DELIVERING EFFECTIVENESS ENHANCING THE GROWTH OF NATURAL EYELASHES IN COLOR COSMETICS FORMULATION: THE MECHANISM OF NATURAL PLANT-BASED ACTIVE INGREDIENTS WITH IN VITRO AND VIVO TESTS CORRELATION

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Abstract

Mascara formulation aims to improve the esthetic appearance of eyelashes temporarily, little is known about the mechanism of action of natural plant-based active ingredients and its effectiveness enhancing the growth and volume increase of natural eyelashes in color cosmetics formulation.

This work aims to study the effectiveness and to evaluate the correlation between in vitro and in vivo methods in the ability to provide eyelash growth.

In vitro tests were performed to evaluate the effect of baicalin on primary human keratinocytes and dermal papilla cell spheroids. Complementary efficacy studies were carried out with the objective of confirming the capacity for growth and volume increase of natural eyelashes through a mascara formulation, considering the use of natural plant-based active ingredients with established concentrations.

There was a significant increase in the expression of the B-catenin and BCL-2 genes, related to the maintenance and prolongation of the anagen phase, and an increase in VGFa. In addition, treatment with the active induced an increase in CK15, demonstrating the potential of the active in aging prevention and hair growth.

The product promoted a statistically significant increase of eyelash length and volume after 45 days of use, providing an increase of 8% eyelash growth and 9% increase of volume.

The combination of in vitro and in vivo tests demonstrates the action mode of the natural plant-based actives, to promote growth and to improve the volume of eyelashes through the prolongation of anagen phase and maintenance of the stem cell potential of follicular cells.

Keywords: eyelash growth, color cosmetics, natural active ingredients, in vitro and vivo tests correlation.

Introduction

Few research has been done about human eyelashes on account that most of the attention has been directed to research on hair for people suffering from scalp hair loss. However, recent discoveries on the role of eyelashes and its distinctive characteristics have led to an increased scientific interest [1]. Eyelashes are an integral part of the lid margin anatomy, contributing to the overall homeostasis of the ocular surface. As such, it is important to maintain their integrity [2,3]. Eyelashes also serve as a protective function by defending the eyes against debris and triggering the blink reflex.

The hair cycle for all hair types is divided into the phases of anagen, catagen, and telogen, but the average length of the cycle and the individual phases varies by body location [1,8]. Though variable, the normal eyelash cycle is estimated to last from 5 to 11 months. The growth phase of eyelash follicles, anagen, lasts approximately 1–2 months [8]. Following anagen, eyelash follicles enter catagen, a transition phase, which lasts approximately 15 days and is the time during which epithelial elements of the follicle undergo apoptosis or programmed cell death. The longest phase of the normal eyelash cycle, telogen or the resting phase, lasts approximately 4–9 months [1,2,3].

The unique properties of eyelashes (e.g., relatively long telogen and short anagen phases compared with scalp hairs, slow rate of growth, and a lack of influence by androgens) can provide for specific aesthetic interventions to improve the appearance of natural eyelashes [6].

Consumers are looking for formulations that go beyond decoration, that can naturally enhance their appearance. Moreover, eyelashes are now considered an important aspect of the facial esthetic and are the object of various beauty treatments to enhance them [5,7]. Prominent eyes and eyelashes are often considered a sign of beauty and can be associated with increased levels of attractiveness, confidence, and well-being [4]. Eyelash mascara aims to improve the esthetic appearance temporarily, providing darken, lengthen, and

thicken eyelashes by using a combination of waxes, pigments, and resins [5]. Little is known about the mechanism of action of natural plant-based active ingredients and its effectiveness enhancing the growth and volume increase of natural eyelashes in color cosmetics formulation [9,10]. To deliver these innovative performances while still maintaining the mascara must-haves was a challenge accepted.

This work aims to study the effectiveness and to evaluate the correlation between in vitro and in vivo methods in the ability to provide eyelash growth of an oil/wax-in water emulsion formulation of mascara. The proposed mechanisms by which the topical application of natural-based ingredients enhances eyelash growth and volume is also approached.

