraphPad Prism version 6.0 (GraphPad Software).

Results

Characterization of Mesenchymal stem cells

Flow cytometric analysis of CD markers (Figure 1) showed that CD105, CD73, and CD29 positive cells were in high levels (90.2 ± 1.2%, 95.3 ± 4.1%, and 85.6 ± 1.6%, respectively), while a small fraction of cells expressed hematopoietic markers, CD34 and CD45 (3.7 ± 0.6% and 6.2 ± 1.1%, respectively).

MTS Assay Analysis

For the determination of cell proliferation, the MTS assay was performed on the third and fourteenth days after the initiation of differentiation (Figure 2). Accordingly, no significant differences were observed between all groups on the third and fourteenth days after the induction ($P > 0.05$).

Intracellular Calcium Mobilization

In different groups, the calcium mobilization assay showed different results within 30 minutes after the induction (Figure 3). After 15 minutes, a high calcium influx was observed in the group that received calcium along with A23187 (Sigma-Aldrich, UK) as an ion carrier. This group reached the top of 146 000 ± 2346 RFU after 30 minutes. High intracellular calcium mobilization was also observed in the calcium group and calcium plus ATRA group. The other two groups, including 0.5ATRA and control groups, showed similar results. In these two groups, no significant increase in the intracellular calcium was observed in the

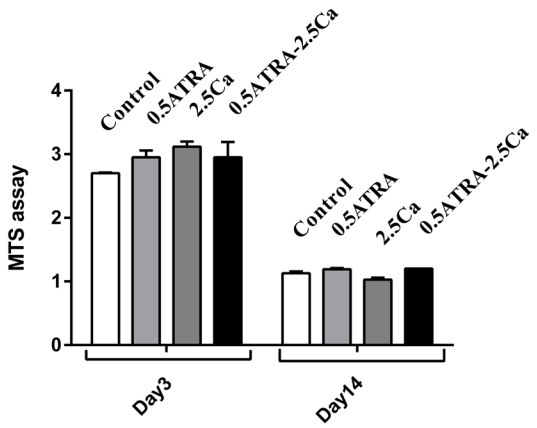

Figure 2. Viability and Cell Toxicity Assays. MTS assay confirms no significant changes among the treated groups after 14 days of treatment. Data are shown as mean ± SEM. C group: control group.0.5AT group: 0.5 µM ATRA. 2.5Ca2 + group: 2.5 mM Ca. 0.5AT-2.5Ca: 0.5 µM ATRA + 2.5 mM Ca2 +

