THE NEW FORMULATIONS OF GINGIVAL RETRACTION SOLUTION: *IN VITRO* STUDY OF PLASMA PROTEIN PRECIPITATION, CYTOTOXIC PROPERTIES AND DETAILED REPRODUCTION WITH AN ADDITION SILICONE IMPRESSION MATERIAL

YONGYUTH IEMPITUKSAKUL

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……
Mr.Yongyuth Iempituksakul
Candidate

……
Clinical. Prof. Chatcharee Suchatlampong,
D.D.S., Grad. Dip. in Cli.Sc.
(Prosthodontics), M.Phil(Dental
Materials). Diplomate Thai Board of
Prosthodontics
Major-advisor

……
Assoc. Prof. Vanida Sangalungkarn,
M.Sc., B.Sc. (in Pharm)
Co-advisor

……
Lecturer Kallaya Suputtamongkol,
D.D.S., M.Sc. (Prosthodontics)
Ph.D. (Ceramics)
Co-advisor

……
Assoc. Prof. Rudee Surarit,
B.Sc., M.S. (Biochemistry)
Ph.D. (Oral Biology)
Co-advisor

……
Prof. Bunchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

……
Assoc. Prof. Potchaman Sinavarat,
B.Sc., D.D.S., M.Sc. (Prosthetic
Dentistry)
Chair
Master of Science Programme in
Prosthodontics
Faculty of Dentistry
Mahidol University

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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science in Prosthodontic
May 27, 2009

……
Mr.Yongyuth Iempituksakul
Candidate

……
Emeritus Clinical Professor Amara Muangmingsuk,
D.D.S., Grad. Dip. in Clin. Sc. (Endodontics),Cert. in Operative Dentistry,M.S.,Diplomate Thai Board of Endodontics
Chair

……
Clinical. Prof. Chatcharee Suchatlampong,
D.D.S., Grad. Dip. in Cli.Sc. (Prosthodontics). M. Phil (Dental Materials). Diplomate Thai Board of Prosthodontics
Member

……
Assoc. Prof. Vanida Sangalungkarn,
M.Sc., B.Sc. (in Pharm)
Member

……
Assoc. Prof. Rudee Surarit,
B.Sc., M.S. (Biochemistry)
Ph.D. (Oral Biology)
Member

……
Lecturer Kallaya Suputtamongkol,
D.D.S., M.Sc. (Prosthodontics)
Ph.D. (Ceramics)
Member

……
Prof. Bunchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

……
Assist. Prof. Theeralaksna Suddhasthira
B.Sc.,D.D.S.Diplomate, American Board of Oral and Maxillofacial
Ph.D. (Biomedical Science)
Dean
Faculty of Dentistry, Mahidol University

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YONGYUTH IEMPITUKSAKUL 5037255 DTPT/M

M.Sc. (PROSTHODONTICS)

THESIS ADVISORY COMMITTEE: CHATCHAREE SUCHATLAMPONG, D.D.S., Grad. Dip. in Cli.Sc. (Prosthodontics) ,M. Phil. (Dental Materials), Diplomate Thai Board of Prosthodontics, VANIDA SANGALUNGKAN, M.SC.(in Pharm), RUDEE SURARIT, M.Sc., Ph.D.(Oral Biology), KALLAYA SUPUTTAMONGKOL, D.D.S., M.Sc.(Prosthodontics), Ph.D.(Ceramics)

ABSTRACT

The objectives of this study were to compare the ability of hemostasis property, cytotoxicity and compatibility with addition silicone impression material of four gingival retraction solutions containing aluminium chloride (A1, A2) and aluminium sulfate (B1, B2) developed by the Faculty of Dentistry, Mahidol University with commercial products containing aluminium sulfate namely Rastringent II ™, Gelcord ™ and aluminium chloride containing Viscostat clear ™. *In vitro* plasma protein precipitation was used to determine the bleeding control ability. The cytotoxicity assay followed the ISO 10993 part 5, 12 using MTT cell viability assay on mouse fibroblast cell line L929. The detailed reproduction with addition silicone impression material was tested according to ISO 4823 using test block for detailed reproduction.

The results showed that Formula A1, B1 and B2 were found to give significantly higher plasma protein precipitation ability (96.77, 96.46 and 96.80%, respectively) while Gelcord ™, Viscostat clear ™ and Rastringent II ™ gave lower protein precipitation (53.84, 26.09 and 21.26% respectively)($p<.05$). Formula A2 gave 43.47% protein precipitation. The cytotoxicity tested showed that Gelcord ™ and Rastringent II ™ could be classified as slightly cytotoxic with cell viability of 76.1% and 66.91% respectively while others were severely toxic. Formula B1 and B2 were less toxic (cell viability of 21.91% and 17.86%) when compared to Formula A1, A2 and Viscostat clear ™ (cell viability of 1.72%, 1.11%, and 2.47% respectively). The impression surface and the detailed reproduction of all groups showed normal detail and surface characteristic after application with retraction solutions followed by rinsing with water for 10 seconds and blow-drying. Rinsing with water was important since without rinsing gels would interfere with detailed reproduction and make the impression surface irregular.

In conclusion, Formula A1, B1 and B2 showed similar ability to precipitate plasma protein. Formula B1 and B2 produced more cytotoxic effect than Gelcord ™ and Rastringent II™. However, the cytotoxicity of Formula A1 was similar to the Viscostat clear ™. Therefore, Formula A1 may be a candidate for further study.

KEY WORDS: GINGIVAL RETRACTION MATERIAL/ ASTRINGENT/ PLASMA PROTEIN PRECIPITATION/ CYTOTOXICITY/ DETAILED REPRODUCTION/ADDITION SILICONE

63 pages

สารละลายแยกเหงือกสูตรใหม่ : สมบัติการตกตะกอนพลาสมาโปรตีน, ความเป็นพิษต่อเซลล์ในห้องปฏิบัติการ และการลอกเลียนรายละเอียดด้วยวัสดุพิมพ์ชนิดแอคดิชันซิลิโคน

THE NEW FORMULATIONS OF GINGIVAL RETRACTION SOLUTION: *IN VITRO* STUDY OF PLASMA PROTEIN PRECIPITATION, CYTOTOXIC PROPERTIES AND DETAILED REPRODUCTION WITH AN ADDITION SILICONE IMPRESSION MATERIAL.

ยงยุทธ เอี่ยมพิทักษ์สกุล 5037255 DTPT/M

วท.ม. ทันตกรรมประดิษฐ์

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ ชัชรี สุชาติล้ำพงศ์ ท.บ., ป.ชั้นสูงวิทยาศาสตร์การแพทย์คลินิค(ทันตกรรมประดิษฐ์), M. Phil. (Dental Materials), อ.ท.(ทันตกรรมประดิษฐ์), วนิดา แสงอลังการ ภ.บ., M.Sc.(in Pharm) , ฤดี สุราฤทธิ์ วท.ม.,Ph. D (Oral Biology), กัลยา ศุพุทธมงคล ท.บ.,วท.ม.(ทันตกรรมประดิษฐ์),Ph.D. (Ceramics)

บทคัดย่อ

วัตถุประสงค์การศึกษาเพื่อเปรียบเทียบสมบัติการห้ามเลือด, ความเป็นพิษต่อเซลล์ และ การลอกเลียนรายละเอียดด้วยวัสดุชนิดแอคดิชันซิลิโคนของวัสดุแยกเหงือกใหม่ 4 ชนิด อะลูมิเนียมคลอไรด์ (สูตรเอ1, เอ2) และอะลูมิเนียมซัลเฟต (สูตรบี1, บี2) พัฒนาโดยคณะทันตแพทยศาสตร์ ม.มหิดล กับวัสดุแยกเหงือกตามท้องตลาด อะลูมิเนียมซัลเฟต (ราสตรินเจนต์ ทู, เจลคอร์ด) และอะลูมิเนียมคลอไรด์ (วิสโคสแตตเคลีย) การตกตะกอนพลาสมาโปรตีนแสดงถึงความสามารถในการห้ามเลือด การทดสอบความเป็นพิษต่อเซลล์โดยการประเมินความมีชีวิตของเซลล์ โดยวิธี เอ็มทีที ตามไอเอสโอ 10993 ส่วน 5 และ 12, การศึกษาการการลอกเลียนรายละเอียดของวัสดุพิมพ์แอดดิชันซิลิโคน ตามไอเอสโอ 4823 โดยใช้โมเดลทดสอบการลอกเลียนรายละเอียด

ผลการศึกษาสูตรเอ1, บี1 และบี2 สามารถตกตะกอนพลาสมาโปรตีนได้มาก มีนัยสำคัญ ร้อยละ 96.77, 96.46 และ 96.80 ตามลำดับ ขณะที่เจลคอร์ด,วิสโคสแตตเคลียและราสตรินเจนต์ทู ให้ผลที่ต่ำกว่า คือ ร้อยละ53.84, 26.09, 21.26 ตามลำดับ ส่วนสูตรเอ2 ตกตะกอนได้ ร้อยละ 43.47 การประเมินพิษต่อเซลล์พบว่าเจลคอร์ดและราสตรินเจนต์ทู ให้ผลเป็นพิษเล็กน้อยโดยมีเซลล์ที่มีชีวิตเหลืออยู่ ร้อยละ 76.1 และ 66.91 ขณะที่สารอื่นให้ผลเป็นพิษระดับรุนแรง สูตรบี1และบี2ให้ผลเป็นพิษน้อยกว่าเมื่อเปรียบเทียบกับสูตรเอ1, เอ2 และวิสโคสแตตเคลีย การลอกเลียนรายละเอียดและลักษณะพื้นผิวของวัสดุพิมพ์จะมีลักษณะปกติและสามารถลอกเลียนรายละเอียดได้เมื่อล้างสารแยกเหงือกออกและเป่าลมก่อนพิมพ์ เนื่องจากวัสดุแยกเหงือกจะมีผลต่อพื้นผิววัสดุพิมพ์ปาก

บทสรุป วัสดุแยกเหงือกสูตรเอ1, บี1 และบี2 ให้ผลไม่แตกต่างในการตกตะกอนพลาสมาโปรตีน ขณะที่สูตรบี1, บี2 ให้ผลเป็นพิษต่อเซลล์มากกว่าเจลคอร์ด และราสตรินเจนต์ทู อย่างไรก็ตามสูตรเอ1ให้ผลเป็นพิษในระดับเดียวกับวิสโคสแตตเคลีย ดังนั้นสูตรเอ1 อาจจะเป็นสูตรที่จะนำไปใช้ในการศึกษาต่อไป

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LIST ABBREVIATIONS

°C	=	Degree Celsius
DMEM	=	Dulbecco’s Modified Eagle Medium
DMSO	=	dimethyl sulfoxide
et al.	=	et alii (and other)
MTT	=	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
g	=	gram
PBS	=	phosphate buffer saline
ml	=	milliliter
µl	=	microliter
conc.	=	concentration

CHAPTER I
INTRODUCTION

In prosthodontic dentistry, indirect restorations such as crown, veneer, partial veneer, inlay and onlay are routinely used to restore defective teeth. These restoration margins are frequently located in the gingival sulcus for esthetic or functional reasons. In these situations, dentists have to make impressions that accurately capture the prepared cervical finishing lines and permit the fabrication of accurate dies on which restorations are fabricated.

The procedure used to facilitate effective impression making with intracrevicular margins called gingival displacement. The goal of the procedure is to reversibly displace the gingival tissue in a latero-vertical direction so that the bulk of impression material can penetrate into the sulcus and capture the margin details (1).

The bulk of impression material is required to obtain minimum accuracy and to improve the tear strength of the material so that it can be removed from the mouth without tearing. The critical sulcular width in this regard appears to be approximately 0.2 mm. A width of less than 0.2 mm results in impressions that have a higher incidence of voids in the marginal area, an increase in tearing of the impression material, and a reduction in marginal accuracy(2).

Another factor in this regard is to ensure that the gingival tissues are in the optimum state of health before making impressions (3) and the optimum position of the margin is 0.5 mm from the healthy free gingival margin or 3.0 to 4.0 mm from the crest of the alveolar bone and must follow the natural scalloped form of the attachment and alveolar housing (4).

The requirements that must be satisfied before effective impression taking are:

1) gingival tissues are healthy and the cervical margins are placed in the appropriate position.

2) tissues covering subgingival margins must be retracted or displaced to provide a sufficient space for the bulk of impression material to penetrate to record the cervical finishing line of the teeth.
3) all hemorrhages must be arrested.
4) all hard and soft tissues should be cleaned and dried.

Donovan et al. (4), Shaw and Krejci (5) reported that most dentists routinely use gingival retraction cords with or without chemical solutions for gingival displacement. The advantages of this method are simplicity, low cost and the capability of combining with other methods to access the tooth or teeth margins.

The disadvantage of the method using the gingival retraction cord with a chemical agent is the side effect from gingival retraction agents that have to contact gingival tissue, include the large amount of agents, the time of contact with gingival tissues and the difficulty in removing the agent by rinse. For these reasons, it will be toxic to the gingival tissue and may lead to gingival inflammation and gingival recession.

