THE EFFECT OF VITAMIN E ON BASIC FIBROBLAST GROWTH FACTOR LEVEL IN HUMAN FIBROBLAST CELL CULTURE.

BY

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IV ABBREVIATIONS

Abbreviation	
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
TRF	tocotrienol rich fraction
CO_2	carbon dioxide
Er:YAG	erbium:yttrium aluminium garnet
TGF-β1	transforming growth factor beta one.
Atm	atmosphere
ELISA	enzyme-linked immunosorbent assay.
IL-1	interleukin-1
TNF –α	tumor necrosis factor – alpha.
mRNA	messenger ribonucleic acid
DNA	deoxyribonucleic acid
DMEM	dulbecco's modified eagle's medium
FBS	fetal bovine serum
DPBS	dulbecco's phosphate-buffered saline
DMSO	dimethyl sulfoxide.

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VII ABSTRAK

Keluaran kosmetic yang mengandungi vitamin E masih lagi belum dibuktikan keberkesanannya dalam merawat parut. Oleh kerana vitamin E adalah anti oksidan larut lemak, maka ia telah dikatakan dapat mempercepatkan penyembuhan luka dan memperbaiki parut. Tocotrienol tergolong dalam kelas analog vitamin E. Walaupun mekanisma serapan dalam badan adalah lebih kurang sama untuk semua analog vitamin E, Tocotrienol adalah lebih banyak dipecahkan berbanding Tocopherol.

Faktor Pertumbuhan 'Basic Fibroblast' (BFGF) menggalakkan pertumbuhan salur darah dan dapat mengawal atur penghasilan kolagen yang berlebihan dimana ia berkemampuan untuk membentuk kolagen yang lebih baik semasa penyembuhan luka. Profail faktor-faktor pertumbuhan sesuatu luka boleh diubah sama ada dengan menambah atau menyekat tindakannya. Penyembuhan luka yang tidak normal berkemungkinan timbul dari sesetengah pengeluran faktor pertumbuhan setempat yang berlebihan atau berkurangan. Justeru dengan itu kita berkebolehan memanipulasikan proses penyembuhan luka.

Kajian ini bertujuan untuk menguji keberkesanan 'Tocotrienol Rich Fraction' (TRF) dalam mengubah tahap kepekatan bFGF di dalam sel fibroblas manusia. Tujuan kajian ini juga untuk menentukan tahap kepekatan bFGF yang dihasilkan dari segi perbezaan masa dan perbezaan kepekatan TRF. Melalui model 'in vitro', sel fibroblas manusia yang normal telah dibiakkan didalam satu peratus serum lembu dan dirawat dengan 0, 30, 60, 100, 120, 180, 200 dan 240μg/ml 'Tocotrienol Rich Fraction' selama 3, 24, 48 dan 72 jam. Kesemua

sel yang dikaji diambil dari kultur tahap 'passage' lima hingga ke lapan, yang dibiakkan di dalam 24 petak-piring pada kepadatan 2 x 10^4 sel dalam satu milileter. Tahap kepekatan bFGF didalam supernatan ditentukan oleh kaedah 'Enzymed-Linked Immunosorbant Assay'(ELISA).

Kajian ini telah membuktikan bahawa TRF berjaya meningkatkan kepekatan bFGF. Penghasilan bFGF adalah maksimum dalam tempoh 24 jam pertama pembiakan sel fibroblas. Lebih banyak kepekatan vitamin E yang ditambah, lebih banyak pula penghasilan bFGF tetapi peningkatan tahap bFGF diantara kepekatan TRF yang berbeza adalah tidak ketara secara statistiknya. Walaubagaimanapun, semakin tinggi kepekatan vitamin E, semakin banyak sel fibroblas yang mati.

Secara kesimpulannya, tahap pengeluaran bFGF oleh sel fibroblast dapat di rangsang oleh kepekatan TRF yang berbeza-beza. Kesan TRF ke atas pembiakan sel adalah bergantung pada kepekatannya dimana pada suatu tahap kepekatan tertentu ia dapat membunuh sel. Dengan meningkatkan bFGF kita berkemungkinan mengurangkan kejadian penyembuhan luka yang tidak normal secara menghalang pembentukan kolagen berlebihan disamping meningkatkan pemecahan kolagen.

