Original Article

Stimulatory effects of collagen production induced by coenzyme Q_{10} in cultured skin fibroblasts

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Coenzyme Q_{10} (CoQ_{10}) is a well-known antioxidant and serves as an essential carrier for electron transport and proton translocation in the mitochondrial respiratory chain. CoQ_{10} has been widely commercially available in Japan as a dietary and health supplement since 2001 and it is used for the prevention of lifestyle-related diseases induced by aging. Recently, it was stated that for Japan, which is facing an aging society, CoQ_{10} has been used in many skincare products. However, the physiological actions of CoQ_{10} in skin fibroblasts are not fully understood. In this study, we examined the effect of CoQ_{10} on cultured human skin fibroblast. In this study, CoQ_{10} treatment increased intracellular CoQ_{10} level and promoted proliferation of fibroblasts. In addition, CoQ_{10} increased mRNA expression of type I, IV, VII collagen, elastin, and HSP47, whereas CoQ_{10} has little effect on mRNA of type II and VIII MMP. These results suggested that CoQ_{10} has the efficacy that it increases collagen production in skin, thereby there is possible of the anti-aging by CoQ_{10} in Japan which reached an aging society, so that it might be based on new physiological function by CoQ_{10}.

Key Words: **coenzyme Q_{10}, collagen, anti-aging, fibroblasts, elastin**

The skin is the body's largest organ with an area of approximately 2 m^2, and it consist of epidermis, dermis and subcutaneous tissue. The epidermis is outermost layers in the skin, and it is composed of stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum germinativum. The dermis is layers of skin between epidermis and subcutaneous tissue, and it consist of connective tissue. The fibroblasts have synthesized extracellular matrix (ECM) such as collagen, elastin, gelatin and fibronectin, which play skin physiological function. Collagen and elastin are the major fibrous proteins as connective tissue in skin. Collagen molecules have three α-chain that form a triple helix, which is one of ECM, has variety types and roles. For example, type I collagen form cross-striated fibrils with an axial periodicity of approximately 67 nm.[1] In addition, a part of basement membrane consists of type IV collagen. Moreover, type VII collagen connect basement membrane to dermis. Collagen fibrils do not stretch, whereas elastin is flexible and elastic fiber, which is structural protein capable of stretching in two dimensions. The basic subunit of elastin fibrils is tropoelastin, it has a molecular weight of about 72,000 and contains about 800 amino acid residues. Like collagen, it is rich in glycine and alanine. Furthermore, Matrix metalloproteinase (MMP) has been known as chemokine and catabolic enzyme of ECM, and it have been included in the group of inflammation enzyme. In particular, MMP play an important role in degradation of collagen. Collagen expression decreased in aged, whereas aging increased level of MMP caused more collagen degradation in skin.[2,3]

It is well known that coenzyme Q_{10} (CoQ_{10}) serves as an essential carrier for electron transport and proton translocation in the mitochondrial respiratory chain, and it is composed of series of enzyme complexes embedded in the lipid bilayer of the inner mitochondrial membrane.[4,5] Besides its role in electron-transfer reactions, CoQ_{10} is an effective lipid soluble antioxidant[6,7] that contribute to exclude a free radical, thereby preventing oxidative damage in the human body. CoQ_{10} in the bodies of human beings is thought to be provided by both dietary intake, such as dietary foods and health supplements, and *de novo* biosynthesis.[8] From these research results, The Ministry of Health, Labour and Welfare in Japan permitted the use of CoQ_{10} as a food additive as long as no claims were made about its pharmacological effectiveness and application, and CoQ_{10} has been widely commercially available in Japan as a dietary and health supplement since 2001 and is used for the prevention of lifestyle-related diseases induced by aging. Recently, it was stated that for Japan, which is facing an aging society, CoQ_{10} has been used in many skincare products and purpose of anti-aging substances. Then, while sales of dietary and health supplement products have been rapidly increasing in Japan, it is essential to supply quality-controlled products for consumers. As one of the causes of this thing, there is Hoppe *et al.*[9] paper that CoQ_{10} is also reported to have anti-aging actions, it also *in vivo* where authors have demonstrated a reduction in skin wrinkles. After it is reported, several reports of CoQ_{10} for the skin have been reported by different investigators.[10–12] However, investigators have used CoQ_{10} of lipid soluble for all those reports. Recently, a part of CoQ_{10} containing products with skincare and health supplement have distributed in Japan is included water soluble CoQ_{10} containing products. Nevertheless, few studies have examined that effect of water soluble CoQ_{10} in skin. In consequence, the physiological actions of water soluble CoQ_{10} in human skin are not fully understood. Therefore, this paper describes that we investigated the effect of water soluble CoQ_{10} in cultured human neonatal dermal fibroblasts.

