Original Article

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Safety and Potential Effects of Cannabidiol on Skin Cells

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Abstract:

Objective: Cannabidiol (CBD), a phytochemical active compound from the *Cannabis sativa L.*, has become a popular ingredient in many industries, especially skincare products. However, the scientific evidence supporting its potential skin benefits and safety concerns are still unclear. Therefore, the aim of this study was to investigate the short-term and long-term cytotoxic effects of CBD and its potential melanin-promoting effect on skin cells in order to deeply evaluate the safety of CBD for use in cosmetics.

Material and Methods: HaCaT keratinocytes and B16F10 melanoma cell lines were cultured and investigated in regard to the cytotoxicity of cannabidiol in various concentrations (0-10 μg/ml) in the short term and long term by sulforhodamine B (SRB) assay and clonogenic assay, respectively. Next, the cellular melanin production was measured by melanin content assay. The expression of the related genes was accessed by qPCR.

Results: The short-term and long-term cytotoxicity studies revealed that CBD at a low concentration was not toxic to skin cells. In addition, CBD could induce melanogenesis in melanocytes by increasing melanin content and upregulating tyrosinase expression. Also, CBD provoked cell proliferation and enhanced vascular endothelial growth factor (VEGF) mRNA expression in keratinocytes.

Conclusion: Our study demonstrated that CBD at a low concentration (0.6 μg/ml) is safe for the skin cells in vitro and should thus also be safe if applied to skin. Additionally, CBD could significantly enhance melanogenesis and cell proliferation, which confirms its potential as a cosmeceutical product.

Keywords: cannabidiol, melanogenesis, proliferation, cytotoxicity, skin

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Introduction

Human skin is the primary organ protecting the human body against the damaging effects of exogenous factors, and also participates in maintaining internal homeostasis. The skin is divided into 3 layers, the epidermis, dermis, and hypodermis. The epidermis is the first layer to usually contact the outer environment and is composed of various cells such as keratinocytes and melanocytes. UV and air pollution are the main factors that increase free radicals or reactive oxygen species (ROS). ROS can provoke intracellular inflammation by inducing cytokine release and are related to control of the senescence-associated secretory phenotype (SASP)[1]. They also affect melanogenesis[2] and decrease cell proliferation, resulting in many skin problems including skin inflammation, deoxyribonucleic acid (DNA) damage, skin aging, and pigment spots. Cannabidiol (CBD) is a non-psychoactive phytochemical compound found in the *Cannabis sativa L.* plant. In recent years, CBD has been increasingly used in skin care products, with claims that it has many benefits including anti-inflammatory, hydrating, moisturizing, wrinkle-reducing, and anti-aging. However, there is little scientific evidence to support these cosmeceutical claims, especially in regard to the chemical's safety and efficacy at a cellular level, which is essential for developing CBD-based cosmeceutical products. Interestingly, recent studies found that CBD has strong antioxidant and anti-inflammation activity in macrophages and fibroblast cells when stimulated with LPS[3] and could protect keratinocyte cell membranes when exposed to UVB and hydrogen peroxide[4]. Furthermore, CBD was nontoxic to melanocyte cells[5], human and mouse macrophages[6], and enhanced keratinocyte viability when exposed to H_2O_2[7]. However, to date, the safety concerns and the supporting evidence related to melanogenesis, and the proliferative properties of CBD have still not been clearly understood. Therefore, this study was done to clarify the effects and to identify the exact molecular mechanisms of CBD that are involved in skin cells in order to assess the safety of CBD and its potential application in cosmeceutical products.

Material and Methods

Cell culture

HaCaT keratinocytes and B16F10 melanoma cell lines were purchased form ATCC (Manassas, VA, USA). The cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), GlutaMAX, and 1% penicillin/streptomycin, and incubated at 37 °C, CO_2 5%, and humidity 95%. The media were replenished daily, and subcultures performed every 70-80% cell confluency. All of the supplements and DMEM were purchased from Gibco (Life Technologies Ltd., Paisley, UK)

Cell viability assays

Briefly, cells were seeded on 96-well plates at a cell density 10,000 cells/well in 100 µl supplemented DMEM medium and incubated at 37˚C, CO_2 5% and 95% humidity for 24 hours. Then 100 µl CBD (Dr. Ehrenstorfer, Augsburg, Germany) was added, in varied concentrations for 24 hours. The samples were removed and the cells were fixed with 10% trichloroacetic acid (TCA) for 1 hour. Then, the cells were washed with deionized water and 0.4% sulforhodamine B (SRB) was added into a 96-well plate for 30 min. After that, the cells were rinsed 3 times with 1% acetic acid and solubilized by 10 mM Tris Base. The absorbance was measured at a wavelength of 564 nm by using a spectrophotometric microplate reader (Infinite® M Plex, Tecan Trading AG, Switzerland) and calculating the percentage of cell viability.

