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EFFECT OF KOREAN BLACK SOYBEAN SEED ON THE CELLULAR PROLIFERATION AND THE PRODUCTION OF TYPE III COLLAGEN IN SKIN FIBROBLAST

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ABSTRACT

Soybeans are one of the major crops for human food resource; protein, lipid, and carbohydrate. In these days, they are widely using for cosmetics to supply phospholipid; natural surfactant. In this study we used black soybean seed in korea and observed many kinds of biochemical constituents; isoflavone, melatonin, crisantemine and calcium in ethanol extract. Also, its extract (we named it Flatonin) has been demonstrated that korean black soybean seed is able to stimulate the proliferation of NIH 3T3 cells and increase the production of type III collagen in NIH 3T3 and Malme-3 (human skin fibroblast) cells. The addition of korean black soybeen to quiescent NIH 3T3 cells resulted in an increase of proliferation which was assayed by MTT method. The maximum effect of korean black soybean was detected in 0.4% korean black soybean treated cells which was comparable to that of 5% serum(96% of 5% serum effect). The addition of korean black soybean to NIH 3T3 and Malme-3 cells also increased the production of type III collagen in both cells. These results indicate that korean black soybean may enhance the repair process after injury and prevent aging processes in connective tissues.

INTRODUCTION

Collagen is the most abundant protein in vertebrate animals and plays a critical role in the structure and biological processes such as development, differentiation and healing after injury. The metabolism of collagen in normal tissues involves a balance between biosynthesis and degradation and control of the balance is of major importance in maintaining normal physiologic condition. There are some types of collagen (1). Collagen I represents 80% of the total dermal collagen in an adult s skin, while collagen II accounts for 15%. The remaining 5% is mainly made up of corresponding type IV and type V collagens. Thick fibers type I collagen are combined with a fine felting of type III collagen, which orientates big collagen I fibers during their growth. More tensile and less fibrous, collagen III is predominant in fetal and postnatal skins and during the wound healing process, i.e. during scarification (2). Thus, type III collagen has been called a restructuring collagen, and is particular to very young skin or skin under repair. Cells having this reconstituted connective support can proliferate, allowing a proportional amplification of chemostatic interactions and providing effective and fast responses during the restoration of tissue. Collagen I is formed later in the human life cycle and then becomes predominant. Fine fibrillar fiber structures, typical to all Juvenile growing dermis, reflect an enrichment in collagen III, which plays a major role in dermal architecture.

Among the numerous modifications of the extracellular matrix during age, collagen synthesis shows a significant decrease the ageing process. Moreover, the ratio of collagen types changes throughout life. Age related changes in the ratio of type I and III collagen have been the subject of several studies. The collagen III content in fetal skin and in newborn infant skin is significantly higher than in adult skin.

Till now, cell growth factor, cytokine and cAMP are known to control the expression of collagen synthesis (3). In this study, korean soybean extract (called Flatonin) to NIH 3T3 and Malme-3 cells also increased the production of type III collagen in both cells. These results indicate that Flatonin may enhance the repair process after injury and prevent aging processes in connective tissues.

MATERIAL AND METHODS

Cell culture

NIH 3T3 mouse fibroblasts (embryo fibroblast, contact-inhibted, NIH Swiss mouse, ATTC CRL 1658) and Malme-3 (skin fibroblast, human, ATTC HTB-10-2) were obtained from the American Type Culture Collection.

Cells were grown in DMEM supplemented with 5% Bovine calf serum(BCS, Hyclone) for NIH 3T3 fibroblasts and Mc Coy s 5a, 15% fetal bovine serum for Malme-3, 100 units/μl penicillin-100mg/ml streptomycin (Gipco) and maintained in an humid atmosphere of 5% CO_2 incubator at 37 C.

MTT assay

NIH 3T3 cells were plated at $2X10^4$/ml cells per well in 24 well culture plates and grown to confluence for 48hrs. The media was changed to fresh DMEM with 0.5% serum and cultivated for 48hrs to go into the quiescent stage. Cells were treated for 24hrs with Flatonin and media was changed to 0.5% serum. Cells were treated with 100μl-MTT solution [5mg MTT, 1ml PBS (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024 % KH_2PO_4, pH7.4)] and incubated in 5% CO_2 at 37 C for 4hrs.

