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Original Article

PLACENTA EXTRACT-LOADED NOVASOME SIGNIFICANTLY IMPROVED HAIR GROWTH IN A RAT *IN VIVO* MODEL

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ABSTRACT

Objective: This study aims to improve the hair-growing effect of bovine placenta extract (PE) by loading it to a novasome vesicle.

Methods: PE-loaded novasome was prepared by thin layer hydration method. A 2^3 full factorial design was used to obtain the most optimum formula by varying the type of surfactants, the type of free fatty acid (FFA), and the ratio of surfactants and FFA. The resulting PE-loaded novasomes were characterized by entrapment efficiency (%EE), particle size (PS), polydispersity index (PDI), and zeta potential (ZP).

Results: PE-loaded novasome composed of Span 60: cholesterol: oleic acid (10:10:3) demonstrated the most optimum characteristics with PS 155.0 nm; PDI 0.139; ZP-63.73 and EE 79.60%. Transmission electron microscopy (TEM) of the most optimum novasome revealed non-aggregating oligo-lamellar nanovesicles. In addition, novasome showed ultra-deformable properties and good stability during 90 d storage at 4 °C. A hair growth study in rats showed that the PE-loaded novasome demonstrated better hair-growing effect compared to PE-loaded liposome and minoxidil 2% solution.

Conclusion: Novasome is a potential carrier for bringing such a big molecule as PE. PE-loaded in novasome showed vesicles with good physical characteristics and *In vivo* assessment demonstrated better hair-growing effect than minoxidil and PE-loaded liposomes.

Keywords: Novasome, Placenta extract, Hair growth, Alopecia

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INTRODUCTION

Hair plays an important role in people's life, including protection, thermoregulation, and social interaction. Hair loss (alopecia) is a minor physiological disorder that affects both men and women, but it can cause significant emotional distress due to a lack of self-confidence [1]. Drug therapy and hair transplantation are some approved methods for treating alopecia.

Minoxidil and finasteride are the most frequently used drugs for treating hair loss. However, these drugs cause some concerning side effects. For example, finasteride can cause sexual dysfunction [2] and external genitalia malformation in the male fetus [1]. Hair transplantation, another alopecia treatment, is a well-established procedure with quite a remarkable result. However, the procedure has some limitations, such as limited transplantable hair, hair survival problem, and high cost [3]. Therefore, alternative therapy to enhance hair growth, especially in alopecia patients, is still necessary.

Placenta extract (PE) is one of the active ingredients that can be used to enhance hair growth. A study by Barat *et al.* showed that bovine PE had the same effect as minoxidil in improving hair growth in women with androgenetic alopecia [4]. Bovine PE contains various bioactive substances, one of which is IGF (Insulin-like Growth Factor), which provides a signal for the growth factor receptor to increase the number and size of hair follicles [5].

Growth factor receptors that promote hair growth are located in dermal papillae in the dermis layer [6], and thus, the applied IGF must reach the dermis layer in order to be able to perform its action. However, IGF is a hydrophilic molecule with high molecular weight [7]. Thus, its penetration to dermis layer of the skin is limited. Therefore, some approaches are required to enhance IGF absorption into the dermis layer. Currently, a cosmetic product containing bovine PE-loaded liposomes has been marketed. However, liposomes have some limitations, such as low entrapment efficiency and active substance distribution [8], as well as a rigid structure [9] which limits their penetration into the skin.

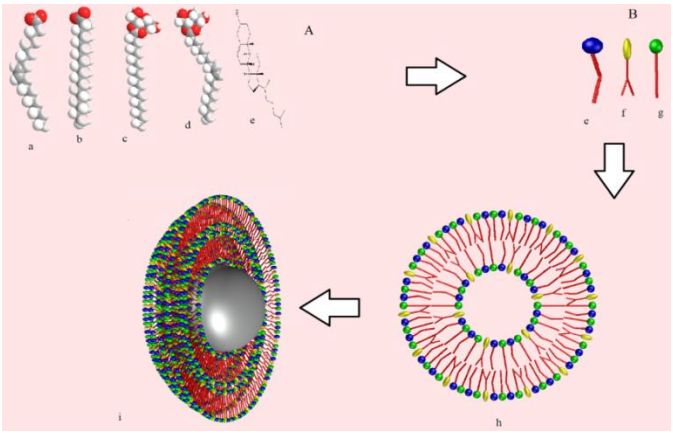


Fig. 1: Novasome structure, A. Molecular structure a. OA; b SA; c. span 60; d. span 80; e. cholesterol; B. modeling molecular (head and tail) e. FFA; f. cholesterol; g. surfactant; h. 2d modeling; i. vesicle modeling

Novasome is a new multi-bilayer vesicle technology that is similar to liposome. However, instead of phospholipid, it involves a combination of surfactant, fatty acid, and cholesterol as the vesicle-forming agent. It has been used as delivery vehicle for a single dose of killed *Mycobacterium bovis* BCG [10]. Novasome technology is considered as one of the effective methods for treating skin problems and has shown minimal side effects and cytotoxicity [11]. Therefore, we suggested that loading PE to novasome could enhance its penetration to skin. The illustration of novasome arrangement is shown on 1.