Clonogenic assay

HaCaT cells were seeded on 12-well plates at a cell density 3,000 cells/well for 24 hours. The various concentrations of CBD were then added for 24 hours and then the media were changed. The complete media was

replenished every 2–3 days for 10 days. Then, the cells were fixed with 3.7% formaldehyde for 20 min at room temperature. After that, the cells were stained with 0.5% crystal violet for 30 min and solubilized with 10% acetic acid. The absorbance was measured at a wavelength of 590 nm by using a spectrophotometric microplate reader.

Melanin content assay

To investigate the melanogenesis activity of CBD, B16F10 cells (100,000) were seeded into a 6-well plate for 24 hours. The cells were treated with CBD for 24 hours before harvesting the pellets. The cell pellets were dissolved with 300 µL of 1 N sodium hydroxide and heated at 95 °C for 1 hour. Then the cell solutions were transferred into 96-well plate and the absorbance of the solution measured at 405 nm wavelength with a spectrophotometric microplate reader (Infinite® M Plex, Tecan Trading AG, Switzerland). The relative melanin content was calculated.

Real-time quantitative PCR (qPCR) assay

Total cell RNA was extracted from the cell pellets using a RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was generated by using a Superscript III reverse transcriptase kit and oligo-dT primers (Invitrogen, Paisley, UK) following the manufacturer's instructions. The real-time analysis was performed on a CFX connect real-time system (Bio-Rad, Hercules, CA, USA). The cDNA obtained amplified the tyrosinase, transforming growth factor beta 1 (TGF- β1), and vascular endothelial growth factor (VEGF) primer with HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) (Solis Biodyne, Tartu, Estonia). The results were normalized using the ribosomal protein L19 mRNA expression as the reference gene.

Statistical analysis

All data are presented as means ± and standard deviations (±S.D.) from triplicate samples of three independent experiments. Statistical significance was calculated using one-way ANOVA between control and treated conditions. A p-value of ≤0.05 was considered as statistical significance between groups.

Results

The cytotoxic effects of cannabidiol on keratinocytes and B16F10 melanoma cells.

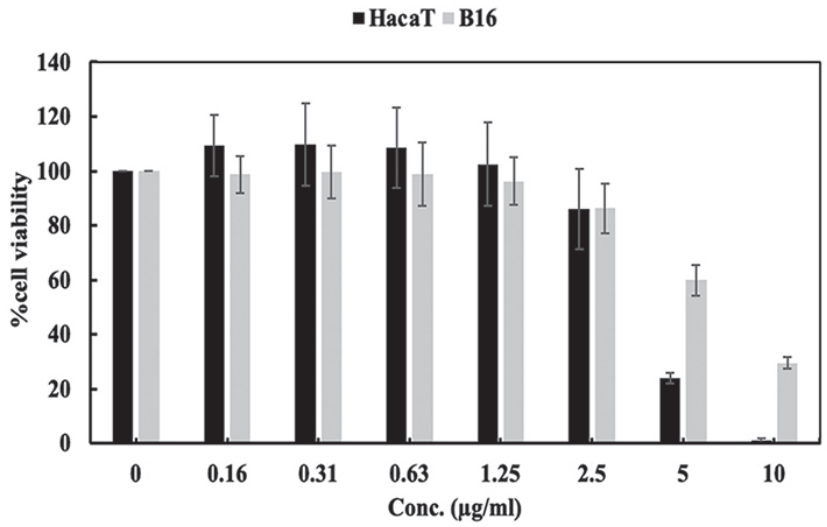
To evaluate the effect of CBD on short-term cytotoxicity, the SRB assay was performed in this study. The keratinocytes and B16F10 melanoma cells were treated with CBD in concentrations of 0–10 µg/mL for 24 hours. The results showed that the concentrations of CBD at 0.16 to 2.5 µg/mL were nontoxic (cell viability >80%) to both keratinocytes and B16F10 melanoma cells (Figure 1).

