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USE OF BUBALINE FIBRIN GLUE FOR THE TREATMENT OF PERIODONTITIS IN EXPERIMENTAL RATS

Miss Poranee Banyatworakul

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Surgery
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ภรณีย์ บัญญัติวรกุล : การใช้กาวไฟบรินจากเลือดกระบือในการแก้ไขภาวะโรคปริทันต์ในหนูทดลอง (USE OF BUBALINE FIBRIN GLUE FOR THE TREATMENT OF PERIODONTITIS IN EXPERIMENTAL RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ชนินทร์ กัลล์ประวิทธ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร. นพดล พิพารัตน์, ผศ. น.สพ. ดร. ชาญณรงค์ รอดคำ, 64 หน้า.

โรคปริทันต์ เป็นการอักเสบของโครงสร้างปริทันต์แบบเรื้อรังซึ่งก่อให้เกิดการสูญเสียฟัน การเลือกใช้สารห้ามเลือด สารเชื่อมติด และสารส่งเสริมการหายของบาดแผล กำลังได้รับความสนใจในการนำมาใช้เป็นวัสดุป้องกันและรักษาโรคปริทันต์ การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อวัดประสิทธิภาพของกาวไฟบรินที่ได้จากเลือดกระบือในการแก้ไขภาวะโรคปริทันต์ในหนูทดลอง หนูทดลองถูกแบ่งออกเป็นสามกลุ่มได้แก่ กลุ่มควบคุม (C) กลุ่มที่ได้รับวัสดุผูกเย็บโดยไม่ได้รับการรักษา (L) และกลุ่มที่ได้รับวัสดุผูกเย็บร่วมกับได้รับการรักษา (LB) การเหนี่ยวนำให้เป็นโรคปริทันต์จะใช้วัสดุผูกเย็บชนิดซิลค์ขนาด 5-0 คล้องรอบฟันกรามล่างซี่แรกทั้งสองข้าง กลุ่มที่ได้รับวัสดุผูกเย็บร่วมกับได้รับการรักษา (LB) จะได้รับการใส่กาวไฟบรินที่ได้จากเลือดกระบือลงในร่องปริทันต์ ตัวแปรในการศึกษาครั้งนี้ คือ การตรวจทางคลีนิก [ดัชนีคราบหินปูน (PI) ดัชนีเหงือกอักเสบ (GI) และดัชนีการคลอนของฟัน (MI)] การวิเคราะห์จากภาพทางจุลกายวิภาค (ระดับการสึกหรอของกระดูกเบ้าฟันจากภาพจุลกายวิภาค ระดับการสึกหรอของเนื้อเยื่อยึดเกาะฟันจากภาพจุลกายวิภาค และคะแนนการอักเสบ) และการแสดงออกของยีนส์ไซโตคายน์ (ไอแอล-1เบต้า ทีเอ็นเอฟ-แอลฟ่า1 ไอแอล-10 พีดีจีเอฟ-เอ และทีจีเอฟ-เบต้า1) วันที่ทำการเก็บข้อมูลตัวแปรที่กล่าวมาในกลุ่มที่ได้รับวัสดุผูกเย็บโดยไม่ได้รับการรักษา (L) และกลุ่มที่ได้รับวัสดุผูกเย็บร่วมกับได้รับการรักษา (LB) คือ วันที่ 1, 7, 8, 14, และ 21 หลังจากการเหนี่ยวนำให้เกิดโรคปริทันต์ นอกเหนือจากนั้น การวิเคราะห์ผลทางจุลชีววิทยา (สายพันธุ์ของแบคทีเรียและจำนวนแบคทีเรียที่นับได้ และวิธีการ disc diffusion) ถูกตรวจสอบในกลุ่มที่ได้รับวัสดุผูกเย็บโดยไม่ได้รับการรักษา (L) ในวันที่ 0, 7, และ 21 หลังจากการเหนี่ยวนำให้เกิดโรคปริทันต์

ผลการทดลองแสดง 11 สายพันธุ์ของแบคทีเรียแบบใช้ออกซิเจน โคลินแบคทีเรียเป็นสายพันธุ์ที่พบได้มากที่สุด (8/24) ตามด้วยสแตปไฟโลค็อกคัสแบคทีเรีย (5/24) จำนวนแบคทีเรียที่นับได้ทั้งหมดเปรียบเทียบระหว่างวันที่ 0 และ 7 และระหว่างวันที่ 7 และ 21 หลังจากการเหนี่ยวนำให้เกิดโรคปริทันต์แสดงความแตกต่างอย่างมีนัยสำคัญ ($P<0.05$) แต่ไม่พบความแตกต่าง ($P\geq0.05$) เมื่อเปรียบเทียบระหว่างวันที่ 0 และ 21 วิธีการ disc diffusion แสดงให้เห็นว่ากาวไฟบรินที่ได้จากเลือดกระบือมีประสิทธิภาพในการต้านจุลชีพต่อเชื้อแบคทีเรีย 6 สายพันธุ์ (6/11) กลุ่มที่ได้รับวัสดุผูกเย็บร่วมกับได้รับการรักษา (LB) ไม่พบความแตกต่างอย่างมีนัยสำคัญระหว่าง PI GI และ MI เมื่อเปรียบเทียบกับกลุ่มที่ได้รับวัสดุผูกเย็บโดยไม่ได้รับการรักษา (L) ระดับการสึกหรอของกระดูกเบ้าฟัน การสึกหรอของเนื้อเยื่อยึดเกาะฟันจากภาพทางจุลกายวิภาค และคะแนนการอักเสบในกลุ่มที่ได้รับวัสดุผูกเย็บร่วมกับได้รับการรักษา (LB) พบว่าต่ำลงเมื่อเปรียบเทียบกับกลุ่มที่ได้รับวัสดุผูกเย็บโดยไม่ได้รับการรักษา (L) ในส่วนของการแสดงออกของยีนส์ กลุ่มที่ได้รับวัสดุผูกเย็บร่วมกับได้รับการรักษา (LB) มีการแสดงออกของยีนส์ไอแอล-1เบต้า ทีเอ็นเอฟ-แอลฟ่า1 ลดลง แต่มีการแสดงออกของยีนส์ไอแอล-10 ยีนส์พีดีจีเอฟ-เอ และยีนส์ทีจีเอฟ-เบต้า1 สูงขึ้นกว่ากลุ่มที่ได้รับวัสดุผูกเย็บโดยไม่ได้รับการรักษา (L) กล่าวโดยสรุปได้ว่ากาวไฟบรินที่ได้จากเลือดกระบือน่าจะเป็นวัสดุทางเลือกเพื่อนำไปใช้ในการป้องกันการพัฒนาของโรคปริทันต์ได้

ภาควิชา ศัลยศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา ศัลยศาสตร์ทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: BUBALINE BLOOD, FIBRIN GLUE, PERIODONTITIS, RAT

PORANEE BANYATWORAKUL: USE OF BUBALINE FIBRIN GLUE FOR THE TREATMENT OF PERIODONTITIS IN EXPERIMENTAL RATS. ADVISOR: ASSOC. PROF. CHANIN KALPRAVIDH, D.V.M., M.S., D.T.B.V.S., CO-ADVISOR: ASSOC. PROF. NOPADON PIRARAT, D.V.M., Ph.D., D.T.B.V.P., ASST. PROF. CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P., 64 pp.

Periodontitis is the chronic inflammation of the periodontal structures resulting in teeth loss. The use of hemostatic agents, sealants or wound healing promoter is now focusing as an alternative prevention and treatment of periodontal diseases. This study aimed to evaluate the efficacy of the bubaline fibrin glue in the treatment of periodontitis in the rat model. Rats were divided into three groups: control (C), ligation without treatment (L), and ligation with bubaline fibrin glue treatment (LB) groups. The periodontitis was induced by 5-0 silk ligature placed around the mandibular first molars. LB group received topical application of the bubaline fibrin glue in the periodontal pocket. The parameters in this study were clinical examination (plaque index; PI gingival index; GI and mobility Index; MI), histological analysis (histological bone loss, histological attachment loss, and inflammation score), and cytokine gene expression analysis (IL-1β, TNF-α, IL-10, PDGF-A, and TGF-β_1). The observations on the aforementioned parameters of L and LB groups were scheduled at 1, 7, 8, 14, and 21 days post ligation (DPL). Moreover, microbiological analysis (bacterial strains and total bacterial count and disc diffusion assay) was examined in L group at 0, 7, and 21 DPL.

