

The effect of *Centella asiatica*, vitamins, glycolic acid and their mixtures preparations in stimulating collagen and fibronectin synthesis in cultured human skin fibroblast

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Abstract: *Centella asiatica* (Linn.) Urban is well known in promoting wound healing and provides significant benefits in skin care and therapeutic products formulation. Glycolic acid and vitamins also play a role in the enhancement of collagen and fibronectin synthesis. Here, we evaluate the specific effect of *Centella asiatica* (CA), vitamins, glycolic acid and their mixture preparations to stimulate collagen and fibronectin synthesis in cultured human fibroblast cells. The fibroblast cells are incubated with CA, glycolic acid, vitamins and their mixture preparations for 48 h. The cell lysates were analyzed for protein content and collagen synthesis by direct binding enzyme immunoassay. The fibronectin of the cultured supernatant was measured by sandwich enzyme immunoassay. The results showed that CA, glycolic acid, vitamins A, E and C significantly stimulate collagen and fibronectin synthesis in the fibroblast. Addition of glycolic acid and vitamins to CA further increased the levels of collagen and fibronectin synthesis to 8.55 and 23.75 $\mu\text{g}/100 \mu\text{g}$, respectively. CA, glycolic acid, vitamins A, E, and C, and their mixtures demonstrated stimulatory effect on both extra-cellular matrix synthesis of collagen and fibronectin in *in vitro* studies on human foreskin fibroblasts, which is beneficial to skin care and therapeutic products formulation.

Keywords: *Centella asiatica*, glycolic acid, vitamins, collagen, fibronectin

INTRODUCTION

CA plant belongs to the family Apiaceae and has been widely used as herbs, salad, vegetables and traditional medicine in East Asia since ancient times. The plant extract is recognized for its ability to promote wound healing and used in pharmaceutical and skin care applications (Loiseau and Mercier, 2000). In skin care products, it has been claimed to renew collagen formation and to restructure the damaged tissue, thereby restoring tissue firmness and skin elasticity as well as improving skin appearance. Studies by Hashim *et al.*, (2011) have shown that CA leaves extract enhanced collagen synthesis and has potential as antioxidant, anticellulite as well as UV protectant. CA extract also has many applications as a topical therapeutic agent. It is used in propriety medicinal products for treatment of cutaneous ulcer, hypertrophic scars, keloids, wound healing disorders and treatment of venous-lymphatic disorder. Although the exact molecular mechanism of action on wound healing is not known, studies showed that the extracts of CA possess good wound healing activity (Hong *et al.*, 2005; Shetty *et al.*, 2006; Hashim, 2011).

Vitamins play an important role in skin health, improvement of wrinkles and wound healing. Therefore, many skin care and wound healing formulations incorporated vitamins such as vitamin A, E and C. Vitamin C (ascorbic acid) plays a vital role in repair of damaged skin and modulates collagen production (Boyera

et al., 1998). This is because it is a cofactor for the lysyl and prolyl hydroxylase enzymes in the collagen synthesis pathway (Gessin *et al.*, 1993). They catalyze the synthesis of hydroxyproline and hydroxylysine in collagen. Therefore, the absence of hydroxyproline in the synthesis pathway will make the collagen structure unstable.

Vitamin A has been shown to affect the endothelial proliferation during wound healing process (Lee *et al.*, 1992). Vitamin A also invigorates the inflammatory response; promote cell epithelialization and the synthesis of collagen (Anstead, 1998). Similarly, vitamin E (alpha-tocopherol), as an antioxidant has a direct affect on the transforming growth factor-mediated protein kinase C activation, which leads to collagen and/or fibronectin synthesis in mesangial cells (Struder *et al.*, 1997). Meanwhile vitamin E prevents the reactive oxygen species (ROS)-induced alteration of collagen synthesis (Tanaka *et al.*, 1993). Autoradiographic, ultrastructural and cell surface studies demonstrated that vitamin A, C, and E are strong regulatory factors for collagen synthesis (Lupulescu, 1994). However, no specific study on collagen and fibronectin enhancement has been conducted on vitamin A and E.

