

Different effects of total flavonoids from *Arachniodes exilis* on human umbilical cord mesenchymal stem cells in vitro

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Abstract

Traditional Chinese medicines are used in promotion of fractured bone healing and bone diseases. Some studies reported total flavonoids from plant can be used as an auxiliary source of exogenous.

Use different methods to identify and verify effects of total flavonoids from *Arachniodes exilis* (TFAE) on human umbilical cord mesenchymal stem cells (HUCMSCs) in vitro.

Concentrations of 1 and 5 $\mu\text{g/mL}$ TFAE significantly increased ALPase activity in HUCMSCs compared to the other concentrations at days 3 and 7 ($P < .05$). RT-PCR showed that expression levels of osteogenic genes (Col1a1, OPN, Runx2 and Osx) were remarkably enhanced in hUCMSCs following treatment with different concentrations of TFAE for 9 days compared with 0 $\mu\text{g/mL}$ TFAE group (control). The results showed that concentration $< 5 \mu\text{g/mL}$ of TFAE induced osteogenic differentiation in HUCMSCs. Alizarin red staining assays revealed that both TFAE and S1191 was significantly decreased (7.80 ± 0.66) compared with the TFAE group (16.00 ± 0.97) ($P < .01$). ALPase activity on days 3 and 7 was relatively lower in HUCMSCs grown in media supplemented with both S1191 and TFAE than that of in TFAE group only. The results indicated that osteogenic markers (Col1a1, OPN, Runx2 and Osx) were significantly downregulated in the TFAE + S1191 group in comparison to the control group. The expressions of Col1a and OPN in the TFAE + S1191 group decreased significantly ($P < .01$) by Western blotting.

TFAE promotes the odonto/osteogenic differentiation of human UCMSCs via activation of ER.

Abbreviations: HUCMSCs = human umbilical cord mesenchymal stem cells, TCMs = traditional Chinese medicines, TFAE = total flavonoids from *Arachniodes exilis*.

Keywords: alizarin red S staining, estrogen receptor, human umbilical cord mesenchymal stem cells, osteogenic, traditional Chinese medicines

1. Introduction

Traditional Chinese medicines (TCMs) are used in promotion of fractured bone healing and bone diseases such as osteoporosis.^[1] Many studies have shown the positive effects of TCM on bone

formation in vitro and in vivo.^[2] The study showed that several TCMs have bone promoting properties, The mechanism of this may be due to the angiogenic effects.^[3]

Recently, some scholars reported the role of total flavonoids in anti-cancer and osteogenic differentiation. Zhou et al found that

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WY and WH had contributed equally to this work.

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Ethics Statement: Umbilical cords (UCs) and informed consent was obtained from delivering mothers of Jiujiang University Subsidiary Hospital. We ensured that participants' rights were protected. In this study, all manipulations with experimental HUCMSCs cells were approved by the Medical Ethics Committee of Jiujiang (Approved ID: 1–2013, 20 February 2013).

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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ethanol extracts from *Arachniodes exilis* have anti-oxidant and hepato-protective roles and could potentially be used in the treatment of liver disease.^[4] We previously reported that total flavonoids from *Arachniodes exilis* (TFAE) enhances the osteogenic capacity of mesenchymal stem cells via BMP signaling.^[5] The roles of flavonoids and polyphenol derivatives of these extracts have been studied, but the biological effects of various extracts are not clear. The study showed that TFAE mediates apoptosis in HepG2 cells by activating MAPK signaling, but the proliferation effect of LO₂ cells is not obvious at appropriate concentrations.^[6] Some researchers proved that flavonoids derived from *Epimedium pubescens* can promote the differentiation of rat bone marrow mesenchymal stem cells into bone cells,^[7] others found that *Epimedium pubescens* flavonoids promotes MC3T3-E1 cell proliferation and osteogenic differentiation by activating ERK-JNK signaling mediated by the estrogen receptor (ER).^[8] The study showed estrogen promotes early osteoblast differentiation.^[9] Little is known about the direct impact of TFAE on human umbilical cord mesenchymal stem cells (HUCMSCs).

TCM have been used in the Chinese population for the treatment of bone diseases and in promoting bone healing for thousands of years.^[10] Shalan NA et al reported Noni leaf and black tea enhance bone regeneration in estrogen-deficient rats.^[11] If TCMS are found to promote bone formation, these can be used in bone grafting materials that could eliminate or decrease the need to use allografts, which may be a source of transmission of disease. In this study, we present evidence that TFAE induces osteogenic differentiation of HUCMSCs. Our study provides support for the use of exogenous growth factors as remedies that could potentially be used to treat bone defects.

2. Materials and methods

2.1. Isolation and culture of HUCMSCs

The research protocol was approved by the ethics committee of the School of Basic Medical Science at Jiujiang University and by the Jiujiang Maternal and Child Health Hospital. Informed consent was obtained from delivering mothers. Umbilical cords (UCs) were processed within 24 hours after delivery. Adherent cells were cultured and isolated using the explant method. Briefly, HUCMSCs were longitudinally dissected from the UC, separating them from the Wharton's jelly tissue coating. HUCMSCs were isolated and cultured following the manufacturer's instructions (Cyagen Biosciences Inc, America).

2.2. Identification of HUCMSCs

HUCMSCs were assessed by flow cytometry to identify typical cell surface epitopes (CD19, CD29, CD90, and CD105). Briefly, HUCMSCs were incubated for 30 minutes at 4°C with the following mouse monoclonal antibodies: phycoerythrin (PE)-conjugated CD29 (No. ab24697 Abcam, UK), CD90 (No. ab3105 Abcam, UK) and CD105 (No. ab11414 Abcam, UK), and fluorescein isothiocyanate [FITC]-conjugated CD19 (ab24936 Abcam, UK). As isotypic controls, we used PE- and FITC-conjugated anti-mouse IgG antibodies (ab6785 Abcam, UK). Dead cells were identified by staining with propidium iodide and excluded from surface epitope analyses. Cells were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

2.3. Preparation of TFAE and determination of the total flavonoids content

TFAE was prepared in Jiujiang Key Laboratory of Translational Medicine (China) according to a previously published method.^[9] The roots of *A. exilis* were collected from Lushan city, Jiangxi province, China. A voucher specimen (No. F1205) has been deposited in the Jiujiang Key Laboratory of Translational Medicine, Jiujiang, Jiangxi, P. R. China. Determination of the Total Flavonoids Content in TFAE. The total flavonoids in TFAE were estimated as rutin equivalent. Briefly, a series of rutin solutions in the concentration range of 0 to 65.0 µg/mL were used for the calibration curve and were prepared as below: 25 mL of rutin samples at certain concentrations was prepared by step by step mixing appropriate volumes of rutin stock solution with 1 mL of 5% (w/v) NaNO₂ solution, 1 mL of 10% Al(NO₃)₃ solution, and 10 mL of a 10% (w/v) NaOH solution, and after each adding, the mixtures were allowed to stand for 6 min. Finally, the mixtures were made up to 25 mL with distilled water and mixed well. The absorbance was measured with a spectrometer (Purkinje General Instrument Co., Ltd, Beijing, China) after 15 min. Similarly, the TFAE sample was prepared and analyzed as above. On the basis of the calibration curve, the total flavonoids content of TFAE was calculated.