This study aims to develop proprietary gingival retraction solutions, designated in viscous and high solution forms, that are kind to the gingival tissue and to eliminate the side effects of the retraction solutions used with the retraction cords (6, 7). Moreover, because of the material viscosity, it can be easily manipulated in the gingival sulcus and improve the ability of bleeding control. Furthermore, the viscous solutions will be easy to clean and maintain the good health of the periodontal tissues. The basic criteria will be achieved by using designated Formula A1 containing 25 % aluminium chloride in aqueous solution, Formula A2 containing 25% aluminium chloride in non aqueous solution and Formula B1 containing of 25% aluminium sulfate in aqueous solution, Formula B2 containing of 25% aluminium sulfate in high viscous aqueous solution. The new gingival retraction solutions can be injected into the patient’s gingival sulcus directly before placing the retraction cord furthermore the high viscous formula will stay at the area of placement for maximum hemostasis. Moreover, the retraction solutions can be used to coat and lubricate the retraction cord before placing into the gingival sulcus. The greatest advantage is after the retraction process is achieved, the agents can be removed from the sulcus easily, so it will be kind for the patient’s gingival tissues by reducing the amount of agents contacting

gingival tissues for a prolonged period of time. Previous knowledge has shown that every retraction agent is acidic (8) and when the acid comes in contact with the teeth or gingival tissues it will present gingival inflammation, gingival recession, and demineralization which cause tooth to become sensitive. Moreover the new gingival retraction solution will be compatible to the addition silicone material without any inhibitory effect to polymerization of addition silicone impression materials. In additions, the new gingival retraction solutions may prove to be useful to the gingival retraction procedure in the future.

Objectives of the study

The objectives of this study are to evaluate

1. the ability of the protein precipitations in the newly-developed gingival retraction solutions. Formula A1, A2, B1, B2 compare with the commercial product; Gelcord ™, Rastringent II ™, Viscostat clear ™ by using *in vitro* plasma protein precipitation test.
2. the cytotoxicity of these four newly- developed gingival retraction solutions compare with the commercial product; Gelcord ™, Rastringent II ™, Viscostat clear ™ .
3. the detailed reproduction with an addition silicone impression material and Type IV model stone .

Research design

Experimental study in laboratory

Hypothesis

1. Newly- developed gingival retraction solutions; Formula A1, A2, B1, B2 and control are not different in ability to precipitate plasma proteins.
2. These four materials are not toxic to L929 fibroblast cells.
3. These four materials are not interfere the detailed reproduction with an addition silicone impression material.

Retraction agents

Newly- developed four retraction solution formulas

1. Formula A1

Formula A1 consists of 25 % aluminium chloride in aqueous solution.

2. Formula A2

Formula A2 consists of 25 % aluminium chloride in non aqueous solution.

3. Formula B1

Formula B1 consists of the 25 % aluminium sulfate in aqueous solution.

4. Formula B2

Formula B2 consists of the 25 % aluminium sulfate in aqueous high viscous solution.

Control groups

The commercial product;

- Rastringent II ™ consists of 25 % aluminium sulfate in solution.
- Gelcord ™ consists of 25 % aluminium sulfate in viscous solution.
- Viscostat clear ™ consists of 25 % aluminium chloride in solution.

CHAPTER II
LITERATURE REVIEW

Gingival displacement is an essential process for fixed prosthodontics, because of dentists have to make impressions that accurately capture the prepared cervical finishing lines and permit the fabrication of accurate dies before restorations are fabricated.

Various methods are used for gingival displacement. These methods can be classified into four categories according to Benson et al (6):

1. Mechanical method
2. Mechanico - chemical method
3. Rotary gingival curettage
4. Electrosurgical method

The method of gingival displacement used by the majority of dentists is the mechanico-chemical method using gingival retraction cords along with hemostatic medicaments (4).

According to the mechanico-chemical method, the cord is inserted into the sulcus to stretch the periodontal fibers through exertion of sustained pressure. Many types of retraction cord are available such as braided, twisted, and knitted cords. Knitted cords tend to be slightly more flexible, and thus easier to place than the braided varieties. Often mechanical pressure from the cord itself is not sufficient for gingival displacement and sometimes after or during tooth preparation, the gingival tissues may have some damage and bleeding, so cords impregnated with certain chemical compounds have been recommended. Furthermore, crevicular seepage can be controlled using these cords with chemical agents. Many chemical agents are recommended and used for the retraction procedure.

The effectiveness of the chemical agents comes from shrinking tissue and stopping bleeding in conjunction with the retraction cords. The cords also help to keep the chemical solutions in contact with the tissues and confine them to application sites.

These chemical agents can arrest excessive bleeding or hemorrhage from small blood vessels or capillaries. They can be classified according to the mechanism of their actions (9).

1) Sympathometic vasoconstrictors stop bleeding action by local vasoconstriction. This agent affects the alpha receptor in the smooth muscle wall of arterioles and causes constriction, resulting in reduced blood flow. Epinephrine is available chemically in two forms: dextrorotary epinephrine and levorotary epinephrine. A commonly used for local vasoconstrictor to stop bleeding in gingival retraction is 0.1 and 8% racemic epinephrine (mixture of the dextrorotary & levorotary forms of epinephrine).

The local use of epinephrine as a gingival displacement or hemostatic medicament has the potential to cause significant systemic side effects. The systemic effects of epinephrine have been studied extensively, and most researchers have concluded that epinephrine should not be used for routine gingival displacement (10, 11).

The absorption and effects of epinephrine from gingival retraction cords is somewhat contradictory. In correlating data from various studies, it is safe to conclude that under certain conditions epinephrine from retraction cords is absorbed systemically. Conditions that limit absorption are not clear, but increased absorption seems to occur with increased exposure of the vascular bed and with an increase in the total amount of epinephrine used. Increased doses may occur with the use of stronger concentrations of medications or with the use of multiple cords when making impressions of multiple prepared teeth.

Other factors related to the total dose of epinephrine received by a patient include epinephrine administered in local anesthetic solution and any endogenous epinephrine that may be secreted by the patient in reaction to stress or discomfort associated with the dental procedures. Epinephrine is contraindicated in patients with hyperthyroidism and in patients taking monoamine oxidase inhibitors or tricyclic antidepressants for depression, ß-blockers, or cocaine. It is also contraindicated in diabetic and cardiovascular patients.

Determining which patients may be classified as cardiovascular patients can be difficult. Although some patients are clearly identified by their medical history, many

patients are unaware of their incipient problems. Even though the majority of dentists routinely take blood pressure and pulse records, resting pulse rates, resting blood pressure records, and resting electrocardiograph records, it was reported that they miss approximately 45% of latent cardiovascular problems (1).

Therefore, recently a majority of dentists avoid using epinephrine to prevent complications in their patients.

2) Astringents are locally acting agents which condense the surface of edematous tissue. A concentrated form of astringent aids in sealing off bleeding areas caused from broken capillaries by precipitating tissue and blood proteins.

The precipitating proteins and blood proteins will cause the mechanical obstruction to hemorrhage from injured blood vessels and inhibiting transcapillary movement of plasma proteins, so it condenses with edematous and hemostasis effects. When applied on moderately inflamed and edematous tissues, they reduce the permeability of the cell membrane, and thus reduced local edema is a result. Simultaneously, when applied on injured vessels; they inhibit capillary blood flow and stop bleeding (9).Since large amounts of these agents cause tissue irritation, only small amounts should be applied to prevent further tissue damage with increased bleeding. Their principal usage in dentistry is to slow or stop capillary bleeding and contraction of gingival tissue before taking an impression (12).

Many astringents are used in gingival displacement procedures such as 100% alum solution (aluminium potassium sulfate), 5 - 25% aluminium chloride, 8- 40% zinc chloride, 13.3% ferric sulfate, aluminium sulfate, 20% and 100% tannic acid solution, and 45% Negatol solution (6).

A review of astringents

The use of ferric sulfate *(13.3%)* for tissue displacement has recently been reported (13). It does not traumatize the tissue as noticeably, and healing is more rapid than using aluminium chloride. Ferric sulfate is compatible with aluminium chloride, but not with epinephrine. Because when used with epinephrine, a massive blue precipitate develops. Ferric sulfate coagulates blood so quickly that it must be placed directly against the cut tissue. If not, the ferric sulfate becomes tied up with the extravasated blood and floats away, leaving a bleeding surface. The recommended use

time is one to three minutes, but can be used up to 10-20 minutes. The resulting tissue displacement is maintained for at least 30 minutes, so that repacking is seldom necessary for multiple impressions. The tissue can temporarily turn to a black or bluish but will be pink again after one or two days. In vitro tests failed to show the corrosive or staining effects on the enamel that had previously been reported with ferric compounds.

Ferric subsulfate, also known as Monsel's solution, has been advocated for use in gingival displacement (13). It is slightly more effective than epinephrine in gingival displacement. Tissue recovery is good, but the solution is messy to use (10). The recommended time of use is three minutes. The literature infers that ferric or ferrous salts should not be used because they are corrosive and injurious to the soft tissues and enamel and they also stain the teeth. These properties are attributed to the high acidity of the solution (6).

Alum (potassium aluminium sulfate) in a 100% saturated concentration has been shown to be only slightly less effective in shrinking the gingival tissues than epinephrine and other retraction agents, but it shows good tissue recovery. Only slight tissue injury was noted within 10 minutes of application, but it was completely healed in 10 days (14). Although alum is kind to the tissue, but the tissue retraction and hemostatic ability is limited. Alum has been recommended for use in place of epinephrine because it is more safe. Cord saturated with saturate alum can be safely left in the sulcus for as long as 20 minutes without adverse effect (14).

Aluminium chloride is one of the most commonly used astringent in the tissue retractions. Uses of concentrations of 5% to 25% have been reported. According to the studies solutions stronger than 10% can produce local tissue destruction (5, 8). A ten minutes application in the sulcus is usually sufficient. Aluminium chloride has not been shown to demonstrate a significantly different inflammatory reaction than alum or 8% racemic epinephrine (15). A permanent crestal gingival loss of 0.1 mm can be expected. No contraindications and minimal systemic effects are known (16). The 25% solution has been advocated for use with other chemical agents because it approximately doubles the hemostatic success of each of the other chemicals studied. A large number of oral health practitioners prefer aluminium chloride-impregnated

cord as the most effective chemical to control bleeding and displace tissues with no resultant tissue damage (15).

Aluminium sulfate causes hemostasis by a weak vasoconstrictor effect in addition to precipitation of the tissue proteins with tissue contraction, inhibited transcapillary movements of plasma proteins and subsequently arrest capillary bleeding. The medication is regarded as safe and devoid of systemic effects when used appropriately (17).

Aluminium sulfate is one of the least irritating of the retraction chemicals currently use and highly recommended. Some practitioners have reported on the good success route of aluminium sulfate for many years. Aluminium sulfate does not ionize as readily as alum and will always have astringent qualities, but never toxic. Usually, it is prepared as a saturated solution while other solutions are prepared by evaporation. Evaporation can cause other retraction chemicals to become concentrated and make them potentially dangerous (14).

Both aluminium chloride and aluminium sulfate can be used as hemostatics like ferric sulfate and ferric chloride. The main difference is that the chlorides tend to be more caustic and corrosive than the sulfates (18). However, both of them have the same clinical effects.

Zinc chloride (bitartrate) has been used in 8% and 40% solutions. Gingival displacement effectiveness of the 8% solution is about equal to that of epinephrine, while the 40% solution is a little more effective. The 8% solution causes severe necrosis of the tissue that dose not heal for 60 days (19). The 40% solution is so caustic that it has been classified as a chemical cautery agent. Because both of these concentrations are escharotics and cause permanent injury to the soft tissue and sometimes to the bone their use is not recommended (10, 19).

Tannic acid (20% and 100%) is less effective than epinephrine but very good tissue recovery (10). Its hemostatic effectiveness is minimal.

Negatol solution is a 45% condensation product of metacresol sulfonic acid and formaldehyde. It provides better retraction than epinephrine. Tissue recovery is poor. It is highly acidic and decalcifies teeth in both 10% and 100% solutions. Besides, it is classified as a chemical cautery agent and not recommended for gingival displacement (6).

A summary of the advantages and disadvantages of hemostatic agents used in conjunction with retraction cords are shown in Table 1.1

Table 2.1 Summary of advantages and disadvantages of chemical agents used in the mechanico chemical method (6).

Drug	Advantages	Disadvantages
Epinephrine 0.1 and 8 %	Good displacement Minimal tissue loss Good response Good hemostasis	Systemic reactions Epinephrine syndrome
Alum 100% (potassium aluminium sulfate)	Minimal tissue loss Extended working time	Less displacement and Hemostasis than epinephrine
Aluminium sulfate 25%	Minimal tissue loss Good hemostasis Good tissue response	may interfere with the polymerization of the addition silicones which would limit their application
Aluminium chloride 5% & 25%	Minimal tissue loss Good hemostasis	Local tissue destruction in concentration >10%
Ferric subsulfate	Good displacement	Complicated to use High acidity Corrosive to tooth structure& Tissues
Ferric sulfate 13.3 %	Good tissue response Compatible with aluminium chloride Good displacement Extended working time	Not compatible with epinephrine Transient tissue Discoloration Unpleasant taste
Zinc chloride 8 % and 40%	Good displacement	Tissue necrosis Permanent tissue injury
Tannic acid 20 % and 100 %	Good tissue response	Less displacement than epinephrine Minimal hemostasis
Negatol 10 % and 100 %	Good displacement	Poor tissue response Corrosive to teeth High acidity

There are several forms of astringents used in dentistry. The most common form used with retraction cord is liquid or solution; the other forms are viscous solution or

gel forms. An advantage of liquid astringent is easy to impregnate with the retraction cord but it is difficult to control bleeding occurred during tooth preparation. The viscous solution form is superior for application e.g. topical application, impregnated or coated with retraction cord. Furthermore, the latter form can prevent the spreading of solution over the gingival tissue.

