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Original Article

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Vanillin increases stem cell signal and cell adhesion in keratinocytes

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ABSTRACT

Introduction: Keratinocyte stem cells, residing in the basal layer of the epidermis, are thought to function in the process of the epidermis renewal and maintain the barrier property of the skin. **Objective:** This study has explored for the first time that vanillin, a natural compound from vanilla cured beans, has the ability to augment the stem cell property and signaling in the human HaCaT keratinocytes. **Methods:** The cell viability effect of vanillin on the keratinocytes was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The Western blot analysis was performed to determine the level of stem cell-mediated proteins, mesenchymal, and epithelial markers. **Results:** Results showed that treatment of the HaCaT cells with vanillin at non-toxic concentrations was able to significantly up-regulate the stemness mediators Oct-4, p-Oct-4 and Nanog. Besides, we investigated the mesenchymal and epithelial makers and found that vanillin could increase the expression of epithelial adhesive protein E-cadherin but has no significant effect on mesenchymal mediator slug. **Conclusions:** As the stem cells, as well as their stemness properties, hold the central functions of skin renewal and repair, information gain from this study may benefit the development of vanillin to be used for skin therapy.

INTRODUCTION

Vanillin (Figure 1a) is the predominant aroma compound of the vanilla cured beans [1]. It can be made synthetically from eugenol or guaiacol and lignin. In analytical chemistry, a flavoring agent process, and in perfumery have used vanillin as a reagent [2]. Essentially, the safety profile of vanillin has been well accepted as the worldwide authorities approved vanillin as a food additive in a safe (generally recognized as safe) status by the US FDA [3]. Vanillin has been established as an anticlastogen, from its properties to suppress gene mutations in both bacterial and mammalian cells [4-7]. Vanillin has been shown to protect against H_2O_2-induced DNA damage in human peripheral blood cells [8]. In addition, vanillin has the ability to protect human keratinocyte stem cells against ultraviolet B irradiation [9].

Human skin is a permeability barrier against harmful pathogen and ultraviolet radiation. Human skin also provides functions in thermoregulation, sensation, and physical

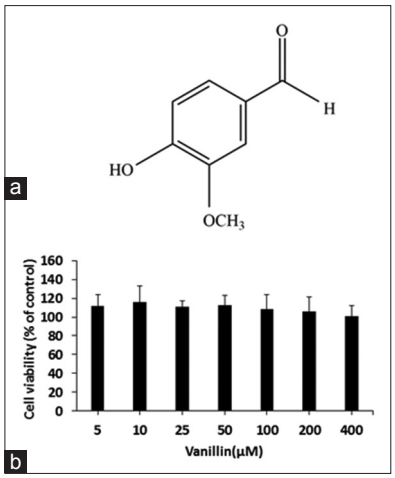

Figure 1: (a) Vanillin structure, (b) the effect of vanillin on viability of HaCaT cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability of HaCaTs were determined after treated with vanillin (0-400 μM) for 24 h. The data represent the means of three independent experiments ± standard deviation. *P < 0.05 versus non-treated control

appearance [10]. Epidermis is the outermost layer of the skin. As the epidermis is the first barrier to encounter damage, it constantly has tissue renewal; the ongoing keratinocyte renewal process is essentially required for sustaining the balance of skin. In the ageing situation, activities and amounts of epidermal stem cells were found to be dramatically reduced [11]. For epidermis layer of the skin, the main function is to provide the strong protective barrier against the loss of moisture and the intrusion of foreign organisms and substances [12]. Thus, maintaining cell stemness might be the good way to keep the nearest level of the healthy skin. It has been reported that Oct-4 and Nanog are the transcription factors which control the stem cell signatures in human [13,14]. Oct-4 orchestrates with Nanog and Sox-2 to activate or hamper genes controlled stem cell identity [13,14]. Phosphorylated Oct-4 (p-Oct-4) is Oct-4 which has been phosphorylated and it has been found that phosphorylated Oct-4 is involved in the process of stem-like cells proliferation [15]. Cell adhesion molecule has also been shown to support the stem cell property of keratinocytes [16]. Furthermore, the loss of E-cadherin, a transmembrane protein functioning in adhering the adjacent cells together has been shown to impair the skin junction formation, deteriorated intercellular adhesion and raises apoptosis rate [16].

