การศึกษาผลของนมอัลมอนด์ต่อความมีชีวิตและการทำหน้าที่ของเซลล์ไฟโบรบลาสท์เอ็นยึดปริทันต์ ในสภาวะจำลองฟันที่หลุดออกจากเบ้า

EFFECTS OF ALMOND MILK ON THE VIABILITY AND FUNCTION OF PERIODONTAL LIGAMENT FIBROBLASTS IN AN IN VITRO TOOTH AVULSION MODEL

PHUTTIKARN SINPREECHANON

GRADUATE SCHOOL Srinakharinwirot University

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พฤติกานต์ สินปรีชานนท์

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PHUTTIKARN SINPREECHANON

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BY

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Author	PHUTTIKARN SINPREECHANON
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Until today, numerous storage media for avulsed tooth preservation have been studied but the ideal solution with all of the essential properties has not been identified. Almond milk is rich in several healthy nutrients with anti-inflammatory and anti-oxidative properties. This study aims to investigate the effects of almond milk on the viability, proliferation and functions of periodontal ligament fibroblasts (PDLF) *in vitro*. Human PDLF were culture media depleted for 5 minutes, stored in Hank's Balanced Salt Solution (HBSS), whole milk, low fat milk or almond milk for one hour at room temperature. Cell viability and proliferation were assessed by MTT assays. Genes expression levels of type I collagen and its modifying enzymes were analyzed by real-time PCR. Collagen matrix production was evaluated by Picrosirius red polarization. Our results showed the overall efficiency of low fat milk in maintaining PDLF viability and proliferation as well as in enhancing the process of collagen matrix production. Additionally, almond milk promoted the highest rate of cell proliferation along with the comparable ability of collagen biosynthesis to the control. Therefore, besides low fat milk, almond milk may potentially be an alternative tooth storage media for PDLF preservation and PDL tissue regeneration.

Keyword : Cell viability, cell proliferation, collagen matrix, dental trauma, storage media, tooth storage, almond milk

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INTRODUCTION

Background

Traumatic injury which causes the tooth avulsion from its socket results in tear of vein, nerve and periodontal ligament (PDL). It will also lead to vitality loss of pulp and periodontal ligament fibroblasts (PDLF). The recommended treatment is immediate replantation of the tooth because leaving the tooth in dry state for long periods of time will increase PDLF cells death. However, it is not always possible to immediately bring the tooth back in place at the scene of accident. Therefore, the method of tooth preservation is very important to the outcome of the treatment (1-3).

Replantation may save the tooth but it is important to realize that at a later stage, some of the replanted teeth have failed in long term and may even be lost or extracted (4). The success of replanting an avulsed tooth mainly depends on the vitality of the PDLF cells on the root surface. If the number of viable PDLF cells is low, it can lead to unfavorable PDL healing and, consequently, post-replantation complications as ankylosis or root resorption. The factors that affect the viability of PDLF are the time between avulsion and replantation, bacteria contamination and property of storage media. Therefore, the avulsed tooth should be stored in a proper medium before tooth replantation by a dentist (1, 2).

Extensive studies have been conducted in search for an ideal media solution to store the avulsed tooth with these characteristics i.e. physiological osmolality, a neutral pH, antimicrobial effects, does not trigger antigen-antibody reactions or post-reimplantation root resorption or ankylosis, should also be easily accessible and inexpensive (2, 5, 6). However, the storage medium which contains all these essential characteristics have not been identified (7, 8).

The effective storage media that the International Association of Dental Traumatology and American Academy of Pediatric Dentistry recommended are Hank's balanced salt solution (HBSS) and milk (4). HBSS has essential characteristics to support

cell survival, by being non-toxic, highly nutritive, has an appropriate pH balance and osmolality (2). HBSS has been commercially released as the Save-A-Tooth® box (Phoenix-Lazerus, Shartlesville, PA, USA) but the usage is still limited because it is not widely available and has inferior efficiency to the original one (9-11). On the other hand, bovine milk is another recommended tooth storage solution with neutral pH, physiological osmolality, abundant nutrients and growth factors, besides it is easily accessible and inexpensive (2, 4, 7, 12). However, there were reports that milk promotes some negative effects on the functions of PDL cells in maintaining or regenerating PDL tissue which may lead to complications such as ankylosis and inflammatory root resorption (13, 14).

Almond milk is a plant-based milk which has become a popular alternative to cow's milk because it contains several healthy nutrients and low saturated fat (15). By its property and being conveniently accessible, almond milk may possibly be an alternative storage media for avulsed tooth. To the best of our knowledge, there is still no report on the use of almond milk as a storage medium for avulsed tooth maintenance.

Regeneration of PDL tissue at the surface of the avulsed tooth is essential for proper healing of the replanted tooth. Type I collagen is the predominant fibrillar collagens which provide form and integrity for PDL tissues (16, 17). Studies have shown that collagen plays critical roles in the periodontal wound healing (18, 19). Collagen-specific modifications of lysine (Lys) residues are considered essential factors for the structural integrity and biomechanical functions of collagen (18, 20). In type I collagen, specific Lys residues are modified by enzymes lysyl hydroxylase (LH), glycosyltransferase 25 domain 1 (GLT25D1) and lysyl oxidase (LOX) (20, 21).

Nowadays, most of the studies on storage media are focusing on its ability to maintain cells viability and promote cells proliferation. The effects of tooth storage media on type I collagen biosynthesis by PDLF have not been directly investigated. Therefore, this study aims to investigate the effect of almond milk on the viability, cell proliferation and collagen biosynthesis of PDLF following an in vitro tooth avulsion simulation.

Objectives of the Study

The purpose of this study is to

1. Evaluate the effectiveness of almond milk in maintaining the viability and cell proliferation of PDLF in tooth avulsion model.
2. Evaluate the effect of almond milk on collagen biosynthesis by PDLF in tooth avulsion model.

Significance of the Study

If almond milk shows effective result in maintaining PDLF viability and proliferation along with promotes collagen synthesis as a suitable storage media compared to cow's milk. Then, almond milk could be recommended as an alternative solution for avulsed tooth's storage.

Scope of the Study

Population

Human PDLF

Research samples

PDLF in culture plates

Variables

1. Independent variables

1.1 Hank's balanced salt solution (Invitrogen, Carlsbad, CA, USA)

1.2 Whole milk, Ultra-High Temperature processed (UHT) (Foremost, Bangkok, Thailand)

1.3 Low fat milk, UHT (Foremost, Bangkok, Thailand)

1.4 Almond milk, unsweetened UHT (Almond Breeze, Bangkok, Thailand)

2. Dependent Variable

2.1 Viability of PDLF

2.2 Proliferation of PDLF

2.3 Expression level of gene COL1A2, PLOD1, PLOD2, PLOD3, GLT25D1 and LOX

2.4 Characteristics of collagen matrix from PDLF

Research Framework

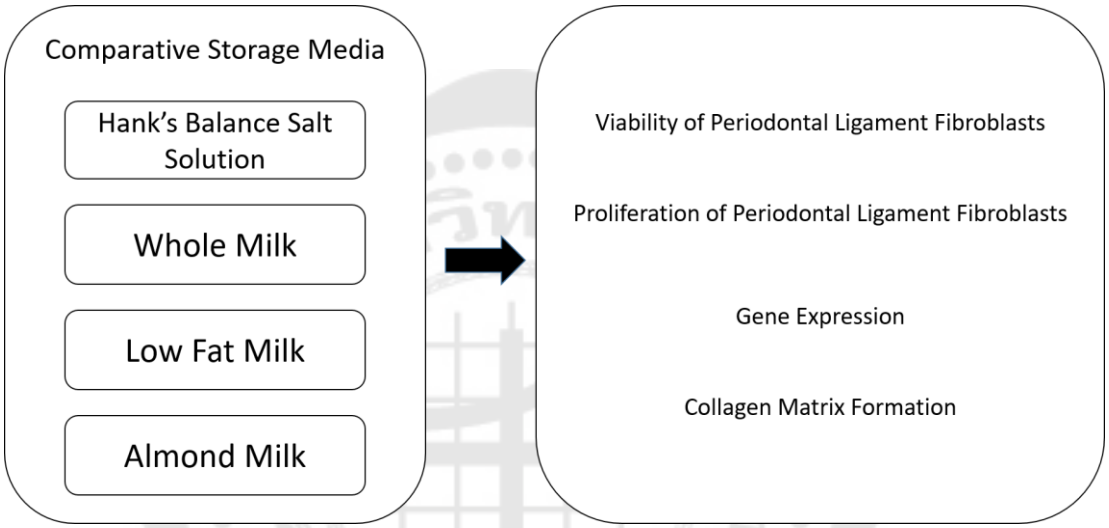

Figure 1 Research Framework

Hypothesis in research

Almond milk is effective in maintaining viability and cell proliferation of PDLF and promote collagen matrix formation by PDLF in tooth avulsion model.

CHAPTER 2

REVIEW OF THE LITERATURE

The researcher studied the related papers and presented in the following topics.

1. Tooth avulsion and management
2. Storage media
3. Almond milk
4. Periodontal healing process
5. Collagen biosynthesis
6. In vitro experiments for storage media evaluation
7. Principle of MTT assay
8. Collagen matrix analysis

Tooth avulsion and management

Tooth avulsion is one of the traumatic dental injury which result in complete displacement of the tooth out of its socket in alveolar bone, compromises the neurovascular supply to the PDL tissue and may also lead to vitality loss of pulp and PDLF. Replantation is the best treatment for avulsed tooth. But in a long term, replantation is likely to cause complications that led the tooth to be extracted afterward. Current guidelines are only recommendations to increase the chances in saving the tooth, if possible, without any treatment complications. The key is extra-alveolar time and damage of tissue around the root. Initial management for avulsed tooth is to pick the tooth by the crown and avoid touching its root. If the tooth is unhygienic, it should be washed by running water at most 10 seconds, then inserted back in its socket. If immediate replantation is impossible, it is suggested to place the avulsed tooth in milk or other suitable media such as Hank's balanced salt solution, tissue culture media or holding it inside the mouth and advise the patients to visit the dentists as soon as possible (1-4).

The most important factor for long-term treatment success, following tooth replantation, is the viability of remaining PDLF on the root surface. Viable PDLF will

function in the PDL repair and regeneration of the damaged part. Necrosis or lack of PDLF leads to ankyloses. Besides, the necrotic cells can cause inflammatory root resorption (22).

The complications after tooth replantation occur by the pathology from pulpal and periodontal reactions which suffer extensive damage during an extra-alveolar period and healing reactions (23). Pulp necrosis is the most common complication (24-26) and can usually be diagnosed after 2–4 weeks with shows apical radiolucency. In rare cases, revascularization of the pulp can occur, provided that replantation is carried out immediately (23). External root resorption can be classified into three various types including surface resorption, replacement resorption/ankylosis and inflammatory resorption (27). Surface resorption is considered a favorable type of periodontal healing because it is not progressive, self-limiting and shows repair with new cementum. It is sometimes seen in radiographs as small excavations on the root surface with normal width of adjacent PDL space (23). Ankylosis is a condition of direct root surface fusion to the alveolar bone during the remodeling process. Its etiology is related to the absence of vital PDL cells coverage on the root surface (22, 23). Radiographically, normal periodontal space disappears and the root substance is replaced with bone. In most cases, ankylosis can be first recognized 6 months or 1 year after replantation (23). Inflammatory resorption is a serious condition that trauma or bacteria contamination could induce resorption cavities on the root surface. This type of resorption is always associated with the presence of a necrosis and infected root canal (23, 28). The resorption process advances towards the root canal and can progress very rapidly until the entire root can be resorbed within a few months (23).

Immediate tooth replantation results significantly in a better PDL healing and risk reduction of root resorption than delayed replantation. Prompt action is needed when placing of avulsed tooth in a suitable storage media to minimize harmful effects and to improve its prognosis (29, 30). It has been reported that tooth replantation is mostly done between 1-4 hours following tooth avulsion. PDL fiber degeneration on the root surface represents the death of PDLF which leads to inflammatory root resorption. This is a major

cause of treatment failure (31). However, some studies indicated that the ability of storage media to preserve viability of PDLF is a more critical prognostic factor than the extra-alveolar time (31, 32).

Storage media

The ideal characteristics of storage media should be as follows maintaining PDLF viability, presenting mitogenic and clonogenic capacity, having compatible physiological pH and osmolality, inducing no antigen-antibody reactions, lowering risk of root resorption, being effective under different conditions and containing antimicrobial property (6).

From the review by Poi et al. (33), according to the efficacy, tooth storage media are classified into 4 groups. First, excellent storage media: Hank's Balanced Salt Solution, organ transplant storage solution such as Viaspan® and Euro-Collin®, Minimum Essential Medium, milk, Propolis and green tea. Second, good storage media: red mulberry, egg white, coconut water, Ricetral. Third, poor storage media: saline, Gatorade® and contact lens solution. The final group, very poor storage media: water and saliva. However, the International Association of Dental Traumatology and American Academy of Pediatric Dentistry recommended HBSS and milk as the effective tooth storage media.

HBSS, a developed solution for cell culture, can preserve tissue for a long duration by its ideal osmolality and pH proper for maintaining cell viability (33). Hwang et al. (34) reported that HBSS could preserve the viability of human PDL cells at over 94% after storage in this media for 24 hours which corresponds to the study of Kransner (3) that showed 90% cell viability after storage in HBSS in 24 hours. Furthermore, the research of Sigalas et al. (35) also showed that HBSS has a better efficacy than other storage media in maintaining cell viability. However, these results was obtained from laboratory environments where it is completely different from the actual site of accident. Since HBSS is not easily available, the commercial product was developed and released as Save-A-Tooth® box. This is a first aid kit that contains HBSS for storage of avulsed tooth, but it showed inferior efficiency than the original HBSS (9-11).

Bovine milk has several characteristics as an excellent storage medium for avulsed teeth. It has isotonic, neutral pH, physiological osmolality, low or no bacterial content, some growth factors and nutrients for cells. It is also widely available and inexpensive. Therefore, milk can be considered as the practical storage medium in most situations (7, 33). Several studies used bovine milk as a storage media for avulsed tooth. They reported that milk could preserve 70 – 90% viability of PDLF for 72 hours (11, 35, 36).

However, each type of bovine milk has a different efficacy as storage media. Marino et al. (37) reported that PDLF viability in regular pasteurized milk and long shelf-life (ultra-high temperature, UTH) milk had no significant differences at any time period. Pearson et al. (38) compared the efficacy of several milk substitutes (Reconstituted powdered milk, evaporated milk and two baby formulas included Similac® and Enfamil®) to whole milk in maintaining the viability of human PDL cells. At 8 hours, all substitutes performed worse than whole milk but Enfamil® was the best in maintaining cell viability at 1 to 4 hours. Another advantage of Enfamil® is that, it is in powder form that does not require special storage and has a shelf life of 18 months. Santos et al. (36) used powdered milk and UHT whole milk as storage media and found similar results. Saluja et al. (39) reported that low fat milk was a superior storage media in maintaining cells viability when compared to culture medium and high fat milk solution at 1, 2 and 6 hours which is consistent with the findings of Harkacz et al. (40) The fat content of milk somehow affected cell viability, in that higher fat level generated more cell damage. And Souza et al. (11) showed at 5°C, the effectiveness of skim milk was significantly better than whole milk at 120 hours but from 2 to 69 hours there was no significant difference.

The reported literature suggests that the presence of antigens in milk may interfere with the function of PDLF when the tooth is replanted (6). Choi et al. (14) demonstrated that exposure of PDL cells to milk is associated with an upregulated expression of several pro-inflammatory proteins and key antioxidant proteins via the activation of Nrf2/ARE pathway (NF-E2-related factor-2 / antioxidant response element pathway) in PDL cells which is a cytoprotective defense mechanism. There are also

studies in mice from Hasan et al. (13) which used HBSS, milk, and egg white as tooth storage media. In the media used for immersion, more PDL cells were remarked in milk than in the other solutions. After transplantation, PDL tissue in the milk group was significantly thinner than the other groups. Moreover, ankylosis was observed in more than half of the specimens submerged in milk. From Immunohistochemistry analysis, periostin and CD68 labeling suggested degradation of the extracellular matrix in the PDL.