Table 2.2 A review of preparation forms of commercial astringent used in dentistry.

Preparation form of astringent	**Active ingredient**	**Other compositions**	**Commercial name/Vender**
Solution form	15% ferric sulfate	unknown	-FS Hemostatic/Premier
	25% aluminium sulfate	unknown	-Rastringent II/Pascal
	25% aluminium chloride	unknown	-Gingi – aid/Gingi-Pak -Hemodent liquid/Premier -Rastyptine/Septodon -Hemoban/Sultan chemists
Viscous solution form	15.5%ferric sulfate 20% ferric sulfate	-unknown thickening agent -other ingredient	-Astringedent/Ultradent -Viscostat/Ultradent
	12.7% iron solution	-unknown thickening agent -other ingredient	-AstringedentX/Ultradent
	25% aluminium chloride	-unknown thickening agent -other ingredient	-Viscostat clear/Ultradent
		-glycerin base -other ingredient	- Hemogin-L/Van R
		-non-aqueous	

		glycerin base -other ingredient	-Styptin/Van R
Gel form	25% aluminium sulfate	-unknown thickening agent -other ingredient	-Gel-Cord/Pascal -StatGel FSProPack/Pascal
	25% aluminium chloride	-non-aqueous glycerin base -unknown thickening agent -other ingredient	-Hemodenttes/Van R

Recently, new gingival retraction materials have been developed to overcome the disadvantages of commonly used retraction solutions. The ability in bleeding control and protein precipitation will be provided by 25 % aluminium chloride or aluminium sulfate contained in the viscous solution and use with retraction cord that will make the opening sulcus clean and dry from bleeding and sulcular fluids.

Moreover, in this study, new gingival retraction solutions will be developed in "viscous" forms that will be used with retraction cord. The nature of the viscous solution makes it easy to expel the vehicle into the gingival sulcus and physically provides easy or helpful lubricate of the gingival retraction cord when put in the sulcus. It is expected that these new viscous solutions can be completely cleaned up from gingival sulcus after the procedure. At the same time, they can stop bleeding and keep the gingival sulcus clean and dry from bleeding and gingival crevicular fluid. Furthermore, since the vehicle is coated on the cord, so it will prevent the clotting removed when the cord is removed.

Many studies reported that the polymerization of polyvinyl siloxane impression materials can be inhibited by sulfur in latex rubber products, and some authors suggested that gingival medications may be the cause of inhibition of the polymerization of polyvinyl siloxane in a manner similar to that of latex (20). The effect of inhibited polymerization of polyvinyl siloxanes is manifested by the appearance of a rippled surface on the set impression material. The surface of the impression material in the areas that were contaminated appears slippery to the touch.

After using these impressions, the rippling is duplicated in the gypsum cast, and the cast may appear wet, wrinkled, or poorly defined. Often, unpolymerized impression materials adhered to the prepared teeth or to the cast when the impression is separated. Regardless, the poorly defined surface detail of the cast should not be compromised and suitable for use in the fabrication of cast restorations. However, Camargo, Chee, and Donovan (21) reported that commonly used gingival retraction medications contain chemically active agents, such as racemic epinephrine, aluminium chloride, aluminium sulfate, aluminium potassium sulfate, and ferric sulfate were not the cause of inhibition of polyvinyl siloxane polymerization.

In this study, new gingival retraction solutions are innovative products. Therefore, these newly developed gingival retraction solutions would be tested *in vitro* to provide basic knowledge regarding bleeding control ability and biological response before applying to humans. Furthermore, the test of compatibility with impression materials would be done to confirm for using in the clinical practice. Therefore

1) Bleeding control ability of new gingival retraction solutions would be determined by the blood plasma protein precipitation test.

2) Biological compatibility with periodontal tissue would be determined by an *in vitro* cytotoxicity test.

3) Compatibility of new gingival retraction solutions with addition silicone impression material would be test.

The bleeding control ability of the agent or the effectiveness of hemostasis property can be tested by the ability of plasma protein precipitation.

The assessment of retraction agents can be done by testing the ability of the agent to perform as a hemostasis agent and its astringent properties, to investigate what extent the retraction agents or astringents have any effectiveness. In the process that we use to test the ability of bleeding control, we will examine at the mechanisms of hemostasis agents that will be the indicators for an effective viable and reliable hemostasis agent. The mechanisms of the astringent that can cause hemostasis comes from the process of precipitate protein in the blood and the protein that aggregate and

will obtulate the pore of the blood vessel, So this process can be used as a test to control bleeding.

Using *in vivo* study, Weir & Williams (16) compared the procedure of retraction by pack cord with non medicated cord, cord with epinephrine and cord with aluminium sulfate followed by evaluation and comparison by a scale to score of bleeding.

However, *in vitro* study evaluation of the hemostasis normally can be checked from the precipitated protein ability. Dobbs (22) explained that commonly used astringents were known as “protein precipitating agents” and that they can shrink tissue and stop bleeding by precipitating action on tissue and blood protein when bleeding occurred from injured blood vessels during the tooth preparation and gingival retraction. The astringent action, manifested by the heavy metal salts in astringents could combine with blood protein to form insoluble compound. This compound causes a mechanical obstruction to hemorrhage and bleeding from the injured blood vessel site. The *in vitro* protein precipitation test was performed by Bradford protein assay. The Bradford protein assay is widely used for determining the protein quantitatively, which is a rapid and sensitive method (23).

This assay is based on the following the quantitation of protein dye binding by the observation that Coomassie brilliant blue G-250 exists in two different color forms, red and blue. The red form is converted into blue when the dye binds to the protein. The protein dye binding complex has a high extinction coefficient and great sensitivity in measurement of protein. The binding of the dye to protein is a very rapid process, and the protein dye binding complex remains dispersed in solution for a relatively long time.

Biological consideration of new gingival retraction solutions

Biocompatibility is the ability of a material to function in a specific application in the presence of an appropriate host response. It is a primary requirement of any dental material that it should be harmless to the patient and to those involved in its manufacture and handling. Gingival retraction materials will come into direct contact with gingival tissue. Therefore, their biocompatibility is primary importance.

In previously published literatures, Kopac et al (7) and Harrison (19) reported *in vivo* cytotoxicity of gingival retraction agents in animals and human. All gingival retraction agents tend to produce transient damage to the sulcular epithelium and underlying connective tissue. Several *in vitro* cytotoxic tests have been used for primary evaluation of gingival retraction materials or agents and reducing the use of animals or human experiments (23). *In vitro* cytotoxicity assessment is the measurement of the leachable toxic products from the test materials on the metabolic function of the target cells. It is an important technique for screening dental materials, particularly in new products and reducing the use of animals or human experiments. There are various advantages of *in vitro* methods; that they can be conducted under controlled laboratory conditions and are also free from the influence of the many variables inherent in animal models, which are complex and often impossible to control. A high level of reproducibility is also obtained from the *in vitro* methods. In addition to controllability and reproducibility, they are rapid and cheap compared to animals studies. Controlled *in vitro* experiments are better standardized than *in vivo* experiments and have no problem in ethics (24, 25, and 26). Kopac et al (18) studied the effects of four chemical agents: 10 % aluminium chloride, 25 % aluminium chloride, 20% aluminium sulfate and 0.05% tetrahydrozoline on cultured cell using three different cytotoxicity tests: dye exclusion test, colony forming ability test and MTT assay. The results found that 25% aluminium chloride was the most toxic. Also, the study indicated that the colony forming ability test and MTT assay were more sensitive and reliable than the dye exclusion test, because the cellular damage caused by the dye exclusion test was underestimated. Lui (27) studied non medicated cord, adrenaline impregnated cord and aluminium sulfate impregnated cord by MTT assay and found that adrenaline impregnated cord was the most toxic. As previously published commonly used gingival retraction materials or agents trend to be toxic on culture cells. Therefore, the objective of the *in vitro* cytotoxicity testing in this study is to identify the toxic reaction of these new gingival retraction solutions to culture cells before they are applied in humans.

This study would be based on International Standards covering dental materials (ISO 10993 part 5) (25) and medical devices (ISO 10993 part 12) (26), which include dental materials. Furthermore, the compatibility with an addition silicone impression

material would be test followed by International Standard for elastomeric impression material (ISO 4823) (28).

CHAPTER III
METHODOLOGY

Materials

Test materials: Formula A1, A2 composed of 25 % Aluminuim chloride in aqueous and non aqueous solution, respectively. Formula B1, B2 composed of 25 % Aluminuim sulfate in aqueous solution and high viscous solution, respectively. Commercial gingival retraction solutions i.e. Rastringent II™ (Pascal company Inc, Washington, USA), Gelcord™ (Pascal company Inc, Washington, USA), Viscostat clear™ (Ultradent products Inc, South Jordan, Utah). Rastringent II™, Gelcord™, Viscostat clear ™ are commercially available topical hemostasis agents widely use in Thailand, they were selected to use as control solutions. Overall compositions were shown in Table 3.1.

Table 3.1: Composition of gingival retraction agents .

Formula	Ingredients		pH
	Active ingredient	Others	
Formula A1	25% Aluminuim chloride	-aqueous vehicle	0.61
Formula A2	25% Aluminuim chloride	-non-aqueous vehicle	0
Formula B1	25% Aluminuim sulfate	-aqueous vehicle(low viscosity)	0.51
Formula B2	25% Aluminuim sulfate	-aqueous vehicle(high viscosity)	0.7
A commercial retraction agent 1 (Rastringent II™ (Lot no.100110))	25% Aluminuim sulfate	-aqueous vehicle -other ingredient	2.3
A Commercial retraction agent 2 (Gelcord ™ (Lot no.100109))	25% Aluminuim sulfate	-aqueous vehicle -other ingredient	2.5
A Commercial retraction agent 3 (Viscostat clear™) (Batch 6408 Lot B39KJ)	25% Aluminuim chloride	-aqueous vehicle -other ingredient	0

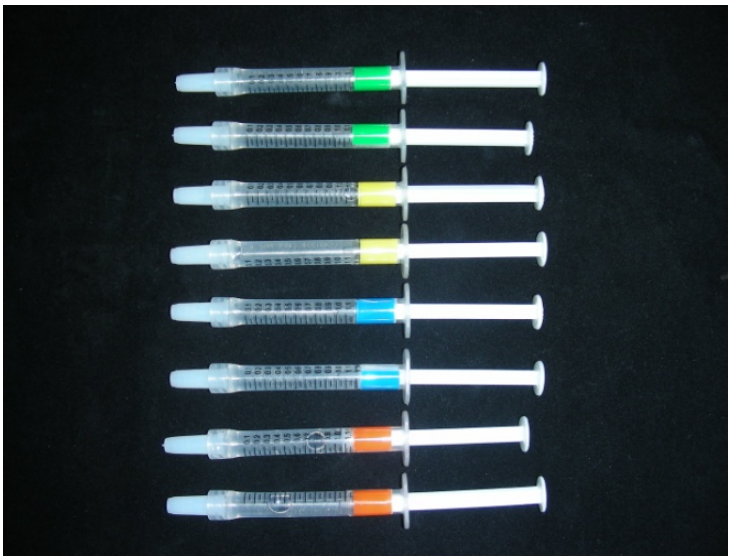

Figure 3.1 Developed gingival retraction agents.

Figure 3.2 A commercial product No. 1: Rastringent II ™.

Figure 3.3 A commercial product No. 2: Gelcord ™.

Figure 3.4 A commercial product No. 3: Viscostat clear ™.

Culture medium, reagents and equipment preparation for cytotoxic testing and plasma protein precipitation testing

Culture medium and reagent preparation

Culture medium is Dulbecco Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island, New York, USA) supplemented with 10% Fetal bovine serum (Hyclone, Logan, Utha) plus 10,000 units/ml penicillin G sodium, Streptomycin sulfate 10,000 µg/ml and 255 µg/ml in 0.85% saline Amphotericin B as fungizone

MTT reagent composes of 10 mg MTT powder (Sigma, St Louis,USA) in 20 ml of 10% complete medium then filtered with filter paper 0.2 micron pore size.

