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ORIGINAL PAPER

A umbilical cord blood-derived exosome product simulates hair growth through laminin V and collagen XVII: an ex vivo study

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Abstract

Hair loss is a prevalent disorder negatively affecting the quality of life and mental health. However, current treatment options are limited, highlighting the urgent need for the development of new therapies. Growing evidence has proven that stem cell-derived nutrient medium and exosome are novel approaches to future hair loss therapy. Limelight (CB-EVs) is an umbilical cord blood-derived exosome product. This study was to investigate the safety and efficacy of Limelight (CB-EVs) for hair growth ex vivo. Our analysis revealed that concentrations of Limelight (CB-EVs) below 5 U/ml demonstrated no cytotoxicity in vitro through MTS assay. In hair follicle culture, concentrations of 0.1, 0.01, and 0.001 U/ml significantly promoted hair elongation while maintaining hair bulb diameter. Analysis of the hair growth cycle indicated a reduced transition from the anagen phase to the catagen phase. Overexpression of Laminin V and Collagen XVII were seen by immunohistochemistry staining. These results endorse the exosome product, Limelight (CB-EVs), offers a safe and effective method for stimulating hair growth ex vivo.

Keywords Hair loss · Exosome · Ex vivo · Limelight (CB-EVs)

Introduction

Alopecia, or hair loss, is a prevalent disorder affecting a significant global population [1, 2]negatively impacting quality of life and mental health [3]. Research indicates that hair loss is primarily influenced by genetic predisposition, aging, and sebum production, among other factors. While current treatments such as minoxidil and finasteride can partially alleviate symptoms, their efficacy is limited and often accompanied by substantial adverse effects [4].

Minoxidil, an FDA-approved topical agent for androgenetic alopecia [5, 6]functions as a potassium channel activator and vasodilator. It prolongs the anagen phase of the hair cycle and promotes thicker growth by enhancing blood circulation to hair follicles [7]. Despite its widespread use, minoxidil has significant drawbacks. Treatment effects are temporary, requiring continuous application for maintenance, which leads to considerable costs over time [8]. Users may also experience side effects ranging from local reactions like scalp irritation, itching, and dryness to systemic effects including dizziness and tachycardia [9, 10]. These limitations highlight the pressing need to explore safer and more efficacious biological therapies for alopecia.

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Mesenchymal stem cells (MSCs) have emerged as promising candidates in tissue regeneration and repair due to their multipotent differentiation potential and paracrine activity [11]. Umbilical cord-derived MSCs (UC–MSCs) are particularly valuable as a cell therapy option because of their abundant supply, low immunogenicity, and robust proliferation capacity [12]. Importantly, research has revealed that the therapeutic efficacy of MSCs is primarily attributed to their secreted bioactive factors rather than direct differentiation capability [13]. This insight has directed research focus on exosomes, key components of MSC secretion. Exosomes are small extracellular vesicles (30–150 nm diameter) containing proteins, mRNAs, miRNAs, and lipids that facilitate intercellular communication by modulating gene expression and signaling pathways in recipient cells [14]. Studies have demonstrated the efficacy of MSC-derived exosomes in wound healing [15]regulation of immune-inflammatory responses [16]and hair growth stimulation [17]. Specifically, these exosomes activate AKT and upregulate Bcl2 expression in dermal papilla (DP) cells, enhancing their proliferation and migration [18]. They also increase the expression of vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1), leading to substantial improvement in follicular unit retention and hair growth.

Despite mounting evidence supporting their therapeutic potential across various medical domains, the clinical utility of exosomes remains constrained by several limitations. Standardized protocols for isolation, purification, identification, and storage are lacking, and challenges persist in accurately quantifying and achieving optimal concentrations [19]. Limelight (CB-EVs), a concentrated form of exosome derived from UC-MSCs, offers improved product stability that may address these limitations. This study aims to assess the impact of Limelight (CB-EVs) on hair follicle length, width, and growth cycle in an ex vivo setting, investigating its potential as a novel therapy to promote hair growth.

Methods and materials

This study has been approved and confirmed by the Ethics Committee of the Korean Skin Research Center of H&Bio Company in Seongnam, Korea. All studies in this manuscript were conducted following the relevant guidelines. The experiments were conducted using tissues obtained from three donors: a 56-year-old female (Donor 1), a 72-year-old female (Donor 2), and a 46-year-old female (Donor 3). From each donor, 8 hair follicles were allocated to each treatment group as technical replicates.

Test materials and test conditions

(1) Untreated group (normal).
(2) Positive Control: Minoxidil 0.20 ppm.
(3) Test group A: Limelight (CB-EVs) 0.001 U/ml.
(4) Test group B: Limelight (CB-EVs) 0.01 U/ml.
(5) Test group C: Limelight (CB-EVs) 0.1 U/ml.

Limelight, a cord blood-derived extracellular vesicles (CB-EVs) product (50 U/vial), was provided by IEUL BIOSCIENCES Inc. (Seoul, Korea) and reconstituted in 1 mL phosphate-buffered saline (PBS) to achieve a final concentration of 50 U/mL, according to the manufacturer's instructions. And then add to the aforementioned solution for different test group and a different concentration. The chosen concentration of minoxidil aligns with previously published studies demonstrating efficacy without cytotoxic effects [20–22].

Human hair follicle dermal papilla cell isolation and culture

Hair scalp tissue, approximately a 2 cm × 2 cm piece, was obtained from Korean adults aged 20–80 years without skin diseases or active hair-loss treatments and was utilized in this study approved by the IRB of KSRC (Korean Skin Research Center) (HBABN01-240403-HRBR-E0013-01). This broad age range was adopted due to limited donor availability and to include diverse adult ages, although we acknowledge that age-related differences in follicle health could introduce variability. Human dermal papilla cells (hDPCs) isolation and culture were the same as what we have done and published [23]. The collected scalp specimens were immediately placed in PBS and transported to the laboratory under sterile conditions. Hair follicles (HFs) were microdissected under a stereomicroscope. The lower portion of the follicle, including the dermal papilla, was carefully isolated using fine forceps and microscissors. Isolated DP tissues were enzymatically digested in a mixed solution of collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and dispase II (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 1–2 h with gentle agitation. Following digestion, the solution was centrifuged at 300 g for 5 min, and the pellet was resuspended in culture medium. hDPCs were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (PS) at 37 °C in 5% CO_2.

Exosome identification

The sizes and concentration of the exosomes in Limelight (CB-EVs) were determined via nanoparticle tracking analysis (NTA; PARTICLE METRIX, Germany) in the original concentration of 50 U/ml. Exosome morphology was determined by transmission electron microscopy (TEM; FEI, Tecnai G2 Spirit BioTwin, USA).

Cell toxicity

DP cells (5000 cells/well) were seeded in 96-well plates and treated with serial dilution of limelight (CB-EVs) in incomplete medium (DMEM + 1%PS) at 0, 5, 10, 15, 25 U/mL for 24 h at 37 °C, 5% CO_2. Then 20 µl of CellTiter 90 AQaueous One Solution reagent (Promega, WI, USA) was added to each well, followed by incubation for 3 h. Cell viability was then assessed by measuring the absorbance at 490 nm using a BioTek Epoch 2 microplate spectrophotometer (BioTek Instruments, VT, USA).

