Adenine, new anti-wrinkle agent.

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Summary

It has been known that adenine is a very important material in living cells. Because, adenine is a member of nucleotide base, so it takes part in DNA, RNA and ATP synthesis. There are many reports that adenine participated in ingredients, especially DNA, RNA, NADH and ATP, affect on the cell. As well adenosine, conjugated adenine to glycoside, was known to anti-wrinkle compound. But there is no report whether adenine shows a good effect on the skin, especially anti-wrinkle. So, in this study, we tested whether adenine affects cell proliferation, collagen synthesis, collagenase synthesis inhibition in human dermal fibroblasts. In addition, we performed clinical study with adenine cream.

Cell proliferation effect was tested by MTT assay. Collagen and collagenase synthesis were measured by Immunoassay with ELISA kit. Clinical study was performed by IECK according to KFDA Functional Cosmetic method.

The results of cell proliferation show that 10^{-6}~10^{-8} % of adenine increases cell proliferation about 50 % compare with non-treated control. At 10^{-7}~10^{-10} %, adenine increases type I collagen synthesis about 50%, decreases type I collagenase about 22% compare with non-treated control. The results of clinical study show that 0.05% adenine treated group reduces wrinkle significantly compare with placebo treated group.

Therefore adenine may be a new anti-wrinkle candidate, through increases cell proliferation and collagen synthesis dramatically. And it decreases collagenase synthesis. So adenine could be used as a new anti-wrinkle agent.

Introduction

Cutaneous aging is a complex of biological process, which affects various layers of the skin, but the major changes are seen in the dermis. There are two independent, clinically and biologically distinct, processes affecting the skin simultaneously. The first is innate or intrinsic aging, "the biologic clock." which affects skin in a manner similar to how it probably affects a variety of internal organs, i.e., by slow, irreversible tissue degeneration. The second process is extrinsic aging, or "photo-aging" the result of exposure to outdoor elements, primarily ultraviolet (UV) radiation[1]. The consequences of innate aging can be observed all over the skin, including sun-protected areas, whereas in sun-exposed areas, particularly on the face and backs of hands, photo-damage is superimposed on the background of the ongoing innate aging process. Consequently, the most noticeable changes on facial and neck skin, result from a combination of intrinsic and extrinsic aging processes; however, it has been suggested that as much as 80% of facial aging is attributable to sun exposure [2].

Wrinkling of the skin, including dryness, roughness and pigmentation is a common phenomenon of aged skin. Dermal-epidermal junctions are also weaker. Striking changes are observed in the dermis. In the dermis, there is a loss of dermal thickness. The concentration of glycosaminoglycans in aged skin becomes progressively lower. There is a decrease in the numbers of elastic fibers and in the skin elasticity. These phenomena are accompanied by a decrease in cell activity of the dermis, including a decrease of proliferation of fibroblasts and a low cellular communication by cytokines. Especially aged skin shows a decrease of collagen production. In the case of photo-aging, the content of collagen is decreased, because of increased degradation of collagen and decreased synthesis of collagen. Decreased collagen affects the dermal architectures and changes stretch and elasticity of skin, which causes fine wrinkles of skin. [3]

Normal human dermis consists primarily of an extracellular matrix of connective tissue, and three major extracellular components have been recognized that contribute to physiological properties of the skin.

Specifically, fibers consist of collagen, an abundant extracellular matrix protein that accounts for about 80% of the dry weight of the skin, provides tensile properties to the dermis, so as to allow skin to serve as a protective organ against external trauma [4]. The elastic fibers, which account for 2-4% of the extracellular matrix in sun-protected skin, form an interconnecting network that provides elasticity and resilience to normal skin[3]. Finally, glycosaminoglycan/proteoglycan macromolecules, even though a minor component accounting for as little as 0.1-0.3% of the dry weight of tissue, play a role in providing hyaluronic acid [5]. Thus processes that alter the relative proportions of these components can result in clinical manifestations that recognized as part of the cutaneous aging process.