Materials and Methods

1. In vitro tests

1.1. Cell Culture

In this study, HEK cells (Human Epidermal Keratinocytes), obtained from Cascade Biologics were used. Cells were maintained in culture with Epilife medium (M-EPI-500-CA – Gibco) containing HKGS (Human Keratinocyte Growth Supplement, S-001-5 – Gibco) at 37°C and 6% CO2. HHDP cells (Human Dermal Papilla Cells) were seed in six-well low attachment plates (Corning, model 3471) at 2.37x105 cells per well in DMEM containing 10% FBS without antibiotic. The cells were kept at 37°C and 6% CO2, for a period of 24h. After this period, the spheroids were transferred to 96-well plates for further testing.

1.2. Gene Expression by qPCR

In this study, HEKn cultures and HHDP spheroids were used, with 10^5 HEKs cells plated in 12-well plates and $3x10^5$ HHDP cells in 6-well Low Binding plates (Corning). The active was prepared at 0,32 mg/mL (0,032%) in DMEM with 10% FBS. After 24 hours of incubation with the actives, RNA extraction was performed with the RNeasy Mini Kit (74106, Qiagen), and the samples were quantified in Nanodrop and preserved at -80°C.

Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (4368814 - Life Technologies) according to the manufacturer's recommendations. For each sample, 200ng of total RNA were considered as a template for cDNA synthesis. Gene expression was detected by the Real Time PCR (qPCR) methodology through the TaqMan system (4444963 - Life Technologies) with inventoried probes (BCL-2, CK15, β-Catenin, VGFa).

All reactions were performed on 384 wells plate in triplicate using the reaction solution described in table 1.

Table 1: qPCR reaction volumes

Reagents	**Volume (µL) for 1 reaction** **384 well plate**
TaqMan Fast Advanced Master Mix (Applied Biosystems)	5,0
TaqMan Gene Expression Assay	0,5
cDNA template	1,0
RNase - free water	3,5
TOTAL	10,0

The runs were performed on the ViiA7 system with the parameters defined on Table 2.

Table 2: qPCR reaction parameters

Parameters	**Polymerase Activation**	**Denaturation**	**Ringing / Extension**
		40 cycles	
Temperature	50°C	95°C	60°C
Time (mm:ss)	02:00	00:01	00:20

The results were analyzed using the "2 -ΔΔCt" (fold change) method based on the Ct values of the samples obtained after the qPCR run [11].Statistical analysis was performed using the ANOVA Test with multiple Dunnet's comparisons based on Fold Change values comparing the samples against the experimental control without treatment.

2. In vivo tests

2.1 Materials

A combination of natural plant-based active ingredients with the following composition: Glycine soybean germ extract (and) Triticum vulgare germ extract (and) Scutellaria baicalensis root extract, and, Hydrolyzed Ceratonia siliqua seed extract (and) Hydrolyzed soy protein (and) Zea mays starch (and) Amaranthus hypochondriacus leaf extract, supplied by Provital Group were evaluated in this study.

An oil/wax-in water emulsion formulation of eyelash mascara, composed by texture modifiers, emollients, emulsifiers, film formers, pigments with the active ingredients previously mentioned were used in this experiment and can be found in Table 3

Table 3. Quantitative formulation with the natural plant-based active ingredients included in this study