Human HF (hair follicle) isolation and culture

The scalp tissue was cleaned with PBS and placed in sterile petri dishes. HFs were isolated based on the dermis under an anatomical microscope. Surrounding fat tissue was meticulously removed from the HF tissue, which was then trimmed to match the length of the DP. The HF organs were cultured in a 37°C, 5% CO_2 incubator using William's E medium supplemented with 2 mM L-glutamine, 10 µg/mL insulin, 10 ng/mL hydrocortisone and 1% PS, which is the same standardized medium commonly used for human hair follicle organ culture [24]. And then the length and root diameter of HFs were assessed on days 0, 3, 6, 9, and 12 of the culture periods through photographic analysis. We extended the culture to 12 days to allow the observation of both early and delayed effects of Limeligh (CB-EVs) treatment on hair cycle progression, although human HFs typically survive ex vivo for about 7–9 days before growth ceases [20, 24, 25].

Measure length and root diameter (hair bulb)

HFs were photographed at 0, 3, 6, 9, and 12 days using a dissecting microscope (SZ51, OLYMPUS, Japan) and ToupLite software (ToupTek, China), and the length and root diameter of HFs were measured using the Image J software (National Institutes of Health, Bethesda, MD, USA).

Growth cycle assessment

HFs were photographed at 0, 3, 6, 9, and 12 days using a dissecting microscope (SZ51, OLYMPUS, Japan) and ToupLite software (ToupTek, China), and hair root portions of the HFs structures were analyzed. It mainly distinguishes between growth period (Anagen) and the regression period (Catagen). We distinguished follicles in the growth phase (Anagen) versus the regression phase (Catagen) based on morphology: anagen follicles continued to elongate and retained a large, pigmented bulb, whereas catagen follicles were identified by cessation of growth, shrinkage of the hair bulb, and formation of a club-shaped hair base [26]. (Fig. 1)

Histopathological evaluation

HFs were fixed in 10% formalin on day 12 of incubation and embedded in paraffin blocks. Sections of 3 µm thickness were prepared, followed by antigen exposure through hydration with 10 mM sodium citrate buffer. Subsequently, the sections were treated with the primary antibody, followed by development using a secondary antibody (Cell Signaling Technology, 8114, MA, USA) containing horseradish peroxidase (HRP) enzyme and diaminobenzidine (DAB) substrate. Control staining was conducted with hematoxylin. Cross-sectional images of the HFs were captured using a light microscope (AX10, ZEISS, Germany),

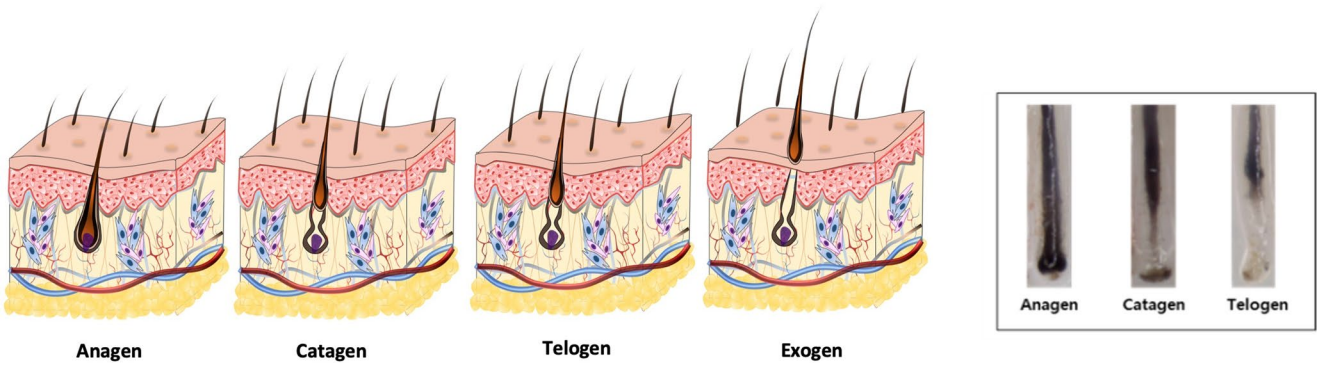

Fig. 1 Identification of HF cycle and representative image of Anagen, Catagen and Telogen of scalp-derived hair follicles. Based on morphological changes, the hair follicle growth cycle was divided into Anagen, Catagen, and Telogen

and the expression of laminin V (Abcam, ab14095, Cambridge, UK) and collagen XVIIa1 (Abcam, ab184996, Cambridge, UK) was quantified using the Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data are presented as mean ± standard error (SE). Statistical analysis was performed using SPSS (v.20, IBM, USA). Normality of data was verified by the Shapiro-Wilk test and kurtosis and skewness. An analysis of Variance (Repeated Measures ANOVA) was used to compare values before and after treatment, and between groups at each time point (significance threshold $p < 0.05$).

The HF length change rate, hair root diameter change rate, and HF growth cycle rate are calculated as follows:

$$\text{HF length change rate} = \frac{\text{HF length at each time point}}{\text{average value of HF length on day 0}} \times 100\%$$

$$\text{Change rate of hair bulb diameter} = \frac{\text{hair bulb diameter at each time point}}{\text{average value of hair bulb diameter on day 0}} \times 100\%$$

$$HF\ growth\ cycle\ ratio = \frac{number\ of\ HF\ in\ anagen\ phase}{total\ number\ of\ HF} \times 100\%$$

$$Change\ rate\ of\ X\ protein\ expression = \frac{X\ protein\ expression\ in\ test\ group\ -\ X\ protein\ expression\ in\ normal\ group}{X\ protein\ expression\ in\ normal\ group} \times 100\%$$

Result

Exosomes in limelight (CB-EVs)

The exosome particle size distribution was assessed through nanoparticle tracking analysis (NTA), revealing an average size of approximately 104.6 nm (Fig. 2a, b). The concentration of exosomes in Limelight (CB-EVs) at 50 U/ml was determined to be 6.2×10^9 particles/ml. Notably, 1 unit of Limelight (CB-EVs) corresponds to approximately 1.2×10^8 exosome particles, as quantified by NTA. Transmission electron microscopy (TEM) further confirmed the typical double-membrane structure characteristic of exosomes (Fig. 2c).

Limelight (CB-EVs) with low cytotoxicity

hDPCs were incubated with Limelight (CB-EVs) at various concentrations (0, 5, 10, 15, and 25 U/ml) for 24 h. The results showed that Limelight (CB-EVs) slightly reduced cell viability by 1.26% −5.66% at low concentration of Limelight (CB-EVs), from 5 to 15 U/ml, compared to the untreated group, and significantly reduced at high concentration (25U/ml). These results indicate that Limelight (CB-EVs) is non-toxic and safe for hDPCs at concentrations below 15 U/ml (Fig. 3).

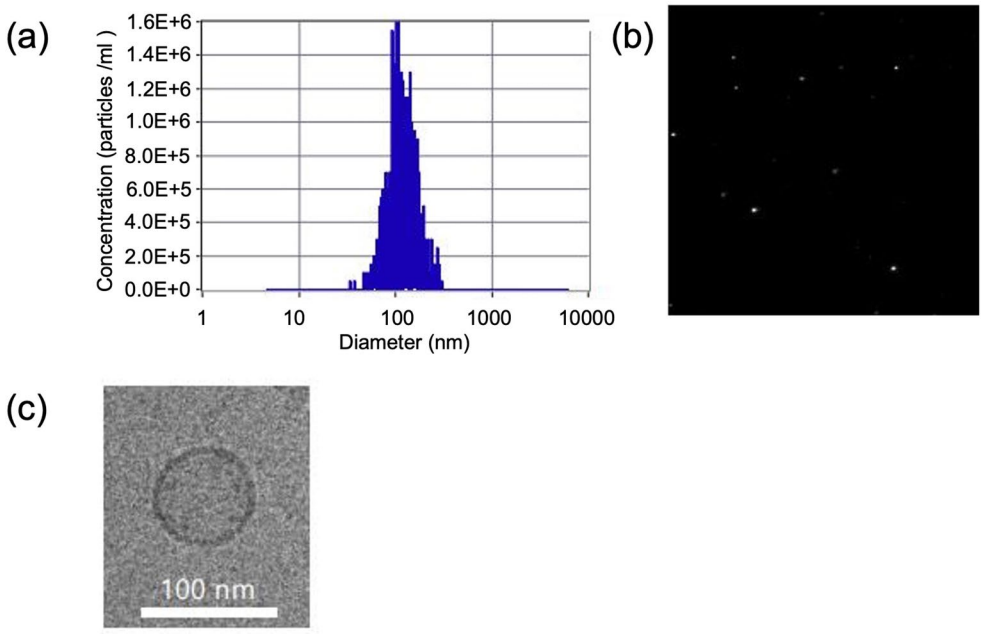

Fig. 2 The characteristics of Limelight (CB-EVs) and exosomes. The particle size distribution in Limelight (CB-EVs) was determined by NTA **a**. Brownian motion image of exosome particles **b**. Morphology of exosomes from Limelight (CB-EVs) observed by transmission electron microscopy **c**. Scar bar, 100 nm

