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Effect of Different Concentrations of Collagen, Ceramides, N-acetyl glucosamine, or Their Mixture on Enhancing the Proliferation of Keratinocytes, Fibroblasts and the Secretion of Collagen and/or the Expression of mRNA of Type I Collagen

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ABSTRACT

The objectives of the study are to survey the effects of different concentrations of collagen, ceramides, N-acetyl glucosamine (NAG), or their mixture on the proliferation of keratinocytes, fibroblasts and on the secretion of collagen and/or the expression of mRNA of type I collagen. The optimal concentrations that facilitated the proliferation of fibroblasts were 49, 2.0, and 24.4 μg/mL, respectively for collagen, ceramides, and NAG. The proliferation percentages were 191%, 170%, and 223%, respectively at the same order in compare to the control. The concentration of the mixture containing 6.1, 0.12 and 6.1 μg/mL, respectively of collagen, ceramides, and NAG facilitated the proliferation of fibroblasts. The optimal concentrations that had highest keratinocytes proliferation were 0.76, 0.001, and 0.76 μg/mL, respectively for collagen, ceramides, and NAG. The proliferation percentages were 242%, 203% and 230%, respectively at the same order in compare to the control. The optimal concentration that effects the highest keratinocytes proliferation by the mixture were 0.38, 0.008 and 0.38 μg/mL for collagen, ceramides and NAG, respectively. The concentrations for fibroblasts to express higher amount of type I collagen mRNA were 6.1, 0.49, and 6.1 μg/mL, respectively for collagen, ceramides, and NAG. The expression levels of type I collagen mRNA were 152%, 160% and 220%, respectively at the same order in compare to the control. However, the expressions of type I collagen mRNA were 180% in compare to the control at the concentration of mixture containing 0.76, 0.015 and 0.76 μg/mL, respectively of collagen, ceramides, and NAG.

Key words: collagen, N-acetyl glucosamine, ceramides, mixture, proliferation, keratinocytes, fibroblasts, mRNA, Type I Collagen, expression

INTRODUCTION

Stratum corneum plays a key role maintaining the moisture level and firmness of the skin. Stratum corneum consists of flattened dead cells, corneocytes, embedded in a complex lipid matrix of ceramides (50%), cholesterol (25%) and free fatty acids (10%), as well as small amounts of cholesterol esters and cholesterol sulfate[1-3]. The dermis is composed largely of collagen protein. Collagen is created by fibroblasts, which are specialized skin cells located in the dermis. Fibroblasts also produce other skin structural proteins, such as elastin, and glucosaminoglycans (GAGs), such as hyaluronic acid. Most of the collagen are organised in bundles running horizontally through the dermis, which are buried in a jelly-like material called extracellular matrix (ECM). Collagen is responsible for the resilience, strength, durability, elasticity, smooth, plump appearance of young healthy skin. Fibroblasts not only function to sustain various organs and tissues as stroma cells but also act directly to regulate adjacent cell behaviors including migration, proliferation, and differentiation. Olivera *et al.*[4] studied the role of ceramides in cellular proliferation and revealed that at low concentrations, ceramides stimulated [^{3}H] thymidine incorporation and acted synergistically with a wide variety of growth factors to induce the proliferation of quiescent Swiss 3T3 fibroblasts. Olivera *et al.*[5] reported that cell-permeable ceramides analogs increase the progression of quiescent Swiss 3T3 fibroblasts through the S phase of the cell cycle leading to an increase in cell division. Wakita

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et al.[6] reported ceramides and sphingosine were intracellular modulators of cell growth and differentiation. The modulating effects were confirmed by the upregulation of cornified envelope formation, synthesis of involucrin and increased transglutaminase activity. Kubomura and Matahira[7] reported that N-acetylglucosamine (NAG) increases the production of hyaluronic acid (HA) and type I procollagens by cultured human keratinocytes in a dose-dependent manner, but has no effect on cell proliferation. Okamura *et al*.[8] investigated the effects of chitin/chitosan and their oligomers/monomers on the proliferation of human keratinocytes using the WST-8 assay. Monomers were reported to cause a significant increase of cell proliferation, and NAG was the most effective.

Petersen[9] studied the effects of different extracellular matrixs on the modulation of the production of collagenase *in vitro*, and the results showed that the keratinocytes cultured with type IV or type I collagen produced more collagenase than those cultured with laminin or in the absence of matrix. Middelkoop *et al*.[10] used three classes of collagenous materials: (1) reconstituted non-crosslinked collagen, (2) reconstituted collagen that was chemically crosslinked with glutaraldehyde, aluminium alginate or acetate, and (3) native collagen fibres, with or without other extracellular matrix molecules (elastin hydrolysate, hyaluronic acid or fibronectin) to evaluate the dermal substitutes on cell proliferation, the rate of degradation of the dermal equivalent, contractibility and *de novo* synthesis of collagen. They revealed that non-crosslinked reconstituted collagen was degraded rapidly by human fibroblasts. The chemically crosslinked materials proved to be cytotoxic. Native collagen fibres were stable.

The above literatures indicated that ceramides will induce fibroblasts proliferation[4] and cell division[5]. It is an intracellular modulator of cell growth and differentiation; NAG will increase the production of HA and type I procollagen[7] and the proliferation of human keratinocytes[8]; collagen will modulate the keratinocytes production of collagen *in vitro*. The objectives of this study are to survey the effects of different concentrations of collagen, ceramides, NAG, or their mixture on the enhancement of the proliferation of keratinocytes, fibroblasts and on the secretion of collagen and/or the expression of mRNA of type I collagen.