The results showed 11 aerobic bacterial strains. Corynebacterium was the most predominate (8/24) followed by Staphylococcal bacteria (5/24). The number of total bacterial count showed significant difference ($P<0.05$) between 0 and 7 DPL and between 7 and 21 DPL, but not between 0 and 21 DPL ($P\geq0.05$). The disc diffusion assay showed the bubaline fibrin glue had a potential antimicrobial activity against 6 bacterial strains (6/11). The LB group did not show the significant difference of PI, GI, and MI when compared with those of the L group. The levels of histological bone loss, histological attachment loss, and inflammation score were lower in the LB group when compared with the L group. For the cytokine gene expression, the LB group had the lower number of IL-1β and TNF-α but the higher number of IL-10, PDGF-A, and TGF-β_1 gene expression when compared with the L group. In conclusion, the bubaline fibrin glue might be an alternative material for preventing the progress of the periodontal diseases.

Department: Veterinary Surgery	Student's Signature .....
Field of Study: Veterinary Surgery	Advisor's Signature .....
Academic Year: 2015	Co-Advisor's Signature .....
	Co-Advisor's Signature .....

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INTRODUCTION

1.1 Importance and Rationale

Periodontal diseases are a group of inflammatory diseases of the periodontal tissue (gingiva, cementum, alveolar bone, and periodontal ligament). It is recognized as one of the most common oral diseases in dogs (Hamp et al., 1984), of which 80% are over 2 years old (Gorrel et al., 2013). The main causative factor of periodontitis is the presence of microorganisms in the mouth forming a plaque adhered on the surface of teeth. Dental calculus is gradually formed by the combination of saliva and plaque leading to gingivitis and periodontitis. In humans, periodontitis induces several systemic diseases including cardiovascular diseases (Desvarieux et al., 2005), rheumatoid arthritis (de Pablo et al., 2009), adverse outcome of pregnancy (Xiong et al., 2006), low birth weight (Saini et al., 2010), diabetes (Taylor et al., 1996), and pancreatitis (Milasin et al., 2011). In dogs, periodontitis can be the cause of systemic diseases including endocarditis (Peddle et al., 2009), chronic azotemic kidney disease (Glickman et al., 2011), and hepatic dysfunction.

Prevention and treatment of periodontal diseases are now being implemented in order to promote good health status. Although dental extraction is the conservative treatment in advanced periodontitis, bleeding, trauma, and host suffering are the major disadvantages. Others are an increased risk of infection, a high cost, and a prolonged hospitalization. Gingivectomy and apically repositioned flap are the alternative surgical techniques to maintain the affected tooth by reducing depth of the periodontal pocket. However, the latter techniques increase the duration of the anesthesia and the surgery, the cost of the treatment, the risk of infection, and the home care period.

Fibrin glue is a novel treatment that can be used for the fixation of tissue in the oral cavity instead of conventional suturing (Yücel et al., 2003). Functions of fibrin glue are hemostatic, sealing, and enhancing wound healing. The stabilized fibrin structure plays a crucial role in platelet aggregation during homeostasis. Platelets are

a reservoir source of growth factors such as platelet derived growth factor (PDGF), transforming growth factor beta-1 (TGF-β_1), vascular endothelial growth factor, and other glycoprotein. These growth factors stimulate the healing of soft and hard tissues, this suggesting a modulatory effects of the inflammatory process by the fibrin adhesive glue. Hence, the commercial fibrin glue could significantly reduce the presence of microorganisms on partial thickness burn wounds in vivo (Lahoda et al., 2006). The antimicrobial activity of GHR28 released from the β-chain of fibrinogen plays a role in inflammation and wound healing (Påhlman et al., 2013). However, commercial fibrin glue (combination of human fibrinogen and bovine thrombin) is very expensive. In consequence, several attempts have been made to find other sources of fibrinogen. Thomazini-Santos et al. (1998) revealed that the bubaline blood prepared by a cryoprecipitate method had the highest fibrinogen level when compared with bovine and ovine blood. Therefore, bubaline fibrinogen might be a potential source of fibrin adhesive glue. Fortunately, there is a high numbers of water buffaloes in Thailand. Production of buffalo bloodderived fibrin glue, named bubaline fibrin glue, could raise the added value of products from water buffalo and corresponds to the incomes received by the farmers. Modification of the sources of fibrinogen by bubaline fibrin glue might be economically cheaper than commercial product. However, the utilization of these products requires further investigation to reduce the risk of viral transmission and hypersensitivity reactions from buffalo to human or other mammals. In cases of periodontitis, the use of the bubaline fibrin glue as a periodontal filler has never been mentioned. Also, there is no information regarding the effectiveness of bubaline fibrin glue on oral wound healing and periodontal diseases as well. Therefore, this study aims to evaluate the efficacy of the bubaline fibrin glue in the treatment of periodontitis in the rat model.

1.2 Objectives of Study

To evaluate the efficacy of the bubaline fibrin glue in rats with induced periodontitis by clinical examination, histological analysis and cytokine gene expression analysis.

1.3 Research question

Can the bubaline fibrin glue improve clinical sign, enhance wound healing, and decrease inflammation of induced periodontitis in rats?

1.4 Hypothesis

The bubaline fibrin glue can be used for the treatment of induced periodontitis in rats.

Keywords: Bubaline blood, Fibrin glue, Periodontitis, Rat

1.5 Advantages of Study

The bubaline fibrin glue could be used for preventing the progression of the periodontal diseases. The production of fibrin glue from bubaline blood could increase the revenue of Thai farmers.

CHAPTER II
LITERATURE REVIEW

2.1 Periodontitis

Periodontitis is the chronic inflammation of the periodontal structures including gingiva, cementum, alveolar bone, and periodontal ligament. It causes redness, inflammation, swelling, loss of functionality, resulting in teeth loss.

Periodontal diseases have multifactorial etiology (AlJehani, 2014) such as bacteria, virus, autoimmune disease, hypersensitivity, and immune system dysfunction (Gorrel et al., 2013). The dental biofilm, consisting of bacteria and their by-products, saliva and inflammatory cells form a dental plaque, is a primary cause of periodontitis. The biofilm covers the supragingiva surface of the tooth leading to gingivitis, which is the earliest sign of the disease. Gram-positive, aerobic bacteria are the predominantly infectious agents. The accumulation of supragingival biofilm will extend to the sulcus and polymorphonuclear cells will get chemotactically attracted to the area resulting in a deepening of the sulcus. The biofilm will gain access to the subgingival region, which has a low oxygen, and will transform itself to be anaerobic.

2.2 Periodontal microorganisms

Periodontitis is usually caused by microorganisms. There are many studies about periodontal microorganisms in canine species. The presence of putative periodontal organisms such as *Porphyromonas gingivalis* (64%), *Campylobacter rectus* (36%), *Actinobacillus actinomycetemcomitans* (24%) and *Prevotella intermedia* (20%) by using PCR method in 25 dogs with periodontitis (Nishiyama et al., 2007). The study was related to Nociti *et al*. (2001), who performed a microbiological evaluation on day 0 and day 30 after induced periodontitis in mongrel dogs by PCR technique. Authors found presence of *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, and *Prevotella nigrescens*. Nemec *et al*. (2013) reported that anaerobic gram negative bacteria were the most predominant

putative periodontal pathogens (*Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*, *Fusobacterium* species, etc.). In summary, gram positive bacteria are often detected during the early stage of periodontitis while gram negative bacteria are dominant in the progression of the diseases. Most of subgingival bacteria in cases of canine periodontitis are gram-positive aerobic bacteria such as *Staphylococcus* and *Streptococcus* spp. Moreover, anaerobic bacteria can be found in the supragingival area (Riggio et al., 2011). However, Periodontal microorganisms vary among different animal species. Oral microbiota isolated from the experimental rats is formed by aerobic or facultative gram-positive cocci (*Streptococcus*, *Staphylococcus*, *Enterococcus*), aerobic or facultative gram-positive rods (*Lactobacillus*, *Corynebacterium*, *Actinomyces*), aerobic or facultative gram-negative cocci (*Neisseria*), obligate anaerobes (*Veillonella*), aerobic or facultative gram-negative rods (*Enterobacteriaceae*) or obligate anaerobes (*Bacteroides*, *Fusobacterium*) (Isogai et al., 1985).