More recently, changes in collagen metabolism have been brought into focus as a major factor leading to photoaging. Specifically, it has been demonstrated that accumulation of elastotic material is accompanied by concomitant degeneration of the surrounding collagenous meshwork, and evidence implicating matrix metalloproteinases (MMPs) as mediators of collagen damage in photoaging has been presented [6,7,8]. MMPs comprise a family of degradative enzymes consist of at least 14 different members with rather broad substrate specificity [9]. Many of these proteases can degrade native collagen fibers, denatured collagen, elastic fibers, various proteoglycans, and fibronectin, among other components of the dermis. It has previously been demonstrated *in vitro* that UV irradiation of fibroblasts in culture enhances the expression of these proteolytic enzymes [10,11]. Loss of collagen can result in a leathery, inelastic, and graphically illustrated in the neck area of an individual spending significant unprotected time outdoors. Collectively, the observations on dermal connective tissue components in innate and photo- aging suggest an imbalance between the biosynthesis and degradation, with less repair capacity on the face of ongoing degradation. This imbalance will eventually lead to loss of recoil, which results fine wrinkles.

It has been known that adenine is a very important material in living cells. Because adenine is a member of nucleotide base, so it takes part in DNA, RNA and ATP synthesis. There are many reports that adenine participated ingredients, especially DNA, RNA, NADH and ATP, affect on the

cell. As well adenosine, conjugated adenine to glycoside, was known to anti-wrinkle compound. But there is no reported whether adenine shows a good effect on the skin, especially anti-wrinkle. So, in this study, we tested whether adenine affects cell proliferation, collagen synthesis, collagenase inhibition in human dermal fibroblasts, and clinical study.

Materials and Methods

Cell Cultures

Fibroblast cultures were initiated from biopsies of normal human skin. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 0.48 mg/ml glutamine and 100 IU/ml penicilline, 50 μg/ml streptomycin, and 10 % fetal bovine serum(FBS, Gibco BRL) at 37°C in a 5% CO_2 humidified atmosphere. This study used between the fourth and seventh passage fibroblasts. Then, fibroblasts were assayed for proliferation, collagen and collagenase synthetic activities as described below.

Assay of Fibroblasts Proliferation

An assay of fibroblasts proliferation was performed with placing 6,000 freshly trypsinized fibroblasts, contained in 200μl of DMEM with supplemented 5% FBS, into 96-well multi-chamber plates and incubated for 18hrs at 37°C in 5% CO_2 atmosphere to permit adherence well bottoms. After adherence, the media were removed and replaced with 200μl fresh DMEM - 5% FBS with different concentrations of adenine. Then the cultures were incubated for an additional 72hrs. After 72hrs incubation, fibroblasts were used for the MTT assay. MTT assay was based on the Mosmann method. The 0.1mg(50μl of 2mg/ml) MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma, U.S.A.) was added to each well. The plates were incubated for an additional 4hrs at 37°C and then all supernatant were removed. After that, 150μl of dimethyl sulfoxide(DMSO) was added to each well. Plates were placed on a plate shaker for 10mins and read immediately at 570nm using multi-microplate reader (Bio-TEK EL310).

Collagen synthesis assay

Human normal fibroblast was inoculated to 24-well microplate ($5x10^4$ cell/well) and cultivated for 24 hours. After the culture, culture medium was changed to serum-free DMEM medium containing adenine in the following and cultivated 72 hours. Control group was cultivated without sample extracts. After the culture, supernatants of each well were collected and amount of procollagen type

I pN-peptide was measured by using type I pN-peptide assay kit (Takara, Kyoto, Japan).

Collagenase synthesis inhibition assay

Human normal fibroblast was inoculated to 24-well microplate ($5x10^4$ cell/well) and cultivated for 24 hours. After the culture, culture medium was changed to serum-free DMEM medium containing sample extracts in the following and cultivated 72 hours. Control group was cultivated without sample extracts. After the culture, supernatants of each well was collected and amount of interstitial collagenase was measured the with MMP-1 human ELISA system (Amersham, Sweden).

Clinical study

We studied the effect of adenine on human skin with thirty-two healthy female volunteers. A cream containing 0.05% adenine was topically applied on the left or right face twice a day for the 8 weeks, while the opposite face received only vehicle for the same period of time.