Long-term effects of cannabidiol in keratinocytes.

The long-term cytotoxicity in keratinocytes was assessed by clonogenic assay for 10 days after being treated with CBD at concentrations of 0, 0.6, 1.25, and 2.5 µg/mL for 24 hours. We found that CBD at 0.6–1.25 µg/mL was nontoxic to keratinocytes in the long term and could induce cell proliferation when activated at a very low concentration (0.6 µg/mL) (Figure 2). Interestingly, the very low concentration (0.6 µg/mL) of CBD did not affect the cells in either the short term or long term.

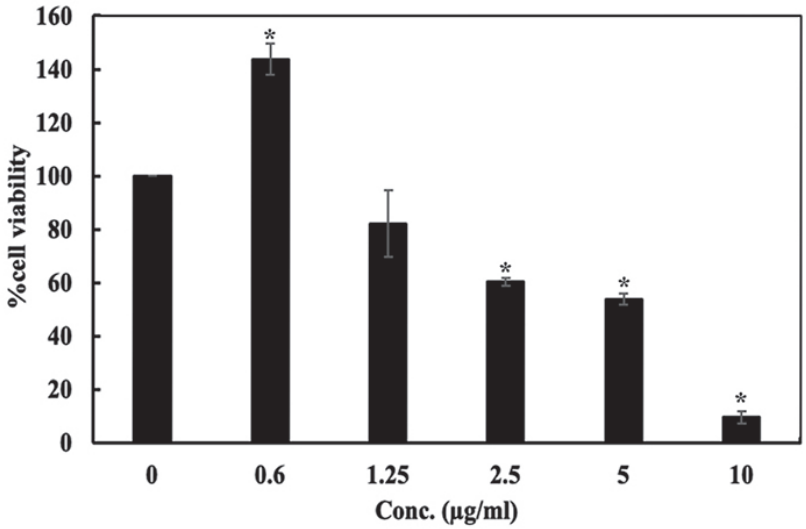
The effect of cannabidiol on the expression of the proliferation gene in keratinocytes.

To identify the exact molecular mechanisms of CBD that are involved in skin cells, the expression of proliferation genes such as TGF-β1 and VEGF was evaluated by qPCR. CBD showed activation effects on mRNA expression levels of the CBD-treated cells by inducing VEGF expression in keratinocytes (Figure 3). However, CBD did not affect the expression of TGF-β1 after CBD treatment in keratinocytes.



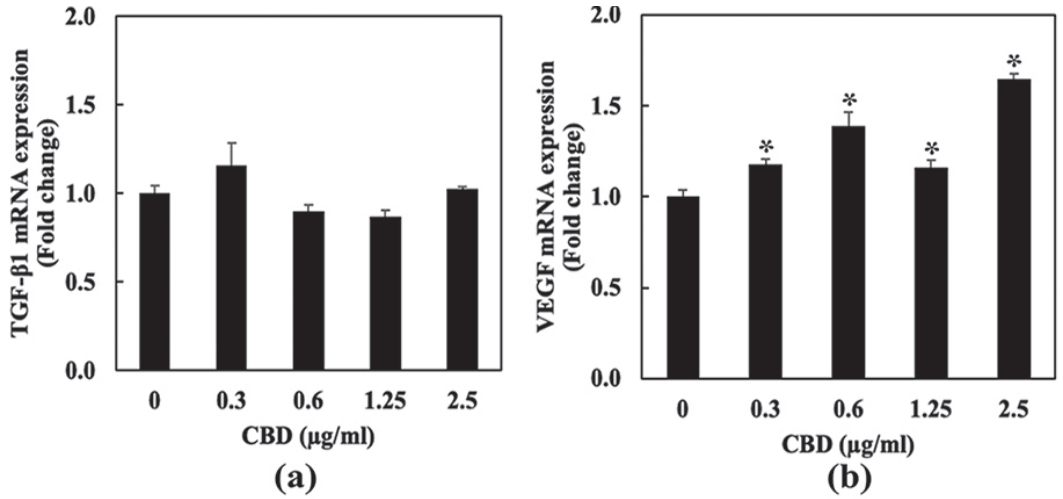
CBD=concentration (ug/mL)

Figure 1 Cell viability of keratinocytes and B16F10 melanoma cells when incubated with cannabidiol (CBD) (0–10 µg/mL) for 24 hours. Each bar represents an average of three independent experiments ± S.D. (n=3) Statistical significance was determined by one-way ANOVA (significant; ****p-value<0.0001 vs. control (0 µg/mL)).