2.3 Staging of periodontal disease

Stage 1 – Early gingivitis: The inflammation is limited to the gingiva, without alveolar bone loss. The clinical signs are gingival redness and swelling due to the formation of plaque and calculus on the tooth. The depth of the pocket is below 2 mm. In dogs, the disease can be reversible with home care control (tooth brushing at least once a day, pellet feeding to decrease the accumulation of dental tartar), supragingival scaling, and polishing treatment.

Stage 2 – Advanced gingivitis: the presence of redness, swelling, inflammation, plaque and calculus that have extended to the root of the teeth can be observed. When probing gingival bleeding can be seen, the pocket having deepened up to 25%. The treatment, including supra- and subgingival scaling and polishing, this followed by the application of local antibiotics, is more complicated than that in stage 1.

Stage 3-4 - Irreversible stage: Stage 3 Early periodontitis. Clinical signs are halitosis, hypersalivation, edema, inflammation, and sometimes root tooth abscess with purulent discharge. Systemic signs include pain, fever, dysphagia, and anorexia.

Severe dental tartar and a probing depth above 4 mm can be recognized during physical examination. In dogs, bleeding occurs when probing and gingivitis is more extensive than in stage 1-2. It is possible to see the bifurcation of the teeth due to the loss of attachment of the alveolar bone, which is about 25-50%. The treatments include supra- and subgingival scaling, polishing to remove heavy dental tartar and root planning to estimate attachment loss. Gingivectomy and apically repositioned flap to reduce the depth of the periodontal pocket are the alternative surgical techniques used in order to maintain the affected teeth. In the cases of owners who are not able to provide home care to the patient, dental extraction should be performed. The clinical signs of stage 4 (Advanced periodontitis) are similar to those of stage 3 but with more than 50% of loss of periodontal attachment. In this case, it is not possible to use the conservative treatment and so dental extraction would be the treatment of choice.

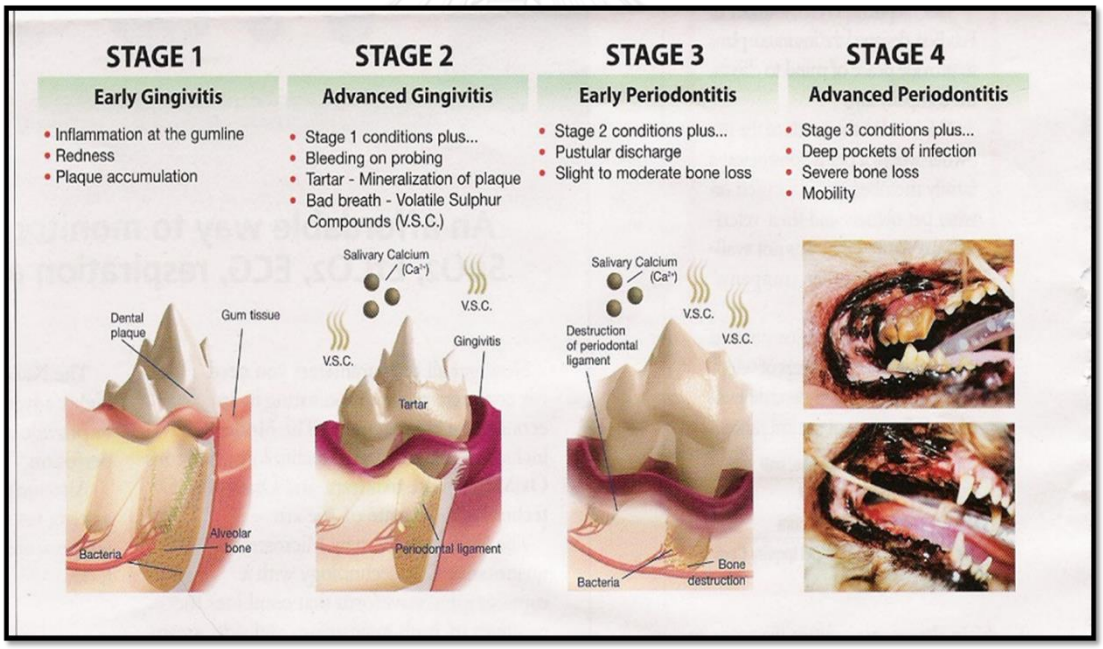

Figure 1 Staging of periodontal disease.

Available from: http://dogsaholic.com/care/periodontal-disease-in-dogs.html

2.4 Wound healing process of oral tissue

The process of wound healing is overlapping including inflammatory, proliferation and maturation phases. The rate and pattern of the healing process

depends on host and surgical factors. The host defense mechanisms in front of a bacterial infection are related to the physical and the chemical barrier, as well as to the cell mediated immune system. The rate of wound healing in soft tissue is faster than in skin and bone.

1) Inflammatory phase (1–2 days) begins immediately after the injury. Platelets are released in order to control bleeding and to stimulate thromboplastin and wound healing modulators such as PDGF (Platelet-Derived Growth Factor), VEGF (Vascular Endothelial Growth Factor), and TGF-β (Transforming Growth Factor-β). PDGF is not only produced from platelets but also produced from macrophages, monocytes, smooth muscle cells and endothelial cells. TGF-β has an important role on wound healing and inflammatory activity and a significant level of its expression has been detected in human gingival fluid and tissue by ELISA (Skalerič et al., 1997). Polymorphonuclear cells (PMN) represent the first line immune cells in circulation, fighting the presence of bacteria by phagocytosis. Macrophages are the second line of cells in the inflammation cascade and produce the following pro-inflammatory cytokines via Toll-like receptor (TLRs): TNF-α (tumor necrotic factor-alpha), IL-1 (Interferon-1), IL-6 (Interferon-6), and IL-8 (Interferon-8). Several literatures reviews regarding pro-inflammatory cytokines in cases of periodontitis. TNF can be present in 2 forms: alpha and beta. The first has a role of PMN migration, cytotoxicity, collagen synthesis, and re-epithelization (Häkkinen et al., 2000) as well as in chemotaxis and in the release of inflammatory mediators and tissue destruction enzymes, the latter being associated with bone destruction. TNF-α expression in rats with periodontitis is significantly higher than normal rats (Nemec et al., 2013; Liao et al., 2014). IL-1, derived from monocytes, macrophages, dendritic cells, plays a significant role in the pathogenesis of periodontitis (Graves and Cochran, 2003), appearing in 2 different forms, alpha and beta. It stimulates the release of PGE_2 (prostaglandin E_2) and MMPs (Matrix metalloproteinases), that have a role in bone resorption. In a study by Nemec et al. (2013), it was reported that blood IL-1β, IL-6 and TNF-α were significantly higher in rats with induced periodontitis than in healthy rats. Similarly, IL-1β and PGE_2 gene expression level of gingival tissue was also significantly higher in chronic periodontitis

analysed by Quantitative TaqMan Real Time-PCR (Polymerase Chain Reaction) (Popova and Mlachkova, 2010; Nemec et al., 2013).

2) Proliferation phase (1 week): phase of blood vessel, fibroblast, smooth muscle cell proliferation, and new bone formation. In this phase, PDGF plays a significant roles, its function being to drive the proliferation of undifferentiated mesenchyme cells, chemoattractant for fibroblast, monocytes, neutrophils, blood vessel formation (angiogenesis), and also to activate collagenase for the remodeling of collagen in late stage of wound healing. Moreover, TGF-β is the anti-inflammatory cytokine that regulates periosteal formation and bone remodeling. The administration via subcutaneous route in rats was reported to increase the number of the osteoblast precursors and to accelerate the formation of new bone (Marcelli et al., 1990).

3) Maturation phase (2–4 weeks): Fibroblasts produce collagen and proteoglycans that provide the strength of the wound. In this phase, trabeculae of osteoid can be seen. The process ends with the remodeling of the granulation tissue.

2.5 Alternative treatments for periodontitis

Dental extraction is a traumatic and painful procedure, resulting in soft and hard tissue trauma, bleeding, and inflammation, this producing suffering of the host. Moreover, it is a time-consuming procedure which implies a high risk of infection, high cost and that requires a long-term hospitalization. Given its disadvantages, researchers have tried to find new modalities or techniques such as pocket reduction surgery, local antibiotics, bone replacement graft, bioactive agents, guided tissue regeneration (Albuquerque et al., 2012), and photodynamic therapy for suppressing periodontopathogenic bacteria (Sigusch et al., 2005) in order to preserve the teeth.

