ผลของวิตามินซีต่อการหายของแผลโดยทดสอบกับเซลล์ไฟโบรบลาสจากเหงือกในห้องปฏิบัติการ

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In vitro Effect of L-ascorbic acid on gingival fibroblast to regulate wound healing.

Miss Tatcha Chaitrakoonthong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Oral and Maxillofacial Surgery
Department of Oral and Maxillofacial Surgery
Faculty of Dentistry
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ทัชฌา ชัยตระกูลทอง : ผลของวิตามินซีต่อการหายของแผลโดยทดสอบกับเซลล์ไฟโบรบลาสจากเหงือกในห้องปฏิบัติการ (*In vitro* Effect of L-ascorbic acid on gingival fibroblast to regulate wound healing.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ทญ. ดร. ภัคสินี กมลรัตนกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ทญ. ดร. รัชนี อัมพรอร่ามเวทย์, 61 หน้า.

เซลล์ไฟโบรบลาสจากเหงือกมีบทบาทสำคัญต่อการหายของแผลในช่องปากจากการเคลื่อนที่ (migration) และการแบ่งตัวเพิ่มจำนวน (proliferation) นอกจากนี้ยังเป็นแหล่งสำคัญในการผลิตเมทริกซ์นอกเซลล์ (Extracellular matrix) โดยเฉพาะอย่างยิ่งคอลลาเจน วิตามินซี (L-ascorbic acid) เป็นสารที่มีหน้าที่หลายอย่างในร่างกายร่างกาย เช่น กระบวนการไฮดรอกซิเลชั่น (hydroxylation) ซึ่งจำเป็นต่อการสร้างคอลลาเจน หลายการศึกษารายงานฎ่าวิตามินซีเป็นสารสำคัญที่ช่วยในกระบวนการหายของแผล โดยการศึกษาทั้งหมดเป็นการได้รับวิตามินซีทางระบบ ปัจจุบันวิตามินซีแบบอมให้ละลายช้าๆในปากเป็นที่นิยมมากขึ้น จึงเป็นที่น่าสนใจว่าวิตามินซีเมื่อสัมผัสแผลโดยตรงอาจจะสามารถส่งเสริมการหายของแผลในช่องปาก โดยผ่านการกระตุ้นการเคลื่อนที่ เพิ่มจำนวนของเซลล์ไฟโบรบลาสได้ วัตถุประสงค์ของงานวิจัยนี้ เพื่อทดสอบคุณสมบัติของวิตามินซีที่มีต่อพฤติกรรมของเซลล์ไฟโบรบลาสจากเหงือกเมื่อสัมผัสวิตามินซีโดยตรง เพื่อประเมินการหายของแผลช่องปากในห้องปฏิบัติการ

วิธีการทดลอง เซลล์ไฟโบรบลาสจะถูกแยกจากเหงือกของอาสาสมัครที่เข้ารับการผ่าฟันคุดที่คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เซลล์ไฟโบรบลาสดังกล่าวจะถูกนำมาล้างด้วยอาหารเลี้ยงเซลล์ที่มีวิตามินซีความเข้มข้นต่างๆ 3 ครั้งต่อวัน ผลของการล้าง วิตามินซี ที่มีต่อการหายในห้องปฏิบัติการจะถูกประเมินด้วย Scratch-test assay ในส่วนของการเพิ่มจำนวนของเซลล์จะถูกวิเคราะห์ด้วย MTT assay และทดสอบการเคลื่อนที่ของเซลล์โดย transwell migration assay และการสารเมทริกซ์นอกเซลล์ ด้วย real time PCR

ผลการวิจัยพบว่าการล้างด้วยวิตามินซีเข้มข้น 10 และ 20 μg/ml กระตุ้นการเคลื่อนที่ของเซลล์ใน transwell migration assay แต่ไม่มีผลต่อการปิดของแผล และการเพิ่มจำนวนเซลล์ในห้องปฏิบัติการ ในขณะที่ความเข้มข้น 50 μg/ml ทำให้แผลปิดช้าลงและลดการเพิ่มจำนวนเซลล์อย่างมีนัยสำคัญทางสถิติ เมื่อเทียบกับกลุ่มควบคุม (ที่ระดับนัยสำคัญที่0.05) ในการทดสอบด้วย Scratch-test assay และ MTT assay ตามลำดับ นอกจากนี้ความเข้มข้นดังกล่าวเพิ่มการแสดงออกของยีน COL1 FN IL-6 และ bFGF อีกด้วย

สรุปได้ว่าในระยะแรกการล้างด้วยวิตามินซีความเข้มข้นต่ำ ช่วยกระตุ้นการเคลื่อนที่ของเซลล์ไฟโบรบลาส แต่เมื่อเวลาผ่านไปการเคลื่อนที่ดังกล่าว ไม่สามารถกระตุ้นให้เกิดการปิดของแผลให้ต่างจากกลุ่มควบคุมได้ ในขณะที่วิตามินซีความเข้มข้นสูงทำให้แผลปิดช้าลงโดยยับยั้งเพิ่มจำนวนของเซลล์ไฟโบรบลาส นอกจากนี้วิตามินซีความเข้มข้นสูงยังช่วยส่งเสริมการสร้างของแมทริกซ์นอกเซลล์ ได้แก่ คอลลาเจน ไฟโบรเนกติน ช่วยในการทำงานของเซลล์อักเสบ และช่วยกระตุ้นการทำงานของเซลล์ไฟโบรบลาส การใช้วิตามินซีความเข้มข้นต่ำนั้นมีความปลอดภัย และสามารถจ่ายให้ ผู้ป่วยหลังได้รับการผ่าตัดในช่องปากได้ แต่ควรเลือกการบริหารยาในรูปแบบและเวลาที่เหมาะสม โดยหลีกเลี่ยงการการสัมผัสวิตามินซีที่มีความเข้มข้นสูงกับแผลโดยตรง อย่างไรก็ตามข้อสรุปนี้ยังต้องการการศึกษาเพิ่มเติมทางคลินิกต่อไป

ภาควิชา	ศัลยศาสตร์	ลายมือชื่อนิสิต	....
สาขาวิชา	ศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล	ลายมือชื่อ อ.ที่ปรึกษาหลัก	....
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5875812832 : MAJOR ORAL AND MAXILLOFACIAL SURGERY
KEYWORDS: VITAMIN C / HUMAN GINGIVAL FIBROBLASTS / WOUND HEALING

TATCHA CHAITRAKOONTHONG: *In vitro* Effect of L-ascorbic acid on gingival fibroblast to regulate wound healing.. ADVISOR: PAKSINEE KAMOLRATANAKUL, D.D.S., Ph.D., CO-ADVISOR: ASSOC. PROF. RUCHANEE AMPORNARAMVETH, D.D.S., Ph.D., 61 pp.

Background and Objective: Besides closing the wound by intrinsic properties like migration and proliferation, gingival fibroblasts produce a major source of extracellular matrix especially collagen and play a major role in oral wound healing. Vitamin C or L-ascorbic acid has diverse functions in the body, including an essential role in hydroxylation reactions which is necessary for collagen formation. Whether L-ascorbic acid can promote gingival wound healing through inducing proliferation of fibroblasts is of our interest. The aim of this study is to evaluate the effect of L-ascorbic acid on gingival fibroblasts behaviors to promote wound healing in vitro.

Materials and Methods: Primary human gingival fibroblasts isolated from gingival tissue from healthy volunteers were rinsed 3 times a day with medium containing L-ascorbic acid of various concentrations. Local effect of L-ascorbic acid rinsing on in vitro wound healing was assessed by mean of Scratch-test assay. Cell migration, cell proliferation and extracellular matrix production were analyzed by transwell-migration assay, MTT assay and real-time RT-PCR respectively.

Result: Rinsing with vitamin C at concentration of 10 and 20 μg/ml accelerated fibroblast migration in transwell-migration assay but no significant effect on cell proliferation and in vitro wound closure. However, rinsing with vitamin C at higher concentration (50 μg/ml) significantly delayed wound closure comparing with the control group (p value=0.05). This data was in accordance with cell viability assessed by MTT assay demonstrating that Vitamin C at concentration above 50 μg/ml significantly reduced fibroblasts proliferation. However, real time PCR demonstrated that vitamin C at 50 μg/ml significantly increased expression of COL1, FN, IL-6 and bFGF, molecules related to wound healing.

Conclusion: Vitamin C at various concentrations differentially affect wound healing capability of gingiva fibroblast. Vitamin C is safe and can be prescribed to patients after oral surgery. However, suitable duration and appropriate drug administration method should be adjusting to maximize it benefit and further clinical study is required.

Department:	Oral and Maxillofacial Surgery	Student's Signature ....
Field of Study:	Oral and Maxillofacial Surgery	Advisor's Signature ....
Academic Year:	2017	Co-Advisor's Signature ....

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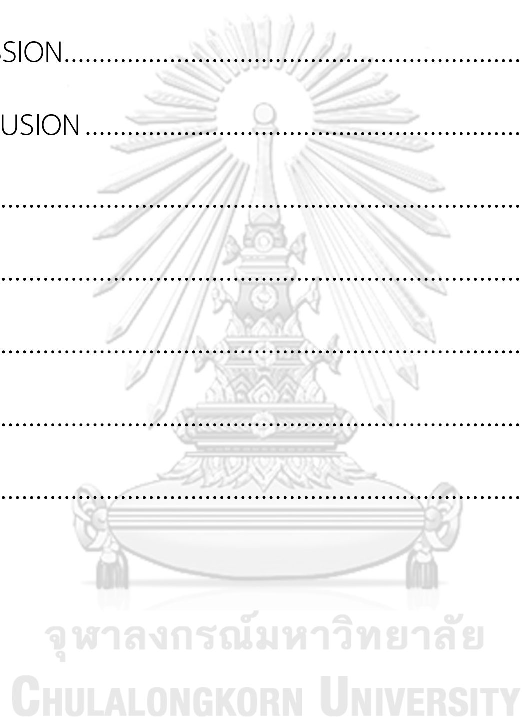

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CHAPTER 1 INTRODUCTION

1.1 Background and rational

Normal wound healing can be classified into four stages with overlapping phases: hemostasis, inflammation, proliferation and remodeling (1-4). Fibroblasts play an important role in the wound repairing process via migrating and proliferating into the wound, synthesizing cytokines and the provisional wound matrix for wound contraction and wound remodeling (5, 6).