Ingredient	Formulation (% w/t)
AQUA (WATER)	44,19342
SYNTHETIC BEESWAX	10,50000
PARAFFIN	7,70000
CI 77499 (IRON OXIDES)	5,00000
ORYZA SATIVA BRAN WAX [ORYZA SATIVA (RICE) BRAN WAX]	3,20000
BUTYLENE GLYCOL	2,99370
GLYCERIN	2,70000
GLYCERYL STEARATE	2,65000
ACRYLATES/ETHYLHEXYL ACRYLATE COPOLYMER	2,33750
PALMITIC ACID	2,22000
VP/EICOSENE COPOLYMER	2,20000
POLYBUTENE	2,00000
STEARIC ACID	1,68000
COPERNICIA CERIFERA CERA [COPERNICIA CERIFERA (CARNAUBA) WAX]	1,50000
PROPANEDIOL	1,15335
AMINOMETHYL PROPANOL	1,07475
PANTHENOL	1,00000
SILICA	1,00000
CI 77266 (NANO) [(BLACK 2) (NANO)]	0,85000
ACACIA SENEGAL GUM	0,66000
PHENOXYETHANOL	0,58500

XANTHAN GUM	0,54000
ETHYLHEXYLGLYCERIN	0,50000
LAURETH-21	0,29750
ARGININE	0,24000
LACTIC ACID	0,22500
SODIUM BENZOATE	0,12500
PEG-40 HYDROGENATED CASTOR OIL	0,11688
HYDROXYETHYLCELLULOSE	0,09300
GLYCINE SOJA GERM EXTRACT [GLYCINE SOJA (SOYBEAN) GERM EXTRACT]	0,09000
TRITICUM VULGARE GERM EXTRACT [TRITICUM VULGARE (WHEAT) GLUTEN EXTRACT]	0,09000
ZEA MAYS STARCH [ZEA MAYS (CORN) STARCH]	0,07000
HYDROLYZED CERATONIA SILIQUA SEED EXTRACT	0,06000
HYDROLYZED SOY PROTEIN	0,06000
COCOS NUCIFERA OIL [COCOS NUCIFERA (COCONUT) OIL]	0,05000
TOCOPHERYL ACETATE	0,05000
LAURIC ACID	0,04000
MYRISTIC ACID	0,04000
GLUCONOLACTONE	0,02250
ARACHIDIC ACID	0,02000
SCUTELLARIA BAICALENSIS ROOT EXTRACT	0,01800
GUAR HYDROXYPROPYLTRIMONIUM CHLORIDE	0,01290
SODIUM DEHYDROACETATE	0,01148
PHENETHYL ALCOHOL	0,00677
CAPRYLYL GLYCOL	0,00545
POLYQUATERNIUM-16	0,00500

POTASSIUM SORBATE	0,00500
POLYQUATERNIUM-7	0,00440
AMARANTHUS HYPOCHONDRIACUS LEAF EXTRACT	0,00200
DISODIUM PHOSPHATE	0,00050
POLYSORBATE 60	0,00050
CALCIUM GLUCONATE	0,00030
SODIUM PHOSPHATE	0,00010

2.2. Methods

In addition to the in vitro tests, complementary efficacy studies were carried out with the objective of confirming the capacity for growth and volume increase of natural eyelashes through a mascara formulation.

The in vivo tests were conducted with 32 female participants, of which 30 completed the assessments. The volunteers had phototypes I to IV, aged between 18 and 55 years and the mascara formulation was applied by each of the participants for 45 days. Facial region images of the participants (Figure A) were captured using the Reveal Image® (Canfield) equipment to determine the size and volume of eyelashes. Images were captured with the following experimental times: D0 (baseline) and D45 (after 45 days of product use).

The data on average length and volume of eyelashes obtained with the Reveal® Equipment, after 45 days of use (D45), were statistically analyzed by paired T test, comparing the condition baseline (D0). These analyzes were performed using Minitab Statistical software 19.

