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Submission date: 24-Dec-2019 03:32PM (UTC+0800)

Submission ID: 1238232360

File name: in_fibroblast_cell_and_its_irritability_test_in_a_mice_model.pdf (3.7M)

Word count: 3970

Character count: 20255

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Abstract:

Background: Coenzyme Q10 is a fat-soluble antioxidant that can help to prevent collagen and elastin damage and avoid wrinkles. Coenzyme Q10 has several disadvantages to be formulated in topical dosage forms, such as low water solubility and large molecular weight. These make coenzyme Q10 retained in the stratum corneum and cause low skin penetration, so proper formulation is required to get products that can penetrate the skin layer. A nanostructured lipid carrier (NLC) consists of a matrix of solid lipids and liquid lipids in a certain amount with nanoparticle size; it may help increase the penetration of active ingredients.

Methods: For the antiaging activity test, mice were grouped into four treatment groups and killed on the 14th day; then the back of the skin was stained with Masson trichrome staining. For the irritation test, the mice were grouped into three groups and killed after 24 h; then the back of the mice was stained with hematoxylin-eosin staining.

Results: The number of fibroblasts in mice with NLC coenzyme Q10 is highest from all test groups. The irritation test results after 24 h of application preparation showed that NLC coenzyme Q10 did not irritate the skin of the back of male mice.

Conclusions: One percent coenzyme Q10 loaded in NLC induced the number of fibroblast cells in the mice model and showed no irritability effect in histopathology preparations.

Keywords: antiaging, antioxidant, collagen, Masson, staining, trichrome

DOI: 10.1515/jbcpp-2019-0320

Received: October 29, 2019; **Accepted:** November 15, 2019

Introduction

UV exposure activated the reactive oxygen species (ROS) that can increase collagen breakdown, decreasing procollagen and hyaluronic acid production in the skin, which caused wrinkles of skin aging [1]. The widely used therapy to overcome this process is antiaging cosmetics. The commonly used antiaging components are retinoids (vitamin A) and their derivatives, vitamin C, vitamin E, coenzyme Q10, α lipoic acid, and vitamin B3 [2], [3], and most of them are antioxidants. Coenzyme Q10 or ubiquinone is one of antioxidants found in every cell of the body. It can inhibit oxidative stress generated by UV light and it is the first antioxidant that is depleted from the skin. Coenzyme Q10 is an antioxidant that can protect the dermal matrix and fibroblast, and increases the collagen and hyaluronic acid in the skin. Unfortunately, the amount of coenzyme Q10 decreases with age [3], [4]. Coenzyme Q10 is reportedly capable of reducing the production of reactive oxygen species and DNA damage induced by UVA radiation in human keratinocytes *in vitro*. It has been shown to reduce UVA-induced matrix metalloproteinase (MMP) in human dermal fibroblasts. It may inhibit the production of interleukin-6, which stimulates fibroblasts in the dermis by paracrine to regulate MMP production, contribute to protecting the skin fibers from degradation, and rejuvenate the wrinkled skin [5]. Coenzyme Q10 has low water solubility (0.193 $\mu\text{g}/\text{mL}$ in water), large molecular weight (863.36 g/mol), and high lipophilicity ($\log P > 10$) [6], which make coenzyme Q10 poorly penetrate the skin. So coenzyme Q10 requires proper formulation to obtain products with good bioavailability that can penetrate the skin layer [6], [7].

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Nanolipid carrier systems such as solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have been developed from nanotechnology-based colloidal systems. The discovery of SLN and NLC has attracted great attention from the pharmaceutical and cosmetics industries. SLN is a spherical particle composed of solid lipids at room temperature with the addition of emulsifier, dissolved in the aqueous phase, while NLC is a refined form of SLN [8]. NLC consists of a matrix of solid lipids and liquid lipids in a certain amount. NLC remains in its solid form by controlling the levels of liquid lipids added to the liquid formulation, so that controlled drug release properties for NLC can be achieved [9]. NLC has a particle size distribution of up to 500 nm. It can reduce the formation of crystals in the lipids and also can increase drug entrapment [10]. The small droplet size of NLC gives a large surface area. It increases the number of active ingredients that can be trapped in it. So the NLC can carry bioactive molecules in a more controlled manner which is expected to release the drug to be greater, and its solubilization power increases [11] and the rate of release (flux) is directly proportional to the concentration so that the greater the active ingredient is trapped in the system, the release flux is also higher [12].

