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Original Article

In vitro induction effect of $1,25(OH)_2D_3$ on differentiation of hair follicle stem cell into keratinocyte

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ARTICLE INFO

Article history:
Received 9 July 2015
Accepted 2 August 2016
Available online 23 March 2017

Keywords:
Hair follicle
Stem cell
Bulge
Keratinocyte
Differentiation
$1,25(OH)_2D_3$

ABSTRACT

Background: Stem cells are characterized by self-renewal and differentiation capabilities. The bulge hair follicle stem cells (HFSCs) are able to convert to epithelial components. The active metabolite of vitamin D, $1,25(OH)_2D_3$, plays important roles in this differentiation process. In the present study has found that $1,25(OH)_2D_3$ induces the HFSCs differentiation into keratinocyte.

Methods: HFSCs are isolated from rat whiskers and cultivated in DMEM medium. To isolate bulge stem cell population, flow cytometry and immunocytochemistry using K15, CD34 and nestin biomarkers were performed. In order to accelerate the HFSCs differentiation into keratinocyte, HFSCs were treated with 10^{-12} M, $1,25(OH)_2D_3$ every 48 h for a week.

Results: Immunocytochemistry results showed that bulge stem cells are nestin and CD34 positive but K15 negative before differentiation. Subsequently flow cytometry results, showed that the expression of nestin, CD34 and K15 were 70.96%, 93.03% and 6.88% respectively. After differentiation, the immunocytochemical and flow cytometry results indicated that differentiated cells have positive reaction to K15 with 68.94% expression level.

Conclusion: It was concluded that 10^{-12} M, $1,25(OH)_2D_3$ could induce the HFSCs differentiation into keratinocytes.

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Peer review under responsibility of Chang Gung University.
http://dx.doi.org/10.1016/j.bj.2016.08.007

At a glance commentary

Scientific background on the subject

Stem cells, particularly adult stem cells, have shown promising results in both translational and clinical applications. Previous studies show that the bulge region of hair follicle contains pluripotent stem cells which have the ability to differentiate into epithelial components. Accordingly we believe that HFSCs may be used for therapeutic purposes in regenerative medicine. Since $1.25(OH)_2D_3$ regulates keratinocytes differentiation, in the present study we sought to investigate the effect of $1.25(OH)_2D_3$on HFSCs differentiation into keratinocytes.

What this study adds to the field

The novelty of our experiment is proving the efficient induction of $1,25(OH)_2D_3$ on HFSCs differentiation. In this study, for the first time we showed that, $1,25(OH)_2D_3$ (10^{-12} M) dissolved in ethanol could direct and accelerate the differentiation of rat HFSCs into keratinocytes cells.

Skin injuries resulting from diabetic ulcer or burns are major healthcare problem [1,2]. Hence, utilization of adult [3] or embryonic [4] stem cells in skin grafting and tissue engineering is very important in regenerative medicine. It is important to direct stem cell differentiation into the keratinocyte lineage in skin grafting and tissue engineering. To avoid immunological rejection, it is essential to utilize autologous skin grafts and to prevent additional surgery according to unaffected skin requirement, it would be preferable to utilize *ex vivo* cultured autologous keratinocytes [5].

Recently, adult stem cells are highly concerned in clinical applications because some of the problems associated with embryonic and fetal stem cells, such as immunological incompatibility are not found in adult stem cells [6,7]. Hoffman demonstrated the pluripotency of hair follicle stem cells use in regenerative medicine, because [1], they are readily available from essentially anyone [2], they are easily cultured and expanded [3], are highly pluripotent [4], they have been demonstrated to be able to support the regrowth of nerves [5], they do not carry the ethical issues that embryonic stem cells and fetal stem cells do [8].

Paus et al. declared that hair follicle adult stem cells are reserved in bulge area of the hair follicle [9]. Bulge region is located between the insertion of the arrector pili muscle and duct of the sebaceous gland [10]. Li et al. showed that the population of cells in the bulge region expresses Nestin (intermediate filament) as a marker of neural stem cells [6–8,11,12]. Trempus et al. first described the expression of CD34 (the surface protein) as a marker of mouse bulge cells of hair follicle [13]. Lyle et al. for the first time reported that K15 (intermediate filament) as a keratinocytes marker is expressed in human bulge cells of hair follicle [14]. Liu et al. also showed that K15 has been expressed in adult mouse bulge cells [15].