The degree of wrinkle improvement was evaluated by 1) replica analysis using Skin-Visiometer SV 400 (C+K electronic GmbH, Germany) 2) eye evaluation of dermatologists and 3) self-evaluation of volunteers.

Statistical Analysis

The statistical analysis of the results was performed using the two tailed t-test (*in vitro* assay) and independent t-test (clinical test) at $p<0.05$.

Results

1. Effect of Adenine on Proliferation in Normal Human Fibroblasts.

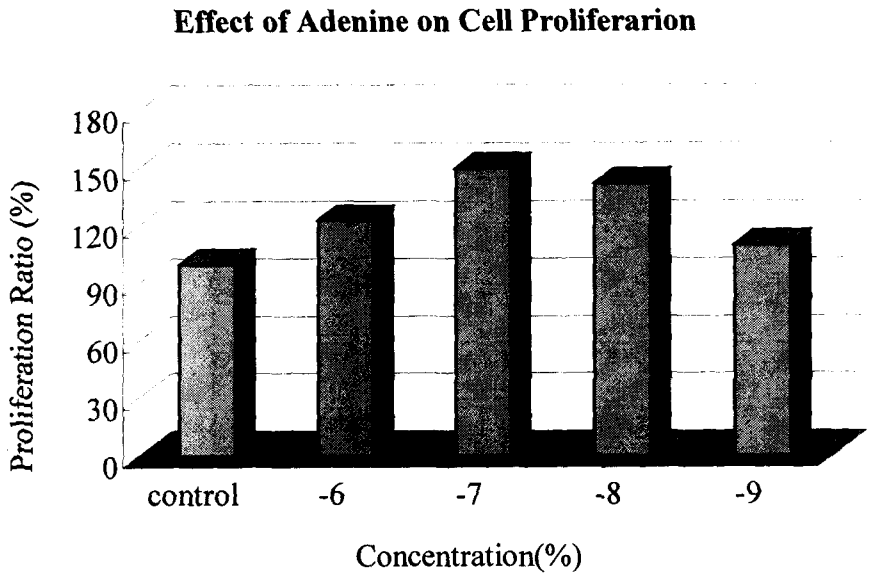

Fig. 1) Normal human fibroblasts were seeding on 96-well plate, and then cultured with DMEM media for 18 hours. Then, adenine was applied 10^{-5}~10^{-12} % on normal human fibroblasts during 72 hours. After 72 hours, media was removed and MTT assay was performed.

2. Effect of Adenine on Collagen Synthesis in Normal Human Fibroblasts.

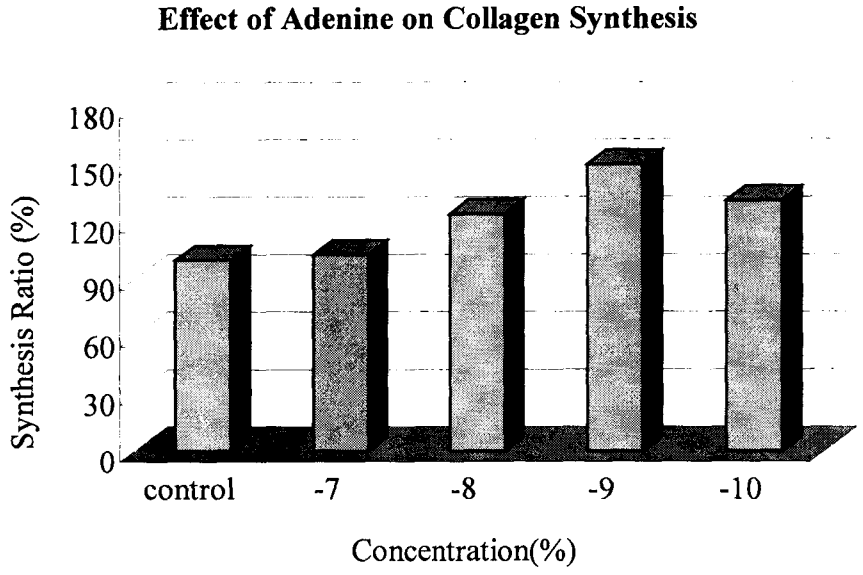

Fig. 2) Normal human fibroblasts were seeded on 96-well plate, and then cultured with DMEM media for 18 hours. Then, adenine was treated 10^{-5}~10^{-12} % on normal human fibroblasts during 72 hours. After 72 hours, media was collected and collagen synthesis assay was performed by procollagen type I pN-peptide immunoassay.