Vitamin C is an essential nutrient for humans because Vitamin C cannot be synthesized endogenously in our body (7). Vitamin C has various biochemical functions including an essential role in hydroxylation reactions necessary for collagen and carnitine synthesis (7, 8). Many studies revealed that vitamin C has been shown to be of the great advantage in wound healing (3, 9-12). In addition, a deficiency of vitamin C impairs wound healing in animals and surgical patients (4, 13, 14). Vitamin C is different from other vitamins because it regulates the formation and maintenance of intercellular matrix and collagen. Thus, the structural integrity of every tissue and organ is dependent on this vitamin. A deficit in vitamin C can lead to structural deterioration and/or aggravation of specific disease states of any tissue, organ, or system (11). Some clinical studies even suggested to prescribe the patients with supplemented systematically vitamin C before surgery (3, 9, 11, 12). However, no clinical studies show the local effect of vitamin c on wound healing when apply

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directly to the wound. *In vitro* studies showed that vitamin C promoted collagen synthesis on human gingival fibroblast, and reduced reactive oxygen species (15). However, the mechanisms how vitamin C regulates gingival fibroblast-mediated wound healing remains unclear. Gingival fibroblasts behavior after exposing to vitamin C at short duration of time to mimic using of oral lozenge have never been studied before. Hence, in this study, we aim to investigate whether brief exposing to vitamin C will promote *in vitro* wound healing by inducing fibroblast proliferation, migration and promote fibroblast function via induction of extracellular matrix and collagen synthesis. This is the first *in vitro* study that applied intermittent rinsing of vitamin c to mimic the administration of oral lozenge on primary human gingival fibroblast culture. Detailed cellular behaviors related to wound healing in term of cell migration, proliferation, extracellular matrix production will also be clarified for the first time.

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1.2 Objective

The aim of this study is to evaluate the local effect of vitamin C on gingival fibroblast mediated wound healing *in vitro* through regulating cell migration, proliferation and extracellular matrix production.

1.3 Research Question

1. Can intermittent rinsing of vitamin c promote oral wound closure *in vitro* via inducing fibroblasts proliferation and/or migration?

2. Can intermittent rinsing of vitamin c promote extracellular matrix activity?

1.4 Hypothesis

H0: Intermittent rinsing of vitamin c cannot promote *in vitro* oral wound closure via inducing cell proliferation, cell migration and/or increasing extracellular matrix activity of gingival fibroblasts.

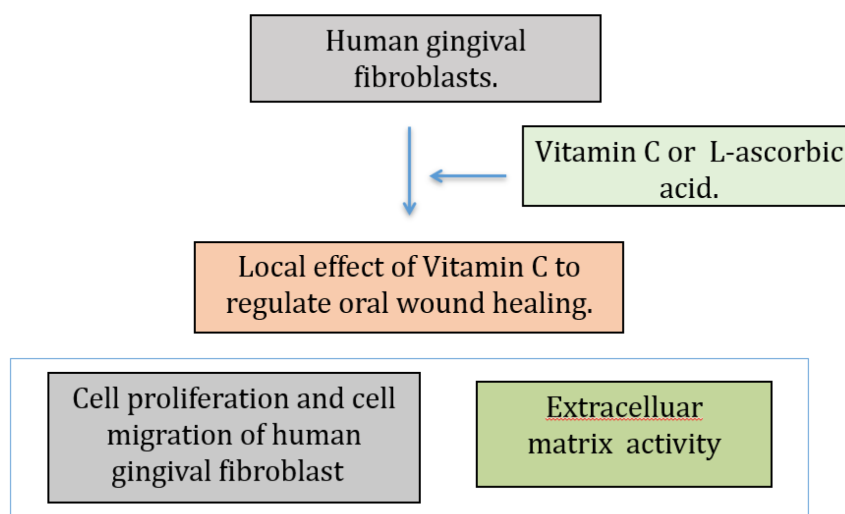
Ha: Intermittent rinsing of vitamin c promotes *in vitro* oral wound closure via inducing cell proliferation, cell migration and/or increasing extracellular matrix activity of gingival fibroblasts.

1.5 Expected benefit

This experiment will provide information about optimal concentration of vitamin C that should be used for activating fibroblast when applied locally. In addition, the detail mechanisms on cellular behaviors will also be clarified. This study will help strengthen the concept that vitamin C should probably be prescribed to patients by means of local application after oral surgery to promote gingival

wound healing. However, further clinical study still needs in order to draw the conclusion.

1.6 Conceptual Framework



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CHAPTER 2 LITERATURE REVIEW

Normal wounds healing can be classified into four distinctive stages with overlapping phases: hemostasis, inflammation, proliferation and remodeling (1-4). Oral soft tissue wound healing proceeds stages along the same principles as in other areas of the body (2). Wound healing starts with blood clot that initially seals the wound in hemostatic phase. Platelets and inflammatory cells are the first cells to arrive and providing key functions and signals which is essential for the influx of important cells like fibroblasts and endothelial cells to the site of injury. Within the first week, blood clot is almost entirely replaced with granulation tissue by the participating of fibroblasts (2, 3, 6).

Fibroblasts play an important role in the wound repairing process via migrating and proliferating into the wound, synthesizing cytokines and the provisional wound matrix including intracellular and extracellular matrix. Finally their phenotype changes for wound contraction and wound remodeling (5, 6).

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The extracellular matrix (ECM) is a composite of collagens and elastic fibers embedded in a viscoelastic gel of proteoglycans, hyaluronan, and assorted glycoproteins. These molecules regulate the biomechanical properties of tissues and their cellular phenotypes. The relative contributions of different ECM molecules vary with tissue type and result in chemical and mechanical properties appropriate to

each environment. Cells interact with specific components of the ECM. These interactions cause, to a large extent, the ability of cells to adhere to that ECM, proliferate, and migrate, as well as to survive and resist cell death(16).

Collagen is secreted by fibroblasts for repair the injured tissue. Collagen is accounting for 30% of the total protein in the body which is the most abandon protein in human. In normal tissues, collagen provides strength, integrity and structure. When tissues are disrupted after injury, collagen is required to repair the defect and restore anatomical structure and function. Adequate production of collagen in the granulation tissue can activate wound contraction which increases the speed of wound closure. When excessive collagen is deposited in the wound site, fibrosis occurs which compromised tissues function. However, if an insufficient amount of collagen is deposited, the wound is weak and prone to rupture (1, 17). Several types of collagen had been reported to involve in soft tissue wound healing at different stages (18). During the healing process, changing from granulation to remodeling phase of wound healing, content of fibronectin and type III collagen decrease. Conversely, content of type I collagen increases (19) and becomes predominant in mature wound. Type III collagen is believed to reduce type I collagen's fibril size (18). In remodeling phase, collagen production will cease, remodel and re-organize (2).

Fibronectins, secreted by fibroblasts, are a group of glycoproteins found in

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plasma(20). The fibronectins play an important role in cell adhesion to biological and material surfaces (19, 20). The adhesion of fibroblasts to fibrin requires fibronectins. Moreover, fibronectins have been found to be chemotactic for fibroblasts. These findings suggested that fibronectins are very important for fibroblast migration into the wound. In addition, fibronectins play a role in the organization of the granulation tissue matrix indicating fibronectin is a main component present during wound repairing (20).

Focal adhesion kinase (FAK) is a member of a growing family of non-receptor protein tyrosine kinases (21, 22). FAK is localized to focal adhesions (21-23) and is centrally implicated in the regulation of cell motility and adhesion (21, 23). In FAK knockout model, they found impairment of embryonic development by loss of mesenchymal cell motility (21) and a decrease in migration speed and directional persistence (22). The results suggested that FAK played an important role in cellular migration (21-23) and re-epithelialization of human wounds (23).

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Basic fibroblast growth factor (FGF-2) and Vascular endothelial growth factor (VEGF) are angiogenesis mediator in human wound. Immediately after injury, FGF-2 is shortly released, providing an early stimulus for initiate wound angiogenesis. As FGF-2 levels decline, VEGF is produced. VEGF provides a sustained stimulus for endothelial cell migration and differentiation into new capillary tubes in proliferation phase of wound healing (24). VEGF is heparin-binding homodimeric protein. VEGF features 5

family members e.g. VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (25). The primary sources of VEGF are fibroblasts and macrophages. Capillary density reaches a maximum shortly after peak of VEGF levels. Thus, the important role for VEGF is a predominate angiogenesis mediator in human wounds (24).

Interleukin-6 (IL-6) is cytokines that produced at the site of inflammation and plays a key role in acute inflammatory response. IL-6 activated monocytes and neutrophils activities. Moreover, IL-6 helps to stimulatory effects on T and B-cell to chronic inflammatory responses (26).

Vitamin C or L-ascorbic acid, a water-soluble vitamin, has various biochemical functions including an essential role in hydroxylation reactions necessary for collagen and carnitine synthesis (7, 8), phagocytosis of polymorphonuclear leukocytes, differentiation of several mesenchymal cell types and antioxidant scavenging of reactive oxygen species (8, 15, 27). In addition, vitamin C can regulate tumor growth and angiogenesis (25).

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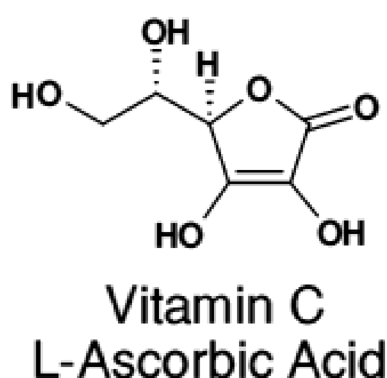

Figure 1 Chemical structure of Vitamin C or L-ascorbic acid.

Vitamin C is an essential nutrient for humans because Vitamin C cannot be synthesized endogenously in our body. The highest concentration of this vitamin is found in the adrenals, ovaries, brain, pituitary gland, liver, spleen, blood cells, extracellular fluid surrounding the lung and eye (8). The recommended daily allowance (RDA) of vitamin C is 60 mg. The dose of treatment is 500-3000 mg per day (11).

