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Title

Improving the anti-ageing activity of coenzyme q10 through protransfersome-loaded emulgel

Type

original-research

Journal

Scientific Reports

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a904d626-ce96-4ebd-9a16-6f2ad0d44cb5

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Submission history

1. **Publishing and rights**

Submission status	Date
Submission is in publishing and rights	29 Dec 2021

2. **Peer review**

Submission status	Date
Submission accepted	29 Dec 2021
Submission under peer review	16 Dec 2021

Submission passed technical check	16 Dec 2021
Revision received	13 Dec 2021
Submission under peer review	14 Sep 2021

3. Technical check

Submission status	Date
Submission passed technical check	14 Sep 2021
Amendment received	14 Sep 2021
Submission is under technical check	01 Sep 2021

4. Submission received

Submission status	Date
Submission received	01 Sep 2021

andang miatmoko <andang-m@ff.unair.ac.id>

Fwd: Your revision is now overdue - Improving the anti-ageing activity of coenzyme q10 through protransfersome-loaded emulgel

7 messages

Bhakti Thakkar <srep@nature.com> Thu, Nov 25, 2021 at 1:34 AM
Reply-To: Bhakti Thakkar <srep@nature.com>
To: andang-m@ff.unair.ac.id

Dear Prof. Miatmoko,

Re: "Improving the anti-ageing activity of coenzyme q10 through protransfersome-loaded emulgel'

On checking our records, I notice that we were due to receive your revision. Your extension has also expired

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On Fri, 5 Nov at 7:31 AM , Andang-m <andang-m@ff.unair.ac.id> wrote:

[External - Use Caution]

Dear Editor,
Regarding the revision of the manuscript, can we get an extension for submission? There are some data that we should collect and summarize to the paper.
We do also need proofreading the manuscript.
Many thanks

On Tue, Oct 19, 2021 at 1:53 PM Scientific Reports <srep@nature.com> wrote:

> Ref: Submission ID a904d626-ce96-4ebd-9a16-6f2ad0d44cb5
>
> Dear Dr Miatmoko,
>
> Re: "Improving the anti-ageing activity of coenzyme q10 through protransfersome-loaded emulgel"
>
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>
> Editor comments
> We regret that the manuscript requires substantial revision for its publication.
>
> Regarding the addition of references:
> Authors do not need to cite the articles listed by reviewer #2.

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Kind regards,

mitsutoshi setou
Editorial Board Member
Scientific Reports

Reviewer Comments:

Reviewer 1
The manuscript was well-written and the in vivo studies of anti-ageing activity and irritability using UV ray ageing-induced male Balb/c mice provided interesting results. However, the following comments need to be addressed.

- The authors should explain how palisade crystalline liquid was detected under a light microscope. Is polarizing light required for the identification of the crystallinity.

- Improve the Resolution of figure 2.

- The quality of figure 4 was very poor. Please improve its resolution.

- The authors should explain why the color of CoQ10 loaded in emulgel is more intense comparing to the others.

- Protransf-CoQ10 shows similar infrared spectroscopical profiles to Protransfersome blank. What is the concentration of CoQ10 loaded? Would it be possible that the concentration of CoQ10 loaded was insufficient for for the FTIR analysis.

- Differential thermal analysis of Blank protransfersome should be presented and discussed.

- The author mention about the protransfersomal gel mask. What is the difference form the normal protransfersomal gel formulation.

- “The Protransf-CoQ10 was dispersed in an emulgel base consisting of Tween 80 and Span 80 to produce Protransf-CoQ10 gel.” The term " emulgel" could lead to the misunderstanding about the composition of the formulation. Normally, gel formulation refers to the three-dimensional polymeric matrix physically or sometimes chemically cross-linked by gelling agents. The author should provide more detail about the polymer used.

- More detail about the in vivo studies of anti-ageing activity and irritability using UV ray ageing-induced male Balb/c mice should be provided in the abstract.

Reviewer 2
Improving the anti-ageing activity of coenzyme q10 through protransfersome-loaded emulgel

Comments to Authors and Editor
The authors presented an interesting “Improving the anti-ageing activity of coenzyme q10 through protransfersome-loaded emulgel”. The purpose of the current study is to improve the skin delivery and stability of CoQ10. Although the concept is interesting, yet this study is poorly designed, and the results and discussion have many flaws. Moreover, some basic studies are missing from the manuscript. The detailed comments are given below.

Minor comments:
1- Grammatical, typographical and spelling mistakes.
2- Before using abbreviation, it should be defined and once defined then should be used throughout the text.
3- Space should be given between number and units i.e. line 387 2mL.
4- Same spelling should be used for aging. Authors have used ageing and aging.
5- Gel preparation method is not clearly defined in methodology section. Please rectify
6- The authors have used two term gel and Emulgel, is it same word or there is any difference? The author should use single term.

Major comments:

7- In abstract the authors claimed stability studies for 14 weeks while in methodology and results the author has reported 28 days’ stability study. On 28 days’ study how can one calculate stability of the formulation? Is it according to ICH guidelines?
8- The authors has mentioned a particle size of 238 nm for simple CoQ10 gel in table 1, Is it possible for plain drug gel to have size in nm? if yes why the authors formulating nano-formulation? Please Justify?
9- The PDI of CoQ10-Ole and Protransf-CoQ10 Gel are same in table 1, while in text the values are different. Also in the same table the authors have reported particle size of 146 nm for CoQ10-Ole, is it possible? Moreover, its difference from the Protransf-CoQ10 Gel seems to be non-significant which needs to be justified.
10- In line 160, the authors have mentioned particle size of 201 nm for the formulation, why the particle size is reduced to 134 nm by loading it into gel? Please give valid reasons in the manuscript.
11- The authors have discussed that loading the CoQ10 in pro-transferosomes will increase its stability while its zeta potential value is low i.e. -11 ± 5.14 mV. Please justify
12- The authors have not provided any figure of DLS results. Please add
13- Carbopol is water soluble, why the authors used two surfactants with it i.e. lipophilic and hydrophilic. Please justify in the manuscript
14- The authors have claimed an entrapment of 45% for the final transfersomal formulation. However, transfersomes have been reported with capability of 97% drug entrapment and

excellent skin deposition. What are the main reasons of low drug entrapment in this system? How the authors justify the superiority of their developed system over the already existed systems. Please discuss in the manuscript.
15- In figure 5, on day 0 the authors have shown difference in the size and pH, is it possible?
16- Table 2 is confusing, why the authors added only CoQ10 in protransferosomes and lipid and surfactant in simple CoQ10 dispersion.
17- In discussion section, the authors claimed the conversion of pro-transferosomes into transferosomes which is in contradiction with the text in introduction in which the author mentioned in situ formation of transferosomes. If the conversion occurs during loading into gel what is the purpose of protransferosomes formulation. Please respond.
18- In figure 6 the collagen density is higher in CoQ10-Ole treated group as compared to Protransf-CoQ10 group while these results are in contradiction with figure 7 results in which authors have shown higher number of fibroblast in Protransf-CoQ10 group. As fibroblast are collagen forming cells if their number is high in Protransf-CoQ10 treated group then collagen density must also be higher. Please respond in the discussion section.
19- Same can be seen in case of normal and CoQ10-Ole group, where difference in collagen density is significant while the number of fibroblast are almost same. Please respond
20- The authors have used the word Protransf-CoQ10 mask in the text repeatedly, but no such information is provided in the results or discussion. How Carbopol gel will form a mask? Please explain.
21- The authors didn't conducted any release study for the prepared formulation, which is an important aspect of drug delivery. Please respond.
22- Authors have not performed any permeation or deposition study please respond why?
23- The author have claimed that due to higher lipid concentration protransferosomes are preferred over transefersomes. However, most of the transfersomes have been reported with the same (85:15) ratio, which same with that used for transferosomesas reported by authors for protransferosomes. Please Justify
24- It is very surprising that the authors have not reported any characterization of the gel, being the final product gel characterization is of prime importance. Please respond.
25- The characteristic of 3 prepared gel i.e. color given in the text are in contradiction with the explanation in the text.
26- Melting point of CoQ10 is 48-52 C°, why the author has selected range of 30-300 C° in DSC study. Also please add Temperature range in Figure 4.
27- It is highly recommended to check the drug content, particle size and zeta potential of the formulation in stability study for at least 3-4 months as per the ICH guidelines.
28- Which animal models were used for Skin irritation and other in vivo studies? Please report in the manuscript.
29- References are missing in methodology section i.e. antiaging study. The authors are advised to add the following references.

https://doi.org/10.1080/03639045.2021.1890768
https://doi.org/10.1155/2021/9968602
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--
Salam,

Andang Miatmoko, PhD., Apt.
Department of Pharmaceutical Sciences
Faculty of Pharmacy, Airlangga University
Nanizar Zaman Joenoes Building
Campus C Airlangga University, Mulyorejo, 60115
Surabaya

Andang MIATMOKO <andang-m@ff.unair.ac.id> Wed, Nov 24, 2021 at 10:43 PM
To: Bhakti Thakkar <srep@nature.com>

Dear Dr. Thakkar,
Many thanks for your email, I am still struggling to collect the data since our experiments have been performed in other laboratories, however I have no courage to ask for a re-extension for submission. Could you please give us some advice? many thanks
[Quoted text hidden]

Bhakti Thakkar <srep@nature.com> Thu, Nov 25, 2021 at 9:39 PM
Reply-To: Bhakti Thakkar <srep@nature.com>
To: andang-m@ff.unair.ac.id

Dear Prof. Miatmoko,

Thank You for getting back to us.

Please do not hesitate if you need any help. We are there to assist you.

We prefer our authors to be completely happy with what they submit and what we can do is grant another grand extension, we have no issue with it.

I look forward to from hearing you.
[Quoted text hidden]

Andang MIATMOKO <andang-m@ff.unair.ac.id> Fri, Nov 26, 2021 at 7:19 PM
To: Bhakti Thakkar <srep@nature.com>

Dear DR Thakkar,
Many thanks for your email. I really appreciate it and thank you very much for your kind help.
Please allow me to finish the revision within 2 weeks, it really helps me a lot
I will send it once the revision is done.
Thank you
[Quoted text hidden]

Bhakti Thakkar <srep@nature.com> Mon, Nov 29, 2021 at 4:37 PM
Reply-To: Bhakti Thakkar <srep@nature.com>
To: andang-m@ff.unair.ac.id

Hello Prof. Miatmoko,

Thank You for your email.

We have extended the date to 13 December 2021.

Kind Regards,
Bhakti Thakkar (Ms.)
Editorial Support at Scientific Reports

[Quoted text hidden]
, Andang-m <andang-m@ff.unair.ac.id> wrote:
[Quoted text hidden]

[Quoted text hidden]
[Quoted text hidden]

[Quoted text hidden]
, Andang-m <andang-m@ff.unair.ac.id> wrote:
[Quoted text hidden]

[Quoted text hidden]

--
Salam,

Andang Miatmoko, PhD., Apt.
Department of Pharmaceutical Sciences
Faculty of Pharmacy, Airlangga University
Nanizar Zaman Joenoes Building
Campus C Airlangga University, Mulyorejo, 60115
Surabaya

--
Salam,

Andang Miatmoko, PhD., Apt.
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Nanizar Zaman Joenoes Building
Campus C Airlangga University, Mulyorejo, 60115
Surabaya

Andang MIATMOKO <andang-m@ff.unair.ac.id> Wed, Dec 15, 2021 at 1:31 PM
To: Bhakti Thakkar <srep@nature.com>

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Reply-To: Bhakti Thakkar <srep@nature.com>
To: andang-m@ff.unair.ac.id

Dear Prof. Miatmoko,

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Editorial Support at Scientific Reports

On Wed, 15 Dec at 6:31 AM , Andang-m <andang-m@ff.unair.ac.id> wrote:

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Salam,

Andang Miatmoko, PhD., Apt.
Department of Pharmaceutical Sciences
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--

Salam,

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Surabaya

andang miatmoko <andang-m@ff.unair.ac.id>

Scientific Reports: Decision on your manuscript

1 message

Scientific Reports <srep@nature.com> Wed, Dec 29, 2021 at 9:06 PM
To: andang-m@ff.unair.ac.id

Ref: Submission ID a904d626-ce96-4ebd-9a16-6f2ad0d44cb5

Dear Dr Miatmoko,

Re: “Improving the anti-ageing activity of coenzyme q10 through protransfersome-loaded emulgel”

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Editor comments
I am happy to accept the revised manuscript.