VIII ABSTRACT

Cosmetic products that contain vitamin E have not been proven effective in the treatment of scars. Since vitamin E is a major lipid soluble antioxidant in skin, it has been thought that it can speed healing and improve the cosmetic outcome of wounds. Tocotrienol is a class of vitamin E analogs. Although the absorption mechanisms are essentially the same for all vitamin E analogs, tocotrienols are degraded to a greater extent than tocopherols.

Basic fibroblast growth factor (bFGF) is angiogenic and effective in down-regulating excess collagen production suggesting a potential role in collagen remodeling during wound healing. It is possible to alter the growth factor profile of a wound either by adding or by blocking the actions of growth factors. Aberrant wound healing may arise from a local overproduction or insufficiency of certain growth factors. Hence we may be able to manipulate the process of wound healing.

The purpose of this study is to evaluate the effectiveness of Tocotrienol Rich Fraction (TRF) in altering the level of basic fibroblast growth factor in human fibroblasts. We also undertake to determine the difference of bFGF level production according to time and various concentration of TRF in this study. In this *in vitro* model, normal human fibroblasts were propagated in one percent bovine serum and treated with 0, 30, 60, 100, 120, 180, 200 and 240μg/ml Tocotrienol Rich Fraction for 3, 24, 48 and 72 hours. Cells were used from 5^{th} to 8^{th} passage and seeded on 24–well plate trays at a concentration of 6 x 10^4 cells per

milliliter. Levels of bFGF in the supernatants were determined by Enzyme-Linked Immunosorbant Assay (ELISA).

This study has demonstrated that TRF stimulated bFGF production by fibroblast. The maximum effect was evident in the first 24 hours of culture. Cells treated with higher concentrations of TRF produced higher levels of bFGF but the rise of bFGF level between the different concentrations of TRF was not statistically significant. However, the viability of fibroblasts was reduced when higher concentrations of TRF were used.

In conclusion, bFGF production by fibroblasts can be stimulated by different concentrations of TRF. The effect of TRF on cell viability is dose-dependent; higher concentration can induce cell death. Methods that increase bFGF may decrease aberrant scar formation by inhibiting excess collagen deposition as well as by increasing collagen degradation.

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Fibroblast Growth factors.

Wound healing is a complex process, which includes inflammation, angiogenesis, new tissue formation, remodeling and finally leading to at least partial reconstruction of the wounded area. Immediately after a surgical incision, the wound is covered with clotted blood containing fibrin and blood cells. The clot, within twenty four hours of its formation is invaded by neutrophils, attracted by locally released inflammatory factors. At this time, there is also mitotic activity of the basal cell layer of the epidermis. By the third day, macrophages are the most common cells in the tissue, rather than neutrophils. Macrophages appear at the site of injury within forty-eight to ninety six hours, and actively participate in the inflammatory and debridement phases (Rohrich and Robinson, 1999). Activated macrophages release various cytokines like interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), fibroblast growth factor (FGF), transforming growth factor alpha (TGF-α) and platelet derived growth factor.

Cytokines are the signaling molecules mediating both pro-inflammatory and anti-inflammatory actions (Kapoor *et al.*, 2006). Besides macrophages, keratinocytes, platelets and damaged endothelial cells also produce fibroblast growth factors. There are at least twenty-two distinct members of the fibroblast growth factor (FGF) family. Most FGFs share an internal core region of similarity (Ornitz and Itoh, 2001). The 2 originally characterized FGFs were identified by biological assay and are termed FGF-1 (acidic-FGF or aFGF) and FGF-2 (basic-FGF or bFGF). Both aFGF and bFGF share similar effects