In a study by Abd-Elal *et al.*, (2016) it was shown that the most optimum formula was the novasome with the ratio of surfactant: free fatty acid: cholesterol 1:1:1 [12], while in a study by Mosallam *et al.*, (2021), the optimal formula was in ratio of 5:2:3, respectively [9]. In this study, we tried to lower the cholesterol concentration to reduce the stiffness and rigidity of the vesicle [13]. The effectiveness of PE-loaded novasomes in penetrating skin and promoting hair growth was then evaluated *in vivo*, compared with PE-loaded liposomes and minoxidil 2% solution.

MATERIALS AND METHODS

Material

Bovine placenta extract (PE) was purchased from orthana kemisk fabrik; span 60, span 80 and cholesterol from techno pharmchem; stearic acid (SA) from J. T Baker, oleic acid (OA) from Tokyo Chemical Industry, disodium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from merck.

Animals

Male sprague dawley rats with body weight between 120 and 150 g were housed at room temperature (25 °C) on a normal day-night cycle (07.00 to 19.00). All animal experiments were carried out after approval of the protocol by The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, Indonesia number 935/UN2. F1/ETIK/PPM.00.02/2022.

Methods

Preparation of PE-loaded novasome

A modified thin-film hydration method was used to encapsulate PE into the novasome. Novasome was formulated by varying the type of surfactant and free fatty acid (FFA), as well as the ratio of surfactant to FFA (table 1). Cholesterol, surfactant, and FFA with amount stated in table 2 were dissolved in 10 ml of dichloromethane in a 250 ml round bottom flask. The solution was then evaporated for an hour under reduced pressure using a rotavapor at 40 °C. The resulting film was hydrated using approximately 10 ml solution of PE in phosphate buffer saline (PBS, pH 7.4) for 1 h at 40 °C aided by tiny glass beads [12]. The temperature employed during hydration has a significant impact on the stability of vesicle and the subsequent active constituent. If the temperature is too high, the vesicle will lose its stability and the encapsulation efficiency will decrease. Moreover, proteins are renowned for their thermal instability [14]. The produced suspension was sonicated in an ice bath using a probe sonicator for 10 min at 30% amplitude [15–17] and then refrigerated overnight at 4 °C to allow maturation.

Preparation of PE-loaded liposomes

We used the same thin-film hydration method as novasome. Phosphatidylcholine and cholesterol (1:1) dissolved in 10 ml of dichloromethane. The solution was then evaporated for an hour under reduced pressure using a rotavapor at 40 °C. The resulting film was hydrated and sonicated with the same procedure as mentioned above in novasome.

Determination of entrapment efficiency (% EE)

Three ml of PE-loaded novasome suspension was centrifuged in an Eppendorf tube at 20,000 rpm at 4 °C for one hour. Total protein in the supernatant (unentrapped) was determined by the bicinchoninic acid (BCA) assay method [18]. The absorbance was read at 562 nm using a spectrophotometer UV-Vis. All step was done in triplicate. %EE of PE was calculated by the following equation:

$$\%EE = \frac{\text{Total amount of BSA} - \text{amount of BSA in the supernatant}}{\text{Total amount of BSA}} \times 100$$

Determination of particle size, polydispersity index, and zeta potential

Particle size (PS), polydispersity index (PDI), and zeta potential (ZP) of the various EP-loaded novasomes formulation were measured by Malvern Zetasizer 2000. The nanodispersion was diluted with purified water before measurements. Measuring the ZP was carried out with the same equipment. Each sample was measured in triplicate. The criteria for the most optimum formulation were set based on the smallest PS and PDI and the highest EE % and ZP [17, 18].

Transmission electron microscopy (TEM)

The morphology of the PE-loaded novasomes was analyzed by TEM. One drop of the undiluted formulation was deposited on a film-coated 200-mesh copper grid, negatively stained with one drop of 2% aqueous solution of phosphotungstic acid (PTA), and allowed to dry prior to TEM observation [17, 18].

Deformability study

The PE-loaded novasomes suspension was diluted five times before extrusion. 1 ml of suspension was passed through a 50 nm fine polycarbonate filter using a mini extruder set (Hamilton). The average particle size and PDI of the vesicle were measured before and after extrusion. Novasome deformability was assessed by comparing the particle size between initial and after extrusion [21].

Stability of novasome

PE-loaded novasomes suspensions were stored at 4 °C for 90 d. %EE, PS, and PDI of the samples were analyzed on day-30 60, and 90 of storage [9].