Almond milk

Almonds have been recognized by the U.S. Food and Drug Administration (FDA) as a nutrient-rich food. They are high in proteins, fiber, vitamins, minerals, and monounsaturated fatty acids but low in carbohydrate. In addition, almonds also possess antioxidative and an anti-inflammatory properties (15). Nowadays, almond milk has become a popular plant-based alternative to cow's milk because it is nutritious and contains no lactose and has minimal saturated fat. Commercially available almond milk appears in a variety of brands and flavors that can be conveniently accessible. Almond milk is the milky white colloid solution obtained from the filtered mixture of grinded almonds with water. To maintain its stability and shelf-life, almond milk is further homogenized with high pressure and pasteurized (41). From thorough search of relevant literatures, there is still no any published studies that investigate the use of almond milk as a storage media for avulsed tooth maintenance.

Periodontium healing process

The periodontium is consists of four principal components, namely gingiva, cementum, alveolar bone and PDL (42). PDL is a mechano-responsive connective tissue that interposed between the root of a tooth and the inner wall of adjacent alveolar socket. The PDL exists in order to maintain mechanical resistance and support of mechanical load from masticatory forces. When a tooth is avulsed, almost all PDL tissue is completely torn. Therefore, the survival of replanted tooth is mainly depending on regeneration or re-establishment of PDL (43, 44).

The healing process is basically the same in all tissues but may vary clinically according to the tissues involved. Thus, wound healing after dental trauma is complicated by the unique anatomy and composition of the periodontium. The wound margins are not two opposing vascular gingival but compose the rigid nonvascular mineralized tooth surface. When periodontal tissue is injured, the healing process starts immediately to restore lost tissues to their original form and function. Regeneration and repair are terms used in healing (42, 44, 45).

Regeneration is the process of damaged tissue renewal to the original one by increase in size and number of cells. Proper tissue regeneration requires a population of precursor cells with the potential to differentiate and replicate. This process can completely restore the structure and functions of the tissue. On the contrary, repair is the process to regain tissue mass, in which its structure and functions may not be restored. Some tissues can be repaired to similar tissue as the original, but in some, abnormal tissue repair can occur through excess collagen deposition leading to scar tissue formation (fibrosis) (42, 45, 46).

Table 1 Periodontal wound healing responses by regeneration and repair (47).

Regeneration	Repair
Formation of new functional attachment, including formation of cementum, PDL and alveolar bone	Formation of long junctional epithelium
	Re-attachment of connective tissue to adjacent root surface
	New bone separation from the root surface by long functional epithelium, accompanied by root resorption, and/or ankylosis

Adapted from Connective Tissue Research. Narayanan AS, Bartold PM. Biochemistry of Periodontal Connective Tissues and their Regeneration: A Current Perspective. Connect Tissue Res. 1996;34(3):191-201. © Taylor & Francis.

Periodontal tissue healing events must progress in an ordered and programmed sequence. Immediately after replantation, a coagulum is found between the two parts of the teared PDL. Proliferation of connective tissue cells and some deposition of collagen matrix can be seen after 3 to 4 days, then the gap in the PDL is eliminated by young connective tissue. The maximum period of collagen synthesis is often seen on day 5 to 6 of healing and gradually decreased. Collagen fibers are randomly arranged pattern with two dominant types, including type I and type III collagen. After 1 week, the connective tissue is reattached at the cementoenamel junction. Gingival collagen fibers are usually spliced, while the infra-bony fibers are united in only a few areas and the first superficial osteoclast can be seen along the root surface. After 2 weeks, collagen fibers are extending from the cementum surface to the alveolar bone. The synthesis and degradation of collagen gradually reaches equilibrium. Type III collagen fibers that were initially produced are eventually replaced by type I collagen which becomes arranged in large collagen bundles and take a specific orientation to provide the healing tissue with increased the tensile strength (23, 46, 48, 49).

Collagen biosynthesis

Collagen is the most abundant structural protein in various connective tissues in the human body, representing about 30% of the total body proteins. At this time, at least 29 genetically distinct types of collagen have been identified in vertebrates and there are encoded by at least 44 genes (50). Fibroblasts are the major type of cells that are responsible for collagen production. In the PDL, which is a dense fibrous connective tissue, the most abundant collagens are presented as fibril and network-like structures. Studies have shown that collagen plays critical roles in the periodontal wound healing (18, 19). Fibril-forming collagens in PDL consisted of approximately 75% of type I, 20% of type

III, and 5% of type V collagens (16, 17). They function as structural scaffold that regulate the form, connectivity and strength of tissue. From this ability, PDL can resist against both tensile and compressive forces. The diameter of collagen fibrils in PDL is smaller than fibrils in other connective tissues because of the high turnover rate and the regulation of collagen synthesis from the non-collagenous components. In general, collagen fibrils in the PDL are not mineralized. They show a high level of glycosylation. But Sharpey's fibers that embedded in bone and cementum have a larger fibril diameter and are partially mineralized. The function of PDL mainly depends on the components that include both collagens and non-collagenous structures. These components have a site-specific composition and structural characteristics that could prevent or promote proper mineralization (18).

The most abundant collagen is type I collagen which provides most tissues and organs with form and mechanical properties. It is a heterotrimeric molecule with triple-helix structures containing three polypeptide chains of two α1 chains and one α2 chain, and is approximately 300 nm in length and 1.5 nm in thickness. The biosynthesis of type I collagen is a long and complicated process involving gene transcription, several post-translational modifications of proα chains, chains association and folding, procollagen processing to a triple-helix, secretion to extracellular matrix, a collagen molecule formation by enzymatic processing, self-assembly into a fibril and progressive covalent cross-linking for stabilization (18, 50). (Figure 2)

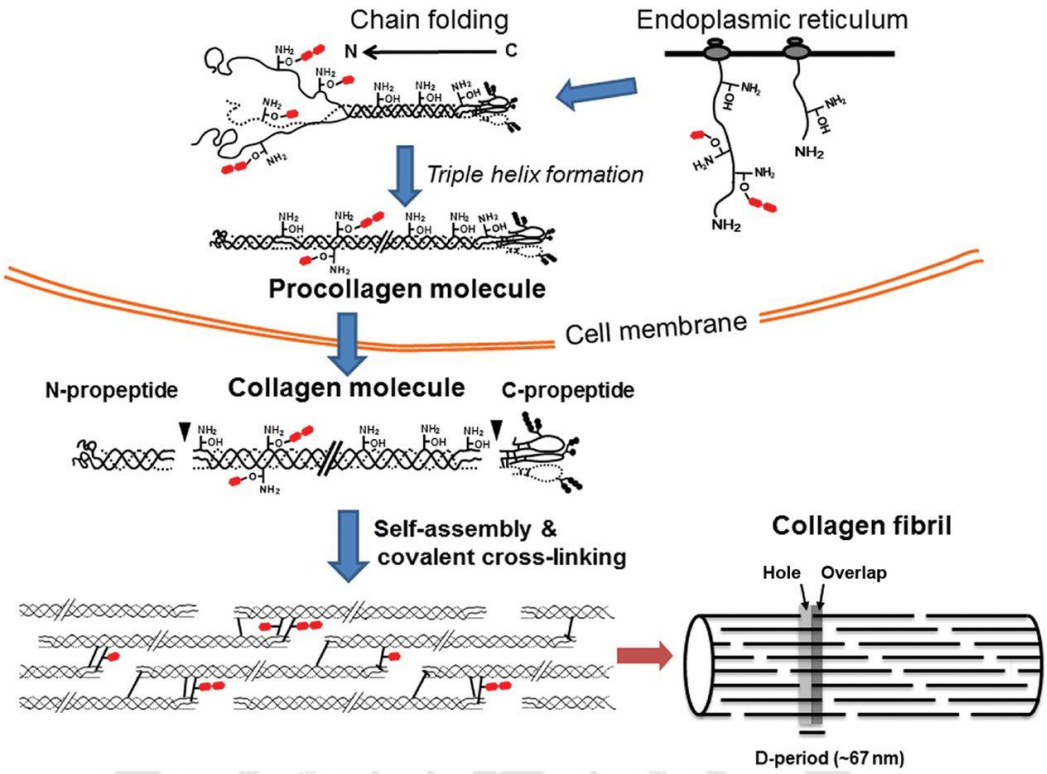

Figure 2 Type I collagen biosynthesis (50).

This research was originally published in Essays in Biochemistry. Yamauchi M, Sricholpech M. Lysine post-translational modifications of collagen. Essays Biochem. 2012;52:113-33. © Portland Press. DOI: 10.1042/BSE0520113.

During biosynthesis, the process that is crucial for the structural integrity and biomechanical functions of collagen fibrils are the various steps intra- and extracellular post-translational modifications. There are many types of enzymes that are involved in the modifications. Most of these enzymes are collagen specific and include their binding molecules and molecular chaperones (18, 50).

As mentioned above, the biosynthesis of type I collagen is a complex process but there are 2 major processes that affect the structure and function of collagen i.e. the formation of polypeptide chains and cross-linking. Numerous studies have shown that the

polypeptide chains formation of type I collagen is encoded by the *COL1A1* and *COL1A2* genes. The *COL1A1* gene translates into α1 chain while the *COL1A2* gene translates into α2 chain. On the other hand, formation of collagen cross-links depends on the expression of collagen modifying enzymes regulating the sequential steps of lysine (Lys) modifications. These modifications occur both intra- and extracellularly. In the cell, the formations lysyl hydroxylase (LH) family are essential for cross-linking pathways regulation. The expressions and functions of LHs determine the quality and completeness of collagen cross-links formation. The LHs enzymes are encoded by procollagen-lysine, 2-oxoglutarate 5-dioxygenase (*PLOD*) genes. Genes encoding the 3 isoforms of LH have been identified (*PLOD1–PLOD3*/LH1-3) and partially characterized. LH1 primarily hydroxylates Lys residues in the triple helical domains of collagens while LH2 specifically hydroxylates the Lys residues in the telopeptide domains. Some of these hydroxylysine (Hyl) residues are further potentially galactosylated by glycosyltransferase 25 domain 1 (GLT25D1) enzyme, encoded by the *GLT25D1* gene. As for LH3, it is a glucosyltransferase enzyme which mediated the glucosylation of galactosyl-Hyl. To initiate collagen cross-linking, Lys and Hyl residues in the telopeptides are oxidative deaminated and converted into the respective aldehyde forms, Lys^{ald} and Hyl^{ald} respectively, by an enzyme lysyl oxidase (LOX) which encoded by *LOX* genes. When aldehyde is formed, the rest of the reactions occurs spontaneous. These aldehydes then spontaneously form covalent bonds with other aldehydes or helical Lys and Hyl residues, resulting the formation of intra- and intermolecular cross-links. Therefore, the *LOX* gene is important at the Initiation stage of the cross-linking process (18, 50-53). In PDL tissue, collagen cross-links are mainly immature bi-functional cross-links that are formed by the condensation reaction between telopeptidyl Hyl^{ald} or Lys^{ald} with the helical Hyl87 residues which are fully glycosylated (16, 52). (Figure 3)

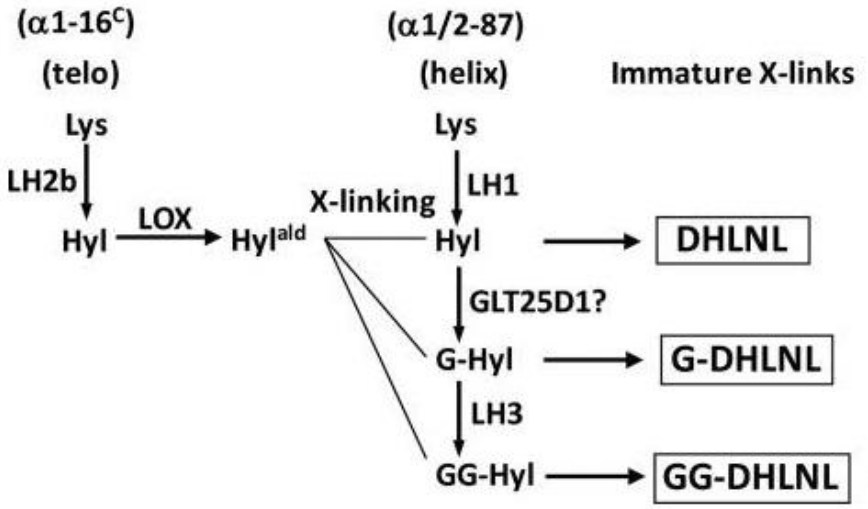

Figure 3 Type I collagen cross-linking pathway (54).

This research was originally published in the Journal of Biological Chemistry. Sricholpech M, Perdivara I, Yokoyama M, Nagaoka H, Terajima M, Tomer KB, Yamauchi M. Lysyl hydroxylase 3-mediated glucosylation in type I collagen: molecular loci and biological significance. J. Biol. Chem. 2012;287(27):22998-3009. © the American Society for Biochemistry and Molecular Biology.

In vitro experiments for storage media evaluation

There were great varieties in experimental methods used for the studies of tooth avulsion in vitro. Different factors in previous reports such as storage media source, dry-time, storage time and temperature led to variable results. Therefore, it was difficult to directly compare studies with another one. There are some examples of the in vitro methods utilized.

Table 2 In vitro experiments used for storage media evaluation

Studies	Sample	Dry-time	Storage time	Temperature
Rajendran et al., 2011 (55)	Extracted tooth	30 minutes	45 minutes	Unspecified
Saini t et al., 2016 (56)	Extracted tooth	20 minutes	30 minutes	Unspecified
Macway - Gomez et al., 2013 (57)	Isolation of PDLF prior to incubation with culture media	No	2, 6, 24, 48 hours	4ºC or 25ºC (Room temperature)
Ulusoy et al., 2016 (58)	Isolation of PDLF prior to incubation with culture media	No	1, 3, 6, 12, 24 hours	37ºC
Adeli et al., 2016 (59)	Isolation of PDLF prior to incubation with culture media	No	1, 2, 4, 24 hours	Unspecified
Yuan et al., 2018 (60)	Isolation of PDLF prior to incubation with culture media	No	1 hours	Room temperature

Principle of MTT assay

The MTT assay is a colorimetric assay that can assess cell metabolic activity based on the reduction of yellow tetrazole MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazoliumbromide) by dehydrogenases in active mitochondria of living cells. The MTT enters the viable cells and passes into the mitochondria. The active metabolism converts MTT into a purple colored formazan product which accumulates as insoluble precipitates in the cells and in the culture medium. The formazan crystals can be solubilized by a variety of solvents such as isopropanol, DMSO and dimethylformamide. Then the solubilized formazan reagent is measured spectrophotometrically. Since only metabolically active cells can reduce MTT, thus the positive absorbance level are markers of cells viability (61).

Collagen matrix analysis

Currently, several methods are being used to analyze collagen matrix such as immunoassays, high performance liquid chromatography (HPLC) or hydroxyproline assay. These methods are quite complex and costly but there is also an easier and more popular way to analyze collagen matrix which is Picrosirius polarization method. The Picrosirius polarization method is a histochemical study of collagen. Because the collagen bundle in PDL tissues are arranged in a parallel orientation and able to reflect light, therefore, the collagen fibers at different development levels can be visualized in different colors through the polarized light microscope. The reflected Red and Yellow color represent thick collagen fibers while green represents thin collagen fibers (62).

CHAPTER 3

METHODOLOGY

Research procedure

1. Population and sampling
2. Experimental procedure
3. Data collection
4. Data analysis

Population and sampling

Population

Human PDLF was isolated from PDL tissue scraped from the middle third of the root surface of healthy premolars which were extracted, with informed consent, according to orthodontics treatment plan. This study was approved by the Ethics Committee of the Faculty of Dentistry, Srinakharinwirot University (DENTSWU-EC27/2560).

Sample selection

PDLF from passage number 5-9 using 3-5 cell lines.

Experimental procedures

Cell Culture

PDLF culture was maintained in Dulbecco Modified Eagle's Medium high glucose (DMEM/HIGH, HyCloneTM) (GE Healthcare, UT, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA, USA), 2mM L-Glutamine (Invitrogen, Carlsbad, CA, USA), 100IU/ml Penicillin, 100ug/ml Streptomycin and 5ug/ml Amphotericin B (Invitrogen, Carlsbad, CA, USA), and incubated in 5% CO_2 atmosphere at 37ºC. PDLF from passages 5-9 were used in this study.