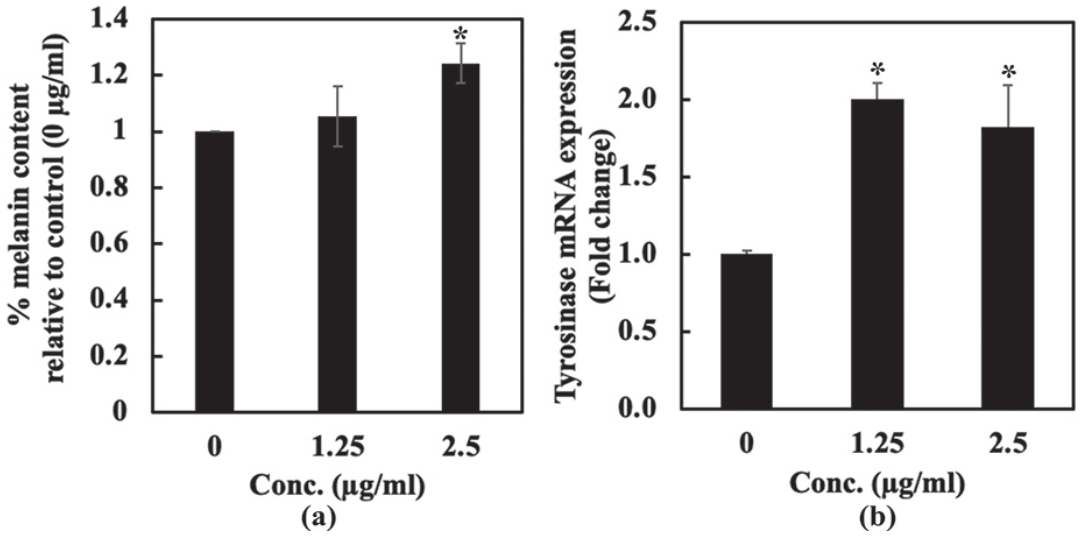
CBD=concentration (ug/mL)

Figure 2 Long-term effect of keratinocytes after treatment with cannabidiol (CBD) for 24 hours and following for 10 days. Each bar represents an average of three independent experiments ± S.D. (n=3). Statistical significance was determined by one-way ANOVA (significant; *p-value<0.05 and ****p-value<0.0001 vs. control (0 µg/mL)).



CBD=concentration (ug/mL)

Figure 3 The mRNA expression of proliferation genes (a) TGF-β1 and (b) vascular endothelial growth factor (VEGF) after treatment with cannabidiol (CBD) in keratinocytes. Each bar represents an average of three independent experiments ± S.D. (n=3). Statistical significance was determined by one-way ANOVA (non-significant; ns, significant; *p-value<0.05, **p-value<0.01, and ****p-value<0.0001 vs. control (0 µg/mL).



CBD=concentration (ug/mL)

Figure 4 The melanin-promoting effect of cannabidiol (CBD) on (a) the melanin content and (b) tyrosinase mRNA expression after being treated with CBD in melanoma cells. Each bar represents an average of three independent experiments ± S.D. (n=3). Statistical significance was determined by one-way ANOVA (non-significant; ns, significant; *p-value<0.05 vs. control (0 µg/mL)).

The effect of cannabidiol on melanogenic activity in B16F10 melanoma cells.

The effect of CBD on melanogenic activity was investigated by measuring the melanin content in the cells. B16F10 melanoma cells were treated with CBD at concentrations of 0, 1.25, and 2.5 μg/mL. The study found that CBD increased the melanin content in B16 melanoma cells in a dose-dependent manner (Figure 4a). After that, the effect of CBD on the expression of the melanogenic-related genes was followed up by qPCR. The relative mRNA expression levels of the CBD-treated cells in various concentrations indicated that CBD could significantly enhance tyrosinase expression levels in the cells (Figure 4b).