In vivo study

Twenty-four rats were acclimatized for two weeks and then randomly divided into four treatment groups of six rats. Group I was normal control, group II was treated with minoxidil hair tonic, group III was treated with PE-loaded liposomes, and group IV was treated with PE-loaded novasomes. A 4×3 cm^2 of dorsal skin was shaved to be used as a testing site testing area. On each testing site, a square with an area of 2×2 cm^2 was marked as the inner area as shown in Fig. 3. One milliliter of the sample was applied once a day for 28 d on the testing site.

The hair growth was observed visually. For a quantitative evaluation, we periodically pulled 25 hair roots on the outer area on days 7, 14, 21, and 28. Hair length, diameter, morphology, and anagen-telogen ratio were measured using a camera-equipped optical light microscope, with a magnification of 40x for hair length and 400x for the other parameter [22]. For hair length and diameter, we measured 6 hair from each rat, and the average value was calculated and expressed as the mean±SD n=6 from 6 rats in each group. For the anagen-telogen ratio, we calculated ratio of anagen and telogen from 25 hair of each rat, and the average value was calculated and expressed as the mean±SD from 6 rats in each group.

Hair weight was determined on day 28 after treatment. It was conducted by weighing whole hairs that were shaved at the drawn test area. Results are expressed as hair weight mean±SD from 6 rats in each group.

RESULTS AND DISCUSSION

Characterization of PE-loaded novasome

Particle size and PDI

The Z-average of all PE-loaded novasomes ranged from 122.9 to 1135.7 nm, as shown in table 3. Among all formulas, N1 (OA: Span 60 1:1) and N2 (SA: Span 80 1:1) demonstrated, respectively, the smallest and largest particle size. ANOVA analysis showed that the type of FFA significantly affects the size of novasomes with a p-value<0.001. Novasomes with OA tend to form smaller vesicles than those with SA. For example, as shown in table 3, formula with OA, N1 (155.0 nm), N4 (122.9), N5 (230,8) and N8 (297.5) was significantly smaller than those with SA, N2 (1135.7), N3 (339,6), N6 (743.4), N7 (553.0). SA has higher melting point (69 °C) than OA (13 °C), which results in higher melting viscosity [23] then, consequently affecting

PS during the film-forming process. Novasomes with SA produced opaque and thicker film; thus, at the hydration procedure, larger particle was formed. The higher melting viscosity also causes lower efficiency in the sonication procedure in PS reduction [23].

The difference in melting point also affects the PDI. Novasomes made with OA produced lower PDI than those made with SA (p-value<0.001). It might be because the SA creates a larger PS and its lower efficiency in the sonication process, resulting in a heterogeneous distribution of PS [23]. As shown in table 3, PDI of the different formulations containing OA were found, N1 (0,139), N4 (0,126), N5 (0,313) and N8 (0,122). That value was significantly lower than those with SA, N2 (0,646), N3 (0,437), N6 (0,550), and N7 (0,396).

In contrast, the type of surfactant and the ratio of surfactant and FFA do not affect PS and PDI significantly, with a p-value of 0.1631 and 0.9333, respectively.

Table 1: Design parameters for full factorial design (2^3)

Independent variable	Level	
Surfactant type	Span 60	Span 80
FFA type	Oleic Acid (OA)	Stearic Acid (SA)
Ratio of surfactant and FFA	1:1	2:1
Dependent variable	Target	
% EE	Maximum	
PS	Minimum	
PDI	Minimum	
ZP	Maximum (Absolute Value)	

Table 2: Formula for PE-loaded novasomes

Composition	Weight (mg)							
	N1	N2	N3	N4	N5	N6	N7	N8
Span 60	200	-	200	-	400	-	400	-
Span 80	-	200	-	200	-	400	-	400
OA	200	-	-	200	200	-	-	200
SA	-	200	200	-	-	200	200	-
PE	200	200	200	200	200	200	200	200
Cholesterol	60	60	60	60	60	60	60	60

OA= oleic acid, SA = stearic acid, PE = placenta extract

Entrapment efficiency

We calculated %EE from all the formulas and found values ranging from 75.50%±0.43 to 80.15%±0.52. The result from our study showed that the type of surfactant affects %EE of the novasomes. Formula with span 60 showed higher EE than those with span 80 (p-value =0,021). Using the same FFA (OA), N1 with span 60 showed a higher %EE than N4 with span 80, 79.60% and 76.88%, respectively.

This result was in agreement with those obtained by Diksha *et al.* [24]. It might be because span 60 has higher Tc (53 °C) than span 80 (-12 °C), thus providing higher %EE [12]. Tc is an essential factor affecting vesicle stability; vesicles consisting of saturated lipids with high Tc were more stable than those consisting of short or low Tc unsaturated hydrocarbons [25].

Another reason is the degree of saturation of the alkyl chain length. Span 80 and span 60 have identical numbers of carbon atoms (17) in their hydrophobic chain; the difference is that in span 80, there is a double chain on the 8th carbon, which makes the chain look bent. This causes irregularities in the vesicles formed and the membrane to be more permeable, resulting in lower adsorption efficiency in span 80 [26].

