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Research Article

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Evaluation of Healing and Proliferation Potential of Hacat Cells with the Use of the Creb Homeopathic Eye Drops

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Abstract

The popularity of homeopathic treatment for diseases has increased due to its widespread accessibility and its minimal or no side effects. Despite these advantages, the scientific evidence regarding its mechanism of action is not entirely elucidated. Thus, in vitro tests serve as primary indicators that can guarantee the initial analysis of the efficacy of medicine in general. The current study evaluated the healing and proliferation potential of HaCat cells using the CREB (Calendula, Ruta graveolens, Euphrasia, and Belladonna) homeopathic eye drops. An in vitro cell migration assay was performed to assess the tissue healing, repair, and regeneration mechanisms induced by the homeopathic eye drops containing Calendula DH3 + Ruta DH3 + Euphrasia DH3 + Belladonna DH3 (CREB). A mechanical scratch was created on the cellular layer of the human keratinocyte cell line (HaCat), and the treatment involved the application of the CREB DH3 eye drops at a concentration of 5 µL/mL. Consequently, the statistical analysis indicated that applying the CREB DH3 eye drops facilitated the closure of the scratch, exhibiting more promising outcomes than the control treatment. Therefore, this medicine emerges as a viable treatment alternative for addressing ocular disorders.

Keywords: HaCat Cells, Homeopathi, Eye

Introduction

The advantages of homeopathy have garnered increasing prominence over the years. Homeopathy was established in 1796 by the German physician Christian Frederich Samuel Hahnemann. This therapeutic approach endeavors to regard the individual as a holistic entity rather than in isolated fragments. Homeopathic medical practice emphasizes the customization of quantity and quality, tailored to the patient's requirements and the availability of personalized remedies. This approach extends to cases of similar pathologies, where medicine selection is based on the distinct attributes of each patient [1].

Although the homeopathic method of treating diseases is linked to the importance of dynamized medicine, introduced to avoid symptomatic diseases, one of its main challenges lies in comprehensively elucidating the mechanisms of action for medicines administered in minimal doses [1]. Nevertheless, the popularity of homeopathy has continued to grow annually, driven mainly by the absence or minimal nature of side effects and the wide array of pharmaceutical formulations of the same medicine accessible in the market. This diversity includes injectable forms of administration, as well as options such as eye drops. Some current research lines are already providing support and validation for experiments related to the mechanisms of action of homeopathic medicines. Nonetheless, it is necessary to undertake further research to substantiate and ensure the level of efficacy and safety of these medicines since the existing studies on this practice identified in the literature could be more extensive in number and scope [2-4].

In vitro assays are critical indicators for the preliminary evaluation of medicine efficacy. These assays constitute robust studies that can be conducted to unravel the cellular mechanisms of action underlying these medicinal interventions. According to international standards, establishing biocompatibility qualification parameters of any materials intended for medical applications is paramount. These parameters can be comprehensively elucidated through in vitro tests [5]. Consequently, the utilization of cell lines has become extensively propagated in scientific research due to the advancements facilitated by various techniques, including their cultivation and application. Cell lines are sources of inexhaustible biomaterials, contributing to their increasing popularity. Another advantageous aspect of their utilization is the avoidance of highly invasive procedures and the circumven-

tion of the need for other animal or human-derived tissues. This approach has been well-regarded from a bioethical standpoint [5].

Under this perspective, the in vitro migration assay employing the human keratinocyte cell line (HaCat) emerges as a prominent tool for assessing the cell migration process and evaluating mechanisms related to wound healing, as well as other tissue repair and regeneration processes. In addition to evaluating the closure of the scratch created during the assay, the test aims to analyze the migration performance of the studied cells [6]. This test has become a preferred choice for assessing the functionality of eye drops, primarily because the HaCat cell line can reconstitute an epidermal tissue in a well-organized manner [7]. This characteristic renders the cell line a promising element in examining keratinization regulation in human cells [8, 9]. Due to its transparent and avascular nature, the cornea is susceptible to various diseases, lesions, and complications, including ulcerative lesions, infections, inflammations, and perforations. All these complications can result in opacification, visual impairment, and diminished visual acuity [10]. For these reasons, the keratinocyte cell line can be employed to simulate this superficial ocular structure.

In order to evaluate the in vitro performance of the homeopathic eye drops formulated with Calendula DH3 + Ruta DH3 + Euphrasia DH3 + Belladonna DH3 (CREB), a cell migration assay using human keratinocyte cells (HaCaT) was conducted.

Method

Cell Culture

HaCat cells (human keratinocytes) were cultivated in 75 cm^2 flasks using DMEM High Glucose culture medium + 10% fetal bovine serum supplemented with antibiotics. The cultivation flasks were incubated at 37 ºC with 5% CO2. The culture medium was replaced every 48 hours until the cells reached a confluence of approximately 80%. Cells were trypsinized and then plated in 12-well plates at a density of 500,000 cells per well. After this procedure, the plates were placed in an incubator at 37ºC with 5% CO2 for 24 hours.