Glycolic acid is commonly used as chemical peeling agent. It has been used in skin care products to improve photo ageing and smooth fine wrinkles (Moy *et al.*, 1996; Funasaka, 2001; Couch and Howard, 2002). However, it may have direct effect on the skin, especially collagen production. Glycolic acid has also been demonstrated to repair photo-damaged hairless mice (Moon *et al.*, 1999).

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The fibroblast is a critical component of granulation tissue and is responsible for the production of collagen, elastin, fibronectin, glycosaminoglycans and proteases. Therefore, the synthesis of collagen and fibronectin is very important in the proliferation phase and in the wound healing process (Romo *et al.*, 2008).

Therefore, the aim of the study is to evaluate the specific effect of CA, vitamins, glycolic acid and their mixtures preparations in enhancing collagen and fibronectin synthesis. The biochemical basis of these treatments on connective tissue metabolism will be determined using cultured fibroblast cells incubated for 48 h.

MATERIALS AND METHODS

Materials

CA extract was obtained from MMP Inc. (South Plainfield, NJ). Vitamin A, C and E, horseradish peroxidase enzyme substrates, ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, hydrogen peroxide, L-glutamine and penicillin-streptomycin antibiotics were purchased from Sigma. Type 1 human collagen standard was purchased from Life Technologies (Rockville, MD). A polyclonal goat antibody to collagen, fibronectin antigen standard and monoclonal antibody (MAB 1926) to fibronectin were from Chemicon International (Temecula, CA). Polyclonal goat anti-human fibronectin, flexible polyvinyl chloride microtiter plates and Horseradish peroxidase-conjugated secondary antibodies were purchased from Foster City, CA; Corning Inc., Corning, NY; and KPL, Kirkegaard and Perry Lab., Gaithersburg, MD, respectively. Tissue culture media, i.e. Dubelcco's Minimal Essential Medium (DMEM) and heat inactivated fetal bovine serum were purchased from Cellgro (Mediatech Inc.). Human foreskin fibroblast cells CCD-1114Sk (CRL 2450) was from ATCC (American Type Culture Collection, Manassas, VA).

Cell cultures

The primary human foreskin fibroblast cells were cultured in Dubelcco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. The confluent foreskin fibroblast cells from passages 4-8 grown in culture plate (100 x 15 mm) were incubated in medium containing the tested materials (CA extract 100 µg/ml, vitamin A 50 µg/ml, vitamin E 50 µg/ml, glycolic acid 10 µg/ml) for 48 h in a 5% CO₂ incubator at 37°C. Vitamin C (25 µg/ml) was used as positive control and solvent 0.2% ethanol was used as untreated control (control). After 48 h of incubation, the culture supernatants were removed and the amount of an extracellular protein i.e. fibronectin, synthesized and secreted into the culture supernatant was measured by sandwich enzyme immunoassay (EIA).

Protein assay

Protein concentration of the cell lysate produced with

hypotonic buffer was determined by Coomassie blue dye binding method (Bradford, 1976) using the dye concentrates from Bio-Rad (Richmond, CA). Bovine serum albumin was used as protein standard.

Direct binding enzyme immunoassay for collagen

Due to the insolubility of collagen at neutral pH, the cellular collagen solubilized with an acetic acid buffer (0.5 M, pH 2.0) was measured by direct binding assay on microtiter plates. The collagen solubilized in acidic buffer was allowed to bind to the microtiter wells for 12-20 h at 4°C. The residual sites in the microtiter plates were blocked with 1% bovine serum albumin (BSA). The solid phase-bound collagen was then detected with a polyclonal goat anti-collagen antibody and the bound goat antibody was monitored with an enzyme-conjugated secondary antibody to the goat immunoglobulin. The enzyme activity of the solid phase-bound secondary antibody was assayed using a chromogenic substrate of ATBS solution. The color intensity was measured at 570 nm with a Bio-Tek plate reader. Delta-Soft computer program was used to convert the optical density units of the samples into µg/100 µg of collagen using log/logit transformation of the standard curve. Sample values were taken from the standard curve which had correlation coefficient >0.95 of the theoretical value.