2.4. Proliferation of HUCMSCs

A CCK-8 assay was performed to detect the effect of TFAE on the viability of HUCMSCs. For this assay, cells were seeded in 96-well plates at 5×10^3 cells/well. After 24 hours, cells were treated with different concentrations of TFAE (1 µg/mL, 5 µg/mL, 10 µg/mL, or 20 µg/mL) for 24 hours, 48 hours, and 72 hours. Control cells were cultured in DMEM/F12 not supplemented with TFAE. 10 µL CCK-8 assay reagent was added to each well, and the plate was incubated for 2 h at 37°C. The absorbance (A) at 450 nm was recorded for each well using a microplate reader. Cell viability (%) was calculated.

2.5. Osteogenic differentiation of HUCMSCs with treatment of TFAE and S1191

HUCMSCs were seeded at 3×10^4 cells/cm² in 6-well plates pre-coated with a gelatin solution. After 24 h, cells were treated with different concentrations of TFAE. An osteogenic differentiation medium from the Human Umbilical Cord Mesenchymal Stem Cell Osteogenic Differentiation Basal Medium kit (HUXUC-90021, Cyagen Biosciences Inc) was used. This medium consisted of DMEM/F12 base medium (Guangzhou Cyagen Biosciences Inc) supplemented with 10% fetal bovine serum (Human Umbilical Cord Mesenchymal Stem Cell Osteogenic Differentiation Fetal Bovine Serum, Guangzhou Cyagen Biosciences Inc), penicillin-streptomycin (100 units/mL and 100 µg/mL, respectively, Guangzhou Cyagen Biosciences Inc), 10 nM dexamethasone (Guangzhou Cyagen Biosciences Inc), 10 mM β-glycerophosphate (Guangzhou Cyagen Biosciences Inc), and 50 µg/mL Ascorbate (Guangzhou Cyagen Biosciences Inc). Four treatment groups were analyzed in triplicate: a control group (cultured in DMEM/F12 not supplemented with the osteogenic differentiation medium), a 0 µg/mL TFAE group (cultured in the osteogenic differentiation medium not supplemented with TFAE), a 1 µg/mL TFAE group (cultured in the osteogenic differentiation medium supplemented with 1 µg/mL TFAE), and

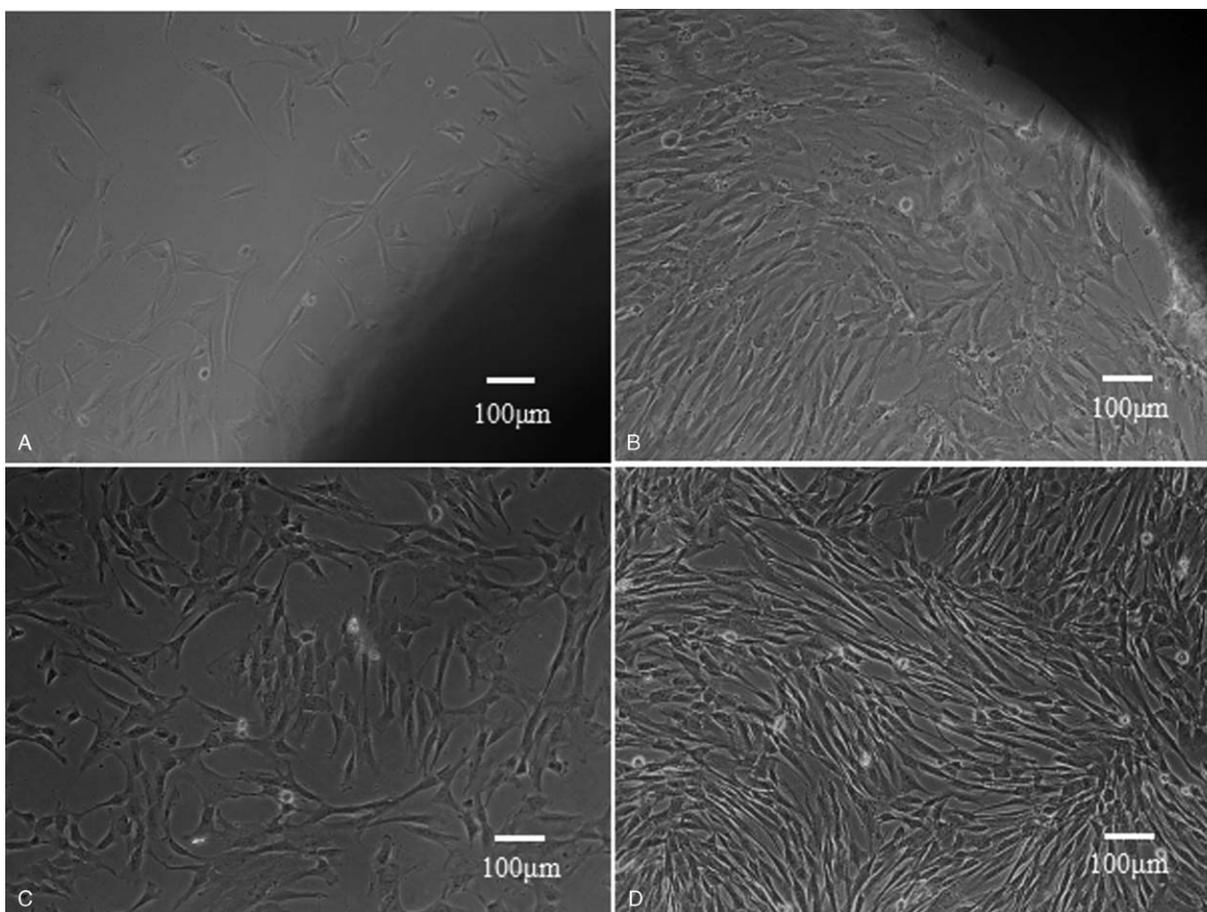


Figure 1. Morphological features of cultured cells derived from human umbilical cord. A. HUCMSCs at primary cultured for 5 d. B. HUCMSCs at primary cultured for 12 d; C. HUCMSCs at passage 1 cultured for 2 d. D. HUCMSCs at passage 3 cultured for 3 d. HUCMSCs = human umbilical cord mesenchymal stem cells.

a 5 µg/mL TFAE group (cultured in the osteogenic differentiation medium supplemented with 5 µg/mL TFAE). The 0 µg/mL TFAE group was used as a positive control.