Plating and Scratching

Following incubation, vertical scratches were created on the cell layer using a sterile pipette tip. Upon completing the scratch procedure in each well, the culture medium was aspirated, and any detached cells were eliminated using PBS medium. Subsequently, a treatment dilution of CREB DH3 eye drops at a concentration of 5 µL/mL was introduced into each well, followed by an incubation period of 48 hours. The control treatment comprised wells containing culture medium without the addition of the eye drops. These control wells were maintained to facilitate result comparison. The wells were examined using an inverted microscope and captured in photographs at time zero, corresponding to the moment immediately after the scratch was made. Following the 48-hour duration, the plate was reexamined using a microscope, and the observed alterations were properly recorded. The culture medium was aspirated from the wells, and the wells were subsequently rinsed with PBS. New photographs were captured subsequent to this procedure in the previously analyzed locations.

Quantification of Closure in the Scratched Areas

The images were organized and subjected to analysis using the ImageJ software to determine the percentage of the closure area. The entire area of the scratch was delineated and computed using this software. Data collection was performed, and the average healed area was calculated via Excel.

Statistical Analysis

Statistical analysis was performed by GraphPrisma Version 9.5.0. Data were analyzed for normality by the Shapiro-Wilk test. Afterward, the Student's T-test was performed to compare the means.

Results and Discussion

The cell migration assay is a commonly employed method to evaluate the efficacy of medicines used for wound closure. In the context of eye drops, evaluating cellular wound closure can be valuable to gauge the product's effectiveness. In the present study, the cell monolayer was deliberately scratched, and the cellular response was assessed upon exposure to the CREB DH3 homeopathic eye drops.

The control treatment, which involved the culture medium without the inclusion of the medicine, exhibited a reduction in the area between the edges both at the initial scratch (time 0) and after 48 hours of incubation (Figure 1). Partial closure of the scratch, resembling a wound, is discernible.

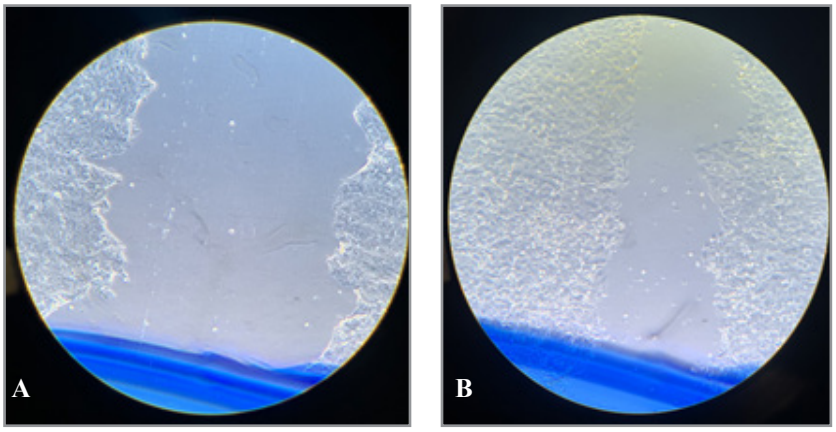

Figure 1: A - Photograph of the control well taken on the day the scratch was performed.
B - Control well photographed 48 hours after incubation in an oven.

Figure 2 demonstrates the reduction in the scratch area 48 hours after cell incubation with the sample of CREB DH3 eye drops.

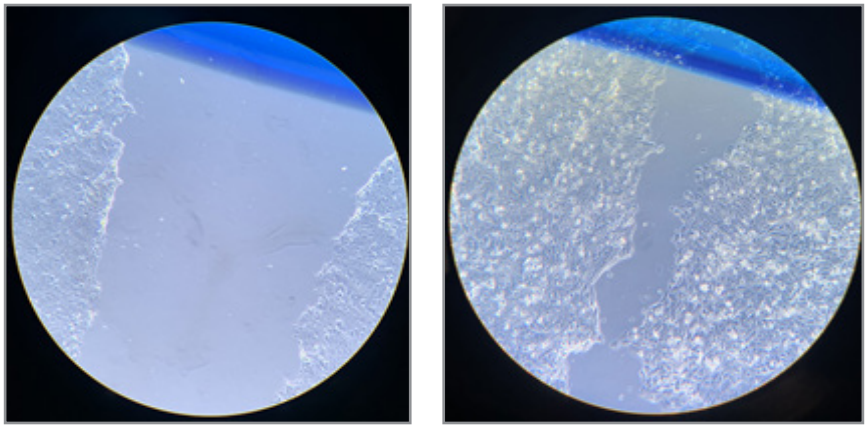

Figure 2: A - Photograph of the well treated with the CREB eye drops taken on the day the scratch was performed. **B -** Photograph of the well treated with the CREB eye drops taken 48 hours after cell incubation in an oven.

