

Activity and Safety of Cinchonine Nanostructured Lipid Carriers as a Hair Growth Stimulant in Mice Model of Androgenetic Alopecia

(Aktiviti dan Keselamatan Pembawa Lipid Berstruktur Nano Sinkonina sebagai Perangsang Pertumbuhan Rambut dalam Model Tikus Alopecia Androgenetik)

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Received: 21 February 2022/Accepted: 9 June 2023

ABSTRACT

Androgenetic alopecia (AA) is a hair growth disorder characterized by hair loss and miniaturization of the size of follicles and dermal papillae. Cinchonine is a quinoline alkaloid that can increase VEGF production and potential as a hair growth stimulant. This study aimed to determine the morphology, penetration (*in vitro*), as well as safety and activity of cinchonine nanostructured lipid carriers (CN-NLC) serum as a hair growth stimulant. Preparation of CN-NLC serum using a combination of micro-emulsification and ultra-sonification methods, characterization of CN-NLC serum included morphology and closed hair follicle diffusion methods. Dermal and eye irritation test using New Zealand rabbit strain with primary irritation index parameters. Hair growth stimulant activity test using Swiss Webster mice strain, induced with the hormone testosterone. Observations were performed at 7, 14, and 21 days with hair length and histology parameters. CN-NLC serum morphology is a spherical shape with size ± 500 nm, diffusion of serum CN-NLC (open hair follicles) of $23.92 \pm 0.84\%$; (close hair follicles) of $11.37 \pm 2.29\%$; and CN solution of $6.00 \pm 0.72\%$. CN-NLC serum are non-irritant with a primary irritation index of 0.0. Activity tests showed hair length on days 7; 14 and 21 were increased by 20.24-23.74%; 33.47-36.43%, and 39.30-39.97% ($P \leq 0.05$). Histological data showed an increase in the number and size of both dermal papillae and hair follicles compared to the control group. CN-NLC serum can improve the penetration of CN into hair follicles. CN-NLC serum is safe and effective as a hair growth stimulant in the treatment of AA.

Keywords: Androgenic alopecia; cinchonine; nanostructured lipid carriers

ABSTRAK

Alopecia androgenetik (AA) adalah gangguan pertumbuhan rambut yang dicirikan oleh keguguran rambut dan pengecilan saiz folikel dan papila dermal. Sinkonina adalah alkaloid kuinolina yang boleh meningkatkan pengeluaran VEGF dan berpotensi sebagai perangsang pertumbuhan rambut. Kajian ini bertujuan untuk menentukan morfologi, penembusan (*in vitro*), serta keselamatan dan aktiviti serum pembawa lipid berstruktur nano (CN-NLC) sinkonina sebagai perangsang pertumbuhan rambut. Penyediaan serum CN-NLC menggunakan gabungan kaedah pengemulsi mikro dan ultra-sonifikasi, pencirian serum CN-NLC termasuk kaedah morfologi dan kaedah penyebaran folikel rambut tertutup. Ujian kerengsaan kulit dan mata menggunakan ketegangan arnab New Zealand dengan parameter indeks kerengsaan primer. Ujian aktiviti perangsang pertumbuhan rambut menggunakan ketegangan tikus Swiss Webster, diinduksi dengan hormon testosteron. Pemerhatian dilakukan pada 7, 14 dan 21 hari dengan panjang rambut dan parameter histologi. Morfologi serum CN-NLC ialah bentuk sfera dengan saiz ± 500 nm, resapan serum CN-NLC (folikel rambut terbuka) sebanyak $23.92 \pm 0.84\%$; (folikel rambut tertutup) sebanyak $11.37 \pm 2.29\%$; dan larutan CN sebanyak $6.00 \pm 0.72\%$. Serum CN-NLC tidak merengsa dengan indeks kerengsaan primer 0.0. Ujian

aktiviti menunjukkan panjang rambut pada hari ke-7; 14 dan 21 meningkat sebanyak 20.24-23.74%; 33.47-36.43% dan 39.30-39.97% ($P \leq 0.05$). Data histologi menunjukkan peningkatan dalam bilangan dan saiz kedua-dua papila dermal dan folikel rambut berbanding kumpulan kawalan. Serum CN-NLC dapat meningkatkan penembusan CN ke dalam folikel rambut. Serum CN-NLC selamat dan berkesan sebagai perangsang pertumbuhan rambut dalam rawatan AA.

Kata kunci: Alopecia androgenetik; pembawa lipid berstruktur nano; sinkonina

INTRODUCTION

Androgenetic alopecia (AA) is a hair growth cycle disorder caused by the sensitivity of the follicles and dermal papilla to androgen hormones, resulting in hair loss and miniaturization (Inaba & Inaba 1996; Santos et al. 2020). The miniaturization of hair follicles and dermal papillae is also exacerbated by genetic factors and vascularization (Blume-Peytavi, Varvar & Annika 2016; Blume-Peytavi et al. 2008). Currently, minoxidil is widely used to treat AA, but long-term use can cause irritation, rashes, dermatitis, and skin sensitivity (Fresta et al. 2020; Satheeshan, Seema & Manjusha 2020). The development of Cinchonine (CN) as a hair growth stimulant for the treatment of AA is a very promising drug candidate (Hariyanti, Damayanti & Darijanto 2020).

CN, a Cinchona alkaloid, has 3 main functional groups, namely the aromatic quinoline ring, the quinuclidine ring, and the methylene alcohol group (Hariyanti et al. 2022). It has the same active site located on the nitrogen atom in the quinuclidine ring and the methylene alcohol functional group, which plays an important role in their pharmacological activity (Hariyanti et al. 2022). Furthermore, CN has been found to have pharmacological activity as a hair growth stimulant. This activity is achieved through the acceleration of hair entering the anagen phase by activating the Wnt/ β -catenin pathway, increasing VEGF (Vascular Endothelial Growth Factor) production (Leveque et al. 2021), and telangiectatic activity by vasodilation of blood vessels (Inaba & Inaba 1996). Therefore, CN must be able to reach hair follicles and dermal papillae to optimize hair growth stimulant activity.

Hair follicles and dermal papillae have unique skin barriers. These include arco infundibulum, which is a stratum corneum on the surface of the infundibulum of diameter $\pm 750 \mu\text{m}$, Tight Junctions (TJs), and sebum (Gorzalanny et al. 2020; Yokouchi & Kubo 2018). Therefore, a suitable delivery system is required to facilitate CN reaching hair follicles and dermal papillae, namely the Nanostructured Lipid Carrier (NLC) (Joshi, Prabhu & Patravale 2019). NLC is the 2nd generation

of Solid Lipid Nanoparticles (SLN), formed from a mixture of solid and liquid lipids with surfactant stabilizers (Patzelt & Lademann 2020). NLC with a particle size of 300 - 640 nm can localize in hair follicles (Lademann et al. 2019, 2015). Therefore, this study aims to determine the safety and activity of CN-NLC serum as a hair growth stimulant in AA.