MATERIALS AND METHODS

1. *Cells*

Human normal skin fibroblasts CCD-966SK and human keratinocytes HEK001 were purchased from Cell Bank of Food Industry Research and Development Institute (FIRDI), HsinChu, Taiwan.

II. *Collagen, N-acetyl-glucosamine, Ceramides and Their Mixture*

Collagen powder, N-acetyl-glucosamine (NAG) powder, ceramides powder and their mixture in liquid form were supplied by Wei Chuan Foods Corporation, Taipei, Taiwan.

Collagen is a hydrolized product from fish collagen with molecular weights from 3 to 7 KDa, protein content higher than 85% and ash content less than 3%, in white powder. Collagen was used without purification.

NAG has purity higher than 95% and ash content less than 0.3%, in white powder. NAG was a hydrolyzed product from chitin oligosaccharide by enzyme and used without purification.

Ceramides brown powder was extracted and refined from rice bran or rice germ. It contains more than 3.0% glycosphingolipids and was used without purification.

The mixture contains 1 g collagen, 1 g NAG, 20 mg ceramides, suitable amounts of flavors, antioxidants, seasonings and water in 20 mL.

III. *Culture of Fibroblasts (CCD-966SK) and Kerationocytes (HEK001)*

Fibroblasts were cultured in minimum essential medium (Eagle) in Earle's BSS with non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum[11]. Kerationocytes were cultured in keratinocytes-serum Free (GIBCO-BRL 17005-042) with 5 ng/mL human recombinant epidermal growth factor (EGF) and 2 mM L-glutamine (without serum). Cultured cells were harvested by trypsinisation and kept frozen in liquid nitrogen. For experiments, fibroblasts and keratinocytes at passage 1 were thawed and cultured in the medium mentioned. Before cells reached confluence, they were trypsinised and plated at a density of about 20,000 cells/mL. The medium was changed every 2 days for the first 6 days and every day after then for 6 more days. Cultures were maintained at 37°C in 8% CO_2. Confluence (%) of epidermial sheets produced by undifferentiated fibroblasts and keratinocytes were evaluated by microscopic observation and cells were counted with a Coulter Counter (Excell-500, Metertech, Taiwan) after trypsinisation.

IV. *Proliferiation Activity: MTT Assay*

The proliferative activity of cultured cells was determined by the MTT[11] colorimetric assay at 24, 36, 48 and 72 hr after seeding with different concentrations of collagen, NAG, ceramides and their mixture. Cells were incubated with a tetrazolium salt solution (MTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma M-2128 for 4 hr at 37°C. The MTT was reduced to insoluble formazan precipitate by mitochondrial succinic dehydrogenase of viable cells. After removal of the medium, ethanol-dimethyl sulfoxide (DMSO) solution (1:1) was added to each well. After complete solubilisation of the dark-blue crystal of MTT formazan, the absorbance of the content of each well was measured at 570 nm in a 96-well microplate reader on a spectrophotom-

eter (Dynatech MR5000, USA). The blank reference was taken from wells without cells, also incubated with the MTT solution.

V. *Expression of Collagen Type I mRNA*

(I) *Total RNA Isolation*

Total RNA isolation Kit of Macherey-Negel was used to lyse the fibroblasts cells[12]. Added were 350 μL buffer RA1 and 3.5 μL β-mercaptoethanol to the cell pellet. The mixture was filtered through a NucleoSpin® Filter unit to reduce the viscosity and clear the lysate, and then centrifuged for 1 min at 11,000 ×g.

Ethanol (70%, 350 μL) was added to the lysate and mixed by pipetting up and down (5 times). For each preparation, one NucleoSpin® RNA II column was placed in a 2-mL centrifuge tube. Lysate was pipetted up and down 2-3 times and load over the column. The column and centrifuge tube was centrifuged for 30 sec at 11,000 ×g. The column was then placed a new collecting tube. MDB (Membrane Desalting Buffer, 350 μL) was added and the tube was centrifuged at 11,000 ×g for 1 min to dry the membrane. For each isolation, 10 μL reconstituted rDNase was added to 90 μL reaction buffer. DNase reaction mixture (95 μL) was applied directly onto the center of the silica membrane of the column. The column was incubated at room temperature for 15 min. Two hundred microliter of buffer RA2 was added to the NucleoSpin® RNA II column and the column was centrifuged for 30 sec at 11,000 ×g. The column was then placed into a new collecting tube. Six hundred microliter of buffer RA3 was added to the column and the column was centrifuged for 30 sec at 11,000 ×g. The flow-through was discarded and the column was placed back into the collecting tube. Another 250 μL of buffer RA3 was added to the column and the column was centrifuged for 2 min at 11,000 ×g to dry the membrane completely. The column was placed into a nuclease-free 1.5-mL microcentrifuge tube, and RNA was eluted in 60 μL of H_2O (RNase-free) by centrifugation at 11,000 ×g for 1 min.