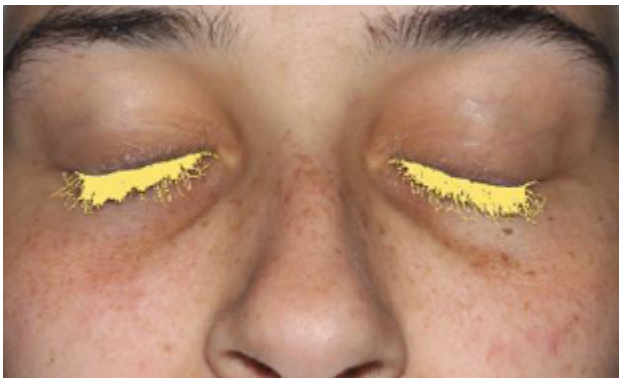

Figure A. Image obtained from the equipment Reveal Image® (Canfield)

Results and Discussion

1. In vitro tests

1.1. Gene expression in vitro assay

With the in vitro tests performed, there was a significant increase in the expression of the β-Catenin in HEKn with a fold change of 1.34 (Table 4). β-Catenin participates in the conical pathway of WNT signaling, it is expressed during the anagen phase, increasing the duration of this phase of the capillary cycle [12]. For both conditions, HEKn and HHDP, that was an increase in BCL-2 (Table 4) (4.16 and 1.27 respectively), a gene that encodes a protein involved in the control of apoptosis, having an anti-apoptotic function [14]. Its expression is increased in the anagen phase of the cell cycle, decreased in the catagen phase and abolished in the telogen phase. Together, these genes are key biomarkers related to the maintenance and prolongation of the anagen phase. It was also detected a significant increase in VGFa (Table 4), a growth factor that induces follicular vascularization helping to prevent apoptosis and plays an important role in the anagen-catagen transition being secreted by the dermal papilla [15]. In addition, treatment with the active induced an increase in CK15, a biomarker of stem cell potential, in neopapalis, demonstrating the potential of the active in aging prevention and hair growth [13]. Cytokeratin 15 is also continuously expressed in the outer sheath of the hair follicle, acting in the formation of the fiber.

Table 4. Fold change values obtained for the genes evaluated in HEK and HHDP

	HEKn	HHDP
β-Catenin	1,34***	0,98
BCL-2	4,16***	1,27***
VGFa	1,87***	2,26***
CK15	1,42	6,70***

*Fold change obtained after 24 hours of treatment. *** refers to statistical significance in relation to control treatment, p<0.001, ANOVA with Dunnet's post-test.*

2. In vivo tests

The product promoted a statistically significant increase of eyelash length and volume after 45 days of use, (Figure B) providing an increase of 8% eyelash growth (Figure C) and 9% increase of volume (Figure D), supporting the benefits promised by the product, expected by the consumers and supporting its value proposition. The product perception evaluation is also a strong indication of the fulfillment of consumer acceptability, presenting favorable purchase results in 87% and proof of the eyelash growth action in 80% (Figure E).

Figure B. Images obtained from survey participants before and after normal conditions of use of the investigational product in the following conditions D0 (baseline) and D45 (final)