To determine the potential involvement of ER in TFAE-mediated osteogenic differentiation of HUCMSCs, we used the ER inhibitor S1191. Four treatment groups were analyzed in triplicate: a 0 µg/mL TFAE group (cultured in the osteogenic differentiation medium not supplemented with TFAE), a 5 µg/mL TFAE group (cultured in osteogenic differentiation medium supplemented with 5 µg/mL TFAE), a 5 µg/mL TFAE+S1191 group (cultured in the osteogenic differentiation medium supplemented with 5 µg/mL TFAE and S1191 (Sigma-Aldrich, USA), and an S1191 group (cultured in osteogenic differentiation medium supplemented with S1191). Media was changed every 3 days. Osteogenic differentiation medium without the TFAE supplement was used as control.

2.6. Alizarin red S staining

Alizarin red S staining was performed to determine bone nodule formation. After 10 days of treatment, cells were washed twice with 1 × PBS and then fixed with 4% formaldehyde solution for 30 minutes. Next, cells were washed twice with 1 × PBS and stained with 1 mL alizarin red solution for 5 minutes. Lastly, cells were rinsed 3 times with 1 × PBS and visualized and imaged under a microscope.

2.7. Alkaline phosphatase activity (ALP)

An ALP assay kit (Maccara Biotechnology Co., Ltd. China, No: CH0101203) was used to determine alkaline phosphatase activity. After 3 or 7 days of treatment, media was aspirated off of the cells, 0.5 ml 0.1% TritonX-100 was added to the cells, and then, the cell lysate was collected using the ALP assay kit according to the manufacturer's recommended protocol. Absorbance values (OD) were recorded at 450 nm, the average absorbance values ($\Delta A/\text{min}$) were determined, and ALP activity was calculated as follows: $\text{ALP}(\Delta\text{U/L}) = \Delta A/\text{min} \times 2713$.

2.8. RNA extraction and RT-PCR analysis

Cells were collected at the appropriate times as described above. Total RNA was extracted using the GREEN spin tissue/cell RNA rapid extraction kit (Zomanbio, China) according to the manufacturer's recommended protocol. The reverse transcriptase polymerase chain reaction was carried out using the HIFIScript 1st Strand cDNA Synthesis Kit (Cwbio, China No. 00081309). Briefly, 2 µg of RNA was incubated with a dNTP mixture and oligo (dT) primers at 65°C for 5 min for denaturation and annealing, samples were reverse-transcribed at 42°C for 15 min, and the reverse transcriptase was inactivated at 95°C for 5 min. Synthesized cDNA was amplified by PCR in the presence of primer pairs specific to genes expressed during differentiation. Reaction mixtures (25 µl total) contained 100 ng of cDNA,

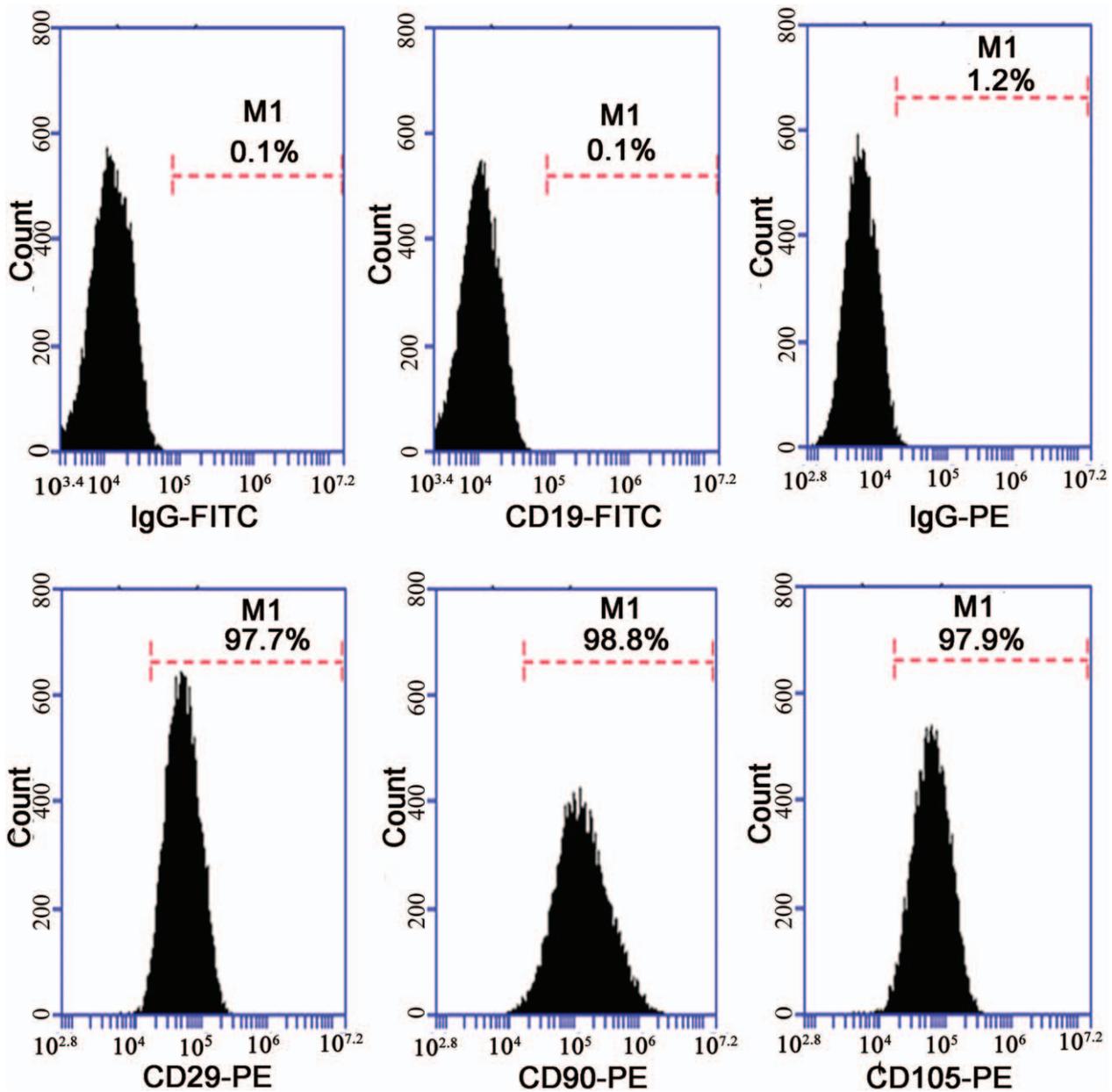


Figure 2. Immunophenotypic characterization of hUCMSCs. For flow cytometry, UCMSCs at passage 5 were dissociated using pancreatin, stained with isotype controls and labelled with antibodies against CD19, CD29, CD90 and CD105 and analysed by flow cytometry. CD = cluster of differentiation, hUCMSCs = human umbilical cord mesenchymal stem cells.