(II) *RT-PCR*

The MasterAmp™ High Fidelity RT-PCR Kit was used[13]. Sixteen microliter of sterile nuclease-free water, 25 μL of MasterAmp 2X RT-PCR PreMix (1X final concentration), 3 μL of RNA template (120 ng) and 1 μL of MMLV-RT Plus (40 units) were pipetted into PCR tube to react for 30 min at 37°C. One microliter of human collagen type I sense fragment, 1 μL of human collagen type I anti-sense fragment, 1 μL of β-actin sense fragment, 1 μL of β-actin anti-sense fragment and 1 μL of MasterAmp TAQurate DNA Polymerase Mix were then pipetted into the same PCR tube to start PCR reaction. A denaturation step was performed by heating at 95°C for 5 min. Followed by 30 cycles of incubation 95°C for 45 seconds, 60°C for 45 sec, 72°C for 45 sec. After 30 cycles reactions finished, the samples were heated at 72°C for 10 min to stop the PCR reaction. The samples may be kept at 4°C overnight or frozen at -20°C.

The relative amount of collagen type I and β-actin determined by measuring the luminosity after electrophoresis.

VI. *Statistical Analysis*

Data of cell proliferation among different mediums were analyzed by the ANOVA test at $P < 0.05$ (SAS, SAS Ltd., NC, USA). The significance of differences between treatments was analyzed using Duncan new multiple range test (SAS). The relationships among cell proliferation were tested using the association and regression analysis at $P < 0.05$ (SAS).

RESULTS

I. *Effect of Concentration of Collagen, Ceramides, NAG and Their Mixture on the Proliferation of Fibroblasts*

The overtime effects of concentrations of collagen (a), ceramides (b), NAG (c) and their mixture (d) on the fibroblasts cell viability were shown in Figure 1. Cell viability of fibroblasts depended on the concentration of collagen in the medium. Between 3.05 and 48.8 μg/mL, cell viability increased, however decreased at a concentration higher than 97.7 μg/mL. Cell viability of fibroblasts depended also on the concentration of ceramides; between 0.12 to 1.95 μg/mL, cell viability increased, however decreased at the concentration higher than 1.95~3.91 μg/mL. Cell viability depended also on the concentration of NAG, increasing between 3.05 to 24.4 μg/mL, but decreasing at concentration higher than 24.4 μg/mL. Cell viability of fibroblasts depended on the dilution fold of the mixture in the medium. Between the 14th and the 16th dilution, cell viability increased with decreasing the dilution fold e.g. increasing the concentration of the mixture. However, at a concentration higher than the 14th dilution of the mixture, cell viability decreased with increasing concentrations of resulted mixture accordingly. At the 14th and 16th dilution, the corresponding concentrations are 6.10 and 1.53 μg/mL respectively for collagen, ceramides (0.12 and 0.03 μg/mL respectively) and NAG (6.10 and 1.53 μg/mL respectively).

Between 24 and 72hr, the cell viability of fibroblasts depended on the cultivation time. At the same concentration of collagen, ceramides, NAG, or their mixture in medium, the cell viability increased between 24 and 48 hr but decreased after 48 hr in the medium containing collagen (Figure 1A), however, the cell viability increased overtime between 24 and 72 hr, in the mediums containing either ceramides (Figure 1B), or NAG (Figure 1C) or the mixture (Figure 1D).

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The effects of various concentrations of collagen, ceramides, NAG and their mixture on the fibroblasts cell viability at 74 hr were shown in Figure 2. Between 14th and 16th series dilution of stock solution, the cell viability increased with concentrations. However, between the 9th and the 13th or the 14th series dilution of stock solution, cell viability decreased with the increasing of concentrations. Between 13th and 14th dilution, the corresponding concentrations of collagen, ceramides and NAG are 12.2~6.1, 0.24 ~0.12 and 12.2~6.1 μg/mL respectively.