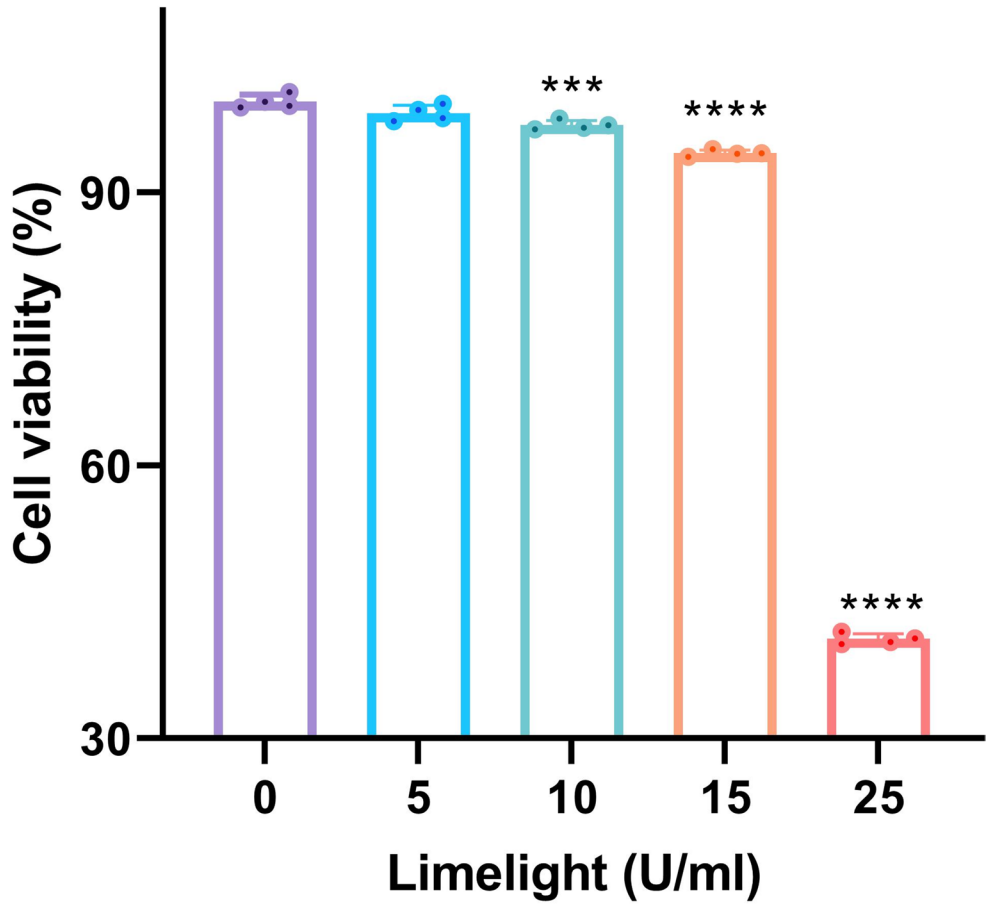

Fig. 3 Cytotoxicity of hDPCs. hDPCs were incubated with different concentrations of Limelight (CB-EVs) for 24 h and cell survival was analyzed by absorbance. $N=3$ per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ compared with untreated group

Limelight (CB-EVs) promotes HF elongation

HF length was measured after treatment with Limelight (CB-EVs) at three concentrations. For HF from Donor 1, Test group A showed significant HF length increases of 26.61%, 34.87%, 35.30%, and 35.30% on days 3, 6, 9, and 12, respectively ($p<0.05$). Test group B demonstrated similar growth with increases of 25.35%, 33.07%, 34.40%, and 35.42% ($p<0.05$). Test group C showed more modest but still significant increases of 17.04%, 23.31%, 23.58%, and 25.69% ($p<0.05$). While positive control group showed HF length increases of 27.96%, 37.78%,38.69%, and 41.98% ($p<0.05$) over the same period. When compared to the normal group after 12 days of HF culture, the Test groups A, B, C, and positive control group exhibited HF length increases of 22.92%, 23.04%, 13.31%, and 29.60%, respectively ($p<0.05$) (Fig. 4).

For experiments on the HF from the Donor 2 and Donor 3, the results were consistent with the Donor 1, showing continuous hair growth across all treatment groups over the 12-day culture period (Figs. 5a and c and 6a and c). But when compared to the normal group after 12 days of HF culture from Donor2, Test group A demonstrated a significant HF length increase of 16.07%($p<0.05$) (Fig. 5b). When compared to the normal group after 12 days of HF culture for Donor 2, Test group B showed a significant HF length increase of 10.69% ($p<0.05$) (Fig. 6b).

Limelight (CB-EVs) maintains hair bulb diameter

For HF from Donor 1, all groups showed decreased hair bulb diameter compared to baseline (day 0). The untreated normal group exhibited the most significant reduction (22.87%, $p<0.05$), while Test groups A and C showed more moderate decreases of 13.56% and 14.04%, respectively. Test group B and positive control group demonstrated a significant reduction of 19.89% and 21.60% ($p<0.05$). After 12 days of HF culture, the test groups maintained larger hair bulb diameters compared to the normal group, with increases of 9.31%, 2.98%, 8.83%, and 1.27% for Test groups A, B, C, and the positive control group, respectively (Fig. 7a, b).

For HF from Donor 2, it showed similar trends. The untreated control group experienced a substantial hair bulb diameter decrease of 27.73% from baseline ($p<0.05$). Test groups A, B, C and positive control also showed significant reductions of 20.04%, 18.32%, 19.27%, and 19.51%