and bind to the same FGF receptor, however bFGF is ten times more potent (Vishnu and Gregory, 2001). Fibroblast growth factor biology involves the interaction between FGF and heparin. This interaction stabilizes the FGFs to thermal denaturation and proteolysis (Ornitz and Itoh, 2001, Nugent and Iozzo, 2000). bFGF is a single-chain polypeptide composed of 146 amino acids. It was originally isolated from bovine brain and pituitary gland. It stimulates the growth of fibroblast and endothelial cells and also stimulates collagen degradation by increasing the synthesis of collagenase. It has been studied as an angiogenic factor in wound healing in humans and rats (Nakae *et al.*, 2006, Kawai *et al.*, 2005). In addition, it stimulates the production of plasminogen activator, leading to breakdown of injured tissue and basement membrane which allows the ingrowth of endothelium (Nugent and Iozzo, 2000). Oda *et al.* (2004) had surgically created mucosal defect to the depth of periosteum in rat palate. They noted remarkable granulation tissue formation and maturation of connective tissue in the wound after the edge was injected with bFGF immediately after surgery compared to the control group. Immunostaining revealed the expression of FGF receptors not only localized to the epithelium but also distributed in the connective tissue. This indicates that bFGF might not only accelerate wound healing through reepithelialization but also might affect a wide range of cells in connective tissues. Nowak *et al* (2000) also showed that normal dermal fibroblasts tend to secrete more bFGF than keloid dermal fibroblast (Nowak *et al.*, 2000).

After angiogenesis, fibroblasts remodel the granulation tissue and transform it to a scar. A scar is a relatively avascular and acellular mass of collagen which serves to restore tissue continuity, strength and function. The tensile strength of wound never reaches that of the

original, leveling off at about 80%. All wounds gain strength at approximately the same rate during the first 14 – 21 days but may diverge significantly afterwards according to the tissue involved. Collagen fibers are largely responsible for the tensile strength of wounds. Ono (2002) has demonstrated that administration of bFGF at the time of wound closure significantly increases the wound breaking strength of the skin of rabbits compared to the control group from 5 weeks after the operation. He also reported significant reduction in scarring in the wounds treated with bFGF compared with those of the control group. Histopathologic examination showed proliferation of blood vessels in the group treated with bFGF in the early bFGF treatment and re-arrangement of collagen fibers. In a study performed by Spyrou and Naylor (2002), early subcutaneous administration of bFGF on incisional wounds in rats exhibited improved architecture of the neodermis that resembled the architecture of normal uninjured skin under microscopic examination. This finding suggests a possible antiscarring effect of bFGF in wound healing.

Many factors can affect the rate of wound healing. Irradiation causes stasis and occlusion of small vessels, with a consequent decrease in wound tensile strength and total collagen deposition. Ionizing radiation probably causes permanent damage to the fibroblasts that lead to chronic ulcers. Hom *et al* (2005), using porcine skin, showed that bFGF message was decreased by 75% in irradiated skin compared with that in non-irradiated skin. They also noted intravenous administration of bFGF increased random skin flap viability and appeared to increase skin flap tolerance to irradiation. Intravenous administration of bFGF to pigs significantly reduced (50%) gastrointestinal side effect from irradiation compared to non-treated controls pigs (Hom *et al.*, 2005). Thus, intravenous bFGF may also provide

a systemic protective effect by reducing radiation enteritis. Hyperbaric oxygen has been advocated to reduce wound dehiscence, infection and delayed healing. Kang *et al.*, (2004), observed increased secretions of bFGF in cells after initial hyperbaric oxygen exposure. Repeated exposure does not appear to maintain elevated bFGF levels in the cell supernatant, suggesting a possible cellular adaptation to the hyperbaric environment. Daily hyperbaric oxygen treatment at 2.0 atmosphere selectively stimulates fibroblast proliferation but after seven days, lower or higher levels of hyperbaric oxygen do not appear to have this effect.

1.2 Fetal wound healing

Fetal dermal wound healing occurs rapidly and results in little or no scarring until the late trimester of gestation (Havlik, 1997). Growth factors play a major role in the healing processes in the fetuses. The five phases of adult healing however, are not applicable to the scarless fetal healing. Fibroblasts are the main effectors of scarless healing in fetal tissue and such healing can also occur outside the fetal environment. The fetal inflammatory response, compared with the adult consists of fewer or lesser differentiated inflammatory cells. Re-epithelialization of an adult wound requires migration of epithelial cells, whereas fetal wounds are reepithelialized by the pulling of epidermal cells across the wound by a purse-string action of actin fibres (Chen and Davidson, 2005). When comparing the fibroblast growth factor that is produced between adult and fetal fibroblasts, Lee *et al* (2000) demonstrated higher levels of acidic Fibroblast Growth Factor, basic fibroblast growth factor and TGF-β-1 expression in fetal fibroblasts. Hanasono *et al*