The photos were analyzed in the ImageJ program, which transformed the cell-free area into numerical values. These data were analyzed and compared to each other. The non-healed area values obtained through the ImageJ software were subjected to a normality analysis, and they were found to conform to a normal (parametric) distribution. The statistical analysis revealed that the CREB DH3 eye drops facilitated a more substantial closure of the scratch area, demonstrating the potential for closure of the simulated wound, which was greater than that observed in the control group (p=0.0082). The result is shown in Figure 3.

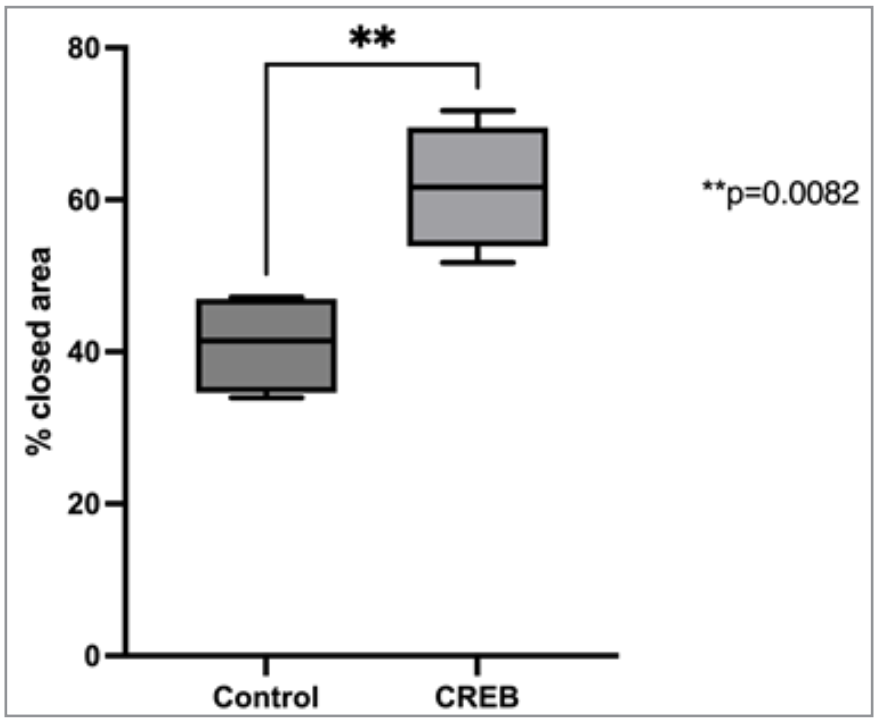

Figure 3: Statistical representation of the percentage of scratch area closure in control samples and samples treated with the CREB DH3 eye drops.

Corneal lesions use the processes of migration, mitosis, and epithelial adhesion for tissue reconstitution. Lesions occurring in the corneal structure trigger both proliferative and inhibitory responses to epithelial migration within the standard conditions. This occurs despite the involvement of diverse cellular layers within the corneal structure, which collectively form the foundation for the aforementioned processes [11, 12].

The selection of the HaCat cell line for this study was based on its suitability as a well-defined experimental model. This choice was motivated by its capacity to effectively elucidate and recognize cellular responses across diverse scenarios, encompassing intracellular signaling, pathway exploration, and responsiveness to various forms of stress. All of this underscores its extensive utilization in in vitro studies. In comparison to the corneal cells

of interest for the tested product, human keratinocytes are similarly organized into flat layers, resulting in an epidermal architecture that closely resembles the normal configuration. In addition, they provide highly conserved differentiation capacity and great genetic stability, ensuring minimal physiological disruptions in the subsequent analyses following the experiments. This cell line also has a high proliferative potential. However, despite the ability of cell proliferation to remain unaffected, the collaborative cell migration process crucial for healing may be hindered, leading to impaired healing activity [13, 14].