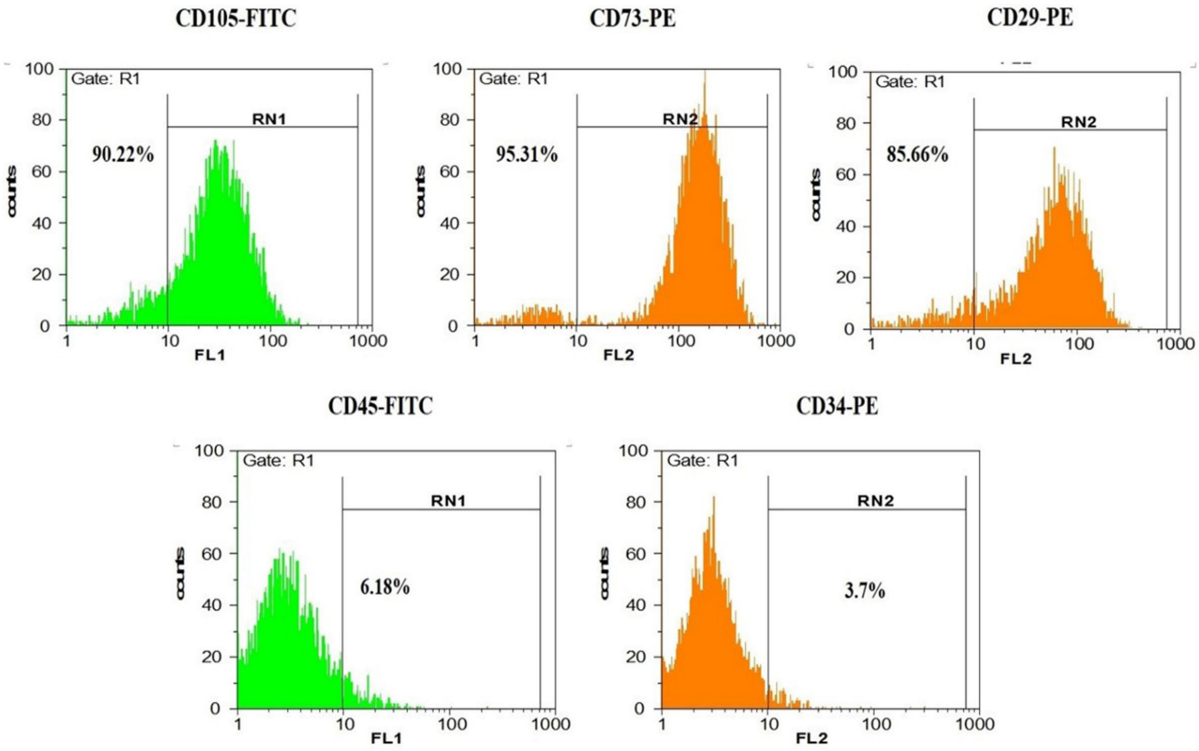

Figure 1. Flow Cytometry Analysis of Human Adipose-Derived Mesenchymal Stem Cell. The human amnion-derived mesenchymal stem cells (hAD-MSCs) at 3rd passage expressed CD105 (90.22 ± 1.2%), CD73 (95.31 ± 4.1%), and CD29 (85.66 ± 1.6%), while CD45 (6.18 ± 1.1%) and CD34 (3.7 ± 0.6%) were poorly expressed. FITC: Fluorescein isothiocyanate. PE: phycoerythrin

defined time period.

Oil Red O Staining

The lipid droplets which indicate the adipocyte formation was lower in the treatment groups than in the control group (Figure 4A). Accordingly, the group that received both ATRA and Ca^{2+} showed the lowest lipid accumulation compared with the other treatment groups.

Triglyceride Content

Triglyceride content was evaluated to confirm the qualitative results of Oil Red O staining (Figure 4B). Similar to the Oil Red O staining results, the TG content was remarkably decreased in ATRA ($P=0.02$), Ca^{2+} ($P=0.03$), and Ca^{2+} plus ATRA ($P<0.001$) groups in comparison with the control group. Interestingly, the combined treatment group (0.5 µM AT-2.5 mM Ca^{2+}) showed a more significant inhibitory effect on TG content than the group that received 2.5 mM Ca^{2+} ($P=0.01$) or 0.5µM ATRA ($P=0.21$) alone.

GAPDH Activity Is Significantly Diminished With Combined Treatment

As shown in Figure 5, lower GAPDH activity was seen in the ATRA ($P=0.04$), Ca^{2+} ($P=0.031$), and Ca^{2+} plus ATRA ($P<0.001$) groups compared with the control group. The rate of GAPDH activity reduction in the combined treatment group (Ca^{2+} plus ATRA) was greater compared to the other two groups. Therefore, the combination of Ca^{2+} and ATRA treatments amplifies their inhibitory effects against GAPDH activity.

Gene Expression Analysis of RAR-α and Calreticulin

Gene expression analysis of RAR-α and calreticulin are shown in Figures 6A and 6B. Unlike the treatment with Ca^{2+} (2.5 mM), treatment with ATRA (0.5 µM) or