Cell Viability Assay

PDLF were trypsinized and seeded in 96-well culture plates at $1x10^4$ cells/well/ 100 µl of culture media and incubated at 37°C with 5% CO_2 for 24 hours for cells attachment. Simulated tooth avulsion was performed by complete removal of culture medium from each well and leaving the plates under hood at room temperature for 5 minutes. Next, 100 µl of each storage media (HBSS, whole milk, low fat milk and almond milk) was added to the wells in quintuplicates and the plates were left at room temperature for 1 hour. PDLF, from which the culture media (DMEM) was not removed, was assigned as the control. After the cells were immersed in the storage media for 1 hour, the solution was completely aspirated and the cells were rinsed twice with 200 µl/well of phosphate buffered saline (PBS). To determine cell viability (Day 0), MTT powder (USB Corporation, Cleveland, OH, USA) was dissolved in PBS at the concentration of 5 mg/ml and mixed, at the ratio of 10:100 µl, with serum-free DMEM. The MTT/serum-free DMEM solution at 110 µl was added to each well. Following the 30 minutes incubation of the plate at 37°C, the solution was carefully aspirated from the wells and 100 µl of dimethyl sulfoxide (DMSO) was then added to dissolve the formazan crystals formed. Cell viability was evaluated by measuring the optical density at 550 nm on a microplate reader (Asys UVM340, Biochrom, Cambridge,UK). Mean absorbance values from each condition were calculated as percentages relatively to the DMEM control.

Cell Proliferation Assay

To evaluate the effect on cell proliferation, PDLF was plated in 96-well culture plates at $1x10^4$ cells/well/ 100 µl of culture media. (3 plates for 3 time points, on day 1, 3 and 5 after the tooth avulsion simulation.) Incubated the plates at 37°C in CO_2 5% for 24 hours to allow cells attachment. Completely remove the medium from each well and leave the plate at room temperature for 5 minutes except the control wells. Next, 100 µl of the test storage media (HBSS, Whole milk, low fat milk and almond milk) was added to the wells in 5 replicates and the plate was kept at room temperature. After the cells were soaked in storage media for 1 hour, the medium was totally removed and the cells were rinsed twice with PBS 200 µl/well, added the fresh culture media 100 µl to each well and

were incubated at 37°C with 5% CO_2. MTT assays were performed to assess the PDLF viability at Day 1, 3 and 5. Cell proliferation were calculated by using slope formula from 2 time points, Day 0 and Day 5, which shown as percentages relatively to the control.

Gene Expression Analysis by Quantitative Real-time PCR

PDLF were seeded at the density of $3x10^5$ cells/well/2 ml of culture media in a 6-well plate and incubated at 37°C in 5% CO_2 for 24 hours to allow cells attachment. Next, the culture media was completely removed from each well except in the well assigned as the control, and the plate was left at room temperature for 5 minutes. Two milliliters of the test storage media (HBSS, whole milk, low fat milk and almond milk) were then added to the cells, stored at room temperature for 1 hour, followed by complete aspiration and rinse with PBS. Fresh culture media was added to the wells and the plate was placed back into the 37°C incubator. After 24 hours, total RNA was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and 1µg of RNA was converted into cDNA with ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA). Quantitative real time polymerase chain reaction (real time PCR) reactions were prepared with LightCycler 480 SYBR Green I master (Roche Diagnostics, Mannheim, Germany), specific primers for type I collagen and its modifying enzymes (table 3) and analyzed with the LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany). Gene expression levels relatively to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were analyzed by using the $2^{-\Delta\Delta CT}$ method and shown as fold change relatively to control.

Table 3 The sequence-specific primers for type I collagen and its modifying enzymes.

Gene		Sequence-specific primer
COL1A2 (63)	Forward	5'-TAATGGGGAAGCTGGATCTG-3'
	Reverse	5'-GTCCCTGAGCACCATTGTTT-3'
PLOD1 (64)	Forward	5'-GAAGCTCTACCCCGGCTACT-3'
	Reverse	5'-CTTGTAGCGGACGACAAAGG-3'
PLOD2 (64)	Forward	5'-GGGAGTTCATTGCACCAGTT-3'
	Reverse	5'-GAGGACGAAGAGAACGCTGT-3'
PLOD3 (64)	Forward	5'-CACTACGGCCAGTGGTCAG-3'
	Reverse	5'-GTGGGCACATTCTCGTAGC-3'
GLT25D1 (65)	Forward	5'-GATGCTGCCTGTGGACGAGTTC-3'
	Reverse	5'-CTCACATAGCCATCGTCTCCTG-3'
LOX (66)	Forward	5'-CGCTGTGACATTCGCTACACAGGAC-3'
	Reverse	5'-CATTGGGAGTTTTGCTTTGCCTTCT-3'
GAPDH (67)	Forward	5'-GCTCTCCAGAACATCATCC-3'
	Reverse	5'-GTGTCGCTGTTGAAGTCAG-3'

Collagen Matrix Analysis by Picrosirius Polarization

For collagen matrix production, PDLF were plated in 8-well cell culture slide (SPL Life Sciences, Korea) at the density of $4x10^4$ cells/ well / 500 µl of culture media. After the 24 hours incubation at 37°C with 5% CO_2, culture media was removed from each well and the culture slide was placed at room temperature for 5 minutes. Then, 500 µl of storage media (HBSS, whole milk, low fat milk and almond milk) were added to each well. PDLF in the well that the culture media was not removed would serve as the control. After the cells were immersed in storage media for 1 hour at room temperature, the solution was completely removed, the cells were washed twice with PBS, and 500 µl of fresh culture media was added to the wells. The culture slide was incubated at 37°C in 5% CO2 and maintained with twice a week change of culture media. After 1 week, PDLF were

further maintained in the culture medium containing 50 µg/ml ascorbic acid to induce collagen matrix production. At the end of 2 weeks, the cell/matrix layers were rinsed with PBS, fixed with 10% formaldehyde for 30 minutes and stained with Picrosirius Red. (0.1% of Sirius red in saturated aqueous picric acid, Bio-optica, Milano, Italy) for 30 minutes. Subsequently, the cell/matrix layers were washed once and stored in distilled water, with rocking motion for overnight, to remove nonspecific binding. The next day, the matrix layers were dehydrated with gradient increase of ethanol and xylene, followed by coverslip mounting and left to dry prior to analysis. The stained collagen matrix layers were observed under a polarized light microscope (Motic BA310E, Motic, Kowloon, Hongkong). Digital RGB images of the matrices were captured in 9 areas/condition (Moticam 580INT, Motic, Kowloon, Hongkong) and subjected to ImageJ software (NIH, Bethesda, MD, USA) to quantitate the area of red and orange color, which represents mature collagen fibers (34, 35). Each color was binarized by using a fixed threshold value and the area of positive pixels was determined (detail method shown in appendix 2). Total red and orange areas from each condition were demonstrated as percentage of area relatively to the control.

Data Collection

1. Evaluate the viability and proliferation of PDLF from the absorbance at 550 nm wavelength in MTT assay.
2. Evaluate the levels of gene expression by performing real-time PCR and comparing the expression using $2^{-\Delta\Delta CT}$ method. (Ct value is the number of cycles required for the signal to cross the threshold)
3. Evaluate the characteristic of collagen matrix by determining calculated the average percentage of mature collagen matrix area from the orange-red color reflection of collagen fibers observed with polarized light microscopy.

Data Analysis

The experiments were repeated at least 3 times to provide enough evidences for accurate and reproducible results. Statistical analyses were carried out with SPSS Statistics for Windows (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). Statistical differences were established with Kruskal-Wallis one-way ANOVA and multiple comparison of the means by Tukey's test. The data were shown as means ± SEM and p value of less than 0.05 was considered significant.

CHAPTER 4

RESULTS

To investigate the effect of almond milk on the viability and functions of PDLF in an in vitro tooth avulsion model. The researcher conducted the research by studying according to the procedures and evaluating accordance with the stated objectives as follows

1. Cell viability
2. Cell proliferation
3. Expression of Genes Encoding Type I Collagen and Its Modifying Enzymes
4. Collagen Matrix Production

Cell Viability

The levels of PDLF viability, following tooth avulsion simulation and storage media exposure for 1 hour, relatively to the DMEM control are shown in Figure. 4. Cell survival among the groups were significantly different ($P = 0.0007$). Whole milk and low fat milk could preserve the viability of PDLF at the level of 87.8% and 90.4% respectively, which were almost comparable to the control (DMEM 100%). There was a significantly lower number of viable PDLF (74%) in almond milk when compared to that of the control ($P < 0.01$). However, there were no significant differences between whole milk, low fat milk and almond milk. On the other hand, HBSS demonstrated the lowest level of cell survival (63.4%) and showed significant differences when compared to the control, whole milk and low fat milk ($P < 0.001$, $P < 0.01$ and $P < 0.01$ respectively).

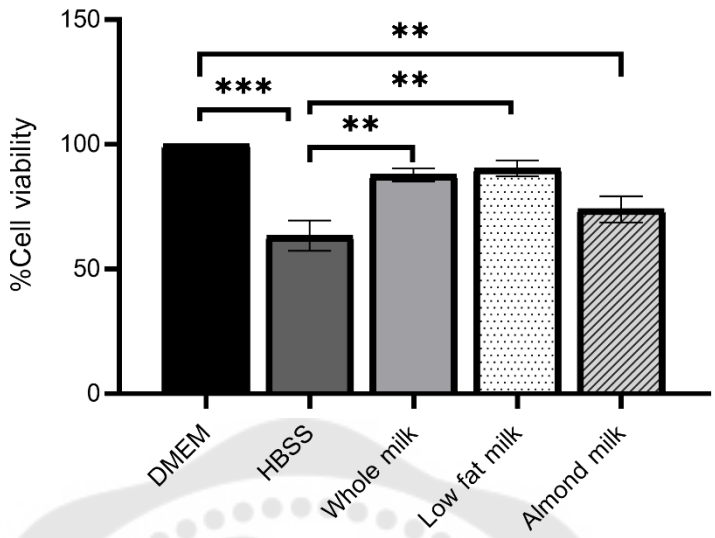

Figure 4 PDLF viability. Bar graph demonstrates percentages of cell viability relatively to the DMEM control. Data are shown as mean ± SEM (standard error of mean) from 5 independent experiments. **, significantly different at $P < 0.01$;
***, significantly different at $P < 0.001$.

Cell Proliferation

The ability of the survived PDLF to proliferate was determined by MTT assays at day 1, 3 and 5 after culture media removal and 1 hour storage of cells in the test solutions. The proliferation patterns of PDLF in each condition were shown as line graph plots in Figure 5.

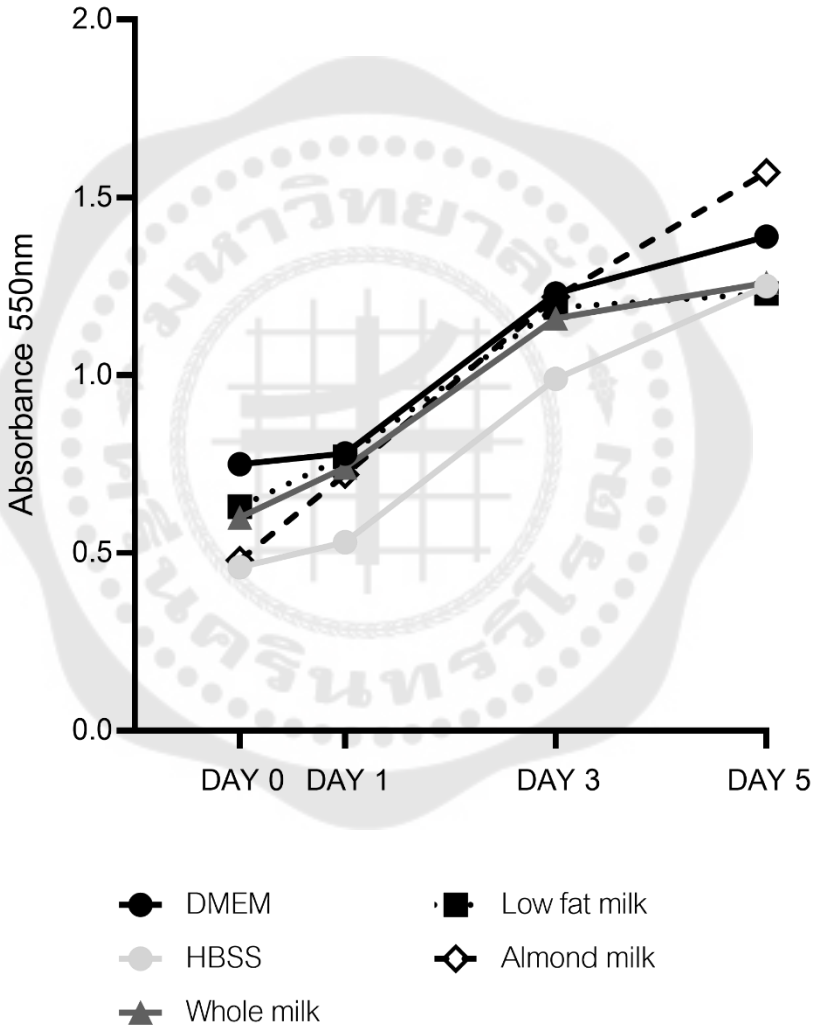

Figure 5 PDLF proliferation pattern. PDLF viability was mesured at Day 0,1, 3 and 5 following culture media depletion for 5 minutes and storage media immersion for 1 hour. Data are shown as mean absorbance values from 1 representative experiment.

The bar graph plots in Figure 6 represent the cell proliferation in percentages relatively to DMEM control. PDLF stored in almond milk demonstrated the highest percentage of cell proliferation (165.20%) which was significantly different from those in the other groups ($P < 0.001$). The proliferation of PDLF previously stored in HBSS (118.6%), whole milk (107%) and low fat milk (102.2%) were somewhat higher than those in the DMEM control (100%) but there were no statistically significant differences between the groups.

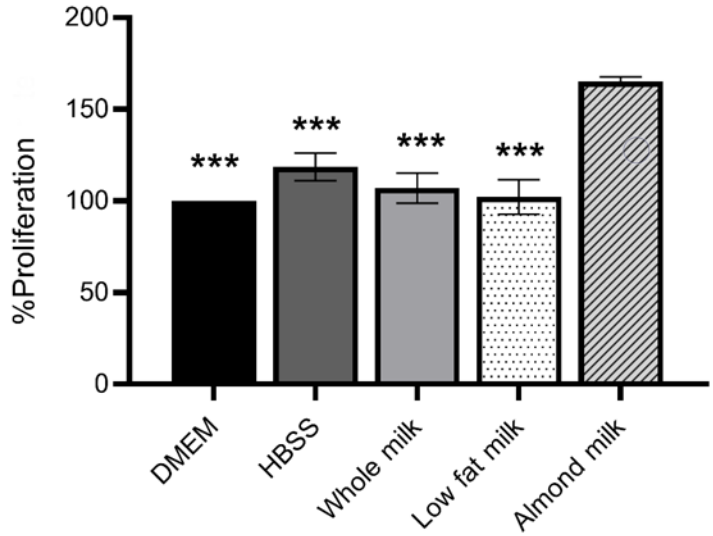

Figure 6 PDLF proliferation. Bar graph demonstrates average percentages of cell proliferation from Day 0 to Day 5 relatively to the DMEM control.
Data are shown as mean ± SEM from 5 independent experiments.
***, significantly different from almond milk at $P < 0.001$.

Expression of Genes Encoding Type I Collagen and Its Modifying Enzymes

The expressions of *COL1A2, PLOD1-2-3, GLT25D1* and *LOX* in PDLF at 24 hours after tooth avulsion simulation and immersion of cells in various storage media for 1 hour were analyzed by quantitative real time PCR. The mRNA expression levels relatively to GAPDH were normalized to the DMEM control. The expression levels of *COL1A2*, encoding type I collagen, among the groups were significantly different ($P = 0.0043$) (Figure 7). From multiple comparison, PDLF previously stored in almond milk showed approximately 50% downregulation of *COL1A2* expression which was significantly different from those placed in low fat milk and the DMEM control ($P < 0.05$). In addition, *COL1A2* expression was also downregulated in PDLF placed in HBSS and whole milk, however, the differences were not statistically significant from the other groups.

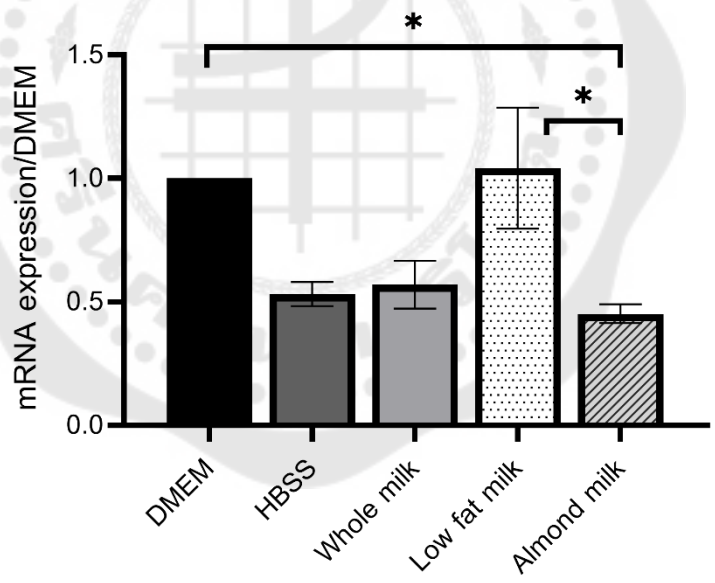

Figure 7 Expression levels of *COL1A2* encoding type I collagen. Bar graph demonstrates the mRNA expression levels of *COL1A2* relatively to the internal control, *GAPDH*, that were normalized to the DMEM control. Data are shown as mean ± SEM from 5 independent experiments. *, significantly different at $P < 0.05$.