Discussion

CBD is a natural cannabinoid found within the cannabis plant. In recent years, CBD, a natural cannabinoid found within the cannabis plant, has been widely used as an active ingredient in various cosmeceutical products. However, the safety and the unclear molecular mechanisms of CBD are still a concern. Previous studies reported that CBD at low concentrations (1-10 μM) had no effect in regard to cytotoxicity in keratinocytes[7] or THP-1 derived-macrophages[6] in a 24-hour period, and human epidermal melanocytes[5] in the short [no hyphen]term [space](~5 days). Similarly, our findings also showed that CBD at low concentrations (0.16-2.5 ug/mL) was not toxic to keratinocytes and melanoma cells in short-term treatment (~24 hours). Interestingly, our long-term cytotoxic study demonstrated that a very low concentration (0.6 μg/mL) of CBD was not toxic and significantly enhanced growth of keratinocytes. Consistent with the previous study about the long-term toxicity and lifespan of C. elegans which found that CBD did not exhibit any toxicity to C. elegans at concentrations of 10, 40, and 100 μM. In addition, CBD at 40 μM also extended the mean lifespan and improved late-stage life activity of C. elegans[8]. This supports our key finding that CBD might have the promising potential to induce cell proliferation and promote some cell activities in the long term, further confirming that CBD is safe and can potentially be safely used for skin application.

Proliferation is a process that increases the cell number by cell division, which is a crucial role of cell regeneration to repair cell damage. One of the important factors to enhance cell proliferation is growth factors such as the transforming growth factor-β (TGF-β) family, vascular endothelial growth factor (VEGF), and the platelet-derived growth factor (PDGF) family. Our results showed that CBD-treated cells at low concentrations (0.3 -2.5 μg/mL) induced VEGF expression in keratinocytes. Consistently, previous studies reported that CBD at low concentrations can promote cell proliferation, migration, and tubulogenesis in human brain endothelial cells via a transient receptor potential (TRP) channel from the vanilloid subfamily (TRPV)[9]. At the molecular level, not only CB1 and CB2 receptors, but CBD actually can also interact with TRPV receptors that present in various types of skin cells such as fibroblasts, keratinocytes, melanocytes, and hair follicles, which are related to many processes in the skin such as cell growth, cell differentiation, and cell inflammatory reactions[10]. Therefore, at the molecular mechanism aspect, we suggest that CBD can enhance skin cell growth through enhancing the expression of VEGF, a key biomolecule related to angiogenesis, cell proliferation and cell migration, via TRPV receptors. VEGF can increase intracellular calcium (Ca^{2+}) leading to regulation of the TRP receptors family, which are Ca^{2+}-permeable ion channels in cells[11,12]. Earlier studies have reported that increased expression of VEGF in keratinocytes promoted the proliferation and remodeling stages of wound healing on skin by stimulating new blood vessel formation to supply oxygen and nutrients needed for skin regeneration[13,14]. Furthermore, another study reported that CBD at high concentrations (more than 2.5 μg/mL)

could decrease the proliferation of canine cancer cells[15], which supports our finding that high concentrations of CBD were toxic to cells.

Additionally, many previous studies reported that ROS are powerful signaling molecules involved in the regulation of a variety of biological processes in cells[16], which are activated by exogenous factors such as UV and air pollution. Melanogenesis is one of the physiological processes that protect the skin from ultraviolet radiation and harmful oxidative stress. Melanin is a pigment produced by melanocytes. The key molecules that affect melanin biosynthesis are tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2), which are melanocyte-specific enzymes triggering melanin production[5,17]. In addition to a safety assessment, our study also found that CBD induced melanogenesis and encouraged the mRNA expression of tyrosinase, which is related to melanin production in melanocytes. Consistent with previous study, CBD has been shown to induce melanogenesis in human epidermal melanocytes by activating the p42/44 MAPK and p38 MAPK pathways through the cannabinoid CB_1 receptor, which leads to inducing tyrosinase expression via the upregulation of melanocyte-inducing transcription factor (MIFT)[5].

Conclusion

Collectively, our results indicate that CBD at a low concentration (0.6 µg/mL) is safe to be used on the skin both in the short and long term. It can also enhance proliferation in keratinocytes via VEGF expression. Additionally, CBD enhanced melanin production and tyrosinase expression. Therefore, we suggest that CBD exhibits potential for melanin production and is safe for use in cosmeceutical products, such as skincare and sun protection products. However, this was a preliminary study on the effects of CBD in vitro. For further studies, we recommend that in vivo studies and clinical trials should be done to confirm the safety of CBD.

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Conflict of interest

There are no potential conflicts of interest to declare.

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