Finally, Cells were solubilized in 1ml DMSO and measured the O.D value at 540nm wavelength.

Western blot assay

Cells treated with Flatonin were added with sample buffer containing 60mM Tris-Cl (pH 6.8), 25% (w/v) glycerol, 2% (w/v) SDS, 14.4mM 2-mercaptoethanol, 0.1%(w/v) bromophenolblue and proteins were extracted. The protein concentration was by using Bio-Rad microassay kit. The quantity finished protein was heated at 95 C for ten minutes and electrophoresis was established in 10% polyacrylamide gel (Hoffer). The gel was electrotransferred by nitrocellulose membrane. Nitrocellulose membrane blotted with protein was treated with blocking buffer containing 10mM Tris-Cl (pH 8.0), 150mM NaCl, 0.05%(v/v) Tween 20, 3% (w/v) nonfat dry milk for one hour.

Subsequently, after put in binding buffer [10mM Tris-Cl (pH 8.0), 150mM NaCl, 0.05% (v/v) Tween 20, 0.05% (w/v) nonfat dry milk] containing mouse anti-type III collagen monoclonal antibody (Oncogen science) for one hour, treated goat anti-mouse IgG labelled with alkaline phosphatase for one hour.

The substrate of alkaline phophatase was X-phosphate and NBT. The result was identified with color reaction.

RESULT AND DISCUSSION

The influence of Flatonin on proliferation of NIH 3T3

To determine the effect of Flatonin on collagen III synthesis in NIH 3T3 fibroblast cells, Cells were grown in the culture medium containing 0.5% serum for two days to go into the quiescent stage. After 0.05%-0.5% of Flatonin was on cells, MTT assay was established. The relative rate of cell proliferation was observed in a dose-dependent manner and the maximum was in 0.4% Flatonin. (Fig.1), which compared to 96 % effect of cells grown in 5% serum media indicating a selective effect of Flatonin and serum on cell growth. According to the morphology of NIH 3T3, globular mitotic cells were increased dose- dependently (Fig. 2). Globular mitotic cells treated with MTT were dyed by the reaction of mitochondrial dehydrogenase (Fig.3). This result indicates that the cells of Fig.2 survive. Malme-3 human cancer cells didn t go into the quiescent in 0.5% serum media, so treated with 5% serum containing 0.3%-0.4% Flatonin for 24 hrs indicating cells growth. The constituents of Flatonin is known to peptide, lipid, carbohydrate, flavonoid, hormonlike and some minerals.

We don t know exactly what kind of constituent stimulate the cell growth but it is possible to stimulate the proliferation of cell by carrying the cell surface signal of peptide, etc. into the intracell. Actually, it is well known that some carbohydrates and minerals control the cell proliferation and specific gene expression by carrying the signal into the nucleus and binding with cell membrane mediated motility receptor.

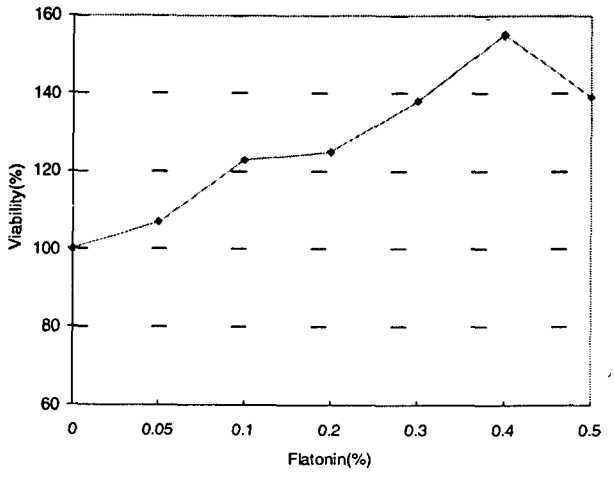

Fig. 1. Effect of Flatonin on the proliferation of NIH 3T3 cells.
Quiescent cells were incubated for 24hrs with various concentration of Flatonin. Proliferation was assayed by MTT assay and presented by percent of 0.5% serum stimulated cells.

