BioFactors

Coenzyme Q_{10} protects against oxidative stress-induced cell death and enhances the synthesis of basement membrane components in dermal and epidermal cells

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Abstract.

Coenzyme Q10 (CoQ10), which has both energizing and anti-oxidative effects, is also reported to have antiaging action, *e.g.*, reducing the area of facial wrinkles. However, the mechanism of its anti-aging activity is not fully established. Here, we examined the effect of CoQ10 on human dermal and epidermal cells. CoQ10 promoted proliferation of fibroblasts but not keratinocytes. It also accelerated production of basement membrane components, *i.e.*, laminin 332 and type IV and VII collagens, in keratinocytes and fibroblasts, respectively; however, it had no effect on type I collagen production in fibroblasts. CoQ10 also showed protective effects against cell death induced by several reactive oxygen species in keratinocytes, but only when its cellular absorption was enhanced by pretreatment of the cells with highly CoQ10-loaded serum. These results suggest that protection of epidermis against oxidative stress and enhancement of production of epidermal basement membrane components may be involved in the antiaging properties of CoQ10 in skin.

Volume 35, Number 5, September/October 2009, Pages 435–441 •
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Keywords: coenzyme Q10, human skin cells, anti-oxidation, basement membrane, incorporation, keratinocyte, fibroblast

1. Introduction

Skin aging can be classified into two types, intrinsic aging and photoaging [1–6]. Intrinsic aging is characterized as an age-dependent deterioration of skin functions and structures, such as epidermal atrophy and epidermal–dermal junctional flattening. Photoaging is well known to be a consequence of chronic exposure of the skin to sunlight. Sun-exposed skin, such as face or neck skin, clearly appears to be prematurely aged in comparison with the relatively sun-protected skin of the trunk or thigh. The histological features of sun-exposed skin include cellular atypia, loss of polarity, flattening of the dermal epidermal junctions (DEJ), decrease in collagen, and dermal elastosis.

The basement membrane (BM) at the DEJ has many functions, of which the most obvious is to tightly link the epidermis to the dermis [7]. The disruption and reduplication of BM at the DEJ in sun-exposed skin is reported to be associated with increased levels of BM-damaging enzymes, such as plasmin and matrix metalloproteinases (MMPs), which degrade BM components (laminin 332—formerly called laminin 5, type IV and VII collagens) [8–10,11,12]. The impairment of BM structure is associated with functional changes of epidermal and dermal cells, and consequently may facilitate aging processes by damaging dermal extracellular matrices (ECMs) and inducing keratinocyte abnormality. So, it is important to promote BM repair by increasing synthesis of BM components such as laminin 332 and type IV and VII collagens in the epidermis and/or the dermis to improve epidermal–dermal communication and skin homeostasis, thereby strengthening defenses against skin aging [13,14].

In the skin, reactive oxygen species are formed inside or around skin cells after exposure to ultraviolet rays, as well as by migrating inflammatory cells and in mitochondria during respiration [15,5,16], and the oxidative stress imposed by these oxygen species is known to damage skin cells and to induce cell death [17,5,18,19]. A decrease in the number of dermal fibroblasts and a decrease in the thickness of epidermis because of reduced epidermal cell proliferation are observed in aged skin [20,21,2,3,22,23]. Therefore, an

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Received 7 June 2009; accepted 23 July 2009
DOI: 10.1002/biof.56
Published online 14 September 2009 in Wiley InterScience (www.interscience.wiley.com)

important target for anti-aging preparations is to protect cells from oxidative stresses.

Coenzyme Q10 (CoQ10), which has both energizing and antioxidative effects [24–28], is expected to have anti-aging properties. It is widely distributed in the living body [27,29,28,30]. With regard to skin, its content of CoQ10 decreases with ageing, and it was reported that wrinkle depth in human facial skin is reduced following application of CoQ10 [31,27,32]. Although an inhibitory effect on UV-induced inflammation, and inhibition of MMP-1 production have been reported [33,32], the mechanism of anti-aging actions of CoQ10 in skin is still not fully established. Therefore, we examined the effects of CoQ10 on the proliferation of fibroblasts and on the production of ECM, such as basement membrane components and dermal collagen, as well as its cell-protective effect against oxidative stress.