Materials and methods

Preparation of coenzyme Q10 in NLC

This formulation contains 1% coenzyme Q10 as an active ingredient, Tween 80 and Span 80 as surfactants, olive oil as liquid lipid, cetyl palmitate as solid lipid, ethanol 96% as cosurfactant, and acetate buffer pH 4.2 ± 0.2 as the water phase. Solid lipid, liquid lipid, and coenzyme Q10 were melted at 60 °C. While, surfactant and the mixture of cosurfactant and acetate buffer were heated at 60 °C in a different beaker glass. After the solid lipid was completely melted and the temperature reached 60 °C, the mixture of lipids was stirred with Ultra-Turrax High Shear Homogenizer at 5000 rpm; then the surfactant was added and mixed well. Finally, the mixture of cosurfactant and acetate buffer was added drop by drop until the end, rising the speed at 16,000 rpm for 3 min.

Physical quality test of coenzyme Q10 in NLC

Physical quality tested from NLC coenzyme Q10 are particle size and polydispersity index using a Delsa Nano Particle Size Analyzer, pH meter, viscosity using a cone and plate viscometer, entrapment efficiency with a centrifugation method, and droplet shape using a transmission electron microscope (TEM).

Animal preparation

The number of fibroblasts and collagen density were evaluated *in vivo* using an animal model, *Mus musculus*. Male mice weighing 30–40 g were used. Under anesthetization with ketamine intraperitoneal, the hair on the back was cleaned with a mechanical hair clipper approximately 2 × 2 cm. Each mouse was given a special cage so that it cannot touch the part to be smeared on the sample to be tested. All procedures of this research have been approved by The Animal Care and Use Committee of Universitas Airlangga, Indonesia, with Ethical Clearance number 645-KE.

In vivo skin fibroblast and collagen density testing

The mice were divided into four groups. The first group was mice treated with coenzyme Q10 in NLC to see the effectiveness of the active ingredient in the preparation, and the second group was mice treated with coenzyme Q10 dissolved in olive oil in order to see the activity of coenzyme Q10 dissolved in the oil phase only. The third group was a control group of mice's skin that was not given any treatment to see the condition of normal mice's skin without any treatment. The fourth and last group was mice that were only exposed to UVB light without any treatment to determine the condition of the skin that is exposed to UV light without any treatment. About 50 mg of the sample was applied to the back skin of mice twice a day (except the third group), 20 min before being exposed to UVB for 20 s (to allow time for absorption of topical material into the skin) and 4 h after irradiation (the formation of ROS starting 4 h after exposure). UV irradiation was done every 2 days, namely on days 1, 3, 5, 7, 9, 11, and 13, with the aim to get rid of the effects of acute irradiation. The application of the preparation was still carried out on days without UV irradiation. After 14 days, the mice were killed by

cervical dislocation. The skin was cut using a microtome and then stained with Masson trichrome staining. The preparations were observed under a light microscope.

***In vivo* skin irritability testing**

The mice were divided into three groups. The first group was mice treated with coenzyme Q10 in NLC, the second group was mice treated with coenzyme Q10 dissolved in olive oil, and the last group was a control group of mice's skin that was not given any treatment to see the condition of normal mice's skin without any treatment. Approximately 50 mg of the test sample was applied to the shaved back skin of mice. The mice were killed by dislocation after 24 h. The cut skin part was soaked with formalin solution for further hematoxylin-eosin staining. The preparations were observed under a light microscope. The observation of skin irritation was performed by histopathology scoring.

Results

Physical quality test of coenzyme Q10 in NLC

NLC coenzyme Q10 has particle size $69.7 \pm 0.93 \mu\text{m}$, with polydispersity index 0.35 ± 0.05 , pH value 4.45 ± 0.006 , and viscosity 387 ± 3.55 . The result of the coenzyme Q10 NLC entrapment efficiency test is $74\% \pm 0.87\%$. It showed high entrapment efficiency ($>70\%$). The morphology of NLC coenzyme Q10 particles showed that the system has spherical particles as shown in Figure 1.