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Improving the anti-ageing activity of coenzyme q10 through protransfersome-loaded emulgel

Qurrota Ayunin[1,2], Andang Miatmoko[3,*], Widji Soeratri[3], Tristiana Erawati[3], Joni Susanto[4], Djoko Legowo[5]

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[2] Study Program of Pharmacy, Faculty of Pharmacy, Hospital Administration, Public Health, and Radiology, Institut Ilmu Kesehatan STRADA, Jl. Manila 37, Kediri, 64133, Indonesia

[3] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Nanizar Zaman Joenoes Building, Campus C Mulyorejo, Surabaya, 60115, Indonesia

[4] Department of Anatomy and Histology, Faculty of Medicine, Universitas Airlangga, Jl. Mayjen. Prof. Dr. Moestopo No. 47, Campus A Mulyorejo, Surabaya, 60132, Indonesia

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Running Title: Improving the anti-ageing activity of coenzyme Q10 through protransfersome

[*] To whom correspondence should be addressed:

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Tel/fax: +62-31-5933-150/+62-31-5935-249

Abstract

Coenzyme Q10 (CoQ10) is a naturally produced organic molecule which acts as an antioxidant agent, including in skin anti-ageing, and plays a major role in the social determinants of health. However, its level in the body will decrease during ageing. Therefore, an external supplement is required to repair damaged skin, especially the skin dermis layer. This study aims to evaluate the use of a protransfersomal emulgel to improve the skin delivery and stability of CoQ10 which demonstrates low water solubility, poor permeability and instability. CoQ10 was initially dissolved in oleic acid at a weight ratio of 1:56. Protransfersome was then loaded with CoQ10 (Protransf-CoQ10) and prepared using a composition of L-α-Phosphatidylcholine and Tween 80 at a molar ratio of 85:15. The Protransf-CoQ10 was dispersed in an emulgel base consisting of Tween 80 and Span 80 to produce Protransf-CoQ10 emulgel. The *in vivo* studies of anti-aging activity and irritability were further evaluated by applying daily 200 mg of emulgels twice a day to a 4 cm^2 section on the back of a UV-ray aging-induced male Balb/c mouse 20 minutes before irradiation. The results showed that Protransf-CoQ10 could transform into transfersomal vesicles with particle sizes of approximately 201.5 ± 6.1 nm and a zeta potential of -11.26 ± 5.14 mV. The dispersion of Protransf-CoQ10 into emulgel base resulted in stable Protransf-CoQ10 Emulgel during 28 days of observation at low temperatures. Moreover, the *in vivo* study revealed that Protransf-CoQ10 Emulgel successfully increases the collagen density and number of fibroblast cells in UV radiation skin-aged induced-mice which reflects its potential for repairing the skin ageing process. In addition, the 24-hour topical application of Protransf-CoQ10 Emulgel showed that no erythema or skin rash was observed during the study. In conclusion, loading CoQ10 into protransfersomal Emulgel successfully enhanced the stability and anti-ageing efficacy enabling its potential use as anti-ageing cosmetics.

Keywords: social determinants of health, coenzyme Q10, protransfersome, emulgel, anti-ageing cosmetics

Introduction

Premature skin ageing occurs because the skin, as the outermost organ, is always directly exposured to oxidants in the environment and is frequently a determining factor in social life. In addition, with increasing age, the activity of mitochondria in the body as a producer of energy in regenerating cells and tissues decreases[1]. Both these internal and external factors cause impaired tissue function and structural changes[2] culminating in skin ageing characterized by thinning of the epidermis and skin dermis and, ultimately, resulting in wrinkles, fine facial lines, and loss of elasticity[3,4]. Skin elasticity is largely dependent upon young collagen fibers and fibroblasts, collagen-producing cells in the dermis layer, whose numbers decrease during the ageing process[5].

Anti-ageing cosmetics have been widely used to promote skin regeneration, especially of the upper skin layers which protect the skin against dehydration, penetration by various microorganisms, allergens, irritants, reactive oxygen species (ROS) and radiation, thereby maintaining healthy skin[6]. Coenzyme Q10 (CoQ10) is one of the natural compounds often employed as an antioxidant, which plays a key role in stabilizing plasma and other intracellular membranes that protect against membrane phospholipid peroxidation[7]. CoQ10 acts by maintaining skin quality against free radicals[3] which have been known to activate the mitogen-activated protein kinase (MAPK) pathway that produces matrix metalloproteinases (MMPs) such as collagenase, thus damaging collagen fibers[8–10]. During ageing, the levels of CoQ10 in organs, including the skin, also decrease with the result that it is necessary to supply CoQ10 to achieve normal levels of between 0.50 and 1.65 μg/mL within the body.

Topical administration of CoQ10 has been shown to be effective in reducing wrinkles in skin that has been exposed to UV rays[3].

CoQ10 demonstrates low solubility in water (0.193 μg/mL) with a large molecular weight of 863.36 g/mol and high lipophilicity with a log P value of 21. This limits its penetration of the skin and explains it tendancy to be deposited in the stratum corneum[11]. Moreover, CoQ10 decomposes when exposed to light[12]. Loading CoQ10 into protransfersome, a vesicular carrier would probably constitute an effective strategy to enhance its biological activity within the skin in addition to increasing its stability.

Protransfersome, one of the provesicular nanocarriers that provides superior skin penetration and high stability, is widely used in transdermal delivery[13]. It possesses a flattened liquid crystal structure which is converted into an ultraflexible vesicle known as transfersome through the absorption of water from the skin during *in situ* hydration[14–16]. Transfersome is known to be an ultradeformable vesicle which is highly flexible and deformable, rendering it capable of passing through three skin penetration pathways[17]. Transfersome can rapidly penetrate the stratum corneum and enter the deeper skin layers via the intercellular lipid of the stratum corneum. It can fuse with the cell membrane, enabling it to enter the transcellular pathway, and is able to penetrate intact through the hair follicle pathway to penetrate the deeper layers of the skin[18–20]. Protransfersome is composed of amphiphatic lipid components such as phosphatidylcholine which, significantly, form double-layer membrane of vesicles, and surfactant as an edge activator that increases the vesicle flexibility or deformability[21]. In general, protransfersome contains a larger number of phospholipids than that present in transfersomes. During the manufacturing process, the protransfersome does not undergo an extrusion process to produce unilamellar vesicles as observed in the transfersome. This is because the protransfersome is a provesicular carrier system which will be converted into transfersome after it comes into contact with water in

situ[22]. Therefore, under a light microscope, the protransfersome can be seen to possess a palisade crystalline liquid form, whereas transfersomes are vesicular when in liquid media[23].

The use of ultradeformable vesicles has successfully improved the skin penetration of drugs and efficacy of anti-ageing properties of certain antioxidant molecules such as tocopherol which, when prepared in transfersome, possess good characteristics with a particle size <100 nm and entrapment efficiency of up to 90%. Moreover, it is well distributed within the skin layer and in vitro tests have proved it biocompatible with keratinocytes and fibroblasts, indicating its protective effect against oxidative damage and the potential for wound healing[24]. Previous reports have evaluated the use of nanocarriers for CoQ10 delivery such as a self-emulsifying drug delivery system (SEDDS)[25], ethosomes[26], transethosomes[27], and microemulsion[28]. The use of transethosomes successfully encapsulated CoQ10 up to 97% in vesicles and produced >95% drug deposition in different skin layers resulting in high efficacy for androgenic alopecia[27]. The low water solubility of CoQ10 frequently limits drug encapsulation efficiency in nanocarriers, thus the use of large amounts of lipid phase or ethanol may improve its loading.

In this study, a protransfersome containing CoQ10 will be prepared for anti-ageing emulgel. The high level of phospholipids contained in protransfersome is intended to improve drug loading. The use of protransfersome in the anti-ageing activity and irritation level of Protransf-CoQ10 emulgel was evaluated *in vivo* using UV-induced aged mice models. This study could represent an attempt to improve CoQ10 anti-ageing activity with the result that is effective, safe and non-irritating.

Results

This study aims to evaluate the potential use of protransfersome for topical delivery of CoQ10 as an anti-ageing agent. This study provides a scientific approach to successfully delivering low water solubility and poor permeable lipophilic substances and nanovesicular carriers specifically designed for anti-ageing cosmetics. The CoQ10 was loaded into protransfersomal emulgel composed of oleic acid containing soluble CoQ10, phospholipids as bilayer-forming lipids, and Tween 80 which acts as the edge activator of bilayer membrane after the protransfersome has been hydrated with skin water *in situ*, before being loaded into an emulgel base. There were improvements in stability and potential efficacy to inhibit premature ageing of the skin in UV-radiation skin aged-induced mice models as demonstrated in this study.

Physical Characteristics and Stability of Protransfersome-Loaded CoQ10 Emulgel

After dissolving the CoQ10 in oleic acid and encapsulated it into protransfersomes composed of phospholipids and Tween 80, the protransfersome-loaded CoQ10 (Protransf-CoQ10) forms a bright orange, viscous, oily liquid, with a distinctive phospholipid smell, and viscous consistency. After hydration with saline, lamellar vesicular structures rapidly formed and were ultimately transformed into transfersome vesicles, as shown in Figure 1.

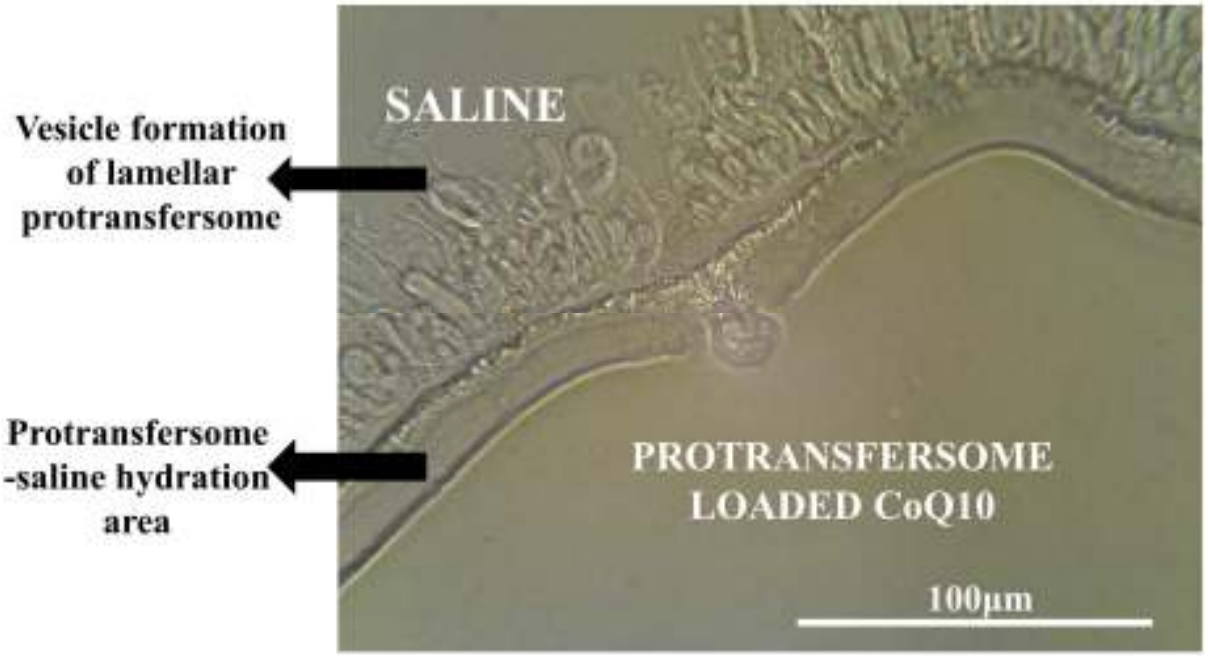


Figure 1. Lamellar structure of liquid crystals of the CoQ10 protransfersome emulgel after adding one drop of saline under an optical microscopy observation at 400x magnification (Scale bar:100 µm).