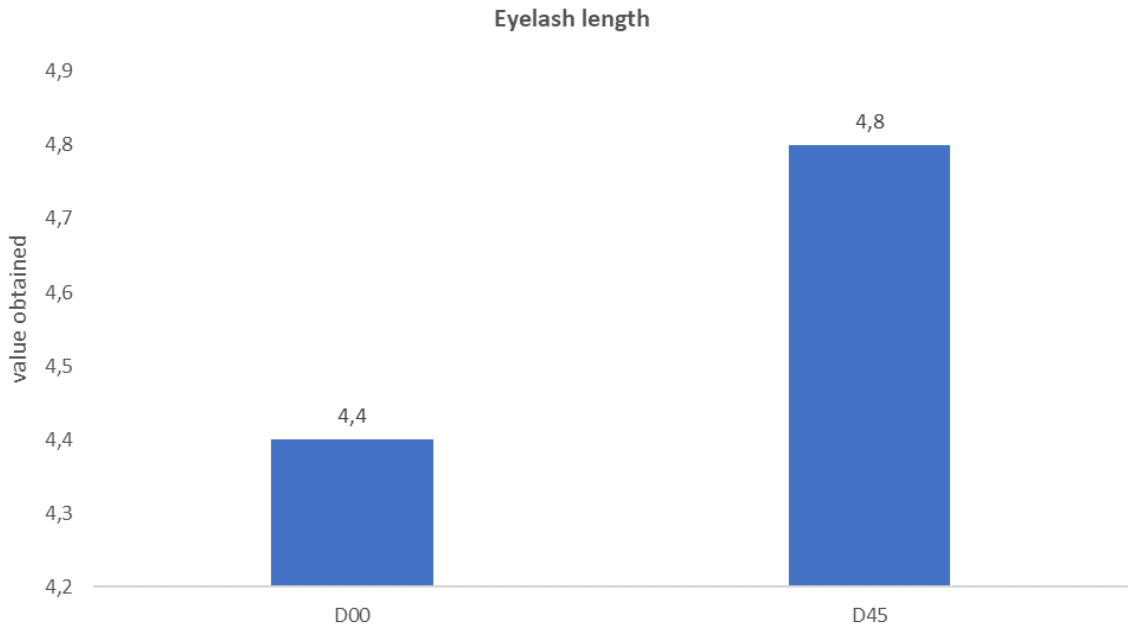

Figure C. Average of the eyelash length data by experimental time obtained through the equipment Reveal Image® (Canfield)

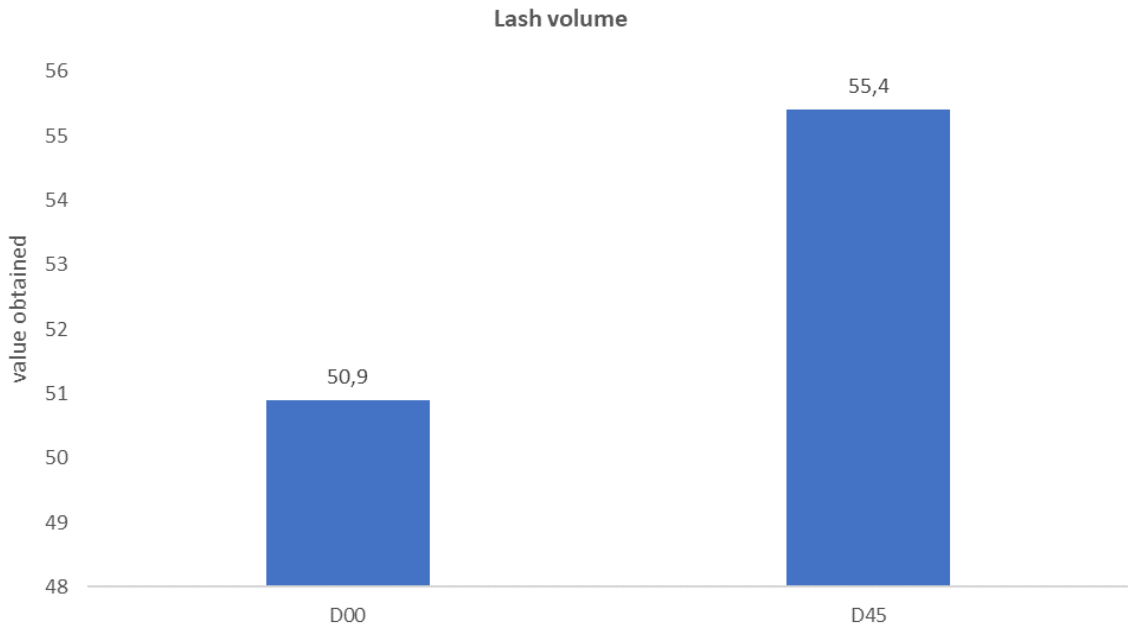

Figure D. Average of the eyelash volume data by experimental time obtained through the equipment Reveal Image® (Canfield)