Effect of vitamin C have been studied both *in vitro*, *in vivo* and in clinical study. *In vitro* studies, vitamin C was tested on many cell types such as fibroblasts bone cells, keratinocytes, endothelial cells and cancer cells.

Vitamin C is required for healing of injured tissue both of soft and hard tissues (10). In 1982, W. M. Ringsdorf et al. reported a case of 8-year boy whose genetic impairment of collagen synthesis was determined as Type VI Ehlers-Danlos syndrome. Treatment with 4000 mg of vitamin C daily was able to improve newly collagen synthesis in this patient (11). The patients who got sufficient vitamin C demonstrated significantly accelerated wound healing (11). Vitamin C should be routinely prescribed in any cases with tissue injury (10). Besides, promoting wound healing property, vitamin C also plays an important role in mediating inflammation through antioxidant activities. These data supported the idea of prescribing vitamin C in addition to standard therapy in order to reduce the adverse effects of inflammation in sepsis and septic shock patients (28)

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Mice receiving higher dose of vitamin C demonstrated greater wound integrity than those receiving the moderate dose, even though the data was not significant (29). In addition, vitamin C deficiency wound had low tensile strength (30). In 2016, study of B. M. Mohammed et al., reported Gluo knockout mice which result in disability of normal vitamin C synthesis. Treatment of the excisional wounds in these mice with vitamin C reveals improve wound matrix deposition and organization. The wounds had reduced expression of pro-inflammatory mediators and higher expression of wound healing mediators (3).

In 2014, K. Omori et al. proposed that mild vitamin C deficiency with metabolic syndrome and severe periodontal infection may associate with the pathogenesis of severe gingival overgrowth in patient (31). In 2016, A. Bikker et al. reported the improvement of surgical wounds after treatment with vitamin C in 4 cases of impair surgical wound healing (14). Vitamin C supplement may accelerate wound healing from tooth extraction and reduce complication comparing with placebo. But the dosage of vitamin C should be concerned (12).

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Effect of vitamin C on fibroblast cell.

Several studies reported that vitamin C promotes fibroblast proliferation (3, 32). Although fibroblast harvested from infant and elderly demonstrated different

potential in cell proliferation, they response to vitamin C in the same way (33). Vitamin C induces a dose-dependent increase in collagen type I by normal human fibroblasts and enhances extracellular matrix contraction (34). Moreover, vitamin C induces the expression of self-renewal genes on human neonatal dermal fibroblasts (3).

Ascorbic acid 2-phosphate (AA2P) is long acting vitamin C derivative that can promote reorganization of ECM from skin fibroblasts in culture by stimulating collagen accumulation in the cell (7). Primary human dermal fibroblasts with long-term exposure to AA2P, increased the expression of genes associated with DNA replication and repair. AA2P increased the mitogenic stimulation of fibroblasts and cell motility in the context of wound healing. AA2P-treated fibroblasts showed faster repair of oxidatively damaged DNA bases (35). In 2011, K. Tsutsumi et al. compared the effect of vitamin C and AA2P on human gingival fibroblast, AA2P is better in reserve the level of vitamin C in the cell, reduce reactive oxygen species, promote collagen I production, inhibit IL-8. This study supported the usage of locally applied vitamin C for treating periodontal disease (15). In addition, in 1998, N. Boyera et al. showed AA2P and vitamin C-glucoside, vitamin C derivatives presenting higher chemical stability in aqueous solution, demonstrated similar biological properties but different potencies (34).

In 2010, H. Staudte et al. reported that vitamin C induce significant higher cell

viability rates and lower apoptosis rates in human gingival fibroblast exposure to P. gingivalis (36). They concluded that vitamin C reduced the cytotoxic effects of *P. gingivalis* and apoptotic effects of *P. gingivalis* on human gingival fibroblast *in vitro*.

Effect of vitamin C on bone cell.

Normal osteogenic media compose of normal media, ascorbic acid 50 μg/ml, β-glycerophosphate and dexamethasone. Only vitamin C in normal media cannot activate differentiation of bone marrow to bone cell (37). However, 30-90 μg/ml of vitamin C in normal media can induce mononuclear cells differentiation to bone cell. Therefore, vitamin C can be use as nutrition supplement in cellular therapy of bone related disease (38).

Effect of vitamin C on Keratinocyte.

Vitamin C promotes differentiation of Keratinocyte via protein kinase C pathway. Vitamin C protects keratinocyte from oxidative stress in inflammatory phase via hydrophilic antioxidant in cell by gain the level of glutathione (39). Moreover, Vitamin C promotes re-attachment of basement membrane in human skin graft, maintains epidermal barrier formation. Vitamin C promotes wound closure and reduces wound

contraction in full thickness flap (32).

Effect of vitamin C on endothelial cell.

Vitamin C reduces endothelial permeability and increase endothelial barrier that opposes the effect of VEGF. In addition, vitamin c inhibits effect of VDGF (40).

Vitamin C inhibits the formation of vessel – like tubular structure of blood vessel. High dose of vitamin C do not inhibit proliferation and migration of endothelial cell. Vitamin C inhibits angiogenesis in animal model via 2 pathways; antioxidant properties and collagen synthesis. Accordingly, vitamin C may use in order to inhibit migration of cancer cell and angiogenic disease (41).

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Effect of vitamin C on stem cell.

Vitamin C induces telomerase activity in periodontal ligament stem cells (PDLSCs). Vitamin C-mediates human PDLSCs sheet enhanced tissue regeneration in nude mice and swine (42).

Effect of Vitamin C on cancer cell.

Vitamin C reduces iron that is an essential factor for function of epigenetic regulators. Vitamin C deficiency is frequently observed in patients with cancer (27). Vitamin C promotes normal self-renewal of normal cell. Vitamin C activates Tet2 that drive cell to cell differentiation or cell death. In leukemia cell, vitamin C regulates hematopoitic stem cell function and suppress leukemogenesis by modulating Tet2 activity (43). Recently, vitamin C are candidate of anti-cancer therapy that have been studied in many types of cancer.

Vitamin C induces cell death through the apoptosis inducing factor in human breast cancer cells but not effect normal breast cell (44). Vitamin C suppresses VEGF via down regulation of COX-2 expression in melanoma cell (25). Vitamin C (0.25-1 mM) induces dose and time dependent inhibition in cell proliferation of acute myeloid leukemia cell. Treated leukemia cell with high dose of vitamin C induced level of glutathione and cysteine in cell (45).

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CHAPTER 3 MATERIAL AND METHODS

3.1 Cell culture

Specimen of gingiva from unerupted mandibular third molar were harvested form healthy young volunteers, aged 18 to 25 years. Their third molar were removed surgically via flap operation according to the indication described elsewhere (46). Immediately, the gingival tissue was transferred in ice-cold storage medium DMEM (Gibco, Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% FBS, 1% L-Gluamine, 0.5 mg/ml gentamicin and 3 mg/ml amphotericin B. The specimen was rinsed twice in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS). The gingival tissues was cut into 1x2 mm pieces with a surgical blade and seeded in DMEM supplemented with 10% FBS, 1% L-Gluamine, 1% antibiotics. The tissue samples were incubated at 37°C humidified atmosphere with 5% CO2, and the medium was changed every 3 days until outgrowing cells reached confluence. The primary human gingival fibroblasts (hGFs) at the 3rd–6th passage was used for the experiments. The patients provided written informed consent for the use of discarded tissues for research purposes. Tissue samples were deidentified and analyzed anonymously. This study was considered by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Thailand (HREC-DCU 2016-097).

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3.2 Scratch-test assay ("wound healing assay")

To evaluate the effect of L-Ascorbic acid on hGFs mediated wound healing *in vitro,* a scratch-test assay was performed. Cells were seeded in a 24-well-plate (39500 cells/cm2). After 24h, a sterile 1 ml pipette tip (blue tip) was used to make a straight scratch line on the monolayer of confluent cells at the bottom of the culture plate (that mimics to wound configuration). The debris were washed away with PBS. The cells were cultured at 37°C, humidified 5% CO2. The cells were rinsed with 0, 10, 20 or 50 µg/ml L-Ascorbic acid in culture medium 7 minutes, 3 times/day. At time points 0, 24 and 48h the "wound" areas were observed and recorded by using an inverted microscope (Eclipse TS100, Nikon, Japan) and a digital camera at the same position of each culture plate. The area of "wound healing" were analyzed using the Image J 1.45S software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) as described previously (47). The remaining "wound" areas between groups were compared.

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3.3 Cell proliferation assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT; #298-931, USB Corporation, Cleveland, OH, USA). The cells were plated at 2x104 cells/cm2 in 24-well plate for 2 plates. After 24h, the

medium of the 1st plate was replaced with 0.5 ml MTT solution and incubated for 30 min at 37°C. The formazan product was dissolved in solubilization/stop solution including 9:1 DMSO and glycine buffer. Using a microplate reader (ELx800, BioTek, Winooski, VT, USA), the optical densities were measured at 570 nm. For the 2nd plate, the cells were rinsed with 0, 10, 20 or 50 μg/ml L-Ascorbic acid 7 minutes, 3 times/day. After 48h, the 2nd plate was subjected to MTT assay as described in the 1st plate process. The difference of absorbance between groups were represent the variety of cell numbers and compare with the 1st plate.

3.4 Transwell migration assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT; #298-931, USB Corporation, Cleveland, OH, USA). The cells were plated at 2x104 cells/cm2 in 24-well plate for 2 plates. After 24h, the medium of the 1st plate was replaced with 0.5 ml MTT solution and incubated for 30 min at 37°C. The formazan product was dissolved in solubilization/stop solution including 9:1 DMSO and glycine buffer. Using a microplate reader (ELx800, BioTek, Winooski, VT, USA), the optical densities were measured at 570 nm. For the 2nd plate, the cells were rinsed with 0, 10, 20 or 50 μg/ml L-Ascorbic acid 7 minutes, 3 times/day. After 48h, the 2nd plate was subjected to MTT assay as described in the 1st plate process. The difference of absorbance between groups were represent the

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variety of cell numbers and compare with the 1st plate.