Pocket reduction surgery

Gingivectomy is performed in cases of gingival hyperplasia or suprabony periodontal pocket. This technique is simple and takes short time to carry out. However, it destroys the healthy attached gingiva tissue and cannot be performed in

cases that absent or less than 2 mm of attached gingiva. Horizontal or vertical bone loss is contraindicated (Holmstrom et al., 2004).

Apically repositioned flap is a common surgical approach to reduce periodontal pocket depth. This technique is important for preserving a keratinized gingiva, as opposed to the gingivectomy method, in which attached gingiva tissue is resected. This method implies an extensive post-operative care.

Local antibiotics

Due to the possible disadvantages the administration of systemic antibiotics can have (drug resistance, drug eruption, or the inconvenience for the owner in terms of the home care), local antibiotics would be of choice. Their commercial products have been used for periodontal treatment, providing preferable outcomes.

Doxycycline hyclate (Doxirobe® Gel) (Zoetis, Canada) was claimed to provide the highest plasma concentration within 6 hours after its appilcation in the periodontal pocket.

Clindamycin hydrochloride (Clindoral®) (Trilogic Pharma, USA) decreased the pocket depth (19%), the gingival index (16%) and the number of bleeding sites (64%) significantly when compared with the control group (Johnston et al., 2011).

Poloxamer gel containing 15% metronidazole had been described to have drug delivery properties when applied in the periodontal pocket in cases of periodontitis in animal. It was found that its level in gingival crevicular fluid was greater than in the bloodstream within 48 hours after its application (Sato et al., 2008).

Chitosan gel can provide many bioactive properties such as wound healing, hemostatic and tissue regeneration. Moreover, Its additive effect when combined with metronidazole in chronic periodontitis patients because of its antimicrobial property (Akıncıbay et al., 2007).

Additionally, as reviewed by Schwach-Abdellaoui et al. (2000), fiber (Hollow fibers, Ethylene vinyl acetate fiber), strips and compacts, films, and injectable systems (Microparticles, Gels) are used to treat periodontal pocket in humans.

Bone replacement graft

A bone graft is applied to restore the lost of alveolar bone and to promote bone formation in cases of periodontitis or periodontal intra-bony defects in humans

(Reynolds et al., 2003). A professional dental cleaning, a dental scaling and a root planning are required prior to the regenerative surgical procedure. The most commonly used graft materials are autograft, allograft, xenograft, and synthetic graft. In this case an inefficient home care would lead to a post-operative infection and wound dehiscence. Thus, the use of bone replacement graft may be limited in small animal surgery.

Bioactive agents

Bioactive agents or bioactive glasses are a group of glass-ceramic biomaterials used as naturally derived scaffold (Pandit et al., 2011). The major compositions of bioactive glasses are calcium and phosphate that are similar to the bone structures. Bioactive agents are indicated to accelerate the bone repairment and healing as a minor invasive periodontal therapy with a short time for healing. In vivo, bioactive glasses are biocompatible with the bone and so can stimulate bone growth more rapidly than other bioceramics (Jones, 2013).

Guided Tissue Regeneration

Guided Tissue Regeneration (GTR) is a procedure that enhances the proliferation of soft tissue (gum) and hard tissue (bone) thought the insertion of a biocompatible material as barrier membranes into the defect between soft and hard tissue. The mucoperiosteal flaps are sutured tightly back in place. Caffesse et al. (1988) reported that GTR had significantly increased the area of attachment of new connective tissue in 7 beagle dogs with periodontitis compared with the control group.

Photodynamic therapy

Photodynamic therapy is a new method for reducing the periodontopathogenic bacteria by photosensitizing them with a diode laser. The performance of this technique decreased significantly the clinical signs of redness and bleeding in dog with periodontitis compared to the control group (Sigusch et al., 2005).

2.6 Development of fibrin glue

Fibrin glue (Fibrin-Fibronectin sealing network) is a biological substances used to create a fibrin clot and is divided into 2 syringes consisting of fibrinogen and

thrombin. Fibrin glue is a local hemostatic agent, a sealant, a drug delivery to target sites, and a promoter of wound healing. Moreover, it plays a crucial role in stimulating the proliferation of macrophage (Van der Ham et al., 1991), fibroblasts and osteoblasts (Yücel et al., 2003), and the induction of the growth factor, angiogenesis, collagen synthesis, and epithelialization (Tasdemir et al., 2011). As demonstrated, fibrin glue is demonstrated has excellent wound healing property, which were observed in the stage of granulation and re-epithelization on incision wound in rats (Aksoy et al., 2009). Michel and Harmand (1990) suggested that the effect of wound healing in the presence of fibrin glue containing thrombin and calcium could stimulate the proliferation of fibroblast and the synthesis of collagen in healthy humans.

The mechanism of action of fibrin glue mimics the last stage of natural clotting cascades. The prothrombin activator, produced on the surface of activated platelets and tissue cells, cleaves prothrombin into thrombin. Then fibrinogen, which is the precursor of fibrin combines with thrombin and turns into fibrin monomers. Thrombin activated factor XIII generates the conversion of fibrin monomers into fibrin polymers, that strengthen and stabilize the fibrin clot by promoting the polymerization (Figure 2).

Fibrin glue has several properties: It is easy to use in excavated lesion, it represent a faster method than conservative sutures, it provides early hemostasis and wound healing, it is biocompatible and biodegradable, it does not generate inflammation or foreign body reaction, it activates the formation of new blood vessel, it enhances the osteogenesis in human maxillary and mandible bone (Marini et al., 1994), and it acts an adhesive for osteoinductive biologic tissue (Abiraman et al., 2002). Fibrin glue is degraded by macrophage and fibroblasts in a period of 10–14 days after its application.

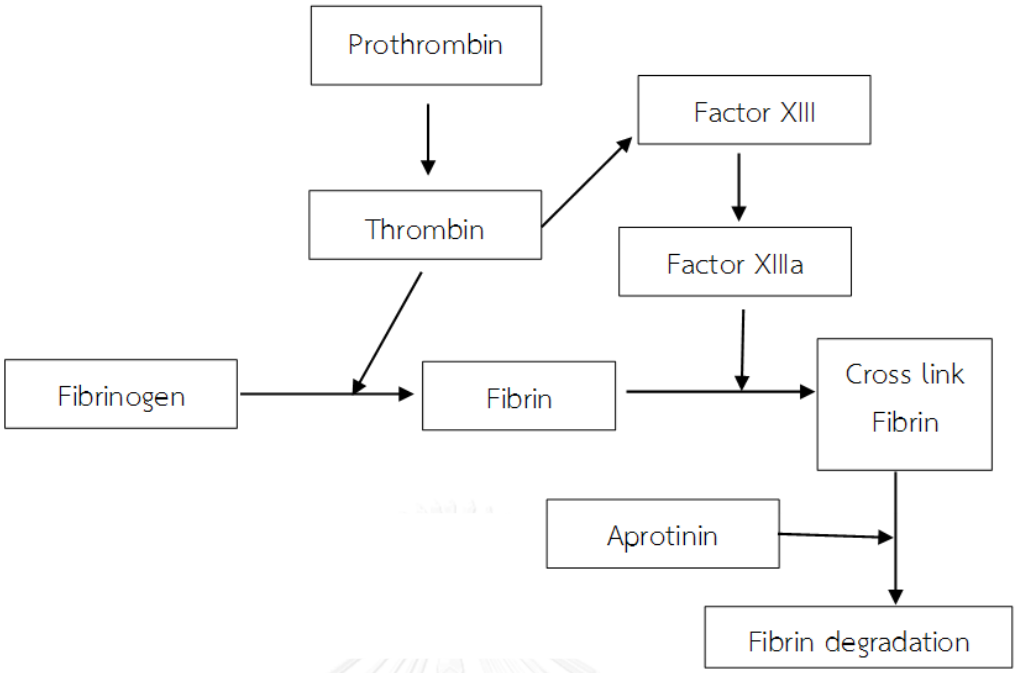

Figure 2 Mechanism of action of the fibrin glue. Adapted from Nieto et al. (2009).

Fibrin glue was first developed for medical procedures in 1909 by Bergel as a hemostatic agent. The composition of the commercial fibrin glue is lyophilised pooled human concentrated fibrinogen and human or bovine thrombin combined with calcium chloride in separate syringes. Moreover, it has several trademarks; TISSEEL® (Baxter Healthcare, Deerfield, Illinois), EVICEL® (Ethicon Johnson & Johnson, Somerville, NJ, USA), TACHOSIL® (Nycomed, Roskilde, Denmark), QUIXIL® (Ethicon, New Brunswick, NJ, USA), and BERIPLAST® (Pharmatek, Istanbul, Turkey).