Sandwich enzyme immunoassay for fibronectin

Microtiter plates were coated with goat anti-human fibronectin immunoglobulin and served as capture antibody. Fibronectin in the culture supernatant was allowed to bind with the capture antibody for 1 h at 4°C. The unbound protein was removed by washing the plates successively with PBS buffer. The solid phase-captured fibronectin was detected with monoclonal antibody to fibronectin, MAB 1926 (1 h at 4°C). The excess antibody was removed by washing and the bound antibody was monitored with an enzyme-conjugated secondary antibody to mouse immunoglobulins. After washing to remove the excess secondary antibody, the conjugated enzyme activity was assayed with a chromogenic substrate, ABTS in the presence of 0.03% hydrogen peroxide in an acidic buffer as described in direct binding assay for collagen. Data reduction was performed using a similar method as described above.

STATISTICAL ANALYSIS

All experiments were carried out in triplicates. Analysis of variance (ANOVA) was performed with SAS software using Duncan's multiple comparison test. The level of significance was set at $P < 0.05$.

RESULTS

Stimulation effect of CA extract, vitamin A, E, C and their vitamin mixtures

After 48 h incubation (table 1), the collagen produced by

CA (4.02 $\mu\text{g}/100 \mu\text{g}$ protein), vitamins A (3.50 $\mu\text{g}/100 \mu\text{g}$ protein), E (3.66 $\mu\text{g}/100 \mu\text{g}$ protein) and C (3.95 $\mu\text{g}/100 \mu\text{g}$ protein) was significantly increased ($P<0.05$) by approximately 2-fold compared with control, while the fibronectin was stimulated approximately by 1.5-fold. The addition of individual vitamins A (50 $\mu\text{g}/\text{ml}$), E (50 $\mu\text{g}/\text{ml}$) and C (25 $\mu\text{g}/\text{ml}$) to CA (100 $\mu\text{g}/\text{ml}$) significantly increased levels of collagen and fibronectin by 30% and 40%, respectively. When the mixture of vitamins A (50 $\mu\text{g}/\text{ml}$), E (50 $\mu\text{g}/\text{ml}$) and C (25 $\mu\text{g}/\text{ml}$) was added to CA (100 $\mu\text{g}/\text{ml}$), it stimulated the synthesis of collagen and fibronectin approximately by 1.5-fold compared to CA alone and when it was compared to control, the collagen and fibronectin levels were significantly stimulated approximately by 3-fold and 2-fold, respectively.

Table 1: Stimulation of CA extract, vitamin A, E, C and their vitamin mixtures on collagen and fibronectin synthesis

Treatments	Collagen ($\mu\text{g}/100 \mu\text{g}$ protein)	Fibronectin ($\mu\text{g}/100 \mu\text{g}$ protein)
Control (untreated)	2.09 ^a ±0.30	10.12 ^a ±0.37
Vitamin C	3.95 ^{b,c} ±0.34	14.92 ^{b,c} ±1.10
Vitamin A	3.50 ^b ±0.35	14.03 ^{b,c} ±1.25
Vitamin E	3.66 ^b ±0.21	13.12 ^b ±0.51
CA (Centella extract)	4.02 ^{b,c} ±0.27	14.22 ^{b,c} ±0.77
CA+Vitamin C	5.11 ^{d,e} ±0.54	18.11 ^d ±1.14
CA +Vitamin A	4.45 ^{c,d} ±0.39	15.15 ^{b,c} ±1.92
CA + Vitamin E	4.80 ^{c,d} ±0.67	16.85 ^{c,d} ±1.96
CA + Vitamin A, E, C	5.88 ^e ±0.59	21.01 ^e ±1.78

Values are expressed as mean \pm SD (n=3, $P<0.05$).