MATERIALS AND METHODS

MATERIALS

Materials used in this study were pharmaceutical grade, including CN (PT. Sinkona Indonesia Lestari, Bandung, Indonesia), stearic acid, oleic acid, polysorbate 80, and glycerin (all from Bratachem, Bandung, Indonesia), deionized water (Amidis, Bandung, Indonesia), testosterone injection (organon, Germany), solid paraffin (Bratachem, Bandung, Indonesia), acetonitrile (Merck, Germany), aqua pro injection (IPHA, Bandung, Indonesia), KH_2PO_4 (Merck, Germany), hematoxylin and eosin (Sigma-Aldrich, Germany). The animal used for the test involved male rabbits of the New Zealand strain of 6, aged around 6-7 months and weighing about 2 kg. Male mice of the Swiss Webster strain, 9 in each group for activity test, aged 9 -10 weeks, with a body weight of 35 - 40 g, were also used.

INSTRUMENT

The instrument used included analytical Balance (mg) (ME 204, Mettler Toledo, Indonesia), Magnetic stirrer (RT 15, IKA, Malaysia), Ultra turrax (T18, IKA, Malaysia), bath sonicator (falc LBS2, Akribis Scientific Ltd, UK), Probe Sonicator (CY-500 Ultrasound Homogenizer, JP Selecta, Spain), HPLC (Waters 2487, Agilent, USA), and Silversil column (C18. 250 \times 4.6 mm, DikmaTech, USA). Other instruments included Binocular Microscope Olympus (CX23, Evident Headquarters, Japan), Transmission electron microscopy (TEM HT7700,

Hitachi, Japan), Franz diffusion cells (Delta laboratorium, Malang, Indonesia), rotary microtome (Minux S700, PT. Indogen Intertama, Indonesia), and glassware that is often used in laboratories.

CN-NLC SERUM FORMULATION

The lipid phase, consisting of 1.8% w/v stearic acid, 0.2% w/v oleic acid, and 0.18% w/v CN, was heated to a temperature of 70 °C. Similarly, the aqueous phase consisting of 3.5% w/v polysorbate 80, 2.5% w/v glycerin, and deionized water up to 100% w/v was heated to a temperature of 70 °C. The aqueous phase was added to the lipid phase and homogenized with a magnetic stirrer for 20 min at 70 °C and 800 rpm. A hot microemulsion was formed after the homogenization with ultra turrax at 8000 rpm for 2 min. This was followed by bath sonication at a frequency of 59 kHz for 15 min, solidification at a temperature of 4 °C for 30 min, and probe sonication for 15 min (pulse on-off 45:15 s, amplitude 60%). The CN-NLC formed was stored overnight at 4±2°C in the refrigerator (Chen et al. 2020; Kakadia & Conway 2018; Kharat & McClements 2019; Pereira et al. 2021; Pires et al. 2019; Santos et al. 2018; Souto 2004; Yazdani-Arazi et al. 2017).

CINCHONINE (CN) SOLUTION FORMULATION

A total of 0.18% CN was dissolved with mixed solvents, such as PEG 400, Ethanol 90%, and deionized water, with a ratio of 40:30:30, and then vortexed for 30 min.

MORPHOLOGICAL ANALYSIS OF CN-NLC SERUM

Morphological observations were carried out using Transmission Electron Microscopy (TEM). CN-NLC serum (±15 µL) was placed on the grid and then analyzed using TEM.

DIFFUSION TEST OF CN-NLC SERUM

The diffusion test using the Franz diffusion cell was carried out using the skin of Swiss Webster male mice. A pair of skins with equal areas were sheared and carefully separated. Excess subcutaneous fat was carefully removed using a scalpel with the pinch method. The skin samples were cleaned of hair residue to open hair follicles by adding a drop of power glue (99.0%, w/w cyanoacrylate) to the skin and covered by a glass slide with slight pressure. After glue polymerization (5 min),

the slide was removed swiftly to expose all follicles. Furthermore, the skin was used as the active and blocked/inactive hair follicles diffusion membrane (Bubić Pajić et al. 2019; Kim et al. 2019a; Kim et al. 2019b).

Nail polish was applied evenly to the skin of active Swiss Webster male mice using a suitable brush to block hair follicles (inactive hair follicles). After 30 s, the area was covered with plastic, nail polish, and pressed into the follicle using a roller. Subsequently, any remaining nail polish/varnish was removed from the skin surface using a glass slide and two strips of tape. Since the skin was inactive, the nail polish/varnish persisted selectively in the follicles (Bubić Pajić et al. 2019; Kim et al. 2019a; Kim et al. 2019b).

Trans follicular diffusion of the sample (CN-NLC serum) was carried out using skin with active or inactive hair follicles using the Franz diffusion cell. The donor compartment (epidermis) was added with 1000 µL of CN-NLC serum. The receptor compartment was added with phosphate-buffered saline (PBS) to represent the subcutaneous tissue and circulating blood. The diffusion test was carried out with a constant stirring speed of 100 rpm and a temperature of 37 ± 2°C. The test was carried out for 8 h with a sampling time interval of 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, and 8 h. A total of 1000 µL of each sample was taken from the receptor and replaced with the same amount of fresh PBS solution. Samples were analyzed using HPLC (Bubić Pajić et al. 2019; Kim et al. 2019a; Kim et al. 2019b; Hariyanti et al. 2023). Phosphate-Buffered Saline pH 7.4: A total of 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄ were dissolved in 800 mL of distilled water, adjusted pH 7.4 with NaOH, and add 1000 mL of distilled water. CN-NLC serum Diffusion test has been approved by the Animal Research Ethics Committee of the Bandung Institute of Technology No. 03/KEPHP-ITB/2-2022.

IN VIVO ASSAY OF CN-NLC SERUM

During the acclimatization process, which lasted for 7 days, all test animals were carefully cared for in an environment with a temperature of 25 ± 2 °C and humidity (RH) of 72 ± 3%, with a cycle of 12 h dark/12 h light. During acclimation, observations were carried out on activities, weight, eating, and drinking habits to ensure that the test animals were in good health.

DERMAL IRRITATION TEST OF CN-NLC SERUM

The test animals were shaved from the scapula (shoulder) area to the groin (lumbar spine) using electric clippers designed for animals. The shaver is cleaned with 70% alcohol before the clippers are used. All test animals were shaved 24 h before the test with an area of approximately 10 × 15 cm, which was divided into four, namely test preparation (CN-NLC serum), blank control (NLC without CN), CN solution preparation, and normal control (not given any treatment). The formulas (each ± 0.5 mL) were applied to a test area of approximately 6 (2 × 3) cm² accompanied by light massage for 3 min, which was then covered with gauze and glued with non-irritant tape. After 4 h, the residue of the test preparation was washed off the skin with water. Observations and calculations of the erythema and edema indices were made at intervals of 1, 24, 48, and 72 h after removing the plaster. Observations were continued until day 14 to determine skin reversibility based on the Globally Harmonized System (GHS) (Jimenez et al. 2021; Kim et al. 2021; Rooney et al. 2021). CN-NLC serum dermal irritation test has been approved by the Animal Research Ethics Committee of the Bandung Institute of Technology No. 04/KEPHP-ITB/2-2022.