Vitamin D is responsible for maintenance of plasma phosphate [16], regulating bone formation and calcium homeostasis [17]. Vitamin D, like other steroid hormones, regulates gene expression in different cell types [18]. Peehl et al. reported that active metabolite of vitamin D, $1,25(OH)_2D_3$ regulates cell proliferation and differentiation of several cell types including keratinocytes [19]. Hosomi et al. found that $1,25(OH)_2D_3$ accelerates terminal differentiation of cultured mouse epidermal cells. Similarly Smith et al. demonstrated this marker is able to enhance human keratinocytes differentiation. Thus, $1,25(OH)_2D_3$ is assumed to have an important role in keratinocytes differentiation [20].

The present study investigates the effect of $1,25(OH)_2D_3$ on HFSCs differentiation into keratinocytes cells for the first time.

Materials and methods

Animals and housing conditions

All animal experiments were carried out according to the guidelines of the Iranian Council for Use and Care of Animals and approved by the Animal Research Ethical Committee of Iran University of Medical Sciences. Male Wistar rats (n = 40, 150–200 g of body weight) were purchased from the Animal Center of Iran University of Medical Science. All rats were permitted free access to food and water at all times and were maintained under light–dark cycles.

Hair follicle isolation and cultivation

The rats were deeply anesthetized with chloroform, and the whisker follicles were dissected as described by Amoh et al. and Gilanchi et al. with modification [6,7,21]. After rinsing the animal's head, with betadine and 70% ethanol for 3 min, the upper lip was completely shaved and trimmed into small pieces. The samples were incubated at 37 °C and 5% CO2 in Collagenase I/Dispase II solution (Sigma–Aldrich) in incubator for 7 min. Then, most of the connective tissue and dermis around the follicles were removed, and whisker follicles were extracted with fine forceps. The follicles were transferred into another sterile 35-mm dish. The bulge region located in the middle third of the hair follicle, were dissected from the upper follicle by making two transversal cuts at the site of the enlargement spots of outer root sheath (ORS) with a fine needle, and the collagen capsule was incised longitudinally. 20–30 isolated bulges submerged in amphotericin B for 3 min. Afterward they were incubated in Trypsin–EDTA 0.25% for 10 min and cut into small pieces, and plated into 25-cm^2 tissue culture flasks (TCFs) pre-coated with collagen type I (Sigma–Aldrich). Before the cultivation, the flasks were pre-incubated with medium and after 2 h the medium was eliminated. Bulges submerged in a 3:1 supplemented mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml

streptomycin), 2.5 mg/ml hydrocortisone, and 0.27 U/ml insulin.

The bulges were allowed to attach to the collagen in 5–7 days. Cells were incubated at 37 °C and 5% CO_2. Sterile conditions were duly observed during all procedures. When primary culture reached 50–80% confluency, cells were subcultured to other collagen coated flask and incubated at 37 °C with same medium.

Cell differentiation

HFSCs were grown in culture as previously described [9,11]. After 10 days of culture, cultured cells were incubated with fresh medium containing $1,25(OH)_2D_3$ (10^{-12} M) dissolved in ethanol. Cells were fed and dosed every 48 h for 1 week. This cell group was compared with control group effectuated only with medium and vehicle effectuated with medium and ethanol without inducer.

MTT assay

To assess the viability of HFSC, 3-(4,5-dimethylthiazol-yl) 2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich) assay was performed. Cells in six groups (containing $1,25(OH)_2D_3$ (10^{-6} M, 10^{-9} M and 10^{-12} M) dissolved in ethanol and DMSO) were seed in 24-well plates. 100 μL MTT with final concentration of 5 mg/mL was added to each well. MTT solution was aspirated after 4 h of incubation in the dark at 37 °C. By addition of 1 mL dimethyl sulfoxide (DMSO), the purple formazan reaction products were dissolved. After shaking the plates, the solution was transferred to a 96-well plate for spectrophotometric analysis. The optical density of the formazan solution was read at 570 nm, on an ELISA plate reader (Dynex MRX). In this assay the greater number of living cells leads to the formation of formazan crystals and makes the optical density increase.