In this study SA containing vesicles showed significantly higher %EE, compared to those containing OA as FFA (p-value=0.001), this is in accordance with research by Abd-elal *et al.* For example, N2 that contain SA showed higher %EE (80.04%) than N4 (76.88%) that contain OA. This may be related to the degree of saturation of the alkyl chain of the FFA, as the degree of unsaturation has a significant effect on vesicle structures [28]. SA and OA have the similar number of carbon atoms (18) in their hydrophobic chain; however, OA has an unsaturated double bond, which affects its Tc. SA with higher Tc (69 °C) formed more stable and less leaky than OA with lower Tc (13 °C), resulting in higher % EE [29].

A significant interaction was observed between surfactant type and FFA type on the %EE (p-value<0.001). Formulas with span 60 showed higher %EE with the presence of SA, i.e., N7 (Span 60: SA 2:1) has %EE 80.15% which is higher than N5 (Span 60: OA 2:1) with %EE 79.37%.

Zeta potential

Vesicle composition and pH value have an effect on zeta potential [30]. Novasomes from all formulas showed negative ZP values ranging from-24.07 to-63.73 mV (table 3), which might be caused by the negative charge of the fatty acid. The highest ZP was shown by N1 with a value of-63.73 mV. Statistical calculations were performed using absolute values (removing the negative sign) and found that FFA type had a noticeable influence on ZP (p-value= 0.004). ZP of the various developed formulation containing OA were found to be-63.73 (N1),-36.17 (N4),-38.37 (N5), and-35.40 (N8). Those values were greater (absolute value) than formulations containing SA-24.07 (N2),-34.97 (N3),-39.97 (N6), and-25.60 (N7). It might be attributed to their hydrophilicity, SA is less hydrophilic compared to OA, which could cause more shield of the negative surface charge, eventually resulting in significant lower ZP values [31].

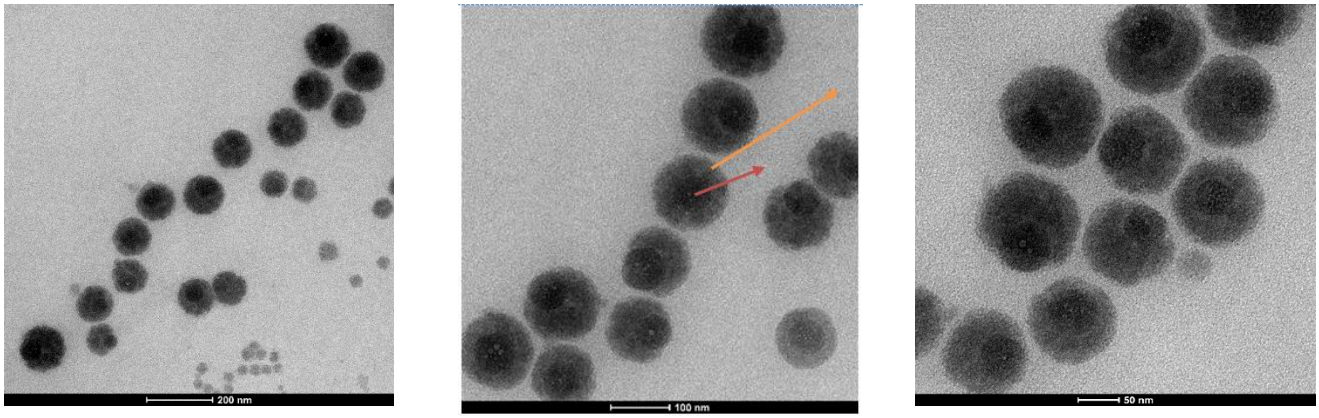
The use of phosphate buffer with pH 7.4 in vesicles had three purposes: increasing %EE, skin permeation, and stability. Generally, electrostatic stabilization requires a ZP of more than+30 mV or below-30 mV because particles with high potentials are less likely to aggregate due to electrical repulsion [32]. The formula with a high ZP value is stable and produces the least sediment [33], and can penetrate optimally through the skin tissue in a relatively short time [34].

Optimization

Among eight formulas, we chose N1 as an optimal formula with PS 155.0±2.84 nm, PDI 0.139±0.05, ZP–63,73±1,80 mV, and %EE 79.60±0.58.