Materials and Methods

Materials and cell culture. CoQ_{10} powder, PureSorb-QTM™40 (P40), which is containing 40 w/v% CoQ_{10}, was kindly donated by Nisshin Pharma Inc. (Tokyo, Japan) for this study.[13] High performance liquid chromatography (HPLC) solvents and ethanol were purchased (HPLC grade) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemi-

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cals used were of analytical grade, available from commercial suppliers. Antibodies against type I collagen was obtained from COSMO BIO Co., Ltd. (Tokyo, Japan). Normal human dermal fibroblast (NHDF) was purchased from Lonza Co. Ltd. (Tokyo, Japan). Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 2% fetal bovine serum (FBS; MP Biomedicals, Illkirch, France), 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin (Nacalai Tesque). The cells were incubated under 37°C in a humidified atmosphere of 5% CO_2 and 95% air in a CO_2 incubator. After 24 h, culture medium was replaced by the DMEM containing 2% FBS and P40, and the cells were subsequently pre-cultured for 1 week in the CO_2 incubator for all experiments in this study.

Measurement of CoQ_{10} levels in human skin fibroblast. CoQ_{10} levels were measured by HPLC-electrochemical detector (HPLC-ECD) with the method of Okamoto *et al.*[14] Intracellualr CoQ_{10} levels were expressed as μmol per mg protein.

Protein assay. Protein assay was performed to normalize with the Bradford method.[15]

Cell proliferation assay. Fibroblasts were seeded on 24-well plates (1.0×10^4 cells per well) and cultured in DMEM containing 2% FBS for 24 h. Culture medium was then changed to DMEM which did not contain FBS for 3 days. After, cell proliferation was determined by cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan).[16] The results were expressed as percentage of untreated control.

Real-time PCR. Total RNA from cultured skin fibroblasts was prepared using a commercial kit (RNeasy Mini Kit; Qiagen, Chatsworth, CA) according to the manufacture's protocol. The mRNA expression was quantified by methods of TaqMan with Real-time reverse transcriptase PCR (RT-PCR). PCR primers were purchased from SIGMA-ALDRICH custom oligonucleotide synthesis service. Standard RT-PCR primers for human COL1: Forward, 5'-GGGATTCCCTGGACCTAAAG-3' and reverse, 5'-GGAACACCTCGCTCTCCA-3' and COL4: forward, 5'-AGGAGAGAAGGGCGCTGT-3' and reverse, 5'-TCCAGGTAA GCCGTCAACA-3' and COL7: Forward: 5'-GCTGGTGCT GCCTTTCTCT-3' and reverse: 5'-TCCAGGCCGAACTCT GTC-3' and MMP2: forward, 5'-CCCCAAAACGGACAA AGAG-3' and reverse, 5'-TGTCCTTCAGCACAAACAGG-3' and MMP8: forward, 5'-TGACAGAGACCTCATTTTCCTATT TA-3' and reverse, 5'-CTGCGTCAATTGCTTGGA-3' and HSP47: forward, 5'-TCCCTCTGAGGCAGTTTCC-3' and reverse, 5'-GCTGCAGGTTTCTTCACCTC-3'.

The PCR was semi-quantitative and the cycling conditions were 95°C for 10 min, 40 cycles of amplification at 95°C for 15 s, 60°C for 1 min, followed by 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Target gene levels were normalized to the housekeeping gene 18sRNA.

Validation by immunostaining. To detect the cellular localization and protein expression of type I collagen, the fibroblasts were grown in 35 mm dish and then treated under the indicated conditions. After fixing with 4% paraformaldehyde for 20 min and washed three times with PBS, the fibroblasts were permeabilized and blocked with 0.2% Triton-X and 1% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 20 min at room temperature. Subsequently, the samples were incubated with primary antibody of type I collagen (1:300) overnight at 4°C. After the fibroblasts were washed three times with PBS, the fibroblasts were incubated with for Alexa Fluor 488 probe (1:500, Invitrogen, Carlsbad, CA) 1 h at RT in dark. The fibroblasts were mounted with DAPI (Dojundo) and imaged by using *in vitro* confocal microscope.

Statistical analysis. Data are expressed as mean ± SD. Differences between the mean values were analysed using the unpaired Student's *t* test: **p*<0.05; ***p*<0.01.