EYE IRRITATION TEST OF CN-NLC SERUM

It was confirmed that the eyes of the test animals were healthy 24 h before applying the preparation. A total of 0.1 mL of the test preparation was applied with a single dose in one eye, and the other was used as a control. Observations on the degree of irritation/corrosion of the conjunctiva, cornea, and iris were made at intervals of 1, 24, 48, and 72 h after applying the preparation. It was continued until day 21 to determine the effect of eye reversibility based on the Globally Harmonized System (GHS) (Kim et al. 2021). The CN-NLC serum eye irritation test has been approved by the Animal Research Ethics Committee of the Bandung Institute of Technology No. 05/KEPHP-ITB/2-2022.

ANDROGENIC ALOPECIA MICE MODEL

Test animals, except for the negative control, were induced with the hormone testosterone through subcutaneous injection. The injection volume was 0.1 mL/day with a dose of 10 mg/kg body weight. Induction was carried out for 20 days until AA occurred. AA was identified based on hair loss and morphology, which became shorter and thinner than the negative control. On the 21st day, the hair on the dorsal part of the animal was shaved to obtain a test area of 2 × 3 cm² (Fu et al. 2021; Kim et al. 2019b; Truong et al. 2017; Wang et al. 2021, 2017; Zhang et al. 2016).

TABLE 1. Group of test animals

Groups	Testosterone induction (10 mg/kg body weight/day)	Formula concentration (%)	The amount of preparation applied (mL)
Negative control (healthy)	No	-	No
Positive control (AA)	Induction	-	No
Blank (NLC)	Induction	NLC	0,1/day
Minoxidil	Induction	2	0,1/day
CN solution	Induction	0,18	0,1/day
CN-NLC serum (1)	Induction	0,18	0,1/day
CN-NLC serum (2)	Induction	0,18	0,1/2 days

ACTIVITY TEST OF CN-NLC SERUM

The rest of the animal's hair was removed with hair removal cream. CN-NLC serum was applied \pm 0.1 mL with a frequency of 1 time/day and 1 time/2 days, followed by a light massage for 3 min. Observations were made for 21 days at intervals of 7, 14, and 21, with the hair length parameter, which is the longest strands. At each sampling point, 3 test animals were randomly selected for sacrifice and histology (Lademann et al. 2019, 2015, 2007; Patzelt & Lademann 2020; Tanaka, Saito & Tabata 1980; Toll et al. 2004)".

DERMAL HISTOLOGY

Mice were randomly selected and sacrificed at each observation at intervals of 7, 14, and 21 days. The skin of each animal's dorsal area (2×3 cm) was shaved and dissected, rinsed with PBS, and fixed in 4% paraformaldehyde buffer at room temperature for a maximum of 24 h. Samples were embedded in solid paraffin, cut using a rotary microtome with a thickness

of 5 μ m, and stained with hematoxylin and eosin (H&E). The CN-NLC serum activity test has been approved by the Animal Research Ethics Committee of the Bandung Institute of Technology No. 03/KEPHEP-ITB/2-2022.

DATA ANALYSIS

The data collected in triplicate ($n = 3$) were presented as mean \pm standard deviation and analyzed statistically using the Kolmogorov Smirnov test, one-way ANOVA ($p \leq 0.05$), and a Post Hoc test (Fisher's LSD) using Minitab Statistical Software (Minitab 21).

RESULTS AND DISCUSSION

MORPHOLOGICAL ANALYSIS OF CN-NLC SERUM

TEM analysis (Figure 1) showed that serum CN-NLC is spherical with a particle size of \pm 500 nm. Particle size plays a significant role in penetration, specifically through hair follicles. This is due to several barriers in hair follicles, including physical and chemical barriers.

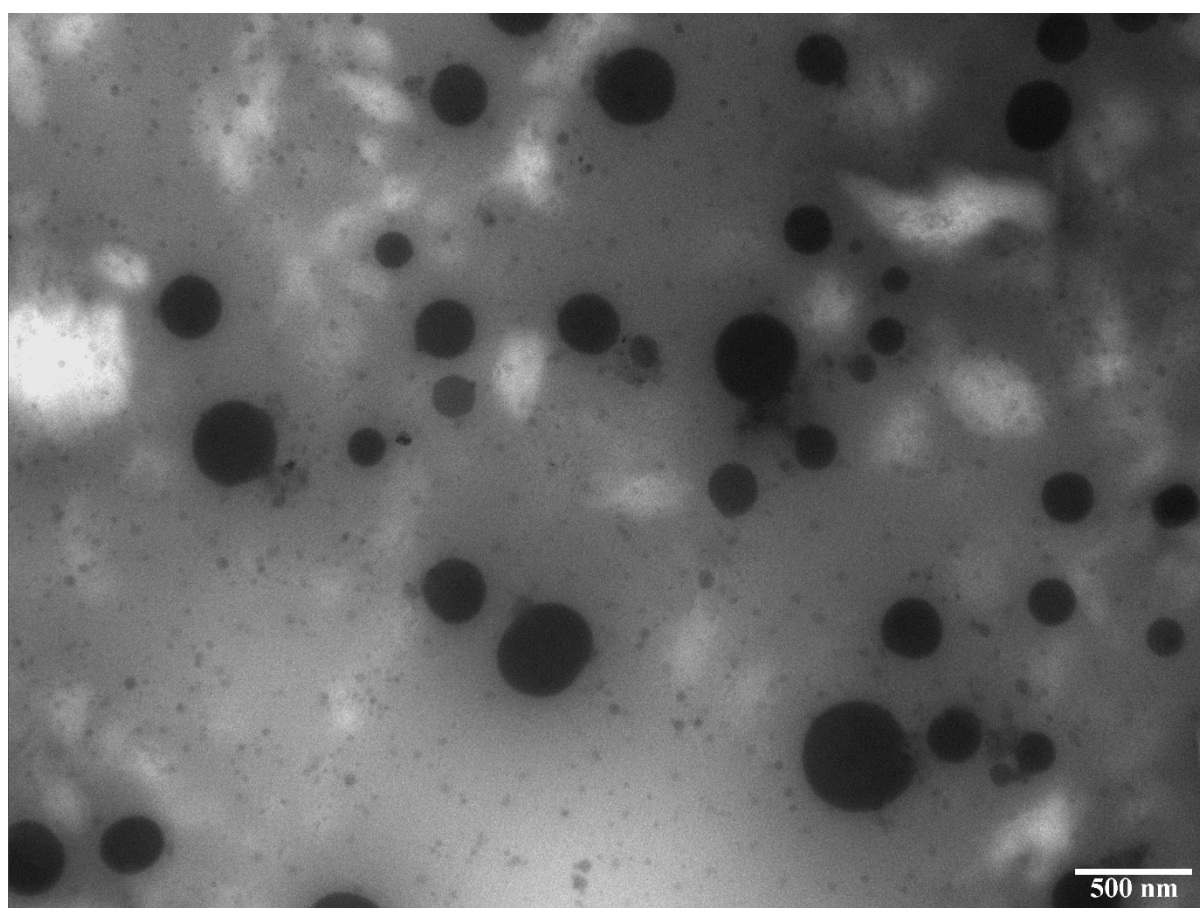


FIGURE 1. Morphology of CN-NLC serum

The physical barriers include the presence of sebum whose flow direction is towards the surface of the skin, the anatomy of hair follicles such as the infundibulum with a diameter of ± 750 nm, arco infundibulum, and TJs. CN-NLC particle size between 300-640 nm has optimal penetration ability into hair follicles. A particle size of <300 nm is generally pushed out toward the skin's surface by the sebum flow, and sizes >640 nm are retained on the surface of the infundibulum (Lademann et al. 2019, 2015).