TEM analysis

To ensure the formation of novasome vesicles, we conducted TEM imaging on N1. Fig. 2 shows that the novasomes obtained from N1 appeared to be non-aggregating oligolamellar nanovesicles with spherical shape and narrow size distribution. The fig. clearly indicates that the diameter of the vesicles was in the nano-range and consistent with PS measurement.



a. **b.** **C**

Fig. 2: TEM Image of N1 formula in magnitude a. 38000 b. 71000 and c. 97000 (Area with two different colors of arrow show different lamellar of bilayer)

Table 3: Characterization result

	Z average (nm)	PDI	ZP (mV)	%EE
N1	155.0±2.84	0.139±0.05	-63.73±1.80	79.60±0.58
N2	1135.7±34.39	0.646±0.29	-24.07±3.35	80.08±0.46
N3	339.6±4.00	0.437±0.09	-34.97±2.75	79.39±0.67
N4	122.9±0.62	0.126±0.02	-36.17±3.04	76.88±0.60
N5	230.8±8.85	0.313±0.04	-38.37±2.47	79.37±0.44
N6	743.4±65.65	0.550±0.36	-39.97±0.93	79.86±0.30
N7	553.0±81.05	0.396±0.14	-25.60±1.41	80.15±0.52
N8	297.5±8,10	0.122±0.05	-35.40±0.87	75.50±0.43

Notes: Data are presented as mean±SD (n=3)

Table 4: Stability study

Day	0	30	60	90
PS (nm)	152.4±1.71	154.8±9.49	157.1±6.07	159.3±13.08
PDI	0.139±0.05	0.290±0.05	0.260±0.06	0.308±0.04
ZP (mV)	-63.73±1.80	60.00±2.40	60.43±3.14	57.67±0.21
%EE	78.90±0.56	78.74±0.46	78.21±0.85	77.51±1.32

Notes: Data are presented as mean±SD (n=3)

Table 5: PS and PDI before and after extrusion

		PS (nm)	PDI
Novasome	Before	154.97±2.84	0.139±0.05
	after	153.97±10	0.181±0.1
Liposome	Before	258.9±3.5	0.276±0.1
	after	101.2±4,3	0.459±0.2

Notes: Data are presented as mean±SD (n=3)

Deformability study

The PE-loaded novasomes obtained from N1 demonstrated ultra-deformable properties. It was shown by the relatively unchanged particle size after extrusion, as shown in table 5. We suggested that the PE-loaded novasomes are quite flexible; therefore, their size remained the same after passing the membrane. With this property, we hypothesized that our PE-loaded novasomes could deal with the skin barrier by penetrating through the paracellular pathway.

Stability study

The PE-loaded novasomes obtained from N1 did not show any clumps and changes in their appearance during storage for 90 d and showed no significant change (p-value>0.05) in EE%, PS, PDI, and ZP compared to the fresh ones. These results indicated good stability of N1 upon storage, as shown in Table 4.

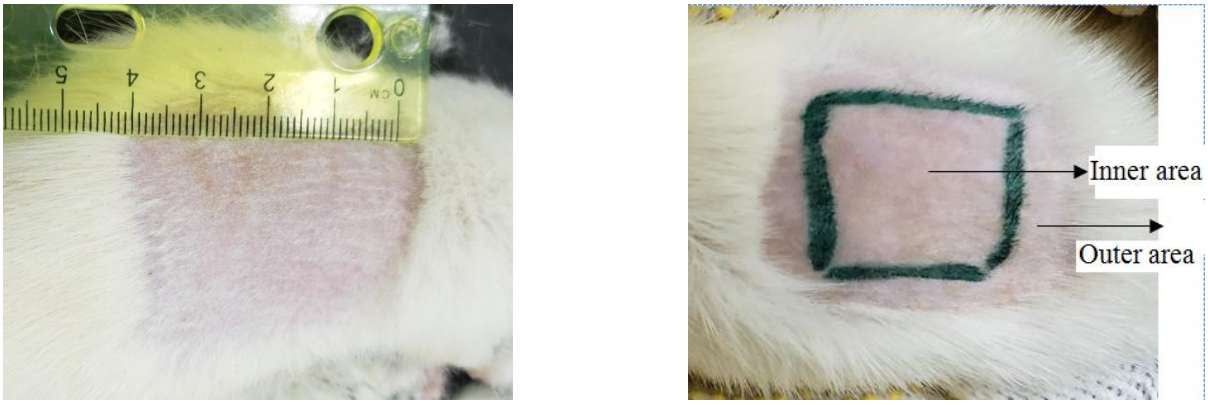
***In vivo* evaluation**

Qualitative evaluation

The hair growth in the rats was observed periodically after treatment (Fig. 4). On the 7th day of treatment, hair growth initiation was observed in the PE-loaded novasomes, PE-loaded liposomes, and minoxidil groups, which was earlier than the negative-control group. Notably, the novasome group showed a more even hair growth compared to the other groups. On day 14th, the negative control group did not show full hair growth, while the other groups demonstrated hair growth since the testing area were fully covered by hair, as shown in Fig. 4. Faster initiation of hair growth occurred in the group treated with PE-loaded novasomes.