As for the genes expression of collagen modifying enzymes, we assessed the expressions of the *PLOD* family, *GLT25D1* and *LOX. PLOD1* expression was comparable among all the storage media conditions (P = 0.2926) (Figure 8).

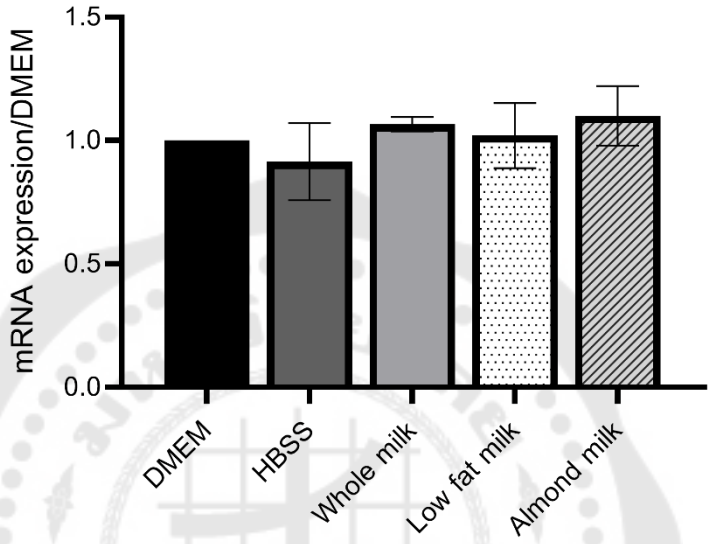

Figure 8 Expression levels of *PLOD1* encoding lysyl hydroxylase 1. Bar graph demonstrates the mRNA expression levels of *PLOD1* relatively to the internal control, *GAPDH*, that were normalized to the DMEM control. Data are shown as mean ± SEM from 5 independent experiments.

The expression levels of *PLOD2* was highest in PDLF exposed to low fat milk which was significantly different from those stored in HBSS and whole milk ($P < 0.001$). Moreover, the expression of *PLOD2* was downregulated in the whole milk group which was significantly different from the almond milk group and DMEM control ($P < 0.05$) (Figure 9).

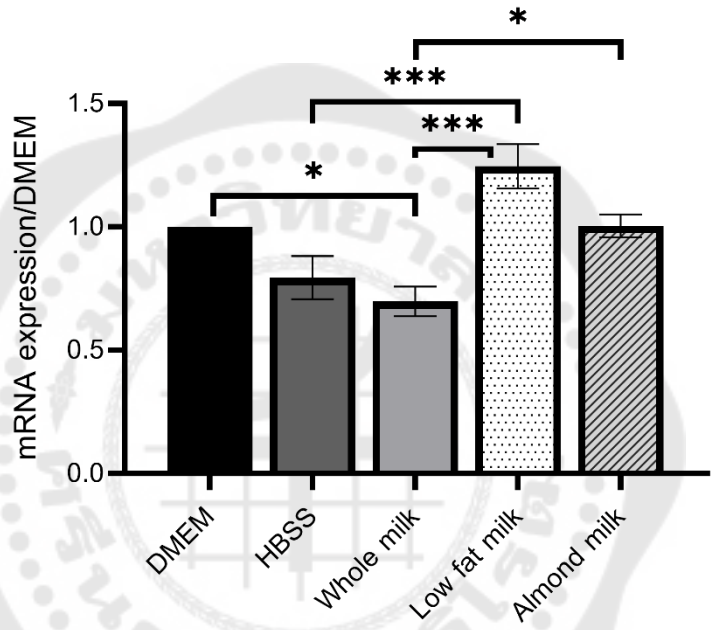

Figure 9 Expression levels of *PLOD2* encoding lysyl hydroxylase 2. Bar graph demonstrates the mRNA expression levels of *PLOD2* relatively to the internal control, *GAPDH*, that were normalized to the DMEM control. Data are shown as mean ± SEM from 5 independent experiments. *, significantly different at $P < 0.05$; ***, significantly different at $P < 0.001$.

As for *PLOD3* expression, there was a slight upregulation in PDLF previously placed in almond milk, however the difference was not statistically significant when compared with the other groups (P = 0.2218) (Figure 10).

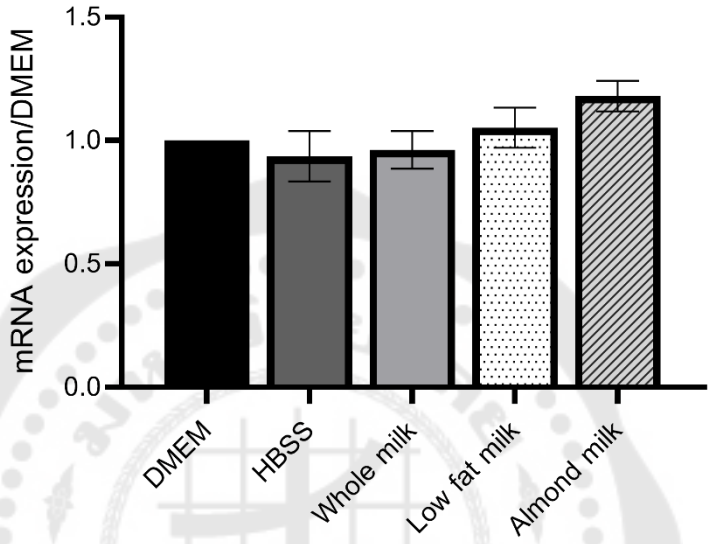

Figure 10 Expression levels of *PLOD3* encoding lysyl hydroxylase 3. Bar graph demonstrates the mRNA expression levels of *PLOD3* relatively to the internal control, *GAPDH*, that were normalized to the DMEM control. Data are shown as mean ± SEM from 5 independent experiments.

Next, the expressions of *GLT25D1* from all storage solutions were significantly different (P = 0.0179). However, the highest expression level was observed in the whole milk group, which was significantly different when compared to those in the DMEM (P < 0.05) and HBSS group (P < 0.01). The expression levels of *GLT25D1* in PDLF placed in low fat milk and almond milk showed an increasing trend, but they were not significantly different from those stored in the other media (Figure 11).

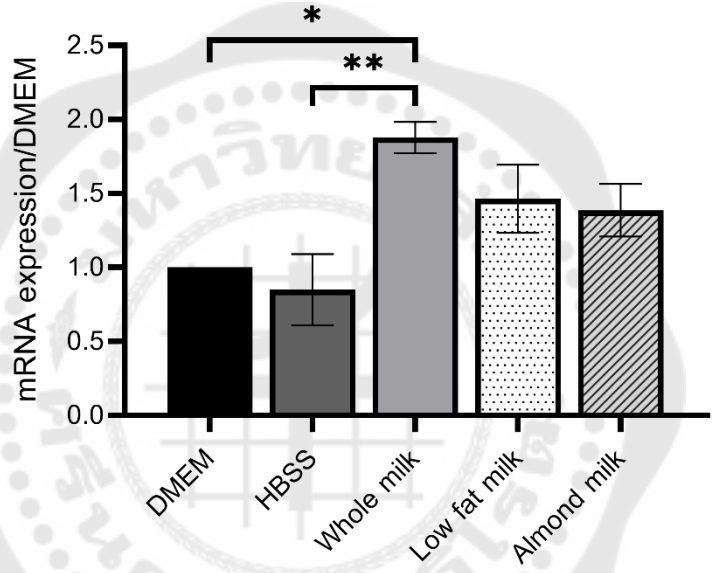

Figure 11 Expression levels of *GLT25D1* encoding glycosyltransferase 25 domain 1. Bar graph demonstrates the mRNA expression levels *GLT25D1* relatively to the internal control, *GAPDH*, that were normalized to the DMEM control. Data are shown as mean ± SEM from 5 independent experiments. *, significantly different at P < 0.05; **, significantly different at P < 0.01.

Lastly, the expressions of *LOX* among the groups were significantly different (P = 0.0113). PDLF immersed in low fat milk showed the highest expression level of *LOX*, which was significantly different from those in whole milk, HBSS and DMEM ($P < 0.05$). Expression of *LOX* in the almond milk group was slightly upregulated as well, however the differences when compared to the other groups were not statistically significant (Figure 12).

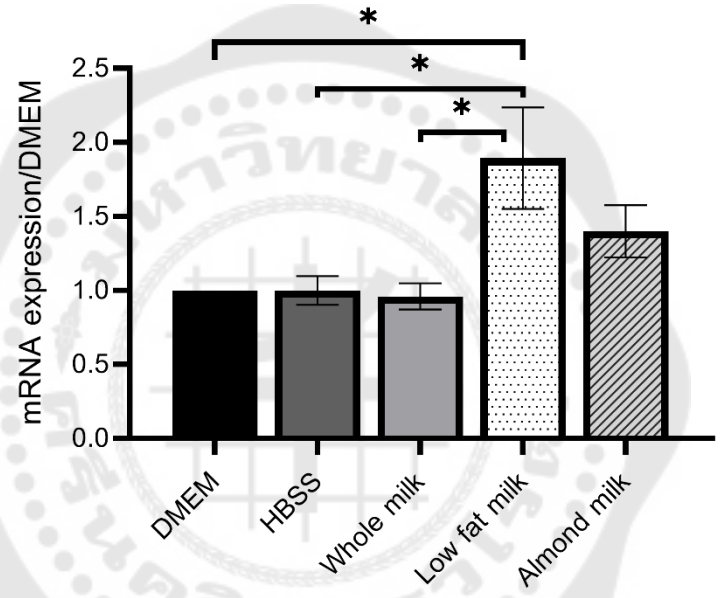

Figure 12 Expression levels of *LOX* encoding lysyl oxidase. Bar graph demonstrates the mRNA expression levels of *LOX* relatively to the internal control, *GAPDH*, that were normalized to the DMEM control. Data are shown as mean ± SEM from 5 independent experiments. *, significantly different at $P < 0.05$.

Collagen Matrix Production

The density and maturity of collagen fibers, produced by PDLF in each storage conditions, were analyzed from the Picrosirius red-stained cell/matrix layers observed under polarized light microscope. A representative set of picrosirius red stained matrices with its quantification of total red and orange color areas are shown in the bar chart (Figure 13). Comparing to the DMEM control (100%), there were more areas of red and orange-stained collagen fibers laid down by PDLF stored in almond milk (103%) and low fat milk (113%), which denoted thicker and more mature collagen fibers. On the other hand, total red and orange-stained areas were lesser in the whole milk (90.8%) and HBSS (79.5%) group, thus implying the production of thinner and less mature collagen fibers.

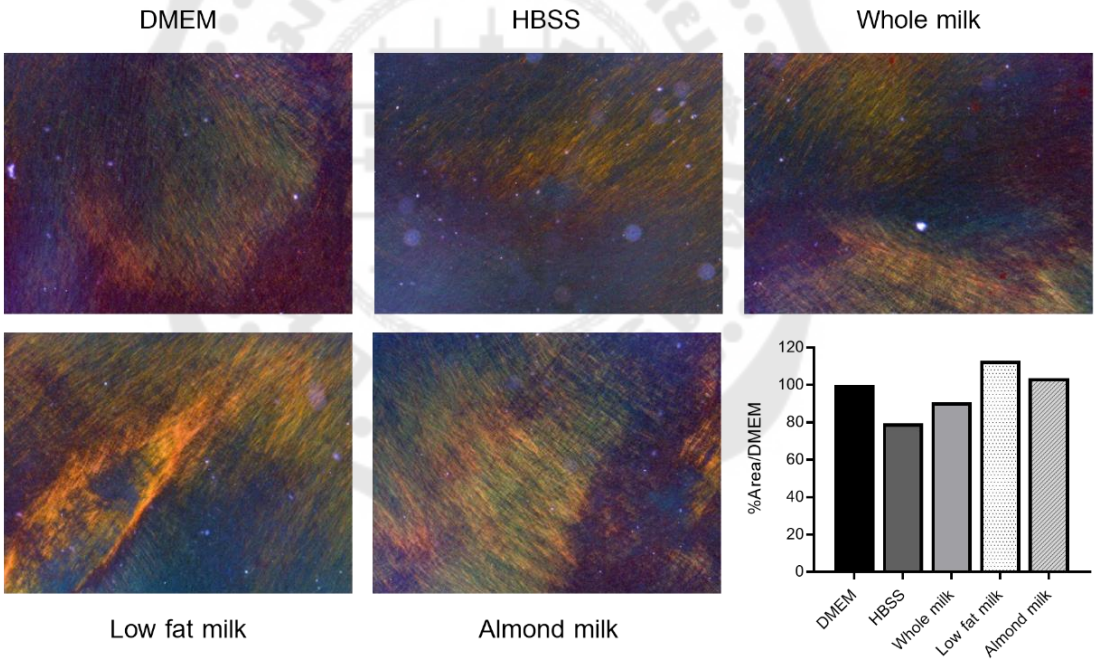

Figure 13 Picrosirius red stained collagen matrices. Representative images of stained collagen matrices observed under polarized light microscope, were shown along with a bar graph showing the amount of red and orange color measured from 9 areas per condition shown as percentages relatively to DMEM.

From 5 independent experiments, the amount of red-orange color areas among the groups were not significantly different (P = 0.0678) However, on average, there is a trend of higher level of red-orange color area in the collagen matrix of the low fat milk group when compared to the comparable levels between the other groups (Figure 14).

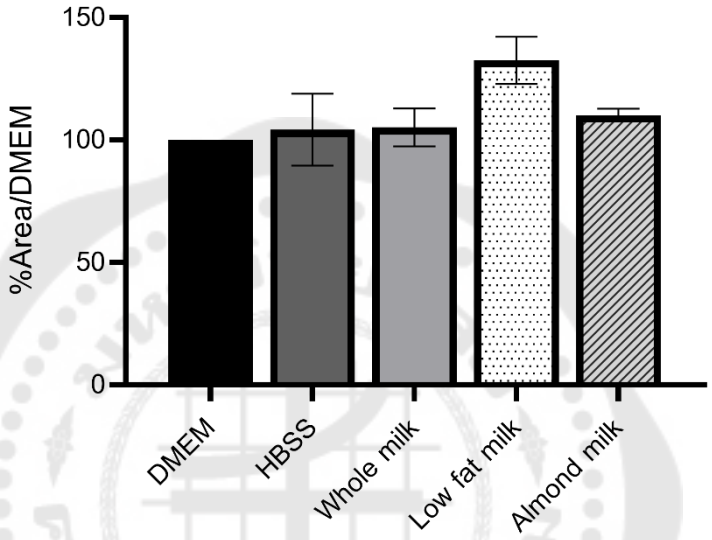

Figure 14 Quantification of red and orange color from picrosirius red stained matrices. The colors area were quantified from 9 regions/conditions and shown as percentages relatively to DMEM. Data are shown as mean ± SEM from 5 independent experiments.

CHAPTER 5

CONCLUSION DISCUSSION and SUGGESTION

The researcher evaluated the effectiveness of almond milk as a tooth storage media. From the results, we can conclude by the topics in the summary as follows.

1. Conclusion
2. Discussion
3. Suggestion

Conclusion

In conclusion, our results indicate that low fat milk has the overall capabilities in maintaining PDLF viability and proliferation, and a higher tendency of modulating the formation of thick and mature collagen matrix by PDLF, in an in vitro tooth avulsion simulation. In addition, storing PDLF in almond milk resulted in significant enhancement in cell proliferation and comparable collagen matrix production to the control. Therefore, almond milk could potentially be an alternative storage media for avulsed tooth.

Discussion

Long-term survival of the replanted tooth mainly depends on optimum regeneration of PDL which may prevent the occurrence of post-replantation root resorption or ankyloses (43). The remaining viable PDLF on the root surface is one of the key factors influencing the success of tooth replantation. Viability of the cells is essentially governed by the extra-alveolar time, damage degree of supporting tissue around the root and tooth storage condition (1, 4). To date, various tooth storage media are still being tested in order to find a proper solution that can preserve the vitality and functions of PDLF, promote PDL regeneration and prevent post-replantation complications. So far, HBSS and milk are still the recommended tooth preserving solutions from their characteristics and capabilities in maintaining the viability of PDLF (4, 7, 8, 12).