The dispersion of Protransf-CoQ10 into the emulgel base (Figure 2A-B) at a weight ratio of 2:1 produced Protransf-CoQ10 Emulgel whose color changes to brownish orange with a reduction in its pungent smell as shown in Figure 2C. CoQ10 dissolved in oleic acid (CoQ10-Ole) was in the form of a bright orange odorless emulgel (Figure 2D) whose character is identical to that of CoQ10 Emulgel except that it is more transparent due to no oleic acid being present in the formula (Figure 2E). The darkening color of Protransf-CoQ10 emulgel probably due to large amount of L-α-Phosphatidylcholine content of which is dark yellow in color[29] and easily oxidized when it is exposed to air in for lengthy periods [30,31].

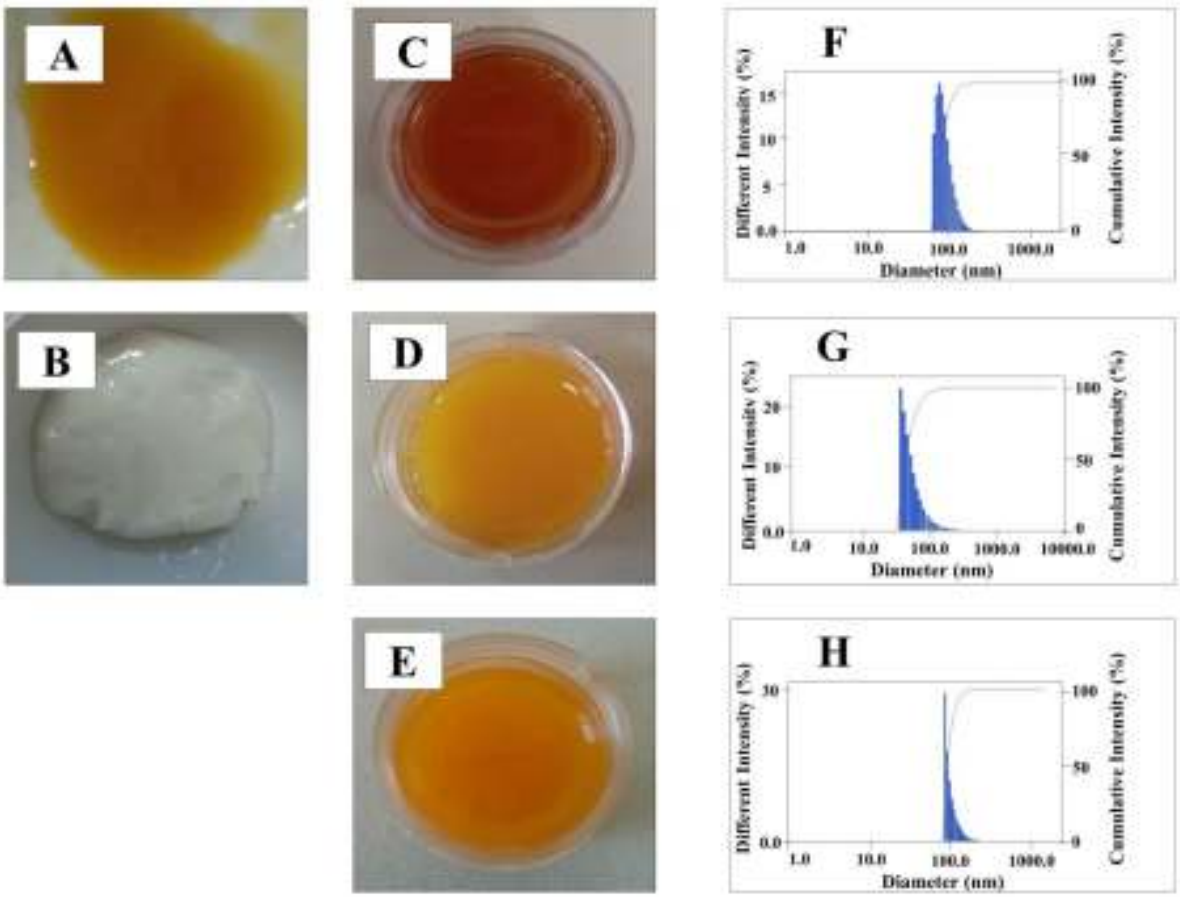


Figure 2. Visual appearance of protransfersomal CoQ10 (Protransf-CoQ10) (A), emulgel base (B), protransfesomal CoQ10 (Protransf-CoQ10) Emulgel (C), CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel (D), and CoQ10 loaded in emulgel (CoQ10 Emulgel) (E). The Intensity distribution of particle of protransfesomal CoQ10 (Protransf-CoQ10) Emulgel (F), CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel (G), and CoQ10 loaded in emulgel (CoQ10 Emulgel) (H).

The particle size and polydispersity index value were further evaluated since they determine the ability of the vesicles to penetrate the deeper layers of the skin. The smaller the particle size of the vesicles, the easier the vesicles are to penetrate. In addition, the smaller the polydispersity index value, the more homogeneous the particle size of the vesicles[16], thus ensuring that a larger number of vesicles penetrate the skin. From the results, it is evident that the entrapment efficiency value of the CoQ10 in Protransf-CoQ10 is comparatively high at

45.64 ± 7.52% with particle size of 201.5 ± 6.1 nm (by manual shaking method), polydispersity index value of 0.229 ± 0.047, and ζ-potential of -11.26 ± 5.14 mV, as presented in Table 1. The manual shaking method of five minutes' duration was reflective of the real situation in which protransfersomes change into transfersomes. The Protransf-CoQ10 Emulgel had the smallest particle size compared to both CoQ10-Ole Emulgel and CoQ10 Emulgel, which were 134.3 ± 4.8 nm < 146.9 ± 1.6 nm < 238.8 ± 3.1 nm, respectively, with intensity distribution of particle presented in Figure 2F-H. The polydispersity index values for Protransf-CoQ10 Emulgel, CoQ10-Ole Emulgel, and CoQ10 Emulgel were 0.291 ± 0.020 < 0.298 ± 0.019 < 0.384 ± 0.010.

Table 1. Particle Size and polydispersity index of CoQ10 loaded in emulgel (CoQ10 Emulgel), CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel. Each value represents the mean ± SD (n = 3).

Formula	Particle size (nm)	Polydispersity index (PDI)
CoQ10 Emulgel	±	± 0.010
CoQ10-Ole Emulgel	±	±
Protransf-CoQ10 Emulgel	134.3 ± 4.8	± 0.020

In order to evaluate any interaction between CoQ10 and protransfersomal matrix, a Fourier Transform Infra Red (FTIR) analysis was further observed. As presented in Figure 3, there were no new absorption bands of functional groups or peak shifts observed for Protransf-CoQ10, which shows similar infrared spectroscopical profiles to Protransfersome blank, while no specific peaks of CoQ10 appear. This result indicates that CoQ10 successfully encapsulated protransfersome and no chemical interaction between the mixtures occurred[32–34].

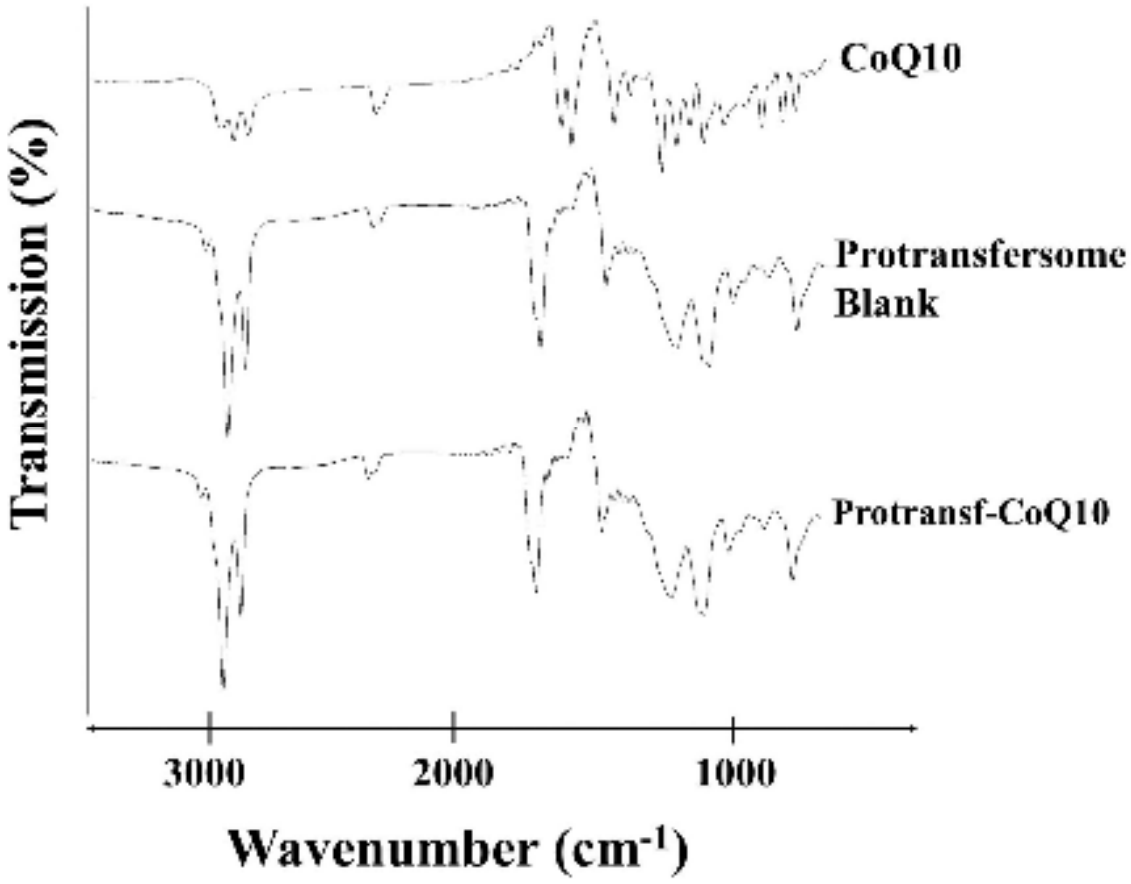


Figure 3. Fourier-transform infrared spectra of Coenzyme Q10 (CoQ10), Blank protransfersome, and protransfersome loaded CoQ10 (Protransf-CoQ10).