(2003) compared the bFGF and TGF-β1 production by fetal, keloid and normal dermal fibroblasts and noted that both keloid and fetal fibroblasts produced significantly more TFG-β1 than normal adult fibroblasts. This findings contrast with the work of Broker *et al* (1999) and Tan *et al* (1993), who concluded that adult fibroblasts demonstrated twice the relative expression of those growth factors compared to that in fetal fibroblasts.

1.3 Aberrant wound healing

Normal wound healing involves anabolic and catabolic processes which maintain equilibrium approximately six to eight weeks after the original injury. When an imbalance occurs between the anabolic and catabolic phases of the healing process, more collagen is produced than is degraded; the scar grows in all directions, elevated above the skin and remains hyperemic. Increase in collagen gene transcription or increased translation of collagen mRNA may contribute to this excess collagen deposition (Tan *et al.*, 1993, Phan *et al.*, 2002). Excessive scar tissue can be classified either as a keloid or hypertrophic scar.

1.3.1 Hypertrophic scar

Hypertrophic scars develop due to delayed epithelial closure and excessive wound tension. Hypertrophic scars are elevated above the skin surface but limited to the initial boundaries of the injury. Early skin grafting may improve the overall appearance of skin in full thickness defect. Hypertrophic scarring may occur at any age or site and tend to regress

spontaneously. It is also associated with a greater propensity for scar contracture than keloids (Miller and Nanchahal, 2005).

1.3.2 Keloids

Keloids proliferate beyond the confines of the original lesion. The natural history is usually more than three months after the wound heals and exhibits no tendency to regress and usually recur after excision. Virtually all abnormal scars are associated with trauma, tattoos, burns, injection and bites. Other local etiologic factors include wound infection, prolonged inflammatory response and a scar placement away from the relaxed skin tension lines (Rohrich and Robinson, 1999). It is clinically difficult to determine if a scar will develop into a keloid or hypertrophic scar (Hom, 2001). Scars in specific sites of the body, including the lower face, pre-sternum, pectoral area of the chest, upper back, ears, neck and deltoid are more likely to develop keloids. Individuals with scars in these high risk anatomical areas, or with history of forming keloid scars, aim to prevent further scarring by avoiding non-essential cosmetic surgery, closing all wounds with minimal tension and using pressure garments for four to six months after injury or surgery.

Several treatment modalities for the management of keloids have been described but with unsatisfying result. Keloid recurrence rate for surgery is with 45 to 100 percent (Niessen *et al.*, 1999). Therefore surgery is usually combined with other treatment modalities such as compression therapy, intralesional steroid, radiation and silicone gel application. Other additional modalities are laser excision, cryotherapy, oral colchicine therapy and interferon

therapy. Simple surgical excision, primary closure and postoperative steroid injection are the most common forms of keloid treatment (Hom, 2001). Certain authors advocate leaving a rim of keloid tissue at the perimeter to minimize recurrence. Lee *et al* (2001) showed that, 12.5% of keloid patients had recurrence after excision of the fibrous core of the keloid and leaving a shell of keloid tissue attached to the normal skin as a flap. Steroid injection acts by inhibiting fibroblast growth, which leads to collagen degradation. However the recurrence rate of intralesional steroid alone was 50 – 100% (Mafong and Ashinoff, 2000). The mechanism of action of silicone gel for the treatment of hypertrophic scars and keloids is still not known. Chang *et al* (1995) showed that hydration effect or water impermeability of the silicone gel was responsible for inhibition of fibroblast proliferation and their production of collagen (Chang *et al.*, 1995). In another study by Hanasono *et al* (2004), silicone gel increased the levels of bFGF level in fibroblast cell culture and this would be expected to reduce collagen proliferation.