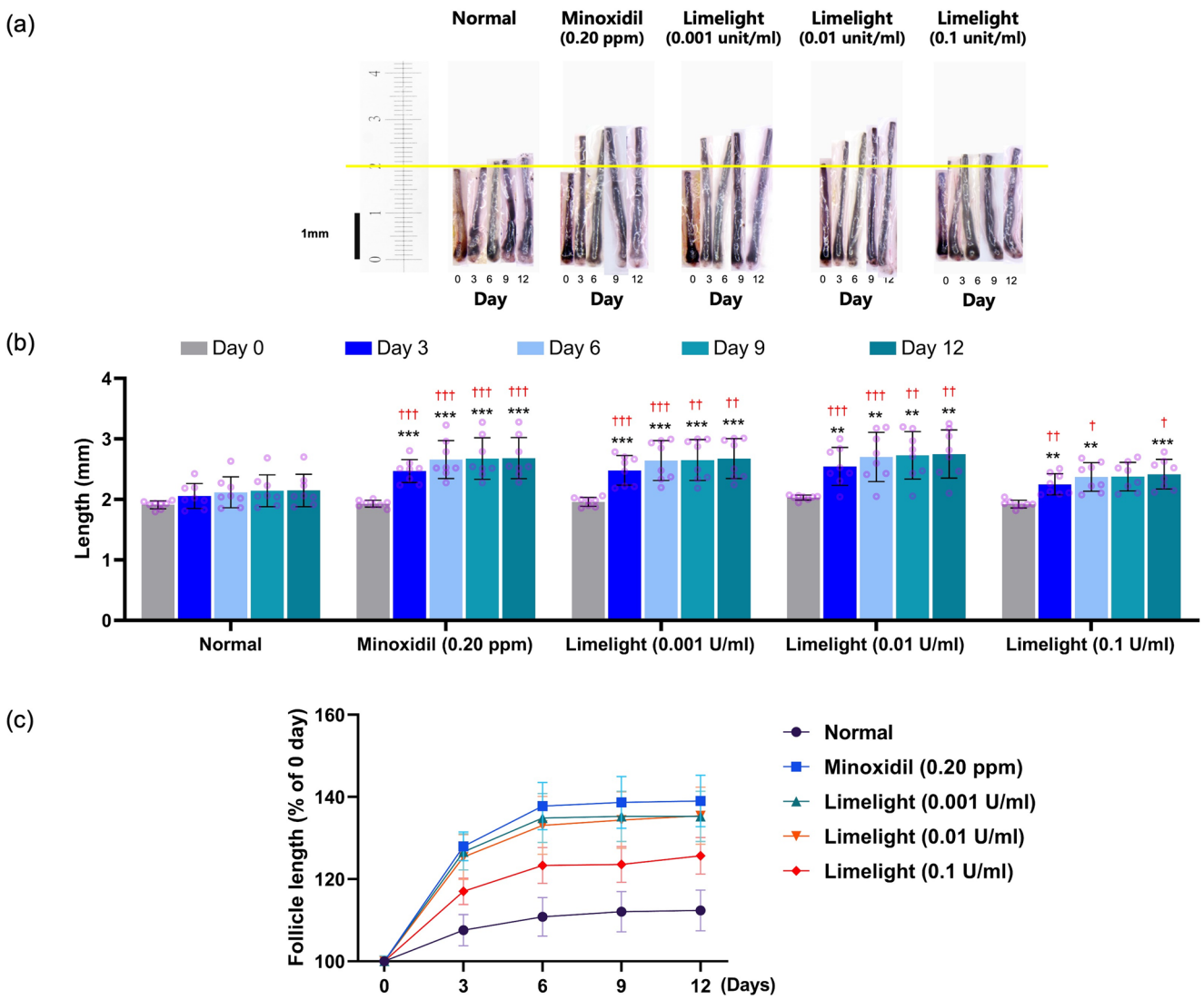

Fig. 4 HF length analysis for Donor 1. Visualization of hair length measurement under different treatments **a**. Quantitative of hair length in a column graph **b**. Quantitative of hair length in a line graph **c**. $N=8$ per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with 0-day point. †$p<0.05$, ††$p<0.01$, †††$p<0.001$ compared with untreated group. Normal: Untreated group; Minoxidil (0.20 ppm): Positive Control group; Limelight (0.001 U/ml): Test group A; Limelight (0.01 U/ml): Test group B; Limelight (0.1 U/ml): Test group C

respectively ($p<0.05$), but maintained hair bulb diameters 7.69%, 9.41%, 8.46%, and 8.22% larger than the control group after 12 days of culture (Fig. 7c, d).

For HF from Donor 3, the normal control group showed a significant hair bulb diameter reduction of 19.57% from the baseline ($p<0.05$). Test groups A, C, and the positive control group experienced more modest decreases of 11.90%, 10.66%, and 9.28%, respectively, while Test group B showed a significant reduction of 15.62% ($p<0.05$). After 12 days of culture, Test groups A, B, C, and the positive control group- maintained hair bulb diameters 7.67%, 3.95%, 8.91%, and 10.29% larger than the control group, respectively (Figs. 7e, f).

Limelight (CB-EVs) maintain anagen phase

Analysis of hair HF growth cycles from 3 donors revealed varying patterns of transition to the catagen phase:

For Donor 1, 25% and 37.5% of follicles in the normal control group transitioned to the catagen phase by days 9 and 12, respectively. Test groups A and C both showed lower transition rates of 25% at both time points, while Test group B and the positive control group exhibited higher rates of 37.5% and 62.5% at both days 9 and 12 (Fig. 8a).

For Donor 2, it demonstrated more pronounced differences. The control group showed high catagen transition rates of 75% on both days 9 and 12. In contrast, Test group A and positive control group maintained significantly lower