Equipments

1. Analytical balance (Precisa 262, SMA - FR Precisa instrument AG, Switzerland, 0.00001 ± 0.00003 µg)
2. Inverted microscope (Nikon model TMS, Nikon Co., Kanagawa, Japan)
3. Vortex mixer (Genie 2® , Scientific Industries Inc., USA)
4. Biohazard cabinet (GELAIRE ®, Esco Biotech Co., USA)
5. Spectrophotometer (Spectronic 501/601®, Milton Roy Co., North Carolina, USA)
6. Centrifugation machine (Hettich Universal 32®, Andreas Hettich GmbH & Co.,Germany)
7. Elisa plate reader (BIO-TEK Instruments Inc,Winooski,VT)

Materials and equipments for detailed reproduction testing

Materials

1. Addition silicone(Silagum®, DMG, Hamburg, Germany, Lot. 574895)
2. Type IV dental stone(Velmix®, Kerr Co., West Collins Orange, CA, USA, Lot. 31008)

Equipments

1. Metal test block with three horizontal (20, 50, 75) and two vertical lines for detail reproductively evaluation (ISO 4823).
2. Microscope (Nikon model TMS, Nikon Co., Kanagawa, Japan)

Methods

1. Protein precipitation test

The bleeding control ability of four experimental solutions and three commercial retraction products; Rastringent ^{TM}, Gelcord ^{TM}, Viscostat clear ^{TM} were determined by *in vitro* human plasma protein precipitation.

a) Plasma preparation

Human blood (10 ml) was taken from a healthy donor aged 29 years old who had not taken any medication known to affect the blood coagulation and platelet function for at least 2 weeks prior to the study. Blood was put into tube with 200 units of heparin as anticoagulant agent then was centrifuged at 3,000 rpm for 10 minutes. The supernatant was collected and kept at 4°C until used (29).

b) Determination of protein

Protein content of the plasma was determined by protein dye binding assay as described by Bradford (23). This assay was based on the determination of optical density of the Coomassie Brilliant Blue G 250 exists in two different color forms; red and blue. The red form was converted into the blue form upon the binding of the dye to protein. The protein dye binding complex has a high extinction coefficient leading to great sensitivity in measurement of the protein. These assays had the standard protein curve (Figure 3.5) which could determine the protein concentration (mg/ml) against the corresponding optical density absorbance.

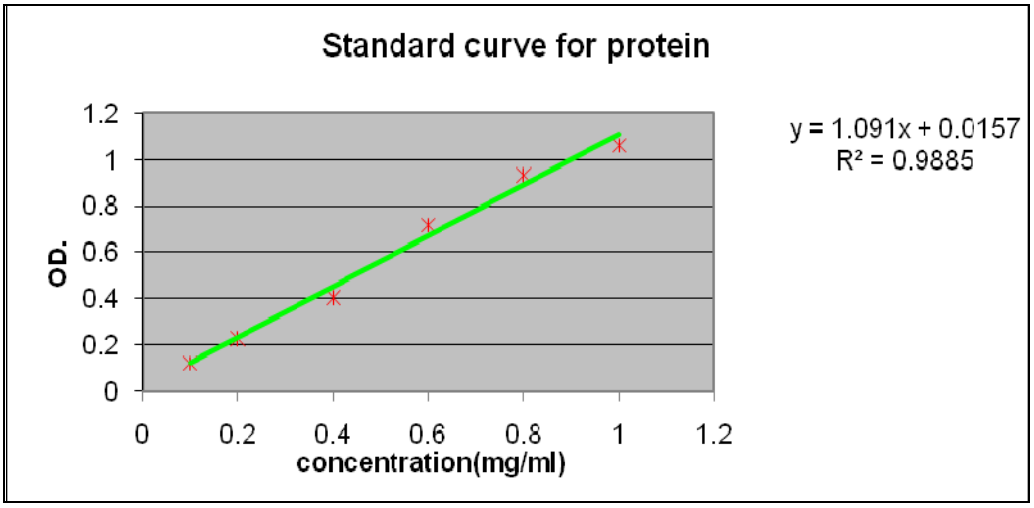

Figure 3.5 Standard curve for Bradford protein assay.

c) Plasma protein concentration determination

Protein concentration was determined by adding 1 ml of 1: 100 diluted plasma into a tube and centrifuged at 3,000 rpm 15°C for 10 minutes. The clear solution after centrifugation was pipetted 100 µl into a clean and dry test tube. Then 5 ml of 1:4 diluted Bio Rad ^{TM} protein dye reagent was added and left at room temperature for 5 minutes. This solution was transferred into the cuvette and measured optical density at 595 nm using a spectrophotometer (Figure 3.6). The optical density of plasma protein was plotted against standard protein curve to determine the plasma protein concentration (mg/ml).

Figure 3.6 Spectrophotometer

d) Plasma protein concentration determination of experimental group

Plasma was diluted 100 folds. 200 µl of each gingival retraction solution Formula A1, A2, B1, B2 and Rastringent II ™, Gelcord ™, Viscostat clear ™ was added into each 1 ml of dilute plasma then left 5 minutes at room temperature. The plasma was centrifuged at 3000 rpm. 15 º C for 10 minutes and the protein concentration of the supernatant was determined according to Bradford (Figure 3.7).

Thirty replications were performed for each experimental group (30).

e) Protein precipitation equation

The percentages of protein precipitation were calculated by using the equation below:

$$\frac{\text{Plasma protein conc. of control} - \text{Plasma protein conc. of experimental group}}{\text{Plasma protein conc. of control}} \times 100$$

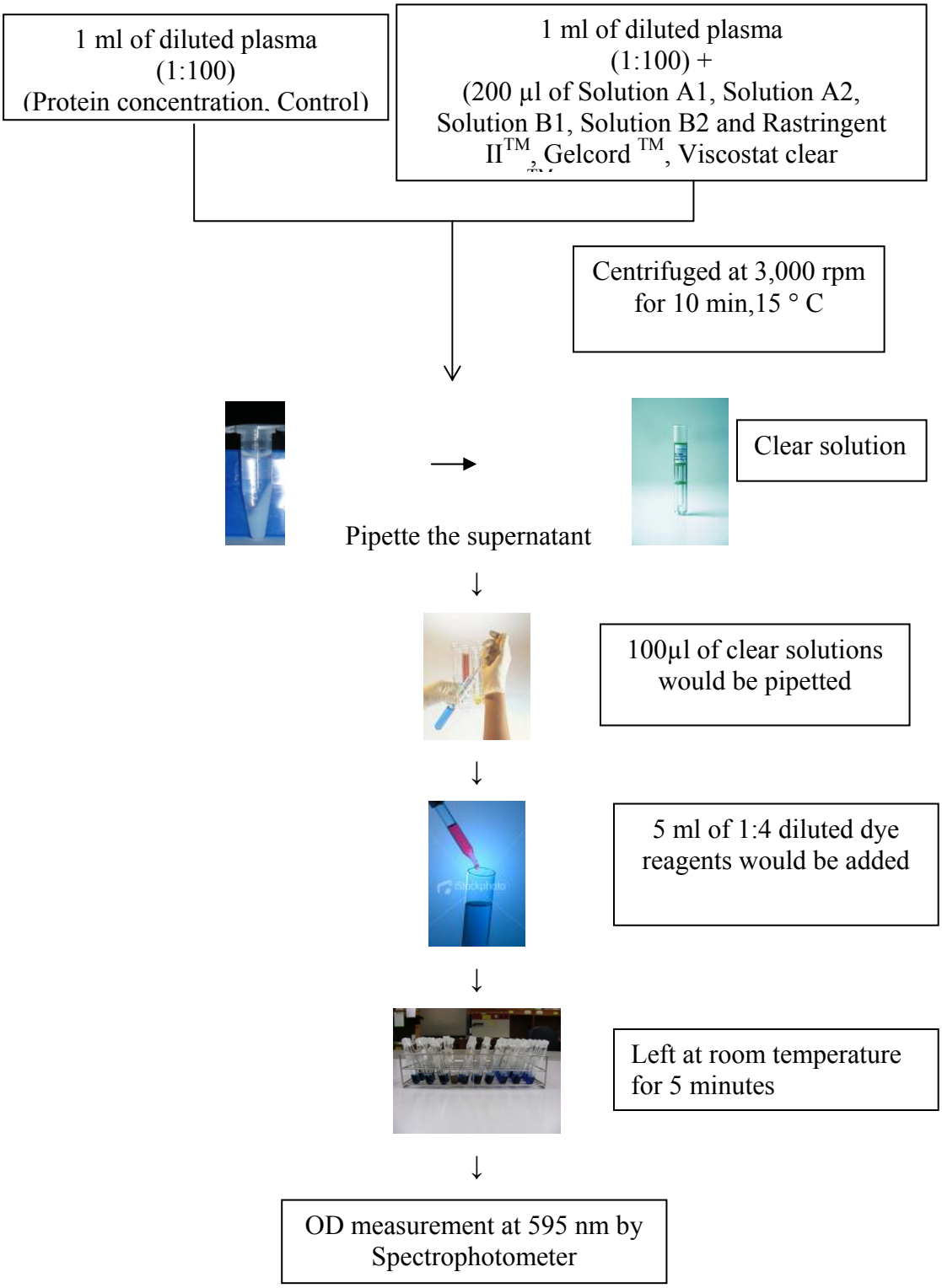

Figure 3.7 Plasma protein precipitation testing diagram

2. Cytotoxicity evaluation

a) Cells culture procedure

L-929 mouse fibroblast cell line (Figure 3.8) were obtained from the cell culture section of the Research unit, Faculty of Dentistry, Mahidol University and used for all experiments. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island, New York, USA) supplemented with 10% Fetal Bovine Serum (Hyclone, Logan, Utha) plus 10,000 units/ml penicillin G sodium, streptomycin sulfate 10,000 μg/ml and 255μg/ml in 0.85% saline amphotericin B as fungizone and incubated in CO_2 incubator (Figure 3.9) at 37°C under a 95% humidified atmosphere and 5% CO_2.

The cell culture was examined daily under an inverted microscope (Figure 3.10) for assessment cell proliferation and bacterial contamination. The medium was changed every other day and the cell suspension was transferred to a new culture flask when cells reached confluency.

b) Subculture procedures

Cells were subcultured after reaching confluency. After removing the complete medium from the tissue culture flask, cells were detached with 5 ml of 0.25% trypsin solution for 1 minute. Then trypsin solution was removed and cells were incubated for 1 minute in the CO_2 incubator at 37°C under a 95% humidified atmosphere and 5 % CO_2. Cells were examined under the inverted microscope to assess their detachment from the culture flask and harvested by vigorous washing with 10 ml. of complete medium. Cells were counted in the haematocytometer under the inverted microscope. The concentration of cell suspension was adjusted by adding the complete medium to gain enough volume for passaging to two new flasks. Cells were subcultured until the amounts of cells were sufficient for the experiment.

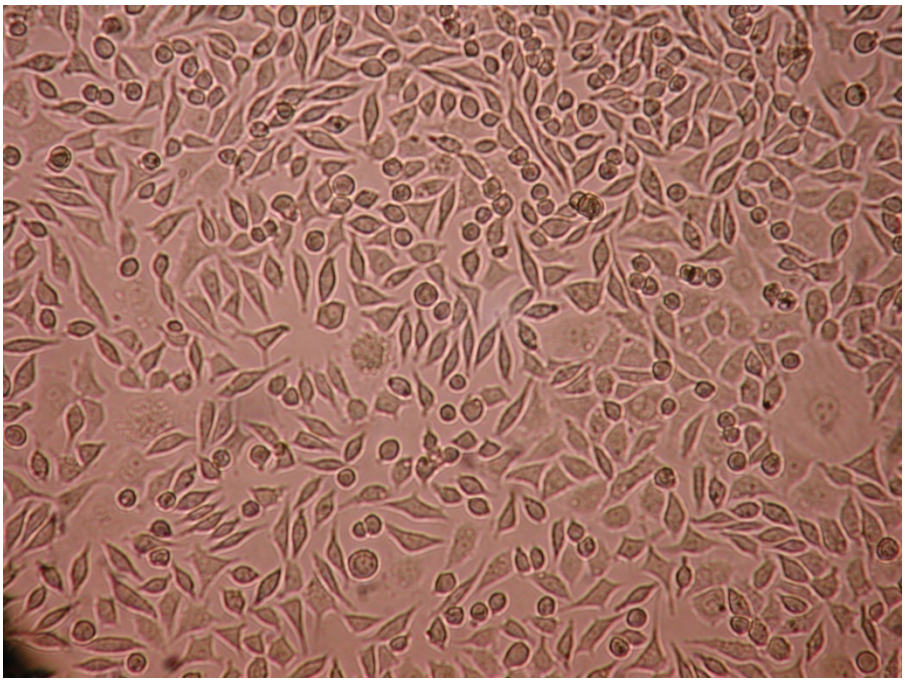

Figure 3.8 L-929 mouse fibroblast cell line (10 X).

Figure 3.9 CO_2 incubator.

Figure 3.10 Inverted microscope.

Sample preparation for serial dilutions (24)

0.2 g of retraction agents was put into the 1 ml of 10% DMEM complete medium. The suspension was mixed by vortex mixer following by centrifugation at 3,000 rpm for 10 min at 15 °C. The supernatant was used for further dilutions of 1: 20 and 1: 30, 1: 40, 1:50 with 10% DMEM complete medium.

c) **Cytotoxicity testing**

Procedure for assessment of the cytotoxicity was performed according to ISO 10993-5 (25).

