Korean J. Ginseng Sci.
Vol. 14, No. 2, 265—273 (1990)

Application of Ginseng Saponin on Cutaneous Disorder, Hyperkeratosis

Hyeyoung Kim(Jun), Sung-Ha Jin and Shin Il Kim
Division of Biochemical Pharmacology, Korea Ginseng & Tobacco Research Institute, Taejon 305-345, Korea

Introduction

Skin is the largest organ of the body and an active site of the biotransformation of a variety of endogenous substances such as steroid hormones, cholesterol, and foreign xenobiotics including drugs and carcinogens (Bickers and Kappas, 1980). The two most important components of skin are the epidermis and the dermis. The epidermis is the surface layer and is essentially a stratified squamous epithelum with a high metabolic rate in direct and continuous contact with the environment (Ackerman, 1978). The use of ginseng for skin-related discorders was very few. The first therapeutic use of ginseng for cutaneous disorder was performed by Russian scientists, Igumnov and Burtkovski (1954); 10% alcohol extract of ginseng roots cured eczema, removing itching but treatment was not consistent. Later, Gredoire (1971) used ginseng root as a component of cold cream to control eczema and wrinkles. In 1980's, antiinflammatory effect of ginseng saponin ointment (Jin and Kim, 1982) and the effects of ginseng powder and ginseng saponin on Candida albicans (Nam and Kim, 1982) have been reported. For cosmetic field, ginseng extract showed certain activating action on sagging and wrinkled skin (Rovesti, 1971) and favorable effects on aging skin, cutaneous elasticity, moisturizing activity on stratum corneum of the epidermis and circulatory derangements as liposome forms (Curri *et al.*, 1986; Gezzi *et al.*, 1986; Bombardelli *et al.*, 1988). Estrone, estradiol, and estriol were isolated from *Panax ginseng* root as cutaneous bioactivation components (Anguelakova *et al.*, 1972) and use of topical ginseng appears to have an estrogen-like effect on genital tissues, contributing to postmenopausal bleeding (Hopkins *et al.*, 1988). These kinds of skin-related studies necessitate rapid and complete separation techniques of epidermis from dermis with a minumum of chemical or thermal exposure, preservation of the *in vivo* levels of macromolecules and small molecules, and reducing concomitant damage to enzymes.

Present study was divided into two studies; Determination of rapid epidermal separation method (study 1) and Ginseng effects on hyperkeratosis (study 2). Study 1 was designed to determine a rapid epidermal separation method in neonatal rat skin by light microscopic appearance and by comparing enzyme activities in epidermis which was separated by heating procedure or microwave irradiation. Study 2 was conducted to investigate the active components in Korean red ginseng on hyperkeratosis through *in vivo* study of fractionated red ginseng extract and to determine the mechanism of action of ginseng by the analysis of epidermal enzyme activities and lipid contents.

Materials and Methods

Materials

n-Hexadecane, triethanolamine, hematoxylin, eosin, lactate dehydrogenase test kit, isocitrate dehydrogenase test kit, glucose-6-phosphate dehydrogenase test kit were obtained from Sigma Chemical Co.(St. Louis, MO). Triglyceride test kit and total cholesterol test kit from Iatron Laboratories, Inc. (Tokyo, Japan) and non-esterified fatty acid test kit

from Nissui Pharmaceutical Co. (Tokyo, Japan) were used. DL-(1-^{14}C) ornithine hydrochloride (58 mCi/nmole) were purchased from Amersham International plc(Buckinghamshire, England). All other reagents were of guaranteed reagent grade commercially available.

1. Determination of epidermal separation method

Animals

Sprague-Dawley rats were obtained from the animal breeding room of the Korea Ginseng and Tobacco Research Institute (KGTRI). Female neonatal rats born *in situ* were allowed to suckle for one day after birth. The neonatal rats were sacrificed and whole skin was excised.

Epidermal-dermal separation procedures

Epidermal separations were performed by using chemicals (dithiothreitol, acetic acid), heating or microwave irradiation. Each method was occurred by following method: Dithiothreitol (DDT) method by Dixit *et al.* (1983); Acetic acid method by Slaga *et al.* (1973); Heating prodedure, Connor and Lowe (1983); Microwave irradiation, Mufson *et al.* (1977).