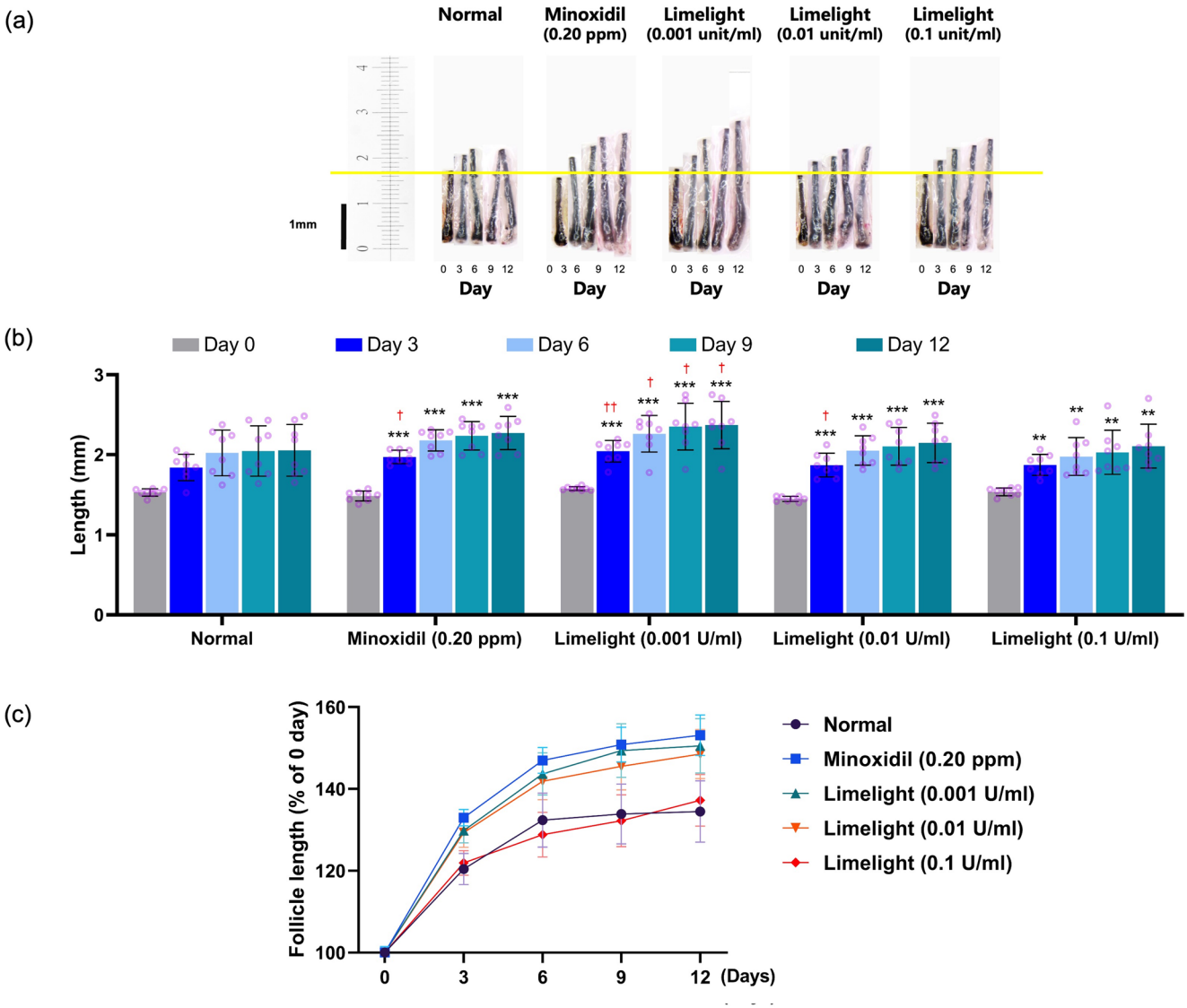

Fig. 5 HF length analysis for Donor 2. Visualization of hair length measurement under different treatments **a**. Quantitative of hair length in a column graph **b**. Quantitative of hair length in a line graph **c**. $N=8$ per group. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with 0-day point. $\dagger p<0.05$, $\dagger\dagger p<0.01$, $\dagger\dagger\dagger p<0.001$ compared with untreated group. Normal: Untreated group; Minoxidil (0.20 ppm): Positive Control group; Limelight (0.001 U/ml): Test group A; Limelight (0.01 U/ml): Test group B; Limelight (0.1 U/ml): Test group C

transition rates of 12.5%, 25% and 25%, 37.5% on days 9 and 12, respectively. Test groups B and C both displayed moderate transition rates of 37.5% and 50% at these time points. By day 12, all test groups maintained significantly lower proportions of follicles in the catagen phase compared to the control group (Fig. 8b).

For Donor 3, the normal group showed catagen transition rates of 12.5% and 37.5% on days 9 and 12, respectively. Test group A maintained consistent transition rates of 25% at both time points. Test group B showed the most delayed transition with 0% on day 9 and 37.5% on day 12, while Test group C exhibited transition rates of 12.5% and 37.5%, matching the control group by day 12. And positive control group showed the transition rates of 25% and 37.5% at the same time points (Fig. 8c).

Limelight (CB-EVs) activates laminin V and collagen XVIIa1

Laminin V and collagen XVII play critical roles in the development and maintenance of hair follicles. Laminin V contributes to epithelial invagination during hair follicle morphogenesis, while COLXVIIa1 is essential for the preservation and function of hair follicle stem cells. These biomarkers were quantified by immunohistochemical staining for the following 12 days of HF culture. Results showed that Laminin V expression increased substantially

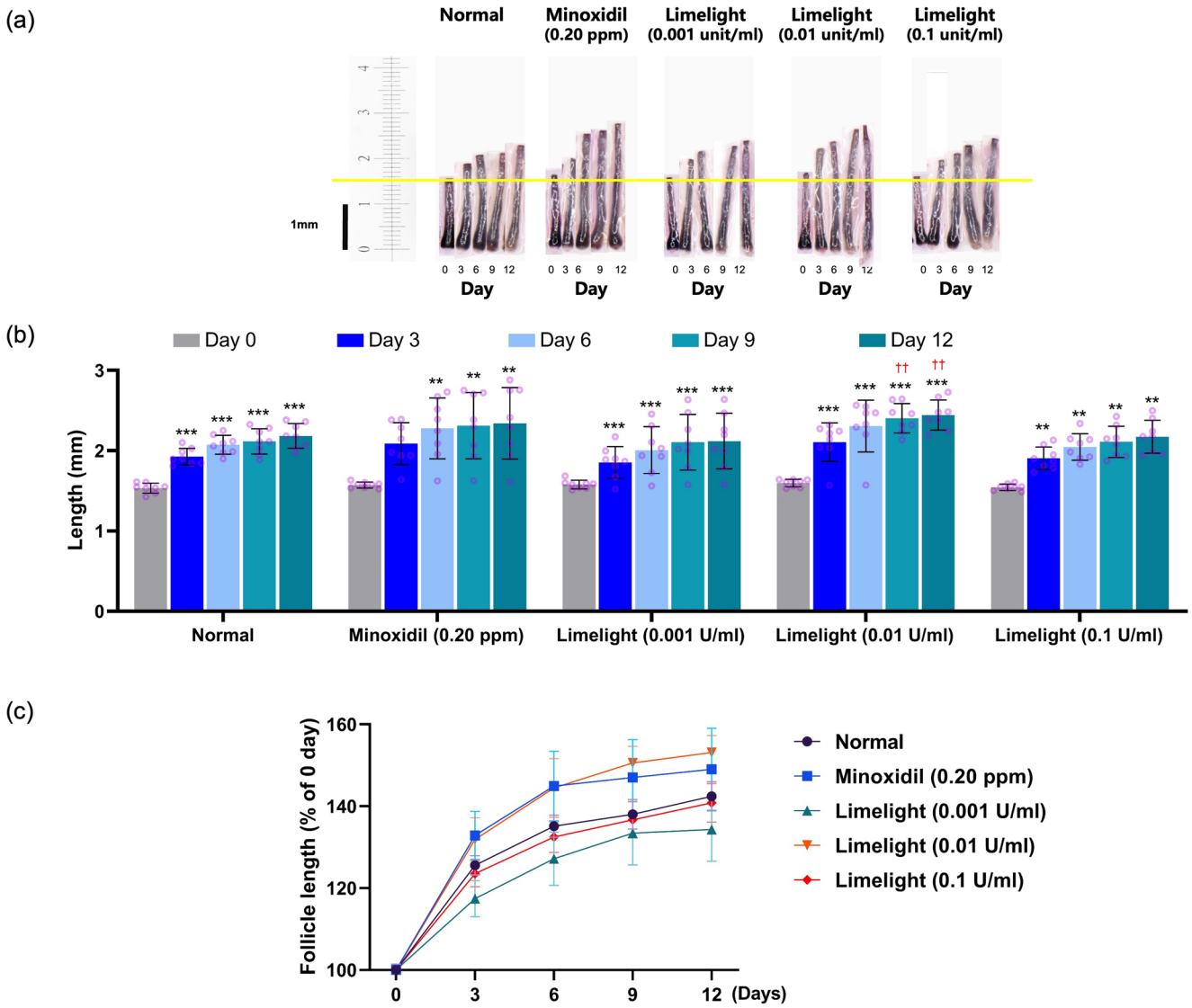

Fig. 6 HF length analysis for Donor 3. Visualization of hair length measurement under different treatments **a**. Quantitative of hair length in a column graph. **b** Quantitative of hair length in a line graph **c**. $N=8$ per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with 0-day point. †$p<0.05$, ††$p<0.01$, †††$p<0.001$ compared with untreated group. Normal: Untreated group; Minoxidil (0.20 ppm): Positive Control group; Limelight (0.001 U/ml): Test group A; Limelight (0.01 U/ml): Test group B; Limelight (0.1 U/ml): Test group C

in all treatment groups compared to the normal control group, with increases of 111.94%, 146.58%, 42.26%, and 126.11% in Test groups A, B, C, and the positive control group, respectively. The increase in Test group B was statistically significant ($p<0.05$). Similarly, Collagen XVIIa1 expression was elevated across all test groups compared to the normal group. Test group C showed a 31.26% increase, while Test groups A, B, and the positive control group demonstrated significant increases of 55.26%, 95.49%, and 69.82%, respectively ($p<0.05$) (Fig. 9). These findings indicated that Test group B showed the highest and statistically significant increase in both Laminin V and Collagen XVII α1 expression. And the concentration of Test B may produce the best results in supporting hair follicle development and stem cell maintenance.