3. Effect of Adenine on Collagenase Inhibition in Normal Human Fibroblasts.

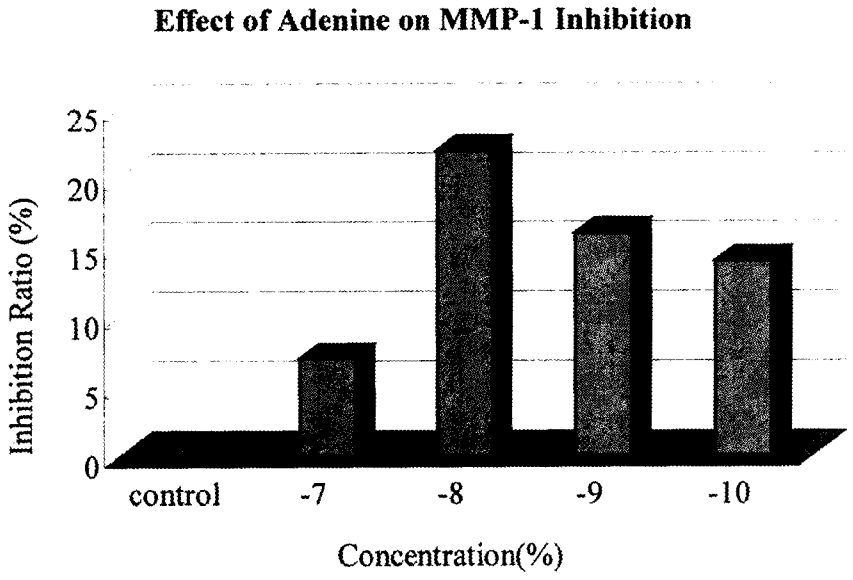

Fig. 3) Normal human fibroblasts were seeding on 96-well plate, and then cultured with DMEM media for 18 hours. Then, adenine was treated 10^{-5}~10^{-12} % on normal human fibroblasts during 72 hours. After 72 hours, media was collected and collagenase inhibition assay was performed by MMP-1 human ELISA system

4. Clinical Study

1) Analysis of replica

Horizontal Analysis

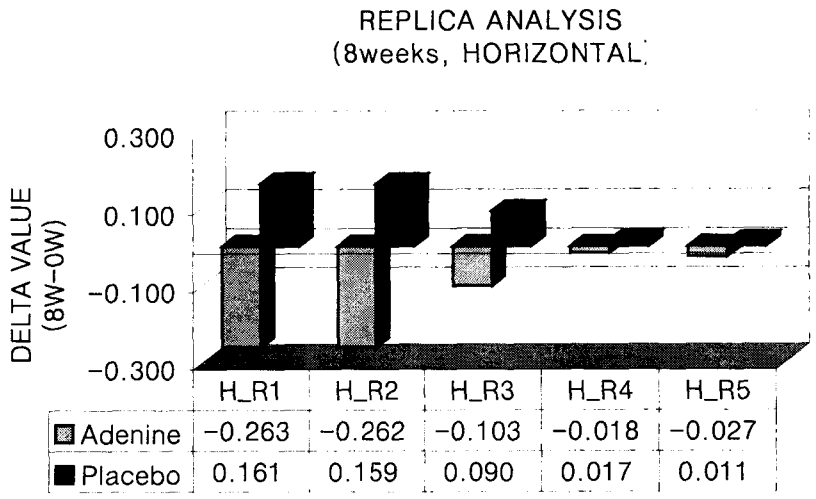

Fig. 4) 0.05% adenine cream and placebo were applied on the left and right face in 32 healthy women. After 8 weeks, Silicone replica was replicated from the crow's feet area on the face. Replica image analysis was performed with C+K Visiometer.