Histological examination (LM)

To monitor the separation techniques, whole skin, epidermal scrapings and dermis were subject to histological examination after exposure to separation procedures. Tissue was fixed in 10% neutral formalin and dehydrated with a graded series of alcohols before embedding in paraffin. Sections 5μ in thickness were stained with hematoxylin and eosin.

Enzymatic determinations

For enzyme assay, each epidermis which was separated by heating method or microwave method, was homogenized in ice-cold 50 mM potassium phosphate buffer, pH7.2, containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA at 0-4°C. Homogenates were centrifuged at 700 × g for 10 min at 0°C and supernatant was used for the assays of lactic dehydrogenase (LDH) and isocitrate dehydrogenase (ICD). The epidermis from 4 animals were pooled and enzyme activities in soluble epidermal extracts were measured by commercial test kits (Sigma Chemical Co., St. Louis, MO). The supernatant fraction obtained after further centrifugation at 30,000 × g for 30 min at 0°C was used for estimation of ornithine decarboxylase (ODC) activity. ODC activity was determned by measuring the release of $^{14}CO_2$ from DL-(1-^{14}C) ornithine, essentially as described by O' Brien *et al.* (1975). Protein concentration in epidermal extract was measured by the method of Lowry *et al.* (1951).

2. Effect of red ginseng components on hyperkeratosis

Ginseng sample preparation

Ginseng saponin was prepared from water-saturated butanol fraction of powdered Korean red ginseng by the method of Sanada *et al.* (1974). For animal treatment, 2% ginseng saponin solution was prepared in 50% ethanol solution (vehicle). 28.6% red ginseng extract which contains 2% saponin was also used. Combination of ethyl ether and ethyl acetate fraction (0.6%) and water-soluble fraction (28%) was made from red ginseng extract and used for animal study.

Animal treatment

Female Hartley guinea pig, 450-500g, were acclimated under conventional laboratory conditions with fresh vegetables for 10 days. One day prior to experiment, dorsal hairs, 8 cm^2, were shaved. 0.5 m*l* of four kinds of ginseng samples, saponin, lipid fraction, extract excluding lipids (water-soluble fraction), red ginseng extract were topically applied to dorsal skin of each guinea pig 1 hr before the application of n-hexadecane (2 m*l*/kg B.W.) every day for 10 days. n-Hexadecane was administered every other day during experimental period. Excisional biopsies were taken from dorsal skins for LM observation, SEM examination and epidermis separation.

Scanning electron microscopy (SEM)

Biopsy materials were pre-fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetraoxide. They were dehydrated with graded alcohols, transferred to isoamyl acetate, dried, mounted, gold-coated and viewed through SEM at 15 kv.

Epidermal enzyme activities and lipid contents

Fig. 1. Light microscoph of newborn rat skin. C, cornified cells; E, epidermis; D, dermis (×100).

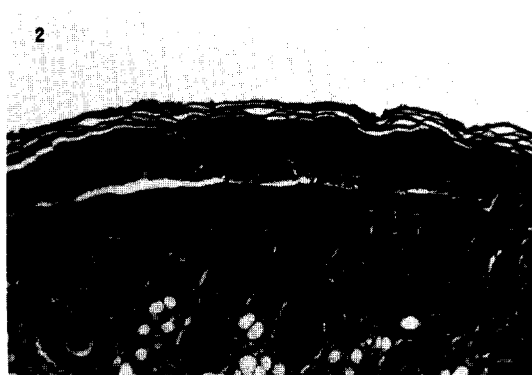

Fig. 2. Whole skin after all separation procedures, but prior to scraping with a scalpel. Epidermal-dermal junction (arrows) was separated (×100).