3.4 Transwell migration assay

Cell migration assay was performed in 24-well size Transwell inserts with 8.0-μm-pore polycarbonate membrane and 0.3cm2 effective growth area (#3422, BD FalconTM Cell Culture Inserts, BD Biosciences, Bedford, MA, USA). hGFs were trypsinized and re-suspended in serum-free media. 1x105 cells in 200 μl of medium were seeded in each insert. One hour after seeding, the rinsing protocol was started. Briefly, the inserts were moved to new 24-well-plates contain 700 μl of 0, 10, 20 or 50 μg/ml L-Ascorbic acid in serum-free medium 7 minutes, 3 times/day. In order to prevent cell proliferation, the migration assay was performed with serum-free medium for only 24 h. On the next day, non-migrated cells from the upper surface of the membrane were carefully removed by a cotton swab. The cells that migrate to the other side of the membrane were fixed in cold methanol for 10 min, stained with 1.4% crystal violet and washed three times with distilled water. Cell migration was evaluated by photomicrographs from five randomly chosen fields (x100) per insert by counting the number of migrated cells and the adhesion area using the Image J 1.45S software as described previously (48). The number of migrated cells and cell area per cell between groups were compared.

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3.5 RNA analysis by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

To detect the quantitative expression of genes that could be involved in extracellular matrix production under L-Ascorbic acid treatment, quantitative real-time RT-PCR was performed. After rinsing with L-Ascorbic acid in culture medium, the total mRNA of hGFs were isolated by using Trizol reagent (#2302700, Prime, Gaithersburg, MD, USA). The lysate was extracted by adding 100 µl chloroform, mixing and centrifuging for 15 min at 12,000 rpm. mRNA was precipitated with 250 µl isopropanol and the pellet were dissolved in nuclease-free water. The amount of RNA was measured using a spectrophotometer (NanoDrop2000, Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized using reverse transcriptase reaction by ImProm-II Reverse Transcription System (#A3800, Promega Corporation, Madison, WI, USA) and quantitative PCR were performed following the manufacturer's manual.

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PCR primer for type I collagen (COL1, F: GCA AAG AAG GCG GCA AA, R: CTC ACC ACG ATC ACC ACT CT), Focal adhesion kinase (FAK, F: CAA TCC CAC ACA TCT TGC TGA, R: AGC CGG CAG TAC CCA TCT ATT), fibronectin (FN, F: GGA TCA CTT ACG GAG AAA CAG, R: GAC ACT AAC CAC ATA CTC CAC), Interluekin6 (IL-6, F: GGA TTC AAT GAG GAG ACT TGC C, R: TCT GCA GGA ACT GGA TCA GG), Basic fibroblast growth factor (bFGF, F: GGC TTC TTC CTG CGC ATC CAC , R: GGT AAC GGT TAG CAC ACA CTC

CT) and Vascular endothelial growth factor (VEGF, F: ATG AGG ACA CCG GCT CTG ACC A, R: AGG CTC CTG AAT CTT CCA GGC A) were used to screen extracellular matrix gene expression. All graphs were analyzed and normalized with the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, F: TGA AGG TCG GAG TCA ACG GAT, R: TCA CAC CCA TGA CGA ACA TGG) using Bio-1D software version 15.03 (Vilber Lourmat, Marne La Vallée, France). Three independent experiments were repeated in each sample to compare the fold change of gene expression.

3.6 Data analysis

All experiments were repeated three times. The data were calculated for the means and standard deviations (SD) for each set of data compose of remaining “wound” area, cell proliferation number, cell migration number and fold change of gene expression. For statistical analysis, all data were tested for normal distribution with Kolmogorov-Smirnov test. If the data is normally distributed, independent samples comparison t-test, one-way ANOVA with Dunnett T3 (unequal variances) or Tukey HSD post-hoc test (equal variances) were used to compare between groups. Conversely, Mann-Whitney U test, Kruskal-Walli’s with Dunn's test were used, if the data is not normal. SPSS v.21 (IBM, New York, NY, USA) was used for calculations with the level of significance being 0.05.

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CHAPTER 4 RESULT

4.1 Scratch-test assay (“wound healing assay”)

Rinsing with vitamin C at concentration of 10 and 20 µg/ml demonstrated no significant effect on *in vitro* wound closure. However, vitamin C at the concentration of 50 µg/ml significantly delayed wound closure comparing with the control group (p value=0.05) (Fig 2).

VitC (µg/ml) / Time(Hrs)	0	10	20	50
0	100	100	100	100
12	96.273	76.093	85.19	81.603
24	78.127	58.667	63.327	81.687
48	48.007	47.24	44.493	82.157

Table 1 percentage of wound remaining area at 12, 24 and 48 hrs.

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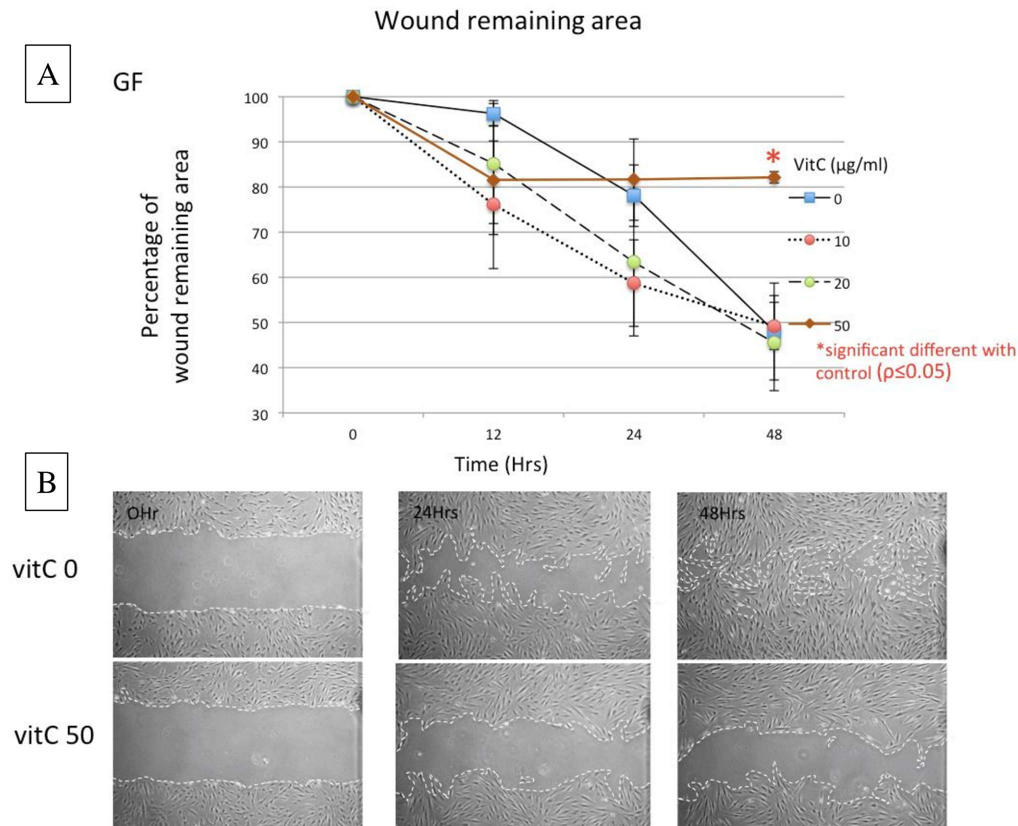

Figure 2 Scratch-test assay

A – Graph show wound remaining area at 48 hrs., rinsing with 50 µg/ml of vitamin C significantly delayed wound closure comparing with the control group (p value=0.05).

B - Scratch-test assay pictures comparing between vitamin C 50 µg/ml and control at 0, 24 and 48 hrs. after rinsing with vitamin C.

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4.2 Cell proliferation assay (MTT assay)

Correspond with wound healing assay, vitamin C at the concentration of 10 and 20 µg/ml demonstrated no significant effect on cell proliferation but vitamin C 50 µg/ml significantly reduce proliferation comparing with the control group (p value=0.05) (Fig 3)

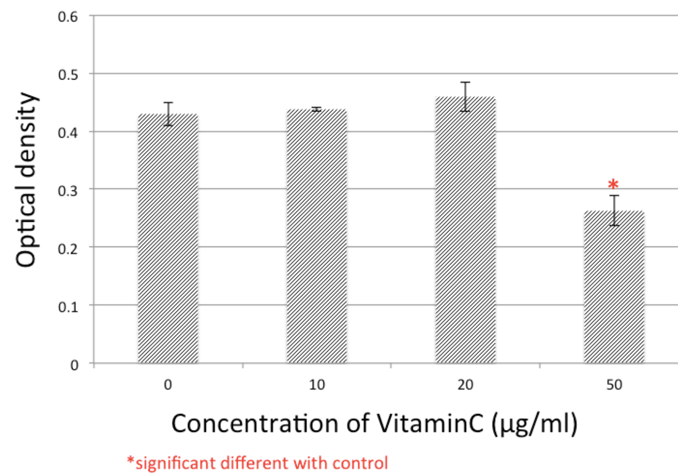

Figure 3 Optical density from MTT assay in various concentration of Vitamin C

4.3 Transwell migration assay

Rinsing with 10-20 µg/ml vitamin C enhance fibroblast migration observed at 24 hrs in a concentration dependent manor (Fig 4). This enhance in cell migration effect was disappear when increasing dose of vitamin C up to 50 µg/ml.