Hair morphology observation, as shown in Fig. 6, showed that the hair in the group treated with PE was arranged in an orderly manner

from hair cuticles and overlapping hair cuticles consisting of flattened layers of keratin. It implies that PE stimulates healthy hair growth, where the cuticle has an essential function to protect the inner cortex, which provides the elastic properties of hair [35].



a b

Fig. 3: Marking of test area on animal

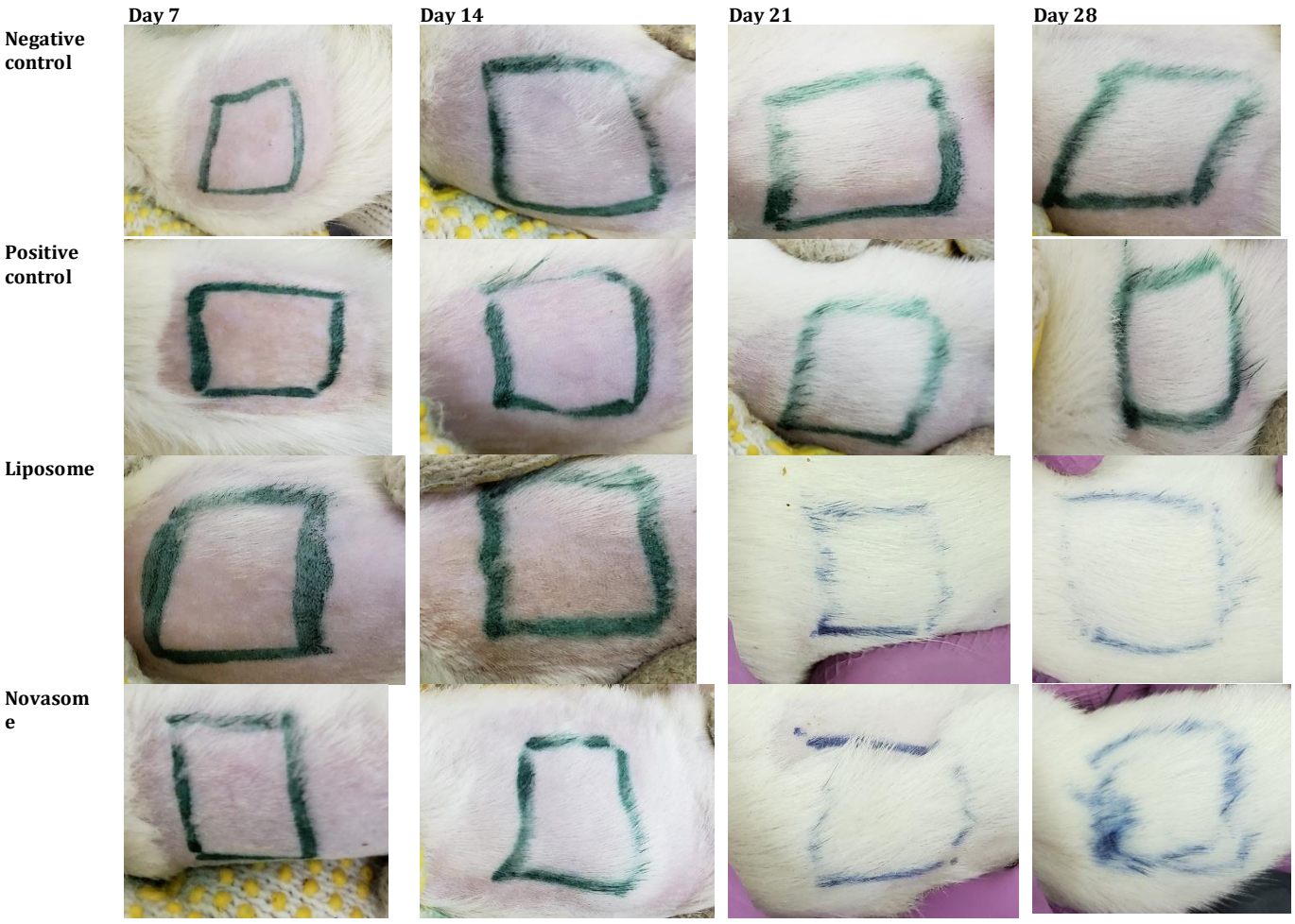


Fig. 4: Hair growth effect of PE in novasome vesicle. The dorsal skin was photographed on days 7, 14, 21 and 28

Quantitative evaluation

Evaluation hair length and hair diameter

To confirm the hair growth effect from all treatment groups, we measured the length of 6 hair from each rat randomly. On the first week after treatment, all groups showed hair growth in the hair length test; novasome showed an average 1.91 mm length; liposome 1.87 mm; positive control 1.92 mm, and negative control 1.88 mm. However, since the length was below 2 mm and the growth was uneven, the growth was not clearly observed in the visual test. On the second week, rats treated with PE-loaded novasomes showed the highest hair growth with 1.54 mm length addition, followed by PE-loaded liposome 0.86 mm, minoxidil (positive) 0.27 mm, untreated 0.15 mm. On last week of treatment Novasome group

showed the longest hair length (14.19 mm) of all other groups (p-value<0.001) where liposome showed a length average 12.54 mm, positive 12.05 mm, negative group 8.50 mm.