Moreover, according to the result of differential thermal analysis, the CoQ10 encapsulation into protransfersome produced changes in the structure of cristallinity. CoQ10 and L-α-Phosphatidylcholine showed sharp endothermic peaks at 53.3 and 112.3ºC, respectively; however, protransfersomal CoQ10 showed weak endothermic peak at 143.9ºC indicating that less ordered crystalline structures were observed as presented in Figure 4.

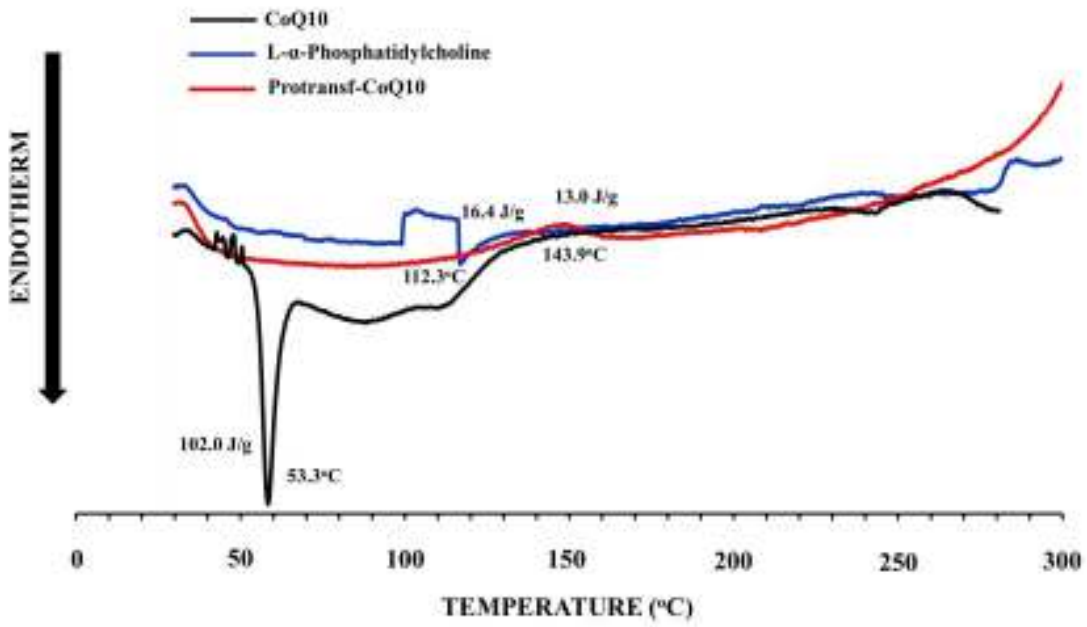
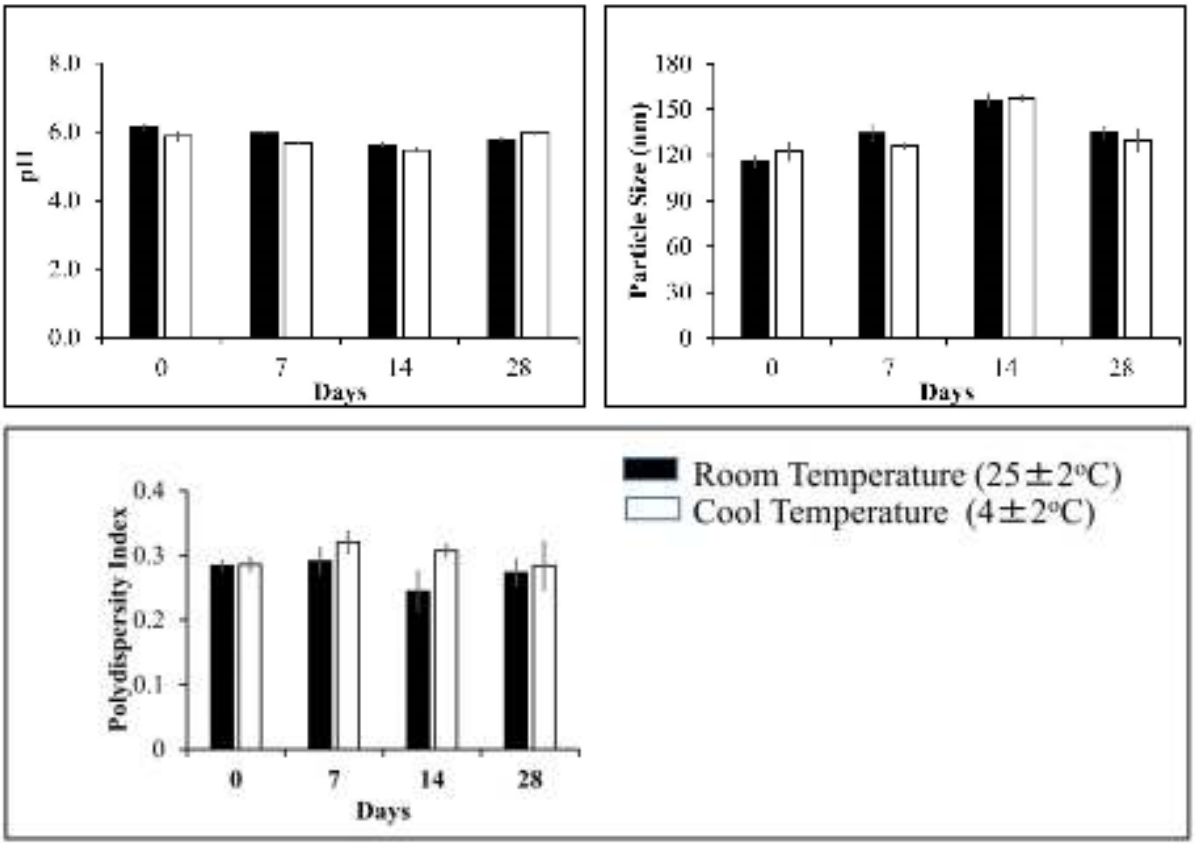


Figure 4. Differential thermal analysis of Coenzyme Q10 (CoQ10), L-α-Phosphatidylcholine as phospholipid component of protransfersome, and protransfersome loaded CoQ10 (Protransf-CoQ10).

A physical stability test was subsequently carried out to determine the physical resistance of the system when stored at different temperatures, namely; room temperature and a lower temperature for 28 days. During the study, the parameters of particle size, polydispersity index, and pH were observed. As seen from Figure 5, the results showed that after a 28-day storage period, there were no significant differences in particle size or polydispersity index ($P<0.05$). On the other hand, a significant difference was observed in the pH during the same period, although the pH value remained within the pH range of the skin. No significant difference existed in the particle size or particle size distribution of the preparation after 28 days of storage.



212

213 **Figure 5.** Evaluation of particle size, polydispersity index, and pH stability of
214 protransfersomal CoQ10 loaded in emulgel (Protransf-CoQ10 Emulgel) during 28 days
215 stored at room (25 ± 2°C) and cool (4 ± 2°C) temperatures.

216

217 ***In vivo* Anti-ageing Activity of Protransfersome-Loaded CoQ10 Emulgel**

218 To evaluate the ability of protransfersomes to topically deliver CoQ10 and produce an
219 effective anti-ageing activity, the Protransf-CoQ10 Emulgel was topically applied for 14 days
220 to the back skin of UV-rays-induced subjects who were subsequently observed for skin
221 histopathology. The control group subjects which received UV rays had the lowest collagen
222 density of 52.30 ± 7.87%, indicating that UV rays damage the collagen in the skin dermis.
223 The administration of both Protransf-CoQ10 Emulgel and CoQ10-Ole Emulgel significantly
224 improved the collagen density of UV-ray radiated subjects' skin as indicated in Figure 6.
225 However, there was no significant difference between these groups. The use of

protransfersomes successfully delivered CoQ10 providing protection against skin damage and repaired that resulting from exposure to UV rays.

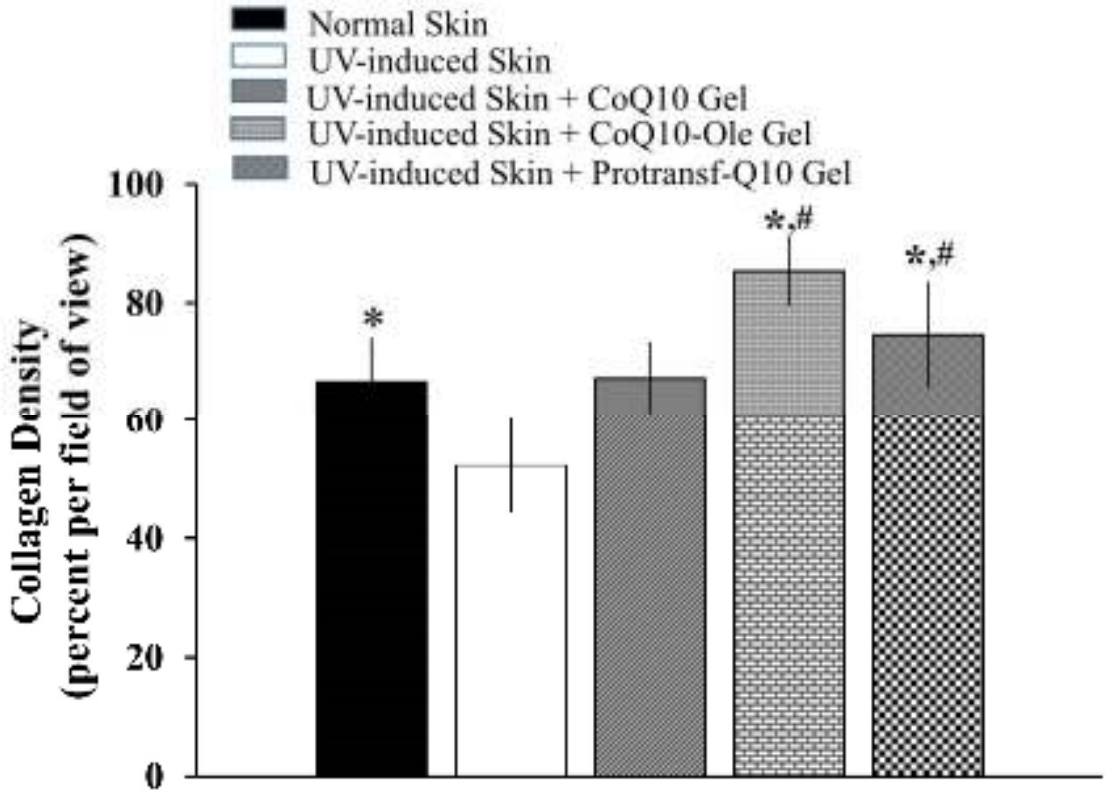


Figure 6. The collagen density of dermis layer of subject's back skin without and with UV-induced photoageing after topically applied with saline (Normal skin and UV-induced skin), CoQ10-loaded Emulgel, CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel once every two days for two weeks. *P<0.05 compared to UV-induced skin, #P<0.05 compared to Normal Skin.

The anti-ageing activity test result was further analyzed by observing the number of fibroblast cells capable of producing collagen. Therefore, the higher the number of fibroblasts, the more collagen was formed. In this study, the assessed fibroblasts were young and light purple in appearance. The results showed that the CoQ10 Emulgel had a significantly different number of fibroblasts compared to the control group, with pro-CoQ10

Emulgel producing the highest number of fibroblasts, which was 31.50 ± 9.48 cells per field view, as indicated in Figure 7. This shows that protransfersomes delivering CoQ10 successfully increase the number of fibroblasts.