Immunocytochemical staining

The attached cells were trypsinized with Trypsin–EDTA 0.25% (Sigma–Aldrich) for 2 min and seeded on collagen-coated plate overnight. After removing culture medium, cells were fixed in 4% paraformaldehyde for 20 min. Then the fixed cells were washed 3 times with PBS for 5 min. Afterward cells were permeabilized using 0.1% Triton X-100 (Fluka, USA) for 20 min. Then cells were blocked in 0.3% normal goat serum (Sigma–Aldrich, USA) for 1 h at room temperature. Subsequently cells were incubated at 4 °C overnight with the following primary antibodies: mouse anti-Nestin monoclonal antibody (1:200, Millipore, USA) mouse anti-CD34 (1:75, Sigma–Aldrich, USA) mouse anti-K15 (1:75, Sigma–Aldrich, USA). The following day, the cells were washed with PBS for 3 × 5 min to remove unbound primary antibodies. Eventually, cells were incubated for at least 1 h at room temperature in the dark with the following secondary antibody: anti-mouse fluorescein isothiocyanate (FITC) conjugate IgG (1:1400; Sigma–Aldrich, USA). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:1000; Invitrogen) for 1 min. After washing, labeled cells were identified using fluorescent microscopy (Olympus IX71).

Flow cytometry

To determine the percentage of cell expressing special markers, flow cytometry technique was performed. Cells were detached from the culture flasks by Trypsin–EDTA 0.25%. Then they were incubated in the same primary antibodies at room temperature for 1 h. After washing with PBS, cells were incubated with secondary antibody FITC (1:1,400, Sigma–Aldrich, USA) for 1 h at room temperature in the dark. Labeled cells were analyzed using flow cytometry technique. Incubated cells with only secondary antibody have been considered as a negative control.

Statistical analysis

All the experiments were performed in triplicate, and the data analyses values were expressed as mean ± SD. One-way ANOVA followed by Tukey's post hoc test were conducted with SPSS version 16.0 software. $p < 0.05$ was considered as statistically significant.

Results

Hair follicle isolation and cell culture

Bulge hair follicle stem cells from dissected rat were successfully isolated and cultured with a modified method. Fig. 1A and B shows an isolated follicle. After 3–4 days of the bulges explantation, cells outgrowth initiated from the isolated bulges [Fig. 2A]. After 8–10 days of proliferation, cells accumulated around the bulge and formed dome shaped cells [Fig. 2B]. Gradually, the cells began to migrate out of the dome-shaped colonies. These results suggest that bulge cells have high proliferation *in vitro* [Fig. 2C]. The bulge stem cells of rat vibrissa follicles were isolated and cultivated in DMEM/F12. To confirm that these cells were primitive stem cells, the immunocytochemistry staining and flow cytometry were performed. The results indicate that bulge cells were Nestin and CD34 positive and K15 negative [Fig. 3].

MTT assay

Cell viability was determined by using MTT chromometry assay to select the most efficient volume of inducer dissolved in suitable solvent. ELISA results (at 570 nm) among six groups of cells respectively treated with $1,25(OH)_2D_3$ (10^{-6} M, 10^{-9} M and 10^{-12} M) dissolved in ethanol and DMSO, showed that, group containing $1,25(OH)_2D_3$ (10^{-12} M) dissolved in ethanol had the highest optical density and the highest cell viability of all other groups (one-way ANOVA and Tukey's test; $p < 0.05$) [Fig. 4].

Quantitative analysis of differentiated cells

To quantify the number of the cells differentiating from HFSCs, immunocytochemical staining using K15 antibody (marker of keratinocyte cells) was performed. On the day 10 after cultivation, treat group cells were fed with $1,25(OH)_2D_3$ (10^{-12} M) dissolved in ethanol every 48 h for 1 week. ICC results on 17 days after cultivation showed that HFSCs differentiated into