DIFFUSION TEST OF CN-NLC SERUM

The diffusion data in Figure 2 showed that the NLC serum delivery system resulted in a CN penetration of $23.92 \pm 0.84\%$ and was higher than conventional CN dosage forms ($6.00 \pm 0.72\%$). This result was consistent with the report of (Yazdani-Arazi et al. 2017) that preparations with an NLC delivery system can penetrate and reach hair follicles. In contrast, solution preparations are only able to reach the surface of the skin or stratum corneum. There was a significant increase in the penetration of CN-NLC serum into open hair follicles

($11.37 \pm 2.29\%$) compared to CN-NLC serum with closed follicles or CN solution. This shows that the NLC drug delivery system is very potent and selective for targeting active substances in hair follicles. A previous study also reported that hair follicles contribute 34 - 60% of the total amount of active substances penetrating the skin (Abd et al. 2018; Mohd et al. 2016; Todo & Mohd 2017; Vitorino, Sousa & Pais 2015).

DERMAL IRRITATION TEST OF CN-NLC SERUM

The dermal irritation test in Table 2 showed that CN was non-irritant with an erythema and edema index of 0, determined by comparing the CN-NLC serum test area to the negative control. The additives used in serum preparations are non-irritant with an erythema and edema index of 0, determined through a comparison between negative and normal control. Therefore, it can be concluded that CN and additional ingredients used in the process of making CN-NLC serum preparations are non-irritating with a primary irritation index of 0.0 (Jimenez et al. 2021; Kim et al. 2021; Rooney et al. 2021).

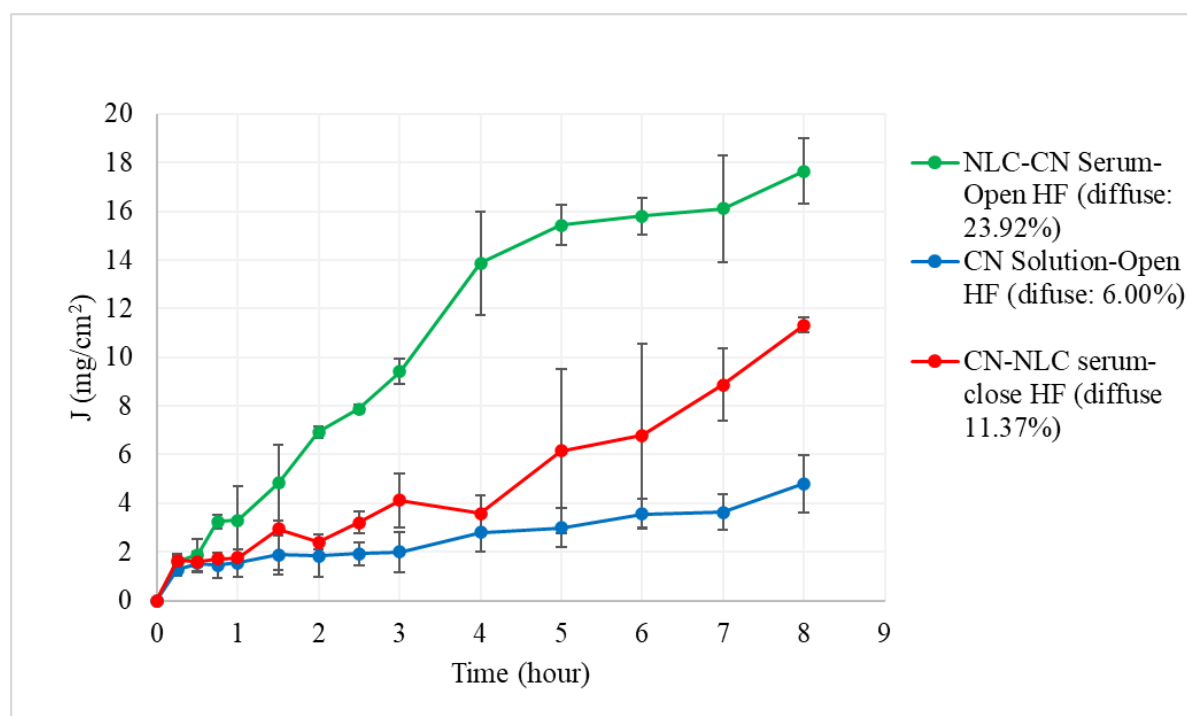


FIGURE 2. Diffusion of CN-NLC serum

TABLE 2. CN-NLC serum dermal irritation test

Group	Hour	Rabbit 1		Rabbit 2		Rabbit 3		Primary irritation index
		Erythema	Edema	Erythema	Edema	Erythema	Edema	
Negative control (healthy)	1	0	0	0	0	0	0	0.0
	24	0	0	0	0	0	0	
	48	0	0	0	0	0	0	
	72	0	0	0	0	0	0	
Blank control (NLC)	1	0	0	0	0	0	0	
	24	0	0	0	0	0	0	
	48	0	0	0	0	0	0	
	72	0	0	0	0	0	0	
CN solution	1	0	0	0	0	0	0	
	24	0	0	0	0	0	0	
	48	0	0	0	0	0	0	
	72	0	0	0	0	0	0	
CN-NLC Serum	1	0	0	0	0	0	0	
	24	0	0	0	0	0	0	
	48	0	0	0	0	0	0	
	72	0	0	0	0	0	0	

EYE IRRITATION TEST OF CN-NLC SERUM

Table 3 shows that all materials used are non-irritant, with a primary irritation index of 0.0.

CN-NLC SERUM ACTIVITY TEST (ANDROGENETIC ALOPECIA MICE MODEL)

The data in Figure 3 showed that the hair length of the negative control area (healthy) was significantly different from the positive (ill), blank, and comparison control, as well as CN solution, CN-NLC 1 serum, and CN-NLC 2 serum ($p \leq 0.05$). This indicates that establishing an AA test model was successful, marked by excess hair loss and miniaturization. The data showed that CN-NLC serum with application 1 time/day and 1 time/2 days at intervals of 7, 14, and 21 days increased by 20.24-23.74%, 33.47-36.43%, and 39.30-39.97%, respectively. Data on the observation of hair length in serum test areas 1 and 2 showed significantly different results compared to the negative, positive, blank, and comparison control, as well as the CN solution ($P \leq 0.05$). However, the CN-NLC 1 serum test area showed no significant difference

in hair length compared to the CN-NLC 2 serum test area. Observations on day 21 showed that the CN-NLC 1 and 2 serum test groups had significantly different hair lengths compared to the negative, positive, blank, comparison control, and CN solution. The hair length of the CN-NLC 1 and 2 serum test groups increased by 39.97% and 39.30%, respectively, compared to the positive control. The minoxidil group experienced skin irritation from the first day of application to the 8th day with varying severity. Furthermore, skin irritation in animals in the minoxidil group causes hair growth and regeneration to stop/decrease. The hair length of the CN-NLC 1 serum test area showed no significant difference compared to the CN-NLC 2 serum test area. The result also showed that the hair length data during the observation interval showed that CN-NLC serum had the potential to be a hair growth stimulant in animal models of androgenic alopecia. However, there was no significant difference in the usage/application interval between 1 and 2 days. Therefore, CN-NLC serum can be used as a hair growth stimulant with intervals of use every 2 days (Leveque et al. 2021; Melincovici et al. 2018; Vanhoutte et al. 2016).