Laser assisted incision have nonspecific to specific thermal tissue reactions and hence minimize scar contraction when compared to incised wounds (Poochareon and Berman, 2003). Nowak *et al* (2000) have showed that superpulsed CO_2 energy may stimulate the release of bFGF in both normal and keloid cells. Cheng *et al* (2001) discovered that combined CO_2 and Er:YAG laser treatment increases the release of bFGF, which has been shown to promote tightly organized collagen bundles and reduce the concentration of TGF-β1

1.4 Cell culture.

Cell culture occurs *in vitro* when animal or plant cells continue to grow if supplied with the appropriate nutrients and conditions. The culture process allows single cells to act as independent units, much like a microorganism, such as a bacterium or fungus. The cells are capable of dividing. They increase in size and can continue to grow until limited by some culture variable such as nutrient depletion. A homogenous population of cells derived from a single parental cell is called a clone. Therefore all cells within a clonal population are genetically identical. The major advantages of using cell culture are the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. The disadvantage is that, after a period of continuous growth, cell characteristics can change and may become quite different from those found in the starting population.

Freshly isolated cultures from mammalian tissues are known as primary cells and the cells that grow out are available for culture. This method is known as explant culture. Most primary cell cultures have limited life span but are more representative of the cell type in the tissue from which they were derived (Lehr, 2002). After a certain number of population doublings, cells will either die out or transform to become a continuous cell line. Continuous cell lines are able to multiply for extended periods of time in vitro and can be expanded and cryopreserved in cell banks. The advantages of continuous cell lines are their faster growth rate to higher cell densities, lower serum requirement, general ease of maintenance in commercially available media and ability to grow in suspension

(Freshney and Masters, 2000). Cell culture-based studies allow evaluation at the cellular level. Therefore manipulations may be performed to evaluate very specific effects.

The primary cell type of the dermis is the fibroblast, a mesenchymally derived cell that migrates through the tissue and is responsible for the synthesis and degradation of fibrous and non-fibrous connective tissue matrix protein (Freunkel and Woodley, 2001). The fibroblasts can generate from any tissues that have been wounded, such as dermis, subdermal fat, fascia, muscle and periosteum (Rovee and Mailbach, 2004). There is great interest in fibroblast regulation because of increase proliferation and synthetic activity in wound healing and during formation of hypertrophic scar. Dozens of investigations have used cell-culture techniques in an attempt to determine why specific wounds have impaired or excessive healing. The use of tissue or cell cultures for the study of keloid scar involves isolating fibroblasts from excised keloid tissue and propagating them in the laboratory using exogenous nutrients and growth factors. Most keloid scar tissue culture models involve the use of serum. Tissue culture studies are less expensive compared with studies performed using animal models.

1.5 Vitamin E

Vitamin E consists of two subfamilies, namely Tocopherols and Tocotrienols, each of which comprises four forms: Alpha (α), Beta (β), Gamma (γ) and Delta (δ). Vitamin E is the major lipid soluble, chain-breaking antioxidant in the body, protecting the integrity of membranes by inhibiting lipid peroxidation. Physiologic effects of vitamin E are

hypothesized to include antioxidant activity, support of female reproductive system function, neuromuscular function, promotion of optimal immune system function, protection of skin from radiation and excessive sunlight and acceleration of wound healing of specific types of wounds (Gray, 2003). Frequently, the term vitamin E is synonymously used with alpha Tocopherol. Vitamin E is essential because the body cannot synthesize its own vitamin E. Foods and supplements must provide it. Absorption of tocopherols is facilitated by concurrent intake and digestion of dietary fat, and both bile salts and pancreatic secretions are necessary for absorption of vitamin E. Tocotrienols are similar to Tocopherols except that they have an isoprenoid tail instead of a saturated phytyl tail. Tocopherols are predominantly found in corn, soybean and olive oil but Tocotrienols are rich in palm oil, rice bran and barley oils. Palm oil is the richest natural source of tocotrienols. Palm oil vitamin E extract consists largely of tocotrienols (70%) and only 30% of tocopherols.