We also observed the diameter of the hair from all treatment groups. As shown in fig. 5, a significant difference in hair diameter average start observed on the second week of treatment, where rats treated with PE-loaded novasomes demonstrated thicker hair diameter (54.75 µm) compared to liposomes (42.36 µm), positive control (18.72 µm) and negative control (15 µm) with p-value 0.003,<0.001 and<0.001 respectively. Then the hair diameter keeps growing until the fourth week of treatment, where novasome group showed the thickest average diameter 120.68 µm, followed by liposome group 90.06 µm, the positive group 49.98 and negative control group 38.59 µm.

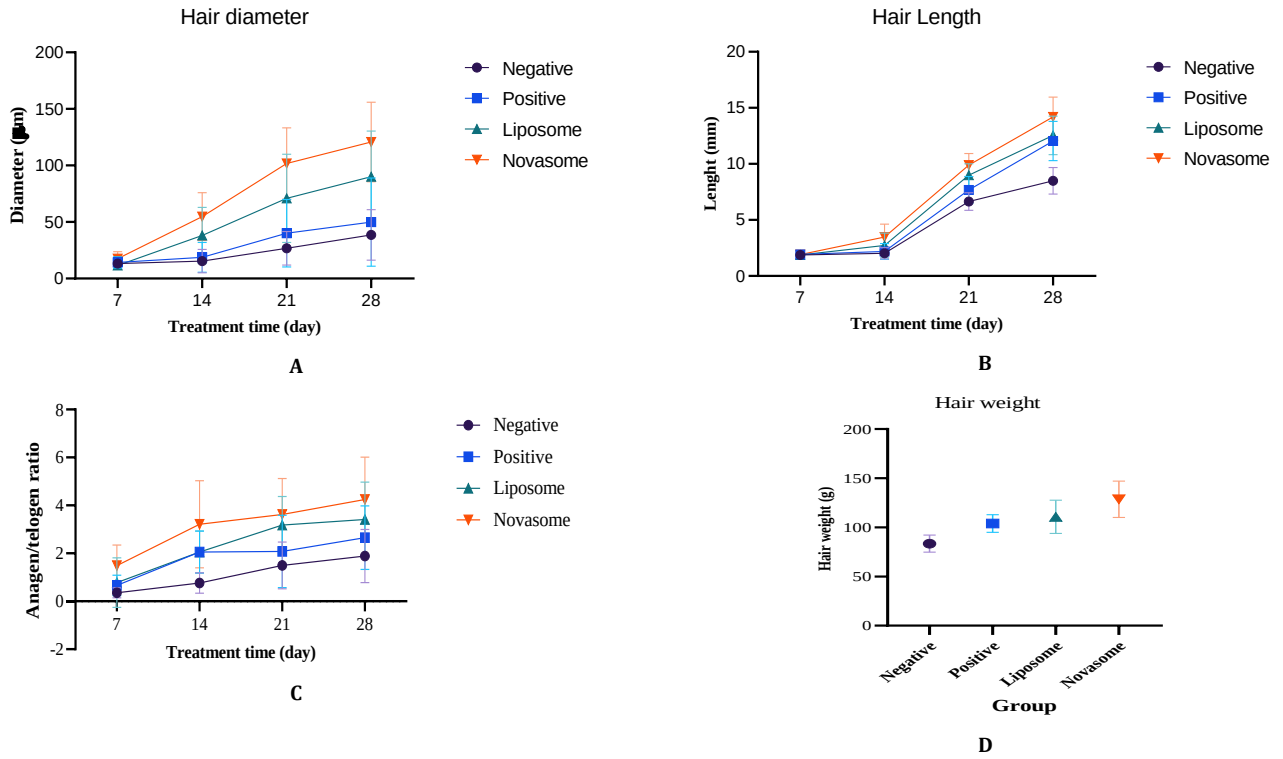


Fig. 5: Evaluation of hair growth parameter in rats, A. Hair diameter B; Hair length; C. Anagen/Telogen Ratio; D. Hair weight (mean±SD, n=6)