TABLE 3. CN-NLC serum eye irritation test

Group	Hour	Rabbit 1				Rabbit 2				Rabbit 3				Primary Irritation Index
		CC	CjC	IC	CE	CC	CjC	IC	CE	CC	CjC	IC	CE	
Control Normal	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0	0	0	0	0	0	0
	72	0	0	0	0	0	0	0	0	0	0	0	0	0
Serum CN-NLC	1	0	0	0	0	0	0	0	0	0	0	0	0	0.0
	24	0	0	0	0	0	0	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0	0	0	0	0	0	0
	72	0	0	0	0	0	0	0	0	0	0	0	0	0

Notes: CC: Corneal Corrosion; CjC: Conjunctival Corrosion; IC: Iris Corrosion; CE: Conjunctival Edema

DERMAL HISTOLOGY

The results of the skin histology in Figure 4 indicated that the anatomy of hair follicles between the negative, positive, and blank control, as well as minoxidil, CN solution, and CN-NLC serum (1 and 2 days), were different on the 7th, 14th, and 21st day of observation. The negative control group showed miniaturization of hair follicles and dermal papillae. This indicates that testosterone induction in forming the AA model is successful. CN-NLC serum test groups 1 and 2 showed that the anatomy was similar/close to the negative control anatomy, where there was an increase in the number and size of follicles compared to the positive, blank, minoxidil, and CN solution. This is consistent with the results of hair length measurements at each observation time. It also indicates that the NLC delivery system effectively delivers and increases CN activity as a stimulant for hair growth and regeneration in AA conditions. The mechanism of CN activity is thought to involve telangiectasia, which is a vasodilation of blood vessels, thereby facilitating the supply of nutrients and oxygen needed for hair growth and regeneration (Vanhoutte et al. 2016). In addition, it stimulates hair follicles and dermal papillae to enter the anagen phase more quickly by activating Wnt/ β -catenin, which plays a significant role in the morphogenesis, development, and growth of hair follicles. Wnt signal activation induces hair placodes development, and β -catenin regulates signaling pathways in dermal papilla cells, such as Fibroblast Growth Factor (FGF7 and FGF10), which play a role in regulating the growth of epithelial cells in hair follicles. Therefore, activating

Wnt/ β -catenin will accelerate the hair cycle entering the anagen phase and increase VEGF production. This will activate eNOS (endothelial nitric oxide synthase), causing vasodilation and increased blood vessel permeability (Leveque et al. 2021; Melincovici et al. 2018; Vanhoutte et al. 2016). Furthermore, increased VEGF has the potential to keep hair roots stronger and prevent loss or death of dermal papilla (Driskell et al. 2011; Hariyanti et al. 2022; Jeong et al. 2022; Ou et al. 2012; Taghiabadi, Nilforoushzadeh & Aghdami 2020).

Dermal papillae are a layer of mesenchymal cells that play an important role in hair growth and regeneration. They also act as a multipotent stem cell reservoir, indicating cells that can differentiate into various other types (Driskell et al. 2011; Taghiabadi, Nilforoushzadeh & Aghdami 2020). The activation of Wnt/ β catenin in the dermal papilla stimulates the Fibroblast Growth Factor (FGF) pathway, which mediates its inductive effect on hair follicles epithelium (Driskell et al. 2011). Dermal papillae contain SOX2 is generally found in Skin-derived Progenitor Cells (SKPs), hence, they can differentiate into other cells, such as smooth muscle cells, adipocytes, fibroblasts, and osteoblasts. SOX2 is expressed in the dermal papillae and SKPs around the dermal papilla cell sheath. Due to the similarity between SKPs and dermal papillae, which both contain SOX2, SKPs may also be activated, causing them to differentiate and form new skin cells, thereby facilitating skin regeneration. In CN-NLC serum test groups (1 and 2), the thickness of the skin layer increased significantly when compared to the positive control, blank, minoxidil, and CN solution.

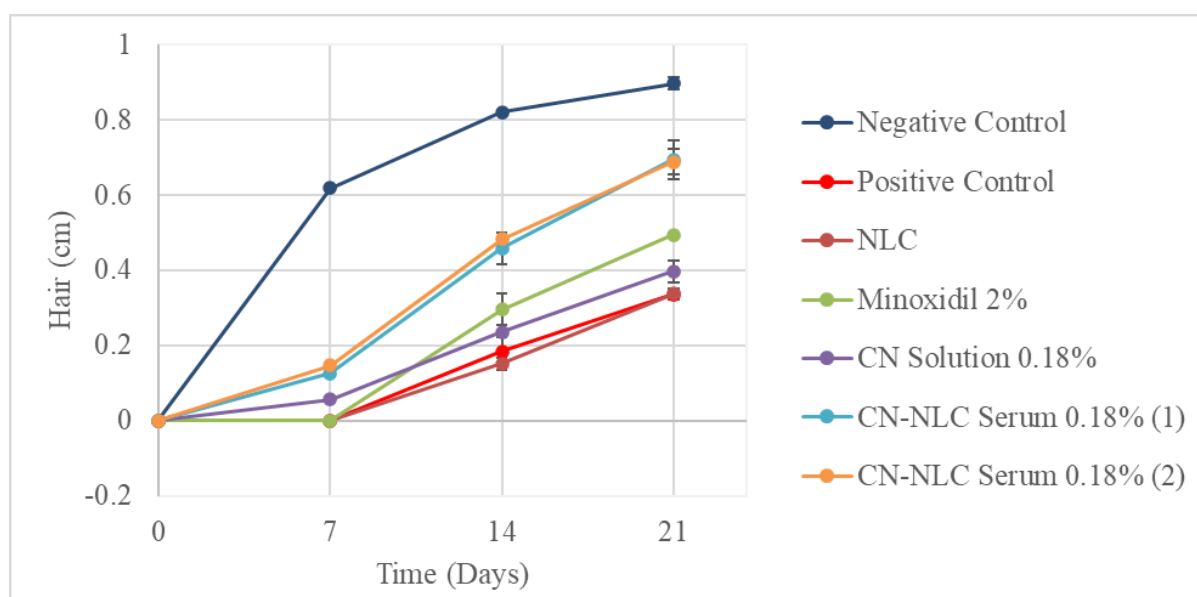


FIGURE 3. Effect of CN-NLC serum on hair length of androgenetic alopecia mice model