Cells viability was determined by using MTT test.

Cell preparation

Monolayer L929 cell cultures in flask 75cm^2 were added with 0.25% Trypsin-vasin 5 ml/flask. The flask was left for 1 minute at the room temperature, then trypsin

solution was removed and further incubation for 1 minute in the CO_2 incubator at 37°C under 95% humidified atmosphere and 5 % CO_2.

Cells were adjusted to the concentration $1x10^5$ cells /ml. Then 200 µl of cell suspension at the 1×10^5 cells/ml concentration were plated into each well of 96 well microtitre tissue plates.

Cytotoxic assay with direct contact

This sensitive colorimetric test was used for the evaluation of viability of cells by measuring dehydrogenase activity as described by Mosmann, 1983 (31) with minor modification. The experiments were performed in 96 well microtitre tissue plates. For the assay, 2×10^4 cells in 10% complete media (10% DMEM) were grown in each well of the 96-well microtiter tissue plates. The cells were incubated in the CO_2 incubator at a 95% humidified atmosphere of air, 5% CO_2 and 37ºC for 24 hours. After 24 hours cells reached confluence and would be monolayer. The media was removed by aspiration and 100 µL of 10% complete media was added to each control well and 100 µL of each retraction agents at one of four different concentrations (stock, 1:20, 1:30, 1:40, and 1:50). The plates were incubated in the CO_2 incubator at a 95% humidified atmosphere of air, 5% CO_2 and 37ºC for another 24 hours.

After 24 hours of exposure to the test materials, the media and the test agents were removed by aspiration and cells were washed twice with PBS 1X solutions followed by the addition of 100 µl of MTT reagent (3-(4, 5-dimethylthiazol-2-yl)-2-5-dyphenyl tetrazolium bromide). Then plates were wrapped with the Aluminuim foil to protect from the light exposure. The plates were incubated in the incubator at 37°C, 95% humidified and 5% CO_2 for 2 hours and washed with 200 µl of PBS 1X, after that 100 µl DMSO (Dimethyl sulphoxide) were added to elute the formazan from the cells. The plates were wrapped again with Aluminuim foil to protect the light exposure and put on the shaker for 30 minutes. After 30 minutes, the absorbance was measured by Elisa reader (Nanomicroplate reader) at 540 nanometers. The DMSO was used as blank. The percentage of the cell viability (%) was calculated. The experiments were performed at least 3 times (27, 30, 32).

The percentages of cell viability was calculated according to the following equation:

$$\frac{\text{OD 540 of tested material} - \text{OD 540 of DMSO}}{\text{OD540 of control- OD 540 of DMSO}} \times 100$$

Cytotoxicity was rated based on cell viability to control as (33)

> 90% cell viability	=	non cytotoxic
60 – 90 % cell viability	=	slightly cytotoxic
30 - 59 % cell viability	=	moderately cytotoxic
<30 % cell viability	=	severe cytotoxic

3. Detailed reproduction and compatibility with an addition silicone impression material

Testing was done to determine whether any of the new gingival retraction solutions inhibit the polymerization of addition silicone impression materials when they were in direct contact.

The experiments were performed using the test block for detail reproduction based on the International Standards Organization specification ISO-4823: Elastomeric impression materials (28) to evaluate the ability of line width reproduced (the groove widths of 20, 50, 75 micrometers) of addition silicone impression material.

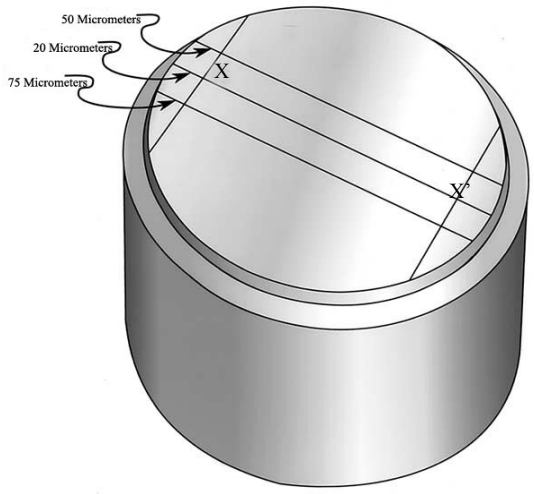

Figure 3.11 Test block for detailed reproduction.

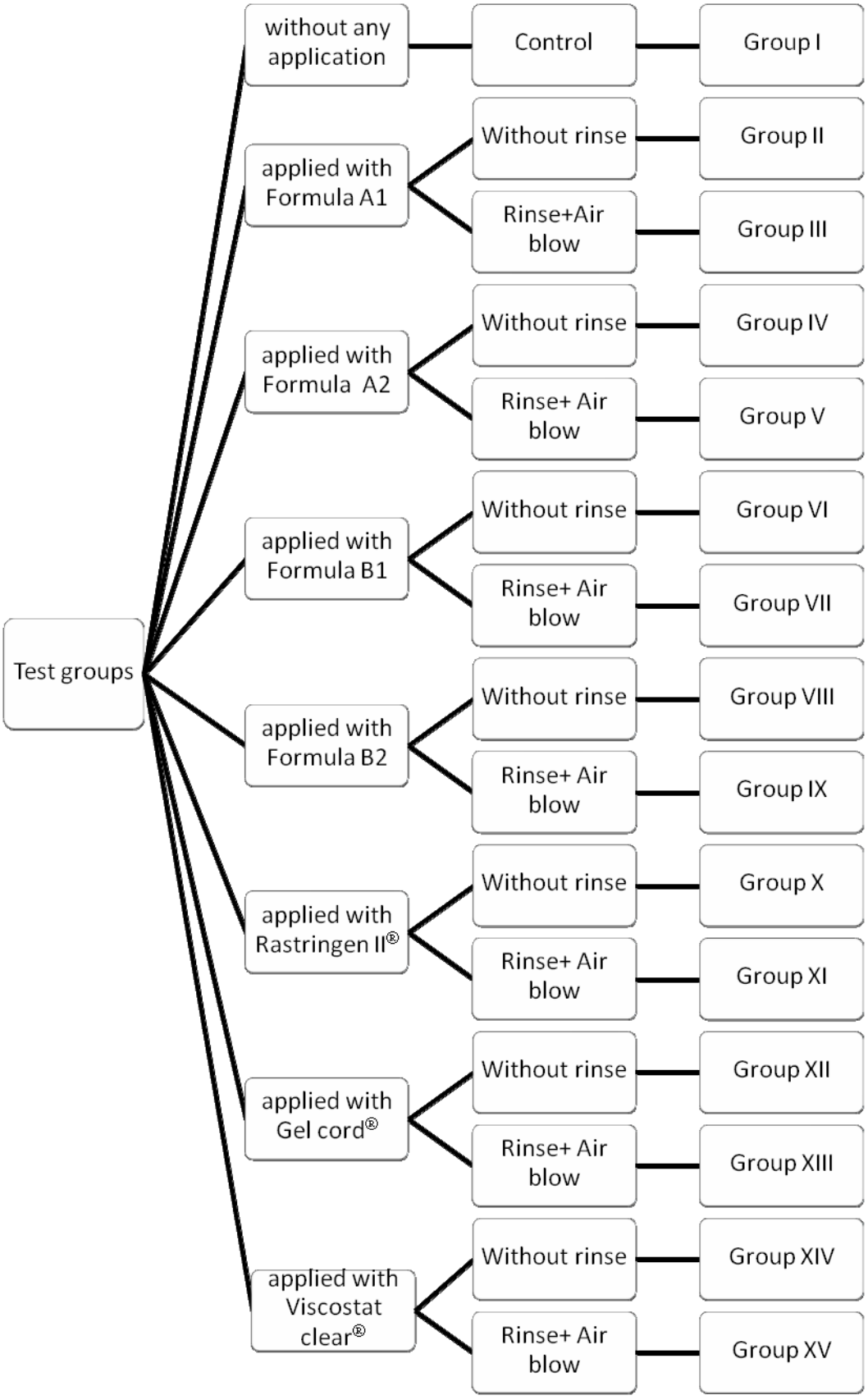

Figure 3.12 Testing protocols for detailed reproduction.

According to ISO 4823 specification, the test blocks were cleaned ultrasonically before each use. After that, test blocks and rings were put into the oven (37 ºC) for dry heat conditioning of the test blocks and rings prior to use. The test agents applied, rinsed and air blown were applied to the test block following with the sample group determination, then the ring mould was seated on the test blocks to form the specimen forming cavity.

Low viscosity of addition silicone impression material (Silagum-Light ™, DMG, Hamburg, Germany (Lot no. 574895) was mixed according to the manufacturer's instruction, and injected into the mold along one side of the cavity to introduce an increment of the mixed material (enough to slightly overfill the cavity), so that it could be directed to first enter the scribed lines 20, 50, and 75 micrometers on one side of the test block. Then, the impression material was covered with polyethylene sheet and gradually forced, via application of pressure applied by the glass plate, to flow into the lines to their opposite ends. Then, this specimen-forming assembly was place in a water bath (35±1°C) for the complete setting of the impression followed by recommended setting time according to the manufacturer's instructions and simulated the intraoral condition. After complete of the water bath treatment, the impression material specimen in the ring mould was separated from the test block forming assembly and the impression surface was flushed with deionized water. Then gentle stream of clean air was used to blow away moisture. The lines on the test specimens were positive copies (raised lines) of the lines scribed in the test block surface.

Phase one of investigation (Detail reproduction of addition silicone impression material)

Surface detail reproductions were evaluated immediately after the impressions had been recovered from the test blocks. The impression was examined under 10X magnification of microscope (Nikon model TMS, Nikon Co., Kanagawa, Japan) for the detail reproduction of the impression and surface characteristics was examined by eye. Evaluation was achieved using two methods.

The first evaluation was performed by an assessment of the continuity of line replication according to ISO 4823. The continuity of three horizontal lines, 20, 50, 75

micrometers were assessed for each specimen. If all of three horizontal lines were reproduced continuously between cross-points in all impressions, these impressions were considered satisfactory. All others were rated unsatisfactory (34).

The second evaluation was performed by an assessment of the surface characteristic of the impression material, the exhibiting surface characteristics such as roughness, oiliness, or slipperiness substance on the surface of the test blocks or adherence of the unpolymerized impression material to the test blocks when the impression was separated from the test blocks. They were rated unsatisfactory, if the entire impression surface was smooth, shiny, and if free of adherence to the test blocks, the impression was rated as satisfactory.

Phase two of this investigation (compatibility with gypsum and line width reproduced)

After evaluating the impression under the microscope, the impression in the ring was clamped with slit mold that was closed during formation of the gypsum models and was poured with Type IV dental stone (Velmix ^{TM}, Lot no. 31008) following the manufacturer's instructions. The dental stone was allowed to set for 45 minutes before separating the model from the impression material. After that, models were examined under 10X magnification for the details (lines) reproduction of the impression to the Type IV dental stone model and surface characteristic of the dental stone model was examined by eye.

The following criterias were used for evaluating the details reproduced by impression materials to the Type IV dental stone models.

- The continuity of the 3 horizontal lines (20, 50, 75 micrometers) were assessed for each specimen. If at least two of three horizontal lines were reproduced continuously between cross-points, this impression was considered satisfactory to reproduce the detail to the Type IV dental stone model. All others were rated unsatisfactory.
- The presence of smoothness on the surface of the Type IV dental stone models were rated as satisfactory. All others were rated unsatisfactory.

Statistical analysis

Statistical analysis of the plasma protein precipitation ability were analyzed by Kruskal Wallis test due to the data did not indicate normal distribution. Results on the differences of the agents using Mann Whitney U Test with $\alpha = 0.05$ were considered as statistically significant. Percentage of cell viability in cytotoxicity test was carried out using one-way analysis of variance (ANOVA) due to the data indicated a normal distribution. Results on the differences of the agents using Tukey test with $\alpha = 0.05$ were considered as statistically significant.

Descriptive statistics were used to describe and compare detail reproduction ability and surface characteristic of the impression materials under the difference surface treatment by difference solutions.

CHAPTER IV
RESULTS

1. Plasma protein precipitation

Bovine serum albumin was used for standard solutions ranging from 0.1 mg/ml to 1 mg/ml. The linear equation is y=1.091x+0.015 with R^2 of 0.988. Each point shows the mean and standard deviation in triplicate.