2. Materials and methods

2.1. Cell proliferation assay

Normal human fibroblasts from foreskin were seeded on a 96-well plate (3.5×10^4 cells/ml) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). After 4 h the concentration of FBS was reduced to 0.25%. After 24 h, CoQ_{10} (Kaneka Co., Osaka, Japan) was added by changing the medium to DMEM containing 0.25% FBS plus CoQ_{10}. The medium was prepared as follows: CoQ_{10} was added and dispersed to 4% Lutrol F 127 (poloxamer 407, BASF, Ludwigshafen, Germany) aqueous solution to obtain transparent 1 mM CoQ_{10} solution. The solution was diluted to designated concentration, and then added into 0.25% FBS-DMEM (final Lutrol F 127 concentration: 0.04%). Then, after 96 h in culture, the amount of DNA in each well was measured using Hoechst 33342 to estimate the number of cells in each well.

2.2. Determination of basement membrane components

For determination of laminin 332, human HaCaT keratinocytes from human foreskin were grown in keratinocyte growth medium (KGM) having a low calcium concentration. Cells were detached with 0.25% trypsin/EDTA, collected by centrifugation, washed and suspended in DMEM-F12 (2:1)-0.1% BSA. Using a 24-well plate, 0.5 ml of the cell suspension (8×10^4 cells/ml) was added to 0.5 ml of the same medium containing twice the desired final concentration of CoQ_{10}. After 24 h incubation, the supernatant and solubilized cell layer of cultured keratinocytes were collected and stored at -20°C.

For determination of type IV and VII collagens, human dermal fibroblasts (6×10^4 cells/ml) were also seeded, incubated and treated with CoQ_{10} in the same way as described for HaCaT keratinocytes, and samples were collected after 48 h incubation.

The contents of laminin 332, type IV and VII collagens were determined by means of sandwich ELISAs [34,35] using appropriate antibodies and expressed as the sum of the amount released into the culture medium and the amount remaining in the cell layer. All data are presented as percent of the control cultured without CoQ_{10}.

2.3. Preparation of highly CoQ_{10}-loaded cells

CoQ_{10} was dissolved in 1,4-dioxane and then added to FBS. FBS containing CoQ_{10} (250 μM) was prepared by mixing at 37°C overnight and used for the preparation of 10% FBS-DMEM containing 25 μM CoQ_{10} ($CoQ_{10}(+)$ medium). Human HaCaT keratinocytes were cultured in $CoQ_{10}(+)$ medium for 14 days, and these highly CoQ_{10}-loaded cells were referred to as $CoQ_{10}(+)$ cells. For some experiments, cells were also cultured in DMEM containing 10% FBS and 12.5 μM CoQ_{10}, and the resulting cells were designated $CoQ_{10}(1/2+)$ cells. As a control, 1,4-dioxane without CoQ_{10} was added to FBS. For comparison, HaCaT keratinocytes were cultured in 10% FBS-DMEM to which 1,4-dioxane or CoQ_{10} was added at 250 μM without the pretreatment. For quantification of the reduced form of CoQ_{10}, $CoQ_{10}H_2$ (ubiquinol), in cells, an HPLC-ECD system (NANOSPACE SI-2, Shiseido, Tokyo, Japan) was used according to the method of Yamashita and Yamamoto with minor modifications [36]. CoQ_{10} in cells (1.5×10^6 cells) or culture medium was extracted with 1 ml of isopropanol in a 1.5-ml polypropylene tube. After gentle mixing and centrifugation at 15,000*g* for 5 min at 4°C, 120 μl of supernatant and 30 μl of water were vortex-mixed, and applied to HPLC-ECD.

2.4. Evaluation of protective effect of CoQ_{10} against oxidative stress-induced cell death

To examine the effect of directly added CoQ_{10} on HaCaT cells, cells (1×10^5 cells/ml) were seeded using 10% FBS-DMEM without any additive. After the cells had adhered, the medium was changed to 0.5% FBS-DMEM containing dioxane solution of CoQ_{10} and the cells were incubated overnight. DMEM containing 50 mM AAPH (2,2′-azobis-(2-amidinopropane) dihydrochloride, Wako Pure Chemical Industries, Osaka) was added to each well, and after 3.5 h, cell viability was measured using Alamar Blue (TREK Diagnostics Systems, Ohio).