II. *Effect of Concentration of Collagen, Ceramides, NAG and Their Mixture on The Proliferation of Keratinocytes*

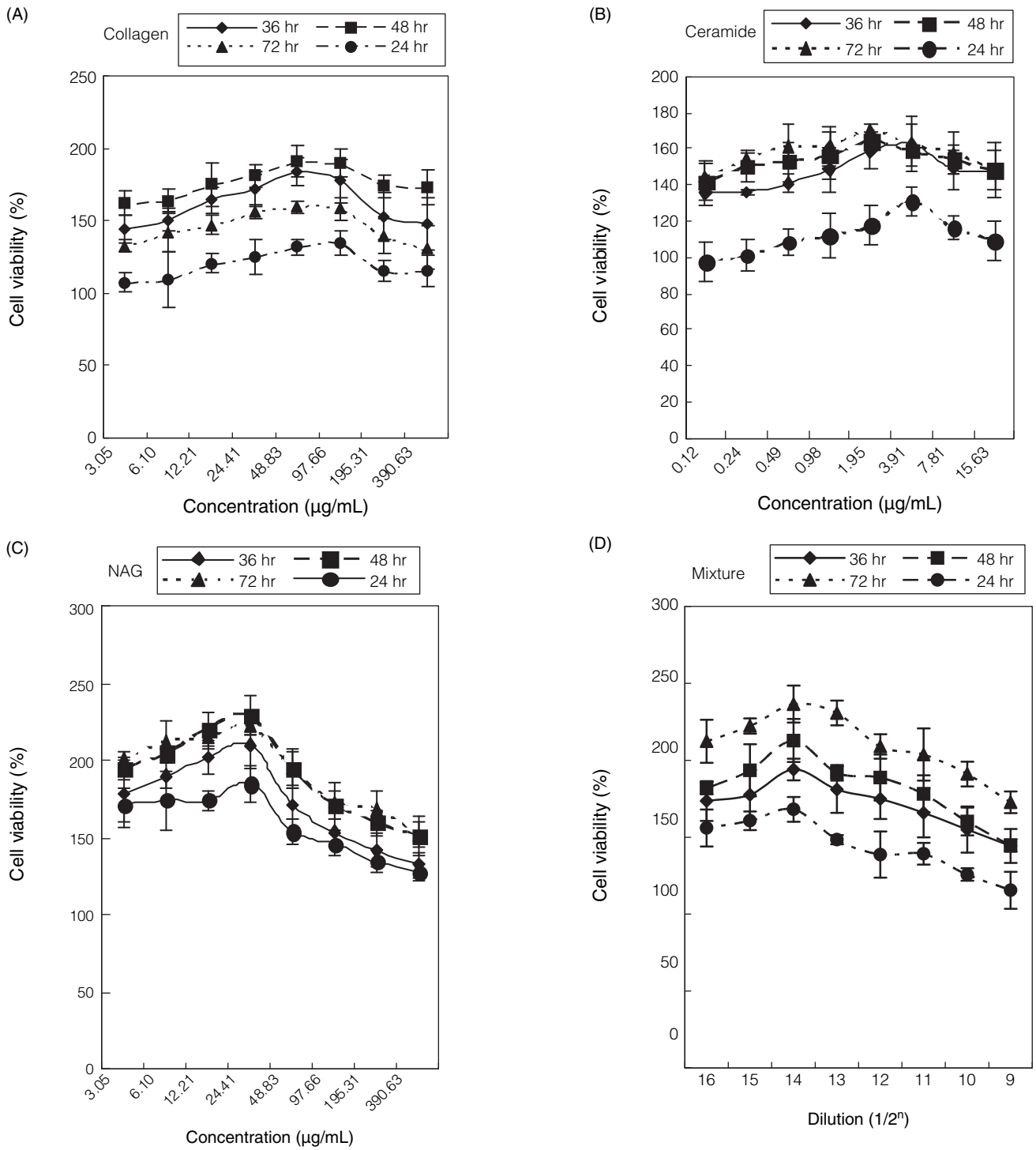

Figure 1. Effect of different concentrations of collagen, N-acetyl-glucosamine, ceramides and their mixture on the fibroblasts cell viability overtime. Cell viability of the control was 100%. Each value represent as means ± S.D. (n = 3).

The overtime effects of concentrations of collagen (a), ceramides (b), NAG (c) and their mixture (d) on the keratinocytes cell viability are shown in Figure 3. Cell viability of keratinocytes depended on the concentration of collagen in the medium. Between 0.19 to 0.76 μg/mL, cell viability increased, however decreased at a concentration higher than 0.76 μg/mL. Cell viability of keratinocytes depended also on the concentration of ceramides; between 0.0002 to 0.0010 μg/mL, cell viability increased, however decreased at a concentration higher than 0.0010 μg/mL. Cell viability depended also on the concentration of NAG, increasing between 0.048 to 0.76 μg/mL, but decreasing at a concentration higher than 0.76 μg/mL. Cell viability of keratinocytes depended on the dilution fold of the mixture in the medium. Between the 18th and the 21st dilution, cell viability increased with decreasing the dilution fold of the mixture. However at the concentration of the resulted mixture higher than the 18th dilution of the mixture, cell viability decreased with the dilution fold. At the 18th and 21st dilution, the corresponding concentrations for collagen 0.381 and 0.048 μg/mL respectively, for ceramides 0.008 and 0.001 μg/mL respectively and for NAG 0.381 and 0.048 μg/mL respectively.

Between 24 and 72 hr, the cell viability of kiratinocytes depended also on the cultivation time. At the same concentration of collagen, ceramides, NAG, and their mixture in medium, the cell viability increased between 24 and 48 hr but decreased after 48 hr (Figure 3).

The effects of different concentrations of collagen, ceramides, NAG and their mixture on the keratinocytes cell viability at 72 hr are shown in Figure 4. Between 18th and 21st series dilution of the stock solution, the cell viability increased with concentrations of the resulted mixture. However, between the 10th and 18th series dilutions of stock solution, cell viability decreased with the increases of concentrations of the resulted mixture. At the 18th dilution, the corresponding concentrations of collagen, NAG, and ceramides are 0.38, 0.38, and 0.008 μg/mL respectively.

III. *Effect of Concentration of Ceramides or NAG on The Amount of Collagen Secretion and on The Expression of Type I Collagen mRNA*

The effects of different concentrations of NAG or ceramides in the culture medium on the amount of collagen secretion (A) and on the expression of type I collagen mRNA (B) are shown in Figure 5. At the concentration of ceramides between 0.244 to 0.031 μg/mL, or between 1.53 to 97.66 μg/mL of NAG, the amount of the secreted collagen increased with the concentration of ceramides or NAG. However, at the concentration of ceramides or NAG higher than 0.244 μg/mL and 97.66, respectively, collagen secretion decreased with the increase of the concentration of the ceramides or NAG. The amount of collagen secreted depended also on the cultivation time. At the same concentration of ceramides or NAG