Results

CoQ_{10} contents in fibroblasts. CoQ_{10} contents in fibroblasts were determined by HPLC-ECD with the method of Okamoto *et al.*[14] In this study, the fibroblasts were supplemented with P40 for a one week. Because, the aim of this study was to confirm whether CoQ_{10} act only by intracellular CoQ_{10} after being incorporated into a cell. The result showed that supplement of P40 also dose-dependently enhanced intracellular CoQ_{10} level in fibroblasts (Fig. 1). Treatments of the same volume of control, i.e., placebo CoQ_{10}, in cultured fibroblasts have no affect intracellular CoQ_{10} levels.

Effect of P40 of proliferation in fibroblasts. It is well known that decrease of cell proliferation is caused by aging or injury. Accordingly, decrease of cell proliferation participate in the aging of skin, and its improvement suggests the possibility of anti-aging effect in skin. The effect of P40 on the cell proliferation of fibroblasts were examined by MTT assay methods.[16] In this study, treatment of 1 μM P40 in fibroblasts has little effect on cell proliferation, whereas 10 μM P40 increased the number of fibroblasts (Fig. 2).

The mRNA level of type I, IV, and VII collagens in fibroblasts. The effect of P40 was examined by measuring mRNA level of collagens which is constituent of the dermis. The mRNA level was determined by methods of TaqMan with RT-PCR. P40 increased mRNA level of type I, IV, and VII collagens of fibroblasts in a dose-dependent manner. It also dose-dependently enhanced these mRNA levels of type I, IV, and VII collagens in fibroblasts (Fig. 3). On the other hand, treatment of

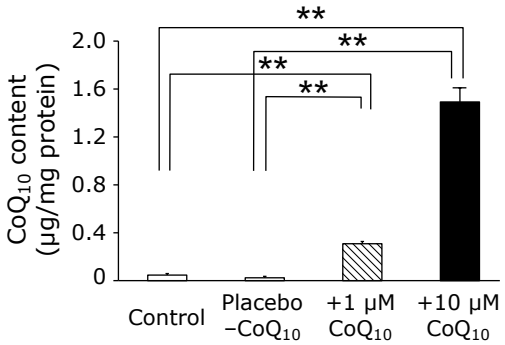

Fig. 1. The fibroblasts were cultured in the presence of P40 for a one week, after, intracellular CoQ_{10} levels are determined by HPLC-ECD methods. Results are means ± SD. $n = 4–7$, **$p<0.01$.

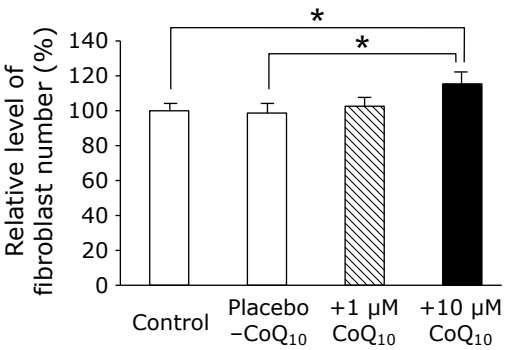

Fig. 2. Cell proliferation in fibroblasts were examined by MTT assay methods. The fibroblasts were treated with 10 μM P40, which increased the number of fibroblasts. Results are means ± SD. $n = 6$, *$p<0.05$.

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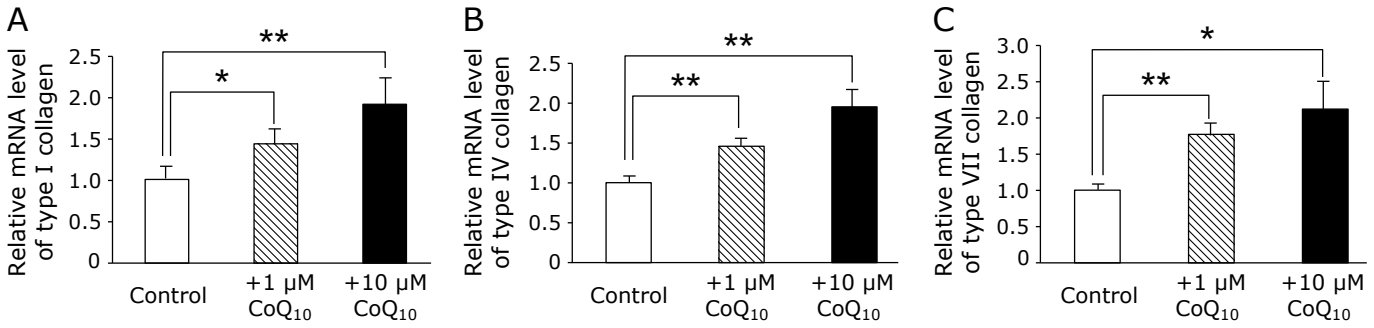