Circular Analysis

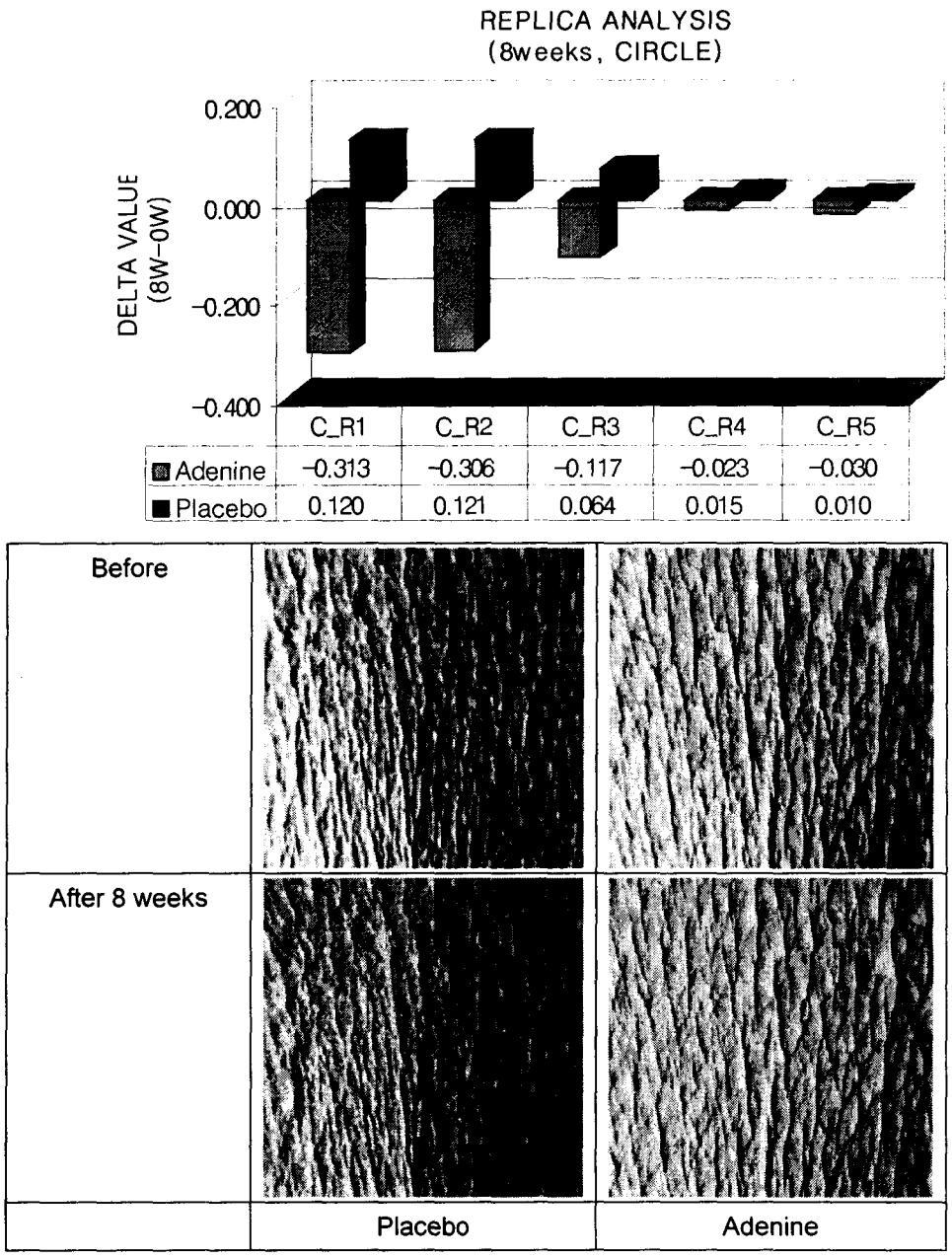

Fig. 5) 0.05% adenine cream and placebo were applied on the left and right face in 32 healthy women. After 8 weeks, Silicone replica was replicated from the crow's feet area on the face. Replica image analysis was performed with C+K Visio meter. (Above- histogram of the replica analysis result. Below- the photography of replica)

2) Clinical evaluation by 2 Dermatologists: on the basis of clinical scores in 13 points