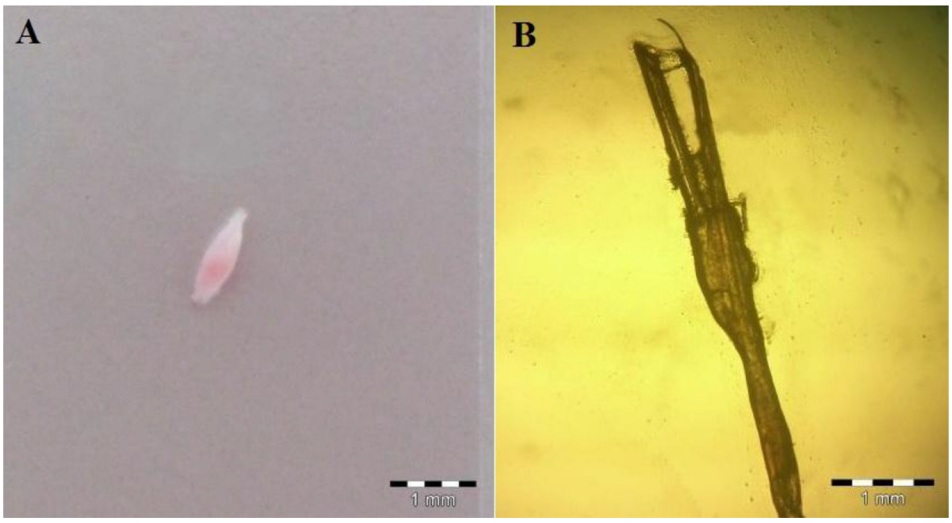

Fig. 1 **Dissection of hair follicle bulge from adult rat whisker follicle**. (A) Dissected hair follicle; (B) hair follicle bulge rolled in capsule. Scale bars = 1000 μm (A, B).

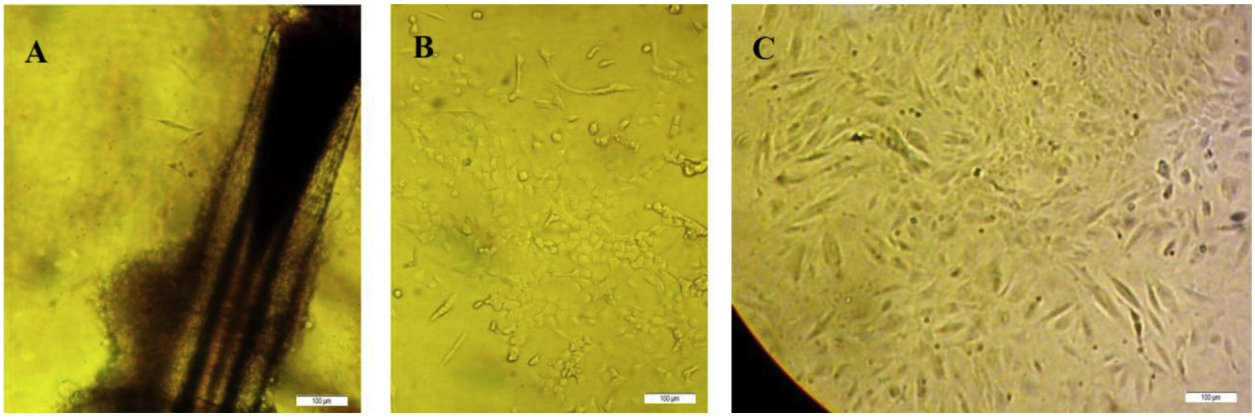

Fig. 2 **The primary culture of bulge cells from rat hair follicles**. (A) The growth of bulge cells after 3–4 days; (B) Stem cells make a dome-shaped and gradually start to migrate after 8–10 days; (C) Cells start to migrate from colonies. Scale bars = 100 μm (A, B, C).

keratinocytes. K15 was expressed in treat group but in control group (effectuated only with medium) and vehicle (effectuated with medium and ethanol without inducer) K15 expression was not found (3 times repeated). The cell reaction was also examined on day 14 after cultivation, since the cell reaction was insignificant in compare with control group; it was not considered (3 times repeated) [Fig. 5]. Flow cytometry technique confirmed these results and showed 68.94%, 11.98% and 10.58% expression of K15 in treat group, control group and vehicle respectively on day 17th (5 times repeated) [Figs. 6 and 7].