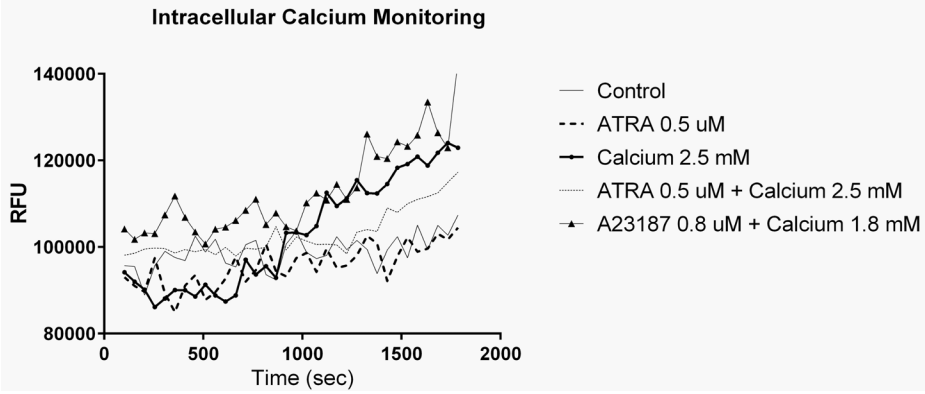

Figure 3. Calcium Mobilization Monitored in Treatment Groups. One hour after the treatments, cells were exposed to Fluo-8 for 30 minutes, and then the signal was captured at excitation/emission (EX/EM) = 480/520 nM. According to the results, the groups that received the extracellular calcium had a transient increase in the intracellular calcium. A23187 (0.8 µM) was used as a positive control to induce calcium intracellular mobilization. The signal was captured every one minute for a total of 30 minutes

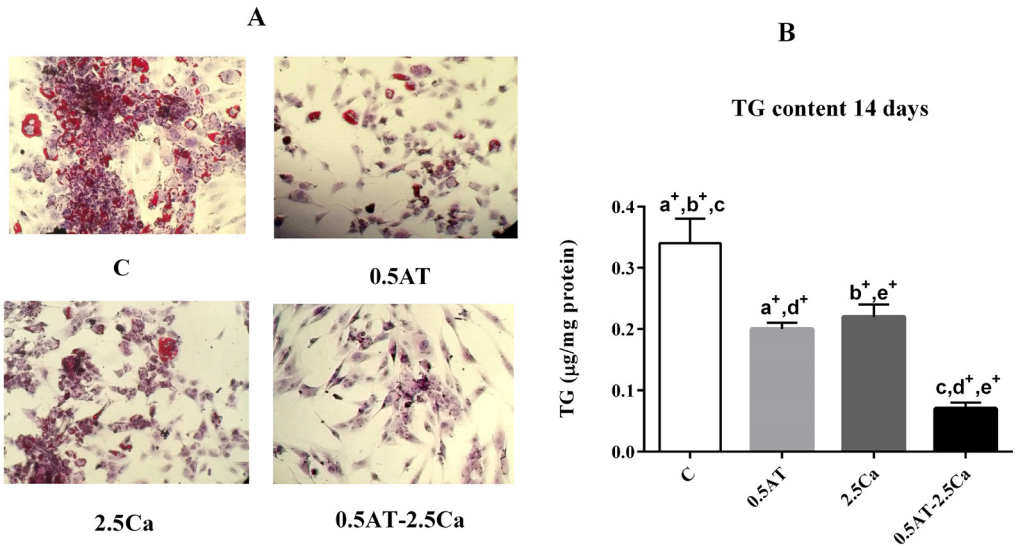

Figure 4. Lipid Droplets Observation and TG Content Evaluation in The Treatment Groups. A) Cells were induced either in differentiation medium (control group) or in differentiation medium containing 0.5 µM ATRA (0.5AT group), 2.5 mM Ca2 + (2.5Ca group), and 0.5 µM ATRA + 2.5 mM Ca2 + (0.5AT-2.5Ca group). A decrease in lipid accumulation was observed in 0.5AT group, 2.5Ca group, and especially in 0.5AT-2.5Ca group (gamma intensity was decreased in this image). B) The significantly lower TG content was seen in all treatment groups versus the control group. The combined treatment group (0.5AT-2.5Ca group) showed a significantly lower TG content compared with 0.5AT group and 2.5Ca group. Data are presented as Mean ± SEM. The similar alphabetic letters a, b, c, d, or e represent the significant differences. $P<0.001$ except ×. $P<0.01$ and + $P<0.05$

Ca^{2+}plus ATRA (2.5 mM Ca-0.5 μM ATRA) increased the gene expression of RAR-α in comparison with the control group (0.5 ATRA group; $P=0.003$ and 0.5At-2.5Ca group; $P<0.001$, respectively). More importantly, the expression of RAR-α in the 0.5ATRA-2.5Ca group was notably higher compared to the 0.5 ATRA group ($P<0.001$) (Figure 6A). Unlike significant changes in RAR-α expression in different groups, calreticulin gene expression showed no significant changes (Figure 6B).