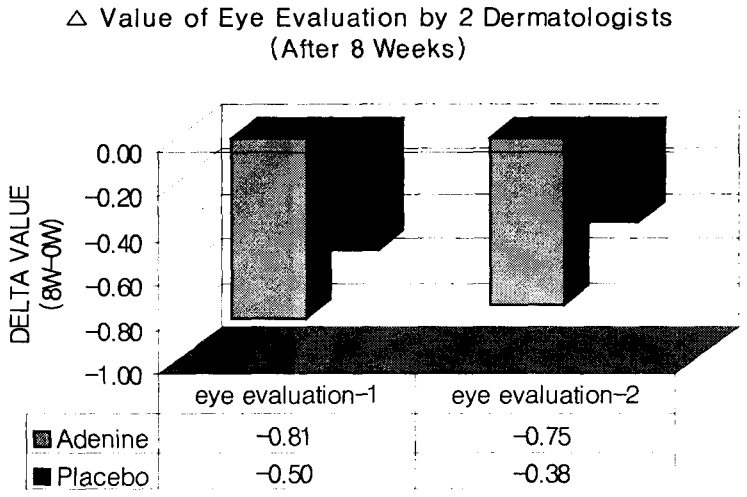

Fig. 6) 0.05% adenine cream and placebo were applied on the left and right face in 32 healthy women. After 8 weeks, Clinical evaluation was performed by 2-dermatologists.

3) Self-assessment questionnaire

Safety

- All of volunteers told us no skin irritation due to the test articles.

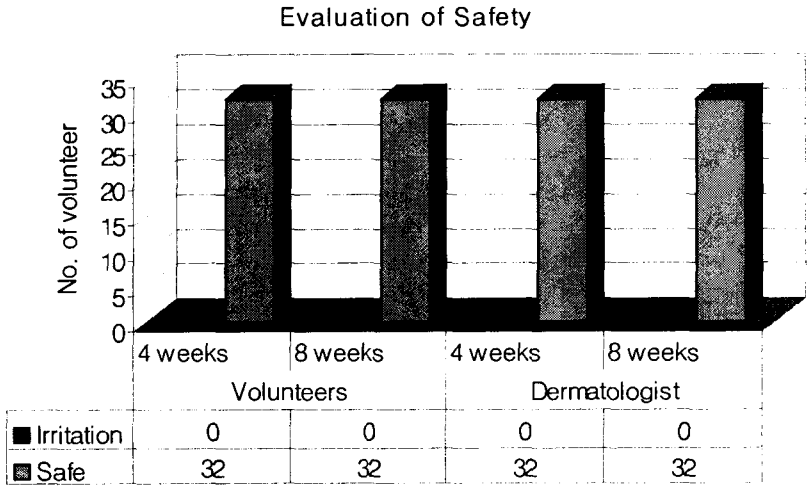

Fig. 7) 0.05% adenine cream and placebo were applied on the left and right face in 32 healthy women. After 8 weeks, volunteers performed Self-assessment questionnaires.

Efficacy

- Do you find the anti-wrinkle effect? If yes, which side do you find?

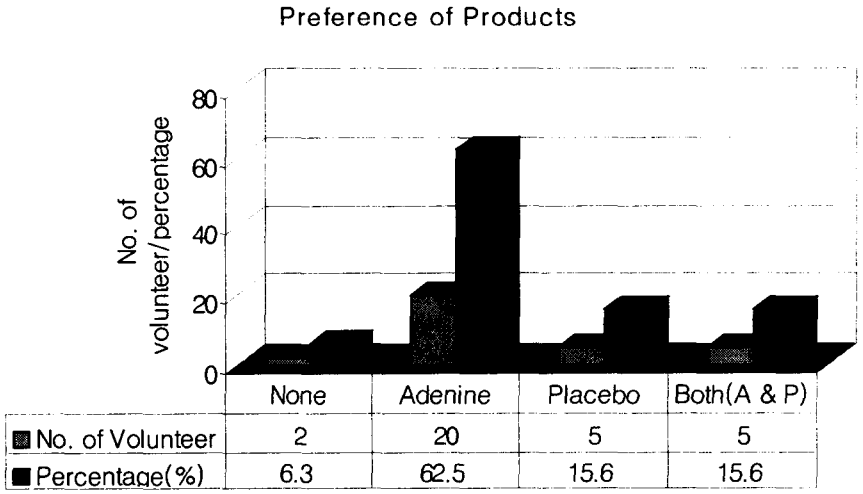

Fig. 8) 0.05% adenine cream and placebo were applied on the left and right face in 32 healthy women. After 8 weeks, volunteers performed Self-assessment questionnaires.