Epidermis was separated by heating procedure (i.e., immersion in 55 °C water for 30 sec, followed by immersion in 0-4 °C water) (Connor and Lowe, 1983) and minced (< 1 mm^3), homogenized in 0.1M triethanolamine buffer (pH 7.6) and centrifuged. Soluble epidermal extract was collected for enzyme analysis and precipitate was dissolved, homogenized in the mixture of chloroform and methanol (2:1, v/v), filtered with glass fiber filter and dried under N_2 gas. Total lipid was measured by weighing method (Christie, 1982). Residue was used for the analysis of triglyceride, total cholesterol and free fatty acid. Epidermal enzymes, lactate dehydrogenase (LDH), isocitrate dehydrogenase (ICD), glucose-6-phosphate dehydrogenase (G6PDH) activities and the contents of triglyceride, total cholesterol and free fatty acids were measured using commercial test kits. Protein concentration was determined by the method of Lowry *et al.* (1951).

Results and Discussion

Determination of epidermal separation method

Mammalian skin contains two major structural components: the outer epidermis and the inner underlying dermis. The epidermis is an actively replicating, compact and stratified squamous epithelium, whereas the dermis has a relatively loose structure consisting of collagen fibers embedded in a ground substrance rich in glycosyl aminoglycans (Bickers *et al.*, 1982). Epidermis is tightly adherent to the dermis and separation without concomitant damage to enzymes, seemed difficult to achieve. In this study, neonatal rat skin was used because it is frequently used in xenobiotic metabolism studies. Neonatal rats take advantages; it is hairless, which precludes the need of shaving, much less resistant to homogenation procedures and minimal environmental pollutant-induced alterations in drug metabolizing enzymes. Fig. 1 shows a 1 day-old newborn rat skin. The whole skin consisted of epidermis(E) and dermis(D). The outermost cornified cells(C) of the epidermis appear anucleated and filled with a fibrous material. Epidermal-dermal junction was separated after all procedures (Fig. 2). After 24h of immersion in 1% acetic acid at 5 °C, cornified cells were cleft from epidermis (Fig. 3). All separation procedures used in the study showed a complete separation of epidermis from dermis (Fig. 4). In general, biochemical determinations on epidermis require harvesting tissue from a large number of animals, and there is thus a corresponding large expenditure of time. With this point of view, heating procedure (55 °C, 30 sec) and microwave irradiation (10 sec) appear favorable in the studies using epidermis and/or dermis.

Lactate dehydrogenase (LDH) is one of the most active enzymes in the skin of primates (Im and Adachi, 1966) and 40 to 70% of glucose is converted to

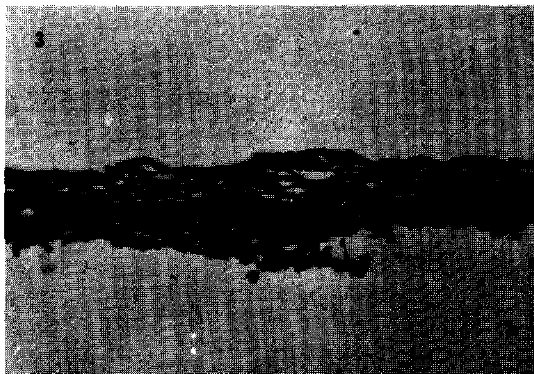

Fig. 3. The separated cornified cells after 24 h of immersion in 1% acetic acid at 5 °C (×100).

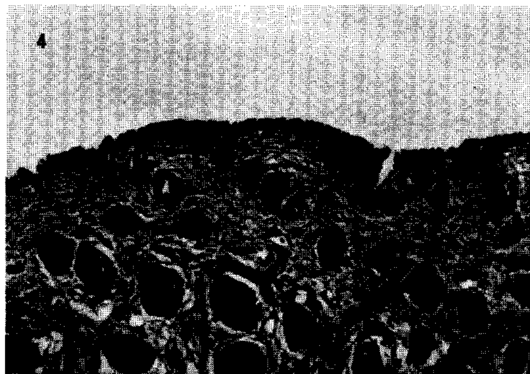

Fig. 4. Epidermis-free surface of the dermis, demonstrating a complete separation of epidermis after scraping (×100).