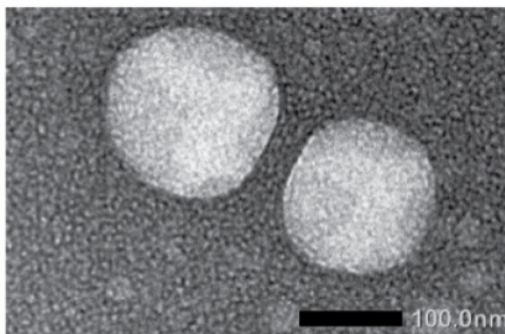


Figure 1: NLC coenzyme Q10 particles with a transmission electron microscope.

***In vivo* skin fibroblast and collagen density testing**

Fibroblast and collagen density from mice skin preparation was observed under a light microscope and is shown in Table 1 and Figure 2.

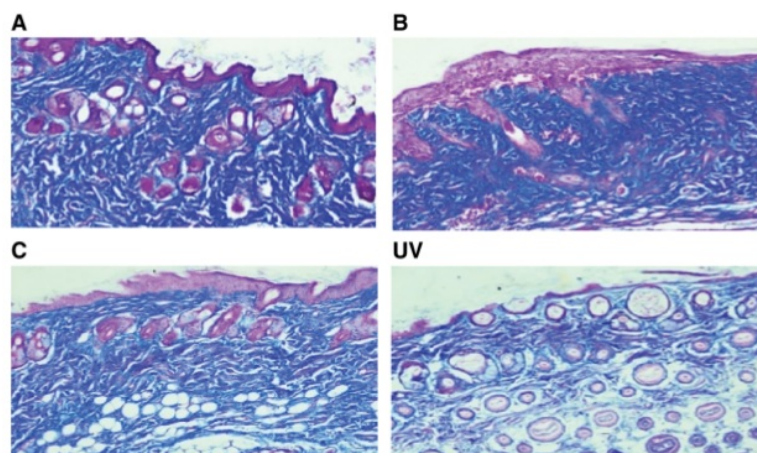


Figure 2: Picture of collagen in histopathology preparation at the back of male mice after 14 days using NLC CoQ10 (A) and the solution of CoQ10 in olive oil (B), also a control group without sample application (C) and a UV control group without sample application (UV). The picture was taken with a light microscope Nikon H600L (scale bar = 100 nm).

Table 1: Result of the number of fibroblasts and density collagen in the back skin of mice after 14 days using NLC CoQ10, and CoQ10 solution in olive oil, the control group of mice without sample application, and the UV control group of mice without sample application.

Group	Number of fibroblast cells	Collagen density
CoQ10 in NLC	94.00 ± 5.13	1.44 ± 0.19
CoQ10 in olive oil	56.87 ± 9.43	1.33 ± 0.33
Control	57.44 ± 23.26	1.44 ± 0.23
UV	30.22 ± 0.51	0.56 ± 0.19

In vivo skin irritability testing

The histopathology scoring of mice is shown in Table 2 and Figure 3.

Table 2: Result of histopathology scoring of mice after 24 h of sample application.

Test parameter	NLC CoQ10	CoQ10 in olive oil	Control
Epidermis			
Liquefaction	0.44 ± 0.49	0.67 ± 0.47	0 ± 0
Subepidermis			
Edema	0.11 ± 0.31	0.44 ± 0.49	0 ± 0
Subepidermis			
Collagen fiber swelling	0 ± 0	0.44 ± 0.49	0 ± 0
Infiltration of inflammatory cell	0.22 ± 0.41	0.11 ± 0.31	0 ± 0
Hypodermis			
Collagen fiber swelling	0 ± 0	0 ± 0	0 ± 0
Infiltration of inflammatory cell	0 ± 0	0 ± 0	0 ± 0
Skin appendages			
Degeneration	0 ± 0	0 ± 0	0 ± 0
Irritation score	0.73 ± 0.51	1.17 ± 0.51	0 ± 0