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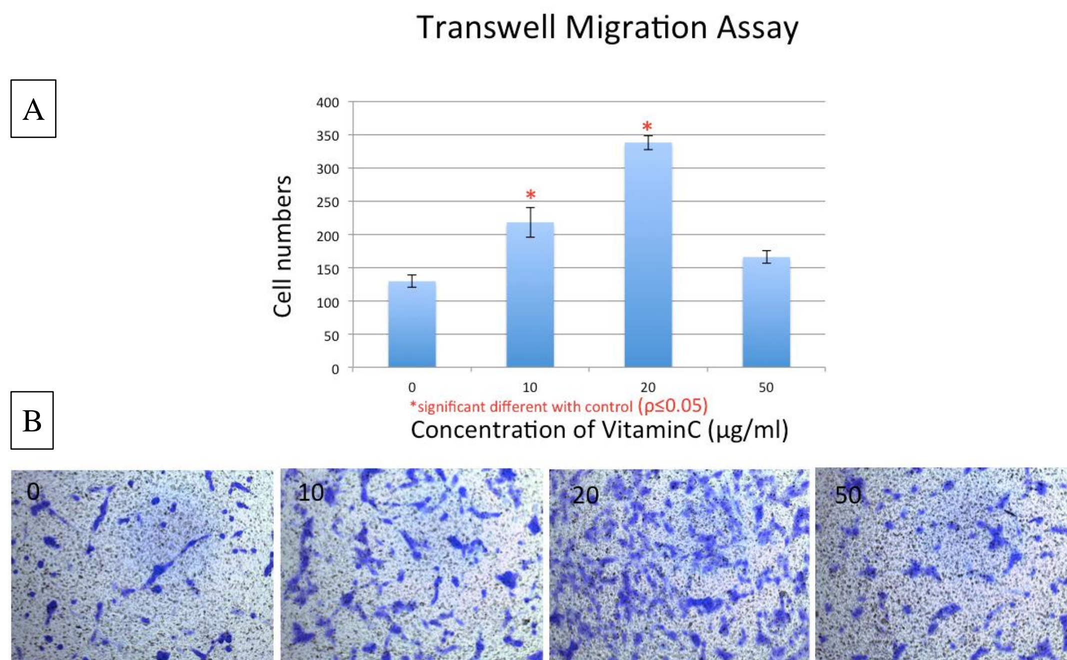

Figure 4 Transwell migration assay

A Cell numbers migrated in various concentrations of vitamin C

B Purple spots in the pictures show migrated cell at 24 hrs.

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4.4 RNA analysis by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

To analyse expression of wound healing related genes, we analyzed gene expression in gingival fibroblasts after rinsing with various concentration of vitamin C. Rinsing with 50 µg/ml vitamin C significantly upregulated the expression of COL1, Fibronectin, IL-6 and bFGF genes compared to those of control group. No significant different in expression level of FAK and VEGF were observed in all groups (Fig 5)

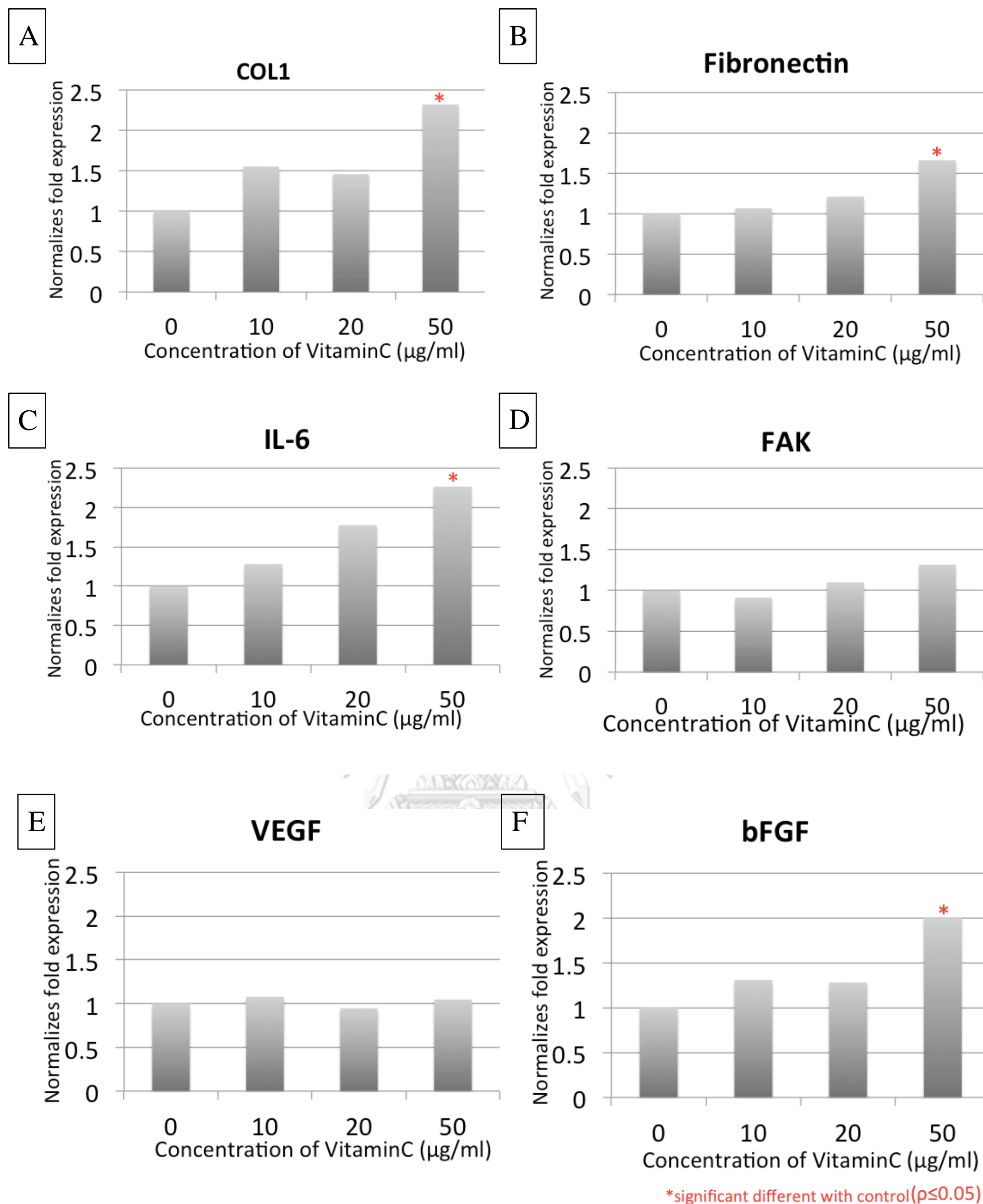

Figure 5 mRNA expression by RT-PCR in rinsing various concentration of vitamin C. A Collagen1, B Fibronectin, C Interleukin-6, D Focal adhesion kinase, E Vascular endothelial growth factor, F Basic Fibroblast growth factor - To analyse expression of wound healing related genes, rinsing with 50 µg/ml vitamin C significantly upregulated the expression of COL1, Fibronectin, IL-6 and bFGF genes compared to control group.

CHAPTER 5 DISCUSSION

This study demonstrated that various concentrations of vitamin c differentially affect behavior of the human gingival fibroblast including proliferation, migration and extracellular matrix activity. In early stage, low dose of vitamin C (10, 20 µg/ml) significantly activated fibroblast migration which result in a trend increase in *in vitro* gingival wound closure, though not significant. However, higher dose of vitamin C (50 µg/ml) delay gingival wound closure by inhibiting proliferation of gingival fibroblast. The result of real-time PCR demonstrated that 50 ug/ml of vitamin C promote production of extracellular matrix like collagen and fibronectin as well as inflammation mediator, IL-6, and bFGF.

Rinsing with vitamin C at lower doses was able to enhance gingival fibroblast migration as observed by transwell migration assay. This effect was not found in higher dose (50 µg/ml) of vitamin C. Rinsing with 50 µg/ml vitamin C significantly reduced cell viability. The effect of 50 µg/ml vitamin C to delayed gingival wound healing in our model might explained by the reduction in cell viability at this concentration.

Study of G.M. Abrahmsohn et al., 1993 demonstrated that vitamin C accelerates wound healing of extraction socket and reduced post-operative complication such as dry socket. This study recommended to prescribe vitamin C to patient after oral surgery. The authors proposed that systemic effect of vitamin C on

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wound healing may be regulated by various pathways which needs further study. Our current study of effect of vitamin C on gingival fibroblast to regulated *in vitro* wound healing is the first study to report local effect of vitamin C on gingival wound healing. Our study try to mimic the usage of vitamin C in the form of oral lozenge that slowly dissolve in the mount. Our pilot study reveals that its take 7 mins for 500 mg lozenge of vitamin C to dissolve in the mount. So, we designed the experiment to mimic this condition by rinsing with various concentrations of vitamin C for a duration of 7 mins, 3 times/day.

The reducing in cell viability in higher concentrations of vitamin C is still controversy. Since raising concentration of vitamin C may bring about the higher acidity which toxic to the cells. However, we did measure the pH in every concentrations of vitamin C in cell culture medium and found no difference in pH, thus ruling out the effect of acidity in our model. Corresponded with our results, there is a report demonstrating that Vitamin C reduced proliferation of many cancer cells by unknown mechanisms but may relate to apoptosis (45) (44). Therefore, the effect of Vitamin C to induce apoptosis is an interesting topic that needs further study.

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Guided Bone Regeneration (GBR) is one of the technique of ridge augmentation. GBR is a surgical procedure that uses barrier membranes to separate bone and connective tissue and epithelium (49). Since Vitamin C control

differentiation of human stem cell to osteoblast, it is a good candidate for bone regeneration material (43),(38). For example, Sreepathy Sridhar et al. use ascorbic acid with scaffolds for improved regeneration potential of fibroblast (50).

It is well known that vitamin C promotes collagen synthesis. Bassem M Mohammed et al. demonstrated that giving vitamin C to mice could promote more collagen synthesis in the wound comparing to control group (3). Tsutsumi K. et al. demonstrated that L-Ascorbic acid 2-phosphate magnesium salt (developed L-ascorbic acid) was more effective in promote collagen I gene expression in human gingival fibroblast comparing with normal L-ascorbic acid (15). Their results are consistent with our study.

Epithelial and fibroblast cell can secrete IL-6 to activate the inflammatory phase of wound healing process (26) IL-6 could also promote epithelium growth in proliferation phase (4). In our study, we found the increase in IL-6 gene expression after rinsing with vitamin C. This effect might be beneficial for promoting wound healing *in vivo*.

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Recently, there are several studies testing the effect of vitamin C to eliminate various types of cancer (25, 27, 43, 45). Our study is the first study that applied locally with shot duration of vitamin C in various concentrations on primary human gingival fibroblast mimic using oral lozenge *in vitro*. Results of this study is topping up the basic knowledge of vitamin c on gingival fibroblasts behavior and

propose vitamin C as an alternative supplement that can prescribe orally to promote oral wound healing.