Fig. 3. Relative mRNA expression of type I, IV, and VII collagens were determined by RT-PCR method and the target gene levels were normalized to the housekeeping gene 18sRNA. (A) The mRNA level of type I collagen was increased by treatment of P40, and it also dose-dependently enhanced in fibroblasts. Results are means ± SD. $n = 3–7$, *$p<0.05$, **$p<0.01$. (B) A part of basement membrane is composed of type IV collagen, and it was increased to treatment of P40 in fibroblasts. Results are means ± SD. $n = 4–5$, **$p<0.01$. (C) Type VII collagen participate to connect basement membrane to dermis in skin. The fibroblasts were treated with more than 1 μM P40 increased mRNA levels of type VII collagen. Results are means ± SD. $n = 3$, *$p<0.05$, **$p<0.01$.

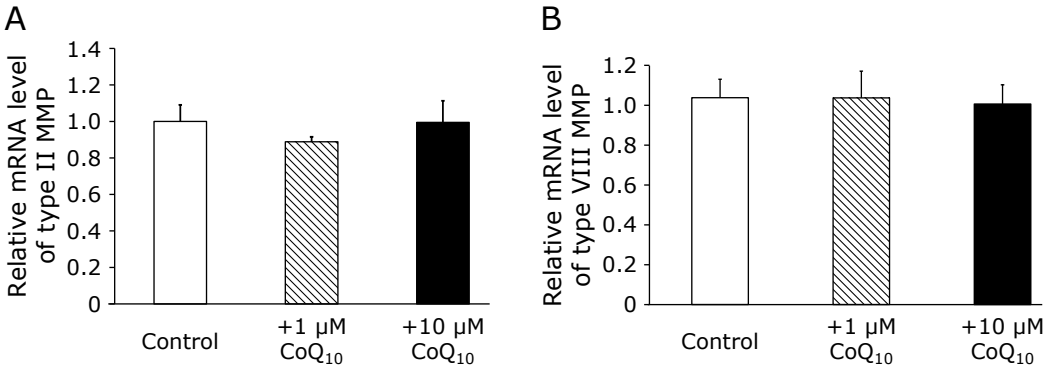

Fig. 4. Relative mRNA expression of type II and VIII MMP were determined by RT-PCR method and the mRNA levels of MMP were normalized to the housekeeping gene 18sRNA. (A) Type II MMP participate in the degradation of type IV and VII collagen. There was no significant difference in mRNA expression of type II MMP between treated of 1 or 10 μM P40 group and control group Results are means ± SD. $n = 3$. (B) Type I collagen is disassembled by type VIII MMP in skin. Treatment of P40 in fibroblasts showed that mRNA level of type VIII MMP were no longer significant compared with control group. Results are means ± SD. $n = 6$.

the same volumes of control, i.e., placebo CoQ_{10}, in cultured fibroblasts have no affect on mRNA levels of type I, IV, and VII levels.

The mRNA level of type II and VIII MMP in fibroblasts. MMP comprise a family of zinc-dependent endopeptidases that consist of more than 21 types MMP in human. In addition, MMP family has been known as catabolic enzyme of collagen. Type IV and VII collagen are degraded by type II MMP, and type I, II, and III collagen are degraded by type VIII MMP.[17] In this study, mRNA expression of type II and VIII MMP were measured by using RT-PCR with TaqMan methods. These results showed that there was no significant difference in mRNA expression of type II and VIII MMP between treated of P40 group and control group (Fig. 4).

The mRNA level of HSP47 and elastin in fibroblasts. The formation of mature collagen is associated with the expression of heat shock protein 47 (HSP47), and it is a collagen-specific chaperone that is essential for the triple helical formation in the endoplasmic reticulum.[18,19] Elastin is flexible and elastic fiber, which is structural protein capable of stretching in two dimensions. In the skin, both of HSP47 and elastin play an important role of physiological action, these are essential molecules for normal skin function. In this result show that P40 increased mRNA level of HSP47 and elastin of fibroblasts in a dose-dependent manner. It also dose-dependently enhanced these mRNA levels of HSP47 and elastin in fibroblasts (Fig. 5).