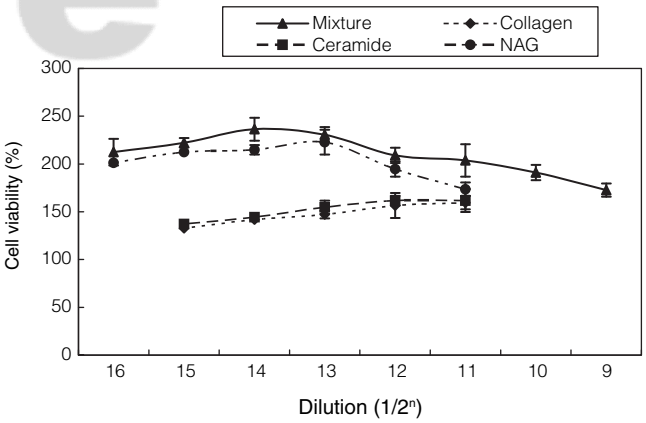

Figure 2. Effect of different concentrations of collagen, N-acetylglucosamine, ceramides and their mixture on the fibroblasts cell viability after 72 hr incubation. Cell viability of the control was 100%. Each value represent as means ± S.D. (n = 3).

in medium, the amount of secreted collagen increased proportional with cultured time between 24 hr and 48 hr (Figure 5A)

The effects of different concentrations of NAG or ceramides on the expression of type I collagen mRNA was revealed in Figure 5B. The results indicated that the concentrations of NAG or ceramides which facilitated the expression of type I collagen mRNA were consistent with the concentrations that enhancing the secretion of collagen. However, the boost effect on the mRNA expression was more pronounced especially by ceramides. The enhancement was nearly double.

IV. *Effects of Different Concentrations of Collagen, Ceramides, NAG or Their Mixture on The Expression of Type I Collagen mRNA after Incubated for 24 hr or 48 hr*

The effects of different concentrations of collagen (A), ceramides (B), NAG (C) and their mixture (D) on the expression of type I collagen mRNA after incubated for 24 hr or 48 hr are shown in Figure 6. The expression of type I collagen mRNA depends on the concentration of collagen in the medium. Between 3.05 and 48.8 μg/mL, the expression of type I collagen mRNA increased, however decreased at a concentration higher than 97.7 μg/mL. The expression of type I collagen mRNA depended also on the concentration of ceramides; between 0.03 to 0.49 μg/mL, expression of type I collagen mRNA increased, however decreased at a concentration higher than 0.49 μg/mL. The expression of type I collagen mRNA depended also on the concentration of NAG increasing between 1.53 to 6.14 μg/mL, but decreasing at a concentration higher than 6.14 μg/mL. The expression of type I collagen mRNA depended on the dilution fold of their mixture in the medium. Between the 18th and the 17th dilution, the expression of type I collagen mRNA increased with the decrease of the number of series dilu-

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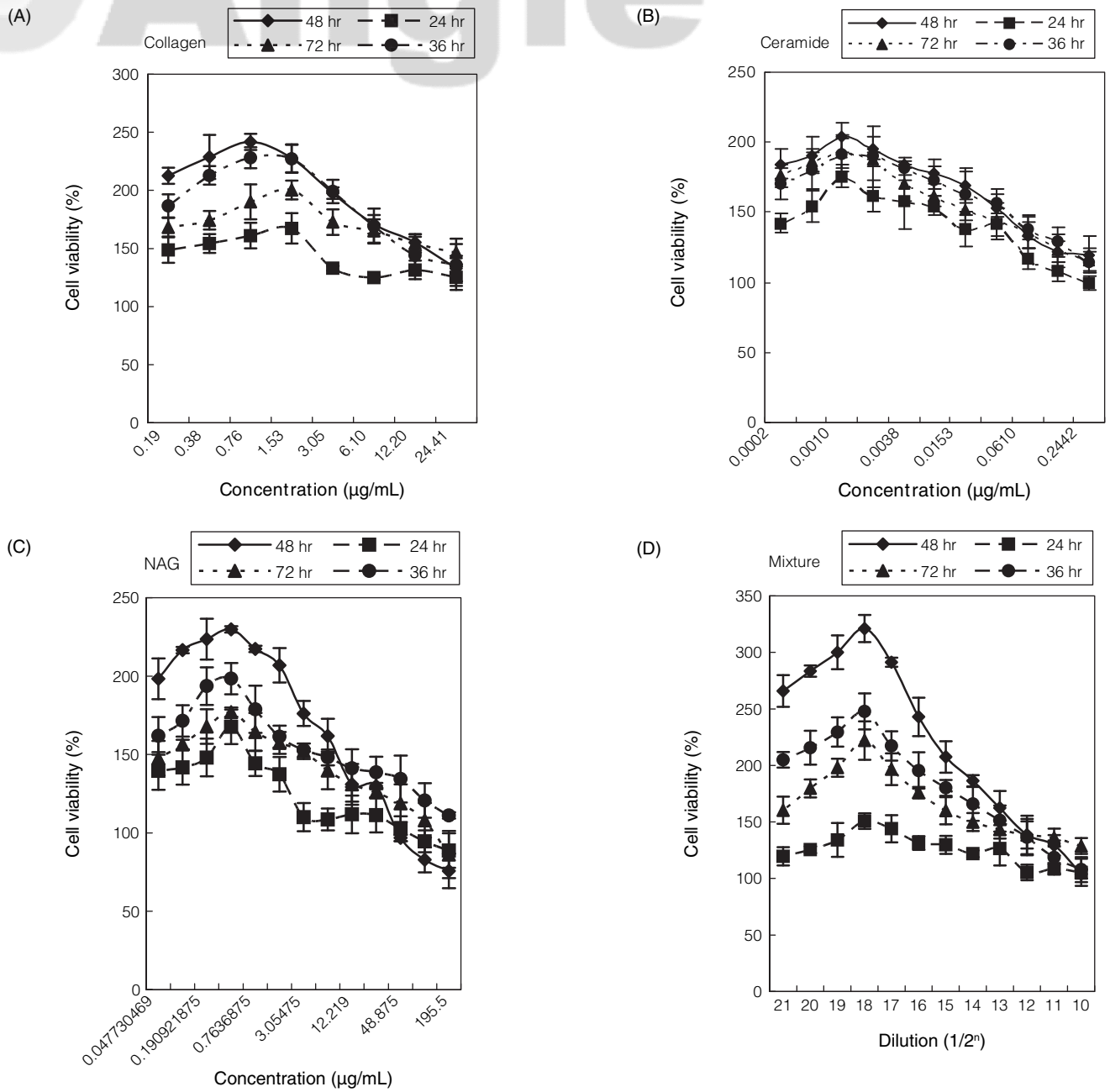