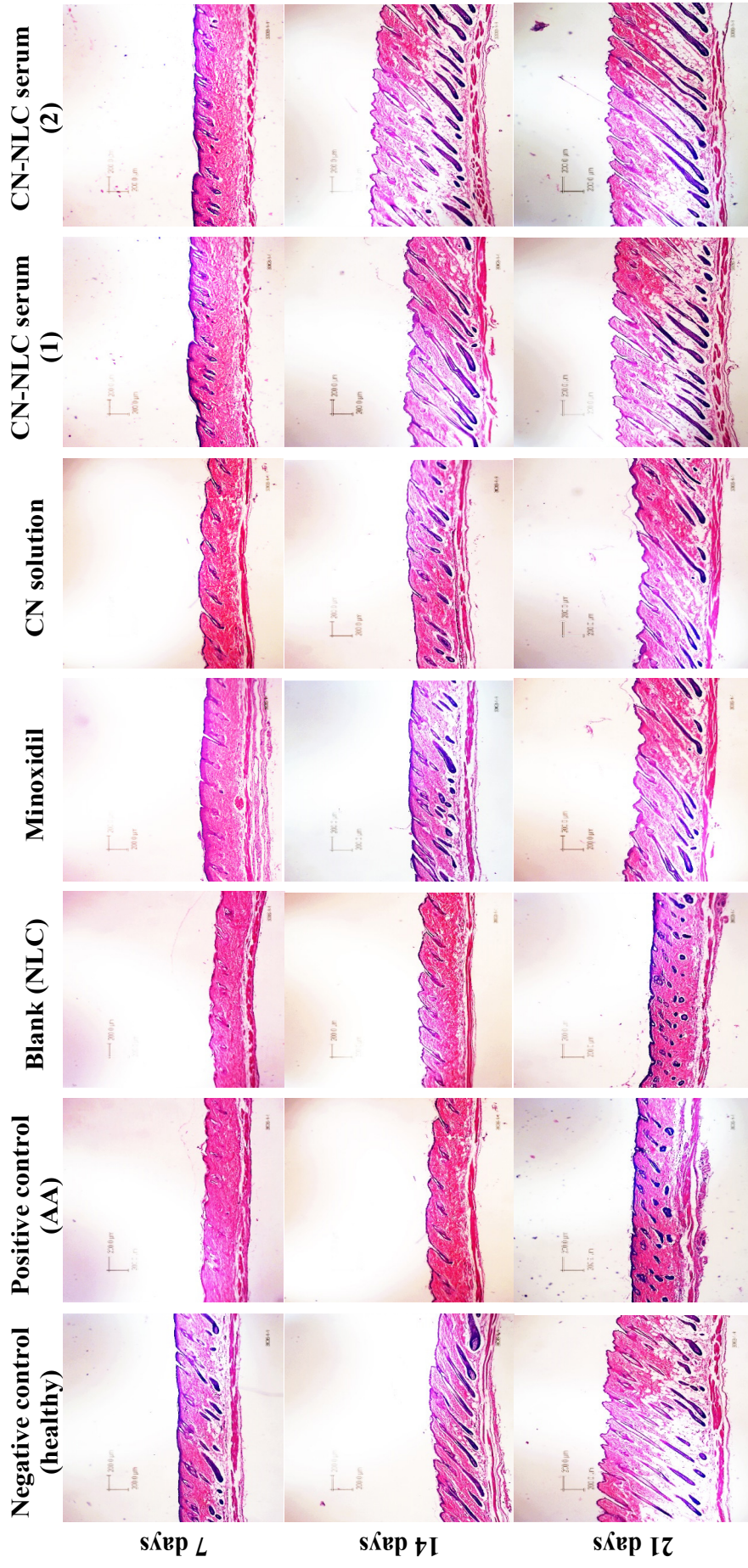


FIGURE 4. Effect of CN-NLC serum on hair follicles and dermal papillae of androgenetic alopecia mice model

CONCLUSIONS

Based on the study data, Nanostructured Lipid Carriers (NLC) is a drug-delivery system capable of penetrating through hair follicles. Therefore, cinchonine loaded in Nanostructured Lipid Carriers has a greater ability to penetrate open hair follicles than CN solution and CN-NLC serum with closed hair follicles. CN-NLC serum is proven safe with primary irritation index of 0.0. CN-NLC serum is effective as a hair growth stimulant in the treatment of AA characterized by increased hair length, number, and size of hair follicles-dermal papillae.

ACKNOWLEDGEMENTS

The authors are grateful to Rachmat Mauludin, Neng Fisheri Kurniati, and Yeyet Cahyati Sumirtapura for the assistance provided in decision-making at every stage of the study analysis.