To examine the protective effect of a high loading of CoQ_{10} against oxidative stress-induced cell death, highly CoQ_{10}-loaded cells {$CoQ_{10}(+)$ cells} or control cells {$CoQ_{10}(-)$ cells} were seeded on a 24-well plate using 10% FBS-DMEM without 1,4-dioxane or CoQ_{10} at 1×10^5 cells/ml for AAPH and t-BuOOH (tert-butyl hydroperoxide, Wako Pure Chemical Industries) and at 8×10^4 cells/ml for hydrogen peroxide (Wako Pure Chemical Industries). After 4 h, the medium was changed to DMEM without FBS and incubation was continued overnight. AAPH, t-BuOOH or hydrogen peroxide was added to each well, and the cell viability was measured using Alamar Blue at 3 h after the addition of AAPH or t-BuOOH, or at 2 and 4 hr after the addition of hydrogen peroxide.

3. Results

3.1. Effect on CoQ_{10} on proliferation of dermal fibroblasts

Because cell proliferation is known to be decreased in aged skin, the effect of CoQ_{10} on the proliferation of dermal fibroblasts and epidermal HaCaT cells was examined. As shown in Fig. 1, CoQ_{10} increased the number of fibroblasts in a dose-dependent manner, whereas HaCaT cells did not respond to CoQ_{10} (data not shown).

3.2. Promoting effect of CoQ_{10} on production of basement membrane components

The effect of CoQ_{10} was examined on the production of laminin 332 in HaCaT keratinocytes and on that of type IV and VII collagens in dermal fibroblasts as basement membrane components. The proteins were determined by means of specific sandwich ELISA with appropriate antibodies. As shown in Fig. 2, CoQ_{10} increased the synthesis of laminin 332 in HaCaT cells in a dose-dependent manner. It also dose-dependently enhanced the production of type IV and VII collagens in dermal fibroblasts (Fig. 3), although it did not affect the production of type I collagen in dermal fibroblasts (data not shown).

3.3. Protective effect of CoQ_{10} against oxidative stress-induced cell death

Because protection of cells against oxidative stress is an important strategy for anti-aging products, the effect of CoQ_{10} on oxidative stress-induced cell death was explored. As shown in Fig. 4, CoQ_{10} did not protect HaCaT cells against cell death induced by the radical initiator AAPH during overnight incubation in DMEM containing 0.5% FBS. Because CoQ_{10} is insoluble in DMEM, its incorporation in cells might have been limited. To increase the incorporation of CoQ_{10} into the cells, CoQ_{10} was premixed with FBS at 37°C overnight, then the mixture was added to DMEM and the cells were cultured in the resulting DMEM containing 10 % FBS highly loaded with CoQ_{10} for 14 days. The incorporation of CoQ_{10} into the resulting highly CoQ_{10}-loaded cells, called $CoQ_{10}(+)$ cells, was confirmed by the detection of an increase of $CoQ_{10}H_2$, the reduced form of CoQ_{10}, in the cells, as measured by using HPLC-ECD (Fig. 5). As shown in Fig. 5, $CoQ_{10}(+)$ cells contained substantially more $CoQ_{10}H_2$ than

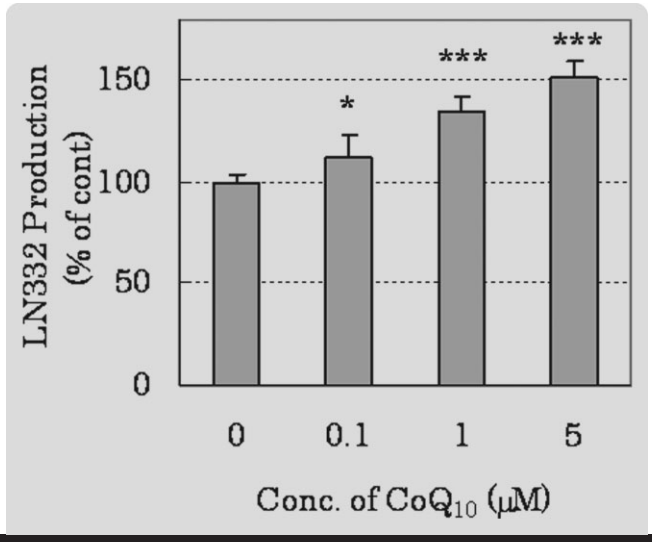

Fig. 2. Effect of CoQ_{10} on laminin 332 (LN332) production in human HaCaT keratinocytes. Mean ± SD, $n = 6$. Unpaired t test, $*P < 0.05$ and $***P < 0.001$.