Due to its composition, the CREB eye drops exhibited promising outcomes in terms of closing the artificially created wound in an in vitro setting. The plant Calendula officinalis is an important ally in the wound healing process, treatment of gastrointestinal disorders, management of hypertension, and addressing comorbidities of the urinary system. These attributes are supported by findings from experimental pharmacological studies [15]. The healing and anti-inflammatory potential of C. officinalis was also observed in studies using its ethanolic extract to evaluate skin wounds in rats. The results indicated a progressive replacement of type III collagen by type I collagen during the process of wound repair. According to Borges et al. (2007), type I collagen is the main constituent of the connective tissue and has superior organization compared to type III collagen [16]. As reported by Gazola et al. (2014), the primary impact on the process of wound healing stems from its capacity to induce the formation of diverse granulation tissue, characterized by the emergence of new blood vessels, connective tissue, fibroblasts, and inflammatory cells. Moreover, the antiseptic actions, which contribute to epithelialization and the regeneration of damaged skin, are noteworthy for their role in fostering the synthesis of glycoproteins, nucleoproteins, and collagen during the process of tissue regeneration. Additionally, the presence of flavonoids in the plant's composition enhances its healing properties [17]. According to Vannier and Poirier (1897), C. officinalis exhibits external and internal effects on all traumatic wounds, stimulating rapid healing and preventing suppuration.

R. graveolens has become a subject of research owing to its extensive utilization in the management of menstrual disorders, venous insufficiency, and skin inflammations [18]. A study evaluating the topical application of rue essential oil (Ruta graveolens) in wound healing in rats revealed that the epidermis of both the experimental and positive groups displayed an increased count of re-epithelialization scores compared to the negative control. Furthermore, the study highlighted that the chemical compound allantoin, which contributes to the healing and astringent effects, is present in the composition of rue leaves. This compound stimulates the formation of granulation tissue [19]. As stated by Vannier and Poirier (1897), Ruta is recommended for addressing adverse effects or recurrent strain affecting the muscles, tendons, and periosteum. In a study conducted to assess the therapeutic efficacy of Ruta graveolens ointment on skin wounds in dogs, Souza et al. (2007) emphasized that this plant contains essential oils that impact capillary permeability. Additionally, the plant possesses several medicinal attributes, including antimicrobial properties. This characteristic appears to be advantageous for the inflammatory phase of wound healing. Rutin, a flavonoid present in Ruta graveolens, is cited as an instance of a compound that directly influences cellular responses [20].

E. officinalis has a range of constituents, including carbohydrates, tannins, alkaloids, sterols, phenolic acids, iridoid glycosides, flavonoids, amino acids, essential oils, as well as vitamins A and C. These components have been evaluated in several studies [21]. One of the studies substantiating the promising effects of applying formulations of Euphrasia officinalis L. as a complementary therapy for ocular disorders was carried out by Paduch et al. (2014). These promising effects are attributed to the combination of ethanol and ethyl acetate extracts, which exhibit potent activity in terms of scavenging free radicals. The aqueous extract of E. officinalis L. demonstrated anti-inflammatory and antimicrobial or immunomodulatory effects due to the presence of active ingredients such as tannins, phenolic acids, etheric oils, resinous substances, and flavonoids. Moreover, the extract contains iridoglycosides (specifically aucubin) which might potentially influence the enhancement of skin cell renewal [22, 23]. This medication is also indicated by Vannier and Poirier (1897) for inflammation in the nose and eyes.

Concerning the effects of Atropa belladonna, its application induces pupil dilation during ophthalmic examinations and is also employed to alleviate pain associated with rashes of cancerous origin. Among its biochemical constituents lies a perilous and toxic alkaloid known as atropine. Handling this substance requires utmost caution and should only be undertaken by skilled professionals to safeguard the patient's well-being [24, 25]. Its therapeutic efficacy was proved by Toporcer et al. (2006) in a study involving rats, which aimed to assess the mechanical attributes of skin wounds after the application of this plant's extract. The study yielded positive outcomes. In this study, it is significant to highlight that the progression of the inflammatory phase exhibited greater swiftness, potentially leading to earlier initiation of collagen synthesis. Furthermore, the extract exhibited antioxidant effects that enhanced cellular proliferation within the injured area, thereby contributing to the expedited process of collagen synthesis [26-28]. As stated by Vannier and Poirier (1897), Belladonna, a member of the Solanaceae family, is recommended for instances characterized by active congestion alongside nervous and vascular excitation. It is especially indicated for acute, sudden, and intense inflammation accompanied by mucous membrane dryness. Additionally, a study using keratinocytes, 3T3 fibroblasts, and endothelial cells demonstrated that wounds treated with A. belladonna extract prompted a shortened inflammation phase. The extract also induced collagen production, resulted in substantially elevated wound stiffness in comparison to control tissues, and fostered growth by stimulating cellular proliferation [29].

Conclusion

This study demonstrates that the homeopathic eye drops CREB DH3, formulated with Calendula DH3 + Ruta DH3 + Euphrasia DH3 + Belladonna DH3 elicit proliferative and migratory effects on HaCat cells (human keratinocytes). This result demonstrates the in vitro potential of action based on its compositional foundation. Hence, the potential utilization of this homeopathic preparation as a supplementary element in the management of ophthalmic conditions can be assessed, thereby enabling the exploration of new in vivo tests for the medicine.

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