The first generation of fibrin glue was collected from plasma derived human fibrinogen and thrombin and was used extensively during around 15 years in Europe. It was not available in the United States because the FDA (Food and Drug Administration) aware about the possible transmission disease such as Hepatitis, HIV, Human Parvovirus, and Prion disease. In 1998, the FDA approved only 3 procedures for using it; in splenic injuries, in cardiopulmonary bypass as a hemostatic agent, and in temporary colostomies anastomosis as a way to decrease the use of suture. Later, autologous blood collected from concentrated fibrinogen combined with bovine thrombin and anti-fibrinolytic agent were developed.

Fibrin glue has been used in several procedure: in the repairment of peripheral nerves (Narakas, 1988), in the reduction of the incidence of arthrofibrosis in total knee arthroplasty (Everts et al., 2007), in ophthalmological procedures (Panda et al., 2009), in urologic (Evans and Morey, 2006), in gastrointestinal system procedures (Mutignani et al., 2006), in oral and maxillofacial surgery (Soffer et al., 2003), and in tonsillectomy (Gross et al., 2001).

Fibrin glue can be produced by two different methods: the cryoprecipitation and the chemical methods (ammonium sulfate, ethanol, polyethylene glycol precipitation). Moreover, many research have been reported that the preparation of fibrin glue by the cryoprecipitation method had many advantages over chemical methods (Silver et al., 1995; Gammon et al., 1997; Mintz et al., 2001).

The use of fibrin glue has been reported on several studies in human and veterinary dentistry. Bösch et al. (1980) investigated the use of fibrin glue in oral surgery in human as heterologous bone grafts retainer for the first time. Jathal et al. (2008) suggested that the fibrin glue could provide more early hemostatic and adhesive effect on periodontal flap surgery in two patients than conventional sutures. In addition, the application of fibrin glue with cancellous bone in mandibular reconstruction in humans can stimulate bone healing by increasing the revasculalization and by improving the fibroblast migration (Toriumi and O'Grady, 1994). Moreover, the application of fibrin glue combined with celluloid splint in dental extraction in patients with bleeding disorder may reduce the cost of dental extraction due to the lack of replacement therapy of blood component it implied (Suwannuraks et al., 1999).

The role of fibrin glue in veterinary dentistry was reported to have positive effects of wound healing and inflammatory reaction in dental extraction procedure in rat with induced periodontitis when compared with a conventional suture. Yücel et al. (2003) suggested that fibrin glue might be a good tissue adhesive in the oral cavity. Moreover, it was claimed to be effective in the alveolar bone regeneration process detected with micro-computed tomography (Micro-CT) and histological analysis as well as enhancing a new bone formation. It is also suitable as biological vehicle for mesenchymal stem cell (Zhang et al., 2013).

Although the commercial fibrin glue has several properties it is costly. Thomazini-Santos et al. (1998) found that the buffalo blood prepared by the cryopicipitation technique had an excellent amount of fibrinogen compared with human, ovine, equine and bovine fibrinogen. Average levels of fibrinogen from several donors were 664.00 mg% of bubaline, 375.50 mg% in humans, 267.70 mg% in ovine, 240.80 mg% in equine, and 218.33 mg % of bovine. Therefore, buffalo blood derived fibrinogen might be a potential source of fibrin adhesive glue. Fortunately, water buffalo can be easily found in Thailand, there amount being of 1,241,896 buffaloes in 2013 (Department of Livestock Development, 2013). However, the number of buffaloes in Thailand obviously decreased the population in the past 12 years. From 1997 (2,293,938) to 2008 (1,359,807), the buffalo population decreased 40.72% so that the author anticipated that the water buffalo in Thailand will play a significant role of tissue adhesives for preventing the progression of the periodontal diseases in medical profession and reducing cost of the treatment. Production of this fibrin glue from buffalo blood could increase Thai farmers' revenue.

CHAPTER III
MATERIALS AND METHODS

3.1 Experimental animals

Thirty-three male Wistar rats (*Rattus norvegicus*) of 60 days of age and a weight 300 ± 50 g were purchased from National Laboratory Animal Center (NLAC), Mahidol University, Nakhon Pathom, Thailand. The rats were housed in the standard cages in the Faculty of Veterinary Science, Chulalongkorn University, Pathumwan, Bangkok, Thailand and were provided with a commercial feed and water ad libitum. All the necessary procedures to minimize pain and discomfort were fulfilled by a researcher. This experiment was a prospective, randomized control trials and the research protocol (No. 1531029) had been approved by Chulalongkorn University Animal Care and Use Committee for the experimental animal welfare under the standard guidelines.

Rats were randomly allocated into three different groups:

1. Control group (C) (n=3)
2. Ligation without treatment group (L) (n=15)
3. Ligation with bubaline fibrin glue treatment group (LB) (n=15)

The C group was not induced to have periodontitis. Clinical examination, histological analysis, and Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) were performed individually study at the end of the study (day 21) and compared with other groups.

The L group was induced to have periodontitis during 7 days by ligating the left and the right mandibular first molars, the study site. The ligatures were left in place for 7 days and removed before dental scaling was performed. The animals were euthanized at the schedule date for clinical examination, sampling for microbiological evaluation, histological evaluation, and cytokine gene expression analysis.

The LB group, the same periodontitis induction procedure as that described for the L group was performed. The bubaline fibrin glue was applied and left in place for

14 days. The animals were euthanized at the schedule date for clinical examination, histological evaluation, and cytokine gene expression analysis were performed.

The scheduled dates as mentioned above for clinical examination, histological evaluation, and cytokine gene expression analysis of L and LB were 1, 7, 8, 14, and 21 days post ligation (DPL).

3.2 Anesthesia protocol

In sampling days each rat using anesthesized with intramuscular ketamine (Hameln Pharmaceuticals GmbH, Germany) at a dose of 0.08 ml/100 g body weight and xylazine HCl (Laboratorios Calier, S.A, Spain) at a dose of 0.04 ml/100 g body weight followed by isoflurance (Baxter®, Puerto Rico) via mask induction. All experimental rats were euthanized with an overdosed anesthetic drug.

3.3 Induction of periodontitis

Periodontitis was induced according to the method of Lu et al. (2013). The sterile 5-0 black braided silk thread (surgical silk suture; Covidien, Dominican) was placed subgingivally around the neck of the left and right mandibular first molars to induce periodontitis. The knot of the ligature was positioned on the mesial site and left in place during 7 days to obtain an accumulation of the biofilm (Figure 3 and 4).