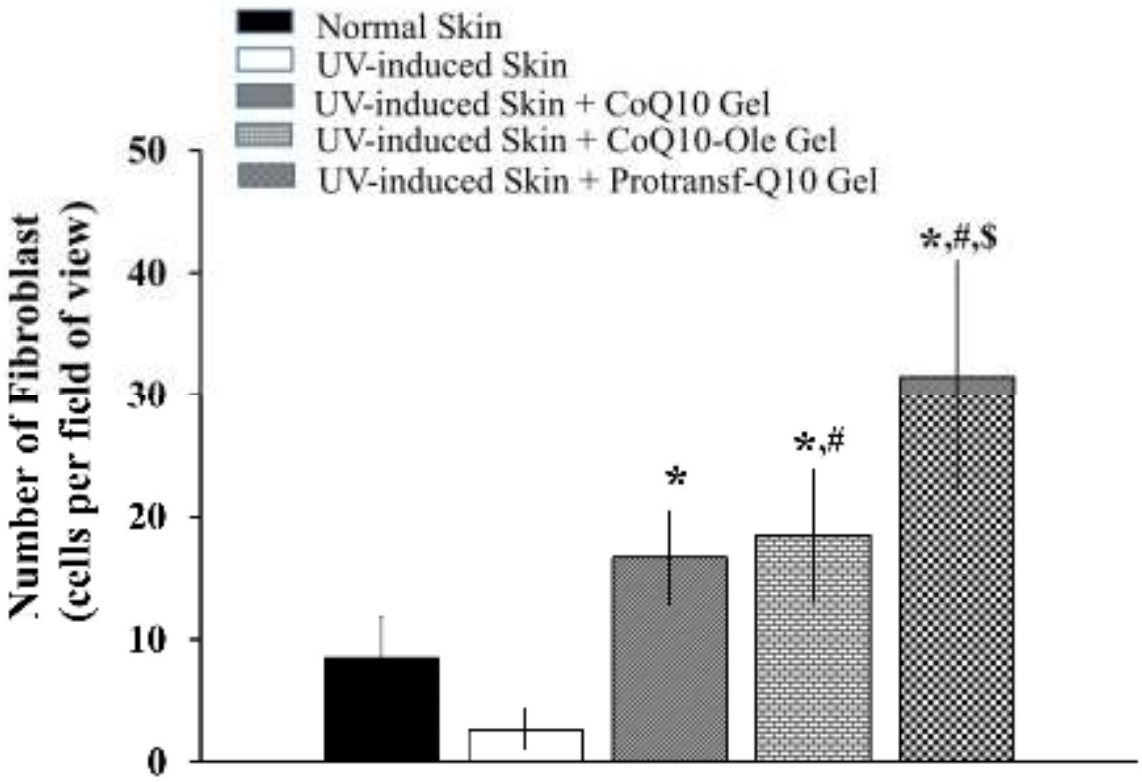


Figure 7. The number of fibroblasts of mice back skin without and with UV-induced photoageing after topically applied with saline (Normal skin and UV-induced skin), CoQ10-loaded Emulgel, CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel once every two days for two weeks. *$P<0.05$ compared to UV-induced skin, #$P<0.05$ compared to Normal Skin, \$ $P<0.05$ compared to CoQ10-Ole treated skin.

***In vivo* Skin Irritation Test**

The safe use of Protransfersome-loaded emulgels in this study was also evaluated by conducting an in vivo irritation test. Epidermis liquefaction, subepidermis edema, collagen

fiber swelling, inflammatory cells infiltration, dan appendages degeneration were observed for determining irritation in model's skin. As presented in Figure 8, there are differences in skin histopathology between normal and UV-induced skin. For further evaluation of severity level of skin irritation, scoring was then determined for each group.

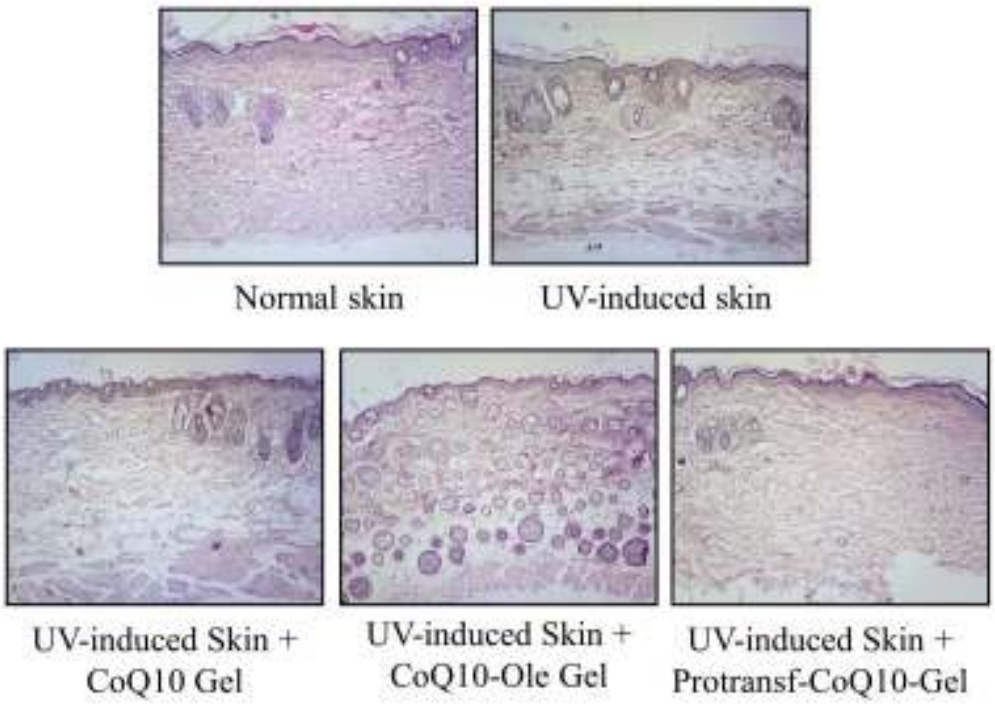


Figure 8. The histopathology of mice back skin stained with Hematoxylin-Eosin without and with UV-induced photoageing at 24 hours after topically applied with saline (Normal skin and UV-induced skin), CoQ10-loaded Emulgel, CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel.

The results of the histopathological scoring of the models' back skin after 24 hours of application showed that CoQ10 Emulgel had an irritation score of 0.52, while CoQ10-Ole Emulgel had one of 1.36, and Protransf-CoQ10 Emulgel one of 0.92 as presented in Figure 9. This result shows that the Protransf-CoQ10 Emulgel does not irritate the skin, while the CoQ10-Ole Emulgel induced mild irritation due to the nature of oleic acid. According to the

Kruskall Wallis statistical test results, there was no significant difference between these emulgel preparations.

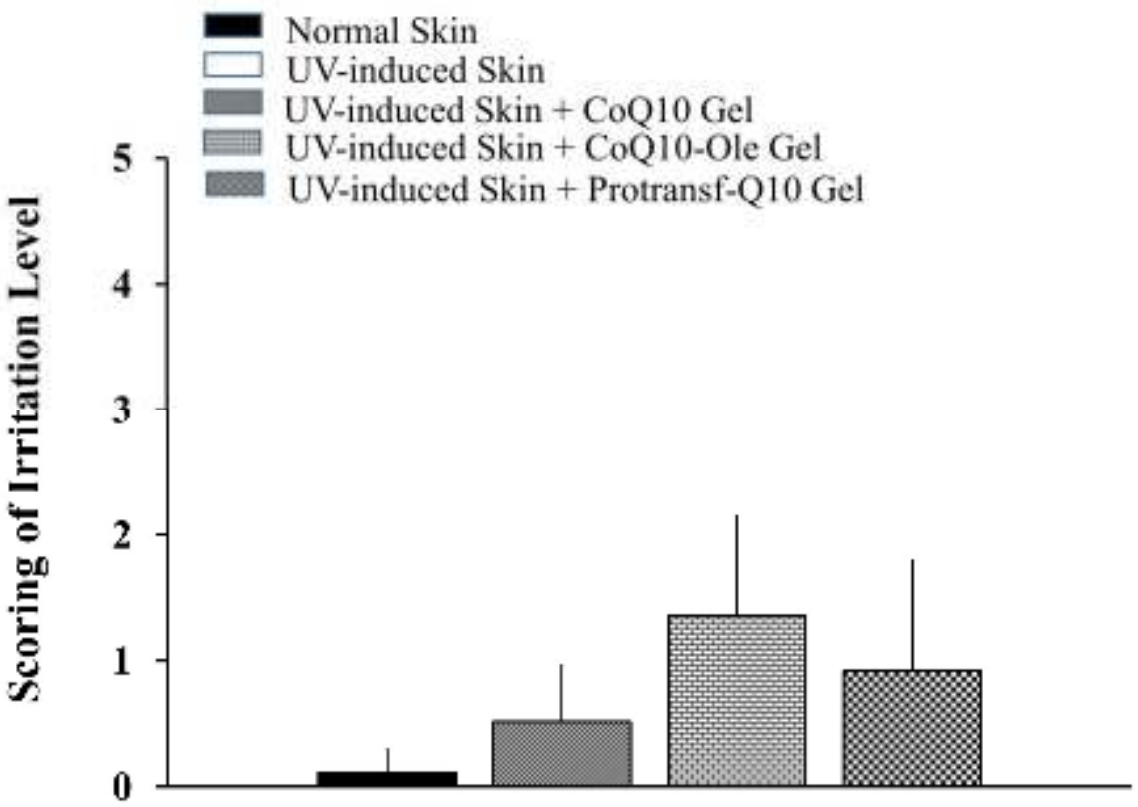


Figure 9. The scoring results of histopathology of mice back skin s without and with UV-induced photoageing at 24 hours after topically applied with saline (Normal skin and UV-induced skin), CoQ10-loaded Emulgel, CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel.

Discussion

In this study, the Protransfersomes and Protransfersomal emulgel preparations for CoQ10 delivery as the active cosmetic ingredient have the potential to inhibit premature ageing of the skin. The main purpose of protransfersome formulation is to significantly encapsulate CoQ10 in order to modify the physicochemical characteristics of CoQ10, rendering it more water dispersible and able to penetrate the skin since high lipophilic CoQ10 demonstrates low water solubility and poor skin penetration. However, the high content of oleic acid, which accounted for approximately 37% of the final weight of protransfersomal emulgel, would render it unacceptable for daily use as a skin cosmetic. Therefore, it was added to emulgel to increase its appropriateness for use. As far as the functional aspects of vesicles are concerned, the formation of transfersome due to hydration of protransfersome by water content in the emulgel base produces ultra-deformable vesicles which allow them to easily penetrate the skin. In addition, previous reports showed that the presence of a gelling agent would act as a steric hindrance which would be adsorbed onto the vesicle surface preventing fusion or aggregation, thus increasing physical stability during storage[35,36]. The addition of lipid vesicles to gel is beneficial for increasing vesicle stability, prolonging drug release, improving dermal permeability, and enhancing drug deposition in the skin [37].