Discussion

Stem cells are highly significant in cell therapy, these cells are able to proliferate and differentiate into specific cell lines. One of the most important strategies in tissue regeneration is to select appropriate stem cell. It is possible to extract adult stem cells from different organs including hematopoietic systems, brain, skin, eyes and gastrointestinal tract by using different techniques. Adult stem cells differentiation potential has provided an opportunity for scientists to utilize these cells in regenerative medicine [22]. Hair follicle stem cells (HFSCs) are major source of pluripotent adult stem cells [8]. Hair follicle bulge has been recognized by Stöhr for over 100 years [23]. Cotsarelis et al. observed label-retaining cells in the bulge region of hair follicles. In recent years several studies have been performed on human hair follicle cells primary culture [24]. Weterings et al. were first grown human hair follicle keratinocytes by explanting pluck scalp hair follicles on bovine eye lens capsules [25]. Likewise, Wells explanted plucked human hair follicles directly on tissue culture plastic [26]. Limat and Noser initiated primary culture of disaggregated hair follicle cells in order to increase the proliferation of primary hair keratinocytes [27]. Morris and Potten reported rat hair follicle growth in primary culture using vibrissa follicles [28]. In this study, rat HFSCs were isolated and cultivated. According to the results of the present and

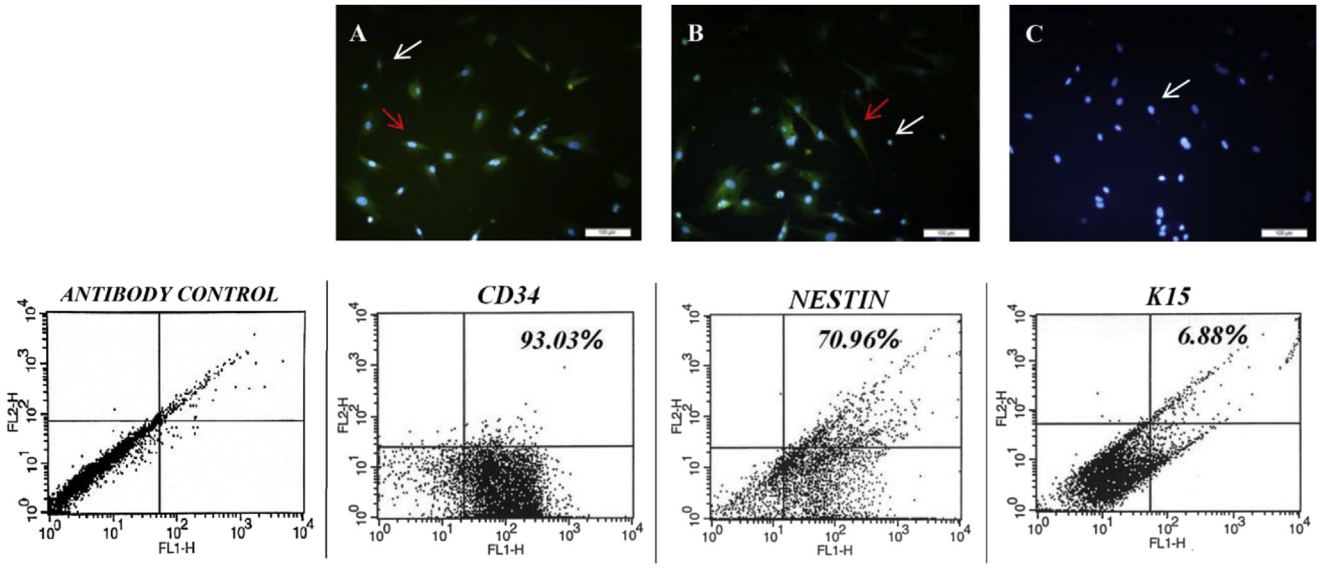

Fig. 3 **Analyzing bulge cells before differentiation**. Before differentiation to determine primarily stem cells nature, bulge cells were analyzed with Nestin, CD34 and K15 antibodies. (A) Hair follicle stem cells has positive reaction in immunocytochemistry staining with CD34 antibody (red arrows) and DAPI nuclear stain (white arrows); (B) Hair follicle stem cells shows positive reaction in immunocytochemistry staining with Nestin antibody (red arrows) and DAPI nuclear stain (white arrows); (C) No positive reaction was observed in immunocytochemistry staining with K15 antibody only nuclei counterstained with DAPI were observed (white arrows). Flow cytometry results show the percentage of CD34 positive, Nestin positive and K15 negative cells. In flow cytometry incubated cells with only secondary antibody have been considered as a negative control. Scale bars = 100 μm (A, B, C).