Discussion

Limelight (CB-EVs) is a Conditioned Media Extraction Solution derived from human umbilical cord blood stem cells. Previous studies have demonstrated the potential of exosomes to enhance hair growth [27] through multiple mechanisms: regulating the hair growth cycle by promoting keratinocyte proliferation and migration, enhancing dermal papilla cell functionality [28]supporting hair follicle stem cell proliferation while inhibiting apoptosis, thereby

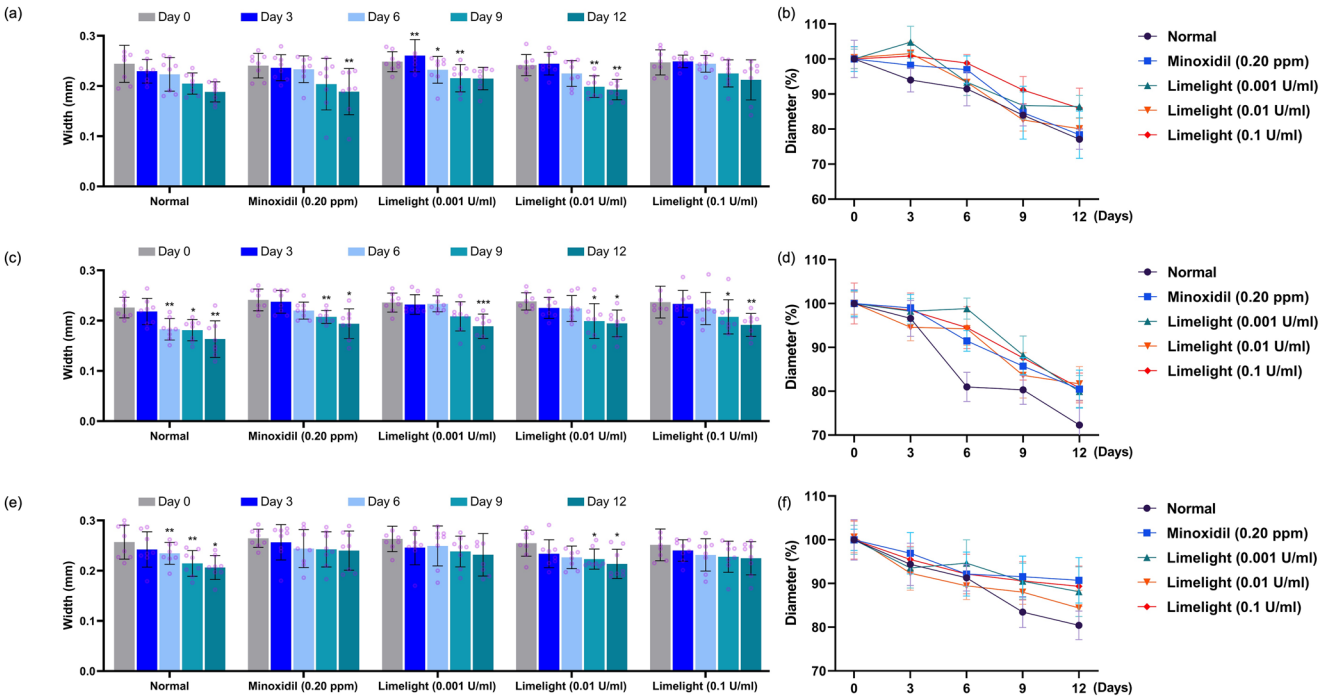

Fig. 7 Hair bulb diameter analysis. Quantitative of hair bulb diameter in a column graph for Donor 1 **a**, Donor 2 **c** and Donor 3 **e**. Quantitative of hair bulb diameter in a line graph for Donor 1 **b**, Donor 2 **d** and Donor 3 **f**. $N=8$ per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with 0-day point. †$p<0.05$, ††$p<0.01$, †††$p<0.001$ compared with untreated group. Normal: Untreated group; Minoxidil (0.20 ppm): Positive Control group; Limelight (0.001 U/ml): Test group A; Limelight (0.01 U/ml): Test group B; Limelight (0.1 U/ml): Test group C

extending the anagen growth phase [29]. Despite these promising findings, the FDA has raised concerns regarding stem cell-derived products. Furthermore, the effects of exosomes sourced from different cell types may vary depending on the specific medical condition being addressed. Limited research has been conducted on the use of umbilical cord blood-derived exosomes for promoting hair follicle growth. Therefore, we conducted ex vivo experiments to assess the safety and efficacy of Limelight (CB-EVs) in promoting hair growth, to generate preclinical evidence to predict its potential in vivo efficacy.

The cytotoxicity of Limelight (CB-EVs) on hDPCs was thoroughly evaluated via MTS assay, revealing that concentrations ranging from 5 to 15 U/ml maintained cell viability comparable to untreated controls ($p>0.05$). In contrast, concentrations of 25U/ml induced progressive cytotoxicity with maximal effect at 25 U/ml ($p<0.01$), establishing the non-cytotoxic threshold below 15 U/ml.

In ex vivo culture experiments with three distinct donors of isolated HFs, administration of Limelight (CB-EVs) resulted in enhanced follicular elongation across all tested concentrations compared to normal controls. Specifically, the group treated with 0.01 U/ml (Test group B) exhibited the most significant increase in hair follicle length. To address the slight variations in baseline lengths among groups, we also compared the percentage of hair elongation base on day 0. However, it is worth noting that the average elongation rate of untreated hair follicles in our study, 0.1–0.2 mm/day, was lower than the 0.3 mm/day reported in the literature during the initial phase of ex vivo culture [30]. This discrepancy may stem from donor-related factors, such as donor age. Nevertheless, our conclusions are primarily drawn from intra-donor comparisons, which remain valid for evaluating relative treatment effects.

Morphometric analysis indicated that although test group B exhibited a slight reduction in hair bulb diameter over time compared to its baseline, it retained a larger diameter than the normal control group by day 12 post-treatment. Furthermore, all Limelight (CB-EVs)-treated groups demonstrated significantly smaller reductions in bulb diameter compared to both the negative and positive control groups ($p<0.05$), suggesting a potential protective effect in supporting a prolonged anagen phase, as hair follicles transitioning into catagen are typically characterized by a marked decrease in bulb size.

Trichological cycle analysis demonstrated that although the proportion of anagen phase follicles in Test group B declined over time, it remained comparable to that of the normal control group at day 12. In contrast, Test group A (0.1 U/mL) significantly prolonged the anagen phase ($p<0.01$). While a sustained high growth rate is a hallmark of active anagen, it is important to note that hair shaft