lactic acid in human and rat skin (Freinkel, 1960; Pomerantz and Asbornsen, 1961). In contrast to most tissues, epidermis converts most of the glucose to lactic acid even in the presence of oxygen (Halprin and Ohkawara, 1966). Isocitrate dehydrogenase (ICD), which belongs to aerobic Krebs cycle and is one of the sources of NADPH in the cell, are highly active in human epidermis (Cruiokshank *et al.*, 1958). As shown in Table 1, epidermal separation method, either microwave irradiation or heating, had no effect on LDH and ICD activities. However, ornithine decarboxylase (ODC) activity in epidermis was significantly different by the separation procedures. ODC is present in all nucleated cells and is the rate-limiting enzyme for synthesis of polyamines (Janne *et al.*, 1978). ODC actitity is normally low in the epidermis but may be elevated during proliferation and can be induced by trophic hormones, carcinogens, tumor promoters, mitogens and by wounding (Conner *et al.*, 1985). In this study, ODC activity was higher in epidermis separated by microwave than by heating. It is a possible reason for that ornithine decarboxylase activity can change markedly and rapidly in response to extracellular signals. Similar observation was reported by Byus *et al.* (1988) in which ODC activity was increased in cultured cells following a transient exposure to microwave field. As a tentative conclusion, heat separation seems to be good for the assay of enzymes and saving time.

Table 1. Comparison of enzyme activities in neonatal rat epidermis separated by microwave irradiation or heat procedure.

Separation procedure	LDH	ICD	ODC
Microwave	5.300±0.210	1.200±0.155	0.4644±0.048
Heat	5.818±0.252	1.636±0.176	0.2339±0.036*

Data represent mean±SE of four animals.
Statistical significant was assessed by student's t-test, *; P<0.005.
Epidermal enzymes are expressed as Sigma unit/μg protein for LDH (lactate dehydrogenase) and ICD (isocitrate dehydrogenase) and nmoles CO_2/30 min/mg protein for ODC (ornithine decarboxylase).

Effect of red ginseng components on hyperkeratosis

Fig. 5 shows the normal skin of guinea pig: the stratum corneum appears as a poorly stained layer of dead and desiccated cells that can flake easily from the epidermis. Vehicle group showed similar histological structure as normal skin (Fig. 6). Hexadecane (HD) induced epidermal hyperplasia, cellular hypertrophy and hyperkeratosis (Fig. 7). The epidermis is divided into four easily recognizable layers; stratum basale(sb), stratum spinosum(ss), stratum granulosum(sg) and stratum corneum(sc). Where the stratum corneum is particularly thick, there is a thin transitional zone between the stratum

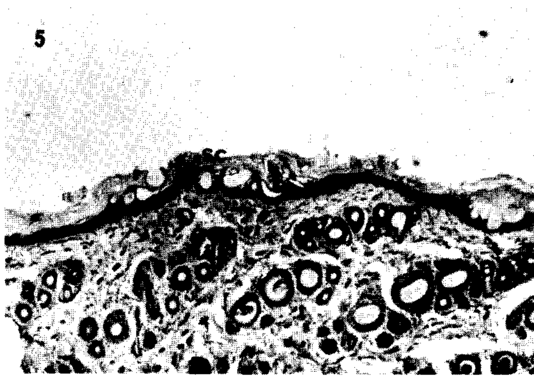

Fig. 5. Light microscoph of normal skin of guinea pig (×40).

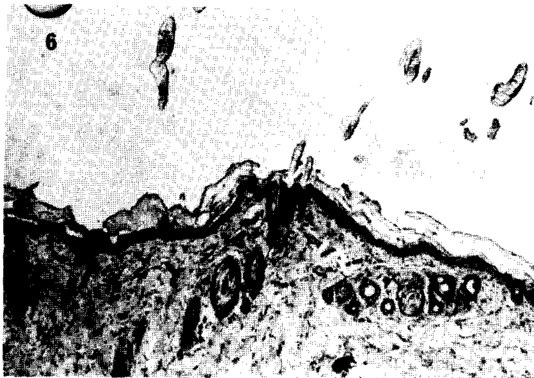

Fig. 6. Whole skin of guinea pig treated with vehicle shows similar histological structure as normal skin (×40).