Despite showing various potential benefits on medicinal use, the effect of vanillin on human keratinocyte stem cells has never been reported. Herein, we revealed that vanillin could increase the stemness markers Oct-4, p-Oct-4 and Nanog, and cell adhesion (E-cadherin) molecule in keratinocytes. Together with it highly safe profile, vanillin could make a good candidate for development to be used for benefiting the healthy epidermis via the enhancement of keratinocyte stemness.

MATERIALS AND METHODS

Vanillin Preparation

Vanillin compound (99.9%, Lot: P500245) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Vanillin solution was prepared according to aseptic technique protocol by dissolving the compound in sterile phosphate buffered saline (PBS).

Cell Culture

Human keratinocyte HaCaT cells were obtained from the Cell Lines Service (CLS, Heidelberg, Germany). The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were maintained in a 37°C humidified incubator with a 5% CO_2 atmosphere. Cells were routinely passaged at preconfluent density using a 0.25% trypsin solution with 0.53 mM ethylenediaminetetraacetic acid (EDTA). DMEM medium, FBS, L-glutamine, PBS, trypsin, and EDTA were purchased from GIBCO (Grand Island, NY, USA).

Cell Viability Assay

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures cellular capacity in reducing MTT (yellow) to purple formazan crystal by mitochondria dehydrogenase enzyme. After specific treatments with vanillin, the cells were incubated with 100 μl of MTT (500 μg/ml) (Invitrogen, Carlsbad, CA, USA) for 4 h at 37°C. The intensity of MTT product was measured at 570 nm using a microplate reader (Anthos, Durham, NC, USA). Cell viability was calculated by the following equation to the relative cell viability of the different treated doses:

$$\text{Cell viability } (\%) = \frac{\text{A570 of treatment}}{\text{A570 of control}}$$

Western Blot Analysis

HaCaT cells were seeded at a density of 5 × 10^4 cells/well onto 6-well plate for 12 h and cultured in the presence of various concentrations of vanillin (0, 50, 100, 200, 400 μM) for 48 h. After washing the cells with PBS, cells were incubated with ice-cold lysis buffer containing 20 mM TrisHCl (pH 7.5), 0.5% Triton X-100, 50 mM sodium fluoride, 150 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche Molecular Biochemicals) for 60 min on ice. Protein content was then evaluated using bicinchoninic acid protein assay kit from Pierce Biotechnology (Rockford, IL, USA). Equal amounts of proteins (100 μg) were heated at 95°C for 5 min with Laemmli loading buffer.

The proteins were then loaded on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis. After separation, proteins were subsequently transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad). Thereafter, the membranes were blocked with 5% skim milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween-20) at room temperature for 1 h. Membranes were washed 3 times in TBST for 5 min and incubated with specific primary antibodies against Oct-4, Nanog, p-Oct-4, E-cadherin, α-tubulin, and glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling, Danvers, MA, USA) at 4°C overnight. Membranes were washed in TBST for 5 min 3 times and incubated with horseradish peroxidase-coupled secondary antibodies for 2 h at room temperature. The immune complexes were detected with chemiluminescence substrate (Supersignal West Pico;

Pierce, Rockford, IL, USA) and quantified using analyst/PC densitometry software (Bio-Rad, Hercules, California, USA).

Statistical Analysis

Data were obtained format least three independent experiments and presented as means ± standard deviation. Statistical analysis was performed using one-way ANOVA with *post-hoc* test at a significance level (α) of 0.05. These analyses were performed using SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Effect of Vanillin on Viability of HaCaT Cells

To evaluate the effect of vanillin on stemness, its concentration effect on HaCaT cell viability was first examined. The non-cytotoxic concentrations of the vanillin were determined by treatment of the human keratinocyte HaCaT cells with vanillin at the concentrations of 0-400 μM and cell viability was determined after 24 h by MTT viability assay. The results showed that treatment of the cells with vanillin caused no significant change in cell viability compared with non-treated control (Figure 1b). This information may help to clarify that the following effects of vanillin on HaCaTs were not a consequence of cytotoxic effect or cell stress.