Gene and Protein Expression of GLUT4 and PPAR-γ2

The protein and gene expression levels of $PPAR\gamma_2$ are shown in Figures 7A and 7C, respectively. As shown in Figure 7A, the protein level in the treatment groups was lower compared to the control group. According to Figure 7C, all of the treatment groups showed down-regulation of $PPAR\gamma_2$ gene expression compared with the control group (group 0.5 ATRA, $P=0.004$; group 2.5 Ca, $P=0.008$; group 0.5 At-2.5 Ca, $P<0.001$). Among treatment groups, the greatest inhibitory effect on $PPAR\gamma_2$ gene expression was detected in the 0.5ATRA-2.5Ca^{2+}group. The protein and gene expression levels of GLUT4 are shown in Figures 7B and 7D, respectively. Both GLUT4 gene and protein expression were remarkably decreased in all treatment groups in comparison with the control group ($P<0.001$; Figure 7D). Furthermore, similar to GLUT4 gene expression that was significantly diminished in the 0.5AT-2.5Ca group compared with 2.5 Ca^{2+}($P=0.012$) and 0.5AT ($P=0.001$) groups, its protein band was also very faint in this group.

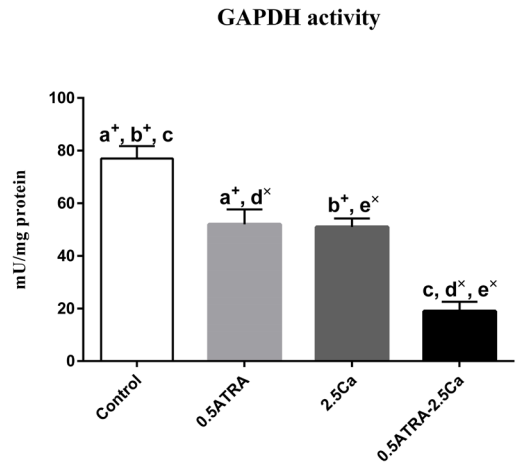

Figure 5. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Activity Assay. A significantly lower GAPDH activity was observed in all treatment groups versus the control group. The combined treatment group (0.5AT-2.5Ca group) showed significantly lower GAPDH activity versus 0.5AT group and 2.5Ca group. Data are presented as Mean±SEM. The similar alphabetic letters a, b, c, d, or e represent the significant differences. $P<0.001$ except×. $P<0.01$ and+$P<0.05$. 0.5ATRA group: 0.5 μM ATRA. 2.5Ca group: 2.5 mM Ca^{2+}. 0.5AT-2.5Ca: 0.5 μM ATRA+2.5 mM Ca^{2+}

Discussion

Both hyperplasia and hypertrophy of adipocytes are involved in the growth of adipose tissue. Many studies have focused on the signaling pathways involved in cellular changes in obesity. In recent years, the discovery of stem cells and their differentiation into a variety of cell types has led scientists to focus on the role of these cells in the activities of the body. Among all progenitor cells, MSCs were considered as a major source of adipocyte generation, and studies have been conducted on various factors affecting this process (27). Therefore, understanding the promoting or inhibiting properties of multiple factors in the regulation of MSC adipogenesis may help us to deal with obesity. Retinoic acid is one of these factors with approved anti-adipogenic activity in the early stages of differentiation in both in vivo and in vitro (28). Calcium is the other factor with controversial roles in the adipogenesis process and development of metabolic disorders that are associated with obesity (29). In the current study, 2.5 mM $Ca^{2+}$and 0.5 μM ATRA inhibited the differentiation of hMSCs to adipocytes. This was confirmed by multiple lines of evidence, from morphology observation to gene expression evaluation. In our experiments, the decrease in differentiation induced by ATRA, through activating its receptor RAR-α, was