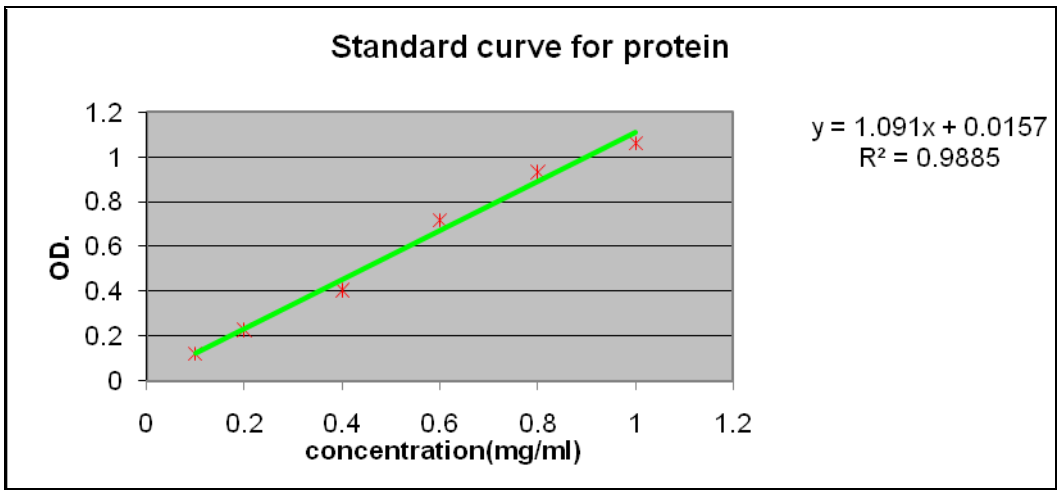

Figure 4.1 Standard curve for Bradford protein assay

Mean plasma protein quantities before and after precipitation with different retraction solutions are shown in Table 4.1. The results were used to calculate the percentages of protein precipitation.

Table 4.1 Mean plasma protein quantities before and after precipitation with different retraction solutions.

Test material	Plasma protein concentration (mean)(mg/ml)	SD	Percentage of plasma protein precipitation
plasma protein 1:100	0.676	0.030	0
Formula A1	0.022[a]	0.016	96.77
Formula A2	0.382	0.017	43.47
Formula B1	0.024[a]	0.015	96.46
Formula B2	0.022[a]	0.017	96.80
Gelcord ™	0.312	0.011	53.84
Rastringent II ™	0.532	0.015	21.26
Viscostat clear ™	0.499	0.016	26.09

Numbers with the same [a] were not significantly different ($p > .05$).

The percentage of protein precipitation was about 96% for Formula B1, Formula A1, and Formula B2 and was 53.8%, 43.4%, 26 % and 21.2% for agents Gelcord ™, Formula A2 , Viscostat clear ™ and Rastringent II ™, respectively. Standard deviations were about 0.01-0.03 for every retraction agent.

Statistical analysis showed that Formula A1, Formula B1, Formula B2 had higher plasma protein precipitation significant different from Formula A2, Gelcord ™, Viscostat clear ™ and Rastringent II ™, respectively ($p<.05$), while Formula B1, Formula A1, and Formula B2 showed no significant different ($p>.05$).

2. Cytotoxicity test

The mean optical density (OD) of seven gingival retraction solutions are shown in Table 4.2 and the percentage of cell viability is shown in Table 4.3.

Table 4.2 Mean optical density of viable L929 mouse fibroblast cells.

Gingival retraction agent	Optical density(OD_{540} nm)	SD
Control	0.769	0.056
Formula A1	0.058[a]	0.033
Formula A2	0.054[a]	0.018
Formula B1	0.215[b]	0.081
Formula B2	0.175[b]	0.044
Gelcord ™	0.600[c]	0.036
Rastringent II ™	0.532[d]	0.024
Viscostat clear ™	0.060[a]	0.002

Superscripts with the same [a] and same [b] were not significantly different ($p>.05$).

The optical density of cell viability was determined after cells were exposed to the tested gingival retraction solution for 24 hours. Mean optical density of agents Formula A1 –Viscostat clear ™ and control group were 0.058, 0.054, 0.215, 0.175, 0.600, 0.532, 0.060 and 0.769, respectively.

Statistical analysis showed that Formula A1, A2, Viscostat clear ™ and Formula B1, B2 showed no significant difference but agents with different superscripts a, b, c, and d were significantly different ($p<.05$).

Table 4.3 Percentages of L-929 mouse fibroblast cell viability and cytotoxicity of seven gingival retraction materials.

Gingival retraction agent	% cell viability	Cytotoxicity
Control	100	Non – cytotoxic
Formula A1	1.72	Severely cytotoxic
Formula A2	1.11	Severely cytotoxic
Formula B1	21.91	Severely cytotoxic
Formula B2	17.86	Severely cytotoxic
Gelcord ™	76.1	Slightly cytotoxic
Rastringent II ™	66.91	Slightly cytotoxic
Viscostat clear ™	2.47	Severely cytotoxic

The percentage of cell viability was calculated in comparison with the control. The percentage of cell viability of Formula A1- Viscostat clear ™ were found to be 1.72%, 1.11%, 21.91%, 17.86%, 76.1%, 66.91%, and 2.47%, respectively.

From the cytotoxicity rating follow by Dahl (37), the Gelcord ™, and Rastringent II ™ showed slightly cytotoxicity, while Formula A1- Formula B2 and Viscostat clear ™ showed severely cytotoxicity.

3. Detailed reproductions

Detailed reproduction with an addition silicone impression material was first evaluated based on ISO-4823 criteria if all 3 horizontal lines are reproduced continuously between cross points, smoothness, shininess, and free of adherence to the test block in all impressions will be rated as satisfactory.

The detailed reproduction of impression material are shown in Table 4.4.

Table 4.4 Detailed reproduction of impression materials.

Category	Line width reproductions			Number of satisfactory impressions	Evaluation
	20µ	50µ	75µ		
Control	√	√	√	5 impressions	Passed
Formula A1, A2, B1, B2, Rastringent II ™, Gelcord ™, Viscostat clear ™ without rinsed	-	-	-	-	Failed
Formula A1, A2, B1, B2, Rastringent II ™, Gelcord ™, Viscostat clear ™ and rinsed	√	√	√	5 impressions	Passed

Criterias for pass is all of impressions can reproduce all of 3 horizontal lines from the metal test block (ISO 4823).

The control and all groups that were applied with retraction solutions then were rinsed for 10 seconds and blow-dried to show the normal appearance of the horizontal lines on the impression surface (all 3 lines) can be reproduced continuously between cross points when evaluated by the naked eye and under microscope. Moreover, the impression surface characteristics showed smoothness, shininess, and free of adherence to the models (Figure 4.2).

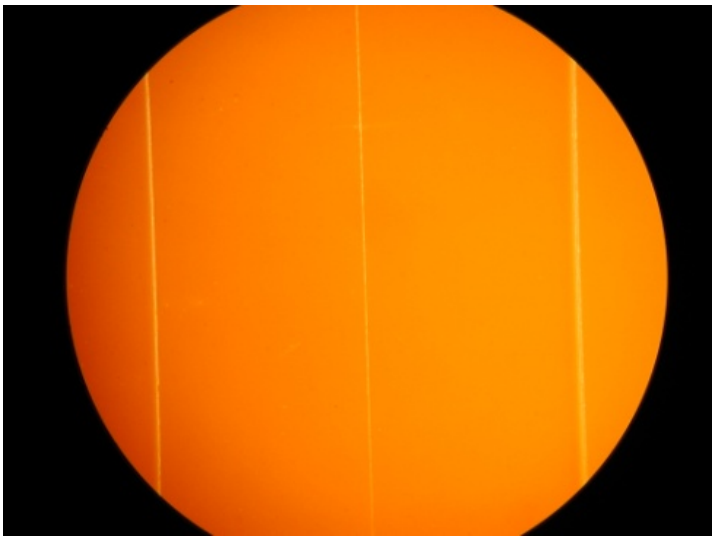

Figure 4.2 Detailed reproduced on the impression in control and rinsed after applied with gingival retraction agent groups (10X).

On the other hand, in the groups that only applied retraction solutions and were blow-dried, the gel still coated the test block. Furthermore, in the group that applied Rastringent II ™, which appeared like small water drops coating the test block. After the impression was taken from these groups, the surface of impressions could not reproduce all 3 horizontal lines from the test blocks. On the other hand, the impression material could reproduce the gel lines appearance similar to those appearing in the test block (Figure 4.3). The impression surface characteristics appeared irregular, all of the impressions were set, but the area that touched the agent was neither shiny nor smoothness (Figure 4.4).

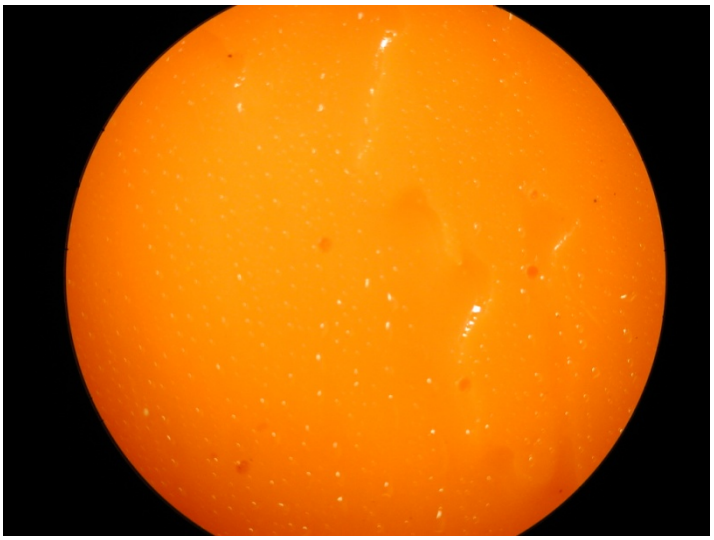

Figure 4.3 Detailed reproduced on the impression in unrinsed after applied with gingival retraction agent group (10X).

Figure 4.4 Impression surface characteristic of unrinsed after applied with gingival retraction agent group.

The detailed reproductions of Type IV stone model are shown in Table 4.5

Table 4.5 Detailed reproduction of Type IV stone model.

Category	Line width reproductions			Number of satisfactory models	Evaluation
	20µ	50µ	75µ		
Control	√	√	√	5 models	Passed
Formula A1, A2, B1, B2, Rastringent II ™, Gelcord ™, Viscostat clear ™ without rinsed	-	-	-	-	Failed
Formula A1, A2, B1, B2, Rastringent II ™, Gelcord ™, Viscostat clear ™ with rinsed	√	√	√	5 models	Passed

Criteria for pass is all of 3 model can reproduce at least 2 of 3 horizontal lines from the impression (ISO 4823).

Regarding the Type IV stone model evaluation by the naked eye and under microscope, it was found that the Type IV stone model could reproduce the horizontal line at least 2 to 3 lines (i.e. 75µ,50µ) from the impression of the control group and the group that applied retraction solutions and were rinsed(Figure 4.5). The surface characteristic of these groups presented smoothness on the surface of the Type IV stone models (Figure 4.6). On the other hand, the group that applied with retraction solutions without rinse found that they could not reproduce the horizontal lines or if reproduced, the lines were not continuous(Figure 4.7). The surface characteristics of the Type IV stone model presented roughness and some porosity on the area that was in contact with the coated retraction agent (Figure 4.8).

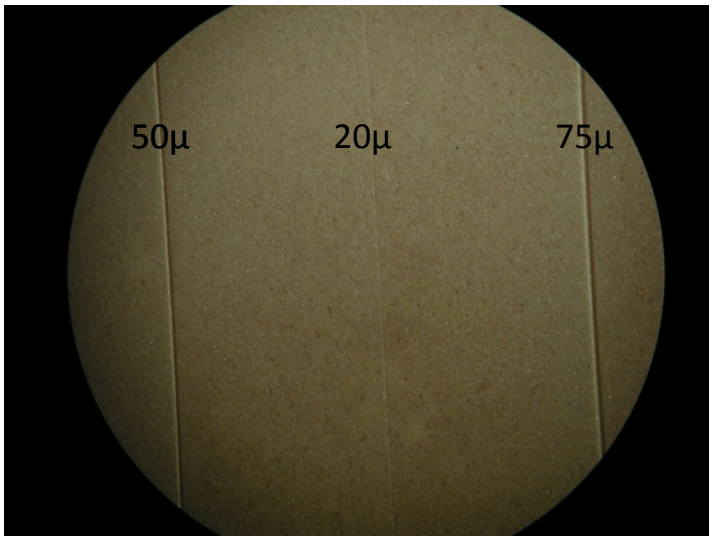

Figure 4.5 Detailed reproduced on the Type IV stone models in control and rinsed after applied with gingival retraction agent groups (10X).

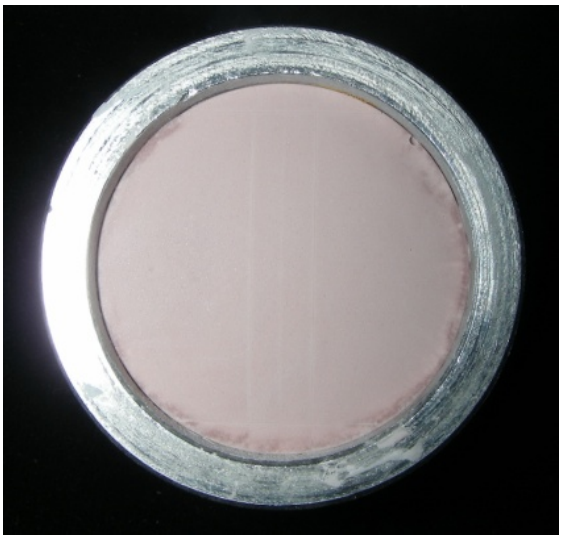

Figure 4.6 Type IV stone models surface characteristic of control and rinsed after applied with gingival retraction agent groups.