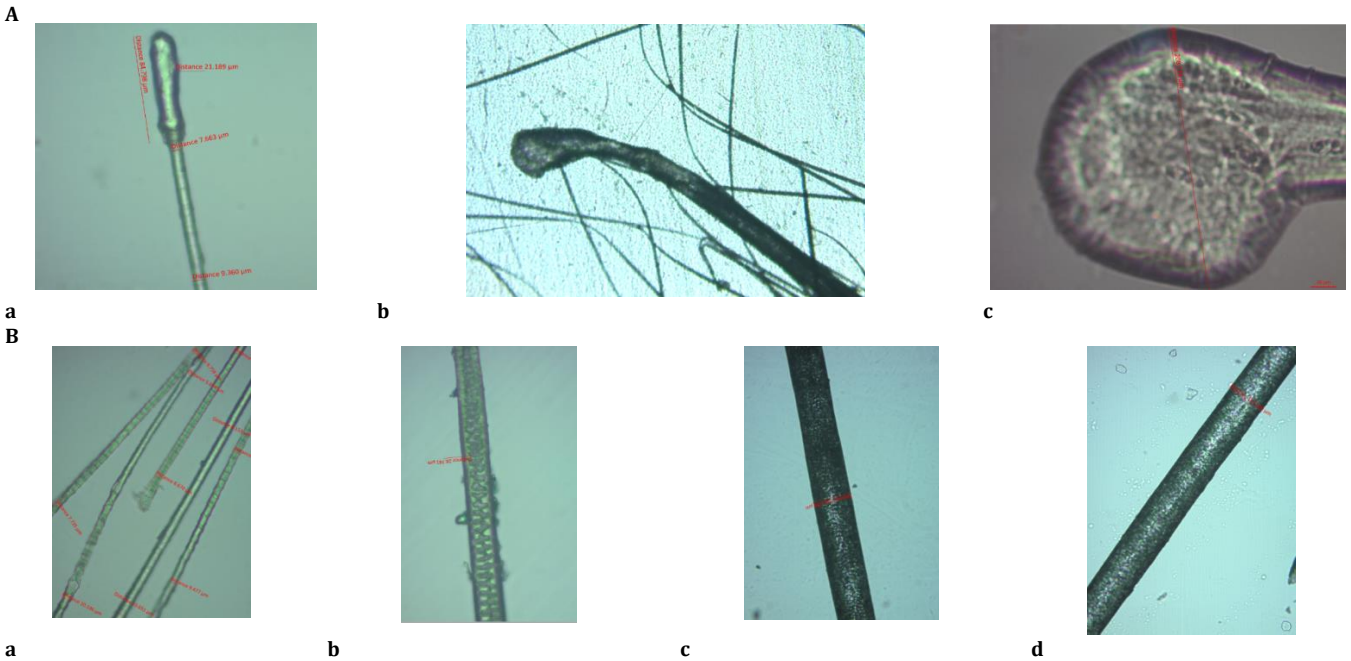


Fig. 6: Microscopic picture of hair, A. Hair root a. Telogen hair b. dan c. Anagen hair; B. Hair shaft each test group a. Negative control group, b. Positif control control, c. liposom control, and d. novasome control

Hair weight evaluation

The hair from the inner testing area was shaved and weighed on day 28 (fig. 5). The novasome group showed an average hair weight of 128.6 mg, which is 17.93 mg higher than liposome groups (110.7 mg). That value is not significantly different with the with p-value=0.109 according to ANOVA test. However, the novasome group showed higher hair weight significantly compared to positive and negative control groups, with a difference 24.67 mg (p-value=0.01) and 45.18 mg (p-value<0.001), respectively. This evaluation implied that novasome was able to promote denser, longer and thicker hair growth in the test area compared to liposome and minoxidil.

Telogen-anagen ratio

The anagen-telogen ratio is an appropriate parameter to determine the hair growth cycle. Hair roots were extracted and examined to estimate cycle status (anagen: telogen difference) [22]. Previous research showed that PE has IGF-1 [36] that stimulates hair follicle growth through anti-apoptosis activity to avoid cell death during the catagen phase and plays a crucial role in the maintenance of hair follicle growth in the anagen phase [37].

The evaluation implied that there was follicular growth with an increase in the number of anagen phase hairs [38]. After seven days of treatment in all groups and continue to increase during treatment in all groups. At the end of the treatment (4^{th} week), PE-loaded novasome treated animals, most of the hair follicles are in anagenic phase. A/T ratio of 4.25 was found in novasome group, whereas, with liposome, it was 3.41; with minoxidil treatment, 2.66 hair were found in the anagenic phase and the untreated animal group was 1.88 (fig. 5). This result indicated that PE loaded in novasomes could improve conversion from telogen to anagen, with better performance compared to minoxidil and PE loaded in liposomes.

From all quantitative evaluation suggest that PE positively influences healthy hair growth, and its administration in novasome preparations appears to enhance its effectiveness because it has an ultra-deformable characteristic that enhance pharmacokinetics/drug biodistribution by promoting intracellular delivery and increasing retention time c. In addition, novasome vesicles could carry large molecules with higher entrapment and pass instinctively through the skin, also aids their rapid penetration through the intercellular lipid of the subcutaneous layer to deliver a higher amount of active ingredients to hair follicle as the target site [39].

CONCLUSION

PE loaded in novasome showed vesicles with good physical characteristics. The optimal formula, N1, contains OA, Span 60, and cholesterol in 10:10:3 proportion, respectively, possessed small PS and PDI, spheric morphology, and high EE% and ZP. *In vivo* assessment revealed that PE-loaded novasomes demonstrated better hair-growing effect than minoxidil and PE-loaded liposomes.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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