- If you have some anti-wrinkle effect, how degree do you satisfy?
  (In case of adenine product)

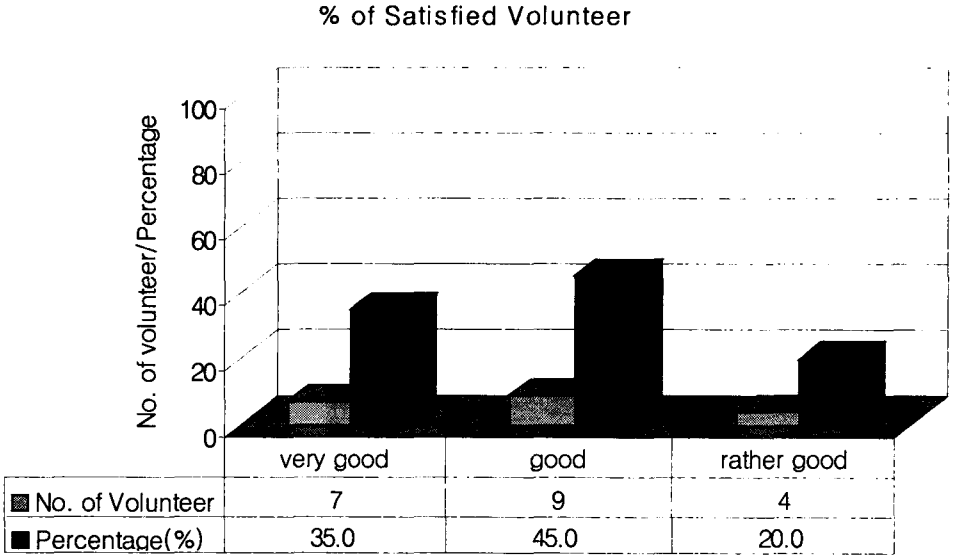

Fig. 9) Percentage and/or No. of volunteer of degree of satisfied among satisfied 20 volunteer in 0.05% adenine.

Conclusions

To identify of adenine's effects on skin aging, we tested *in vitro* assay and clinical test. In *in vitro* assay, whether adenine had effects on proliferation of fibroblasts, on collagen and collagenase synthesis in human fibroblasts. Compared with proliferate activity of non-treated fibroblasts, adenine showed 123±0.09%, 150±0.08% and 142±0.06% proliferation at 10^{-6}%, 10^{-7}% and 10^{-8}%(Fig 1). In collagen synthesis, adenine stimulated collagen protein synthesis 124±0.02%, 150±0.03% and 130±0.02% at 10^{-8}%, 10^{-9}% and 10^{-10}% in fibroblasts cultures (Fig. 2). In collagenase synthesis, adenine inhibits collagenase synthesis 21.7±0.04%, 15.8±0.05% and 13.9±0.04% at 10^{-8}%, 10^{-9}% and 10^{-10}% in fibroblasts culture (Fig. 3).

In clinical study, replica analysis was shown to significant decrease of wrinkle at horizontal and circle analysis after 8 weeks treatment (Fig. 4,5). Replica image picture was shown to significant decrease as well (Fig. 5). At clinical evaluation by 2 dermatologists, adenine 0.05% cream was shown to significant decrease wrinkle by eye evaluation. At self-assessment questionnaire, volunteer hasn't show skin irritation. As well adenine cream was more efficacy than placebo.

In the results of the *in vitro* experiments, we have shown that adenine stimulated fibroblasts proliferation and collagen production in fibroblasts, as well adenine inhibits collagenase synthesis in fibroblasts. At clinical results, replica analysis, dermatological observation and volunteer's self-assessments were shown to significant decrease wrinkle after 8 weeks treatment of adenine cream. Therefore, we can consider that adenine could be used as a powerful anti-aging material.

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