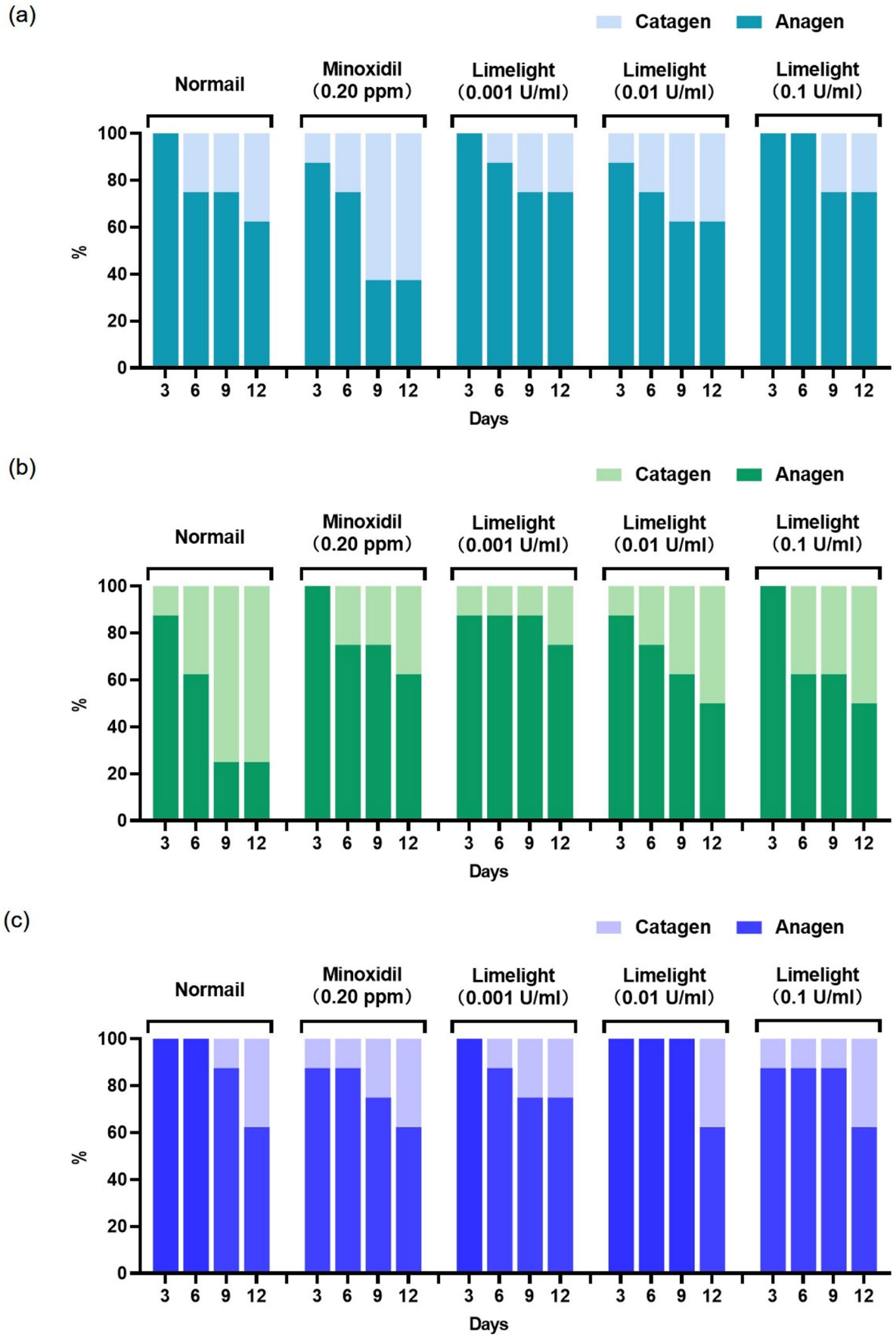
(a)
Catagen
Anagen
Normail
Minoxidil (0.20 ppm)
Limelight (0.001 U/ml)
Limelight (0.01 U/ml)
Limelight (0.1 U/ml)
%
Days
(b)
Catagen
Anagen
Normail
Minoxidil (0.20 ppm)
Limelight (0.001 U/ml)
Limelight (0.01 U/ml)
Limelight (0.1 U/ml)
%
Days
(c)
Catagen
Anagen
Normail
Minoxidil (0.20 ppm)
Limelight (0.001 U/ml)
Limelight (0.01 U/ml)
Limelight (0.1 U/ml)
%
Days

Fig. 8 Hair growth cycle analysis. Proportion of hair follicles in anagen (growth phase) versus catagen (regression phase) for Donor 1 **a**, Donor 2 **b**, and Donor 3 **c**. $N = 8$ per group. Normal: Untreated group; Minoxidil (0.20 ppm): Positive Control group; Limelight (0.001 U/ml): Test group A; Limelight (0.01 U/ml): Test group B; Limelight (0.1 U/ml): Test group C

elongation may persist at a slower rate during early catagen due to passive extrusion of the developing club hair. Thus, a gradual reduction in elongation rate, rather than an abrupt cessation, may indicate the onset of catagen. In our study, all Limelight (CB-EVs) treated groups exhibited continued hair shaft elongation between days 9 and 12, although the rate of elongation decreased markedly during this period. This pattern suggests that, under ex vivo culture conditions, some follicles may have entered early catagen after day 9, which confirms the changes we observed under microscope.

Laminin V, a pivotal extracellular matrix protein, maintains dermal-epidermal junction integrity while facilitating cellular proliferation, adhesion, and migration processes [31]. Its deficiency leads to epidermolysis, highlighting its importance in cutaneous stabilization during development [32]. Several studies have confirmed its contributory role in follicular growth promotion by activating the FAK/PI3K/AKT pathway to enhance migration and proliferation of hDPCs [33, 34]. Our research findings indicated that Limelight (CB-EVs) significantly upregulated Laminin V expression by day 12 post-treatment, with test group B (0.01 U/ml) exhibiting maximal induction ($p < 0.005$).

Concurrently, Collagen XVII, a transmembrane hemidesmosomal component, mediates keratinocyte-basement membrane adhesion and is critical for hair follicle stem cell maintenance. Age-related dysfunction of hair follicle stem cells is characterized by the accumulation of DNA damage, leading to upregulation of neutrophil elastase. This enzyme degrades Collagen XVII, resulting in the transformation of HFSC into epidermal keratinocytes with consequent follicular miniaturization and ultimately hairless epidermal transformation [35]. Immunohistochemical analyses demonstrated that treatment with Limelight (CB-EVs) increased the expression of Collagen XVIIa1 in a dose-dependent manner by day 12.

Nevertheless, we acknowledge that follicle viability and anagen integrity may progressively decline after 7–9 days of ex vivo culture [24]. This limitation could influence the interpretation of certain structural changes observed at later time points. Meanwhile, the morphological features of many follicles at day 12 deviated from canonical anagen or catagen stages. Therefore, the increased Laminin V and Collagen XVII staining observed in treated follicles at day 12 must be interpreted with caution, as it may partly reflect catagen-associated changes due to prolonged culture.

Several prior studies have questioned the suitability of the human hair follicle ex vivo model for evaluating minoxidil's effects. Magerl et al. observed that minoxidil failed to significantly promote hair elongation or prolong anagen in organ-cultured scalp follicles [25]. They hypothesized that this discrepancy from ex vivo results could stem from the inherent limitations, including the lack of a vascular supply and limited drug penetration, as well as the use of fully grown anagen VI follicles and suboptimal drug metabolism or dosing. This casts some doubt on the standard organ culture's sensitivity to minoxidil. On the other hand, evidence also suggests the model is not universally unresponsive: Kwon et al. reported that when follicles are selected in an early anagen stage, minoxidil treatment can significantly increase hair shaft growth ex vivo [20]. This indicates that with careful experimental design, the organ culture can recapitulate minoxidil's hair growth-promoting activity. In our study, we included minoxidil at 0.2 ppm (1 μM) as a positive control, aligning with previously published studies demonstrating efficacy without cytotoxic effects [21, 22].

In line with our investigation, recent research has demonstrated that exosomes derived from UC-MSCs modulate the stemness of hair follicle stem cells via the RAS/ERK pathway, facilitating hair proliferation [28, 36]. This modulation further influences the hair growth cycle by enhancing the proliferation and migration of keratinocytes within hair follicles. Previous studies have indicated that exosomes from bone marrow mesenchymal stem cells stimulate the proliferation and migration of DP cells through AKT phosphorylation and elevated Bcl-2 levels, while also upregulating VEGF and IGF-1 expression in DP cells to promote scalp angiogenesis and hair growth [18]. Likewise, exosomes derived from adipose tissue can impact DP cell behavior by modulating miR-22 levels post-transcriptionally, activating the Wnt/β-catenin pathway, and regulating cell proliferation, migration, and apoptosis [37, 38]. Notably, dermal papilla cells, a distinct subset of mesenchymal stem cells, release exosomes that upregulate β-catenin and Sonic hedgehog (SHH), thereby fostering hair follicle development both in vivo and in vitro [39]. Exosomes derived from diverse sources have demonstrated potential in promoting hair growth [40]. However, the unique advantages of umbilical cord blood-derived exosomes warrant emphasis. UC-MSCs exhibit superior proliferative capacity and lower immunogenicity [41, 42]. The standardized production process of Limelight (CB-EVs) may also ensure more consistent outcomes, though batch-to-batch variability in exosomal cargo requires further validation.