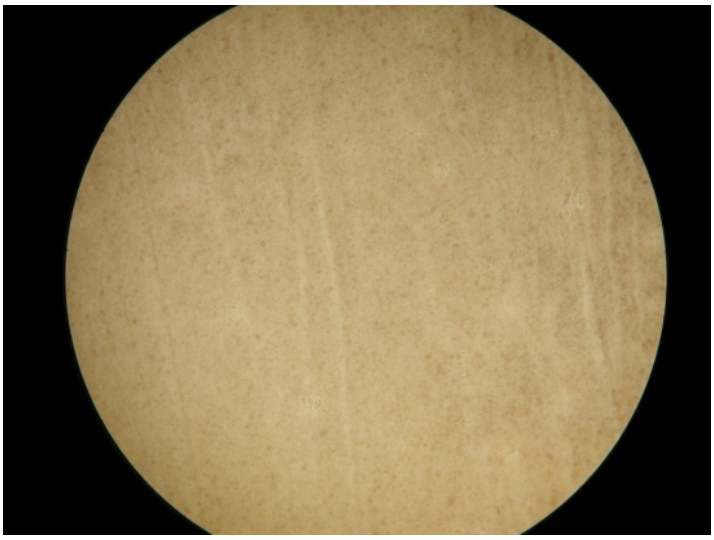

Figure 4.7 Detailed reproduced on the Type IV stone models in unrinsed after applied with gingival retraction agent group (10X).

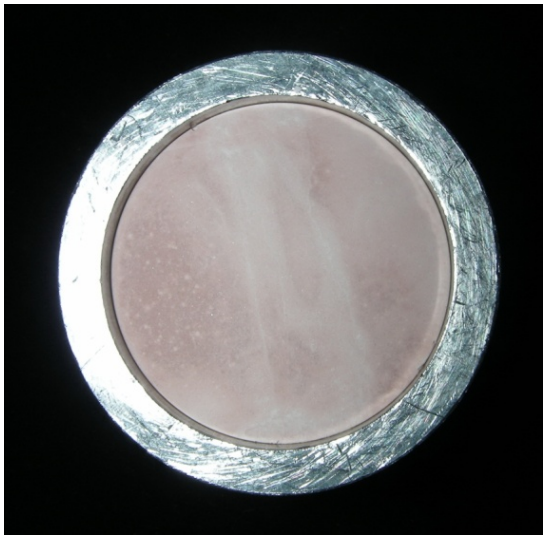

Figure 4.8 Type IV stone models surface characteristic of unrinsed after applied with gingival retraction agent group.

CHAPTER V
DISCUSSION

According to the criteria for gingival displacement procedures described by Nemetz (4), any gingival displacement method should be as follows:

1) able to create sufficient lateral and vertical space between the gingival finishing line and the gingival tissue to allow the margin of the prepared tooth to be recorded in an impression.
2) able to provide absolute control of bleeding and gingival crevicular fluid.
3) able to provide biological compatibility with periodontal tissue without significant irreversible soft or hard tissue damage.
4) not produce any potentially dangerous systemic effect.
5) compatible with an addition silicone impression material without inhibiting polymerization or interfering with the detailed reproduction of addition silicone impression material.

In order to achieve these criteria, the new gingival retraction solution was developed. However, the basic properties, the ability to control bleeding and biocompatibility of these materials have been ascertained in this research.

1. The ability to control bleeding

The ability to control bleeding is one of the important properties of the newly developed gingival retraction solution. The best method to evaluate the ability of bleeding control in the gingival displacement procedure should be done clinically. For example, Wier and Williams (16) studied the ability of bleeding control in teeth prepared for crown restoration and placed the gingival retraction cord with different conditions and evaluated the bleeding control clinically. However, the development of

new materials have to be tested *in vitro* for their properties and toxicity before human experimentation can be performed.

In order to control bleeding one mechanism proposed was plasma protein precipitation (22). In bleeding process, blood clotting will occur after blood coagulation factor aggregated and blood plasma proteins precipitated out from the whole blood. Therefore plasma protein precipitation is one of the tests that can be used to evaluate the bleeding control property.

According to the study of Ciancio and Bourgault (12), the ability to control bleeding of gingival retraction solution was achieved by acting of an astringent component. It is well known that the astringents are protein precipitating agents. The mechanism of bleeding control involved the tissue protein or blood protein precipitation or agglutination by astringent, thus astringent will play an important role in occluding the capillary orifices resulting in the reduction of blood flow and reduced bleeding(13,22). In this, the *in vitro* protein assay described by Bradford (23) was used to determine the plasma protein concentration before and after precipitation by the gingival retraction solution. The percentages of plasma protein precipitation were calculated from the amount of plasma protein after contact with the gingival retraction solution divided by the amount of plasma protein before precipitation x 100.

From the result of this study, the percentages of plasma protein precipitation of 25% aluminium chloride in aqueous vehicle, 25% aluminium sulfate in aqueous vehicle (low viscosity and high viscosity) showed the highest percentages of protein precipitation in the range of 96.5-96.8% with no statistical significant difference. On the other hand, the protein precipitation ability for 25% aluminium chloride in non aqueous vehicle and commercial products (Gelcord®, Rastringent II®, Viscostat clear®) were 53.8%, 43.4%, 26% and 21.2%, respectively.

The *in vitro* protein precipitation with retraction agent using similar procedure had also been reported by Pongkasamwiwat (30) using serum to test the ability of the retraction agent to precipitate protein and referred to the bleeding control ability. From that study, the retraction materials used were developed in the 2 paste form and compared to commercial product Expasyl ™ and the ability in serum protein precipitation of those materials were 5.5-93.05 %. However, previous study from Pongkasamwiwat cannot be compared to this study due to different form of the

retraction agents. Furthermore, the platelets and clotting factors are found in plasma rather than serum, therefore it is more suitable to use plasma for this assay for evaluate the ability of blood protein precipitation.

The most powerful retraction agents for protein precipitations were 25% aluminium sulfate and 25% aluminium chloride in aqueous vehicle. Free ions of heavy metal were suggested to be potent protein precipitation since they can bind to the negative charge in protein molecule and made protein less active to water (35). The high protein precipitation ability may come from the ability of aluminium that can dissociate in the aqueous solution and then the aluminium which contain positive charges will bind to the negative charges in the proteins and protein will be precipitated from solution.

The phenomenon that proteins are precipitated from aqueous solution by high concentrations of neutral salts can be called the salting-out phenomenon. Mostly di- and trivalent ions are more effective than univalent ions(35). In this study both aluminium sulfate and aluminium chloride in aqueous formulas showed similar result as protein precipitations.

These results may not only due to the ability of metal cations to neutralize charges but also due to the pH of aluminium sulfate and aluminium chloride solutions. The effect of the pH on protein solubility has already been noted (36), and this effect, i.e. minimal solubility at or near the isoelectric point, applies regardless of the precipitating agent used: neutral salts, organic solvent, etc. Some proteins, such as casine of milk, can be precipitated by adjusting the pH of the solution to the pH at the isoelectric point. This procedure is described as isoelectric precipitation. Apart from these, the low pH may also break H – bond or weak bond in the protein molecule and causes protein denaturation which protein appears as precipitation. As noted that the commercial products of aluminium sulfate in which pH higher than newly developed product showed lower protein precipitation ability. Therefore, the lower pH of the newly developed retraction agents have stronger effect to plasma protein precipitation as can be seen in the aluminium sulfate group(pH were about 0.5-0.7) when compared with the commercial product which pH were about 2.3-2.5.

From this study the most powerful retraction agents for bleeding control were found to be the newly developed products; 25% aluminium sulfate and the 25% aluminium chloride in aqueous vehicle.

2. Cytotoxicity evaluation

The biological compatibility and toxicity aspects of gingival retraction material are also important in relation to their clinical usage, because gingival retraction materials may induce destruction on sulcular epithelium and produce unfavorable effects to periodontal tissue (7, 15, 19). Therefore, the newly developed gingival retraction solutions should provide acceptable biological compatibility with periodontal tissue. The *in vitro* cytotoxicity testing is an important screening step for new materials before use in humans. In addition, *in vitro* methods are simple, reproducible, cost effective and suitable for the evaluation of basic biologic properties of dental materials (24). In this study, serial dilutions and MTT assays were used to evaluate the cytotoxic effect of four newly developed gingival retraction solutions and commercial products. MTT assays were colorimetric methods for measuring the number and activity of viable cells. This assay measured the conversion of yellow water - soluble MTT dye into a purple formazan product by active mitochondria. MTT assays for cytotoxicity testing of chemical gingival retraction material had also been reported by Kopac et al (18) and Lui et al (27). The advantages of this assay are rapidity and precision. Furthermore, MTT assay have been accepted by ISO and has been widely used for determination of cytotoxicity testing(25,26).

The result of this study showed the cell response with all seven tested gingival retraction solutions at 30 folds dilution revealed statistical differences in optical density when compared to the control group and among tested groups ($p < .05$).

Furthermore, most of all solutions studied were found to be severely cytotoxic except Rastringent II® and Gelcord® were found to be slightly toxic.

In this study, two chemical compounds were used: 25% aluminium chloride (in formula A1, A2, Viscostat clear®) and 25% aluminium sulfate (in formula B1, B2, Rastringent II®, Gelcord®). The pH of all retraction solutions were acidic range from 0-2.5, especially the new products and Viscostat clear® with a pH close to 0. The pH

of retraction solution may play important role to the cytotoxic to the culture. Kopac et al (18) studied the cytotoxic effects of low acidic pH, 25% aluminium chloride, pH 1.8 and 10% aluminium chloride, pH 2.3 to Chinese hamster lung diploid fibroblast; v 79 using MTT assay, found that lower acidic pH of 25% aluminium chloride showed a higher cytotoxic effect than 10% aluminium chloride. This result indicated that the pH of aluminium chloride astringent may be the one of the factor that effect on the toxicity of the cultured cells. Furthermore, the newly developed retraction solution in high and low viscosity of 25% aluminium sulfate groups showed lower pH levels(0.5-0.7) when compared with commercial 25% aluminium sulfate in gel and solution(2.3-2.5). Therefore, it is not surprise that the result presented higher cytotoxicity than commercial material.

The aluminium concentration may be another factor that affect to the cytotoxic property. There are the study by Okazaki (37) found that aluminium can cause cytotoxicity at the concentration of 0.2 ppm. of aluminium in cultured L929 mouse fibroblasts. When compared to our retraction agent that would have the aluminium concentration about 2.5 ppm. in the cultured L929 mouse fibroblast, then more cytotoxic was found. Moreover, the length of time that the retraction agent contact the cultured cells in this study was larger than the previous study by Kopac et al(18) which the retraction agent contacted the cells for only 1 minute but in this study the retraction agent contacted the L929 cell for 24 hours according to ISO 19993-5, It was not surprise that higher cytotoxicity level was found.

In this study, most of the gingival retraction solutions were found to be severely cytotoxic to cells. The final flushing with water should be sufficient to remove all residual chemical retraction agents and careful management of gingival retraction cords should lower the risk of potential gingival tissue damage during clinical application procedures and lower the side effects to the cell and tissue regeneration, thus increasing the success of prosthodontic procedure.

3. The ability of detailed reproductions

Clinically, the gingival retraction procedure is an essential step needed before taking impression for fabricating models used for fabricating the restorations. The

material used in this procedure has to be compatible with the impression material and must not interfere with the detailed reproduction of the impression. Moreover, it should not affect the models poured from the impression.

Polyvinyl siloxane or addition silicone impression material is widely used for impression procedure because it demonstrated dimensional accuracy, exhibited the ability to accurately reproduce the dimensions of impressed surfaces (34). However, there are some controversies about the effect of the gingival retraction agent to the addition silicone. The previous study by Camargo, Chee, and Donovan (21) reported that commonly used gingival retraction medications contain chemically active agents, such as racemic epinephrine, aluminium chloride, aluminium sulfate, aluminium potassium sulfate, and ferric sulfate were not the cause of inhibition of polyvinyl siloxane polymerization. In contrast with the study of O'Mahony et al. (21,38) found by *in vitro* study that ferric subsulfate, ferric sulfate, and aluminium chloride interfered with the quality of reproduction of polyvinyl siloxane impression, possibly due to the amount of sulfur that delayed or inhibited polymerization.

The side effects of the composition of gingival retraction solution to the addition silicone impression material are still unknown. Thus, in this study the surface characteristics and detailed reproduction ability were evaluated according to ISO(4823; Elastomeric impression materials) by using the metal test blocks to evaluate the surface characteristics and line width reproduced on the elastomeric impression material and the reproduction of the detail when making Type IV stone model.