Validation by immunostaining of collagen type I in fibroblasts. Protein expression of type I collagen in fibroblasts were determined by immunofluorescence. Collagen molecules have three α-chain that form a triple helix, and it play an important role in skin functions. The role of type I collagen is well known to provide physical strength to tissue as the major components of ECM. These results showed that P40 increased collagen protein levels in skin fibroblasts (green fluorescence). (Fig. 6).

Discussion

CoQ_{10} is essential constituent components in mitochondrial respiratory chain, which play an important role of ATP synthesis and antioxidant effect. In Japan, CoQ_{10} has been widely commercially available as a dietary and health supplement since 2001 and is used for the prevention of lifestyle-related diseases induced by free radicals and aging. Meanwhile, CoQ_{10} in the bodies of human beings is thought to be provided by both dietary intake, such as dietary foods and health supplements, and *de novo* biosynthesis,[8] and CoQ_{10} has a widespread distribution in human tissues, but the abundance of CoQ_{10} are different between each tissue.[20–22] In particular, CoQ_{10} levels in skin is very low compared with heart, liver, muscle and brain tissues. Consequently, it is assumed that exogenous CoQ_{10} play an important role for skin functions. However, CoQ_{10} is practically insoluble in water, thereby CoQ_{10} having a low absorption property from the

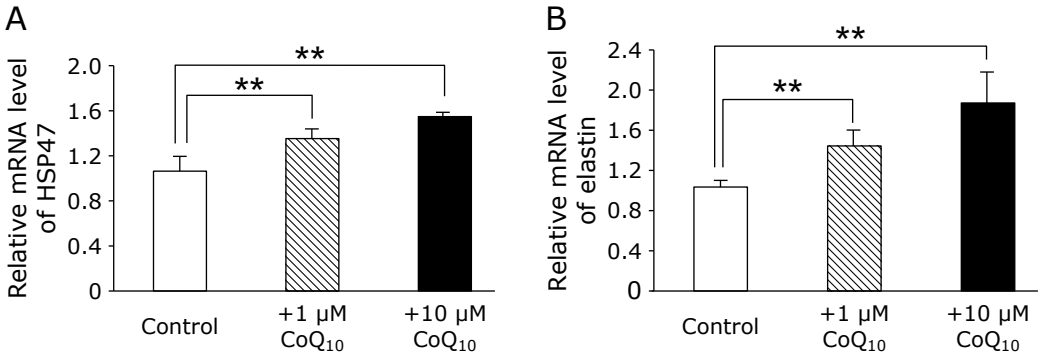

Fig. 5. Relative mRNA expression of HSP47 and elastin were determined by RT-PCR method and the target gene levels were normalized to the housekeeping gene 18sRNA. (A) The mRNA level of HSP47 was increased by treatment of P40, and it also dose-dependently enhanced in fibroblasts. Results are means ± SD. $n = 4–5$, $^{**}p<0.01$. (B) Treatment of P40 in fibroblasts showed to increase mRNA expression of elastin in a dose-dependent manner. Results are means ± SD. $n = 6$, $^{**}p<0.01$.

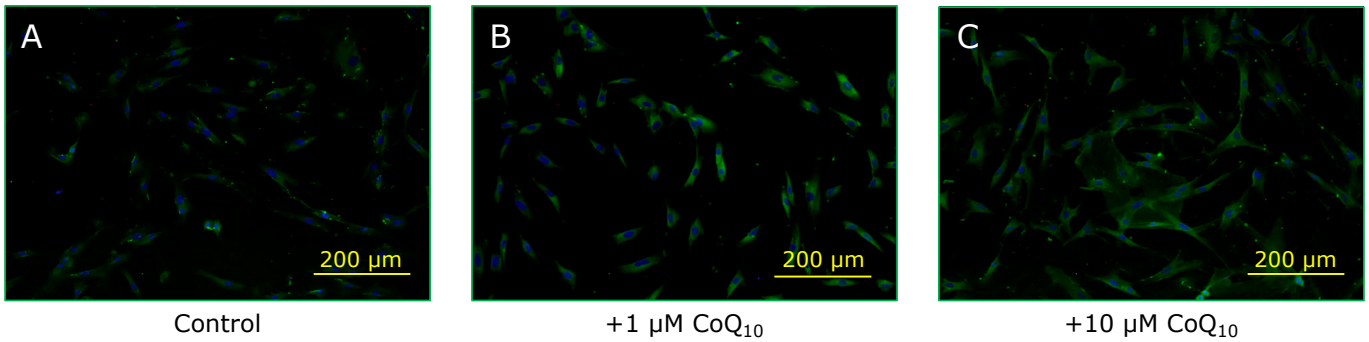

Fig. 6. Type I collagen protein expression in fibroblasts was determined by immunofluorescence, cell nuclei are stained blue with DAPI, and type I collagen protein is stained green with Alexa Fluor 488 probe. Scale bar shows 200 μm.

digestive tract and skin.