Nevertheless, each of them has some disadvantages in that HBSS is not easily accessible at the scene of accidents while milk has been shown with some negative effects on the cells and the formation of PDL tissue (13, 14).

From previous studies, the efficacy of various storage media in maintaining PDLF viability in vitro were mainly determined either by testing the storage solution on cultured PDL cells or by direct immersion of extracted tooth in the media and followed by viable cells quantification (8, 12). Discrepancies in the performance of various storage media between different studies may arise from the storage method chosen and other experimental factors including exposure duration, temperature, various source of storage media and the assessment methods (2, 7, 12). In this study, the efficiency of almond milk and cow's milk were investigated in maintaining the viability and functions of cultured PDLF following a tooth avulsion simulation in vitro. In most studies using this particular model, a dry-time period was not included in the experimental methods. Following cell attachment to the plate, the culture media was removed and immediately replaced with the test solutions (35, 58, 68-73). On the contrary, a dry-time period was added to better mimic the avulsed tooth condition by complete removal of the culture media for 5 minutes followed by immersing the cells in various storage media for 1 hour at room temperature. The vitality of cells placed in both whole and low fat cow's milk were significantly higher than those in HBSS. These outcomes agree with previous reports, using similar experimental method, showing that milk was more capable than HBSS in maintaining PDLF viability in vitro (58, 60, 68, 70). However, in some studies, the efficacy of milk is either lower than (34, 35) or comparable (69, 71-73) to HBSS. Interestingly, the proliferative ability of the survived PDLF was rarely assessed in previous studies. However, we have observed that PDLF stored in both types of cow's milk had comparable proliferation when compared to the control. Our result is consistent with the report by OIKARINEN et al. (74) showing that, comparing to the control, low fat milk did not alter the proliferative capacity of PDLF. On the contrary, SIGALAS et al. (35) have demonstrated the lowest cell number increase in PDLF stored in low fat milk at room temperature. The variabilities in the outcomes observed in previous reports and ours may result from the

different origins and components in the milk tested. By immersing PDLF in almond milk, slightly lower cell survival was detected when compared with those in cow's milk and the DMEM control, but still higher than in the HBSS group. But, surprisingly, PDLF previously placed in almond milk showed the highest cell proliferation among all the test solutions and control. This significant increase in cell number would certainly be advantageous for periodontal tissue regeneration which may promote the success of tooth replantation.

Type I collagen is the most predominant fibrillar collagen which provides tensile strength to the PDL tissue (16, 17). The capability of the survived PDLF at the root surface of avulsed tooth to produce strong collagen matrix may ensure long-term success following tooth replantation. In this study, we have evaluated the effects of different types of milk on the mRNA expression levels of type I collagen and their modifying enzymes in PDLF, along with the maturity of the collagen matrices formed. Comparing to the DMEM control, the expression of *COL1A2* which encodes the pro-alpha2 chain of type I collagen was comparable in PDLF stored in low fat milk while it was down-regulated in the cells placed in HBSS, whole milk and almond milk. Considering the lowered *COL1A2* expression observed in some storage conditions, lower collagen protein production might be expected. However, studies have shown that there is an unmatched temporal expression levels of collagen gene and the amount of collagen protein deposition in the extracellular matrix. Collagen mRNA expression is high during the early stages of culture and gradually decrease overtime, while collagen protein in the matrix is initially low and slowly increase thereafter (75). The inconsistency occurred may be due to the complex sequential steps of post-translational modifications of the procollagen **α**-chains, which are crucial for collagen fibrillogenesis and cross-links formation (76). Therefore, to fully elucidate the effects of storage media on collagen biosynthesis by PDLF, in our study, mRNA expression of collagen modifying enzymes were also analyzed. The modifications of specific helical Lys residues in type I collagen are sequentially modulated by LH1, GLT25D1 and LH3 encoded by *PLOD1*, *GLT25D1* and *PLOD3* respectively. From our results, the expressions of *PLOD1* among the storage media groups may reflect the minimally-altered levels of Lys hydroxylation into Hyl. In the next step of Hyl

galactosylation, the significant upregulation of *GLT25D1* in PDLF stored in whole milk may imply the increase levels of galactosyl-Hyl. Consequently, addition of glucose units to the galactosyl-Hyl residues, catalyzed by LH3, could be enhanced in the whole milk group as well. Possibly, it could be due to the potential increase in the substrate, galactosyl-Hyl residues, along with the essentially unchanged levels of LH3 from the comparable expression levels of PLOD3 among the groups. Previous studies have identified 5 specific loci with various degree of Hyl glycosylation in type I collagen α-chains, in which 2 of those sites, α1-Hyl87 and α2-Hyl87, are involved in the formation of intermolecular cross-links (54, 77). In type I collagen of human PDL, those two helical cross-linking sites were found to be 100% di-glycosylated (52). Thus, increase level of the potential Hyl galactosylation enzyme, GLT25D1, would not affect these two sites but may increase the level of Hyl glycosylation in other remaining sites. Until today, the biological roles of mono- or di-glycosylated Hyl, which are presented at varying levels at those specific sites, are still not clearly elucidated. But, with the bulky feature of sugar attachments and the specific sites on collagen α-chains, it has been proposed that glycosylated Hyl may be involved in the process of collagen fibrillogenesis, cross-linking, protein-protein interaction etc. Modifications at the telopeptidyl Lys residues are modulated by LH2 which hydroxylates Lys residues and LOX which converts Lys and Hyl into their aldehyde forms prior to the initiation of collagen cross-links formation (20). Therefore, from our results, significant upregulation of *PLOD2* and *LOX* expressions in PDLF previously stored in low fat milk could be indicative of a potential increase in the formation of Hyl^{ald} derived collagen cross-links which promote collagen stability.

To determine if the survived PDLF from each storage conditions still retain the PDL tissue regenerative potential, picrosirius red staining was utilized to evaluate the density and maturation of the collagen matrices formed. Picrosirius red stained collagen fibers, when observed under polarized light microscope, generate colors spectrum from green, yellow, orange to red which represent increase in thickness and maturity of collagen fibers, respectively (62, 78). From our study, PDLF previously placed in low fat milk demonstrated a clear trend of forming extracellular matrix with dense and mature

collagen fiber, as shown with the higher degree of red-orange color area of picrosirius red stain. In addition, the collagen matrix formed by PDLF stored in almond milk showed comparable maturity and fiber thickness as the DMEM control. Considering the real time PCR result, we have seen significant upregulation of *PLOD2* and *LOX* expressions in the low fat milk group, whereas in the almond milk group, both their expressions were considered comparable to the control. From these two experiments, it is evident that the expression levels of *PLOD2* and *LOX*, which encode enzymes controlling collagen cross-linking patterns and quantity, demonstrated a positive correlation to the thickness and maturity of the collagen matrix formed by PDLF. This finding is in agreement with the report by KAKU et al. (79) showing that, excessive occlusal loading increases the genes expression and activities of LH2 and LOX which are associated with the formation of thick and mature collagen matrix in PDL tissue. These outcomes underscore the important roles of specific post-translational modifications of collagen in regulating the formation of strong extracellular matrix for PDL tissue regeneration.

When the fat content in milk was taken into consideration, in this study, we have compared the efficacy of whole milk (4% fat content) a low fat milk (1% fat content) and almond milk (1% fat content with 0g of saturated fat) as potential tooth storage media. The nutrition components in all the three types of milk are shown in Table 4. From our results, whole milk and low fat milk showed comparable performances in maintaining PDLF viability and proliferative capacity. Our finding is in accordance with the result from ULUSOY et al. (58) which showed that fat content did not significantly affect PDLF viability, but in contrast to other studies that demonstrated superior efficacy of low fat milk over whole milk (39, 40).

Table 4 Nutrition information of the different types of milk in this study

Nutrition information (per 180 ml)	Whole milk (4% fat)	Low fat milk (1% fat)	Almond milk (1% fat)
Total fat	7g	2g	2g
Saturated fat	4.5g	1g	0g
Unsaturated fat	2.5g	1g	2g
Protein	5g	5g	2g
Carbohydrate	8g	7g	0g
Sugar	8g	7g	0g

As for the effect on type I collagen biosynthesis, low fat milk is better than whole milk in upregulating the expressions of *PLOD2* and *LOX*, which encode essential enzymes controlling the pattern and quantity of collagen cross-links (50). This phenomenon is associated with the formation of a, somewhat, thicker and more mature collagen matrix by PDLF stored in low fat milk. Our result does not go along with the study by OIKARINEN et al. (74) showing that cell previously placed in low fat milk secreted slightly lower level of collagen in the culture medium, when compared to the control. The discrepancy between the two studies may result from the different methods and levels of assessment in the process of type I collagen synthesis. Regarding the reported negative effects of milk on PDL cells/tissue, CHOI et al. (14) have shown that there are significant upregulations of inflammatory cytokines, chemokines and matrix degradation proteins and downregulations of extracellular matrix related genes i.e. *COL1A1*, *COL3A1* in PDLF stored in milk. In another study by HASAN et al. (13), the regenerated PDL tissue at root surface of the replanted tooth, following immersion in milk, was thinner and showed lower expression of periostin, which is a protein involved in the process of collagen assembly. In these two studies, whole milk was used, and both showed potential detrimental effects to the process of collagen synthesis. Similar to these studies, we have seen that in PDLF stored in whole milk, the expressions of *COL1A2* and *PLOD2* was downregulated and the

formed collagen matrix was not as thick as that from the low fat milk group. Collectively, these results of ours and previous studies may be indicative of the adverse effects of whole milk, or possibly high fat content, on the functions of PDLF and PDL tissue regeneration.

In almond milk, the total fat content is at similar level to low fat milk (1% fat), but most of it is unsaturated fat since it contains 0 g of saturated fat. Considering the superior effects of low fat content observed in cow's milk, the low total fat content which is mainly the healthy mono-unsaturated fat in almond milk was initially expected to be increasingly beneficial to the PDLF. However, the effects of almond milk on PDLF viability and collagen biosynthesis, on average, are not as effective as low fat milk. Most likely, it could be due to the lower amount of protein and carbohydrate contents in almond milk (Table 4), which may be essential for maintaining cellular functions. Despite the low expression of *COL1A2*, the normal or slightly upregulated collagen modifying enzyme genes and the surprisingly highest cell proliferation in PDLF placed in almond milk, may contribute to the formation of collagen matrix with comparable thickness and maturity to the control.

Suggestion

The outcome of present study is just a demonstration of the potential abilities of almond milk as a storage medium for avulsed teeth, from experiments were performed in the laboratory. In the real situation, there are numbers of uncontrolled variables such as extra-alveolar time, contamination, temperature, individual cellular and tissue response, etc. Therefore, further studies should be performed in animal models.

Another suggestion is about the commercially available products. Various brands of both bovine and almond milk in the market may have some different nutritional compositions which can affect the experimental outcomes.

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APPENDIX

Appendix 1 Certificate of Approval, Faculty of Dentistry, Srinakarinwirot university

เลขที่รับรอง DENTSWU-EC27/2560

เอกสารรับรองโครงการวิจัยที่เกี่ยวกับงานวิจัยในมนุษย์
คณะทันตแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

ชื่อโครงการ (ชื่อภาษาไทย) การศึกษาผลของนมอัลมอนด์ต่อความมีชีวิตและการทำหน้าที่ของเซลล์ไฟโบรบลาสท์เอ็นยึดปริทันต์ ในสภาวะจำลองฟันที่หลุดออกจากเบ้า

(ชื่อภาษาอังกฤษ) EFFECT OF ALMOND MILK ON THE VIABILITY AND FUNCTION OF PERIODONTAL LIGAMENT FIBROBLASTS IN AN *IN VITRO* TOOTH AVULSION MODEL

ชื่อหัวหน้าโครงการ ผศ.ดร.ทพญ. มานิสา ศรีชลเพ็ชร์
สังกัดหน่วยงาน ภาควิชาศัลยศาสตร์และเวชศาสตร์ช่องปาก คณะทันตแพทยศาสตร์ มศว
ระยะเวลารับรอง เดือนธันวาคม 2560 – เดือนพฤศจิกายน 2561

เอกสารที่รับรอง

1. แบบข้อเสนอโครงการวิจัย Exemption review

รับรองโดย คณะกรรมการพิจารณาจริยธรรมในการทำวิจัย ประจำคณะทันตแพทยศาสตร์

ลงนาม.....
(ผศ.ดร.ทพญ.ปรมาภรณ์ จิ๋วพัฒนกุล แก้วมณี)
ประธานคณะกรรมการพิจารณาจริยธรรมในการทำวิจัย
คณะทันตแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
วันที่ 29 S.A. 2560

ลงนาม.....
(ผศ.ดร.ทพ.ณัฐวุธ แก้วสุทธา)
คณบดีคณะทันตแพทยศาสตร์
วันที่ 29 S.A. 2560

หมายเลขรับข้อเสนอ dentswu-ec-in 24/2560

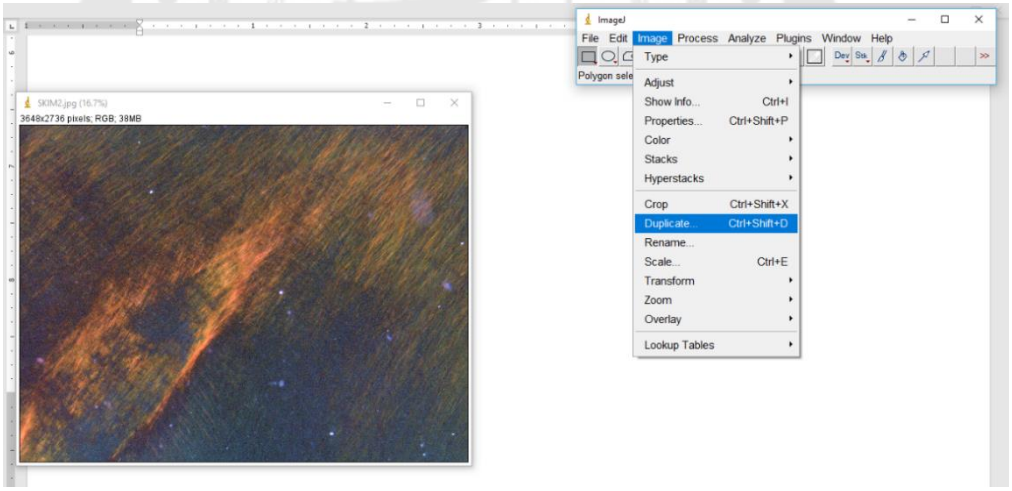
Appendix 2 ImageJ quantification of Polarized Picrosirius red-stained color

1. PDL cell/matrices cultured on culture slides were stained with Picrosirius red and coverslip mounted.

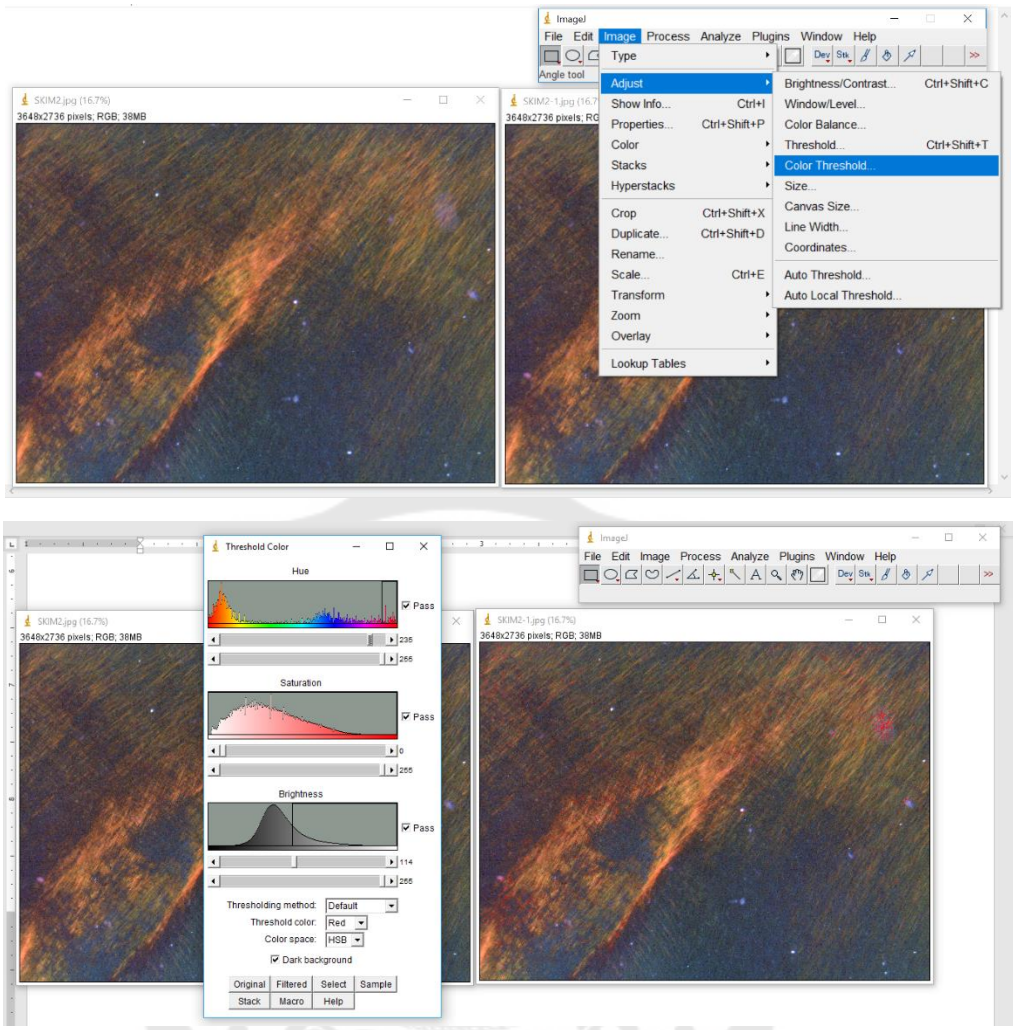
2. Collagen matrix could be observed using polarized light microscope which would show the birefringent color of green-yellow representing immature thin collagen fibers, and orange-red representing mature thick collagen fibers. Digital images (9 regions/condition) were captured at magnification of 4X.