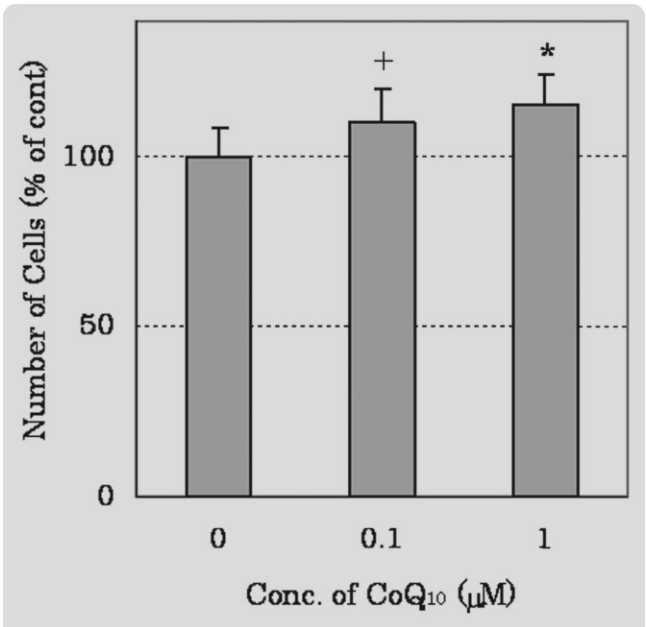

Fig. 1. Effect of CoQ_{10} on proliferation of fibroblasts. Mean ± SD, $n = 6$. Unpaired t test, $+P < 0.1$ and $*P < 0.05$.

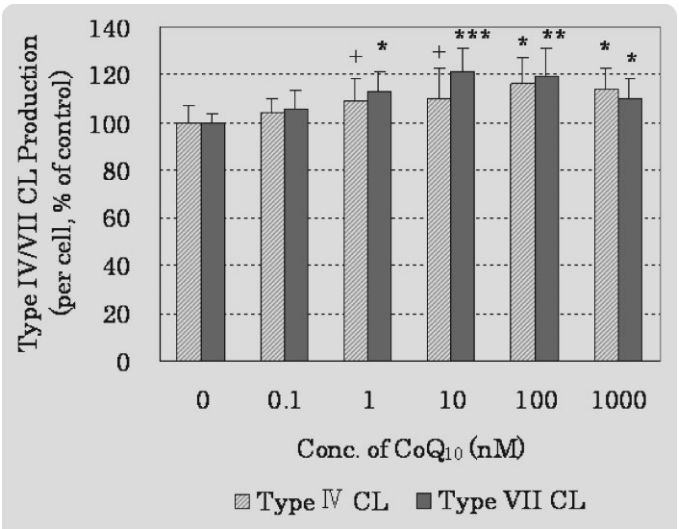

Fig. 3. Effect of CoQ_{10} on type IV and VII collagen (CL) production in human fibroblasts. Mean ± SD, $n = 6$. Unpaired t test, $+P < 0.1$, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

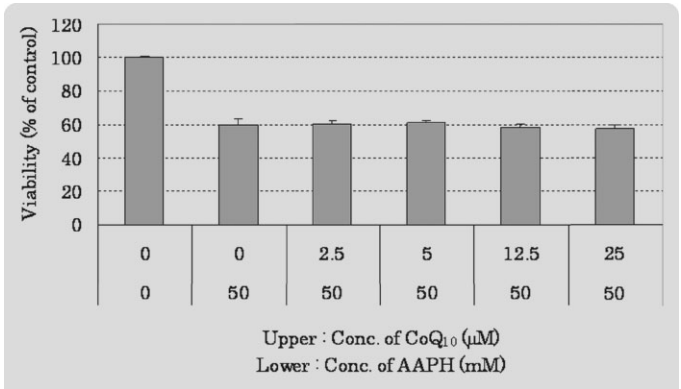

Fig. 4. Effect of directly-added CoQ_{10} on viability of AAPH-treated human HaCaT keratinocytes. HaCaT cells were seeded using 10% FBS-DMEM without any additive. After the adherence of cells, the medium was changed to 0.5% FBS-DMEM containing CoQ_{10} which was dissolved in dioxane and added into medium and then cells were incubated overnight. DMEM containing 50 mM AAPH was added to each well, and after 3.5 h, cell viability was measured using Alamar Blue. Mean ± SD, $n = 4$.