Recently, for the improvement of absorption, it has been developed the various CoQ_{10} formulations (e.g. micellization,[23] water soluble,[24,25] and reduced form[26]). Then, it is possible that water soluble CoQ_{10} (P40) indicate good absorption from the digestive tract and skin, and CoQ_{10} levels in serum and skin increase compared with using lipid soluble CoQ_{10}. Besides, according to previous paper, low levels of CoQ_{10} are found in several diseases[8] and the CoQ_{10} level in the body has been reported to decrease after the age of 20 years.[27] Furthermore, it is well known that decrease of cell proliferation and collagen synthesis participate in the aging of skin, thus, we examined that effect of CoQ_{10} in human skin fibroblasts. Before the addition of P40 in cultured fibroblasts, we attempted to dissolve CoQ_{10} in ethanol or DMSO instead of P40. However, even though the concentration of 1 μM CoQ_{10} dissolve in ethanol or DMSO, these solvents indicated toxic effects to fibroblasts.

In this study, intracellular CoQ_{10} level increased by the addition of 1 or 10 μM P40, and 10 μM P40, and enhanced cell proliferation of skin fibroblasts. A number of studies with using lipid soluble CoQ_{10} suggested that CoQ_{10} has anti-aging effect in skin fibroblasts by increased type IV collagen expression and inhibition of UV-induced ROS and MMP-1 production.[9–11] Accordingly, after addition of P40 for 1 week, we confirmed on the expression level of collagen and MMP in fibroblasts by methods of TaqMan with RT-PCR. Like similarly to previous papers, mRNA expression of type IV and VII collagen were elevated by treatment of 1 or 10 μM P40 for 1 week.

However, P40 had no impact on mRNA expression of type II and VIII MMP that is known as a collagen degrading enzyme. These results suggested that P40 has been participating in collagen synthesis, it hasn't been involved in degradation of collagen in absence of ROS.

As mentioned above, CoQ_{10} is confirmed to stimulate of type IV and VII collagen synthesis by addition P40 in skin fibroblasts, whereas interestingly, P40 increased type I collagen in this study. Previously studies[11,12] have shown that CoQ_{10} had no effect on type I collagen production in fibroblasts. The reason is an unknown, however, we infer that there is different point of collagen synthesis mechanism between lipid soluble CoQ_{10} and water soluble CoQ_{10}. Collagen is the most abundant protein in human body, constituting from 25% to 35% of the whole bodies protein content. Among the collagen types, type I collagen is the most ubiquitous and abundant in human body, and it is a typical fibril-forming collagen and a major component of the ECM. Therefore, effect of P40 is considered to very important by increase in synthesis of type I collagen in skin fibroblasts. A treatment of human skin fibroblasts with P40 induced mRNA level of elastin and HSP47. Elastin is flexible and elastic fiber, which is structural protein capable of stretching in two dimensions, and HSP47, participate in collagen maturation, are collagen-specific molecular chaperone that is essential for the triple helical formation.[28] Hence, not only increased production of collagen, P40 may play an important role in regulating physiological function according to increased expression mRNA of elastin and HSP47 in skin fibroblasts. Collagen and elastin are important fibrous protein of dermis to maintain normal skin structure and function. Accordingly, decrease of these fibrous proteins are known to be involved in formulation of wrinkle, which are accelerated by UVB and aging. In addition,

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Inui *et al.*[10] have reported that collagen is degradated via increasing MMP expression by UVB, and CoQ_{10} inhibit the upregulation of MMP expression, thus CoQ_{10} act on collagen to protect in presence of oxidative stress. Moreover, cell proliferation in skin fibroblasts is evoked by addition to CoQ_{10}, so that skin fibroblasts could more produce of collagen and elastin in dermis.

These results suggested that P40 has the efficacy that its increase collagen production of fibroblasts, thereby there is possible of the anti-aging by P40 in Japan which reached an aging society, so that it might be based on new physiological function by water soluble CoQ_{10}.

Acknowledgments

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Conflict of Interest

No potential conflicts of interest were disclosed.

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