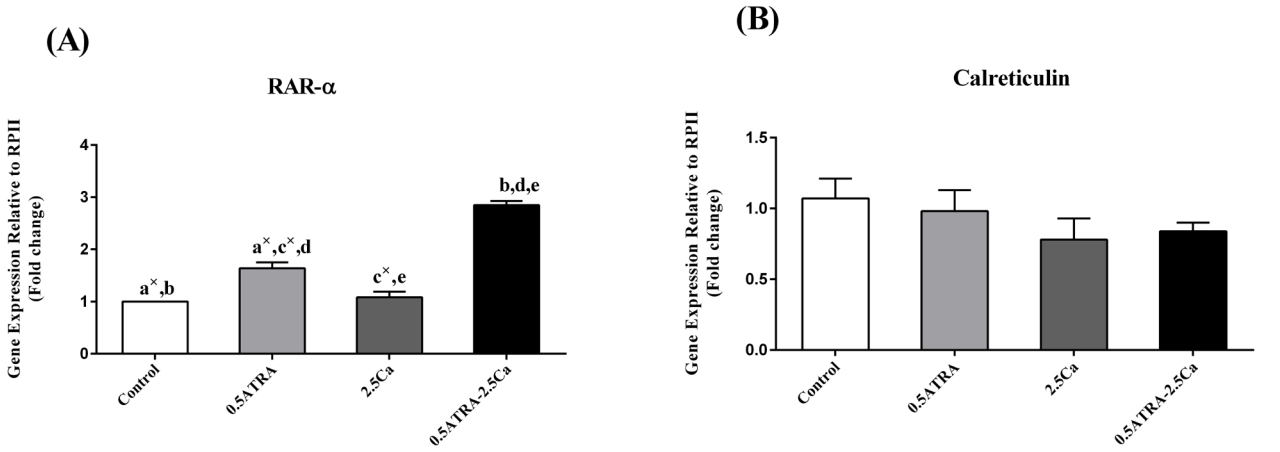

Figure 6. Gene Expression of (A) RAR-α and (B) Calreticulin. While calreticulin gene expression was not significantly changed between different groups, RAR-α gene expression was significantly higher in 0.5AT group and 0.5AT-2.5Ca group versus the control and 2.5Ca group. The similar alphabetic letters a, b, c, d, or e represent the significant differences. $P<0.001$ except×. $P<0.01$ and+$P<0.05$. Data are presented as mean±SD. 0.5ATRA group: 0.5 μM ATRA. 2.5Ca group: 2.5 mM Ca^{2+}. 0.5AT-2.5Ca: 0.5 μM ATRA+2.5 mM Ca^{2+}