12.5 μ l of SinoBio 2 \times Taq Master Mix and 0.4 μ M primers. Primers used in the experiment were as follows: Col1a1, 5'-CAAAGGCAATGCTCAAACAC-3' (forward) and 5'-GACG-CAGGACAGACTAGGAG-3' (reverse); OPN, 5'-ACACATAT-GATGGCCGAGGTGA-3' (forward) and 5'-GTGTGAGGT-GATGTCTCGTCTGTGA-3' (reverse); Osx, 5'-GCCAGAA-GCTGTGAA ACCTC-3' (forward) and 5'-GCTGCAAGCTCT-CCATA ACC-3' (reverse); Runx2, 5'-CCACCTCTGA-CTTCTGCCTC-3' (forward) and 5'-GACTGGCGGGGTG-TAAGTAA-3' (reverse); ER α , 5'-TGCCAAGGAGAC TCGTA-3' (forward) and 5'-CCTCTTCGGTCTTTTCG-TATCC-3' (reverse); ER β , 5'-TCCTATGTAGACAGCCACC-

ATG-3' (forward) and 5'-GCCACCTTCCAAG TTAGTGAC-3' (reverse). GAPDH was used as a control. Primers for GAPDH were: 5'-AGGTCGGAGTCAACGGATTTG-3' (forward) and 5'-AGGCTGTTGTCAT ACTTCTCAT-3' (reverse). The PCR products were separated by electrophoresis on 2.0% agarose gels.

2.9. Western blot

The cells were lysed with immunoprecipitation assay buffer containing protease inhibitors. The protein concentrations were examined using the BCA Protein Assay kit (Vazyme Biotech, China). 40 μ g of proteins were separated by sodium dodecyl

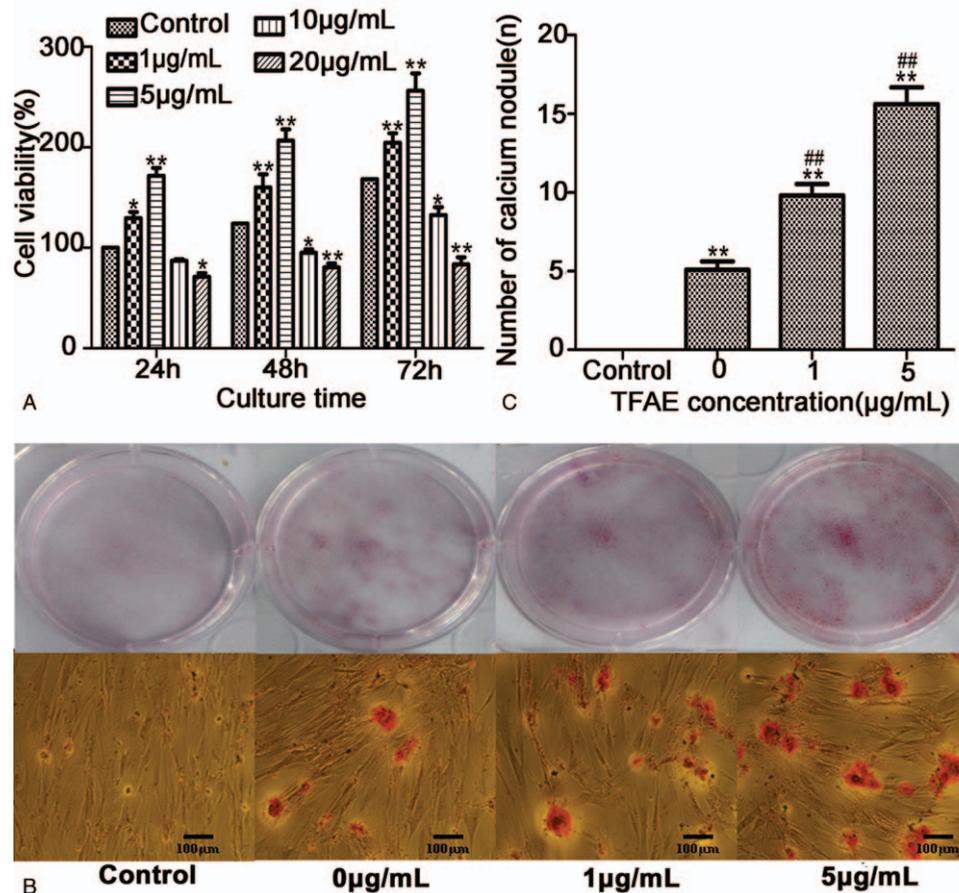


Figure 3. A. The effect of TFAE on proliferation of HUCMSCs. Values were presented as mean \pm SD, * $P < .05$, ** $P < .01$ compared with control group at each time point, (n = 3); B. Alizarin red S staining in HUCMSCs cultured on different concentrations of TFAE at 10 days after induction. C. Calcium nodule was counted. Values were presented as mean \pm SD, * $P < .05$, ** $P < .01$ compared with control group, # $P < .05$, ## $P < .01$ compared with 0 μ g/mL group (n = 3). HUCMSCs = human umbilical cord mesenchymal stem cells.

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a piece of polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.5% of Triton X-100) for 1 hour at room temperature, and then incubated with the appropriate primary antibody against Col1a1 (No.ab21286 Abcam) and OPN (No. ab75285 Abcam) or GAPDH (No. 10494-1-AP Proteintech, China) overnight at 4°C. The membranes were washed 3 times in TBST, followed by incubation with the appropriate horseradish peroxidase-linked secondary antibodies (No. 10285-1-AP Proteintech, China) for 1 hour at room temperature. The specific proteins on the blots were developed with enhanced chemiluminescence (Vazyme Biotech, China) and visualized as the bands on the CL-XPosure Film (Thermo Fisher Scientific).

2.10. | Statistical analysis

Statistical analyses were performed using the SPSS 19.0 statistical software package (SPSS Inc.). The data was expressed as the mean \pm standard deviation (SD). Differences between groups were analyzed using the Student *t*-test or 1-Way ANOVA. $P < .05$ was considered to be statistically significant.

3. Results

3.1. Isolation and culture of HUCMSCs

HUCMSCs migrated from the adherent UC tissue fragments after 3 to 5 days. We successfully isolated HUCMSCs from UCs after culturing for 10 to 14 days. The cells displayed similar fibroblast-like spindle-shaped morphology and were not significantly different from the source material (Fig. 1A–D).

3.2. Identification of HUCMSCs

HUCMSCs at passage 5 were dissociated using pancreatin, stained with isotype controls and labeled with antibodies against CD19, CD29, CD90, and CD105 for flow cytometry. The results showed that over 95% of the cells were positive for the CD90, CD29, and CD105 surface markers. Over 98% of the cells were negative for CD19 (Fig. 2).