3. Quantification of orange-red color in each captured region using ImageJ software (NIH)

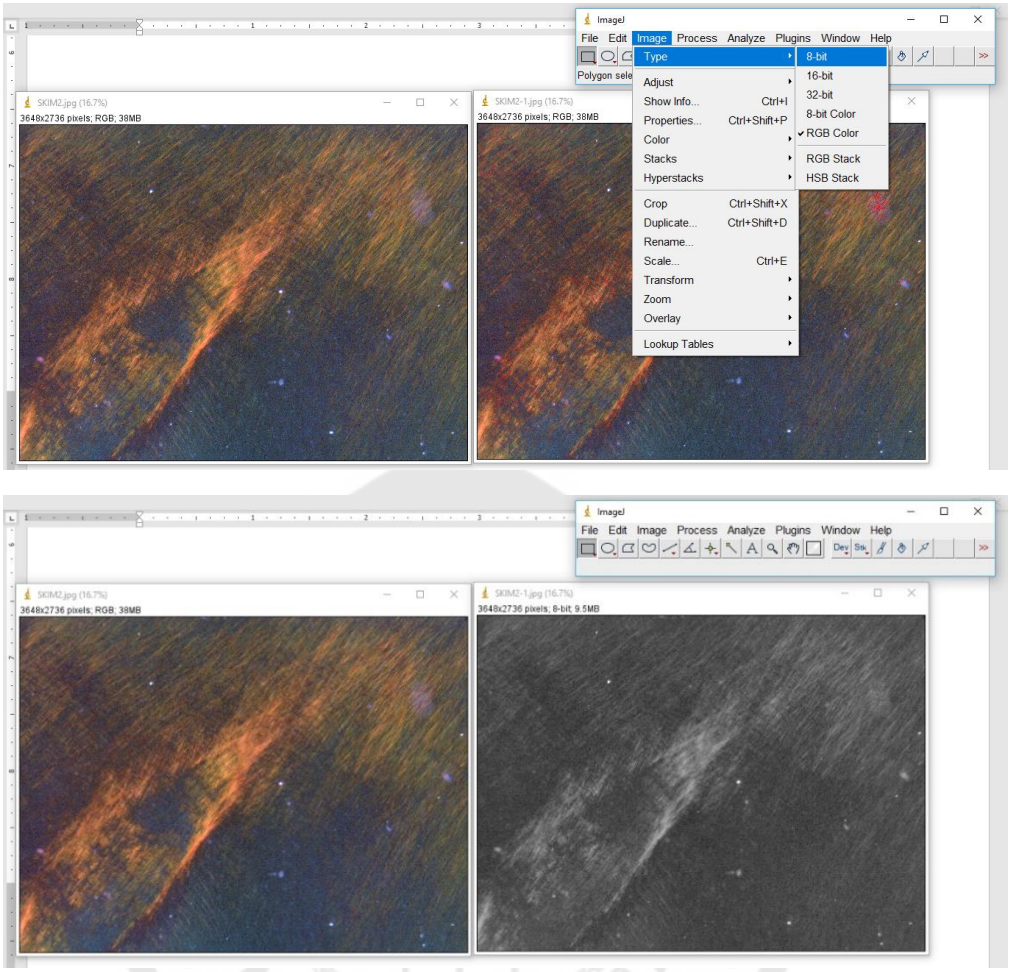
4. Open image in RGB format and make duplicate by select the “Image” tab on the ribbon, then click “Duplicate”.



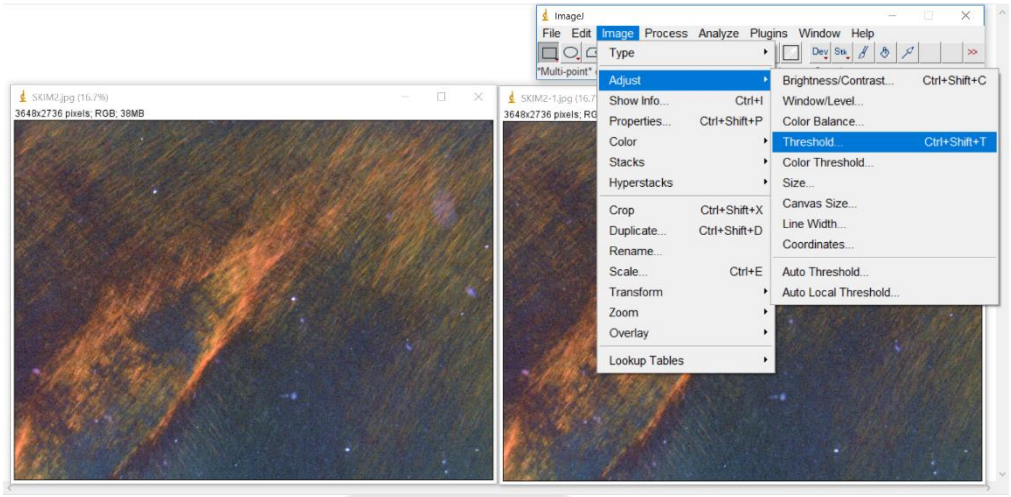
5. On the “Image” menu, point to “Adjust” and then click “Color Threshold”. Adjust the upper slider in the Hue portion to determine the red color area to quantify (move to the right). The area with red color in the image will be highlighted in red and can be compared to the original image. Record the color threshold number which will be used when analyzing the other images in this set of experiment (Red: upper 235, lower 255)

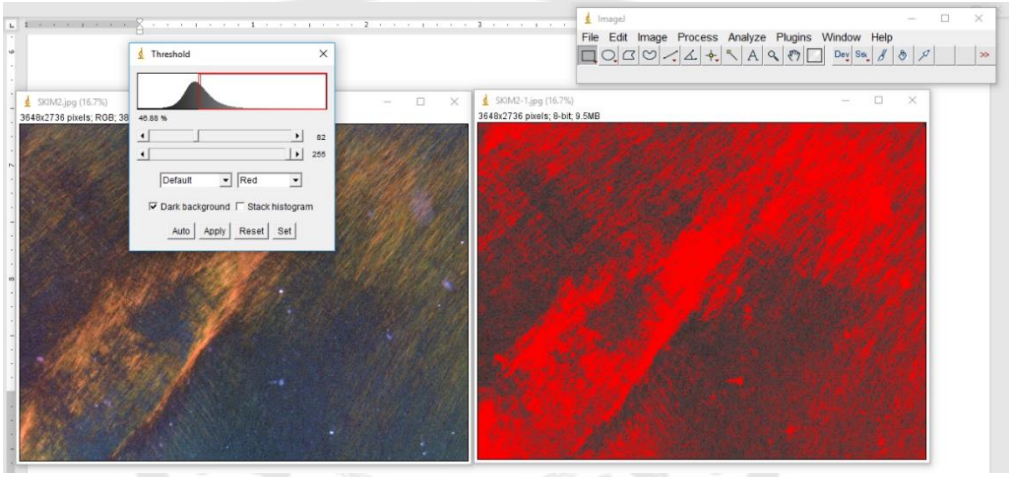


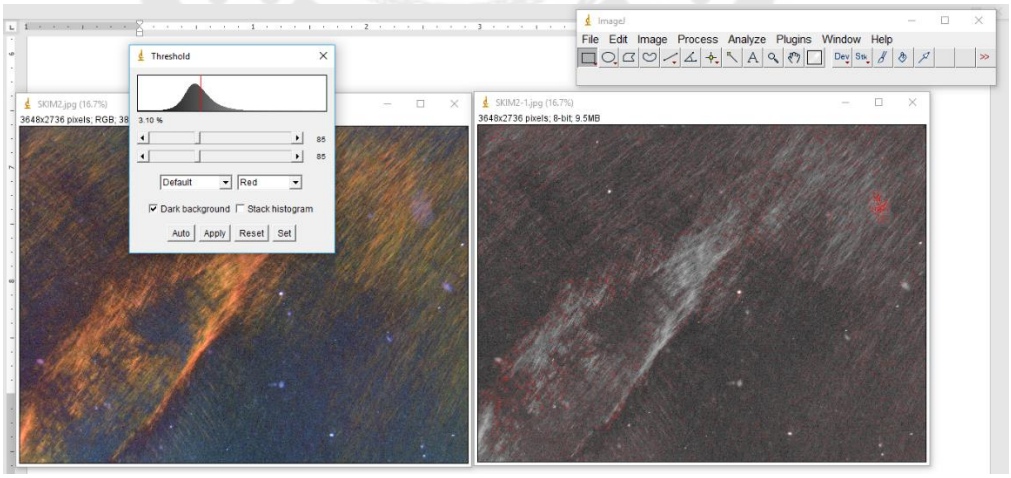
6. Close the Threshold color box and change the RGB image, of the red color selected, into 8-bit type by select “Image”, point to “Type” and then click “8-bit”. The image will turn into grayscale.

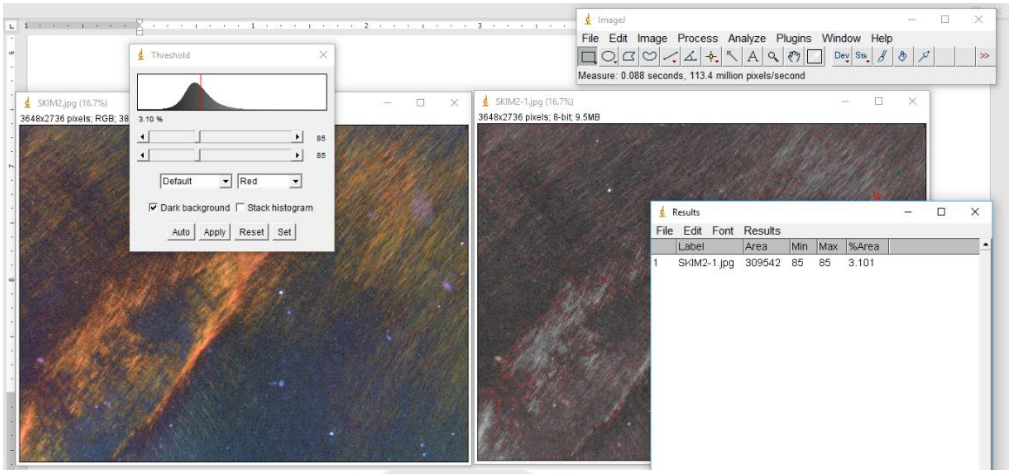


7. On the “Image” menu, point to “Adjust” and then click “Threshold”. The threshold box will come up with a vertical line marked at the point in the histogram where red color was determined in the previous step. Move both the upper and lower sliders to that vertical line point. The area with red color will be highlighted and can be compared with the original image. Press “M” on keyboard to measure the area fraction (%Area) of the threshold (red color) in the image and the result box will pop-up.

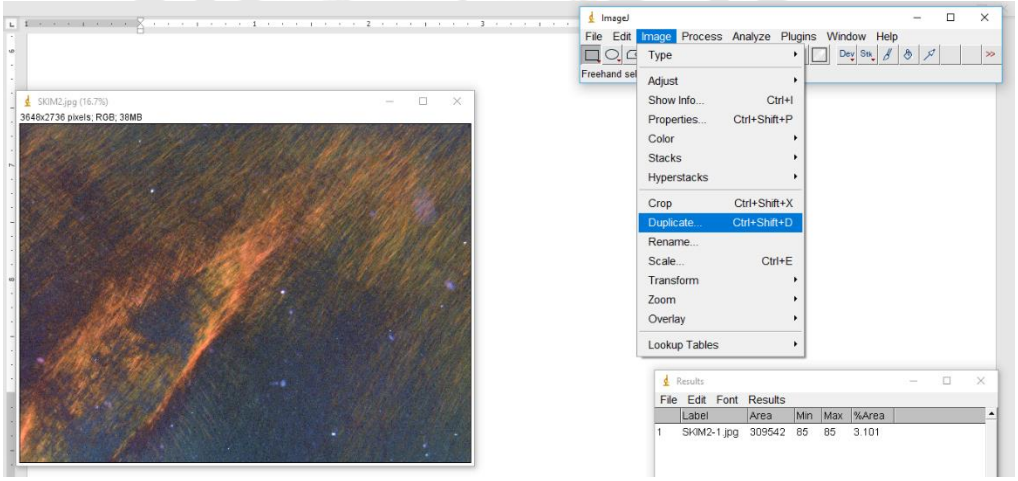

ImageJ
File Edit Image Process Analyze Plugins Window Help
Type
Adjust
Show Info... Ctrl+I
Properties... Ctrl+Shift+P
Color
Stacks
Hyperstacks
Crop Ctrl+Shift+X
Duplicate... Ctrl+Shift+D
Rename...
Scale... Ctrl+E
Transform
Zoom
Overlay
Lookup Tables
Brightness/Contrast... Ctrl+Shift+C
Window/Level...
Color Balance...
Threshold... Ctrl+Shift+T
Color Threshold...
Size...
Canvas Size...
Line Width...
Coordinates...
Auto Threshold...
Auto Local Threshold...
SKIM2.jpg (16.7%)
3648x2736 pixels; RGB; 38MB
SKIM2-1.jpg (16.7%)

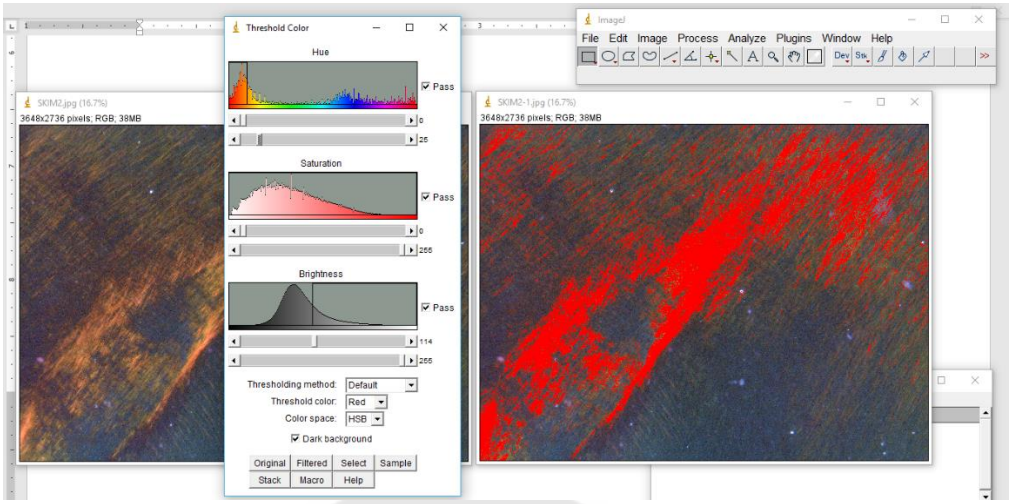

Threshold
Default
Red
Dark background
Stack histogram
Auto
Apply
Reset
Set
SKIM2-1.jpg (16.7%)
3648x2736 pixels; 8-bit; 9.5MB