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CHAPTER 6 CONCLUSION

Lower dose of vitamin C significantly activated fibroblast migration but not affect *in vitro* gingival wound closure. Higher dose of vitamin C delay wound closure by inhibiting proliferation of gingival fibroblast but still promote ECM and inflammatory cytokines production. Vitamin C is safe and can be prescribed to patients after oral surgery but suitable dosage and methods of administration should be adjust to maximize it benefit.

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APPENDIX

Consent Form

เอกสารยินยอมเข้าร่วมการวิจัย (Consent Form)

การวิจัยเรื่องผลของวิตามินซีต่อการหายของแผลโดยทดสอบกับเซลล์ไฟโบรบลาสจากเหงือกในห้องปฏิบัติการ

ข้าพเจ้า (นาย/ นาง/ นางสาว/ เด็กชาย/ เด็กหญิง)...

อยู่บ้านเลขที่..........................ถนน.......................ตำบล/แขวง...

อำเภอ/เขต......................จังหวัด.........................รหัสไปรษณีย์...

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้

1. ข้าพเจ้าได้รับทราบรายละเอียดข้อมูลคำอธิบายสำหรับอาสาสมัครที่เข้าร่วมในการวิจัย รวมทั้งได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการทำวิจัย อันตรายหรืออาการที่อาจเกิดขึ้นจากการทำวิจัย หรือจากยาที่ใช้รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียดและมีความเข้าใจดีแล้ว
2. ผู้วิจัยรับรองว่าจะตอบคำถามต่างๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจไม่ปิดบังซ่อนเร้นจนข้าพเจ้าพอใจ
3. ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับและจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้องกระทำได้ เฉพาะกรณีจำเป็น ด้วยเหตุผลทางวิชาการเท่านั้น และผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆ จากการวิจัยดังกล่าว ข้าพเจ้าจะได้รับการรักษาพยาบาลโดยไม่คิดมูลค่า
4. ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมในโครงการวิจัยนี้เมื่อใดก็ได้และการบอกเลิกการเข้าร่วมการวิจัยนี้จะไม่มีผลต่อการรักษาโรคที่ข้าพเจ้าจะพึงได้รับต่อไป

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ข้าพเจ้าจึงสมัครใจเข้าร่วมโครงการวิจัยนี้ตามที่ระบุในเอกสารข้อมูลคำอธิบายสำหรับอาสาสมัครและได้ลง นามในใบยินยอมนี้ด้วยความเต็มใจ และได้รับสำเนาเอกสารใบยินยอมที่ข้าพเจ้าลงนามและลงวันที่ และเอกสารยกเลิกการเข้าร่วมวิจัย อย่างละ 1 ฉบับ เป็นที่เรียบร้อยแล้ว ในกรณีที่อาสาสมัครยังไม่บรรลุนิติภาวะจะต้องได้รับการยินยอมจากผู้ปกครองด้วย

ลงนาม.. (อาสาสมัคร) (..) วันที่......./................/................	ลงนาม.. (ผู้ปกครอง) (..) วันที่......./................/................

ลงนาม... (ผู้วิจัยหลัก) (...) วันที่......./................./.................	ลงนาม... (พยาน) (...) วันที่......./................./.................

ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนาม หรือประทับลายนิ้วหัวแม่มือขวาของข้าพเจ้าในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม... (อาสาสมัคร) (...) วันที่......./................./.................	ลงนาม... (ผู้ปกครอง) (...) วันที่......./................./.................
ลงนาม... (ผู้วิจัยหลัก) (...) วันที่......./................./.................	ลงนาม..(พยาน) (...) วันที่......./................./.................

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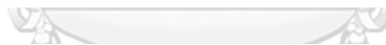
Statistic Result

1. Scratch-test assay (“wound healing assay”)

Tests of Normality

	GF	Kolmogorov-Smirnova			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
At12Hrs	GF5	.119	18	.200*	.943	18	.330
	GF6	.185	18	.105	.916	18	.109
	GF3	.151	18	.200*	.938	18	.264
At24Hrs	GF5	.095	18	.200*	.979	18	.942
	GF6	.134	18	.200*	.948	18	.391
	GF3	.138	18	.200*	.954	18	.498
At48Hrs	GF5	.168	18	.192	.881	18	.027
	GF6	.301	18	.000	.825	18	.003
	GF3	.130	18	.200*	.946	18	.372

*. This is a lower bound of the true significance.



ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
At12Hrs	Between Groups	1546.245	5	309.249	3.744	.028
	Within Groups	991.218	12	82.602		
	Total	2537.463	17			
At24Hrs	Between Groups	2119.007	5	423.801	5.161	.009
	Within Groups	985.357	12	82.113		
	Total	3104.364	17			
At48Hrs	Between Groups	332.012	5	66.402	28.520	.000
	Within Groups	27.939	12	2.328		
	Total	359.951	17			

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
At12Hrs	0	10	20.18000	8.93801	.282	-9.8421	50.2021
		20	11.08333	8.93801	.810	-18.9387	41.1054
		50	14.67000	8.93801	.590	-15.3521	44.6921
		70	20.88000	8.93801	.252	-9.1421	50.9021
		100	16.51667	8.93801	.474	-13.5054	46.5387
	10	0	-20.18000	8.93801	.282	-50.2021	9.8421
		20	-9.09667	8.93801	.903	-39.1187	20.9254
		50	-5.51000	8.93801	.988	-35.5321	24.5121
		70	.70000	8.93801	1.000	-29.3221	30.7221
		100	-3.66333	8.93801	.998	-33.6854	26.3587
	20	0	-11.08333	8.93801	.810	-41.1054	18.9387
		10	9.09667	8.93801	.903	-20.9254	39.1187
		50	3.58667	8.93801	.998	-26.4354	33.6087
		70	9.79667	8.93801	.874	-20.2254	39.8187
		100	5.43333	8.93801	.988	-24.5887	35.4554
	50	0	-14.67000	8.93801	.590	-44.6921	15.3521
		10	5.51000	8.93801	.988	-24.5121	35.5321
		20	-3.58667	8.93801	.998	-33.6087	26.4354
		70	6.21000	8.93801	.979	-23.8121	36.2321
		100	1.84667	8.93801	1.000	-28.1754	31.8687
	70	0	-20.88000	8.93801	.252	-50.9021	9.1421
		10	-.70000	8.93801	1.000	-30.7221	29.3221
		20	-9.79667	8.93801	.874	-39.8187	20.2254

		50	-6.21000	8.93801	.979	-36.2321	23.8121
		100	-4.36333	8.93801	.996	-34.3854	25.6587
	100	0	-16.51667	8.93801	.474	-46.5387	13.5054
		10	3.66333	8.93801	.998	-26.3587	33.6854
		20	-5.43333	8.93801	.988	-35.4554	24.5887
		50	-1.84667	8.93801	1.000	-31.8687	28.1754
		70	4.36333	8.93801	.996	-25.6587	34.3854
At24Hrs	0	10	19.46000	7.49187	.171	-5.7046	44.6246
		20	14.80000	7.49187	.408	-10.3646	39.9646
		50	-3.56000	7.49187	.996	-28.7246	21.6046
		70	11.21000	7.49187	.673	-13.9546	36.3746
		100	2.29000	7.49187	1.000	-22.8746	27.4546
	10	0	-19.46000	7.49187	.171	-44.6246	5.7046
		20	-4.66000	7.49187	.987	-29.8246	20.5046
		50	-23.02000	7.49187	.080	-48.1846	2.1446
		70	-8.25000	7.49187	.872	-33.4146	16.9146
		100	-17.17000	7.49187	.268	-42.3346	7.9946
	20	0	-14.80000	7.49187	.408	-39.9646	10.3646
		10	4.66000	7.49187	.987	-20.5046	29.8246
		50	-18.36000	7.49187	.214	-43.5246	6.8046
		70	-3.59000	7.49187	.996	-28.7546	21.5746
		100	-12.51000	7.49187	.573	-37.6746	12.6546
	50	0	3.56000	7.49187	.996	-21.6046	28.7246
		10	23.02000	7.49187	.080	-2.1446	48.1846
		20	18.36000	7.49187	.214	-6.8046	43.5246
		70	14.77000	7.49187	.410	-10.3946	39.9346
		100	5.85000	7.49187	.966	-19.3146	31.0146
	70	0	-11.21000	7.49187	.673	-36.3746	13.9546
		10	8.25000	7.49187	.872	-16.9146	33.4146

		20	3.59000	7.49187	.996	-21.5746	28.7546
		50	-14.77000	7.49187	.410	-39.9346	10.3946
		100	-8.92000	7.49187	.833	-34.0846	16.2446
	100	0	-2.29000	7.49187	1.000	-27.4546	22.8746
		10	17.17000	7.49187	.268	-7.9946	42.3346
		20	12.51000	7.49187	.573	-12.6546	37.6746
		50	-5.85000	7.49187	.966	-31.0146	19.3146
		70	8.92000	7.49187	.833	-16.2446	34.0846
At48Hrs	0	10	-1.23333	5.35025	1.000	-19.2044	16.7377
		20	8.51333	5.35025	.618	-9.4577	26.4844
		50	-34.15000*	5.35025	.000	-52.1211	-16.1789
		70	-34.15000*	5.35025	.000	-52.1211	-16.1789
		100	-36.00000*	5.35025	.000	-53.9711	-18.0289
	10	0	1.23333	5.35025	1.000	-16.7377	19.2044
		20	9.74667	5.35025	.488	-8.2244	27.7177
		50	-32.91667*	5.35025	.001	-50.8877	-14.9456
		70	-32.91667*	5.35025	.001	-50.8877	-14.9456
		100	-34.76667*	5.35025	.000	-52.7377	-16.7956
	20	0	-8.51333	5.35025	.618	-26.4844	9.4577
		10	-9.74667	5.35025	.488	-27.7177	8.2244
		50	-42.66333*	5.35025	.000	-60.6344	-24.6923
		70	-42.66333*	5.35025	.000	-60.6344	-24.6923
		100	-44.51333*	5.35025	.000	-62.4844	-26.5423
	50	0	34.15000*	5.35025	.000	16.1789	52.1211
		10	32.91667*	5.35025	.001	14.9456	50.8877
		20	42.66333*	5.35025	.000	24.6923	60.6344
		70	.00000	5.35025	1.000	-17.9711	17.9711
		100	-1.85000	5.35025	.999	-19.8211	16.1211