did $CoQ_{10}(-)$ cells or cells cultured with 10% FBS-DMEM containing only 1,4-dioxane. Cells cultured in DMEM containing premixed 10% FBS and 12.5 μM CoQ_{10} ($CoQ_{10}(1/2+)$ cells) contained about 60% as much $CoQ_{10}H_2$ as $CoQ_{10}(+)$ cells. These results suggest that CoQ_{10} was indeed incorporated into the cells and reduced to $CoQ_{10}H_2$. Furthermore, $CoQ_{10}(+)$ cells incorporated 2.6-fold more $CoQ_{10}H_2$ than did cells cultured in 10% FBS-DMEM containing CoQ_{10} without pre-mixing. These results showed that premixing of CoQ_{10} with FBS could be a good method for enhancing incorporation of CoQ_{10} into cells.

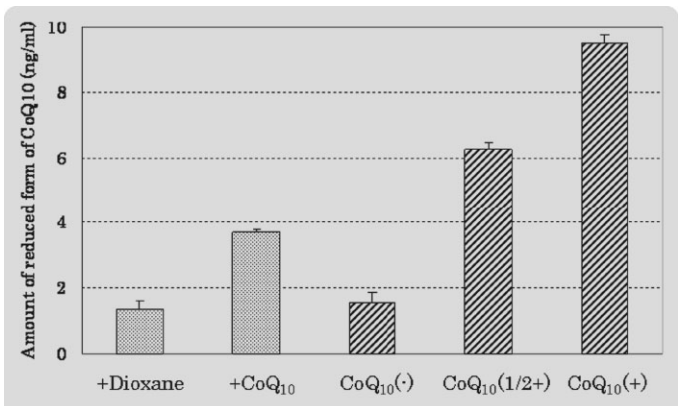

Fig. 5. Amount of reduced form of CoQ_{10}, $CoQ_{10}H_2$, in keratinocytes after direct addition of dioxane or CoQ_{10} to the medium (*dotted column*) and in keratinocytes cultured in medium highly loaded with CoQ_{10} by premixing FBS and CoQ_{10} as described in the text (control $CoQ_{10}(-)$, $CoQ_{10}(1/2+)$, and $CoQ_{10}(+)$ cells, *hatched columns*). Mean ± SD, $n = 3$.

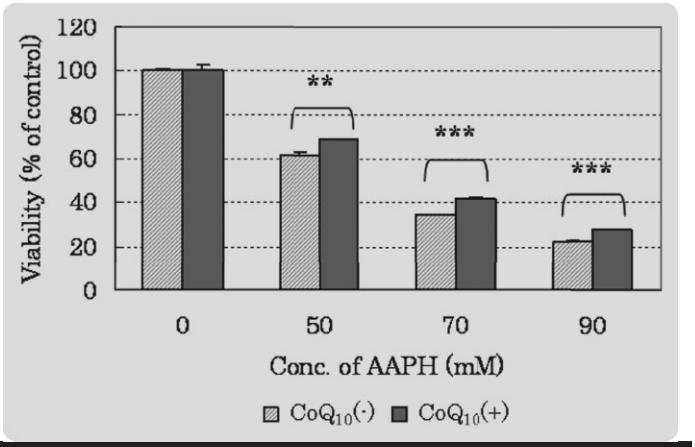

Fig. 6. Inhibitory effect of CoQ_{10} on AAPH-induced cell death of human HaCaT keratinocytes incubated in medium highly loaded with CoQ_{10} ($CoQ_{10}(+)$). Control: $CoQ_{10}(-)$. Mean ± SD, $n = 3$. Unpaired t test, $^{**}P < 0.01$ and $^{***}P < 0.001$.