Stimulation effect of glycolic acid with CA extract and vitamin C

As demonstrated in figs. 1 and 2, the collagen and fibronectin synthesis were stimulated significantly compared with control. The control synthesized collagen of 2.09 $\mu\text{g}/100 \mu\text{g}$ protein and fibronectin of 10.12 $\mu\text{g}/100 \mu\text{g}$ protein, whereas fibroblast treated with glycolic acid produced collagen of 4.15 $\mu\text{g}/100 \mu\text{g}$ protein and fibronectin of 14.92 $\mu\text{g}/100 \mu\text{g}$ protein. Thus, glycolic acid significantly stimulated the synthesis of collagen and fibronectin by approximately 2-fold. When glycolic acid was added to CA extract, the collagen was significantly ($P<0.05$) stimulated by approximately 3-fold. The stimulation of collagen and fibronectin was further enhanced when the fibroblast culture was incubated with the mixture containing CA (100 $\mu\text{g}/\text{ml}$), glycolic acid (10 $\mu\text{g}/\text{ml}$) and vitamin C (25 $\mu\text{g}/\text{ml}$) where the results illustrated the highest stimulation of collagen (4-fold) and fibronectin (2.5-fold) compared to the other treatments.

DISCUSSION

The present investigation was undertaken to determine the biochemical effect of CA, glycolic acid, and vitamins A, E and C on connective tissue protein metabolism at cellular level. An *in-vitro* model using the human foreskin

fibroblast culture was chosen to study the various elements in incubated cultured cells for 48 h. Collagen was detected using polyclonal goat anti-collagen antibody in the direct binding EIA whereas fibronectin was detected using monoclonal antibody to fibronectin, MAB 1926 in the sandwich EIA.

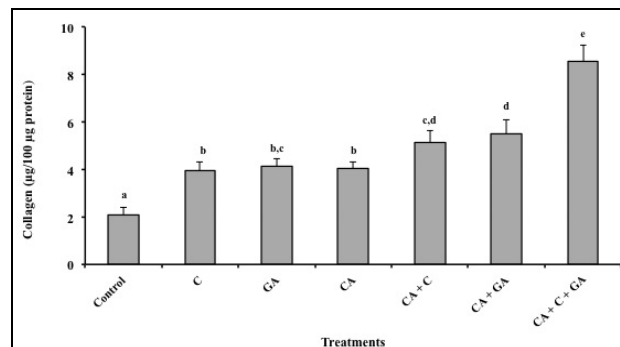


Fig. 1: Stimulation of collagen synthesis using CA (100 $\mu\text{g}/\text{ml}$), vitamin C (25 $\mu\text{g}/\text{ml}$), glycolic acid (10 $\mu\text{g}/\text{ml}$) and their mixtures.

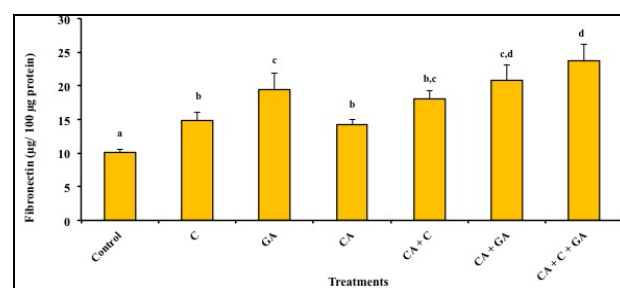


Fig. 2: Stimulation of fibronectin synthesis using CA (100 $\mu\text{g}/\text{ml}$), vitamin C (25 $\mu\text{g}/\text{ml}$), glycolic acid (10 $\mu\text{g}/\text{ml}$) and their mixtures.

CA extract has been used in cosmetic, topical therapeutic preparation and as a potential wound healing agent (Hong *et al.*, 2005; Shetty *et al.*, 2006). Therefore, further studies were done to ascertain the effect of various treatment of CA with glycolic acid and vitamins for skin care and wound healing formulations. In this study, the stimulation of collagen synthesis was found to begin at a concentration of CA between 50 and 100 $\mu\text{g}/\text{ml}$ in the presence of 5% FBS.