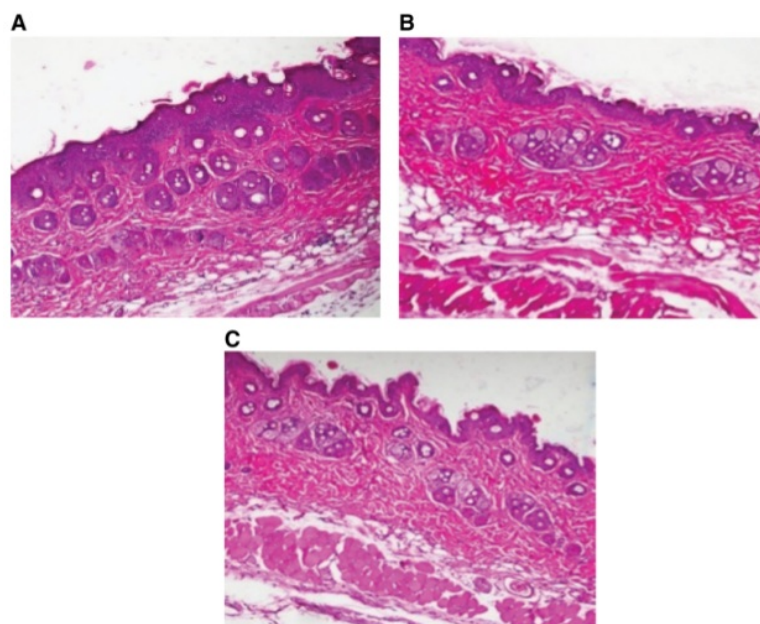


Figure 3: Microscopic images of mice skin backs after 24 h of coenzyme Q10 application in (A) NLC, (B) olive oil, and (C) controls; pictures were taken using the Nikon H600L light microscope at 100× magnification.

Discussion

NLC consists of solid fat, liquid fat, surfactant, cosurfactant, and water phase. Liquid fat used in this study was olive oil and the solid fat used was cetyl palmitate. There are three largest fatty acid contents in olive oil, namely oleic acid, palmitic acid, and linoleic acid, which in topical preparations can be used as emulsifying agents and enhancers to increase the penetration of transdermal preparations by increasing the bioavailability of water-insoluble drug ingredients and modifying the structure of stratum corneum [13]. To create a stable system, surfactants and cosurfactants are needed in right composition. Surfactants are used to reduce surface tension and interface tension. Based on the head group, surfactants are further divided into four surfactants, namely anionic, cationic, nonionic, and zwitterion surfactants [14]. The NLC formulation was made using a combination of nonionic surfactants, namely Tween 80 and Span 80. Nonionic surfactants were chosen because they are safer than other surfactants in terms of skin irritation [15], [16]. Meanwhile, cosurfactants chosen tend to have shorter C atoms, for example, ethanol, which also has the effect of increasing penetration in transdermal preparations [13]. This short chain alcohol was chosen to obtain NLC with a small particle size.

The size of the coenzyme Q10 NLC particle has been following the requirements of a diameter of 40–1000 nm with the polydispersity index value close to 0, which means homogeneous size. The value of pH NLC coenzyme Q10 was 4.4, which is similar to the pH of the buffer used in this study. The buffer pH 4.4 was used to maintain the stability of the active ingredient. Moreover, the pH value below the pKa of the active ingredient aimed to minimize the ionization of the active ingredient so that the active ingredient would remain in its molecular form and easily penetrate the skin. Besides, pH measurements are also needed to prevent irritation and dryness of the skin. NLC coenzyme Q10 has a pH value of around 4.45 ± 0.006 . The result of the coenzyme Q10 NLC entrapment efficiency test is $74\% \pm 0.87\%$. It showed quite high entrapment efficiency (>70%). The morphology of NLC coenzyme Q10 particles is analyzed using the TEM. Photographs taken with the TEM show that the system has spherical particles, which produce smaller contact area, thus preventing aggregation and friction between each other. From the results of the physical quality test of the preparations, it is known that the preparations have met the requirements for pre-clinical activity testing.