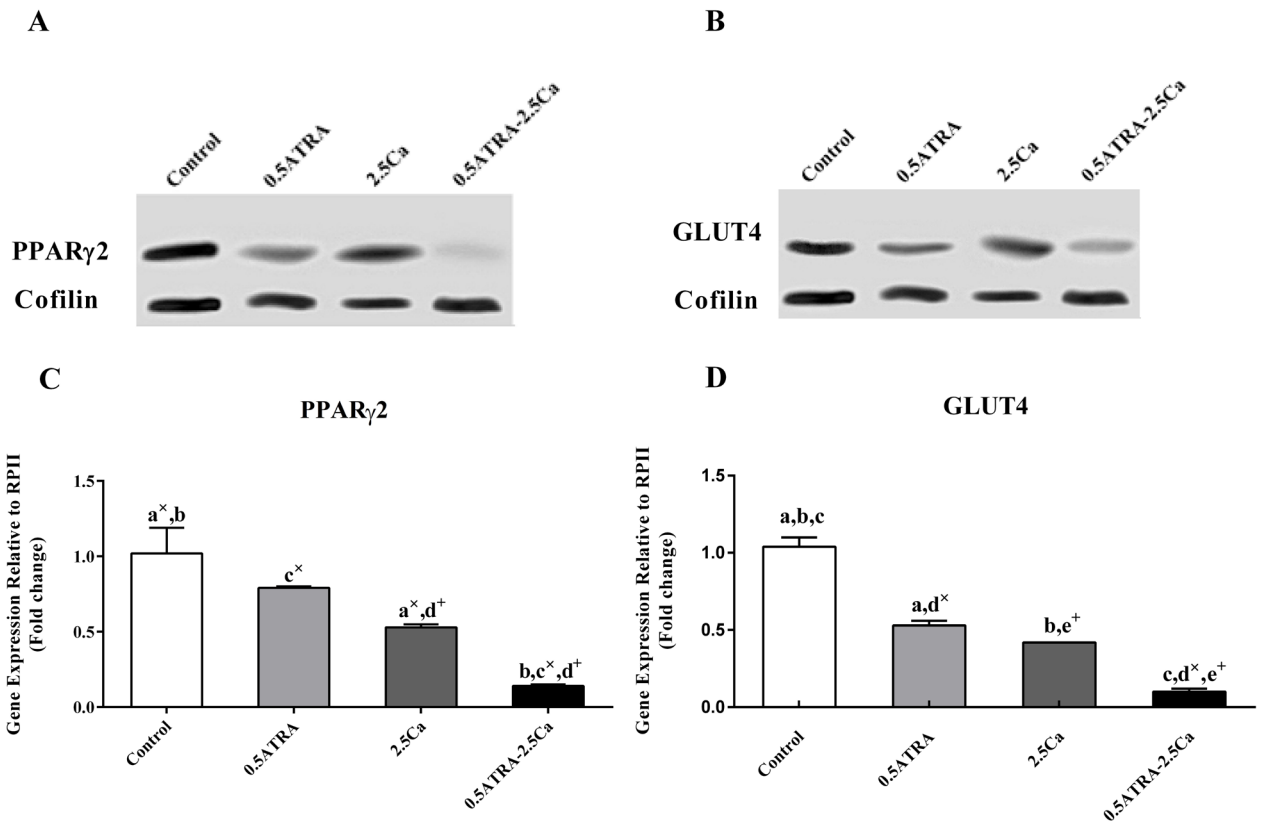

Figure 7. (A) The protein level of PPARγ2. (B) The protein level of GLUT 4. (C) The gene expression level of PPARγ2. (D) The gene expression level of GLUT4. A, C) The gene and protein level of PPARγ2 was significantly lower in all treatment groups than the control group. Also, the combined treatment group (0.5AT-2.5Ca group) showed a significantly lower PPARγ2 gene and protein expression compared with other treatment groups. (C, D) The significantly lower GLUT4 gene and protein level was seen in all treatment groups versus the control group. The combined treatment group (0.5AT-2.5Ca group) showed the lowest amount of GLUT4 gene and protein expression among treatment groups. The similar alphabetic letters a, b, c, d, or e represent the significant differences. $P<0.001$ except ×. $P<0.01$ and + $P<0.05$. Data are presented as Mean ± SD. 0.5ATRA group: 0.5 µM ATRA. 2.5Ca group: 2.5 mM Ca^{2+}. 0.5AT-2.5Ca: 0.5 µM ATRA + 2.5 mM Ca^{2+}

shown by a remarkable reduction of PPARγ2 and GLUT4 expression. Down-regulation of PPARγ2 decreased GLUT4 gene expression and consequently glucose uptake. We also found that ATRA treatment significantly attenuated GAPDH activity. GAPDH, an ultimate adipogenic marker, provides glycerol for the synthesis of triglycerides (24).

Two classes of nuclear hormone receptors including RA receptors (RARs) and retinoid X receptors (RXRs) are involved in the retinoic acid function. Among all receptors, the prominent role of RARs against adipogenesis was reported (30). Among all RARs isoforms, RARα and RARγ are implicated to be more important in the inhibition of adipocyte differentiation (31).

During the differentiation of murine MSCs to adipocytes, when cells are exposed to retinoic acid, an increase in gene expression of RAR-β occurs; however, no change in the RAR-α and RAR-γ has been reported (30).

It has been mentioned that a threshold level of RAR-α and RAR-γ is necessary for retinoic acid to show its inhibitory effect against adipogenesis (32). According to previous studies, the threshold level of RAR-α was detected in the primary stage of 3T3-L1 adipocyte differentiation (33). However, in our experiment, the higher expression of RAR-α gene was observed with ATRA treatment even after 14 days, which indicates the involvement of this receptor even in the last stages of the differentiation. The stimulatory effect of 0.1 and 1 µM ATRA on gene expression of RAR-α concurrent with its inhibitory effect on PPAR-γ gene expression has also been demonstrated in 3T3-L1 adipocyte differentiation (34). Therefore, the discrepancy of these results in higher gene expression of RAR-α in the primary or last stages of adipogenesis, may be the result of variations in cell type and unknown extrinsic factors that are involved in such a process. On the other hand, the inhibitory effect of calcium against adipogenesis was also determined by significant down-regulation of both PPAR-γ2 and GLUT4 genes and finally attenuation of GAPDH activity. In adipocyte differentiation, calcium seems to play different roles via interaction with a variety of receptors and kinases. It has been reported that calcineurin, a calcium-dependent serine/threonine phosphatase, mediates Ca^{2+}dependent inhibition of adipogenesis in the early stages of differentiation in the 3T3-L1 cell line. In contrast, calcineurin has also mediated the terminal phase of adipocyte differentiation (35). Accordingly, the biphasic regulatory effect of calcium on human adipocyte differentiation has also been shown in another study (19). However, our results are in line with the previous