previous studies of Nobakht et al. and Amoh et al. bulge cells express the stem cell marker CD34 but not the keratinocytes marker (K15) [11,21,29–36]. Li et al. showed that HFSCs can also be Nestin positive [11,12,21,29–32,37]. In recent years, most of researches have focused on the factors that affect the proliferation and differentiation of stem cells. In our previous studies the effects of silibinin, nt-3 and seladin-1 (DHCR24) on hair follicle stem cells differentiation into neural-like cells were evaluated [11,21,31]. Since keratinocytes play an important role in skin wound repair, directing stem cell differentiation into the keratinocyte is very considerable [38]. Therefor directing HFSCs into keratinocytes was the aim of present study. Growth factors, cytokines or chemical factors can be used to induce keratinocytes differentiation *in vitro*. [5] Active metabolite of vitamin D, $1,25(OH)_2D_3$ is able to regulates gene expression in different cell types including tumor cell lines [39], hematopoietic cells [40], neural stem cell, oligodendrocyte [41], NHK cells [42], osteogenic, adipocytic [43], human fibroblast [39], melanoma cells, lymphocytes and keratinocytes [19]. In the field of dermatology, $1,25(OH)_2D_3$ plays an important role in keratinocytes differentiation [44]. Matsumoto et al. used flow cytometry and immunofluorescent staining of involucrin and demonstrated that $1,25(OH)_2D_3$ (10^{-6} M) had the highest influence on human skin keratinocyte differentiation [16]. In the present experiment using $1,25(OH)_2D_3$ (10^{-12} M), differentiation of bulge HFSCs was directed into keratinocytes. After inducing bulge cells with $1,25(OH)_2D_3$ (10^{-12} M), on day 17 after cultivation, the results of flow cytometry and immunocytochemistry staining indicate that, K15 as a marker of keratinocytes was expressed in treat group. MTT assay as a quantitative assay for cell viability are the main method *in vitro* to test the biocompatibility and cytotoxicity of biomaterials and the different dose of growth

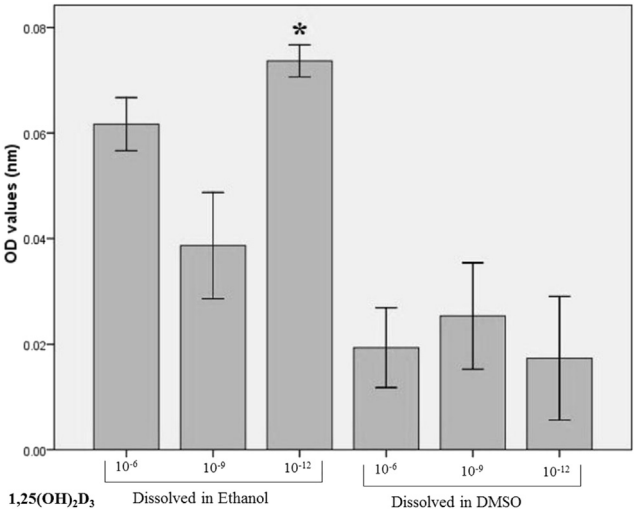

Fig. 4 **Cell viability assay results**. Cell viability in the six co-culture groups was assayed. The optical density of the formazan solution was read at 570 nm, on an ELISA plate reader. Asterisk demonstrate that cell viability of the group containing $1,25(OH)_2D_3$ (10^{-12} M) dissolved in ethanol was significantly higher than the other groups (one-way ANOVA, Tukey's test; $p < 0.05$). Error bars represent means ± SD (n = 3).