The dermis is the layer that is just below the epidermis layer. It is formed from many amorphous intercellular substances that act as “cement” in all components of the dermis. The main cell in the dermis is fibroblast. This cell produces an intercellular component known as collagen fibers. Collagen and elastin are proteins in the form of fibers. The fibers are intertwined with one another and make the dermis strong and elastic. Collagen fibers provide strength to the skin, whereas elastin fibers thinner than collagen are responsible for skin elasticity

and have the ability to return to their original shape after stretching. If both of these fibers are damaged, for example, due to the influence of age or excessive exposure to sunlight, the skin may become saggy, may not return to its original shape after stretching, and looks thin and wrinkled [17]. The collagen density test with the number of fibroblasts was performed *in vivo* on the skin of the back mice for 14 days. Fibroblasts are the most common cells found in connective tissue and synthesize some components of the extracellular matrix, such as collagen. The more amount of fibroblasts it has, the more collagen it will form. In the calculation of the number of fibroblasts, it was found that the group of mice given NLC coenzyme Q10 had a higher amount of fibroblasts (94.00 ± 5.13) than the group given coenzyme Q10 in olive oil (56.87 ± 9.43), control group (57.44 ± 23.26), and the UV group (30.22 ± 0.51). This suggests that the coenzyme Q10 prepared in the NLC delivery systems has the same effectiveness in the number of fibroblasts and can also increase the number of fibroblasts in the dermis of mice skin. Meanwhile, the dissolved coenzyme Q10 in olive oil has the same amount of fibroblasts as the control group, indicating that coenzyme Q10 is incapable of increasing the amount of fibroblasts if it is not helped by a particular delivery system.

The antiaging activity test was followed by scoring in the collagen density test in the same histopathology preparation with the number of fibroblasts. The collagen density test was performed by histopathology scoring, with detailed scores: score 0 means that the mice's skin has a collagen density of 25%–50%, score 1 means collagen density 50%–75%, and score 2 means collagen density >75%. The results of the histopathology test of the back skin of mice with Masson trichrome dye showed that the group of mice given NLC coenzyme Q10 had a higher collagen density score than the group of mice given coenzyme Q10 solution in olive oil, the group of mice that were not treated (control), and the group of mice which were only exposed to UV rays. From the results of scores on collagen density and microscopic images, it can be seen that mice groups exposed only to UV rays had the lowest scores (0.56 ± 0.20), suggesting that UV rays can damage collagen in the dermis of the skin. This is in contrast to the groups treated with coenzyme Q10 prepared in NLC and dissolved in olive oil; the collagen density of these three groups tends to be the same as the control group. From these results it can be concluded that coenzyme Q10 can protect against the adverse effects of UV rays.

Safety of cosmetics use is one of the purposes for such preparations. One of the safety features of topical preparations can be demonstrated by the absence of skin irritation on use. Examination of skin irritation can be done with histopathological observation. Examination of skin irritation by histopathological observation was carried out by the microscopic tissue scoring study using a light microscope. The results of histopathological scores of the back skin of mice after 24 h of dosage application showed that NLC coenzyme Q10 had an average score of 0.73 ± 0.51 so as not to cause structural changes in the back skin of mice, while coenzyme Q10 dissolved in olive oil had a mean score averaging 1.17 ± 0.51 and the control group had a score of 0 ± 0 . This indicates that the NLC system has less irritation risk than the active ingredient solution in olive oil. This is probably due to the addition of cetyl palmitate, which is a solid fat that has the ability of a moisturizer to minimize the occurrence of irritation.

The results show that the NLC of coenzyme Q10 enhances the quality of the particulate appearance of the dosage form. The *in vivo* test shows that NLC improves the skin fibroblast count, the collagen density, and decreases the irritability of the dosage form as compared to those that are olive oil based. Future plans include carrying out further tests regarding the stability of the preparations and the acceptability assays. The results of this study can be one of the potential antiaging cosmetics that can fight fine lines and wrinkles in a relatively short period of time.

Conclusions

One percent coenzyme Q10 loaded in NLC induced the number of fibroblast cells in the mice model and showed no irritability effect in histopathology preparations.

Acknowledgments

Thanks to Yayasan Bhakti Wiyata who allowed finishing and publishing this research. This paper has been presented at the 8th APPEN Conference and 2nd HPC Conference on 8–9 October 2019, Universitas Airlangga, Surabaya, Indonesia.

Research funding: None declared.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Ethical approval: All procedure of this research has been approved by The Animal Care and Use Committee of Universitas Airlangga, Indonesia, with Ethical Clearance number 645-KE.

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