Figure 3. Effect of different concentrations of collagen, N-acetyl-glucosamine, ceramides and their mixture on the keratinocytes viability overtime. Cell viability of the control was 100%. Each value represent as means ± S.D. (n = 3).

tion of the mixture. However, at a concentration higher than the 14th dilution of the mixture, the expression of type I collagen mRNA decreased accordingly. At the 18th and 17th dilution, the corresponding concentrations are for collagen 0.38 and 0.76 μg/mL respectively, for ceramides 0.008 and 0.015 μg/mL respectively and for NAG 0.38 and 0.76 μg/mL respectively.

Between 24 and 48 hr, the Expression of type I collagen mRNA of fibroblasts depended also on the cultivation time. At the same concentration of collagen, ceramides, NAG, and their mixture in medium, the expression of type I collagen mRNA was higher for those incubated for 48 hr than that for 24 hr (Figure 6).

DISCUSSION

As shown in Figure 1A at collagen concentration between 3.1 and 48.8 μg/mL, fibroblasts cell viability increased with the concentration of collagen. This may be due to that collagen was degraded rapidly by human fibroblasts to enhance the cell proliferation[10]. At cerami-

des concentration between 0.12 to 1.95 μg/mL, fibroblasts viability increased (Figure 1B). This may be due to that ceramides analogs stimulated [^{3}H] thymidine incorporation and induced the proliferation of fibroblasts[4] or cell-permeable ceramides analogs and sphingomyelinase increased the progression of fibroblasts through the S phase of the cell cycle leading to an increase in cell division[5]. It is shown in Figure 1C that between 3.05 to 24.4 μg/mL, fibroblasts cell viability increased, it may be owing to that NAG caused a significant increase of human keratinocytes proliferation[8] via increasing the production of HA and type I procollagens that hydrolyzed collagen to provide nutrients for fibroblasts proliferation[7].

As shown in Figure 1, that cell viability decreased at collagen concentration higher than 97.7 μg/mol, or ceramides concentration higher than 1.95~3.91 μg/mol, or NAG concentration higher than 24.4 μg/mL, or mixture with their respective composition of collagen, ceramides, and NAG higher than 6.1 μg/mL, 0.12 μg/mL and 6.1 μg/mL. This may be due to that space stress upon cell reached confluence, and/or due to depression of the nutrients and/or toxicity of metabolic wastes[14].

The optimal concentration ranges that facilitate fibroblasts proliferation are 97~48, 3.9~1.95, 24.4 ~12.2 μg/mL for collagen, ceramides, or NAG respectively. The proliferation increase percentages were 191%, 170%, and 223% respectively. The optimal concentration ranges of the mixture that facilitated the proliferation of fibroblasts for collagen, ceramides, and NAG were 6.1, 0.12, and 6.1 μg/mL respectively. To exert the similar effect, the amount of collagen in the mixture was 1/16~1/8 of its single nutrient. For ceramides the amount in the mixture was 1/32~1/16 and for NAG was 1/4~1/2 to their single nutrient in the medium.

As shown in Figure 3A, at collagen concentration between 0.19 and 0.76 μg/mL, keratinocytes cell viability increased. This may be due to that human keratinocytes could produce collagenase[9] or synthesize and secrete procollagenase[9]. Collagenase or procollagenase degraded

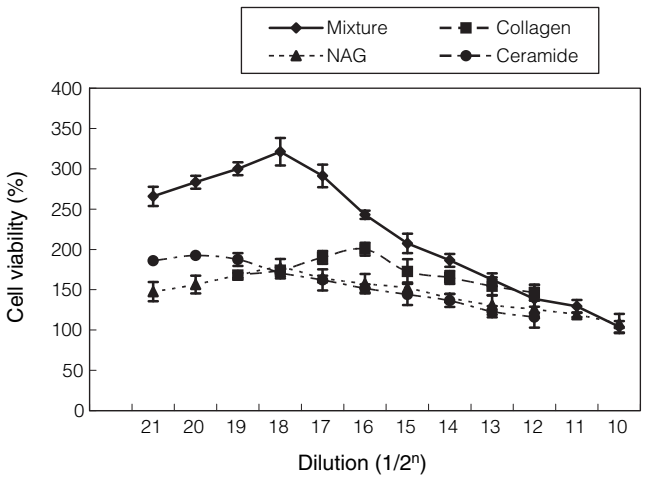

Figure 4. Effect of different concentrations of collagen, N-acetyl-glucosamine, ceramides and their mixture on the keratinocytes viability after 72 hr incubation. Cell viability of the control was 100%. Each value represent as means ± S.D. (n = 3).