Effect of Vanillin on HaCaT Cell Proliferation

The effect of vanillin on the proliferation of HaCaT cells is shown in Figure 2. Cells were exposed to various concentrations of vanillin (0-400 μM) for 72 h, and cells were cultured in normal growth condition for 72 h. Results indicated that all tested concentrations of vanillin did not affect the proliferative activity of the cells. This information further informed that vanillin used in these experiments had no proliferative effect.

Vanillin Increases Expression of Stem Cell Markers

To prove the effect of vanillin on the increase of stemness of keratinocytes, the expression of stem cell key molecular markers was investigated. The Oct-4, Nanog, and p-Oct-4 expressions have been recognized as the indicators for stemness of cells [17,18]. The cells were treated with vanillin in dose-dependent manners and the expression level of Oct-4, Nanog and p-Oct-4 was determined by Western blot analysis. The results showed that vanillin increased the cellular level of p-Oct-4 in keratinocyte at the concentration of 200-400 μM (Figure 3a and c). Furthermore, the level of Oct-4 and Nanog was significantly increased in the 400 μM vanillin-treated cells (Figure 3a, b and d). These data indicated that vanillin increased the stemness of keratinocyte cells.

Vanillin Increases Expression of Cell Adhesion E-cadherin

To further provide additive information about vanillin in regulation of epithelial to mesenchymal transition (EMT) and cell adhesion, the cells were treated with non-toxic concentrations of vanillin as previously described, and the expression of EMT and adhesion was investigated by Western blot analysis. E-cadherin expressions have been recognized as the indicators for adhesion strength [16]. Results indicated that vanillin increased E-cadherin in a concentration dependent manner at 48 h of treatment compared with non-treated control (Figure 4). However, there was no significant change in the level of EMT marker slug. These data indicated that vanillin increased the adhesion strength of keratinocyte cells.

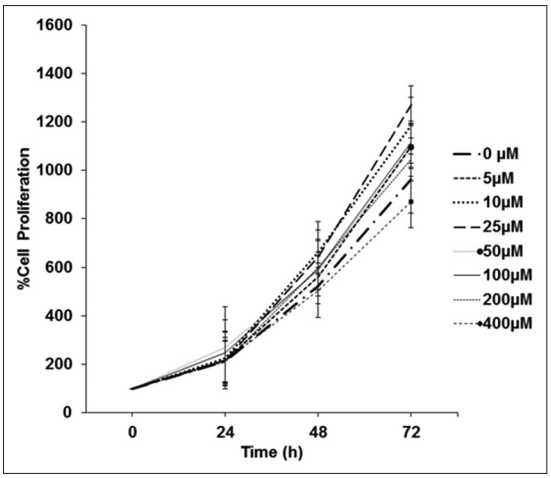

Figure 2: Effect of vanillin on HaCaT cells proliferation. Cells were treated with vanillin (0-400 μM) for 72 h, after that cells were replated. HaCaT cells proliferation at 0, 24, 48, 72 h was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The data represent the means of three independent experiments ± standard deviation. *$P < 0.05$ versus non-treated control

DISCUSSION

Epidermal stem cells residing in the basal layer [19], corporate in the epidermal proliferative unit which is a three-dimensional organized microenvironment surrounding one single stem cell. Epidermal stem cells are the key factor in maintaining epidermal homeostasis via the cell renewal process and cytokine production [11]. Such stem cells have the self-renewal ability along human lifespan. Here, we found the effect of the safety compound vanillin in augmenting the stem cell signals in human keratinocyte cells.