3.3. The effect of TFAE on proliferation of HUCMSCs

The effect of TFAE on the proliferation of HUCMSCs was evaluated using the CCK-8 assay. Cell viability was also examined using the CCK-8 assay. The viability reflected the

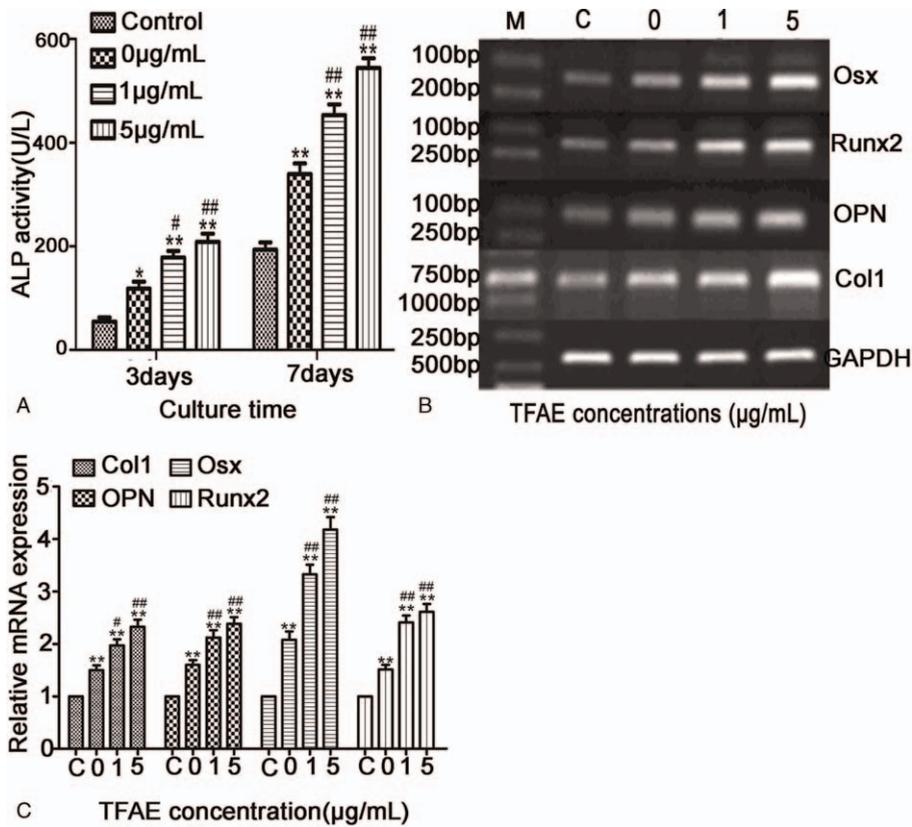


Figure 4. A. Alkaline phosphatase (ALP) activity in different concentrations of TFAE -treated HUCMSCs. Values were presented as mean ±SD, **P* < .05, ***P* < .01 compared with control group at each time point, #*P* < .05, ##*P* < .01 compared with 0 µg/mL group at each time point, (n=3); B. RT-PCR demonstrated gene expression in different concentrations of TFAE-treated hUCMSCs. the expressions of Col1a1, OPN, Runx2 and OsxmRNA (electrophoretogram). C. Relative expression Col1a1, OPN, Runx2 and OsxmRNA, Values were described as mean ±SD, **P* < .05, ***P* < .01 compared with control group, #*P* < .05, ##*P* < .01 compared with 0 µg/mL group (n=3). HUCMSCs = human umbilical cord mesenchymal stem cells.

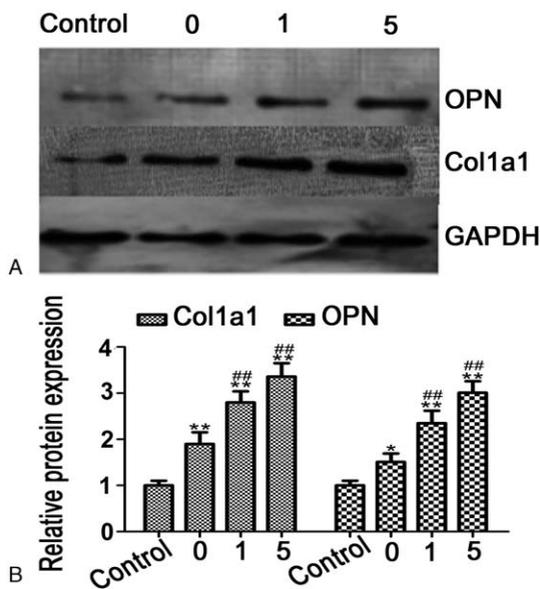


Figure 5. A. Effect of TFAE on expression level of osteogenesis-specific protein Col1a1, OPN in different concentrations of TFAE-treated HUCMSCs. B. Relative expression Col1a1 and OPN. Values were described as mean ±SD, **P* < .05, ***P* < .01 compared with control group, #*P* < .05, ##*P* < .01 compared with 0 µg/mL group (n=3). HUCMSCs = human umbilical cord mesenchymal stem cells.

metabolic activity of living cells, and the data was expressed as a percentage of the untreated control. HUCMSCs were treated with TFAE (1 µg/mL, 5 µg/mL, 10 µg/mL, or 20 µg/mL) for 24 hours, 48 hours, and 72 hours. The results indicated that concentrations of TFAE >5 µg/mL significantly inhibited cell proliferation (*P* < .05), while concentrations of TFAE < 5 µg/mL significantly promoted the proliferation of HUCMSCs (Fig. 3A).

3.4. Effects of TFAE on osteogenic differentiation of HUCMSCs

To study the effect of different concentrations of TFAE on osteogenic differentiation of HUCMSCs, cells were treated with TFAE for 10 days and then stained with alizarin red to analyze calcification levels. Alizarin red staining revealed that calcification was increased in a concentration-dependent manner after treatment. 5 µg/mL TFAE was determined to be the optimal concentration and was used for the following experiments (Fig. 3B, C).