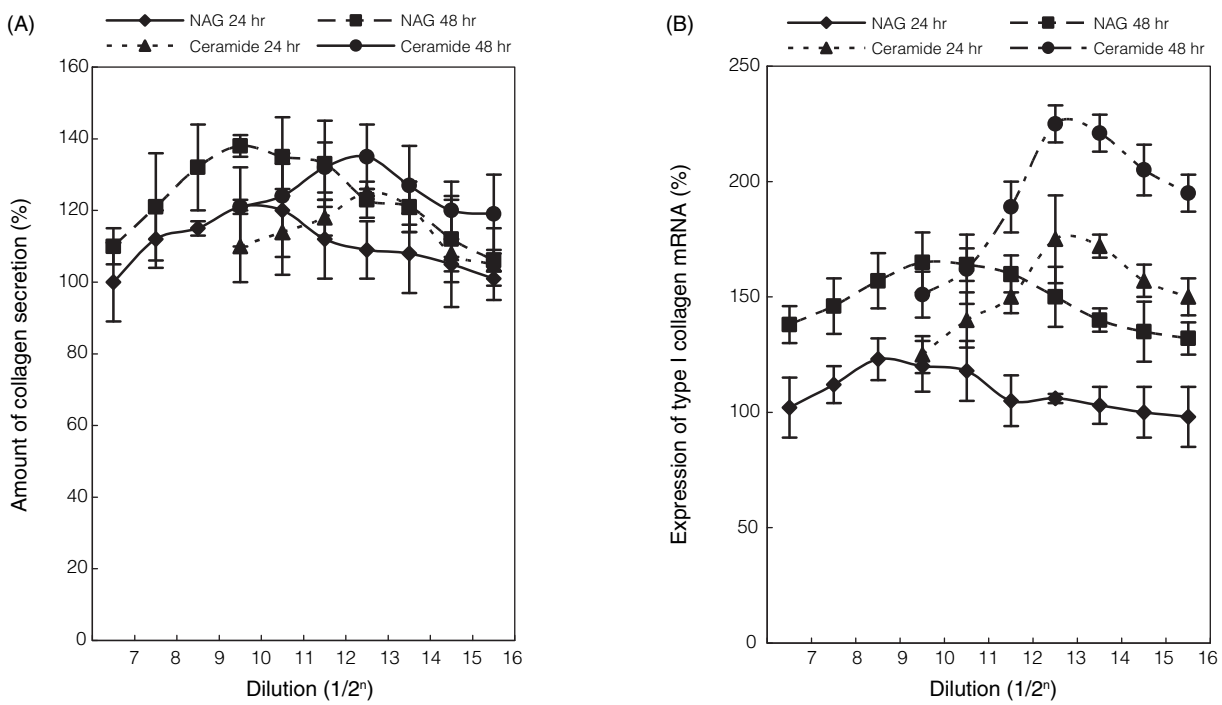

Figure 5. Effect of different concentrations of N-acetyl-glucosaminea and ceramides on the amount of collagen secretion (A) and expression of type I collagen mRNA (B) after 24 and 48 hr incubation. Each value represent as means ± S.D. (n = 8).