Protransfersomes have been developed as the nanometer-sized carrier form of transfersome provesicles and have a higher phospholipid content compared to transfersomes. This enables the protransfersome system to demonstrate greater entrapment efficiency due to a higher number of vesicles formed that are subsequently available for encapsulating drugs, thus providing high stability when compared to the transfersome system [23]. Protransfersomes are able to carry active ingredients through the skin pores into the deeper layer. The protransfersome system analyzed in this study has positive characteristics including nanometer size, and thick consistency resulting from its large phospholipid content. When the

304 protransfersome is observed using a light microscope, a palisade lamellar structure appears in
305 the form of liquid crystals. This is due to differences in the degree of hydration of surfactants
306 and phospholipid molecules triggered by solvent limitations. The protransfersome forms as a
307 mixture of flat liquid crystals resembling palisade and vesicular lamellae linked together[39].
308 The percentage of entrapment efficiency (EE%) of the protransfersome system is a parameter
309 used to predict the stability of the dispersion[40] describing the amount of drug present in the
310 vesicle[41]. In this study, the EE% value was comparatively large because it corresponded to
311 the phospholipid content in the formula[42] and the tendency of CoQ10 to be retained in the
312 phospholipid membrane due to its lipophilic properties[39].
313 To improve acceptability, the protransfersome was formulated as an emulgel
314 preparation incorporating the use of an emulgel as the gel base. In this study, three types of
315 emulgels were developed and evaluated for their anti-ageing and irritability activity, namely;
316 Protransf-CoQ10 emulgel, emulgel loaded CoQ10 which was previously dissolved in oleic
317 acid (CoQ10-Ole emulgel) and CoQ10 dispersed in an emulgel base (CoQ10 emulgel).
318 During the homogenization method for preparing necessary samples the particle size
319 test involves manual shaking which is considered to closely replicate real-life conditions. The
320 particle size of the emulgel loaded Co-Q10 remained in the nanometer range, indicating that
321 adding emulgel base to the particle size of Protransf-CoQ10 had no effect. The particle size
322 of Protransf-CoQ10 Emulgel is smaller than that of Protransf-CoQ10 itself. This indicates
323 that the particles have turned into transfersome vesicles because they have been partially
324 hydrated by the presence of water in the emulgel base. The decreased vesicle size of
325 protransfersomal CoQ10 after dispersion into the emulgel base is probably due to the
326 shearing stress that occurs during the incorporation of Protransf-CoQ10 into hydrated
327 Carbopol-based emulgel. This causes the small vesicles formed and the emulgel matrix to be
328 adsorbed onto the vesicle surface, preventing vesicle fusion or aggregation[35,36], while

spontaneous hydration of protransfersome produces larger vesicles than those resulting from dispersion into emulgel. When compared to the particle sizes of CoQ10-Ole Emulgel and Co-Q10 Emulgel, those of all three emulgels-loaded CoQ10s can be measured in nanometers. The order of particle size from the smallest to the largest is Protransf-CoQ10 Emulgel < CoQ10-Ole Emulgel < Co-Q10 Emulgel. Co-Q10 Emulgel is the largest in size because CoQ10 is only dispersed in the emulgel base, while Protransf-CoQ10 Emulgel and CoQ10-Ole Emulgel had similar particle size and PDI probably due to CoQ10 solubility in Oleic Acid for both formulas[43]. From the results of the polydispersity index, it is evident that all particles have a uniform size distribution. This indicates that the preparation will be stable during storage because it reduces the tendency for particle aggregation which causes the system to become unstable.

A test was carried out to determine the physical stability of Protransf-CoQ10 emulgel when stored at different temperatures, namely; room temperature and colder temperatures for 28 days and whether differences in particle size, polydispersity index, and pH existed. There was no significant difference in particle size, polydispersity index, and pH of Protransf-CoQ10 emulgel during the study period.

The results of the anti-ageing activity of CoQ10 loaded in emulgel and evaluated for skin collagen density confirmed CoQ10-Ole Emulgel as having the highest percentage of collagen density, followed by Protransf-CoQ10 Emulgel. However, no significant difference existed between these groups (P>0.05). These two groups demonstrated significant improvement in collagen density compared with the control group whose subjects had been exposed to UV and who recorded the lowest density value. This is probably due to soluble CoQ10 in Oleic Acid loaded into emulgel had been easily released from emulgel than that of Protransf-CoQ10 Emulgel, which the formation of vesicle during hydration results in semipermeable bilayer membrane as water diffusion-limiting barriers for CoQ10 release. The

low collagen density has been known caused by imbalance between collagen synthesis by fibroblasts and collagen degradation of UV irradiation, while collagen synthesis is proportionally relate to fibroblasts resident[44]. Moreover, collagen synthesis by fibroblast will actively occur on the 4th day of 21 days[45]. The faster CoQ10 release from CoQ10 Ole Emulgel will stimulates fibroblast proliferation which increase expression of collagen matrix[46], while the late CoQ10 release from Protransf-CoQ10 Emulgel will result in delayed effects on fibroblast-stimulated collagen synthesis.

On the other hand, the Co-Q10 Emulgel-treated group had similar collagen density to that of normal mice, indicating that UV light damages collagen in the skin dermis. It has been known that UV-irradiation damage dermal collagen and elastin fibers[47], while CoQ10 increased the collagen content through decrease of MMP-1 protein level in mice exposed with UV-B[48]. CoQ10 also promotes the fibroblast proliferation[49]. However, it seems that the fibroblast stimulation process to produce collagen matrix between normal and CoQ10-treated groups is different. This situation differed from that of the group treated with CoQ10 in the emulgels. From these results, it can be concluded that CoQ10 provides protection against the ageing effects of UV rays.

The anti-ageing activity test was further evaluated for the number of fibroblasts in the skin tissues. Fibroblasts are cells capable of producing collagen. In this case, the assessed fibroblasts were young and light purple in color. The higher the number of fibroblasts, the more collagen was formed. The results showed that the CoQ10 emulgels had a significantly different number of fibroblasts compared to the control group, with the Protransf-CoQ10 Emulgel having the highest number, which was 31.50 ± 9.48% per field view. This indicates that CoQ10 is able to increase the number of fibroblasts.

The safety of these anti-ageing emulgels was further evaluated by an irritancy test. The results indicated that the Protransf-CoQ10 Emulgel produced no signs of irritation in the skin

tissues observed, while the CoQ10-Ole Emulgel induced mild skin irritation due to the nature of oleic acid.

Protransf-CoQ10 Emulgel has potential as an anti-aging product. However, information is lacking about both the drug release profile and its dermal penetrability which supports the theory that protransfersome and its incorporation into emulgel could prove a useful model for developing skin anti-aging cosmetics. Moreover, both the ability of protransfersome and protransfersomal emulgel to maintain drug stability and the physicochemical properties of the forms of skin dosage need to be evaluated for drug levels during study periods in line with ICH guidelines. Therefore, the product development involved could be comprehensively analyzed.

Conclusions

The results of this study indicate that emulgel-loaded protransfersomes, employed as delivery carriers of CoQ10, possess positive physical properties, thereby increasing anti-ageing activity with a low skin irritancy score. Proposing the incorporation of protransfersomal emulgel into cosmetics requires further studies especially on the acceptability test in humans and stability tests for longer storage times. From the results of this study, although the primary nature of CoQ10 severely limits its skin delivery, protransfersome provides potential benefits when used as a delivery system for active cosmetic ingredients within skin ageing therapy.

Methods

Materials

In this study Coenzym Q10 (CoQ10) was obtained from Kangcare Bioindustry Co. Ltd. (Nanjing, China). L-α-Phosphatidylcholine is a product of Sigma-Aldrich (Buchs,

Switzerland). Tween 80 and Span 80 were both purchased from Enviro Prima Co. Ltd. (Tangerang, Indonesia). The oleic acid used in this study was acquired from Brataco Co. Ltd. (Surabaya, Indonesia). All other reagents were of the available pharmaceutical and analytical grades.

Preparation of CoQ10-loaded protransfersome (Protransf-CoQ10)

The protransfersome was composed of L-α-Phosphatidylcholine, Oleic Acid, and Tween 80 as shown in Table 2 and prepared with modifications by the method previously reported by Gupta (2012)[15]. Initially, CoQ10 was stirred until completely dissolved in a mixture of oleic acid and Tween 80. Finally, L-α-Phosphatidylcholine was added and stirred until dissolved to produce Protransf-CoQ10.

Preparation of emulgel containing CoQ10-loaded protransfersome (Protransf-CoQ10 Emulgel)

A CoQ10-loaded protransfersome emulgel was prepared by adding the Protransf-CoQ10 to the emulgel base with a final CoQ10 content of 1%. The emulgel base was produced using Carbopol 940 added to a combination of Tween 80 and Span 80 (1:1) to form a homogenous emulgel base with the addition of Triethylamine (TEA) to adjust the pH to 6.0±0.2. Protransf-CoQ10, CoQ10 solution in oleic acid, and CoQ10 powder were subsequently added to this emulgel base and mixed homogenously to produce Protransf-CoQ10 emulgel, CoQ10-Ole emulgel, and CoQ10 emulgel, respectively.

Table 2. Formulation of CoQ and protransfersomal CoQ10-loaded emulgels.

Component	**Amount in Formula (%)**

	Protransf-CoQ10 Emulgel	CoQ10-Ole Emulgel	CoQ10 Emulgel
Coenzyme Q10	1.0	1.0	1.0
L-α-Phosphatidylcholine	24.9	-	-
Oleic Acid	37.2	37.2	-
Tween 80	4.3	4.3	-
Emulgel base	Up to 100.0	Up to 100.0	Up to 100.0

Evaluation of physical characteristics

The evaluation of physical characteristics includes particle size, polydispersity index, ζ-potential, microscopic observation, entrapment efficiency, and physical stability during storage.

The dispersion of Protransf-CoQ10 into an emulgel base at a weight ratio of 2:1 produced Protransf-CoQ10 emulgel whose color changes to brownish orange and the reduction on its pungent odor. Meanwhile, the CoQ10 dissolved in oleic acid (CoQ10-Ole) emulgel had an odorless, jelly-like consistency and was bright orange in color. These characteristics were identical to those of CoQ10 emulgel, although the latter had a more transparent appearance due to the absence of oleic acid from the formula.

Evaluation of particle size and ζ-potential were respectively carried out using a Delsa™ Nano Submicron Particle Size Analyzer (California, USA) and light scattering and electron scattering methods. Approximately 50mg of CoQ10-loaded protransfersome and emulgels were resuspended in 5 mL of 0.9% NaCl. The samples were then prepared using the manual shaking method for five minutes[23]. The suspension was further diluted by pipetting 150 µL of sample and added with 2 mL of deionized water (Otsuka Indonesia, Lawang, Indonesia) for sample measurement.

The Protransf-CoQ10 was observed microscopically to evaluate its transformation ability in relation to transfersome vesicles by placing a small amount of sample on a glass slide and covering it with a cover glass. A drop of 0.9% NaCl saline solution was added to the other side of the cover slip's cavity[38]. The evaluation was conducted using an optical microscope before, during, and after addition of 0.9% NaCl at 400x magnification.