granulosum and the stratum corneum known as the stratum licidum(sl). Hexadecane treated skin had stratum lucidum(sl, an arrow) which was found neither in normal nor in vehicle-treated skin. Hexadecane is reported to induce hyperplasia and hyperkeratinization to mammalian skin by increasing epidermal mitotic activity and provids a model for studying the keratinization process (Krk and Hoekstra, 1964). Topical application of red ginseng saponin (20 mg/kg B.W.) reduced the thickness of epidermis and the numbers of horny cell layers of stratum corneum induced by hexadecane (Fig. 8). However, the type of keratin was still laminated form (arrows) while normal skin has weave type of keratin as shown in Fig. 5 and Fig. 6. Treatment of red gin-

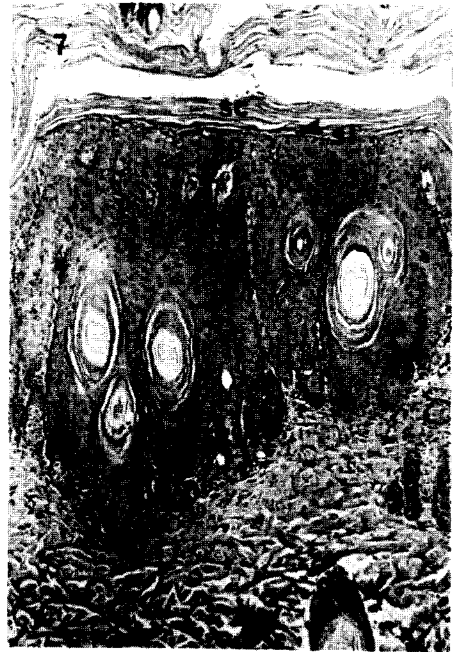

Fig. 7. Hexadecane treatment stimulates repreated mitosis in stratum basale (sb), resulting in epidermal hyperplasia and hyperkeratosis (×40).

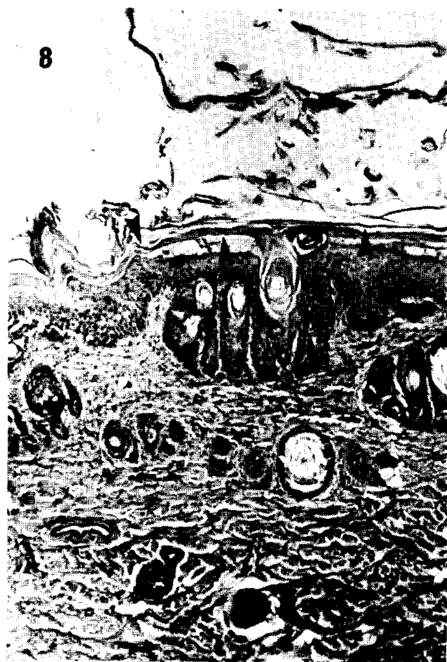

Fig. 8. Topical application of red ginseng saponin with hexadecane (HD) reduces the thickness of epidermis and the numbers of horny cell layers (×40).

Fig. 9. Light micrograph of whole skin treated with red ginseng lipid fraction and HD. Similar hyperkeratosis is seen as hexadecane treated skin (×40).

Fig. 11. Treatment of red ginseng extract with hexadecane (HD) demonstrates hyperkeratosis (×40).

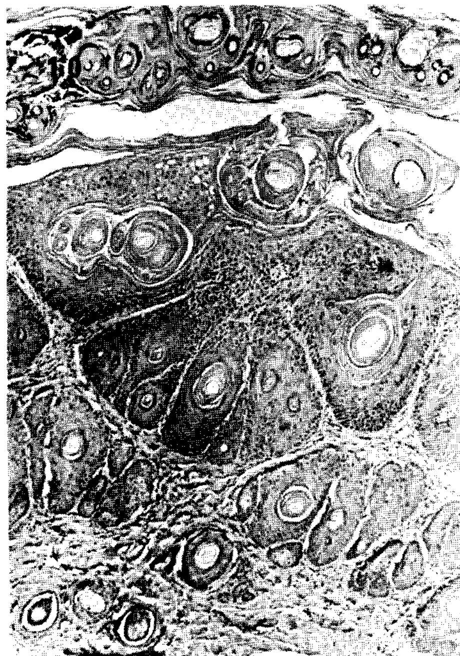

Fig. 10. Whole skin of animals treated with red ginseng extract excluding lipids and HD (×40).