The methods of testing in this study were the indirect technique for investigation the effect retraction agent to the impression material and the effect of the impression taken from the test blocks and treated with the retraction agents to the type IV model stone. Moreover, the method that applies retraction solution on the surface of the test block without rinse simulates the worst situation that may happen after retraction procedure and does not clean prepared teeth well. Our study showed that the unapplied group (control) and all groups that were applied with retraction agents, but rinsed and blow-dried, showed the detail of the metal test block can be reproduced all these lines 20, 50, 75 µ continuously on the impression surface when evaluated by the naked eye and under 10X magnification of microscope according to ISO 4823 for elastomeric

impression materials. Moreover, the impression surface characteristics showed smoothness, shininess, and were free of adherence to the models. It means that even treated the models with retraction agent and cleaning with normal rinsing by water and blown drying did not affect the impression material and detailed reproduction.

On the other hand, in the groups that only applied the retraction agent and only blow-dried, the solution still coated the test blocks. After the impressions were taken from these groups, the impression surfaces could not reproduce all details from the test block. Moreover, the impression surface characteristics appeared irregular, due to the retraction agent that still coated the test block. Although all of the impressions were set, the area that contacted the agent were not shiny.

The study by O'Mahony et al that tested the effect of retraction agents (aluminium chloride, ferric sulfate and ferric subsulfate) to addition silicone impression material using metal test blocks according to ISO 4823. The metal test block was contacted to the retraction agent using gauze that soaked in the retraction agent. After that medium body of polyvinyl siloxane impression was taken from the metal model and the impression was evaluated under 10X magnification using low angle illumination. The result showed that all retraction agents clearly affected the quality of the impression, and the surface detail reproduction was adversely affected by all of the retraction agents, similar to this study. Moreover, the aluminium chloride had distinctly difference effect from that of ferric sulfate and ferric subsulfate. Aluminium chloride produced an extremely rough, melted appearance; whole sections of the lines were completely obliterated. Although the impressions of these treated test blocks were considered unacceptable, ferric sulfate and ferric subsulfate medicaments had a less severe adverse effect on the impressions than aluminium chloride. The horizontal lines were distinct in most areas of the impressions, except in very small areas, where remnants of the liquid were incorporated into the lines or edges of the lines(38).

It remains unclear as to whether the medicament had an additional affect on the addition silicone impression material or whether the addition silicone impression material accurately recorded the contaminated metal test block models. Either way, the result suggested that during the retraction procedure, it is critical that the clinician should remove all retraction agents from the preparation sites prior to making

impression with addition silicone by rinsing with water for accurate detailed reproduction of the impression.

This in vitro investigation should be viewed cautiously because laboratory testing cannot simulate clinical situations exactly (26). In this investigation, impressions were made of standardized stainless steel test block models. Although the surfaces of the metal test block models were calibrated for precise comparisons, they did not resemble the behavior of oral tissues. For example, metal test block models do not absorb liquids. However, this laboratory research primary focused on the basic properties and the effect to the material so the study should be performed clinically before using in the daily practice.

The compatibility of the impression material after contact with the retraction agent and Type IV model stone is also another factor that should be investigated. If the retraction agent has some effect to the impression material and the effect can be transferred to the Type IV model stone, the restoration from that model will not achieve in sufficient quality.

According to this study, all of the models poured from the impression in control and in the groups that were rinsed after applying retraction agents showed that they could reproduce the detail of horizontal lines at least 2 to 3 lines (i.e. 75µ, 50µ). The surface characteristic of these groups presented the normal surface of the type IV stone models. On the other hand, the groups that had impressions applied with retraction agents and without rinse could not reproduce the horizontal lines or if reproduced, the lines were not continuous. The surface characteristics of the type IV stone models presented roughness and some porosity on the area that was in contact with the coated retraction agent.

Our study confirms that the most important step before taking impression is the removal of the retraction agents that may coat the teeth to prevent and reduce the side effects to the impression and affect the models that will be poured from that impression.

Finally, from this study, the retraction agent that showed good plasma protein precipitation, cytotoxicity comparable to the commercial product(Viscostat clear ^{TM}) and does not affect the addition silicone impression material with water rinse before taken impression is 25% aluminium chloride in aqueous vehicle solution. Whereas, the

retraction agent contains 25% aluminium sulfate groups and the 25% aluminium chloride in non aqueous formula provide unsatisfactory result in cytotoxic property. The development of aluminium sulfate retraction agent required more research.

CHAPTER VI
CONCLUSIONS

1. The retraction agent, contain 25% aluminium chloride in aqueous vehicle formula provided good plasma protein precipitation, cytotoxicity level comparable to the commercial product(Viscostat clear ™) and does not affect the addition silicone impression material with water rinse before taken impression.
2. The retraction agent contains 25% aluminium sulfate tested in this study provided unsatisfactory result. The development of aluminium sulfate retraction agent required more research.
3. Therefore, *in vivo* and clinically study should be performed for 25 % aluminium chloride in aqueous vehicle before clinical use.

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APPENDIX

1. The determination of plasma protein precipitation of gingival retraction solutions

Optical Density							
Positive control	Formula A1	Formula A2	Formula B1	Formula B2	Gelcord ™	Rastringent II ™	Viscostat clear ™
0.706	0.062	0.457	0.063	0.061	0.369	0.605	0.572
0.738	0.061	0.46	0.063	0.062	0.373	0.604	0.575
0.725	0.061	0.458	0.065	0.061	0.371	0.603	0.576
0.730	0.061	0.462	0.068	0.062	0.368	0.607	0.570
0.742	0.063	0.455	0.067	0.061	0.370	0.606	0.575
0.753	0.032	0.408	0.034	0.031	0.331	0.560	0.527
0.755	0.031	0.406	0.033	0.036	0.332	0.566	0.530
0.746	0.037	0.407	0.035	0.035	0.339	0.549	0.525
0.760	0.034	0.409	0.038	0.032	0.336	0.554	0.528
0.750	0.033	0.411	0.033	0.029	0.338	0.544	0.524
0.725	0.061	0.456	0.064	0.060	0.376	0.611	0.574
0.731	0.066	0.460	0.067	0.061	0.371	0.603	0.577
0.727	0.062	0.457	0.062	0.063	0.367	0.608	0.573
0.721	0.059	0.451	0.066	0.064	0.372	0.609	0.571
0.735	0.060	0.453	0.070	0.062	0.377	0.610	0.576
0.743	0.049	0.411	0.054	0.042	0.341	0.551	0.528
0.741	0.047	0.421	0.042	0.044	0.338	0.553	0.519
0.736	0.051	0.418	0.049	0.045	0.345	0.552	0.522
0.731	0.044	0.435	0.058	0.041	0.339	0.559	0.520
0.738	0.042	0.421	0.046	0.045	0.340	0.558	0.511
0.765	0.061	0.418	0.060	0.064	0.367	0.612	0.582
0.745	0.066	0.422	0.065	0.066	0.375	0.614	0.584
0.744	0.065	0.425	0.058	0.072	0.374	0.613	0.586
0.780	0.054	0.445	0.059	0.067	0.366	0.607	0.588
0.741	0.069	0.455	0.067	0.062	0.375	0.608	0.581
0.764	0.051	0.410	0.059	0.054	0.365	0.615	0.572
0.746	0.056	0.412	0.052	0.056	0.368	0.616	0.565
0.742	0.055	0.415	0.058	0.052	0.374	0.626	0.557
0.785	0.054	0.435	0.059	0.057	0.365	0.628	0.575
0.743	0.059	0.445	0.057	0.054	0.369	0.618	0.568

2. The determination of optical density of in vitro cytotoxicity test

Optical Density							
control	Formula A1	Formula A2	Formula B1	Formula B2	Gelcord ™	Rastringent II ™	Viscostat clear ™
0.7509	0.060	0.053	0.125	0.207	0.542	0.562	0.061
0.7509	0.055	0.055	0.325	0.166	0.625	0.531	0.058
0.7509	0.055	0.054	0.151	0.121	0.578	0.525	0.061
0.7509	0.061	0.058	0.180	0.150	0.607	0.556	0.065
0.7509	0.062	0.054	0.276	0.241	0.609	0.502	0.058
0.7605	0.060	0.053	0.127	0.207	0.542	0.566	0.061
0.7605	0.055	0.055	0.332	0.165	0.640	0.525	0.058
0.7605	0.055	0.054	0.154	0.120	0.579	0.531	0.061
0.7605	0.061	0.058	0.184	0.149	0.620	0.545	0.064
0.7605	0.062	0.054	0.282	0.240	0.620	0.501	0.058
0.7665	0.060	0.053	0.127	0.205	0.546	0.563	0.061
0.7665	0.055	0.055	0.336	0.162	0.652	0.524	0.059
0.7665	0.055	0.054	0.155	0.118	0.581	0.547	0.061
0.7665	0.062	0.058	0.186	0.148	0.626	0.534	0.064
0.7665	0.063	0.054	0.285	0.238	0.635	0.480	0.058

Nonparametric statistic tests for protein precipitation

Kruskal-Wallis Test of optical density of plasma protein precipitation

Ranks

TRT		N	Mean Rank
OD	Control	30	225.50
	Formula A1	30	44.05
	Formula A2	30	135.50
	Formula B1	30	50.57
	Formula B2	30	41.88
	Gelcord ™	30	105.50
	Rastringent II ™	30	192.53
	Viscostat clear ™	30	168.47
	Total	240	

Test Statistics [a,b]

	DIFF
Chi-Square	223.332
Df	7
Asymp. Sig.	.000

a Kruskal Wallis Test
b Grouping Variable: VAR00002

Parametric statistic tests for MTT

One way analysis of variance test of optical density and multiple comparison of *in vitro* cytotoxicity test

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.520	6	.753	169.977	.000
Within Groups	.434	98	.004		
Total	4.954	104			

Multiple Comparisons

Dependent Variable: OD
Tukey HSD

(I) agent	(J) agent	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
		Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound
1	2	.158267(*)	.024309	.000	.08509	.23145
	3	.040667	.024309	.635	-.03251	.11385
	4	.162333(*)	.024309	.000	.08915	.23551
	5	-.355467(*)	.024309	.000	-.42865	-.28229
	6	-.317533(*)	.024309	.000	-.39071	-.24435
	7	.156600(*)	.024309	.000	.08342	.22978
2	1	-.158267(*)	.024309	.000	-.23145	-.08509
	3	-.117600(*)	.024309	.000	-.19078	-.04442
	4	.004067	.024309	1.000	-.06911	.07725
	5	-.513733(*)	.024309	.000	-.58691	-.44055
	6	-.475800(*)	.024309	.000	-.54898	-.40262
	7	-.001667	.024309	1.000	-.07485	.07151
3	1	-.040667	.024309	.635	-.11385	.03251
	2	.117600(*)	.024309	.000	.04442	.19078
	4	.121667(*)	.024309	.000	.04849	.19485
	5	-.396133(*)	.024309	.000	-.46931	-.32295
	6	-.358200(*)	.024309	.000	-.43138	-.28502
	7	.115933(*)	.024309	.000	.04275	.18911
4	1	-.162333(*)	.024309	.000	-.23551	-.08915
	2	-.004067	.024309	1.000	-.07725	.06911
	3	-.121667(*)	.024309	.000	-.19485	-.04849
	5	-.517800(*)	.024309	.000	-.59098	-.44462
	6	-.479867(*)	.024309	.000	-.55305	-.40669
	7	-.005733	.024309	1.000	-.07891	.06745
5	1	.355467(*)	.024309	.000	.28229	.42865

	2	.513733(*)	.024309	.000	.44055	.58691
	3	.396133(*)	.024309	.000	.32295	.46931
	4	.517800(*)	.024309	.000	.44462	.59098
	6	.037933	.024309	.707	-.03525	.11111
	7	.512067(*)	.024309	.000	.43889	.58525
6	1	.317533(*)	.024309	.000	.24435	.39071
	2	.475800(*)	.024309	.000	.40262	.54898
	3	.358200(*)	.024309	.000	.28502	.43138
	4	.479867(*)	.024309	.000	.40669	.55305
	5	-.037933	.024309	.707	-.11111	.03525
	7	.474133(*)	.024309	.000	.40095	.54731
7	1	-.156600(*)	.024309	.000	-.22978	-.08342
	2	.001667	.024309	1.000	-.07151	.07485
	3	-.115933(*)	.024309	.000	-.18911	-.04275
	4	.005733	.024309	1.000	-.06745	.07891
	5	-.512067(*)	.024309	.000	-.58525	-.43889
	6	-.474133(*)	.024309	.000	-.54731	-.40095

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

Homogeneous Subsets

OD

Tukey HSD

	N	Subset for alpha = .05		
agent	1	2	3	1
4	15	.05480		
2	15	.05887		
7	15	.06053		
3	15		.17647	
1	15		.21713	
6	15			.53467
5	15			.57260
Sig.		1.000	.635	.707

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 15.000.

BIOGRAPHY

NAME	Mister Yongyuth Iempituksakul
DATE OF BIRTH	7 February 1979
PLACE OF BIRTH	Rayong, Thailand
INSTITUTIONS ATTENDED	Khonkean University, 1996 Dental of Doctor Surgery Mahidol University, 2009 Master of Science (Prosthodontics)
POSITION & OFFICE	Bangkok International Dental Center, Din Daeng, Bangkok, Thailand Position: Dentist Pattaya International Hospital Pattaya, Chonburi, Thailand Position: Dentist
HOME ADDRESS	23/23 Moo 3, Samet, Mueng, Chonburi, Thailand