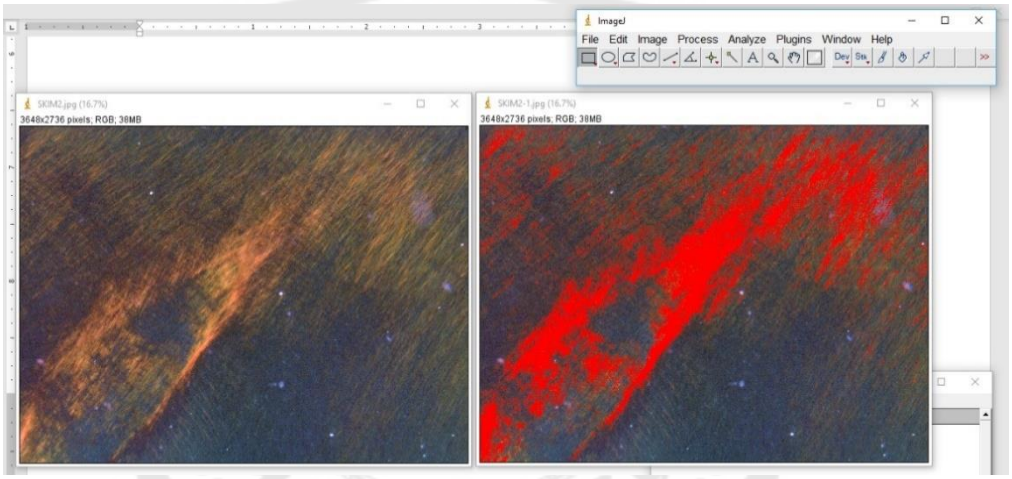

Threshold
Default
Red
Dark background
Stack histogram
Auto
Apply
Reset
Set
SKIM2-1.jpg (16.7%)
3648x2736 pixels; 8-bit; 9.5MB

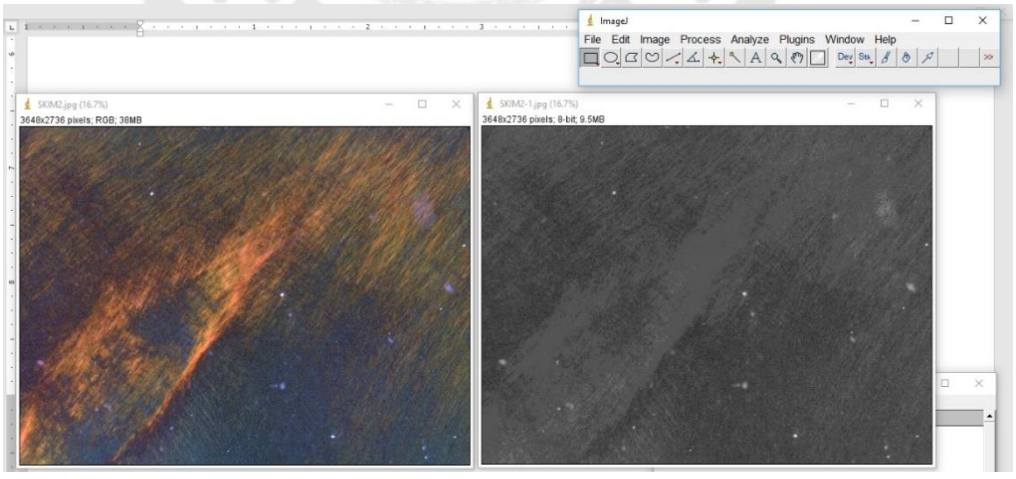


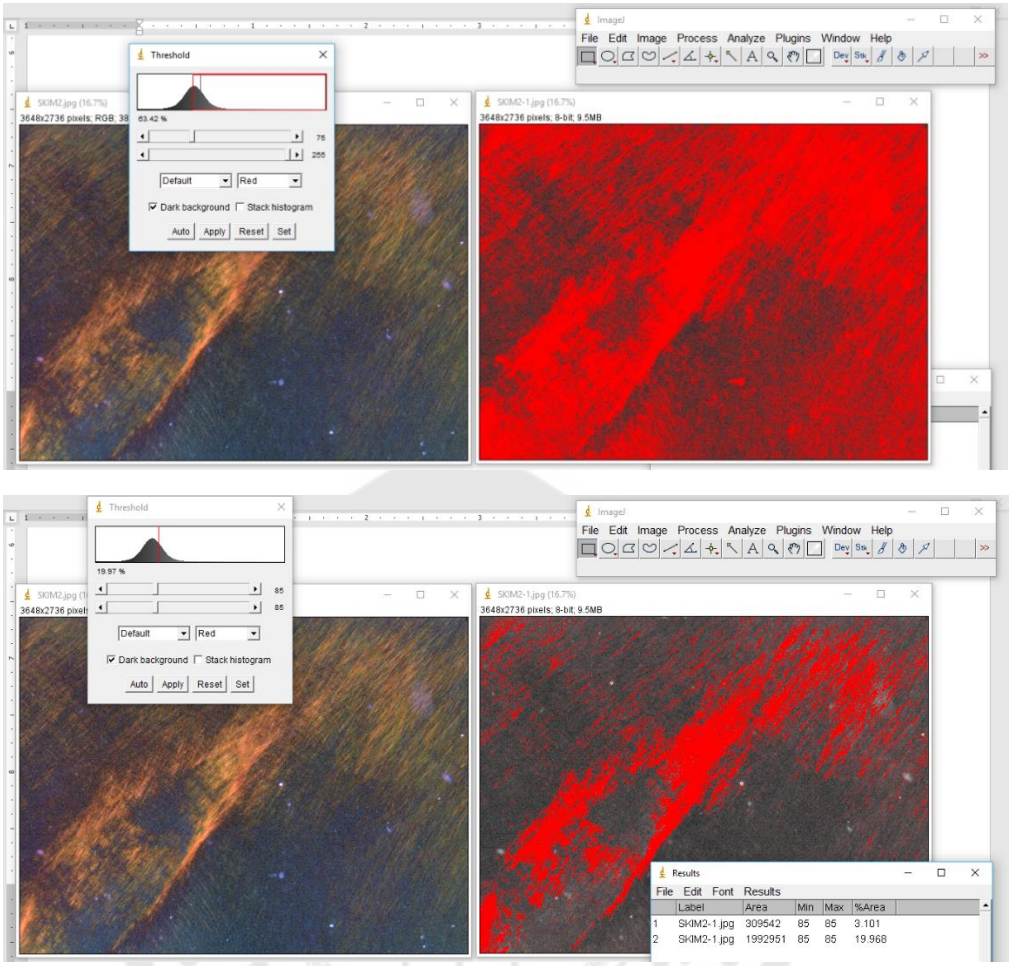
8. Close the threshold box and the red color analyzed image. Make another duplicate image to analyze orange color by adjusting the color threshold lower slider to the left. Compare the highlighted orange color area to the original image and specify the threshold number to use across the images in the same set of experiment. (Orange: upper 0, lower 25) Quantify the orange color using the same steps as stated above.




Threshold Color
Hue
Pass
Saturation
Pass
Brightness
Pass
Thresholding method: Default
Threshold color: Red
Color space: HSB
Dark background
Original
Filtered
Select
Sample
Stack
Macro
Help
ImageJ
File Edit Image Process Analyze Plugins Window Help
SKIM2.jpg (16.7%)
3648x2736 pixels; RGB; 38MB
SKIM2-1.jpg (16.7%)
3648x2736 pixels; RGB; 38MB

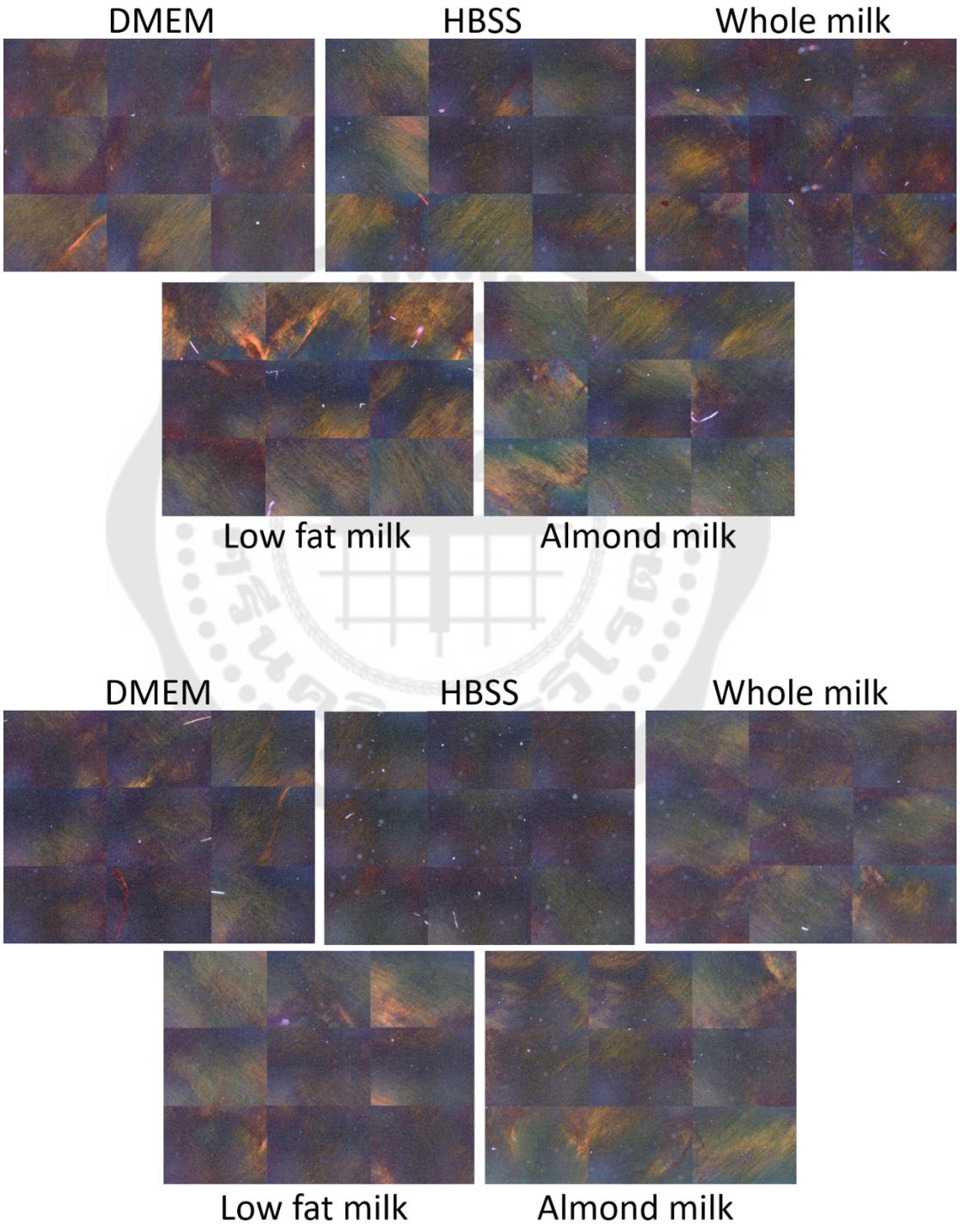

ImageJ
File Edit Image Process Analyze Plugins Window Help
SKIM2.jpg (16.7%)
3648x2736 pixels; RGB; 38MB
SKIM2-1.jpg (16.7%)
3648x2736 pixels; RGB; 38MB

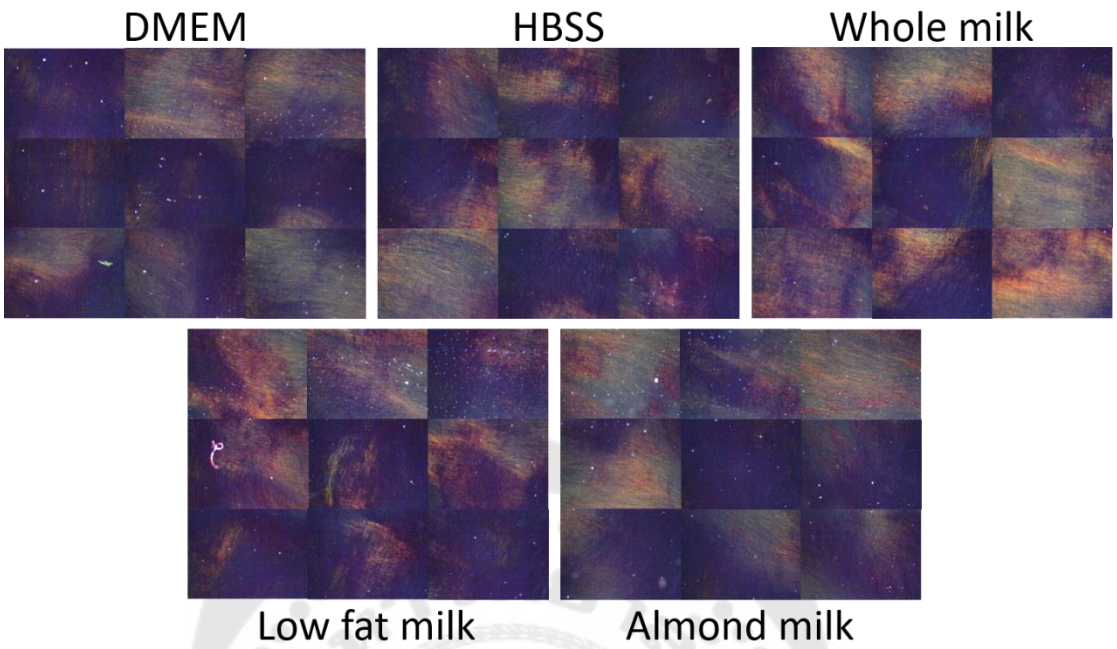

ImageJ
File Edit Image Process Analyze Plugins Window Help
SKIM2.jpg (16.7%)
3648x2736 pixels; RGB; 38MB
SKIM2-1.jpg (16.7%)
3648x2736 pixels; 8-bit; 9.5MB