seng lipid fraction (Fig. 9), red ginseng extract excluding lipids (Fig. 10) and red ginseng extract (Fig. 11) with hexadecane demonstrated similar epidermal hyperplasia and hyperkeratosis as hexadecane-treated group (Fig. 7). Kirk and Hoekstra (1964) reported that n-hexadecane-treated hyperkeratosis with epidermal proliferation in guinea pig skin was similar to that seen in ichthyosis vulgaris in human. From this point of view, red ginseng saponin may be therapeutically valuable for some cutaneous disorders caused by epidermal hyperplasia.

A high-power SEM view of the stratum corneum surface of hexadecane treated skin showed many desquamating horny cells protruded around the base of hair shaft (Fig. 12). Treatment of red ginseng saponin gave less protruding horny cells and well-arranged stratum corneum surface (Fig. 13). Flower like appearance of horny cells around hair shaft was demonstrated in red ginseng extract-treated skin (Fig. 14).

Hexadecane treatment significantly increased epidermal enzyme activities such as lactate dehy-

Fig. 12. SEM view of hexadecane-treated skin shows many desquamating horny cells protruded around the base of hair shaft (×260).

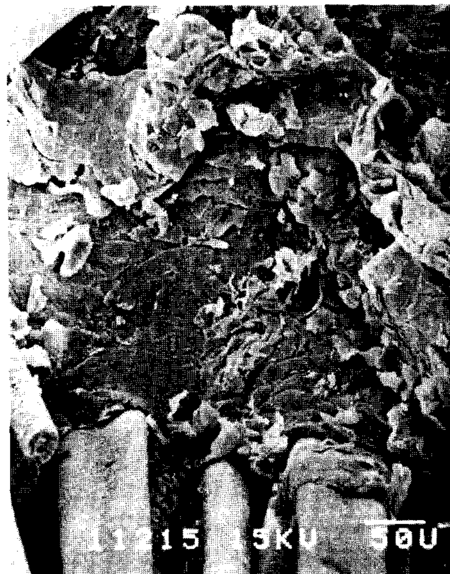

Fig. 13. The surface of dorsal skin treated with red ginseng saponin and HD shows less protruding horny cells and well-arranged stratum corneum (×260).

drogenase (LDH), isocitrate dehydrogenase (ICD) and glucose-6-phosphate dehydrogenase (G6PDH) (Fig. 15). LDH is quite active in mammalian epidermis (Im and Adachi, 1966) and reflects epidermal metabolic activity. ICD belongs to Krebs cycle which is highly active in skin as in other mammalian tissues (Cruiokshank *et al.*, 1958). Glucose-6-phosphate dehydrogenase is a key enzyme of the hexose monophosphate shunt pathway, in which ribose and NADPH are produced and used for cell metabolism. The activity of this enzyme is correlated to epidermal cell proliferation (Ohkawara, 1968). With this regards, hexadecane produced epidermal cell proliferation possibly by activating enzyme activities. Among red ginseng samples, only saponin significantly prevented abnormally increased enzyme activities in epidermis while others had no effect.

As shown in Fig. 16, total lipid content in epidermis was significantly increased by hexadecane treatment. The order of increment was as follows: free fatty acids (5.8 times of vehicle value), cholesterol (2.5 times) and then triglyceride (1.3 times). Accumulation of lipids in epidermis might be caused by highly proliferative epidermal cells which are stimulated by hexadecane application. In late stage of keratinization, epidermal cells are somewhat re-

Fig. 14. Flower-shape appearance of horny cells was shown in the stratum corneum of guinea pig skin treated with red ginseng extract and HD (×40).