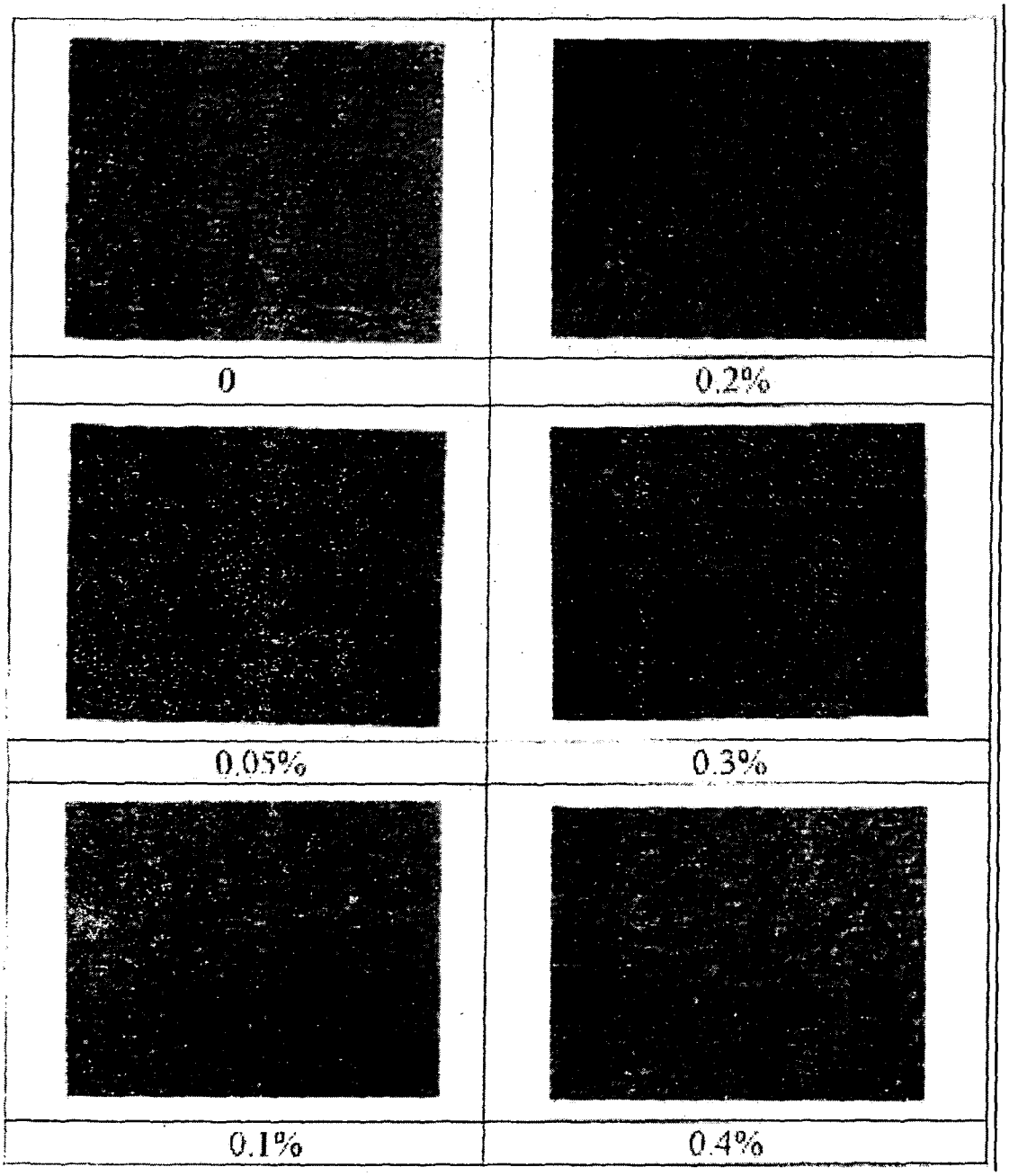

Fig. 2. Effect of Flatonin on the morphology of NIH 3T3 cells.
Quiescent cells were incubated for 24hrs with various concentration of Flatonin and observed under phase contrast microscope(100X). Arrow head indicates the mitotic cell.