In agreement with the alizarin S staining results, ALPase activity was initially detected on days 3 and 7 after treatment with different concentrations of TFAE in HUCMSCs. Concentrations of 1 and 5 µg/mL TFAE significantly increased ALPase activity in HUCMSCs compared to the other concentrations at days 3 and 7 (*P* < .05) (Fig. 4A). RT-PCR showed that expression levels of

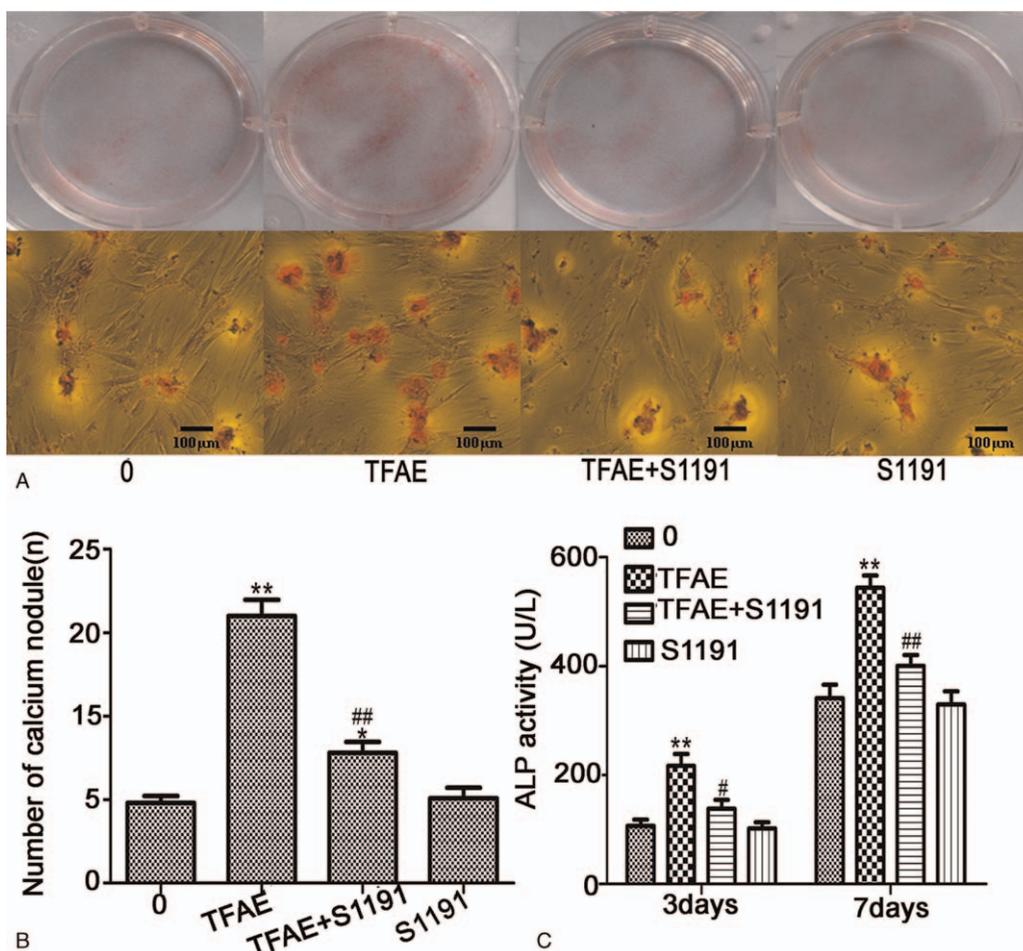


Figure 6. A. Alizarin red S staining in HUCMSCs cultured on concentrations of TFAE (5 $\mu\text{g}/\text{mL}$) and S1191 at 10 days after induction. B. Calcium nodule was counted. Values were described as mean \pm SD, * $P < .05$, ** $P < .01$ compared with 0 $\mu\text{g}/\text{mL}$ group, ## $P < 0.01$ compared with TFAE group, (n=3). C. Alkaline phosphatase (ALP) activity in HUCMSCs cultured on concentrations of TFAE (5 $\mu\text{g}/\text{mL}$) and S1191 at 3 days and 7 days after induction, * $P < .05$, ** $P < .01$ compared with 0 $\mu\text{g}/\text{mL}$ group at each time point, # $P < .05$, ## $P < .01$ compared with TFAE group at each time point, (n=3). HUCMSCs = human umbilical cord mesenchymal stem cells, TFAE = total flavonoids from *Arachniodes exilis*.

osteogenic genes (Col1a1, OPN, Runx2 and Osx) were remarkably enhanced in hUCMSCs following treatment with different concentrations of TFAE for 9 days compared with 0 $\mu\text{g}/\text{mL}$ TFAE group (control). In particular, expression levels of Col1a1, OPN, Runx2 and Osx in HUCMSCs treated with 5 $\mu\text{g}/\text{mL}$ TFAE were significantly higher on day 9 than those of 0 $\mu\text{g}/\text{mL}$ TFAE group (control). Expression levels of Col1a1, OPN, Runx2 and Osx could be upregulated by treatment with as little as 5 $\mu\text{g}/\text{mL}$ (Fig. 4B, C).

Western blotting was employed to detect HUCMSC Col1a1 and OPN protein levels 7 days after treatment with different concentrations of TFAE (1 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$). Col1a1 and OPN protein levels were normalized to the internal control GAPDH (Fig. 5A, B). The results showed that the expressions of Col1a1 and OPN protein was significantly increased ($P < .01$) in the groups treated with 1 and 5 $\mu\text{g}/\text{mL}$ TFAE compared with the control group. Compared with the positive control, the Col1a1 and OPN protein levels were further increased after 7 days of treatment with 1 or 5 $\mu\text{g}/\text{mL}$ TFAE, ($P < .01$). The results showed that concentration $< 5 \mu\text{g}/\text{mL}$ of TFAE induce osteogenic differentiation in HUCMSCs ($P < .01$). The results showed that

concentration $< 5 \mu\text{g}/\text{mL}$ of TFAE induced osteogenic differentiation in HUCMSCs

3.5. Estrogen receptor(ER) involvement in TFAE-mediated osteogenic differentiation of HUCMSCs

To determine the involvement of ER potential in TFAE-mediated osteogenic differentiation of HUCMSCs, we investigated the effects of an inhibitor of ER (S1191, a special inhibitor of ER) on osteogenic differentiation of HUCMSCs by Alizarin red S staining, ALP activity assays and analysis of expression levels of odonto/osteogenic genes. Calcification levels in HUCMSCs treated with TFAE for 10 days were identified by alizarin red staining. Four groups were included in the staining assays: a control group (0 $\mu\text{g}/\text{mL}$ TFAE), a TFAE group (5 $\mu\text{g}/\text{mL}$ TFAE), a TFAE + S1191 group (5 $\mu\text{g}/\text{mL}$ TFAE) and an S1191 group (0 $\mu\text{g}/\text{mL}$ TFAE). Alizarin red staining assays revealed that staining in the TFAE group (16.00 ± 0.97) was higher than in the control group (4.80 ± 0.41) and that the staining in HUCMSCs treated with both TFAE and S1191 was significantly decreased (7.80 ± 0.66) compared with the TFAE group (16.00 ± 0.97) ($P < .01$).