REFERENCES

- Abd, E., Benson, H.A.E., Roberts, M.S. & Grice, J.E. 2018. Follicular penetration of caffeine from topically applied nanoemulsion formulations containing penetration enhancers: *in vitro* human skin studies. *Skin Pharmacology and Physiology* 31(5): 252-260. <https://doi.org/10.1159/000489857>
- Blume-Peytavi, U., Varvar, K. & Vogt, A. 2016. *The Alopecias Diagnosis and Treatments*, edited by Bouhanna, P. & Bouhanna, E. Boca Raton: CRC Press.
- Blume-Peytavi, U., Tosti, A., Whiting, D.A. & Trüeb, R.M. 2008. *Hair Growth and Disorders. Hair*. Springer. <https://doi.org/10.1007/978-3-540-46911-7>
- Bubić Pajić, N., Ilić, T., Nikolić, I., Dobričić, V., Pantelić, I. & Savić, S. 2019. Alkyl Polyglucoside-Based Adapalene-Loaded Microemulsions for Targeted Dermal Delivery: Structure, Stability and Comparative Biopharmaceutical Characterization with a Conventional Dosage Form. *Journal of Drug Delivery Science and Technology* 54. <https://doi.org/10.1016/j.jddst.2019.101245>
- Chen, Y., Feng, X., Zhao, Y., Zhao, X. & Zhang, X. 2020. Mussel-inspired polydopamine coating enhances the intracutaneous drug delivery from nanostructured lipid carriers dependently on a follicular pathway. *Molecular Pharmaceutics* 17(4): 1215-1225. <https://doi.org/10.1021/acs.molpharmaceut.9b01240>
- Driskell, R.R., Clavel, C., Rendl, M. & Watt, F.M. 2011. Hair follicle dermal papilla cells at a glance. *Journal of Cell Science* 124(8): 1179-1182. <https://doi.org/10.1242/jcs.082446>
- Fresta, M., Mancuso, A., Cristiano, M.C., Urbanek, K., Cilirzo, F., Cosco, D., Iannone, M. & Paolino, D. 2020. Targeting of the pilosebaceous follicle by liquid crystal nanocarriers: *in vitro* and *in vivo* effects of the entrapped minoxidil. *Pharmaceutics* 12(11): 1127. <https://doi.org/10.3390/pharmaceutics12111127>
- Fu, D., Huang, J., Li, K., Chen, Y., He, Y., Sun, Y., Guo, Y., Du, L., Qu, Q., Miao, Y. & Hu, Z. 2021. Dihydrotestosterone-induced hair regrowth inhibition by activating androgen receptor in C57BL6 mice simulates androgenetic alopecia. *Biomedicine and Pharmacotherapy* 137: 111247. <https://doi.org/10.1016/j.biopha.2021.111247>
- Gorzelanny, C., Mess, C., Schneider, S.W., Huck, V. & Brandner, J.M. 2020. Skin barriers in dermal drug delivery: Which barriers have to be overcome and how can we measure them? *Pharmaceutics*. <https://doi.org/10.3390/pharmaceutics12070684>
- Hariyanti, H., Sophi Damayanti & Sasanti Tarini Darijanto. 2020. Formulation and Activity test of cinchonine niosomes as hair growth stimulants. In *EBSCO Industries, Inc.* Vol. Suppl, p. 47.
- Hariyanti, H., Kurniati, N.F., Sumirtapura, Y.C. & Mauludin, R. 2023. Development and validation of an analytical method for the determination of nanostructured lipid carrier's cinchonine used direct method modified by liquid-liquid extraction using high-performance liquid chromatography. *Journal of Research in Pharmacy* 27(2): 913-923. <https://doi.org/10.29228/jrp.371>
- Hariyanti, H., Mauludin, R., Sumirtapura, Y.C. & Kurniati, N.F. 2022. A review: Pharmacological activities of quinoline alkaloid of *Cinchona* sp. *Biointerface Research in Applied Chemistry* 13(Issue 4): 3. <https://doi.org/10.33263/BRIAC134.319>
- Inaba, M. & Inaba, Y. 1996. Therapy for androgenetic alopecia. In *Androgenetic Alopecia*. Tokyo: Springer. https://doi.org/10.1007/978-4-431-67038-4_25
- Jeong, G., Shin, S.H., Kim, S.N., Na, Y., Park, B.C., Cho, J.H., Park, W-S. & Kim, H-J. 2022. Ginsenoside Re Prevents 3-Methyladenine-induced catagen phase acceleration by regulating Wnt/ β -catenin signaling in human dermal papilla cells. *Journal of Ginseng Research* 47(3): 440-447. <https://doi.org/https://doi.org/10.1016/j.jgr.2022.11.002>
- Jimenez, W., Gonzalez, E., Murphy, V.A. & Bauta, W. 2021. Evaluation of dermal corrosion and irritation by cytochrome in rabbits. *Toxicology Reports* 8: 1527-1529. <https://doi.org/10.1016/j.toxrep.2021.07.021>
- Joshi, M.D., Prabhu, R.H. & Patravale, V.B. 2019. Fabrication of nanostructured lipid carriers (NLC)-based gels from microemulsion template for delivery through skin. In *Methods in Molecular Biology* 2000: 279-292. https://doi.org/10.1007/978-1-4939-9516-5_19
- Kakadia, P.G. & Conway, B.R. 2018. Solid lipid nanoparticles for targeted delivery of triclosan into skin for infection prevention. *Journal of Microencapsulation* 35(7-8): 695-704. <https://doi.org/10.1080/02652048.2019.1576796>
- Kharat, M. & McClements, D.J. 2019. Fabrication and characterization of nanostructured lipid carriers (NLC) using a plant-based emulsifier: Quillaja Saponin. *Food Research International* 126: 108601. <https://doi.org/10.1016/j.foodres.2019.108601>