The viability of $CoQ_{10}(+)$ cells was significantly higher than that of control cells after treatment with AAPH (Fig. 6). These results show that the amount of CoQ_{10} incorporated into cells when CoQ_{10} was directly added to the medium was small, and that CoQ_{10} can significantly suppress cell death induced by reactive oxygen species when it is highly

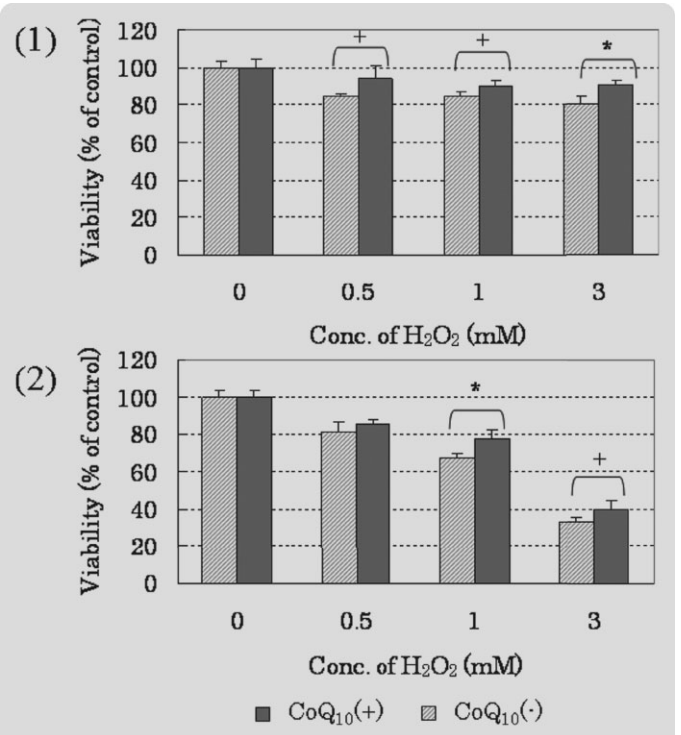

Fig. 7. Inhibitory effect of CoQ_{10} on hydrogen peroxide-induced cell death of human HaCaT keratinocytes. (1, 2) 2 and 4 h after addition of hydrogen peroxide, respectively. Mean ± SD, $n = 3$. Unpaired t test, $^{+}P < 0.1$ and $^{*}P < 0.05$.

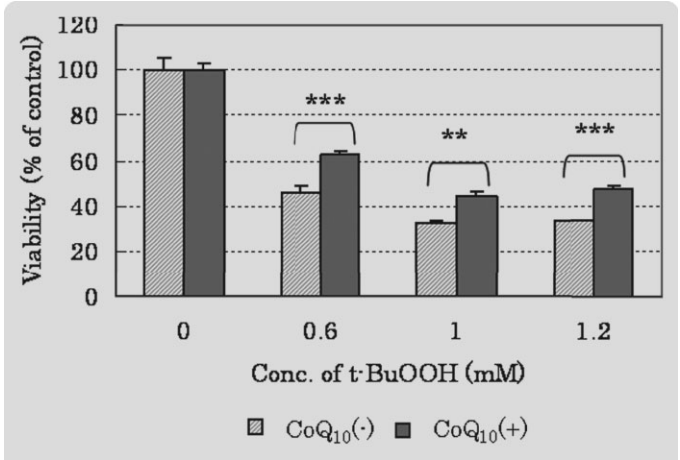

Fig. 8. Inhibitory effect of CoQ_{10} on t-BuOOH-induced cell death of human HaCaT keratinocytes. Mean ± SD, $n = 3$. Unpaired t test, $^{**}P < 0.01$ and $^{***}P < 0.001$.

loaded into the cells. The viability of $CoQ_{10}(-)$ cells and $CoQ_{10}(+)$ cells treated with hydrogen peroxide and t-BuOOH is shown in Figures 7 and 8, respectively. Viability of $CoQ_{10}(+)$ cells was again significantly higher than that of $CoQ_{10}(-)$ cells in both cases, showing that CoQ_{10} suppressed cell injury induced by these oxidative stresses.

4. Discussion

We examined the protective effect of CoQ_{10} against oxidative injury and its effect on the synthesis of basement membrane constituents in keratinocytes and fibroblasts.