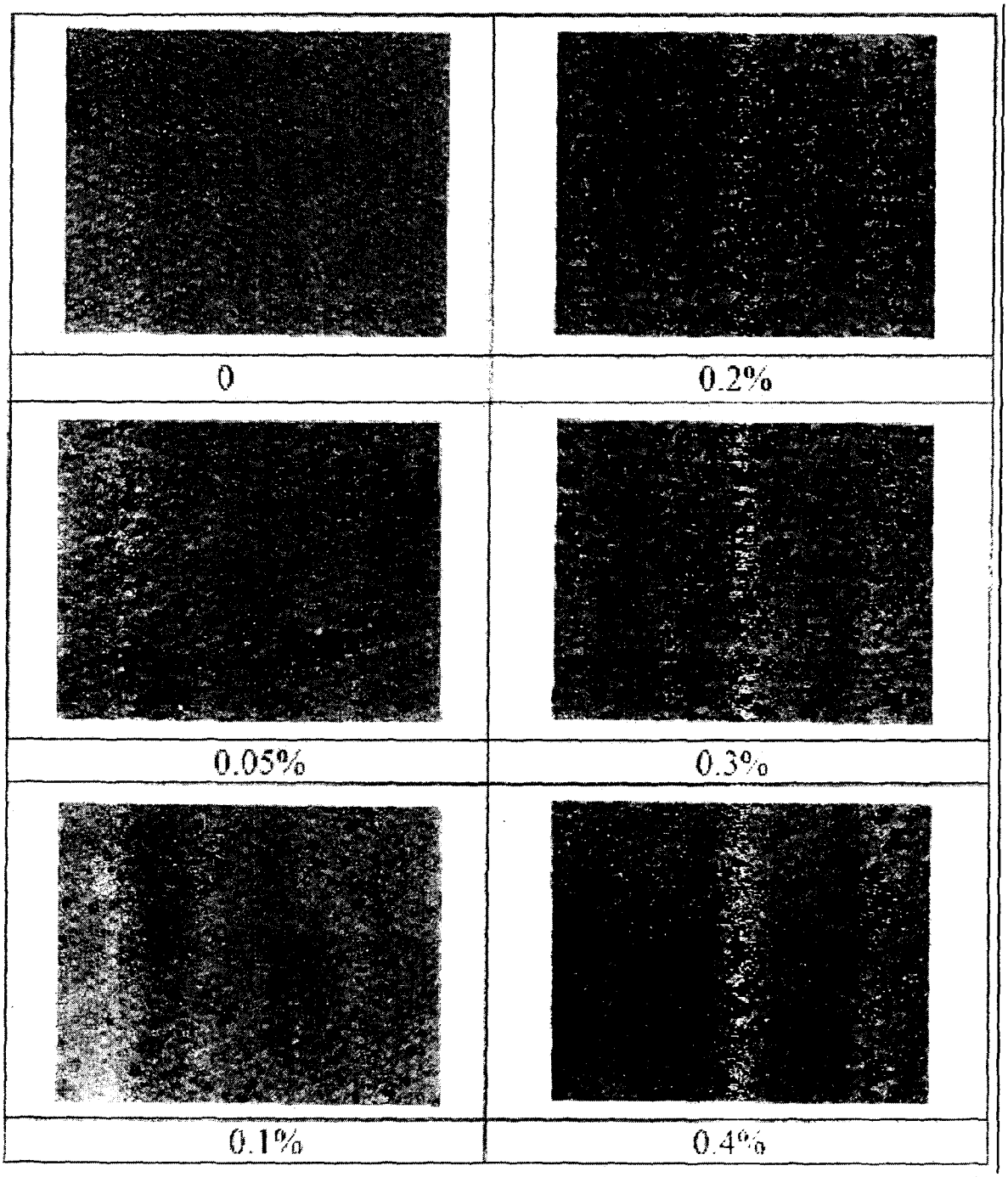

Fig. 3. MTT assay.
NIH 3T3 cells were treated as described in Fig.2 and MTT was added.
Stained cells were observed under phase contrast microscope(100X).