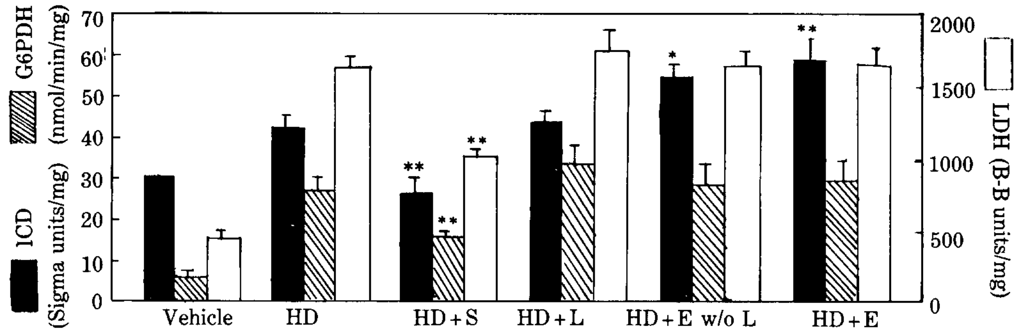

Fig. 15. Effects of red ginseng components on epidermal enzyme activities. Enzyme activities are measured in soluble epidermal extract from two pieces of dorsal skin areas (1.5 cm^2). Values are mean ± SE of 6 animals. An asterisk indicates values significantly different from hexadecane (HD)-treated animals by student's t-test, *; P<0.01, **; P<0.005, S; saponin, L; lipid, E w/o L; extract excluding lipid, E; red ginseng extract.

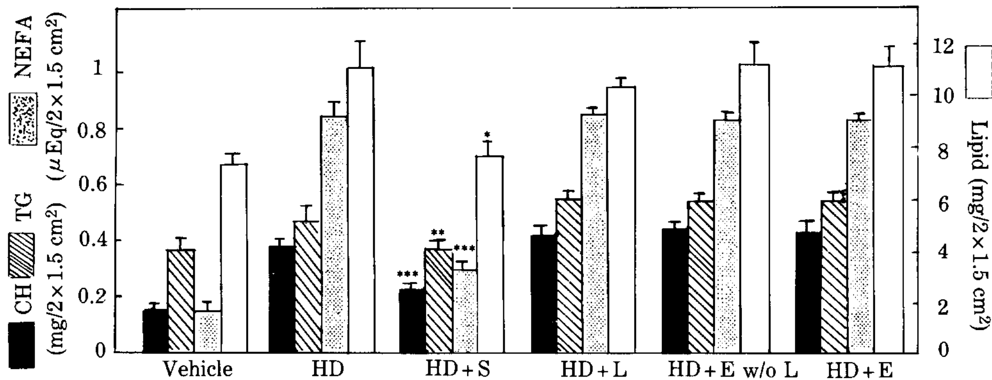

Fig. 16. Effects of red ginseng components on epidermal lipid contents. Lipid contents were determined in precipitates of epidermal homogenates from two pieces of dorsal skin areas (1.5 cm^2). Values are mean ± SE of 6 animals. An asterisk indicates values significantly different from hexadecane (HD) treated animals by student's t-test, * p<0.01, ** p<0.05, *** p<0.005 S: saponin, L: lipid, E w/o L: extract excluding lipid, E: red ginseng extract.

moved from dermis, which is their source of nutrients, and the subcellular membranes begin to disintegrate. The biologically valuable fatty acids that make up these membranes are oxidized by the remaining mitochondria to yield the adenosine triphosphate required to complete keratinization process. The leftover fatty acids then make their small contribution to the surface lipid (Nicolaides, 1974). This report could explain large amounts of free fatty acids in hexadecane-treated epidermis *in vivo*. Topical application of red ginseng components showed similar effects on epidermal lipids as their effects on epidermal enzyme activities. Only ginseng saponin significantly reduced the contents of epidermal lipids induced by hexadecane; total lipid and triglyceride levels were similar to the levels before the treatment of hexadecane.

It is thought that red ginseng saponin has preventive effect on experimentally induced hyperkeratosis possibly by controlling the enzyme activities involved in cellular metabolism and resulting in reduced amounts of abnormal epidermal lipids.

References

1. Ackerman, A.B.: Histologic Diagnosis of Inflammatory Skin Diseases (Ackerman, B.A.(ed.), Lea & Febiger, Philadelphia), p.4 (1978).