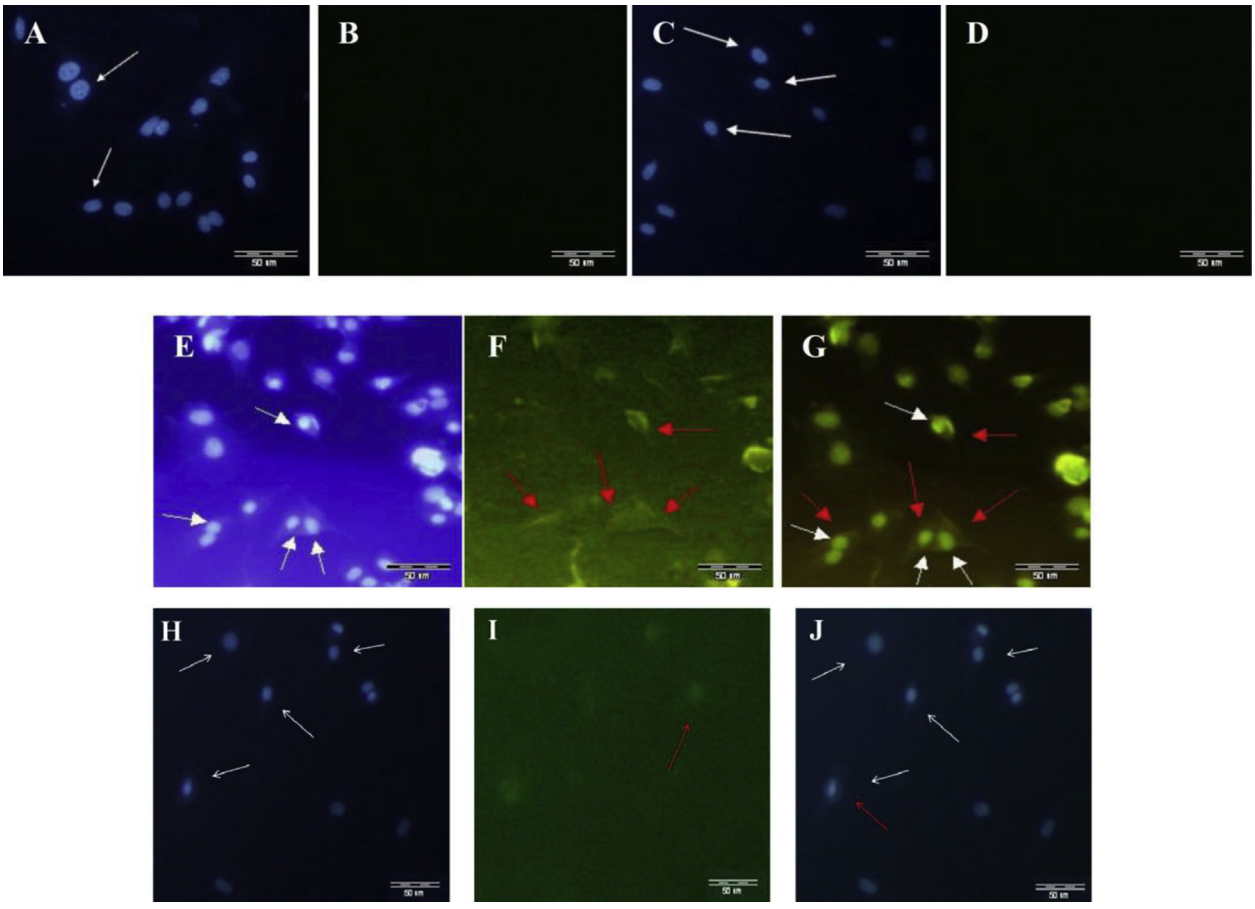

Fig. 5 **Immunocytochemistry results after induction of 1,25(OH)$_2$D$_3$.** After differentiation, to evaluate the roles of 1,25(OH)$_2$D$_3$ on hair follicle bulge stem cell differentiation into keratinocytes, immunocytochemical staining using K15 antibody was performed. (A, B) On day 17 no differentiated cell was detected in the control group, only nuclei counterstained with DAPI were observed (white arrows); (C, D) On day 17 no differentiated cell was detected in the vehicle group, only nuclei counterstained with DAPI were observed (white arrows); (E, F, G) On day 17 differentiated cells (red arrows) and nuclei counterstained with DAPI (white arrows) were observed in treat group; (H, I, J) On day 14 the cells reaction were insignificant in compare with control group, only nuclei counterstained with DAPI were observed (white arrows). Scale bars = 50 μm (A, B, C, D, E, F, G, H, I, J).