	70	0	34.15000*	5.35025	.000	16.1789	52.1211
		10	32.91667*	5.35025	.001	14.9456	50.8877
		20	42.66333*	5.35025	.000	24.6923	60.6344
		50	.00000	5.35025	1.000	-17.9711	17.9711
		100	-1.85000	5.35025	.999	-19.8211	16.1211
	100	0	36.00000*	5.35025	.000	18.0289	53.9711
		10	34.76667*	5.35025	.000	16.7956	52.7377
		20	44.51333*	5.35025	.000	26.5423	62.4844
		50	1.85000	5.35025	.999	-16.1211	19.8211
		70	1.85000	5.35025	.999	-16.1211	19.8211

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

Dunnett T3

Dependent Variable	(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
At12Hrs	0	10	20.18000	8.31967	.492	-50.6258	90.9858
		20	11.08333	7.85173	.840	-55.0676	77.2343
		50	14.67000	7.18260	.608	-44.7779	74.1179
		70	20.88000*	1.79238	.010	9.3561	32.4039
		100	16.51667	8.02367	.604	-51.3476	84.3809
	10	0	-20.18000	8.31967	.492	-90.9858	50.6258
		20	-9.09667	11.21046	.994	-67.1323	48.9389
		50	-5.51000	10.75242	1.000	-61.8019	50.7819
		70	.70000	8.19984	1.000	-73.7507	75.1507

		100	-3.66333	11.33155	1.000	-62.2519	54.9253
	20	0	-11.08333	7.85173	.840	-77.2343	55.0676
		10	9.09667	11.21046	.994	-48.9389	67.1323
		50	3.58667	10.39458	1.000	-50.3805	57.5538
		70	9.79667	7.72465	.887	-60.1477	79.7410
		100	5.43333	10.99258	1.000	-51.3731	62.2398
	50	0	-14.67000	7.18260	.608	-74.1179	44.7779
		10	5.51000	10.75242	1.000	-50.7819	61.8019
		20	-3.58667	10.39458	1.000	-57.5538	50.3805
		70	6.21000	7.04345	.979	-57.2536	69.6736
		100	1.84667	10.52506	1.000	-52.9436	56.6369
	70	0	-20.88000*	1.79238	.010	-32.4039	-9.3561
		10	-.70000	8.19984	1.000	-75.1507	73.7507
		20	-9.79667	7.72465	.887	-79.7410	60.1477
		50	-6.21000	7.04345	.979	-69.6736	57.2536
		100	-4.36333	7.89935	.999	-75.9656	67.2390
	100	0	-16.51667	8.02367	.604	-84.3809	51.3476
		10	3.66333	11.33155	1.000	-54.9253	62.2519
		20	-5.43333	10.99258	1.000	-62.2398	51.3731
		50	-1.84667	10.52506	1.000	-56.6369	52.9436
		70	4.36333	7.89935	.999	-67.2390	75.9656
At24Hrs	0	10	19.46000	6.78011	.302	-17.6911	56.6111
		20	14.80000	10.19629	.830	-55.0923	84.6923
		50	-3.56000	6.55017	1.000	-38.8640	31.7440
		70	11.21000	4.20590	.408	-19.2950	41.7150
		100	2.29000	4.50685	1.000	-25.1987	29.7787
	10	0	-19.46000	6.78011	.302	-56.6111	17.6911
		20	-4.66000	10.89524	1.000	-69.2140	59.8940

		50	-23.02000	7.59253	.251	-62.3080	16.2680
		70	-8.25000	5.69486	.827	-54.0456	37.5456
		100	-17.17000	5.92060	.348	-58.8698	24.5298
	20	0	-14.80000	10.19629	.830	-84.6923	55.0923
		10	4.66000	10.89524	1.000	-59.8940	69.2140
		50	-18.36000	10.75365	.726	-83.5695	46.8495
		70	-3.59000	9.50925	1.000	-87.2104	80.0304
		100	-12.51000	9.64614	.880	-92.3196	67.2996
	50	0	3.56000	6.55017	1.000	-31.7440	38.8640
		10	23.02000	7.59253	.251	-16.2680	62.3080
		20	18.36000	10.75365	.726	-46.8495	83.5695
		70	14.77000	5.41906	.407	-28.2139	57.7539
		100	5.85000	5.65582	.959	-33.1319	44.8319
	70	0	-11.21000	4.20590	.408	-41.7150	19.2950
		10	8.25000	5.69486	.827	-37.5456	54.0456
		20	3.59000	9.50925	1.000	-80.0304	87.2104
		50	-14.77000	5.41906	.407	-57.7539	28.2139
		100	-8.92000	2.60254	.203	-23.5708	5.7308
	100	0	-2.29000	4.50685	1.000	-29.7787	25.1987
		10	17.17000	5.92060	.348	-24.5298	58.8698
		20	12.51000	9.64614	.880	-67.2996	92.3196
		50	-5.85000	5.65582	.959	-44.8319	33.1319
		70	8.92000	2.60254	.203	-5.7308	23.5708
At48Hrs	0	10	-1.23333	6.88627	1.000	-45.4404	42.9738
		20	8.51333	8.69412	.978	-36.4063	53.4330
		50	-34.15000	6.22866	.130	-90.1223	21.8223
		70	-34.15000	6.22866	.130	-90.1223	21.8223
		100	-36.00000	6.19303	.121	-93.1708	21.1708
	10	0	1.23333	6.88627	1.000	-42.9738	45.4404

		20	9.74667	6.81681	.841	-33.7698	53.2631
		50	-32.91667*	3.11087	.029	-58.4413	-7.3921
		70	-32.91667*	3.11087	.029	-58.4413	-7.3921
		100	-34.76667*	3.03890	.032	-62.3641	-7.1692
	20	0	-8.51333	8.69412	.978	-53.4330	36.4063
		10	-9.74667	6.81681	.841	-53.2631	33.7698
		50	-42.66333	6.15178	.083	-97.9008	12.5741
		70	-42.66333	6.15178	.083	-97.9008	12.5741
		100	-44.51333	6.11570	.079	-100.9627	11.9360
	50	0	34.15000	6.22866	.130	-21.8223	90.1223
		10	32.91667*	3.11087	.029	7.3921	58.4413
		20	42.66333	6.15178	.083	-12.5741	97.9008
		70	.00000	1.02614	1.000	-5.3012	5.3012
		100	-1.85000	.78125	.486	-7.3205	3.6205
	70	0	34.15000	6.22866	.130	-21.8223	90.1223
		10	32.91667*	3.11087	.029	7.3921	58.4413
		20	42.66333	6.15178	.083	-12.5741	97.9008
		50	.00000	1.02614	1.000	-5.3012	5.3012
		100	-1.85000	.78125	.486	-7.3205	3.6205
	100	0	36.00000	6.19303	.121	-21.1708	93.1708
		10	34.76667*	3.03890	.032	7.1692	62.3641
		20	44.51333	6.11570	.079	-11.9360	100.9627
		50	1.85000	.78125	.486	-3.6205	7.3205
		70	1.85000	.78125	.486	-3.6205	7.3205

*. The mean difference is significant at the 0.05 level.

2. Cell proliferation assay (MTT assay)

Tests of Normality

	conc	Kolmogorov-Smirnova			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
MTT	0	.216	9	.200*	.880	9	.155
	10	.217	9	.200*	.864	9	.105
	20	.216	9	.200*	.918	9	.375
	50	.168	9	.200*	.933	9	.507
	70	.138	9	.200*	.941	9	.593
	100	.182	9	.200*	.949	9	.684

*. This is a lower bound of the true significance.

ANOVA

MTT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.320	5	.064	34.485	.000
Within Groups	.089	48	.002		
Total	**.409**	**53**			

Multiple Comparisons

MTT

Dunnett T3

(I) conc	(J) conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
00	10	-.0042222	.0231577	1.000	-.082455	.074011
	20	-.0087778	.0270657	1.000	-.101421	.083865
	50	.1166667*	.0175570	.000	.053806	.179527
	70	.1471111*	.0177702	.000	.083925	.210297
	100	.1737778*	.0188437	.000	.108488	.239068
10	00	.0042222	.0231577	1.000	-.074011	.082455
	20	-.0045556	.0276153	1.000	-.098688	.089577
	50	.1208889*	.0183929	.001	.054644	.187134
	70	.1513333*	.0185966	.000	.084807	.217860
	100	.1780000*	.0196250	.000	.109614	.246386
20	00	.0087778	.0270657	1.000	-.083865	.101421
	10	.0045556	.0276153	1.000	-.089577	.098688
	50	.1254444*	.0231208	.004	.040074	.210815
	70	.1558889*	.0232831	.001	.070392	.241386
	100	.1825556*	.0241125	.000	.096114	.268997
50	00	-.1166667*	.0175570	.000	-.179527	-.053806
	10	-.1208889*	.0183929	.001	-.187134	-.054644
	20	-.1254444*	.0231208	.004	-.210815	-.040074
	70	.0304444	.0108535	.151	-.006227	.067116
	100	.0571111*	.0125342	.006	.014248	.099974
70	00	-.1471111*	.0177702	.000	-.210297	-.083925
	10	-.1513333*	.0185966	.000	-.217860	-.084807
	20	-.1558889*	.0232831	.001	-.241386	-.070392
	50	-.0304444	.0108535	.151	-.067116	.006227
	100	.0266667	.0128312	.488	-.017014	.070347
100	00	-.1737778*	.0188437	.000	-.239068	-.108488

10	-.1780000*	.0196250	.000	-.246386	-.109614
20	-.1825556*	.0241125	.000	-.268997	-.096114
50	-.0571111*	.0125342	.006	-.099974	-.014248
70	-.0266667	.0128312	.488	-.070347	.017014

*. The mean difference is significant at the 0.05 level.