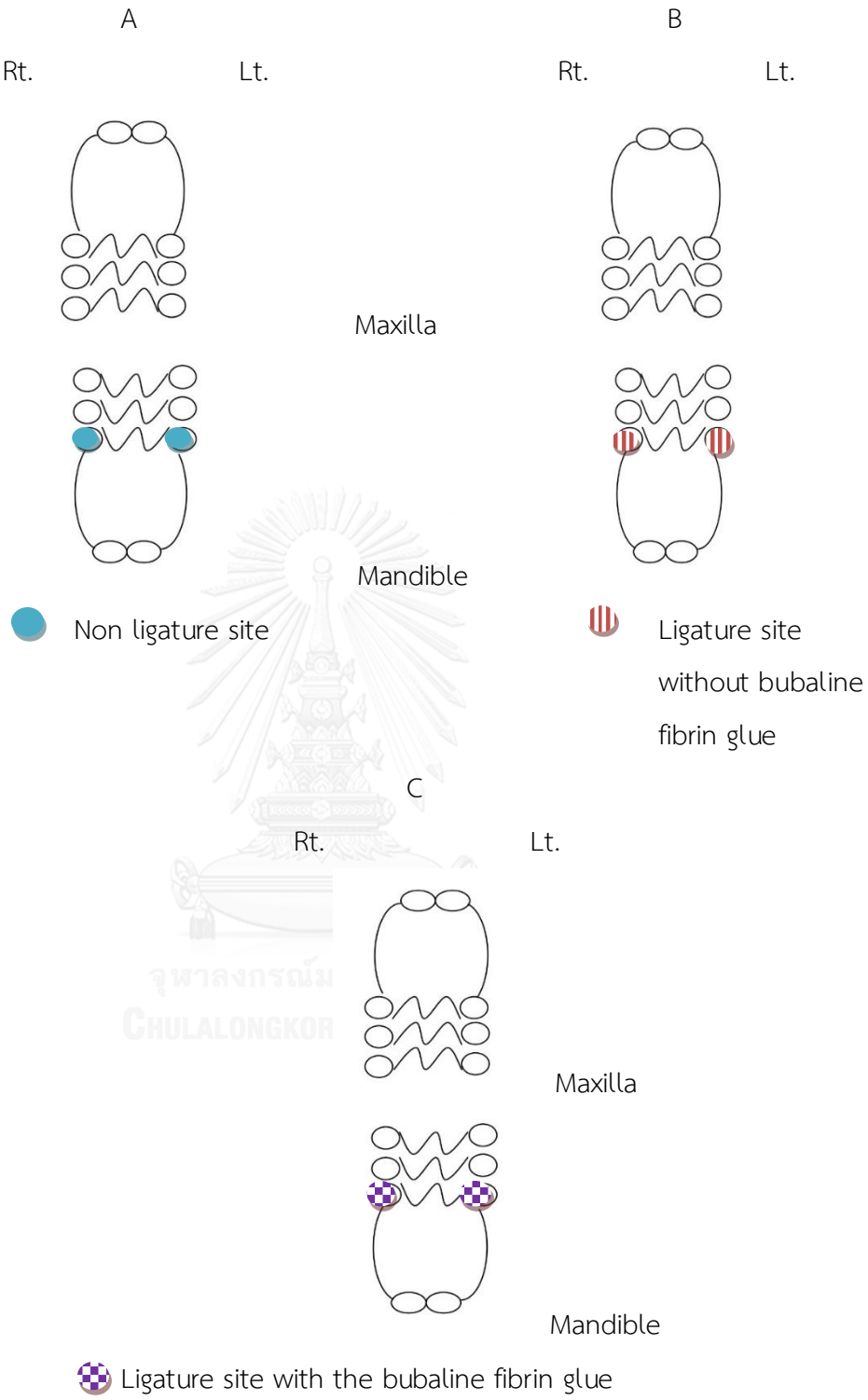

Figure 3 Location of ligature placement. (A) C group, (B) L group, and (C) LB group.

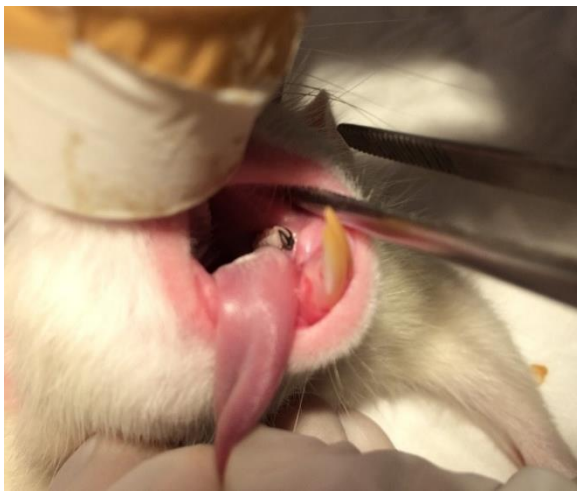

Figure 4 Induction of periodontitis by ligation technique on first molar.

3.4 Bubaline fibrin glue preparation

The procedure of fibrin glue preparation was modified from that of Thorn et al. (2004) (Figure 5). The 350 ml of blood were taken from jugular vein of buffalo, preserved in 49 ml of citrate phosphate dextrose (CPD) and centrifuged at 1500 rpm at room temperature for 30 minutes in order to discard the platelet rich plasma (PRP) and the packed red cells (Figure 6). The platelet rich plasma was divided into two compartments: 20 ml of PRP were mixed with 180 ml of 2.84 mM citric acid and then cryocentrifuged at 3,000 x *g* at 4°C for 5 minutes (Figure 7). Once the supernatant was discarded the remaining part was mixed with 1.14 ml of $CaCl_2$ plus 0.7 ml of 75 mM $NaHCO_3$ (Figure 8) before the clot formation occured (Figure 9). Finally, the thrombin was removed and kept in -80°C (Figure 11A). The remaining PRP was mixed with 3.2 ml of tranexamic acid plus 12 ml of 99% cold ethanol and then incubated in an ice water bath at 0°C for 30 minutes (Figure 10A) and cryocentrifuged at 3,000 x *g* at 0°C for 20 minutes (Figure 10B). The fibrinogen was mixed with 5 ml 0.9% NaCl, then thawed at 37°C, and kept at -80°C (Figure 11B).

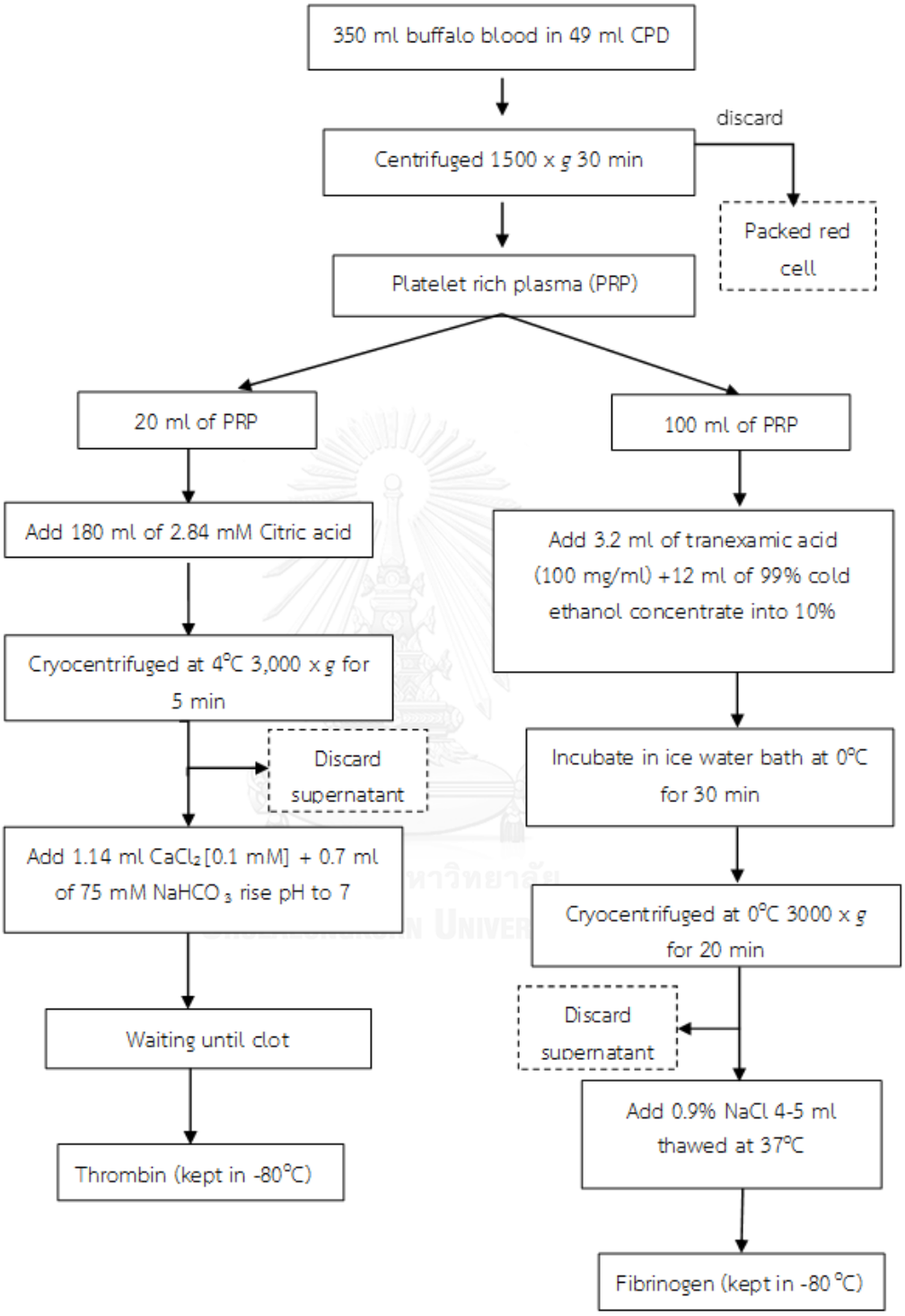

Figure 5 Preparation of the bubaline fibrin glue. Modified from Thorn et al. (2004).