investigation regarding that a higher Ca^{2+}concentration (more than 2.5 mM) inhibits PPARγ gene expression, thus inhibiting the differentiation to adipocyte (36). Calreticulin is another protein contributing to the exchange of the Ca^{2+}pool, thereby down-regulating the critical pre-adipogenic transcription factors via calcineurin and calmodulin/CaMKII pathway (37). In the present study, according to the evaluation of calcium mobilization by calcium probe, fluo-8, the addition of 2.5 mM extracellular calcium could increase the intracellular calcium, but not as much as the calcium ionophore (A23187). Therefore, this calcium concentration could not change calreticulin gene expression or prevent adipocyte differentiation by the commitment of calreticulin. As mentioned before, the higher calcium level may have a more inhibitory effect on such mechanism. Previously, it has been shown that thapsigargin, an inhibitor of Ca^{2+}ATPase, induces calreticulin expression and inhibits the lipogenesis process. Therefore, this revealed the importance of calcium as an adipogenesis inhibitor and the participation of calreticulin in this process (19). In the present investigation, the inhibition of adipogenic differentiation was observed in the combination treatment of ATRA and calcium. Concerning the results of microscopic examination, gene and protein expression of PPARγ2 and GLUT4, and triglyceride assay, we can conclude that the combination of $Ca^{2+}$and ATRA may exhibit a cumulative effect on the inhibition of adipogenesis. This cumulative inhibitory effect was also observed in the last stages of differentiation by prevention of cellular GAPDH activity. There was no change in the gene expression of calreticulin after the combined treatment of $Ca^{2+}$and ATRA, which can be indicative of another mechanism involved in this superior inhibitory effect.

Interestingly, ATRA plus Ca^{2+}treatment induced significantly higher gene expression of RAR-α compared with each treatment alone. This result showed a common pathway of their inhibitory mechanism. It has been recently recommended that the accumulation of calcium in the cytosol can strengthen the retinoic acid signaling pathway by increasing its receptor (RAR-α) (20). Therefore, calcium influx could protect the RAR-α from degradation by proteasomes. In another way, Ca^{2+}dependent phosphatidylserine alters the stabilization of RAR-α and influences the ATRA signaling (38,39). Overall, it is clear that co-treatment of ATRA and Ca^{2+}may modify the ATRA receptor (RAR-α) and finally increases its survival.

Although these results could improve our understanding of adipogenesis, more mechanisms need to be clarified in this regard. To evaluate the physiological role of ATAR and Ca^{2+}, the normal range of these molecules in the human body was used in the present study (40,41). Therefore, no significant changes in proliferation were observed in the period of treatment. In this regard, a higher dose of these molecules may show different results of viability and differentiation rate.

Conclusion

In conclusion, our results revealed that co-administration of 2.5 mM $Ca^{2+}$and 0.5 µM ATRA showed more profound anti-adipogenesis effects compared with each treatment alone. These inhibitory effects were observed in the morphological changes, Oil Red O staining, and triglyceride content. At the molecular level, the attenuation of GLUT4 and $PPAR\gamma_2$ gene expression and finally the GAPDH activity were also observed after these treatments. Increased gene expression of RAR-α by ATRA treatment and further enhancement of this receptor by combined treatment of ATRA and calcium indicated the cumulative effect of these two compounds. Although the results of this study showed the effects of these two compounds on the metabolism of stem cells, more extensive studies should be performed due to the contrasting effects of these compounds on adipocyte differentiation.

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Competing Interests

The authors declare that they have no competing interests.

Ethical Approval

This study was approved by the Ethics Committee of Hamadan University of Medical Sciences. No Issue was claimed because of the *in vitro* design.

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