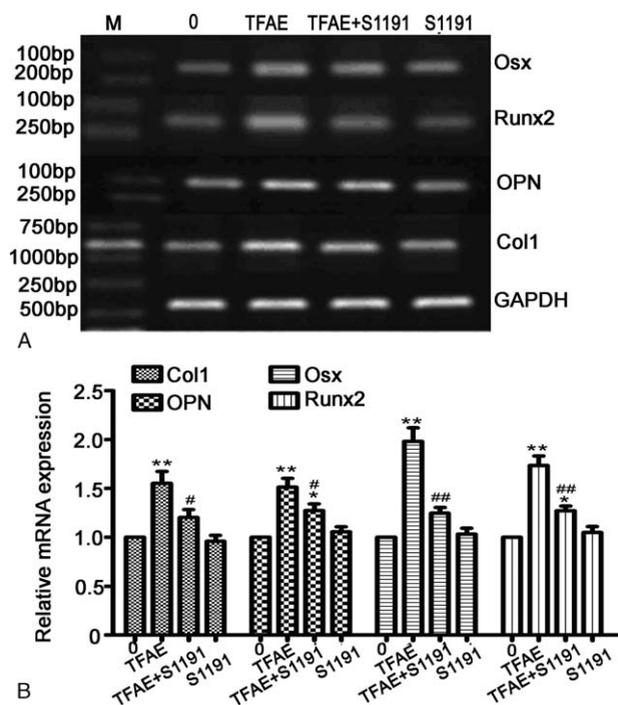


Figure 7. RT-PCR demonstrated gene expression in hUCMSCs cultured on concentrations of TFAE (5 μg/mL) and S1191 after induction. A. the expressions of Col1a1, OPN, Runx2 and Osx mRNA. B. Relative expression Col1a1, OPN, Runx2 and Osx mRNA. Values were described as mean ± SD, **P* < .05, ***P* < .01 compared with 0 μg/mL group, #*P* < .05, ###*P* < .01 compared with TFAE group, (n=3). HUCMSCs = human umbilical cord mesenchymal stem cells, TFAE = total flavonoids from *Arachniodes exilis*, RT-PCR = reverse transcription polymerase chain reaction.

The staining level in the S1191 group (5.10 ± 0.62) was found to be lower than that of the TFAE+S1191 group (7.80 ± 0.66) (*P* < .05) (Fig. 6A, B).

ALPase activity was initially detected on day 3 in HUCMSCs and was increased on day 7 for all treatment groups. The ALPase activity for HUCMSCs was significantly higher in the TFAE group than that in the control group on days 3 and 7 (*P* < .05). However, ALPase activity on days 3 and 7 was relatively lower in HUCMSCs grown in media supplemented with both S1191 and TFAE than that of in TFAE group only. No significant differences were observed between HUCMSCs treated with S1191 only and the control groups. These data indicate that ER is involved in TFAE-mediated osteogenic differentiation of HUCMSCs (Fig. 6C).

To further understand ER involvement in TFAE-mediated osteogenic differentiation of HUCMSCs, we performed transcript-level analyses by RT-PCR. Transcripts specific for osteogenic differentiation (Col1a1, OPN, Runx2 and Osx) were analyzed in cells in the control (0 μg/mL TFAE), the TFAE (5 μg/mL TFAE), the TFAE+S1191 (5 μg/mL TFAE) and the S1191 groups (0 μg/mL TFAE). The results indicated that osteogenic markers (Col1a1, OPN, Runx2 and Osx) were significantly downregulated in the TFAE+S1191 group in comparison to the control group. Furthermore, S1191 clearly inhibited the expressions of Col1a1, OPN, Runx2 and Osx (Fig. 7A, B).

Next, we examined the expressions of Col1a1 and OPN by Western blotting. The cells incubated with TFAE after 14 days, the expression levels of Col1a1 and OPN were significantly

increased in the TFAE group compared with the control group (*P* < .01). Compared with the TFAE group, the expressions of Col1a and OPN in the TFAE+S1191 group decreased significantly (*P* < .01). However, there was no difference between the control group and the S1191 group. These results demonstrate that ER is involved in TFAE-mediated osteogenic differentiation of HUCMSCs (Fig. 8A, B).

3.6. Effect of ER inhibitor on ER expression in TFAE-mediated osteogenic differentiation of HUCMSCs

Since TFAE has been shown to promote the osteogenic differentiation of HUCMSCs we further examined the effect of the ER inhibitor (S1191) on TFAE-mediated osteogenic differentiation of HUCMSCs. We observed the effects of TFAE on the mRNA expression of ERα and ERβ in TFAE-mediated odonto/osteogenic differentiation of HUCMSCs. The results showed that there was no significant effect of TFAE on ERα and ERβ mRNA expression levels after the cells treated with 1 or 5 μg/mL TFAE, which suggested that TFAE does not change the mRNA expressions of ERα and ERβ, and that the ER protein is involved in TFAE-mediated osteogenic differentiation of HUCMSCs (Fig. 8C, D).

4. Discussion

In recent years, human umbilical cords have become an attractive source of mesenchymal stem cells (MSCs) for tissue regeneration because of no ethical considerations. MSCs are considered a promising therapeutic tool for diseases including heart failure, kidney injury, and myasthenia gravis.^[12,13] We previously reported that TFAE enhances the osteogenic capacity of mesenchymal stem cells.^[5] The potential beneficial biological effects of dietary flavonoids should obviously be a key concept in studies of these compounds.^[14] However, the effects of TFAE on the osteogenic differentiation of HUCMSCs is unclear. Although effects of flavonoids have been extensively studied in vitro and in vivo, this study is the first to demonstrate that TFAE promotes the differentiation of HUCMSCs into bone cells.

In the present study, high TFAE concentrations (>10 μg/mL) significantly inhibited proliferation of HUCMSCs, while low concentrations of TFAE regulated cell proliferation in a dose-dependent manner. To elucidate the effects of TFAE on the osteogenic differentiation of HUCMSCs, we used TFAE at a concentration <5 μg/mL to stimulate differentiation of HUCMSCs in vitro. Under such conditions, HUCMSCs can retain a stable capacity for growth, guaranteeing sustainable differentiation and enhancing ALP activity as well as the odonto/osteogenic potential of the HUCMSCs without affecting their proliferation. As osteoblastic-specific markers, Runx2 and Osx are early-stage markers of osteoblastic differentiation,^[15-17] the expressions of Runx2 and Osx have been found to be increased in TFAE-treated HUCMSCs, indicating that TFAE concentration under 5 μg/mL promote osteoblastic differentiation.