The influence of Flatonin on synthesis of Collagen III

More than 10 types of collagen have been reported and known to have a tissue-specific distribution in vertebrate organisms (4). For example, type I collagen is the most prevalent type and distributes ubiquitously in many tissues including dermis, bone and blood vessel, while type III collagen is a major constituent of newly bone fibroblast in dermis. Collagen plays a critical role in the structure and biological processes such as development, differentiation and healing after injury. The metabolism of collagen in normal tissues involves a balance between biosynthesis and degradation and control of the balance is of major importance in maintaining normal physiologic condition (5). Current studies on collagen metabolism revealed that fibroblast, major collagen-producing cells, tightly control the amount and type of collagen they produce (6).

As a result of study, we knew that the cell proliferation and collagen synthesis had a tight relationship. Therefore, we performed following experiment because Flatonin was possible to control collagen synthesis by showing similar proliferation effect to serum in NIH 3T3 cells. After treating Flatonin 0.1%- 0.4% in NIH 3T3 cells or 0.05%-1% in Malme-3 cells and establishing western blot assay by using anti-type III collagen antibody, scanned by densitometer.

As a result, collagen production was increased in a Flatonin dose-dependently in NIH 3T3 fibroblast (Fig.4) and Malme -3 cells (Fig. 5).

This suggests that treatment with Flatonin effects the induction of synthesis of collagen III in NIH 3T3 fibroblasts and Malme-3 cells. We think that this is accompanied by anti-aging effect in skin, furthermore, in cosmetics .

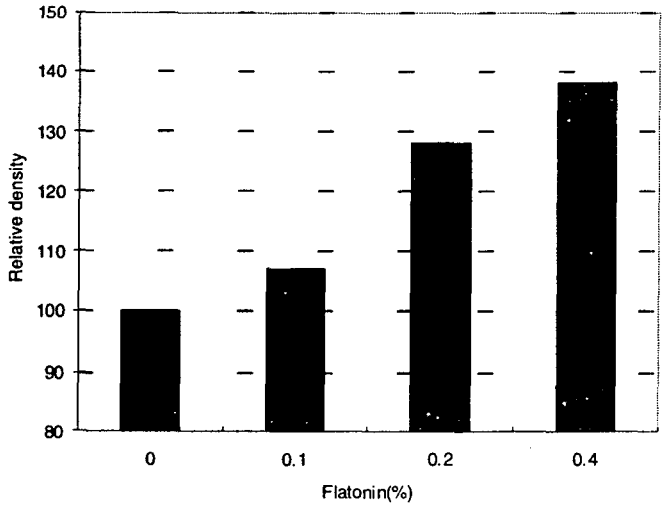

Fig. 4. Western blot analysis. The effect of Flatonin on the expression of type III collagen protein.
Quiescent cells were incubated with Flatonin for 24 hrs and total protein was prepared electrophoresed on 7.5% polyacrylamide gel and incubated with mouse anti-type III collagen monoclonal antibody. The same gel was stained with coomassie blue R-250. Densitometric scanning of the blot was presented.

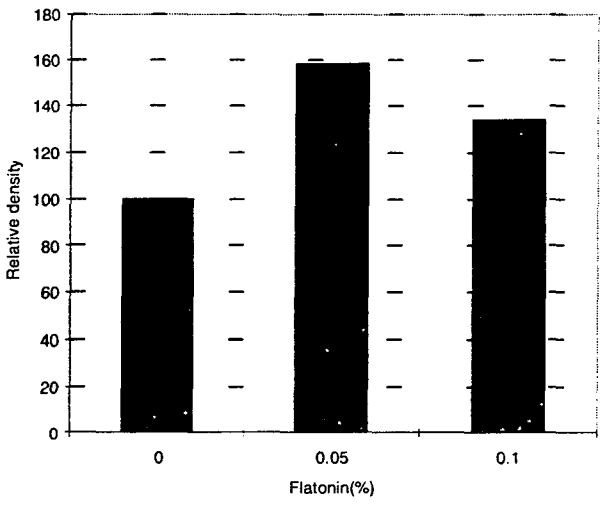

Fig. 5. Western blot analysis. Malme-3 cells were incubated with Flatonin for 24hrs. Total protein was extracted and western blot was performed as described in methods. Densitometric scanning of the blot was presented.

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