2. Anguelakova, M., Rovesti, P. and Colombo, E.: *Parf. Cosm. Sav. France,* **2,** 555 (1972).
3. Bickers, D.R. and Kappas, A.: Extrahepatic Metabolism of Drugs and Foreign Compounds (Gram, T.E. (ed.), Spectrum Publications, New York), p.295 (1980).
4. Bickers, D.R., Dutta-Choudhury, T. and Mukhtar, H.: *Mol. Pharmacol.,* **21,** 239 (1982).
5. Bombardelli, E., Curri, S.B. and Gariboldi, P.L.: Proc. 5th Intl. Ginseng Sym. Seoul, 11-17 (1988).
6. Byus, C.V., Kartun, K., Pieper, S. and Adey, W.R.: *Cancer Res.,* **48,** 4222 (1988).
7. Christie, W.W.: Lipid Analysis (Christie, W.W. ed.), (Pergamon Press, NY) p.17 (1982).
8. Connor, M.J. and Lowe, N.J: *Cancer Res.,* **43,** 5174 (1983).
9. Connor, M.J., Ashton, R.E. and Lowe, N.J.: *J. Pharm. Exp. Ther.,* **237,** 31 (1985).
10. Cruickshank, C.N.D., Hershey, F.B. and Lewis, C.: *J. Invest. Dermatol.,* **39,** 33 (1958).
11. Curri, S.B., Gezz, A.,Longhi, M.G. and Castelpietra, R.: *Fitoterapia,* **57,** 217 (1986).
12. Dixit, R., Mukhtar, H. and Bickers, D.R.: *J. Invest. Dermatol.,* **81,** 369-375.
13. Freinkel, R.K.: *J. Invest. Derm.,* **34,** 37-45.
14. Gezzi, A., Longhi, M.G., Mazzoleni, R. and Curri, S.B.: *Fitoterapia,* **57,** 15 (1986).
15. Gredoire, N.: Abstracts of Korean Ginseng Studies (The Research Institute, Office of Monopoly, Republic of Korea eds.), (Sam-Hwa Printing Co., Seoul), p.168 (1971).
16. Halprin, K.M. and Ohkawara, A.: *J. Invest. Dermatol,* **47,** 222 (1966).
17. Hopkins, M.P., Androff, L. and Benninghoff, A.S.: *Am. J. Obstet. Gynecol.,* **159,** 1121 (1988).
18. Im, M.J.C. and Adachi, K.: *J. Invest. Dermatol.,* **47,** 286 (1966).
19. Igumnov, A.N. and Burtkovski, I.Y.: Abstracts of Korean Ginseng Studies (The Research Institute, Office of Monopoly, Republic of Korea eds.), (Sam-Hwa Printing Co., Seoul), p.169.
20. Janne, J., Poso, H. and Raina, A.: *Biochem. Biophys. Acta.,* **473,** 241 (1978).
21. Jun, J.W. and Kim, J.H.: *Korean J. Dermatol.,* **20,** 375 (1982).
22. Kirk, D.L. and Hoekstra, W.G.: *J. Invest. Derm.,* **43,** 93 (1964).
23. Lonry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: *J. Biol. Chem.,* **193,** 265-275.
24. Mufson, R.A., De Young, L.M. and Boutwell, R.K.: *J. Invest. Dermatol.,* **69,** 547 (1977).
25. Nam, K.H. and Kim, S.N.: *Korean J. Dermatol.,* **20,** 83-88 (1982).
26. Nicholaides, N.: *Science,* **186,** 19 (1974).
27. O'Brien, T.G., Simsiman, R.C. and Boutwell, R.K.: *Cancer Res.,* **35,** 1662-1670.
28. Ohkawara, A.: *Japanese J. Derm.* (Series B), **78,** 493 (1968).
29. Rovesti, P.: *Aromi Saponi Cosmetici Aerosol,* **53,** 203 (1971).
30. Pomerantz, S.H. and Asbornsen, M.T.: *Arch. Biochem.,* **93,** 147 (1961).
31. Sanada, S., Kondo, N., Shoji, J., Tanaka, O. and Shibata, S.: *Chem. Pharm. Bull.,* **22,** 421 (1974).
32. Slaga, T.J., Bowden, G.T., Shapas, B.G. and Boutwell, R.K.: *Cancer Res.,* **33,** 769 (1973).