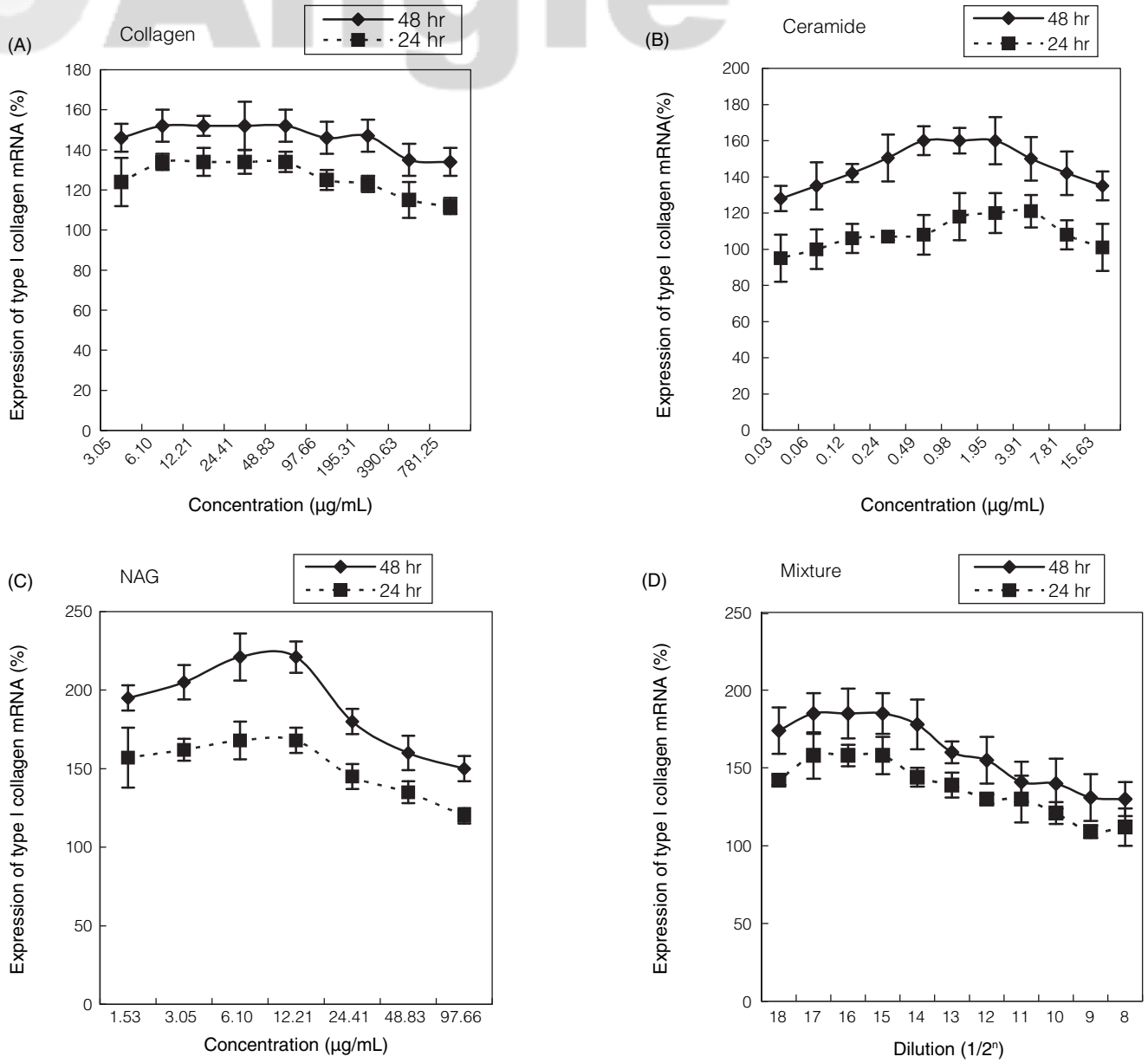

Figure 6. Effect of different concentrations of collagen, N-acetyl-glucosamine, ceramides or their mixture on the expression of type I collagen mRNA after 24 or 48 hr incubation. Each value represent as means ± S.D. (n = 8).

collagen and supplied nutrients for cell proliferation, also shown in Figure 3B at ceramides concentration between 0.0002 to 0.001 μg/mL, keratinocytes viability increased. This may be due to that the growth and differentiation of keratinocytes are partially regulated by ceramides and sphingosine[6]. They reported that short-chain cell-permeant analogs of ceramides significantly promoted human keratinocytes, the squamous cell carcinoma cell line, DJM-1, differentiation. Generally, these activities were greater for those longer N-acyl carbon chain ceramides. As shown in Figure 3C at NAG concentration between 0.048 to 0.76 μg/mL, keratinocytes cell viability increased. This may be due to that NAG caused a significant increase of human keratinocytes cell proliferation[8].

Keratinocytes viability decreased at concentration of collagen, ceramides, and NAG higher than 0.76, 0.001, or 0.76 μg/mL respectively, or concentration of their resulted mixture higher than 0.038, 0.008 and 0.038 μg/mL respectively for collagen, ceramides, and NAG as shown in Figure 3. This may be due to that space stress resulted from cell confluence, or due to depression of the nutrients, and/or due to toxicity of metabolic waste mentioned previously.

The optimal concentration ranges with the highest keratinocytes proliferation for collagen, ceramides, or NAG was 1.53~0.76, 0.001, and 0.76 μg/mL respectively. The proliferation percentages were 242%, 203% and 230% respectively. The optimal concentration ranges that enhance the proliferation of keratinocytes for their mixture

(their individual concentration of collagen, ceramides, and NAG were 0.38, 0.008 and 0.38 μg/mL respectively). To exert similar effect, the amount of collagen in the mixture was 1/2~1/4 to its single nutrients. For ceramides the amount in the mixture was 8 times and was 1/2 for NAG to its single nutrient in the medium.

As shown in Figure 2, cell viability of fibroblasts vs. dilution fold of stock mixture solution was consistent with that of NAG solution. However, cell viability vs. number of series dilution of stock mixture solution did not consistent with those of collagen solution or ceramides solution. The optimum concentration for fibroblasts to have the highest cell viability was at 11th series dilution for collagen solution and 20th series dilution for ceramides solution instead of between 13th and 14th series dilution for NAG and stock mixture solution. The cell viability of NAG solution alone was close to that of the mixture between 11th and 16th dilution. Cell viability of collagen solution or ceramides solution between 11th and 15th dilution were lower than that of the mixture. The results indicated the dominant role of NAG on cell viability of fibroblst than those of collagen or ceramides. Cell viabilities of keratinocytes vs. dilution fold of stock mixture solutions were consistent with that of NAG Solutions as shown in Figure 4. However, cell viability vs. number of series dilution of stock mixture solution did not consistent with those of collagen or ceramides solution. The optimum concentration for kerationcytes to have the highest cell viability was at 16th series dilution for collagen solution and 20th series dilution for ceramides solution instead of 18th series dilution for NAG and stock mixture solution. However, the cell viability of keratinocytes in each solution was significantly lower than that in their mixture eg. 300% vs ca. 200% for collagen or ceramides solution; ca. 170% for NAG solution. The results indicated the cooperative role of collagen, ceradides and NAG in the mixture on the cell viability of keratinocytes.

As shown in Figure 6, the optimal concentration ranges to have expression of type I collagen mRNA for collagen, ceramides, or NAG are 6.1, 0.49, and 6.1 μg/mL respectively. The expressions of type I collagen mRNA was 152%, 160% and 230% respectively. The optimal concentration ranges for the optimal expression of type I collagen mRNA in their mixture were 0.76 μg/mL, 0.015 μg/mL and 0.76 μg/mL for collagen, ceramides and NAG, respectively. To have similar effect, the amount of collagen in the mixture was 1/8 of its single nutrients. For ceramides the amount in the mixture was 1/32 and was 1/8 for NAG of its single nutrient in the medium.

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