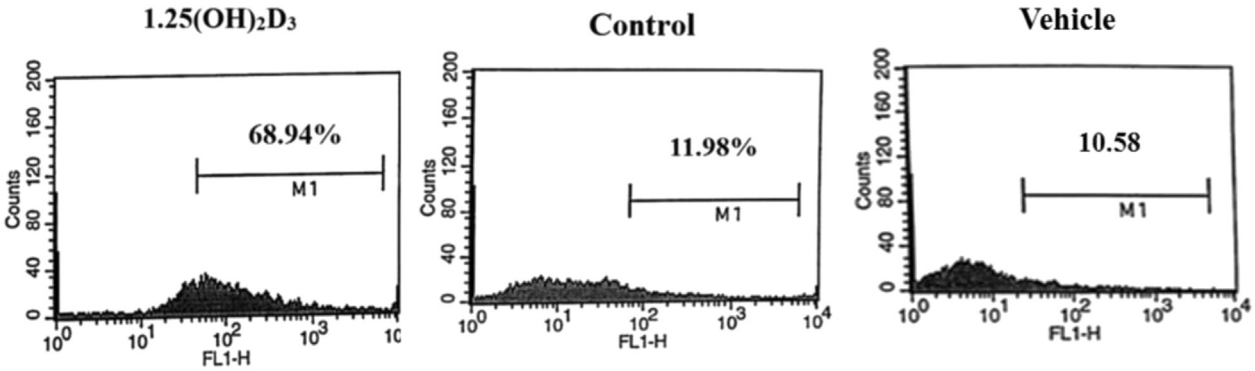

Fig. 6 **Flow cytometry results after induction of 1,25(OH)$_2$D$_3$.** 17 days after co-culture to evaluate the role of 1,25(OH)$_2$D$_3$ on hair follicle bulge stem cell differentiation into keratinocytes flow cytometry technique using K15 antibody was performed. Flow cytometry results show percentage of differentiated cells. Results show 68.94%, 11.98% and 10.58% expression of K15 in treat group, control group (effectuated only with medium) and vehicle (effectuated with medium and ethanol without inducer) respectively.

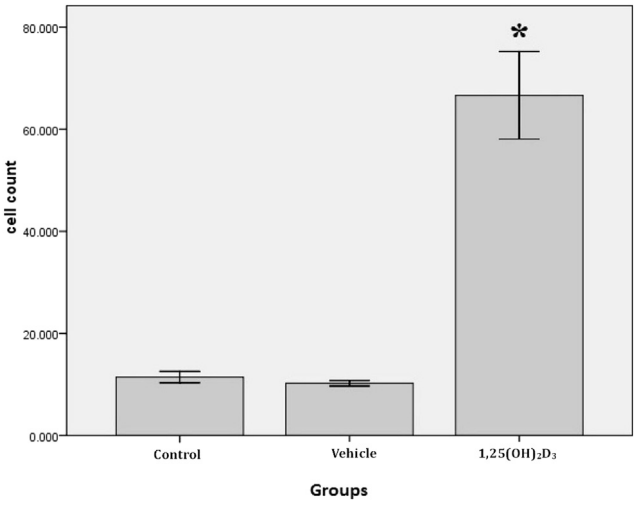

Fig. 7 **Flow cytometry results after induction of $1,25(OH)_2D_3$.** 17 days after co-culture, the average percentages of differentiated cell were evaluated by flow cytometry technique using K15 antibody. Asterisk indicates that the number of the keratinocyte differentiating from the HFSCs in treat group was more than control group (effectuated only with medium) and vehicle (effectuated with medium and ethanol without inducer) (one-way ANOVA, Tukey's test; $p < 0.05$). Error bars represent means ± SD (n = 6).

factors [45,46]. In this study the results of the MTT assay as shown in Fig. 4, demonstrated that all examined doses (10^{-6} M, 10^{-9} M and 10^{-12} M) of $1,25(OH)_2D_3$ dissolved in DMSO had cytotoxicity for HFSC and also 10^{-6} M and 10^{-9} M of $1,25(OH)_2D_3$ dissolved in ethanol were not suitable and efficient but $1,25(OH)_2D_3$ (10^{-12} M) dissolved in ethanol had no cytotoxicity and was suitable for HFSC culture. According to these results $1,25(OH)_2D_3$ can be a factor for inducing differentiation of bulge stem cells into keratinocytes.

Conclusion

The results indicate that $1,25(OH)_2D_3$ (10^{-12} M) dissolved in ethanol could direct and accelerate the differentiation of rat HFSCs into keratinocytes cells.

Conflicts of interest

The authors indicate no potential conflicts of interest.

Acknowledgments

This study was financially supported by a grant no. 23016 from Iran University of Medical Sciences. Also, we would like to thank the Dept. of Anatomy, Iran University of Medical Science, Tehran, Iran.

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