In stem cell research, transcription factors namely Oct-4, Nanog and p-Oct-4 have been accepted as a dominant stemness mediator and were widely used as biomarkers for human stem cells detection [20]. Oct-4 is the essential signal which governs the pluripotency of stem cells [20]. In pluripotent cells, Oct-4 is normally found numerously, while it is dramatically depressed on the process of cell differentiation [17,21]. Moreover, Oct-4 collaborates with Nanog, Sox-2 and other core transcriptional factors, to activate essential RNAs for pluripotency of the stem cells [14]. Our study found that vanillin has risen up the cellular levels of Oct-4, Nanog and p-Oct in HaCaT keratinocytes, indicating the augmentation effect of the compound on keratinocyte stemness. Hence, to sustain self-renewal, stem cells must have niche anchoring via adhesion molecules [16]. Moreover, another important role of adhesion molecules is to regulate stem cell function - retention, division, and exit [22]. Therefore, stem cell-niche adhesion is crucial for stem cell self-renewal and is dynamically regulated.

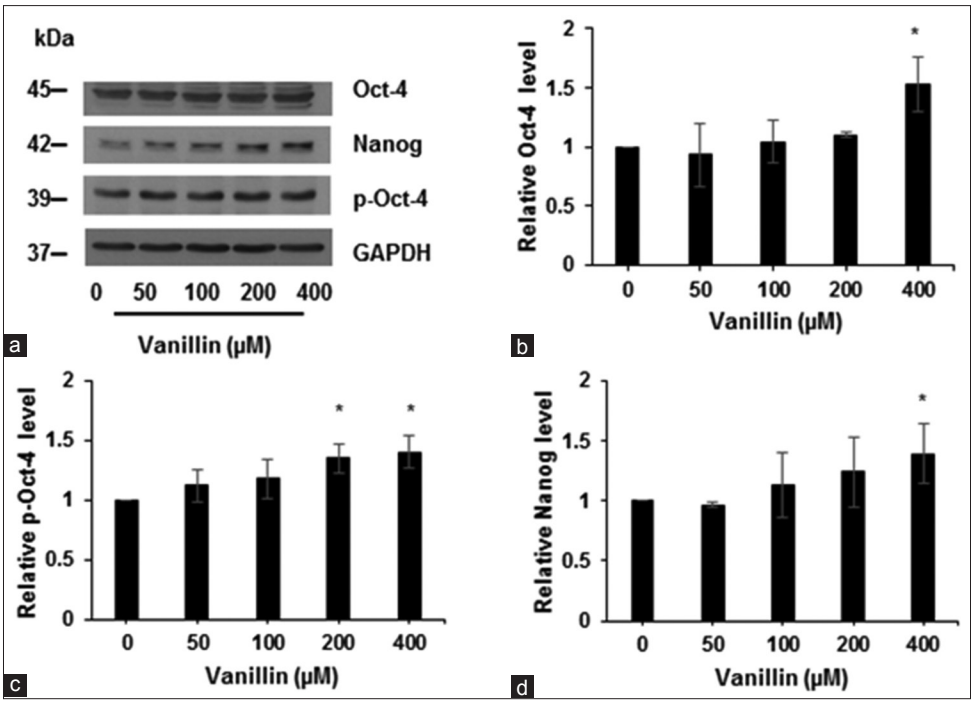

Figure 3: Evaluation of stemness markers. HaCaT cells were treated with vanillin for 48 h. (a) The expression levels of stemness markers in HaCaT cells were determined by western blotting, (b) relative Oct-4 levels, (c) relative p-Oct-4 levels, and (d) relative Nanog levels were quantified by densitometry. Data represent mean ± standard deviation ($n = 3$). *$P < 0.05$ versus non-treated control