3. Transwell migration assay

Tests of Normality

		Kolmogorov-Smirnova			Shapiro-Wilk		
	patient	Statistic	df	Sig.	Statistic	df	Sig.
Number	GF5	.190	12	.200*	.867	12	.060
	GF3	.215	12	.131	.852	12	.039
	GF6	.235	12	.067	.862	12	.052

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

ANOVA

Number

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1867032.917	3	622344.306	123.506	.000
Within Groups	40312.000	8	5039.000		
Total	1907344.917	11			

Multiple Comparisons

CellNumber

Tukey HSD

(I) Conc	(J)	Mean	Std. Error	Sig.	95% Confidence Interval

	Conc	Difference (I-J)			Lower Bound	Upper Bound
0	10	-443.00000*	57.95976	.000	-628.6075	-257.3925
	20	-1043.66667*	57.95976	.000	-1229.2742	-858.0591
	50	-182.33333	57.95976	.054	-367.9409	3.2742
10	0	443.00000*	57.95976	.000	257.3925	628.6075
	20	-600.66667*	57.95976	.000	-786.2742	-415.0591
	50	260.66667*	57.95976	.009	75.0591	446.2742
20	0	1043.66667*	57.95976	.000	858.0591	1229.2742
	10	600.66667*	57.95976	.000	415.0591	786.2742
	50	861.33333*	57.95976	.000	675.7258	1046.9409
50	0	182.33333	57.95976	.054	-3.2742	367.9409
	10	-260.66667*	57.95976	.009	-446.2742	-75.0591
	20	-861.33333*	57.95976	.000	-1046.9409	-675.7258

*. The mean difference is significant at the 0.05 level.

Multiple Comparison

CellNumber

Dunnett T3

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	10	-443.00000*	70.84176	.043	-857.3191	-28.6809
	20	-1043.66667*	40.60241	.000	-1223.9652	-863.3682
	50	-182.33333*	37.51148	.035	-346.2297	-18.4370
10	0	443.00000*	70.84176	.043	28.6809	857.3191
	20	-600.66667*	72.88042	.016	-996.9863	-204.3471
	50	260.66667	71.20471	.144	-149.8833	671.2167
20	0	1043.66667*	40.60241	.000	863.3682	1223.9652

	10	600.66667*	72.88042	.016	204.3471	996.9863
	50	861.33333*	41.23240	.000	679.3548	1043.3119
50	0	182.33333*	37.51148	.035	18.4370	346.2297
	10	-260.66667	71.20471	.144	-671.2167	149.8833
	20	-861.33333*	41.23240	.000	-1043.3119	-679.3548

*. The mean difference is significant at the 0.05 level.

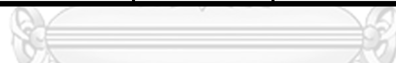
4. RNA analysis by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

4.1 Collagen I

ANOVA

COLI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.039	3	.013	5.050	.030
Within Groups	.021	8	.003		
Total	.060	11			



Multiple Comparisons

COLI

Tukey HSD

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	10	-.0477130417	.0416690102	.674	-.181151880	.085725797
	20	-.0462579853	.0416690102	.694	-.179696824	.087180853
	50	-.1561085637*	.0416690102	.023	-.289547402	-.022669725
10	0	.0477130417	.0416690102	.674	-.085725797	.181151880
	20	.0014550563	.0416690102	1.000	-.131983782	.134893895
	50	-.1083955220	.0416690102	.117	-.241834360	.025043316
20	0	.0462579853	.0416690102	.694	-.087180853	.179696824

	10	-.0014550563	.0416690102	1.000	-.134893895	.131983782
	50	-.1098505783	.0416690102	.111	-.243289417	.023588260
50	0	.1561085637*	.0416690102	.023	.022669725	.289547402
	10	.1083955220	.0416690102	.117	-.025043316	.241834360
	20	.1098505783	.0416690102	.111	-.023588260	.243289417

*. The mean difference is significant at the 0.05 level.

4.2 Fibronectin

ANOVA

FN

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	8.915	.006
Within Groups	.000	8	.000		
Total	.000	11			

Multiple Comparisons

FN

Tukey HSD

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	10	-.0012163560	.0024882581	.959	-.009184634	.006751922
	20	-.0037429710	.0024882581	.478	-.011711249	.004225307
	50	-.0116868393*	.0024882581	.007	-.019655118	-.003718561
10	0	.0012163560	.0024882581	.959	-.006751922	.009184634
	20	-.0025266150	.0024882581	.746	-.010494893	.005441663
	50	-.0104704833*	.0024882581	.013	-.018438762	-.002502205
20	0	.0037429710	.0024882581	.478	-.004225307	.011711249
	10	.0025266150	.0024882581	.746	-.005441663	.010494893

	50	-.0079438683	.0024882581	.051	-.015912147	.000024410
50	0	.0116868393*	.0024882581	.007	.003718561	.019655118
	10	.0104704833*	.0024882581	.013	.002502205	.018438762
	20	.0079438683	.0024882581	.051	-.000024410	.015912147

*. The mean difference is significant at the 0.05 level.

4.3 Interleukin-6

ANOVA

IL6

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	4.363	.042
Within Groups	.000	8	.000		
Total	.000	11			

Multiple Comparisons

IL6

Tukey HSD

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	10	-.0003663607	.0004937461	.878	-.001947509	.001214788
	20	-.0010143930	.0004937461	.246	-.002595542	.000566756
	50	-.0016534260*	.0004937461	.041	-.003234575	-.000072277
10	0	.0003663607	.0004937461	.878	-.001214788	.001947509
	20	-.0006480323	.0004937461	.581	-.002229181	.000933116
	50	-.0012870653	.0004937461	.116	-.002868214	.000294083
20	0	.0010143930	.0004937461	.246	-.000566756	.002595542
	10	.0006480323	.0004937461	.581	-.000933116	.002229181

	50	-.0006390330	.0004937461	.591	-.002220182	.000942116
50	0	.0016534260*	.0004937461	.041	.000072277	.003234575
	10	.0012870653	.0004937461	.116	-.000294083	.002868214
	20	.0006390330	.0004937461	.591	-.000942116	.002220182

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

IL6

Dunnett T3

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	10	-.0003663607	.0003232571	.812	-.001783019	.001050298
	20	-.0010143930	.0006419096	.611	-.004890041	.002861255
	50	-.0016534260*	.0002584298	.021	-.002902389	-.000404463
10	0	.0003663607	.0003232571	.812	-.001050298	.001783019
	20	-.0006480323	.0006486790	.866	-.004444997	.003148932
	50	-.0012870653	.0002748134	.060	-.002660303	.000086172
20	0	.0010143930	.0006419096	.611	-.002861255	.004890041
	10	.0006480323	.0006486790	.866	-.003148932	.004444997
	50	-.0006390330	.0006189307	.851	-.004883083	.003605017
50	0	.0016534260*	.0002584298	.021	.000404463	.002902389
	10	.0012870653	.0002748134	.060	-.000086172	.002660303
	20	.0006390330	.0006189307	.851	-.003605017	.004883083

*. The mean difference is significant at the 0.05 level.

4.4 Focal adhesion kinase

ANOVA

FAK

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	1.368	.320
Within Groups	.000	8	.000		
Total	.000	11			

Multiple Comparisons

FAK

Tukey HSD

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	10	.0015706027	.0037941130	.975	-.010579483	.013720688
	20	-.0018052263	.0037941130	.962	-.013955312	.010344859
	50	-.0057155663	.0037941130	.477	-.017865652	.006434519
10	0	-.0015706027	.0037941130	.975	-.013720688	.010579483
	20	-.0033758290	.0037941130	.810	-.015525914	.008774256
	50	-.0072861690	.0037941130	.292	-.019436254	.004863916
20	0	.0018052263	.0037941130	.962	-.010344859	.013955312
	10	.0033758290	.0037941130	.810	-.008774256	.015525914
	50	-.0039103400	.0037941130	.737	-.016060425	.008239745
50	0	.0057155663	.0037941130	.477	-.006434519	.017865652
	10	.0072861690	.0037941130	.292	-.004863916	.019436254
	20	.0039103400	.0037941130	.737	-.008239745	.016060425

4.5 Vascular endothelial growth factor (VEGF)

ANOVA

VEGF

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	.792	.531
Within Groups	.000	8	.000		
Total	.000	11			

Multiple Comparisons

VEGF

Tukey HSD

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	10	-.0010584363	.0012359827	.827	-.005016488	.002899615
	20	.0007249113	.0012359827	.933	-.003233140	.004682963
	50	-.0006367150	.0012359827	.953	-.004594767	.003321337
10	0	.0010584363	.0012359827	.827	-.002899615	.005016488
	20	.0017833477	.0012359827	.510	-.002174704	.005741399
	50	.0004217213	.0012359827	.985	-.003536330	.004379773
20	0	-.0007249113	.0012359827	.933	-.004682963	.003233140
	10	-.0017833477	.0012359827	.510	-.005741399	.002174704
	50	-.0013616263	.0012359827	.699	-.005319678	.002596425
50	0	.0006367150	.0012359827	.953	-.003321337	.004594767
	10	-.0004217213	.0012359827	.985	-.004379773	.003536330
	20	.0013616263	.0012359827	.699	-.002596425	.005319678

4.6 Basic Fibroblast growth factor

ANOVA

bFGF

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	7.813	.009
Within Groups	.000	8	.000		
Total	.000	11			

Multiple Comparisons

bFGF

Tukey HSD

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	10	-.0030144213	.0020992381	.514	-.009736921	.003708078
	20	-.0027290437	.0020992381	.588	-.009451543	.003993456
	50	-.0097549097*	.0020992381	.007	-.016477409	-.003032410
10	0	.0030144213	.0020992381	.514	-.003708078	.009736921
	20	.0002853777	.0020992381	.999	-.006437122	.007007877
	50	-.0067404883*	.0020992381	.049	-.013462988	-.000017989
20	0	.0027290437	.0020992381	.588	-.003993456	.009451543
	10	-.0002853777	.0020992381	.999	-.007007877	.006437122
	50	-.0070258660*	.0020992381	.041	-.013748365	-.000303367
50	0	.0097549097*	.0020992381	.007	.003032410	.016477409
	10	.0067404883*	.0020992381	.049	.000017989	.013462988
	20	.0070258660*	.0020992381	.041	.000303367	.013748365

*. The mean difference is significant at the 0.05 level.

VITA

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