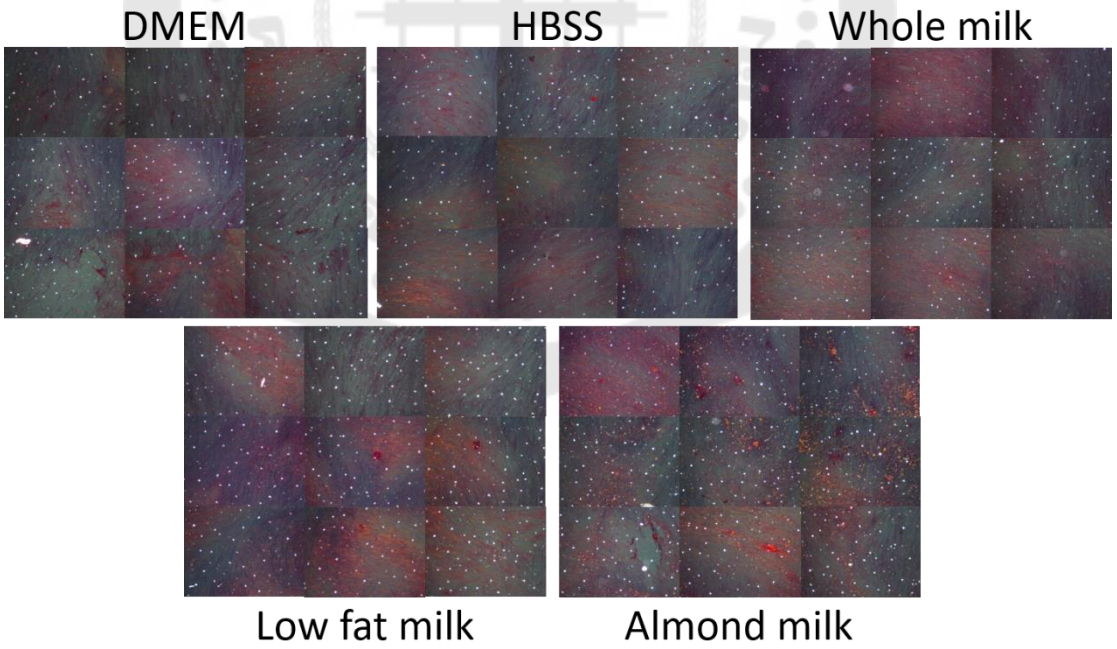


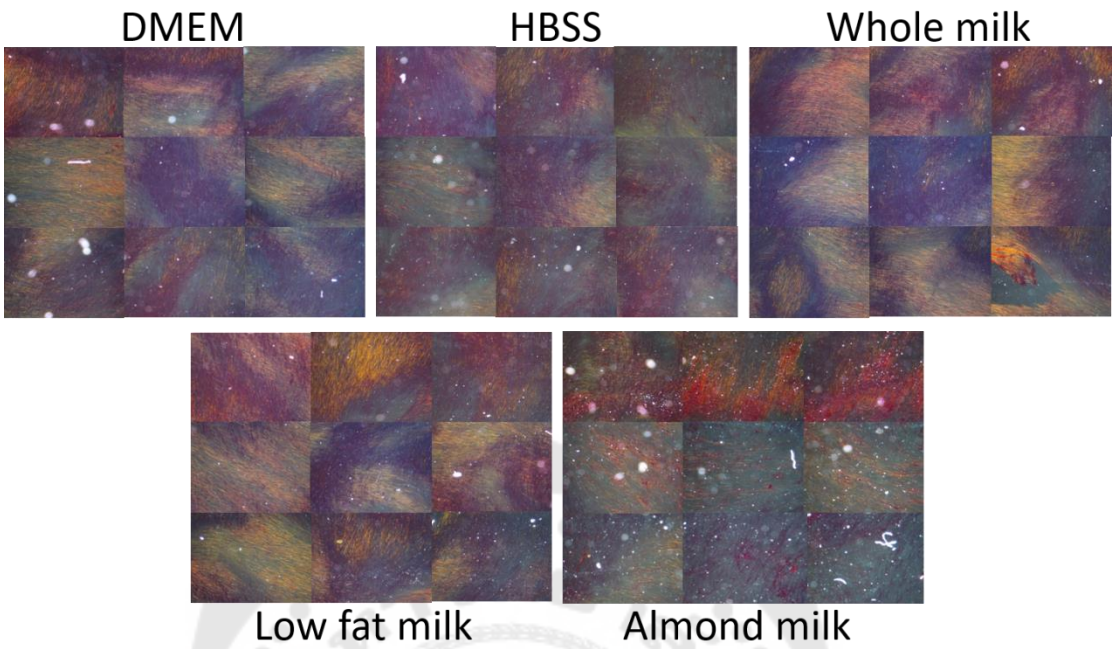
9. Add the %Area of Red and Orange color in the same image/region and sum up the Red and Orange area of 9 representative regions in each condition (Total Red and Orange area) and calculated as percentage of control (%DMEM)

Appendix 3 Images of stained collagen matrices observed under polarized light microscope from 5 independent experiments.




DMEM
HBSS
Whole milk
Low fat milk
Almond milk


DMEM
HBSS
Whole milk
Low fat milk
Almond milk


DMEM
HBSS
Whole milk
Low fat milk
Almond milk

Appendix 4 Statistical results calculated by SPSS of Cell viability

Test Statistics[a,b]

	Cell viability
Chi-Square	19.125
df	4
Asymp. Sig.	.001

a. Kruskal Wallis Test

b. Grouping Variable: Treatment group

Multiple Comparisons

Dependent Variable: Cell viability
Tukey HSD

(I) Treatment group	(J) Treatment group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DMEM	HBSS	36.6000*	5.71874	.000	19.4874	53.7126
	WHOLE MILK	12.2000	5.71874	.245	-4.9126	29.3126
	SKIM MILK	9.6000	5.71874	.468	-7.5126	26.7126
	ALMOND MILK	26.0000*	5.71874	.002	8.8874	43.1126
HBSS	DMEM	-36.6000*	5.71874	.000	-53.7126	-19.4874
	WHOLE MILK	-24.4000*	5.71874	.003	-41.5126	-7.2874
	SKIM MILK	-27.0000*	5.71874	.001	-44.1126	-9.8874
	ALMOND MILK	-10.6000	5.71874	.373	-27.7126	6.5126
WHOLE MILK	DMEM	-12.2000	5.71874	.245	-29.3126	4.9126
	HBSS	24.4000*	5.71874	.003	7.2874	41.5126
	SKIM MILK	-2.6000	5.71874	.990	-19.7126	14.5126
	ALMOND MILK	13.8000	5.71874	.153	-3.3126	30.9126
SKIM MILK	DMEM	-9.6000	5.71874	.468	-26.7126	7.5126
	HBSS	27.0000*	5.71874	.001	9.8874	44.1126
	WHOLE MILK	2.6000	5.71874	.990	-14.5126	19.7126
	ALMOND MILK	16.4000	5.71874	.064	-.7126	33.5126
ALMOND MILK	DMEM	-26.0000*	5.71874	.002	-43.1126	-8.8874
	HBSS	10.6000	5.71874	.373	-6.5126	27.7126
	WHOLE MILK	-13.8000	5.71874	.153	-30.9126	3.3126
	SKIM MILK	-16.4000	5.71874	.064	-33.5126	.7126

Based on observed means.
The error term is Mean Square(Error) = 81.760.

*. The mean difference is significant at the .05 level.

Appendix 5 Statistical results calculated by SPSS of Cell proliferation

Test Statistics[a,b]

	Cell proliferation
Chi-Square	14.279
df	4
Asymp. Sig.	.006

a. Kruskal Wallis Test

b. Grouping Variable: Treatment group

Multiple Comparisons

Dependent Variable: Cell proliferation
Tukey HSD

(I) Treatment group	(J) Treatment group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DMEM	HBSS	-18.6000	9.39234	.311	-46.7054	9.5054
	WHOLE MILK	-7.0000	9.39234	.943	-35.1054	21.1054
	SKIM MILK	-2.2000	9.39234	.999	-30.3054	25.9054
	ALMOND MILK	-65.2000*	9.39234	.000	-93.3054	-37.0946
HBSS	DMEM	18.6000	9.39234	.311	-9.5054	46.7054
	WHOLE MILK	11.6000	9.39234	.732	-16.5054	39.7054
	SKIM MILK	16.4000	9.39234	.430	-11.7054	44.5054
	ALMOND MILK	-46.6000*	9.39234	.001	-74.7054	-18.4946
WHOLE MILK	DMEM	7.0000	9.39234	.943	-21.1054	35.1054
	HBSS	-11.6000	9.39234	.732	-39.7054	16.5054
	SKIM MILK	4.8000	9.39234	.985	-23.3054	32.9054
	ALMOND MILK	-58.2000*	9.39234	.000	-86.3054	-30.0946
SKIM MILK	DMEM	2.2000	9.39234	.999	-25.9054	30.3054
	HBSS	-16.4000	9.39234	.430	-44.5054	11.7054
	WHOLE MILK	-4.8000	9.39234	.985	-32.9054	23.3054
	ALMOND MILK	-63.0000*	9.39234	.000	-91.1054	-34.8946
ALMOND MILK	DMEM	65.2000*	9.39234	.000	37.0946	93.3054
	HBSS	46.6000*	9.39234	.001	18.4946	74.7054
	WHOLE MILK	58.2000*	9.39234	.000	30.0946	86.3054
	SKIM MILK	63.0000*	9.39234	.000	34.8946	91.1054

Based on observed means.
The error term is Mean Square(Error) = 220.540.

*. The mean difference is significant at the .05 level.

Appendix 6 Statistical results calculated by SPSS of COL1A2 Expression/DMEM

Test Statistics[a,b]

	COL1A2 EXPRESSION
Chi-Square	11.375
df	4
Asymp. Sig.	.023

a. Kruskal Wallis Test

b. Grouping Variable: Treatment Group

Multiple Comparisons

Dependent Variable: COL1A2 EXPRESSION
Tukey HSD

(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DMEM	HBSS	.4680	.17095	.083	-.0436	.9796
	WHOLE MILK	.4300	.17095	.127	-.0816	.9416
	SKIM MILK	-.0420	.17095	.999	-.5536	.4696
	ALMOND MILK	.5480*	.17095	.032	.0364	1.0596
HBSS	DMEM	-.4680	.17095	.083	-.9796	.0436
	WHOLE MILK	-.0380	.17095	.999	-.5496	.4736
	SKIM MILK	-.5100	.17095	.051	-1.0216	.0016
	ALMOND MILK	.0800	.17095	.989	-.4316	.5916
WHOLE MILK	DMEM	-.4300	.17095	.127	-.9416	.0816
	HBSS	.0380	.17095	.999	-.4736	.5496
	SKIM MILK	-.4720	.17095	.079	-.9836	.0396
	ALMOND MILK	.1180	.17095	.956	-.3936	.6296
SKIM MILK	DMEM	.0420	.17095	.999	-.4696	.5536
	HBSS	.5100	.17095	.051	-.0016	1.0216
	WHOLE MILK	.4720	.17095	.079	-.0396	.9836
	ALMOND MILK	.5900*	.17095	.019	.0784	1.1016
ALMOND MILK	DMEM	-.5480*	.17095	.032	-1.0596	-.0364
	HBSS	-.0800	.17095	.989	-.5916	.4316
	WHOLE MILK	-.1180	.17095	.956	-.6296	.3936
	SKIM MILK	-.5900*	.17095	.019	-1.1016	-.0784

Based on observed means.
The error term is Mean Square(Error) = .073.

*. The mean difference is significant at the .05 level.

Appendix 7 Statistical results calculated by SPSS of PLOD1 Expression/DMEM

Test Statistics[a,b]

	PLOD1 EXPRESSION
Chi-Square	4.949
df	4
Asymp. Sig.	.293

a. Kruskal Wallis Test

b. Grouping Variable: Treatment Group

Appendix 8 Statistical results calculated by SPSS of PLOD2 Expression/DMEM

Test Statistics[a,b]

	PLOD2 EXPRESSION
Chi-Square	17.957
df	4
Asymp. Sig.	.001

a. Kruskal Wallis Test

b. Grouping Variable: Treatment Group

Multiple Comparisons

Dependent Variable: PLOD2 EXPRESSION
Tukey HSD

(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DMEM	HBSS	.2060	.09270	.212	-.0714	.4834
	WHOLE MILK	.3020*	.09270	.029	.0246	.5794
	SKIM MILK	-.2460	.09270	.098	-.5234	.0314
	ALMOND MILK	-.0040	.09270	1.000	-.2814	.2734
HBSS	DMEM	-.2060	.09270	.212	-.4834	.0714
	WHOLE MILK	.0960	.09270	.836	-.1814	.3734
	SKIM MILK	-.4520*	.09270	.001	-.7294	-.1746
	ALMOND MILK	-.2100	.09270	.197	-.4874	.0674
WHOLE MILK	DMEM	-.3020*	.09270	.029	-.5794	-.0246
	HBSS	-.0960	.09270	.836	-.3734	.1814
	SKIM MILK	-.5480*	.09270	.000	-.8254	-.2706
	ALMOND MILK	-.3060*	.09270	.026	-.5834	-.0286
SKIM MILK	DMEM	.2460	.09270	.098	-.0314	.5234
	HBSS	.4520*	.09270	.001	.1746	.7294
	WHOLE MILK	.5480*	.09270	.000	.2706	.8254
	ALMOND MILK	.2420	.09270	.106	-.0354	.5194
ALMOND MILK	DMEM	.0040	.09270	1.000	-.2734	.2814
	HBSS	.2100	.09270	.197	-.0674	.4874
	WHOLE MILK	.3060*	.09270	.026	.0286	.5834
	SKIM MILK	-.2420	.09270	.106	-.5194	.0354

Based on observed means.
The error term is Mean Square(Error) = .021.

*. The mean difference is significant at the .05 level.

Appendix 9 Statistical results calculated by SPSS of PLOD3 Expression/DMEM

Test Statistics[a,b]

	PLOD3 EXPRESSION
Chi-Square	5.710
df	4
Asymp. Sig.	.222

a. Kruskal Wallis Test

b. Grouping Variable: Treatment Group

Appendix 10 Statistical results calculated by SPSS of GLT25D1 Expression/DMEM

Test Statistics[a,b]

	GLT25D1 EXPRESSION
Chi-Square	11.929
df	4
Asymp. Sig.	.018

a. Kruskal Wallis Test

b. Grouping Variable: Treatment Group

Multiple Comparisons

Dependent Variable: GLT25D1 EXPRESSION

Tukey HSD

(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DMEM	HBSS	.1498	.24808	.973	-.5926	.8922
	WHOLE MILK	-.8788*	.24808	.016	-1.6212	-.1364
	SKIM MILK	-.4648	.24808	.362	-1.2072	.2776
	ALMOND MILK	-.3874	.24808	.537	-1.1298	.3550
HBSS	DMEM	-.1498	.24808	.973	-.8922	.5926
	WHOLE MILK	-1.0286*	.24808	.004	-1.7710	-.2862
	SKIM MILK	-.6146	.24808	.136	-1.3570	.1278
	ALMOND MILK	-.5372	.24808	.233	-1.2796	.2052
WHOLE MILK	DMEM	.8788*	.24808	.016	.1364	1.6212
	HBSS	1.0286*	.24808	.004	.2862	1.7710
	SKIM MILK	.4140	.24808	.474	-.3284	1.1564
	ALMOND MILK	.4914	.24808	.310	-.2510	1.2338
SKIM MILK	DMEM	.4648	.24808	.362	-.2776	1.2072
	HBSS	.6146	.24808	.136	-.1278	1.3570
	WHOLE MILK	-.4140	.24808	.474	-1.1564	.3284
	ALMOND MILK	.0774	.24808	.998	-.6650	.8198
ALMOND MILK	DMEM	.3874	.24808	.537	-.3550	1.1298
	HBSS	.5372	.24808	.233	-.2052	1.2796
	WHOLE MILK	-.4914	.24808	.310	-1.2338	.2510
	SKIM MILK	-.0774	.24808	.998	-.8198	.6650

Based on observed means.

The error term is Mean Square(Error) = .154.

*. The mean difference is significant at the .05 level.

Appendix 11 Statistical results calculated by SPSS of LOX Expression/DMEM

Test Statistics[a,b]

	LOX EXPRESSION
Chi-Square	12.999
df	4
Asymp. Sig.	.011

a. Kruskal Wallis Test

b. Grouping Variable: Treatment Group

Multiple Comparisons

Dependent Variable: LOX EXPRESSION
Tukey HSD

(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DMEM	HBSS	.0000	.25774	1.000	-.7713	.7713
	WHOLE MILK	.0400	.25774	1.000	-.7313	.8113
	SKIM MILK	-.8940*	.25774	.018	-1.6653	-.1227
	ALMOND MILK	-.4000	.25774	.543	-1.1713	.3713
HBSS	DMEM	.0000	.25774	1.000	-.7713	.7713
	WHOLE MILK	.0400	.25774	1.000	-.7313	.8113
	SKIM MILK	-.8940*	.25774	.018	-1.6653	-.1227
	ALMOND MILK	-.4000	.25774	.543	-1.1713	.3713
WHOLE MILK	DMEM	-.0400	.25774	1.000	-.8113	.7313
	HBSS	-.0400	.25774	1.000	-.8113	.7313
	SKIM MILK	-.9340*	.25774	.013	-1.7053	-.1627
	ALMOND MILK	-.4400	.25774	.452	-1.2113	.3313
SKIM MILK	DMEM	.8940*	.25774	.018	.1227	1.6653
	HBSS	.8940*	.25774	.018	.1227	1.6653
	WHOLE MILK	.9340*	.25774	.013	.1627	1.7053
	ALMOND MILK	.4940	.25774	.341	-.2773	1.2653
ALMOND MILK	DMEM	.4000	.25774	.543	-.3713	1.1713
	HBSS	.4000	.25774	.543	-.3713	1.1713
	WHOLE MILK	.4400	.25774	.452	-.3313	1.2113
	SKIM MILK	-.4940	.25774	.341	-1.2653	.2773

Based on observed means.
The error term is Mean Square(Error) = .166.

*. The mean difference is significant at the .05 level.

Appendix 12 Statistical results calculated by SPSS of %Area/DMEM

Test Statistics[a,b]

	% AREA
Chi-Square	8.744
df	4
Asymp. Sig.	.068

a. Kruskal Wallis Test

b. Grouping Variable: Treatment Group

VITA

NAME	Phuttikarn Sinpreechanon
DATE OF BIRTH	20 October 1988
PLACE OF BIRTH	Narathiwat
INSTITUTIONS ATTENDED	Srinakharinwirot University
HOME ADDRESS	1588/488 Villa Asoke condominium Makkasan Ratchathewi Bangkok 10400