Compared to the control, the synthesis of collagen and fibronectin was markedly enhanced in fibroblast cell treated with CA (Fig. 1 and 2). The fibroblast culture results of CA were consistent with those obtained previously from skin cell studies (Maquart *et al.*, 1990; Tenni *et al.*, 1988). In contrast, most researchers reported that CA extract stimulate collagen synthesis (Maquart *et al.*, 1990; Bonte *et al.*, 1994; Maquart *et al.*, 1999; Hashim *et al.*, 2011). None of them conducted any studies on the stimulation of fibronectin synthesis. Vitamin C is the best-known stimulator of collagen synthesis (Barnes *et al.*, 1975) and essential cofactor for lysyl and prolyl

hydroxylase, which are the two enzymes essential for collagen biosynthesis (Pinnel, 1985). As such in our study, vitamin C was used as a positive control as well as vitamin treatment to the cells. CA with the addition of vitamins mixture A, C and E did not improve the augmented synthesis brought by the addition of vitamin C alone. This suggested that supplementing CA extract with vitamin C alone was sufficient to augment the extracellular protein synthesis, particularly for collagen. However, the addition of vitamin A, C and E to CA might be useful for other beneficial effects in cosmetic preparation and wound healing formulations that we had not tested for, such as free radical induced skin damages.

The wound healing activity of extract from CA were confirmed by several other investigators in wound healing studies on rats (Shetty *et al.*, 2006; Bonte *et al.*, 1994; Maquart *et al.*, 1999). In other studies, it was reported that the healing properties of CA are attributed to the presence of asiaticoside which is one of the active components in CA extract (Shukla *et al.*, 1999; Hashim *et al.*, 2011). In animal model, the asiaticoside speeds up wound healing by stimulating the production of peptidic hydroxyproline content, collagen, angiogenesis, epithelization and improve tensile strength (Bonte *et al.*, 1994; Maquart *et al.*, 1999). The same effect of stimulating the production of the peptidic hydroxyproline to increase the collagen synthesis in wounds was also demonstrated by the presence of asiatic acid and madecassic acid in CA extract (Bonte *et al.*, 1994; Maquart *et al.*, 1999). As asiaticoside stimulates the production of antioxidant content at the beginning of healing process, this activity may contribute a significant factor to the healing properties (Shukla *et al.*, 1999). Therefore, further study at the cellular level could be carried out on the active components of CA.

In this evaluation, the glycolic acid study confirmed previous reports which showed that glycolic acid stimulated the synthesis of collagen in culture fibroblast cells (Moy, 1996) and tretinoin stimulated collagen synthesis in photo aged human and hairless mouse skin (Kligman, 1996). The glycolic acid and vitamin A stimulated fibroblasts without causing significant damage to the cells and connective tissue as the concentration used does not cause toxic effect to the cells. The effect on collagen and fibronectin production has a direct stimulatory effect and not a specific damage effect on fibroblast. The fibroblast is a critical component of granulation tissue and is responsible for the production of collagen, elastin, fibronectin, glycosaminoglycans and proteases. Fibronectin is a cell-surface protein that enables cells to interact with the intracellular matrix. Therefore the synthesis of collagen and fibronectin is very important in the proliferation phase and in the wound healing process (Moon, 1999).

CONCLUSION

CA extract, glycolic acid, vitamin A, E and C demonstrated stimulatory effect on both extracellular matrix synthesis of collagen and fibronectin in the *in vitro* studies on human foreskin fibroblasts. The complimentary addition of glycolic acid, vitamin A, C and E further augmented the extracellular protein synthesis. The level of collagen in the mixture containing CA extract, vitamin C and glycolic acid had the highest stimulation of collagen (4-fold) and fibronectin (2.5-fold) compared to other treatments. These results could explain their significant benefits as ingredients in skin care and therapeutic products formulation. By working with the optimum concentration of CA extract, glycolic acid and vitamins, we are stimulating the fibroblasts without causing significant damage to cells and connective tissues.

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