The EE% was measured for CoQ10 loaded in protransfersome by means of UV-Vis spectrophotometry[16]. Approximately 100mg of Protransf-CoQ10 was weighed, and then hydrated with 2 mL phosphate buffered saline (PBS) pH 7.4 and sonicated for 30 minutes until homogeneous. The suspension formed was then centrifuged at 3,000 rpm for 30 minutes to obtain supernatant and sediment in a 10 mL glass tube. The sample was prepared by taking 1.5 mL of supernatant and then dissolved in 2 mL methanol, added to 2 mL PBS pH 7.4 and, finally, sonicated for 15 minutes. The sediment was dissolved in 1.5 mL methanol, added to 2 mL of PBS and sonicated for 15 minutes. The absorbance of each sample was measured by UV-Vis spectrophotometry at a wavelength of 275 nm. The EE% of CoQ10-loaded in protransfersome was calculated by means of the following equation:

$$\% \text{ EE} = \frac{CoQ10 \text{ levels in supernatant}}{CoQ10 \text{ levels in supernatant} + CoQ10 \text{ levels in sediment}} \text{ x } 100 \% \qquad \text{(Eq. 1)}$$

In order to evaluate whether any chemical or physical changes occurred in samples, spectroscopical and thermal analysis were further investigated. The spectroscopical analysis was evaluated using a Fourier Transformed Infra-Red analysis by using Spectrophotometer ECO ATRS Bruker Alpha II (Germany). About 1 mg sample was analyzed at wavenumbers of 450 to 4000 cm^{-1}. While, the thermal analysis was evaluated using *Differential Thermal Analysis* (DTA) instrument (Mettler Toledo FP 85, Switzerland). About 3-5 mg samples was

put into crucible sample pan. The sample was then subsequently heated from 30 to 300ºC at a heating rate of 10ºC per minutes.

Moreover, a stability test of the Protransf-CoQ10 emulgel was carried out by storing the samples at in the dark at room temperature (24 ± 2ºC) and, subsequently, a cold temperature (4 ± 2ºC) for 28 days[50–52]. The emulgel was evaluated for physical characteristics, i.e., pH and particle size, on the 28th day after preparation.

In vivo study of anti-ageing in UV-rays ageing induced mice

The *in vivo* anti-ageing activity was evaluated using Balb/c mice (*Mus musculus*) within the terms of a study protocol approved by The Ethics Commission of Faculty of Veterinary Medicine, Universitas Airlangga (Certificate number 2.KE.016.02.2020 dated February 4, 2020). All methods were performed in accordance with ARRIVE guidelines and relevant regulations[53]. Within this research, two types of study involving the uses of experimental models were evaluated, firstly, anti-ageing activity as indicated by collagen density and number of fibroblasts, and, secondly, a safety test incorporating irritancy scoring of skin tissue. The effect of the Protransf-CoQ10 emulgel was compared with those of CoQ10-Ole and CoQ10 emugels. Each group comprises of 4 mice as the study model. Prior to the study, the hair on the models' backs was trimmed with mechanical hair clippers, ensuring that their skin was not injured during this process. Each model was housed in a separate cage to prevent their touching the part to be smeared with the sample.

Anti-ageing Activity Test

The anti-ageing activity test was evaluated to establish the parameters of collagen fiber density and the number of fibroblasts. The study was carried out by applying 200 mg of the emulgels twice a day every day to a 4 cm^2 area of previously shaved skin on the models'

495 backs. The sample was applied 20 minutes before UV irradiation, in order to provide time for
496 absorption into the skin, and four hours after irradiation which is the point at which the
497 formation of Reactive Oxygen Species commences. An 80 mJ/cm^2 dose of UV light was
498 administered at an irradiation distance of 15 cm for 21 minutes. UV irradiation was carried
499 out once every two days, namely; on days 1, 3, 5, 7, 9, 11, and 13, with the models
500 subsequently being left for 24 hours on completion of the irradiation process to overcome the
501 effects of acute irradiation[54]. Sample application was also conducted on days when the
502 models were not exposed to UV irradiation. After 14 days, the models were sacrificed by
503 dislocation with the skin tissues being subsequently excised to produce a tissue section using
504 a microtome. To evaluate the collagen density, the tissue section was stained with Masson
505 trichrome staining, while for the observation of fibroblasts, the skin tissue section was stained
506 with Hematoxylin-Eosin Staining. The tissue section was then observed with a light
507 microscope (Olympus CX 31 Camera DP 22) using Cellsen Standard Software. Collagen
508 density was measured by histochemical scoring, while the number of fibroblasts was
509 calculated by digital analysis using Adobe Photoshop and Image J software. Density
510 measurement involved measuring the area of collagen coir and comparing it with the field of
511 view. The denser the coir collagen, the higher the density value, and vice versa. Calculation
512 of the density value was completed by means of calculating the area of the field of view and
513 the black colored area using Image J software calibrated fin advance or each degree of
514 magnification. The comparison of the black stained area with the field of view produced the
515 density value.
516
517 ***In vivo* skin irritancy evaluation of proransfersome loaded CoQ10 in emulgels**
518 In order to observe the irritant effects of CoQ10-loaded in proransfersome and emulgels,
519 histopathological changes in the skin tissues of each model after a 24-hour period of exposure

were observed. Firstly, the back hair of the models had been shaved. Approximately 200 mg of the sample was then applied to a 2x2 cm^2 area of skin on their backs. Twenty-four hours after application, the models were sacrificed by dislocation. Skin was excised with a microtome before being immersed in a formalin solution and stained with hematoxylin-eosin. The preparations were observed with a light microscope to assess the degree of skin irritation by means of histopathological scoring. Histological change data is semi-qualitative and features five variables, namely; epidermis liquefaction, subepidermal edema, collagen fiber swelling, inflammatory cell infiltration, and degeneration of the appendages in hair vesicles. The scoring method comprised a score of 0 = normal skin, 1 = mild irritation, 2 = moderate irritation, and 3 = severe irritation[55]. The data from each sample consisted of the mean value of the variable score for each of the five different fields of view at 100x and 400x magnification. All examinations involved the use of an ordinary light microscope (Nikon H600L, equipped with a 300 megapixel DS Fi2 digital camera and Nikkon Image System image processing software).

Statistical analysis

The data in this study consisted of three replicates. In order to test the significance of differences in the data relating to Protransf-CoQ10 emulgel, CoQ10-Ole emulgel, and CoQ10 emulgel, a statistical analysis was performed using the one-way variant analysis (ANOVA) method. After the normality and homogeneity of the data had been tested, a Post Hoc Tukey HSD test was administered. If the *P value* < 0.05, then a significant difference between the results of the tests performed existed. However, if the data was not normally distributed and homogeneous, the data would be analyzed using non-parametric statistics by means of the Kruskall Wallis method and, subsequently, a Post Hoc Mann Whitney U test. If the *P* value < 0.05; then a significant difference existed.

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Author Contributions

Qurrota Ayunin: 1) conception and design of the work, data acquisition, data analysis and interpretation; 2) Drafting the article; 3) Final approval of the version to be published; 4) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Andang Miatmoko: 1) conception and design of the work, data acquisition, data analysis and interpretation; 2) critically revising the article for important intellectual content; 3) Final approval of the version to be published; 4) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Widji Soeratri: 1) data analysis and interpretation; 2) critically revising the article for important intellectual content; 3) Final approval of the version to be published; 4) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Tristiana Erawati: 1) data analysis and interpretation; 2) Final approval of the version to be published; 3) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Joni Susanto: 1) data analysis and interpretation; 2) Final approval of the version to be published; 3) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Djoko Legowo: 1) data analysis and interpretation; 2) Final approval of the version to be published; 3) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Financial Disclosures

None

Ethical Conduct of Research Statement

The animal study procedures were performed in accordance with the ethical clearance issued by The Ethics Commission of Faculty of Veterinary Medicine, Universitas Airlangga (Certificate number 2.KE.016.02.2020 dated February 4, 2020)

Competing Interest

The authors declare no competing interest

737 **Figure Legends**

738

739 **Figure 1.** Lamellar structure of liquid crystals of the CoQ10 protransfersome emulgel after
740 adding one drop of saline under an optical microscopy observation at 400x magnification
741 (Scale bar:100 μm).

742

743 **Figure 2.** Visual appearance of protransfersomal CoQ10 (Protransf-CoQ10) (A), emulgel
744 base (B), protransfesomal CoQ10 (Protransf-CoQ10) Emulgel (C), CoQ10 dissolved in oleic
745 acid (CoQ10-Ole) Emulgel (D), and CoQ10 loaded in emulgel (CoQ10 Emulgel) (E). The
746 Intensity distribution of particle of protransfesomal CoQ10 (Protransf-CoQ10) Emulgel (F),
747 CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel (G), and CoQ10 loaded in emulgel
748 (CoQ10 Emulgel) (H).

749

750

751 **Figure 3.** Fourier-transform infrared spectra of Coenzyme Q10 (CoQ10), Blank
752 protransfersome, and protransfersome loaded CoQ10 (Protransf-CoQ10).

753

754 **Figure 4.** Differential thermal analysis of Coenzyme Q10 (CoQ10), L-α-Phosphatidylcholine
755 as phospholipid component of protransfersome, and protransfersome loaded CoQ10
756 (Protransf-CoQ10).

757

758 **Figure 5.** Evaluation of particle size, polydispersity index, and pH stability of
759 protransfersomal CoQ10 loaded in emulgel (Protransf-CoQ10 Emulgel) during 28 days
760 stored at room (25±2°C) and cool (4±2°C) temperatures.

761

Figure 6. The collagen density of dermis layer of subject's back skin without and with UV-induced photoageing after topically applied with saline (Normal skin and UV-induced skin), CoQ10-loaded emulgel, CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel once every two days for two weeks. *P<0.05 compared to UV-induced skin, #P<0.05 compared to Normal Skin.

Figure 7. The number of fibroblasts of mice back skin without and with UV-induced photoageing after topically applied with saline (Normal skin and UV-induced skin), CoQ10-loaded emulgel, CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel once every two days for two weeks. *P<0.05 compared to UV-induced skin, #P<0.05 compared to Normal Skin, $ P<0.05 compared to CoQ10-Ole treated skin.

Figure 8. The histopathology of mice back skin stained with Hematoxylin-Eosin without and with UV-induced photoageing at 24 hours after topically applied with saline (Normal skin and UV-induced skin), CoQ10-loaded Emulgel, CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel.

Figure 9. The scoring results of histopathology of mice back skin s without and with UV-induced photoageing at 24 hours after topically applied with saline (Normal skin and UV-induced skin), CoQ10-loaded Emulgel, CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel.

Table Legends

Table 1. Particle Size and polydispersity index of CoQ10 loaded in emulgel (CoQ10 Emulgel), CoQ10 dissolved in oleic acid (CoQ10-Ole) Emulgel, and protransfesomal CoQ10 (Protransf-CoQ10) Emulgel. Each value represents the mean ± SD (n = 3).

Table 2. Formulation of CoQ and protransfersomal CoQ10-loaded emulgels.

1. **Answer:**

However, the use of optical microscopy, under plain and polarized light, is generally used for detecting liquid lamellar crystalline of protransfersome as reported in Jain et al., 2003 and Vora et al, 1998. In the study of physical characteristics of protransfersome, the photomicrography analysis is intended to observe before and after in situ hydration as a proof that the vesicle formation has occurred.

4.answer:

In addition, there probably oxidation caused by air exposure occured during the preparation causing darkening color of the Protrans-CoQ10 loaded in emulgel, as we have no use of antioxidant on the formula. It has been reported that L-α-phosphatidylcholine can be more easily oxidized preventing free radicals bad effects to drugs (Bandarra *et al.* 1999; Cui and Decker 2016).

Line 148-150: We have added a sentence into the paragraph: "The darkening color of Protransf-CoQ10 gel probably due to large amount of L-α-Phosphatidylcholine content of which has dark yellow color(Sigma Aldrich 2018) and easily oxidized when it is exposed to air in for long periods (Bandarra *et al.* 1999; Cui and Decker 2016)."