1.5.1 Tocotrienol and antioxidant effect

Tocotrienols are powerful antioxidants. Antioxidants are compounds that prevent cell damage caused by activity of free radicals. Free radicals are reactive compounds generated during normal biological processes and their induction is accelerated by exposure to ultraviolet radiation, pollution, cigarette smoke and other environmental and biological stress factors. High levels of free radicals in the body can break down cell membranes and damage cell DNA. Free radicals cause not only ageing but also other degenerative processes such as cancer, arteriosclerosis and other chronic diseases. Tocotrienols also

have anticancer and cholesterol lowering properties that are more potent than tocopherols (Sen *et al.*, 2004). Alpha- tocopherols have been the focus of research because it is the predominant form in human and animal tissues. However in many studies, α-tocotrienol has been found to be a better antioxidant than α-tocopherols. (Osakada *et al.*, 2003, Osakada *et al.*, 2004, Mazlan *et al.*, 2006). A number reasons have been given that contribute to its higher antioxidant activity compared to α-tocopherols, including a more uniform distribution in the membrane lipid bilayer, a more efficient interaction of the chromanol ring with lipid radicals and ease of transfer are more readily between membranes than are tocopherols. (Saito *et al.*, 2004, Theriault *et al.*, 1999)

Vitamin E, as an antioxidant, has been marketed commercially and vitamin E is often added to food materials, cosmetics and pharmaceuticals. Medical professionals and lay-people have suggested vitamin E for the treatment of burns, surgical scars and other wounds but there is very little objective evidence for the use of vitamin E as a way to improve the cosmetic appearance of scars. A direct role for vitamin E in wound healing is less clear. Vitamin E may assist in wound healing through direct effects on tissue repair and regeneration and indirectly via beneficial effects on immune function. Vitamin E may help minimize the damage and potential healing of wounds from radiation source (Gray, 2003). It may have alternative effects on different types of wounds and in the presence of other nutrients (Mackay and Miller, 2003). The antioxidant membrane stabilizing effect of vitamin E also extends to the lysomal membranes, which places vitamin E in a group with glucocorticoids (Havlik, 1997). In a study done by Galeano *et al* (2001), Raxofelast (Vitamin-E analogue) reversed wound healing deficit in diabetic mice by inhibiting lipid

peroxidation, neutrophil infiltration, edema, stimulating re-epithelialization, neovascularization and proliferation of fibroblast. The beneficial effects of this vitamin-E analogue on wound healing were indicated by the increase in both the breaking strength and collagen content. Systemic vitamin E and glucocorticoids inhibit the inflammatory response and collagen synthesis, thereby possibly impeding the healing process (Mackay and Miller, 2003).

1.5.2 Vitamin E and cardiovascular diseases

Vitamin E has been focused on several studies of cardiovascular diseases due to its potent antioxidant ability in lipid environments. One of the most consistent findings in dietary research is that those who consume higher amounts of fruits and vegetables have lower rates of heart disease and stroke as well as cancer (Kline *et al.*, 2001, Mackay and Miller, 2003). Boaz *et al* (2000) had supplemented 800 IU/day of vitamin E to 97 hemodialysis patients and placebo in 99 patients. They found a 40% reduction in composite cardiovascular disease end points (myocardial infarction, peripheral vascular disease, ischemic stroke and unstable angina) in hemodialysis patients treated with high doses of vitamin E during two years of follow-up. Oral administration of vitamin E also improved the impaired endothelium-dependent vasodilatation in patients with coronary spastic angina (Motoyama T. *et al.*, 1998). In an animal study, Mishima *et al* (2003) injected intravenously different types of vitamin E isoforms in mice immediately, before and three hours after occlusion of middle cerebral artery. The results showed that α-tocotrienol, α-tocopherol and γ-tocotrienol significantly decreased the size of cerebral infarcts one day

after the middle cerebral artery occlusion. Their results suggest that those vitamin E isoforms are potent and effective agents for preventing cerebral infarction.

Topical administration of antioxidants is one approach to diminish oxidative injury. Skin is exposed to oxidative stress from a variety of environmental insults. Ozone can oxidize the lipids present in the upper layers of skin and deplete all various forms of vitamin E that were applied topically to the skin. Thiele *et al* (1997) showed that lipid peroxidation in cutaneous tissue can be attenuated by vitamin E applications. Tocopherols and tocotrienols in murine skin, applied topically or derived from diet were significantly depleted by ultraviolet response to an oxidative stress, suggesting that these vitamin E forms protect against ultraviolet-irradiation induced damage (Traber *et al.*, 1997).