- Kim, J.H., Na, J., Bak, D-H., Lee, B.C., Lee, E., Choi, M.J., Ryu, C.H., Lee, S., Mun, S.K., Park, B.C., Kim, B.J. & Lee, H.S. 2019a. Development of Finasteride Polymer Microspheres for Systemic Application in Androgenic Alopecia. *International Journal of Molecular Medicine* 43(6): 2409-2419. <https://doi.org/10.3892/ijmm.2019.4149>
- Kim, M.H., Kim, K.T., Sohn, S.Y., Lee, J.Y., Lee, C.H., Yang, H., Lee, B.K., Lee, K.W. & Kim, D.D. 2019b. Formulation and evaluation of nanostructured lipid carriers (NLCs) of 20(s)-protopanaxadiol (PPD) by box-behnken design. *International Journal of Nanomedicine* 14: 8509-8520. <https://doi.org/10.2147/IJN.S215835>.
- Kim, S.H., Park, M.K., Seol, J.K., Im, J.M., Park, H.S., Seo, H.S., Park, H.J. & Nah, S.S. 2021. Evaluation of potential eye or skin irritation/corrosion in rabbit exposed to TiO₂ photocatalyst (GST). *Environmental Health and Toxicology* 36(3): e2021022-0. <https://doi.org/10.5620/eaht.2021022>
- Lademann, J., Darvin, M.E., Meinke, M.C. & Patzelt, A. 2019. Characterization of penetration of particles after dermal application. In *Nanocosmetics*, edited by Cornier, J., Keck, C., van de Voorde, M. Springer, Cham. https://doi.org/10.1007/978-3-030-16573-4_11
- Lademann, J., Knorr, F., Richter, H., Jung, S., Meinke, M.C., Rühl, E., Alexiev, U., Calderon, M. & Patzelt, A. 2015. Hair follicles as a target structure for nanoparticles. *Journal of Innovative Optical Health Sciences* 8(4): 1530004. <https://doi.org/10.1142/S1793545815300049>
- Lademann, J., Richter, H., Teichmann, A., Otberg, N., Blume-Peytavi, U., Luengo, J., Weiss, B., Schaefer, U.F., Lehr, C-M., Wepf, R. & Sterry, W. 2007. Nanoparticles - An efficient carrier for drug delivery into the hair follicles. *European Journal of Pharmaceutics and Biopharmaceutics* 66(2): 159-164. <https://doi.org/10.1016/j.ejpb.2006.10.019>
- Leveque, M., Mas, C., Haure, M., Lejeune, O., Duplan, H., Castex-Rizzi, N. & Bessou-Touya, S. 2021. 601 hair growth properties of *Cinchona succirubra* extract, *Leontopodium alpinum* extract and manganese pca in human hair follicle dermal papilla cells. *Journal of Investigative Dermatology* 141(5): S104. <https://doi.org/10.1016/j.jid.2021.02.629>
- Melincovici, C.S., Boşca, A.B., Şuşman, S., Mărginean, M., Mişu, C., Istrate, M., Moldovan, I.M., Roman, A.L. & Mişu, C.M. 2018. Vascular endothelial growth factor (VEGF) - Key factor in normal and pathological angiogenesis. *Romanian Journal of Morphology and Embryology* 59(2): 455-467.
- Mohd, F., Todo, H., Yoshimoto, M., Eddy Yusuf & Sugibayashi, K. 2016. Contribution of the hair follicular pathway to total skin permeation of topically applied and exposed chemicals. *Pharmaceutics* 8(4): 32. <https://doi.org/10.3390/pharmaceutics8040032>
- Ou, H.C., Keating, S., Wu, P., Simon, J.A., Raible, D.W. & Rubel, E.W. 2012. Quinoline ring derivatives protect against aminoglycoside-Induced hair cell death in the zebrafish lateral line. *JARO - Journal of the Association for Research in Otolaryngology* 13(6): 759-770. <https://doi.org/10.1007/s10162-012-0353-0>
- Patzelt, A. & Lademann, J. 2020. Recent advances in follicular drug delivery of nanoparticles. *Expert Opinion on Drug Delivery* 17(1): 49-60. <https://doi.org/10.1080/17425247.2020.1700226>.
- Pereira, M.N., Tolentino, S., Pires, F.Q., Anjos, J.L.V., Alonso, A., Gratieri, T., Cunha-Filho, M. & Gelfuso, G.M. 2021. Nanostructured lipid carriers for hair follicle-targeted delivery of clindamycin and rifampicin to hidradenitis suppurativa treatment. *Colloids and Surfaces B: Biointerfaces* 197: 111448. <https://doi.org/10.1016/j.colsurfb.2020.111448>
- Pires, F.Q., da Silva, J.K.R., Sa-Barreto, L.L., Gratieri, T., Gelfuso, G.M. & Cunha-Filho, M. 2019. Lipid nanoparticles as carriers of cyclodextrin inclusion complexes: A promising approach for cutaneous delivery of a volatile essential oil. *Colloids and Surfaces B: Biointerfaces* 182: 110382. <https://doi.org/10.1016/j.colsurfb.2019.110382>
- Rooney, J.P., Choksi, N.Y., Ceger, P., Daniel, A.B., Truax, J., Allen, D. & Kleinstreuer, N. 2021. Analysis of variability in the rabbit skin irritation assay. *Regulatory Toxicology and Pharmacology* 122: 104920. <https://doi.org/10.1016/j.yrtph.2021.104920>
- Santos, G.A., Angelo, T., Andrade, L.M., Silva, S.M.M., Magalhães, P.O., Cunha-Filho, M., Gelfuso, G.M., Taveira, S.F. & Gratieri, T. 2018. The role of formulation and follicular pathway in voriconazole cutaneous delivery from liposomes and nanostructured lipid carriers. *Colloids and Surfaces B: Biointerfaces* 170: 341-346. <https://doi.org/10.1016/j.colsurfb.2018.06.037>
- Santos, A.C., Pereira-Silva, M., Guerra, C., Costa, D., Peixoto, D., Pereira, I., Pita, I., Ribeiro, A.J. & Veiga, F. 2020. Topical minoxidil-Loaded nanotechnology strategies for alopecia. *Cosmetics* 7(2): 21. <https://doi.org/10.3390/COSMETICS7020021>
- Satheeshan, K.N., Seema, B.R. & Manjusha, M.A.V. 2020. Development and evaluation of VCO based herbal hair tonic. *Journal of Pharmacognosy and Phytochemistry* 9(2): 485-493. <https://www.phytojournal.com/archives/2020/vol9issue3/PartH/9-3-5-112.pdf>
- Souto, E. 2004. Development of a controlled release formulation based on SLN and NLC for topical clotrimazole delivery. *International Journal of Pharmaceutics* 278(1): 71-77. [https://doi.org/10.1016/s0378-5173\(04\)00153-x](https://doi.org/10.1016/s0378-5173(04)00153-x)
- Taghiabadi, E., Nilforoushzadeh, M.A. & Aghdami, N. 2020. Maintaining hair inductivity in human dermal papilla cells: A review of effective methods. *Skin Pharmacology and Physiology* 33(5): 280-292. <https://doi.org/10.1159/000510152>
- Tanaka, S., Saito, M. & Tabata, M. 1980. Bioassay of crude drugs for hair growth promoting activity in mice by a new simple method. *Planta Medica* 40(Suppl.): 84-90. <https://doi.org/10.1055/s-2008-1075009>
- Todo, H. & Mohd, F. 2017. Related topic: Drug permeation through hair follicles. In *Skin Permeation and Disposition of Therapeutic and Cosmeceutical Compounds*, edited by Sugibayashi, K. Tokyo: Springer. pp. 399-409. https://doi.org/10.1007/978-4-431-56526-0_36

- Toll, R., Jacobi, U., Richter, H., Lademann, J., Schaefer, H. & Blume-Peytavi, U. 2004. Penetration profile of microspheres in follicular targeting of terminal hair follicles. *Journal of Investigative Dermatology* 123(1): 168-176. <https://doi.org/10.1111/j.0022-202X.2004.22717.x>
- Truong, V.L., Bak, M.J., Lee, C., Jun, M. & Jeong, W.S. 2017. Hair regenerative mechanisms of red ginseng oil and its major components in the testosterone-induced delay of anagen entry in C57BL/6 mice. *Molecules* 22(9): 1505. <https://doi.org/10.3390/molecules22091505>.
- Vanhoutte, P.M., Zhao, Y., Xu, A. & Leung, S.W.S. 2016. Thirty years of saying NO: Sources, fate, actions, and misfortunes of the endothelium-derived vasodilator mediator. *Circulation Research* 119(2): 375-396. <https://doi.org/10.1161/CIRCRESAHA.116.306531>.
- Vitorino, C., Sousa, J. & Pais, A. 2015. Overcoming the skin permeation barrier: Challenges and opportunities. *Current Pharmaceutical Design* 21(20): 2698-2712. <https://doi.org/10.2174/1381612821666150428124053>.
- Wang, Z.D., Feng, Y., Sun, L., Gan, J., Li, X., Ding, W.F. & Chen, X.M. 2021. Anti-androgenetic alopecia effect of policosanol from chinese wax by regulating abnormal hormone levels to suppress premature hair follicle entry into the regression phase. *Biomedicine and Pharmacotherapy* 136: 111241. <https://doi.org/10.1016/j.biopha.2021.111241>
- Wang, Z.D., Feng, Y., Ma, L.Y., Li, X., Ding, W.F. & Chen, X.M. 2017. Hair growth promoting effect of white wax and policosanol from white wax on the mouse model of testosterone-induced hair loss. *Biomedicine and Pharmacotherapy* 89: 438-446. <https://doi.org/10.1016/j.biopha.2017.02.036>
- Yazdani-Arazi, S.N., Ghanbarzadeh, S., Adibkia, K., Kouhsoltani, M. & Hamishehkar, H. 2017. Histological evaluation of follicular delivery of arginine via nanostructured lipid carriers: A novel potential approach for the treatment of alopecia. *Artificial Cells, Nanomedicine and Biotechnology* 45(7): 1379-1387. <https://doi.org/10.1080/21691401.2016.1241794>
- Yokouchi, M. & Kubo, A. 2018. Maintenance of tight junction barrier integrity in cell turnover and skin diseases 27(8): 876-883. *Experimental Dermatology* <https://doi.org/10.1111/exd.13742>
- Zhang, B., Zhang, R.W., Yin, X.Q., Lao, Z.Z., Zhang, Z., Wu, Q.G., Yu, L.W., Lai, X.P., Wan, Y.H. & Li, G. 2016. Inhibitory activities of some traditional Chinese herbs against testosterone 5 α -reductase and effects of *Cacumen platycladi* on hair re-growth in testosterone-treated mice. *Journal of Ethnopharmacology* 177: 1-9. <https://doi.org/10.1016/j.jep.2015.11.012>.

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