In 2019, the FDA raised concerns regarding stem cell products, especially those derived from human tissues such as umbilical cord blood. Limelight (CB-EVs) is an umbilical cord blood-derived exosome cosmetic product. In this

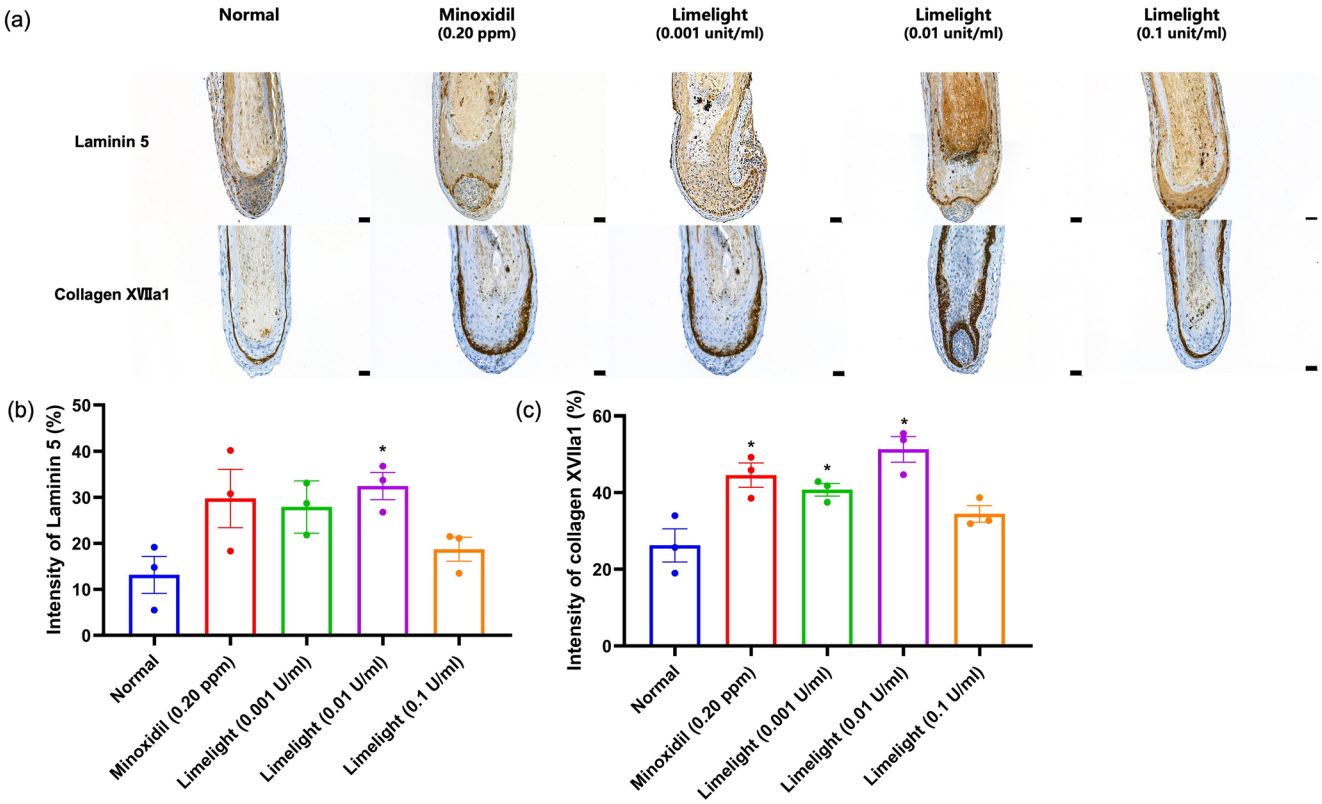

Fig. 9 IHC staining of HF. The HF organ staining of Laminin V (upper raw) and Collagen XVIIa1 (lower raw) **a**. Quantification analyses of intensity of Laminin V in the HF **b**. Quantification analyses of intensity of Collagen XVIIa1 in the HF **c**. Scale bar = 50 μm. $N = 8$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with untreated group. Normal: Untreated group; Minoxidil (0.20 ppm): Positive Control group; Limelight (0.001 U/ml): Test group A; Limelight (0.01 U/ml): Test group B; Limelight (0.1 U/ml): Test group C

study, we sought to evaluate the biological effects of Limelight on hair follicle growth through ex vivo experimentation. Additionally, we aimed to perform a preliminary assessment of its safety, which may serve as foundational data for potential future preclinical or clinical investigations under appropriate regulatory oversight. Meanwhile, more and more clinical studies have recently demonstrated the safety of exosomes in stimulating hair growth without significant adverse effects [27, 43, 44]. However, these studies primarily consist of small cohort and self-controlled experiments conducted in laboratory settings, lacking randomized controlled double-blind trials. We anticipate that advancements in exosome isolation techniques and ongoing research will lead to the development of standardized, safe, and efficacious exosome products for clinical use. Our research aligns with this perspective, as we have conducted ex vivo experiments to support this notion. Subsequent animal studies and randomized controlled double-blind clinical trials are planned to further investigate this potential.

Future investigations necessitate expanded cohorts encompassing both healthy subjects and alopecia patients, complementary in vivo experimentation addressing scalp microvasculature modulation, animal models incorporating microneedle delivery systems, and prospective randomized controlled clinical trials to advance this promising therapeutic strategy from ex vivo foundation to clinical application.

Conclusion

Our research focuses on finding solutions to the problem of thinning hair and preventing hair loss. In this study, we investigated the effects of Limelight (CB-EVs), an exosome product derived from human umbilical cord blood-derived mesenchymal stem cells, on HFs ex vivo. Our results demonstrate that this product stimulates HF elongation, preserves HF structure, and reduces the transition of HFs to the catagen phase, and this was associated with increased Laminin V and Collagen XVII staining in the follicle's lower region. These outcomes indicate that the product represents a secure and efficient approach to promoting hair growth ex vivo. Based on our results, we recommend an optimal concentration of approximately 0.01 U/ml for Limelight (CB-EVs), as this dose maximized hair growth benefits (including extended anagen duration) without causing cytotoxic effects.

Author contributions Chan Yeong Heo and Seungchan, Kim conceptualized the study and designed it. Hye Won Park collected and prepared the samples and did the ex vivo experiment. Thuy-Tien Thi Trinh did the in vitro experiment. Data collection and analysis were performed by Caijun Jin, Teak In Oh and Pham Ngoc Chien, with Xinrui Zhang, Yongxun Jin, Linh Thi Thuy Le, and Nguyen Ngan Giang in a supporting role. The first draft of the manuscript was written by Caijun Jin and Hye Won Park. Teak In Oh and Pham Ngoc Chien critically reviewed the manuscriptAll authors commented and confirmed the final version of the manuscript. All authors read and approved the arrangement.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest Seungchan Kim is the Director of IEUL Plastic Surgery Clinic, which is developing the Limelight (CB-EVs) product. All other authors declare no conflict of interest.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Korean Skin Research Center of H&Bio Company in Seongnam, Korea (HBABN01-240403-HRBR-E0013-01).

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