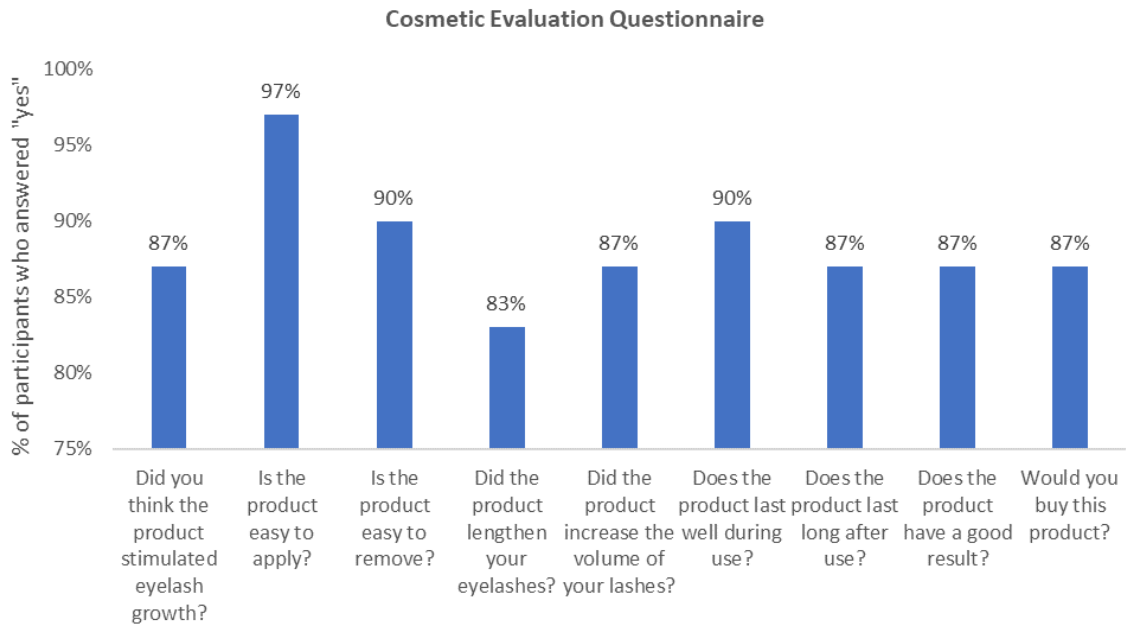

Figure E. Survey volunteers' responses to the questionnaires applied after 45 days of using the product

The biofunctional ingredients studied, composed of Glycine soybean germ extract and Triticum vulgare germ extract for example, is believed to improve intracellular metabolism. Amaranthus hypochondriacus leaf extract and Hydrolyzed Ceratonia siliqua seed extract, which are rich in flavonoids and antioxidants and have a wide range of essential amino acids that increase the density of eyelashes, and finally, Scutellaria baicalensis root extract, a flower that has the power to naturally accelerate the hair growth cycle. The innovative mechanism of action of this combination prolongs the anagen phase of the eyelashes, inducing the growth of the lashes length, in addition to strengthening their anchorage in the follicle, preventing hair loss through the reduction of the telogen phase, protecting its integrity.

Each of the methods explored in this study have its own difficulties and limitations. In vitro tests can mimic certain properties of human skin, but cannot fully replicate it. In vivo test is time/resource consuming and demands a high number of individuals to minimize effects of human-to-human variability in the statistical power of the study.

As future work, the effect of other than baicalin natural plant-based ingredients, will be analyzed on primary human keratinocytes and dermal papilla cell spheroids allowing methods to be directly compared.

Conclusion

Eyelashes have an important role in determining beauty, and as such, prominent eyelashes are a highly sought-after attribute.

The combination of in vitro and in vivo tests demonstrates the action of the natural plant-based actives present in the color cosmetic formulation, to promote growth and improve the volume of eyelashes through the prolongation of anagen phase and maintenance of the stem cell potential of follicular cells.

It is believed that this study opens up a new frontier for makeup with valuable knowledge for the development of new color cosmetics with benefits consumer centric.

Acknowledgments

The authors would like to thank Grupo Boticário and Provital Group for the resources allocated to this research project.

Conflict of Interest Statement

NONE.

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