The poor solubility of CoQ_{10} leads to an inefficient incorporation into cells when it is simply added to the culture medium. As shown in Fig. 4, simple addition of CoQ_{10} to the medium did not have a cell-protective effect against AAPH, although a water-soluble compound, carnosine, was clearly protective [37]. CoQ_{10} must be incorporated into cells and converted to the reduced form to be effective. When CoQ_{10} was simply added to the medium, the $CoQ_{10}H_2$ content of cells rose to 2.7 times that in control cells. On the other hand, when CoQ_{10} and serum were premixed, and the cells were cultured in medium to which the mixture had been added, the $CoQ_{10}H_2$ content rose to 6.0 times that of the control. A similar method of improving uptake has been reported for insoluble vitamin E by Takahashi et al. [38], who reported increased levels in whole cells, and in mitochondria and endoplasmic reticulum. Our finding that CoQ_{10} significantly suppressed oxidative injury of human HaCaT keratinocytes is consistent with previous reports showing a protective effect of CoQ_{10} on cells or tissues exposed to hydroxyl radical and peroxyl radical [39–42]. The ROS-generator t-BuOOH is known to induce both the diminished cell viability and nuclear DNA strand cleavages of BAE-2 endotheliocytes, which are protected dose-dependently with L-ascorbic acid-2-*O*-phosphate [43]. The cytoprotection is reported to be attributed to a decrease of intracellular ROS including hydroperoxide and hydrogen peroxide with L-ascorbic acid-2-*O*-phosphate. Therefore, the intracellularly-incorporated CoQ_{10} may be effective by inhibiting the generation of ROS and inhibit t-BuOOH-induced-cell injury. As it is well known that oxidative stress promotes skin aging, CoQ_{10} might ameliorate skin aging via its antioxidative effect.

Hoppe et al. [27] reported that CoQ_{10} increased the proliferative ability of in vitro aged fibroblasts that had been repeatedly subcultured. We also showed that it promoted proliferation of normal human fibroblasts. The proliferation of keratinocytes and fibroblasts is mainly controlled by growth factors. Some growth factors like epidermal growth factor are able to enhance the proliferation of both keratinocytes and fibroblasts, whereas other growth factors such as keratinocyte growth factor act on keratinocytes but not on fibroblasts. Like transforming growth factor beta, CoQ_{10} increased the proliferation of fibroblasts but not that of keratinocytes. Although its precise mechanism remains unclear, CoQ_{10} might enhance the signaling to proliferate only in fibroblasts. This cell-energizing property might involve enhanced ECM production or enhanced repair of cell injury. Hoppe et al. also found that CoQ_{10} increased production of glycosaminoglycan in their in vitro aged fibroblasts. We confirmed that CoQ_{10} increased production of type IV and VII collagens, but it had no effect on type I collagen. It also promoted laminin 332 production of HaCaT keratinocytes. The promoting effects on fibroblast proliferation and production of basement membrane constituents were apparent even without the use of our method to increase CoQ_{10} incorporation into cells, suggesting that even low concentrations of CoQ_{10} may be effective. A possible mechanism would be the induction of gene expression, but further study is needed to examine this idea.

It is known that laminin 332 promotes basement membrane repair. Reduplication and disruption of basement membrane in sun-exposed skin worsens with advancing age and is apparent even at an early stage, so chronic inflammation might easily be induced [8,9,13,16]. As CoQ_{10} has both an antiinflammatory effect [44,45] and a promoting effect on ECM production, its application to skin should tend to normalize the condition of the basement membrane. CoQ_{10} might also inhibit transfer of MMPs to dermis, blocking ECM damage, and thereby reducing the extent of wrinkle formation. Therefore, CoQ_{10} should be an effective ingredient in antiaging products for human skin.

In conclusion, we showed here that CoQ_{10} increases production of basement membrane components, increases fibroblast proliferation, and protects cells against oxidative stress, in addition to its known antioxidative, antiinflammatory, dermal glycosaminoglycan-increasing, and MMP-1-inhibitory actions. All these activities may contribute to its powerful antiaging action in skin.

Acknowledgements

The authors are very grateful to Ms. Tomoe Yoshikawa and Ms. Yuriko Yamamuro for technical assistance.

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