Figure 6 Platelet rich plasma (right side) and buffalo whole blood (left side).

Figure 7 Platelet rich plasma (PRP) after cryocentrifuged at 3,000 x *g* at 4°C for 5 minutes.

Figure 8 Platelet rich plasma (PRP) after discarded supernatant for a thrombin preparation.

Figure 9 Thrombin was harvested from the supernatant.

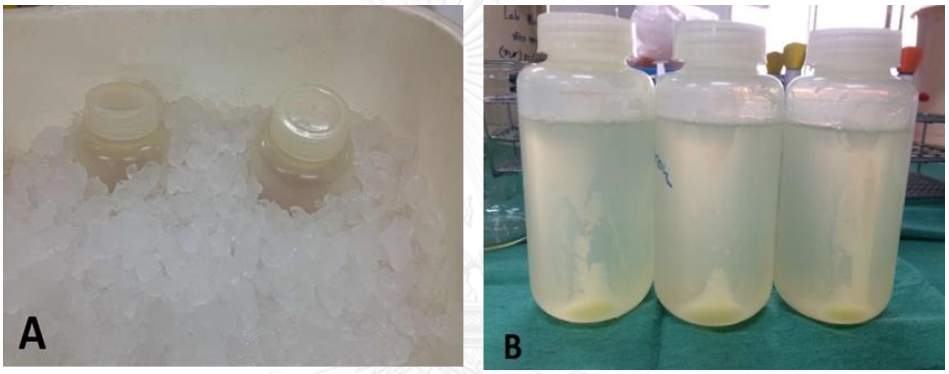

Figure 10 (A) Platelet rich plasma (PRP) mixed with tranexamic acid then incubated in ice water bath. (B) Harvesting of fibrinogen from the solution cryocentrifuged at 3,000 x *g* at 0°C for 20 minutes.

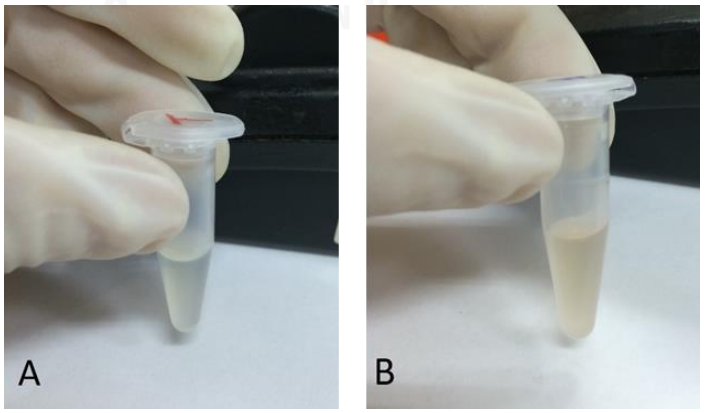

Figure 11 Bubaline fibrin glue (A) thrombin. (B) fibrinogen.

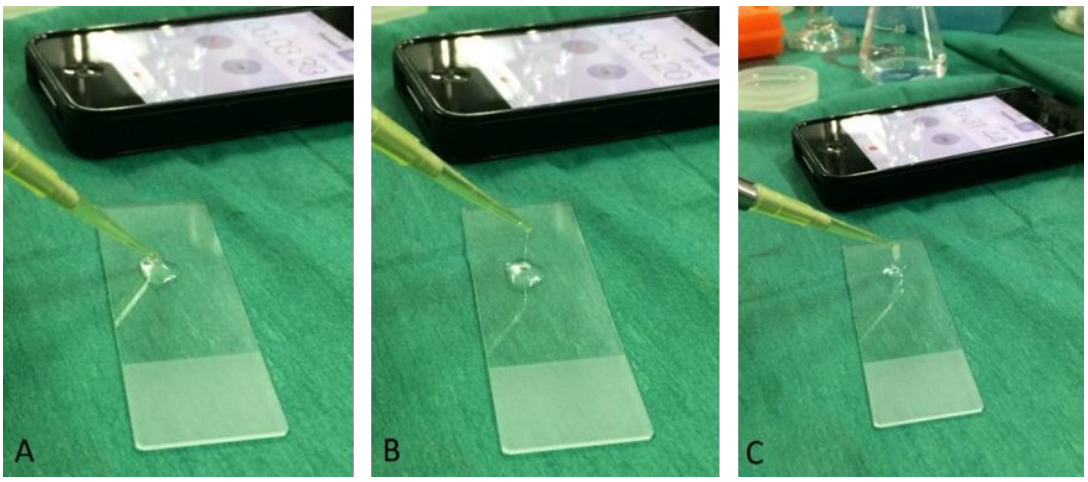

Figure 12 Clotting formation of the bubaline fibrin glue. (A) immediately. (B) 6 seconds later. (C) complete fibrin clot 31 seconds later.

3.5 Bubaline fibrin glue administration

After the ligation and the formation of the biofilm, the bubaline fibrin glue was administered to all animals of LB group.

After 7 DPL, rats were anesthesized with sedative and isoflurance by mask induction. The oral cavity was rinsed with 0.9% normal saline before performing a dental scaling with a magnetic ultrasonic scaler (DTE D7, USA) and it was then dried with an air-water syringe (Pearl®, Thailand). The bubaline fibrin glue was gently applied with a blunt cannula inserted into the periodontal pockets of the left and right mandibular first molars (n=9, l=18), allowing it to solidity for approximately 30–60 seconds. The evaluation was assessed at 8, 14, and 21 DPL.

3.6 Microbiological analysis

Microbiological analysis was carried out in the animals from L group at 0, 7, and 21 DPL (n=3, l=6). The samples from the individual site were kept in a separated container and the samples of the non-ligature and of the ligature induced conditions were compared during 21 days.

3.6.1 Subgingival microbial pathogen collection

Subgingival plaque samples were collected from the ligatures in the first molars to identify the microbial pathogen present. Firstly, the oral cavity was rinsed with 0.9% normal saline before the ligature was removed with forceps. Teeth were scaled with an ultrasonic scaler (DTE D7, USA) and dried with air-water syringe (Pearl®, Thailand). The ligatures were placed in 200 µl of pH 7.4 phosphate buffered saline (PBS). Secondly, the sample was divided into three compartments: 1) 50 µl was streaked on the trypticase soy agar (TSA; Difco™, Becton, Dickinson and Company, USA) supplemented with 5% sheep blood and incubated at 37°C for 24 hours, after which pure colonies were picked up for identification by VITEK2 technology (Biomerieux, USA); 2) 100 µl of the remaining sample were kept in -80°C for further investigation; 3) The final 50 µl remaining were streaked on the plate count agar (PCA; Difco™, Becton, Dickinson and Company, USA) in order to carry out total bacterial count by pour plate technique (Wohlsen et al., 2006).

3.6.2 Disc diffusion assay

The density of the bacterial suspension was adjusted by comparison with 0.5 McFarland standard (1.0×10^8 CFU/ml) and was then streaked on the Muller Hinton agar (MHA; Difco™, Becton, Dickinson and Company, USA) plates. The sterile blank paper discs (Oxoid, UK) impregnated with strains of bacteria were placed on the surface of MHA plates. Each tested plate consisted of two positive controls [(standard commercial antibiotic discs, Enrofloxacin 5 µg (Oxoid, UK), and Ceftiofur 30 µg (Oxoid, UK)], two negative controls (PBS and a mixture of calcium chloride, sodium bicarbonate, and sodium chloride), and one treated with the bubaline fibrin glue. *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were used as quality control organisms. Enrofloxacin and ceftiofur antibiotics were used as control antibiotics. The plate was incubated at 37°C for 18 to 24 hours depending on the species of bacteria. After the incubation the inhibition zone of each plate was examined by using the caliper. The examination was done per triplicate to ensure the accuracy of the test.

3.7 Clinical examination

Clinical examination in C group (n=3, l=6), L group (n=15, l=30), and LB group (n=15, l=30) were performed using standard digital photographs.

In C group, the samples were evaluated at 21 DPL while in L and LB group the samples at 1, 7, 8, 14, and 21 DPL.

3.7.1 Plaque index (PI)

The presence or absence of plaque on six different parts of the surfaces of the teeth was assesses by using PI according to Zhang et al. (2013).