Runx2 overexpression can induce the differentiation of MSCs into osteoblast lineages in vitro,^[18,19] while Osx is highly expressed in functional odonto/osteoblasts.^[20] Thus, upregulation of Runx2 and Osx in HUCMSCs after TFAE treatment (>5 μg/mL) suggests that TFAE induces the biological differentiation changes in HUCMSCs. Our results are consistent with previous reports.^[5] Col1a1 and OPN are considered to be end-stage markers of bone formation, closely related to bone

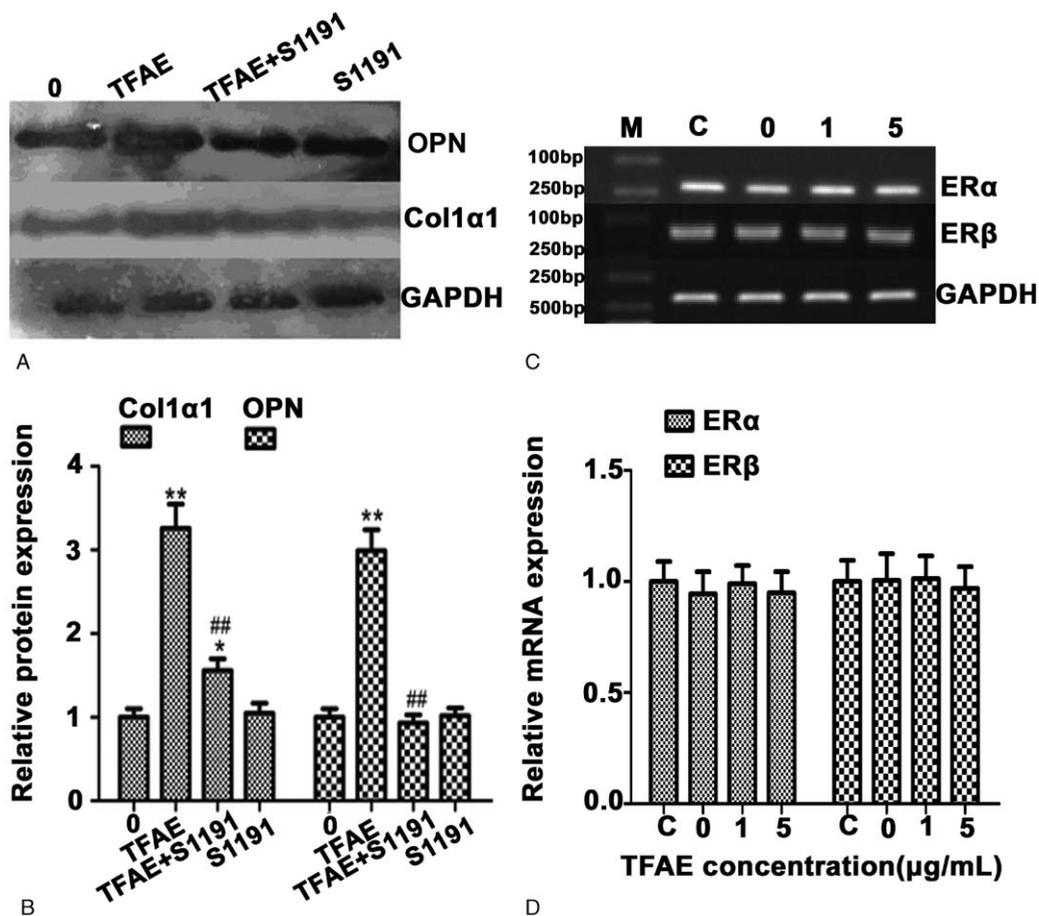


Figure 8. Expression level of osteogenesis-specific protein Col1a1 and OPN of each group was demonstrated in hUCMSCs cultured on concentrations of TFAE (5 μg/mL) and S1191 after induction by western blot. A. the expressions of Col1a1 and OPN. B. Relative expression Col1a1 and OPN. C. gene expression of ER was demonstrated by RT-PCR in different concentrations of TFAE-treated hUCMSCs. the expressions of ERα and ER β mRNA (electrophoretogram). D. Relative expression of ERα and ER β mRNA. Values were described as mean ± SD, **P* < .05, ***P* < .01 compared with control group, #*P* < .05, ##*P* < .01 compared with 0 μg/mL group (n=3). HUCMSCs = human umbilical cord mesenchymal stem cells, RT-PCR = reverse transcription polymerase chain reaction.

maturation.^[21] The upregulation of Col1a1 and OPN further suggested that TFAE promotes the differentiation of HUCMSCs into bone cells. Western blotting for Col1a1 and OPN confirmed these observations.

Understanding the effects of TFAE on the differentiation of HUCMSCs provides us with the opportunity to explore the molecular mechanisms of TFAE-mediated osteoblastic differentiation. Okazaki et al^[22] reported that estrogen can promote the differentiation of rat BMSCs through ER. Studies have found that some plant sources of phytoestrogens have the same effect as estrogen did. Some flavonol compounds have a phytoestrogens effect^[23,24] Song^[9] et al found that epimedium promotes MC3T3 E1 cell proliferation and can be mediated by ER via activation of ERK and JNK signaling pathways, promoting osteogenesis differentiation.

In this study, we found that a specific ER inhibitor (S1191) can reverse the osteogenic differentiation of HUCMSCs caused by TFAE treatment. The results of Alizarin red S staining and ALP activity assays suggest that ER is activated in TFAE-treated HUCMSCs. Furthermore, inhibition of ER by S1191 treatment dramatically suppressed the mRNA expressions of the odonto/osteogenic markers Runx2, Osx, Col1a1, and OPN. To this regard, we next analyzed protein levels of the osteogenic Col1a1

and OPN after ER suppression by S1191. The results were consistent with the results of the mRNA analyses. Some reports have shown that S1191 competitively inhibits binding of oestradiol to the ER. S1191 blocks nuclear localization of ER by impairing receptor dimerization,^[25] and binds to ERα leading to decreased ERα stability and increases turnover of the ERα protein through enhanced proteasomal degradation.^[26,27] In addition, S1191 down-regulates androgen receptor expression and diminishes androgenic responses in LNCaP human prostate cancer cells.^[28] It is interesting to note the effects of S1191 on the expression of osteogenic markers in this study. These data suggest that ER is involved in TFAE-mediated osteogenic differentiation of HUCMSCs. However, we found that mRNA expression level of ER was not affected in TFAE-treated HUCMSCs. Some studies indicated that flavonoids may reduce estrogen receptor binding capacity by binding to nuclear type II estrogen binding sites.^[29,30] Studies have reported that the majority of ER α and β are found in the cytoplasm and nucleus. The cytoplasmic estrogen-ER complex translocates into the nucleus and binds to estrogen response elements located in the target gene promoters, stimulating gene transcription.^[31,32] Our results indicate that the binding of ER to TFAE influences gene transcription in TFAE-mediated osteogenic differentiation of HUCMSCs but that TFAE

does not affect ER expression at the mRNA level. Further investigation of interactions between TFAE and ER is warranted. Although the underlying mechanism remains unclear, this information may provide new insights. Based on the present findings, it can be inferred that ER plays a pivotal role during committed differentiation of TFAE-treated HUCMSCs.

5. Conclusions

TFAE promotes the odonto/osteogenic differentiation of human UCMSCs via activation of ER. The findings provides a strong evidence that the therapeutic application of TFAE results in the HUCMSCs's osteogenic differentiation. Further studies are warranted to investigate other potential mechanisms associated with TFAE-mediated osteogenic differentiation of HUCMSCs.

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