4. **Answer:**

Regarding the concentration of CoQ10 in this formula, there was about 1%w/w, which is actually sufficient for analysis. On the other hand, CoQ10 encapsulation into protransfersome probably responsible for peak disappearance of CoQ10 in the Protransf-CoQ10 spectra profile, which makes it similar to that of Protransfersome blank, as previously reported. In addition, it may be the interaction occurs physically, with less or no chemical interaction causing no appearance of peak absorbances were observed (Kamaraj *et al.* 2017a; Miatmoko *et al.* 2021)

Miatmoko et al., 2021 reported that specific peak of Primaquine and Chloroquine were disappeared due to their encapsulation into inner phase of liposomes. Other reports also showed that if there are physical interactions between drugs and mesoporous silica, the spectra of drug will decrease or less sharp compared with native drug (Budiman 2019).

"This result indicates that CoQ10 succesfully encapsulated into protransfersome and no chemical interaction occurred in the mixtures (Kamaraj *et al.* 2017b; Budiman 2019; Miatmoko *et al.* 2021)."

5. **Answer:**

In this study, protransfersomal gel mask refers to semisolid emulgel formulation contained protransfersome intended use for facial mask purpose , while the normal protransfersome gel is similar to this gel mask either as emulgel or hydrophilic gel, which only requires gelling agents; however, there are some others main functional components of gel mask are required, such as moisturizers, fragrance, etc. However, this study is still the initial research for developing gel mask, which we intend to produce such products for, therefore, we replaced the use of "gel mask" terms in the manuscript.

6. **Answer:**
Many thanks for the comments. Emulgel is an emulsion transformed to a gel by gelling agent" (Ibrahim *et al.* 2017), therefore in this manuscript we used term emulgel due to the addition of emulgators or surfactants, in addition to gelling agent, to stabilize Protransfersome-CoQ10 dispersion. During pre-formulation study, without emulgators addition, the gel was unstable indicated by separation of oil phase due to large amount of hydrophobic oleic acid, which was about 37% in the final weight of protransfersomal gel. The gelling agent used is Carbopol, a methacrylate polymer that has been widely used for topical gel due to its acceptable textures and rigid consistency in neutral pH. The Carbopol itself could entrap oil phase in little amount, however when the amount is getting high, its hydrophilic nature could not stabilize the system, therefore the use of emulgator is urgently required. After adding some emulgators, the gel was stably formed and more acceptable due to watery consistency of this protransfersomal gel.

7. **Answer:**
Line 37-39: "The *in vivo* studies of anti-ageing activity and irritability were further evaluated by applying 200 mg of the emulgels twice a day every day to a 4 cm^2 back area of UV-ray ageing-induced male Balb/c mice at 20 minutes before irradiation."

Reviewer 2

1. Answer:
Line 368-371: we have added a sentence as the following: "The emulgel base was produced by using Carbopol 940 added with a combination of Tween 80 and Span 80 (1:1) to form a homogenous emulgel base with addition of Triethylamine (TEA) for adjusting pH to 6.0 ± 0.2."

3. Answer:
According to ICH guideline, there are accelerated, immediate and long term stability study, which requires 0, 3, and 6 months for accelerated test. However, we referred to other reports regarding development transfersomes and ethosomes which were stored at 4°C and at room temperature (25°C) for 30 days, and monitored every 10 days for physical stability (visual observation and mean size determination) and for drug entrapment efficacy and drug (Bragagni *et al.* 2012). Other reports also evaluated physical stability test including organoleptic testing (discoloration, odor, phase separation and clarity) and pH of the preparation at the room temperature (28^{o}C ± 2^{o}C), high temperature (40^{o}C ± 2^{o}C), and low temperature (4^{o}C ± 2^{o}C) for two weeks (Annisa *et al.* 2019). Therefore, in this study, to determine the stability of protransfersomal emulgels, we evaluated the physical stability for 28 days at cool and room temperature.

4. Answer:
Regarding this result, the presence of surfactant i.e. Tween 80 and Span 80 in the emulgel base may contribute to particle reduction of insoluble CoQ10, however we have no data of COQ10 dispersed in Carbopol base gel to support this opinion. Although CoQ10

particle had size in nanometer, its high hydrophobicity would prevent its penetration to dermis, as well as low stability protection against oxidation thus causing low efficacy in antiageing study in UV-irradiated mice.

5. Answer:
Regarding the similar particle size of CoQ10-Ole Emulgel to Protransf-CoQ10 Emulgel, it could be due to CoQ10 was dissolved in Oleic Acid contained in both emulgels, thus making the particle size is defined by Oleic acid dispersion in the emulgel base.

Line 312-314: while Protransf-CoQ10 Emulgel and CoQ10-Ole Emulgel had similar particle size, as well as PDI probably due to CoQ10 solubility in Oleic Acid for both formulas(Seo *et al.* 2009).

6. Answer:
Line 308-314: "The decreased vesicle size of protransfersomal CoQ10 after dispersion into the emulgel base probably be due to the shearing stress occurs during incorporation Protransf-CoQ10 into hydrated Carbopol-based emulgel causing the vesicle formed into small size and the emulgel matrix would adsorbed onto the vesicle surface, preventing vesicle fusion or aggregation (Gupta and Trivedi 2014, 2015); while, spontaneous hydration of protransfersome produce vesicle in larger size than that of dispersion into emulgel."

7. Answer:
Increasing stability of CoQ10 referring to its entrapment into protransfersome, which hydrophobic molecules of CoQ10 would be entrapped within hydrophobic stack of phospholipids in protransfersome system, or when protransfersome transforms into transfersome vesicle due to water hydration in emulgel, CoQ10 will be entrapped within hydrophobic bilayer membrane, thus preventing direct oxidation against free oxygen. However, in this study, we have not determined yet the level of CoQ10 after storage. In addition, the zeta potentials reflect the stability of vesicle-vesicle and vesicle-medium interaction; also predict the tendency of particle to aggregate. However, the incorporation into carbopol emulgel matrix have been proven to improve vesicle physical stability (Gupta and Trivedi 2014, 2015), thus the zeta potential actually less significantly affects it. On the other hand, the low value of zeta potential provide advantages for improved skin penetration of the vesicles (Gillet *et al.* 2011).

8. Answer:
The use of surfactants in this study is intended to form emulgel, with Carbopol as the gelling agent. Emulgel is an emulsion transformed to a gel by gelling agent (Ibrahim *et al.* 2017), therefore in this study, the addition of emulgators or surfactants, in addition to gelling agent, is intended to stabilize Protransfersome-CoQ10 dispersion. During pre-formulation study, without surfactants addition, the gel was unstable indicated by separation of oil phase due to large amount of hydrophobic oleic acid, which was about 37% in the final weight of protransfersomal gel. The gelling agent used is Carbopol, a methacrylate polymer that has been widely used for topical gel due to its acceptable textures and rigid consistency in neutral pH. The Carbopol itself could entrap oil phase in

little amount, however when the amount is getting high, its hydrophilic nature could not stabilize the system, therefore the use of surfactant is urgently required. However, to produce stable emulgel system, we calculated the Hydrophilic-Lipophilic Balance (HLB) value by considering the oil and water phase, thus making us to use both hydrophilic and lipophilic surfactants. After adding these surfactants, the gel was stably formed and more acceptable due to watery consistency of this protransfersomal gel.

9.Answer:
The entrapment efficiency of some hydrophobic drugs into transfersome could be high if the drug ratio used is low, generally less than 10% molar ratio to the total lipid. Even though, in many cases, according to the previous study we used, hydrophobic drug encapsulation into transfersome only provide no more than 30% of drug encapsulation. On the other hand, encapsulation of hydrophilic drugs such as doxorubicin into liposomal vesicle could produce almost 100% of drug encapsulation by active loading at drug to lipid ratio of 1:5.
Adding drugs into protransfersome successfully increase the amount of encapsulated drugs, without any drug precipitation occurs, as shown in our previous study (Miatmoko *et al.* 2015). In general, transfersome contains total lipid, including phospholipids and surfactants, of 8 mg/mL, which increasing the total lipid will cause lipid or vesicles tend to aggregate, while for protransfersome, it can be formulated up to 50 mg total lipid per mL, which much higher than that of transfersome.

Line 292-296: Protransfersomes have been developed as the nanometer-sized carrier form of provesicle of transfersome and have a higher phospholipid content compared to transfersomes. This enables the protransfersome system to have a higher entrapment efficiency due to more number of vesicles formation encapsulating drugs, thus providing high stability when compared to the transfersome system (Miatmoko *et al.* 2015).

10.Answer:
In this evaluation, we prepared the samples with three replications, and then we divided them for each storage conditions. The differences in size and pH at day 0 occurred because we measured each sample, thus the data reflects variations of measurement.

12. Answer:
The main purpose of protransfersome formulation is to highly encapsulate CoQ10, which is intended to modifiy physicochemical characteristics of CoQ10, making it to more water dispersible and easily penetrate the skin since high lipophilic CoQ10 has low water solubility and poor skin penetration. However, the high use of Oleic Acid in protransfersome would cause inacceptable daily use for skin cosmetics; therefore, we further added it into emulgel, to produce high acceptance for use. In the functional aspects of vesicle, the formation of transfersome due to hydration of protransfersome by water content in emulgel base still results in ultradeformable vesicles which allow them to easily penetrate across the skin.

In addition, previous reports showed that the presence of gelling agent would act as steric hindrance which will be adsorbed onto vesicle surface preventing fusion or aggregation thus increasing physical stability during storage (Gupta and Trivedi 2014, 2015). The addition of lipid vesicles into gel are beneficials for increasing vesicle stability, prolonging drug release, improving dermal permeability, and enhance drug deposition in skin (Ibrahim *et al.* 2017).

13. Answer:
Previously, the product is intended to be used as a facial mask for anti-ageing, however, regarding the use as mask, we have no data support it i this sudy. Therefore, we replaced the word of mask with emulgel, for general use.

14. Answer:
Surely we agree that the drug release profile would determine the delivery of drug to target site, however, in this study, we focused on the use of protransfersome as deformable vesicle for delivering CoQ10 into dermis site by evaluating its antiageing activity through analysis of collagen density and fibroblast counts. The drug release has not been performed yet and it becomes the lack of this study which need to be further determined.

Line 361-368: Protransf-CoQ10 Emulgel produce potential use for antiageing products, however, there are some lack informations about drug release profile as well as dermal penetrability to support the idea how the protransfersome and its incorporation into emulgel would become good model for developing skin antiageing cosmetics. Moreover, the ability of protransfersome and protransfersomal emulgel in maintaining drug stability and also physicochemical properties of skin dosage forms need to be evaluated for drug levels during study periods referring to ICH guideline. So, the product development could be comprehensively analyzed.

15. Answer:
Actually we also performed skin penetration study for 3 hours application; however, there was an accident that haematoxyllin-Eosin was added to the tissue section, so we could not observe nile red-labelled Protransf-CoQ10 gel, as shown in the figures below.

But, the key point of anti-ageing efficacy was well observed by determining collagen density and fibroblast counts, thus enabling to conclude the study.

17. Answer:
In the DSC study, we would like to evaluate the effects of incorporation CoQ10 into protransfersome and it can be seen in Figure 4 that the CoQ10 encapsulation into protransfersome produced changes in the structure of cristallinity. In addition, the melting points of pure L-α-Phosphatidylcholine is 226 or 236^{o}C. However, in this study, we used egg yolk L-α-Phosphatidylcholine with the purity of about 60%, therefore, there is no sharp peaks observed in this temperature. The use of wide range i.e. 30-300^{o}C is intended

to observe whether there are some changes in higher melting points, as observed in our previous studies (Miatmoko *et al.* 2021).