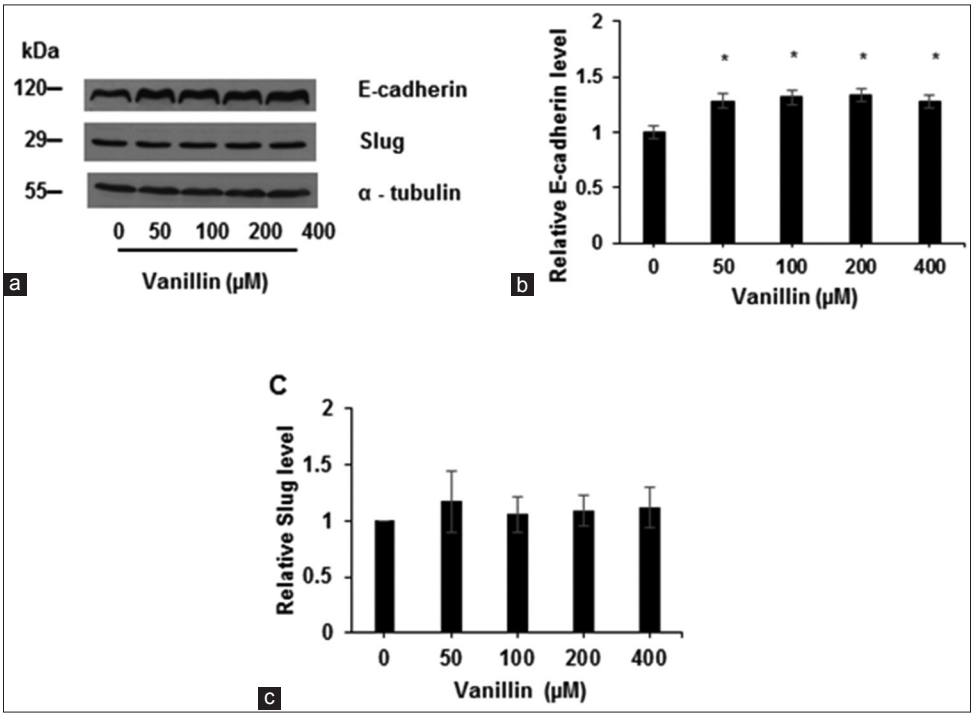

Figure 4: Evaluation of epithelial to mesenchymal transition (EMT) markers. HaCaT cells were treated with vanillin for 48 h. (a) The expression levels of EMT markers in HaCaT cells were determined by western blotting, (b) relative E-cadherin levels, and (c) relative slug levels were quantified by densitometry. Data represent mean ± standard deviation ($n = 3$). *$P < 0.05$ versus non-treated control

Special adhesive characteristics of epidermal stem cells that distinct from their more differentiated progeny [23] are composed of (1) Increased cell-cell cohesiveness and (2) increased extracellular matrix adhesiveness [24-28]. These characteristics are mainly regulated by cadherins for cell-cell interactions and integrins for regulate cell-matrix interactions.

By using invented skin-specific RNAi technology [29], it was proved that when only E-cadherin is suppressed *in vitro*,

deceleration of epidermal sheet formation was found. When both E- and P-cadherins are blocked, expanding of defects to adherens junctions, desmosomes, tight junctions, and cortical actin dynamics was exhibited. In, E-cadherin inhibition caused impaired junction formation, deteriorated intercellular adhesion and raises apoptosis rate. Therefore, the active compound like vanillin could benefit the skin barrier function via the positive regulation on E-cadherin protein.

As we found that vanillin increased E-cadherin expression in human keratinocytes, through this manner, the strength of cohesion between cells is increased, consequently. Besides, the stem cells could tightly incorporate to the surrounding cells and receive signals from their niches more effectively. As stem cell-niche adhesion is crucial for stem cell self-renewal, increase expression of adhesion molecule in the niche might affect stemness marker to raise in the same way. In the aging period, it is hard to avoid the condition of age-associated inflammation skin. In such a situation, activities and amounts of epidermal stem cells have been found to be dramatically reduced [11]. Decrease stem cell function and population from age-associated inflammation skin have been reported to deteriorate skin function [11]; therefore, enhancement of cell adhesion and stemness by vanillin observed in this work may attenuate the defect skin in such a process of inflammation and aging.

CONCLUSION

We demonstrated that vanillin has a potential to increase cell-cell cohesion and stemness in human keratinocyte cells. Cell-cell cohesion is essential for skin barrier function, being strength niches and benefit stem cell property. As the stemness of keratinocytes is an important factor determining the function of epidermis as well as it regeneration processes, vanillin may be a potential candidate for enhancement of the skin protective and repair functions. In conclusion, this study provides for the first time the interesting effects of vanillin on cell stemness enhancement and cell adhesion warranting the further investigations of developing this compound for therapeutic and cosmeceutical applications.

ACKNOWLEDGMENT

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