Score 0: no plaque.

Score 1: mild plaque on free gingival margin and the surface of the tooth.

Score 2: moderate plaque on the surface of the tooth and deposits in the periodontal pocket.

Score 3: severe plaque on the gingival margin and abundant plaque in the periodontal pocket.

3.7.2 Gingival index (GI)

The inflammation of the gingiva was examined in four different parts of the surfaces of the teeth (mesial, distal, buccal, and lingual sites) for the color, the density, and the consistency using GI described by Zhang et al. (2013).

Score 0: no inflammation and healthy periodontium.

Score 1: mild inflammation with slight change in the color of the gingiva and no bleeding when probed.

Score 2: moderate inflammation with a significant change in the color and the consistency of the gingiva as well as bleeding while probed.

Score 3: severe inflammation with a significant change in the color, the consistency, and the density of the gingiva as well as spontaneous bleeding.

3.7.3 Mobility index and furcation involvement (MI)

The mobility of the teeth was assessed following the method by Kerry et al. (1982) and Xu and Wei (2006).

Score 0: no mobility.

Score 1: slightly mobile (the bucco-lingual direction).

Score 2: moderate mobility (the bucco-lingual and the mesio-distal directions).

Score 3: severe mobility (the bucco-lingual, the mesio-distal, and the vertical directions).

3.8 Histological analysis

Lesions were observed by means of histological analysis in C group (n=3, l=6), L group (n=15, l=30), and LB group (n=15, l=30).

In C group the tissue sample was taken on day 21, while in L and LB group the samples were taken at 1, 7, 8, 14, and 21 DPL.

The animals were euthanized with an overdose of anesthetic. The jaws were removed and fixed with 10% neutral buffered formalin for 48 hours (Semenoff et al., 2008), decalcified in 10% nitric acid, dehydrated in ethanol solution, embedded in paraffin, sectioned in 5 μm in a mesio–distal direction, and stained with hematoxylin and eosin (H&E). Sections consist of the first and the second molars, the interproximal alveolar bone crest, and the root pulp chambers were examined under a light microscopic (AmScope, USA).

3.8.1 Measurement of histological bone loss (Semenoff et al., 2008)

Calculation of the distance between the cemento–enamel junction (CEJ) and the alveolar bone crest (BC): CEJ-BC.

3.8.2 Measurement of histological attachment loss (Semenoff et al., 2008)

Calculation of the distance between the cemento–enamel junction (CEJ) and the periodontal ligament (PL): CEJ-PL.

3.8.3 Inflammation and fibrosis scoring (Yücel et al., 2003)

Score 0: no inflammatory cells, fibroblasts, and fibrocytes.

Score 1: inflammatory cells, fibroblasts, and fibrocytes found 1-35% of the fields.

Score 2: inflammatory cells, fibroblasts, and fibrocytes found in 36-70% of the fields.

Score 3: inflammatory cells, fibroblasts, and fibrocytes found over 70% of the fields.

3.9 Cytokine gene expression analysis

Gingival tissue was collected from C group (n=3), L group (n=15), and LB group (n=15). The samples were analyzed by qRT-PCR for the evaluation of the pro-inflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). The anti-inflammatory cytokines including interleukin-10 (IL-10), platelet-derived growth factor-A (PDGF-A), and transforming growth factor-β_1 (TGF-β_1) gene expression level.

3.9.1 RNA extraction protocol

The gingival tissue was collected from the lingual site of the gingiva following the method of Takahashi et al. (1994). The tissue was then analyzed for IL-1β, TNF-α, IL-10, PDGF-A, and TGF-β_1 gene expression level. RNA isolation with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions was carried out by homogenizing with the gingival tissue after washing them with ethanol. The sample was dissolved and stored at -80°C. RNA was purified with RNAse-free DNAse I (Ambion, USA) according to Dantas et al. (2012). The purity and the concentration of the RNA samples were measured using a Nanodrop spectrophotometer (Thermo Scientific, USA) after DNAse digestion.

3.9.2 Primer design

Transcript sequences of the IL-1β, TNF-α, IL-10, PDGF-A, and TGF-β_1 were obtained from *Rattus Norvegicus* genome transcript sequences via NCBI (USA). IL-1β (Accession No. NM_031512), TNF-α (Accession No. AJ002278), IL-10 (Accession No. NM_012854), PDGF-A (Accession No. NM_022595), and TGF-β_1 (Accession No. NM_021578). Primers were designed using Primer 3 Plus software version 2.3.7 (USA)

and then using BLAST analysis (USA). The primers sequences of the IL-1β, TNF-α, IL-10, PDGF-A, TGF-β_1, and β-actin gene were shown in table 2. β-actin gene was used as the endogenous control (a house-keeping gene) (Endo et al., 2013).

3.9.3 Reverse transcriptase (RT–PCR)

cDNA was produced by cDNA reverse transcription kit (ImProm-II™ Reverse Transcription System, Promega, USA) according to the manufacturer's instructions. The reverse transcription was performed on a thermo-cycler (Applied Biosystems, USA). The PCR amplification conditions consisted of initial denaturation of the cDNA by heating. This step can help DNA polymerase activation. Primers annealing allowed primers change to single-stranded DNA template and primer extension. Amplification cycles were shown in table 1.

3.9.4 Quantitative PCR (qPCR)

Real-time qPCR assays were performed in duplicate using a Swift Spectrum™ 48 Real-Time Thermal Cycler (Esco Healthcare, Singapore). The reaction mixture (20 µl) contained 1X KAPA SYBR® Fast qPCR Kit Master Mix (2X) Universal (Kapabiosystems, Massachusettes, USA), 50 ng of template DNA, 200 nM of the forward and reverse primers, and 1X ROX Low.

The conditions for qPCR were 95°C for 3 min (1 cycle) followed by 95°C for 3 sec, 60°C for 20 sec (40 cycles), and 72°C for 30 sec (1 cycle).

Table 1 Amplification cycles of IL-1β, TNF-α, IL-10, PDGF-A, TGF-β_1 and β-actin.

Gene	Denaturation	Annealing	Extension
IL-1β	40 cycles at 94°C 30 s	40 cycles at 55°C 30 s	40 cycles at 72°C 10 s
TNF- α	50 cycles at 95°C 30 s	50 cycles at 55°C 30 s	50 cycles at 72°C 6 s
IL-10	40 cycles at 94°C 45 s	40 cycles at 54°C 50 s	40 cycles at 72°C 55 s
PDGF-A	40 cycles at 94°C 45 s	40 cycles at 57°C 50 s	40 cycles at 72°C 55 s
TGF-β_1	40 cycles at 94°C 15 s	40 cycles at 57°C 15 s	40 cycles at 72°C 30 s
β-actin	45 cycles at 95°C 10 s	45 cycles at 54°C 10 s	45 cycles at 72°C 10 s

Table 2 Primer sequences for IL-1β, TNF-α, IL-10, PDGF-A, TGF-β1, and β-actin.

Gene	Forward (5'-3')	Reverse (5'-3')	Length (bp)
IL-1β	GACTTCACCATGGAACCCGT	GGAGACTGCCCATTCTCGAC	104
TNF-α	CTGTGCCTCAGCCTCTTCTC	ACTGATGAGAGGGAGCCCAT	126
IL-10	TTGAACCACCCGGCATCTAC	CCAAGGAGTTGCTCCCGTTA	91
PDGF-A	GTCAGGGCTAGTGCCCATTT	ACGTCTTGTCTGGGTGATGC	84
TGFβ_1	CACTCCCGTGGCTTCTAGTG	GGACTGGCGAGCCTTAGTTT	145
β-actin	TGTTGCCCTAGACTTCGAGCA	GGACCCAGGAAGGAAGGCT	155

3.10 Statistical analysis

Microbiological, clinical and histological data were expressed as mean ± SE, The cytokine gene expression data was expressed as $\log_{10}(2^{-\Delta\Delta Ct})$.

The comparisons within L group regarding the microbiological analysis were performed using the one-way ANOVA followed by Bonferroni test as post hoc test.

The comparisons between L and LB groups at 1, 7, 8, and 14 DPL regarding the clinical examination and the histological analysis were performed using student's T test or Mann-Whitney U test.

The comparisons between C, L, and LB groups at 21 DPL regarding the clinical examination and the histological analysis were performed using the one-way ANOVA followed by Bonferroni test as post hoc test or Kruskal–Wallis test.

The comparison between L and LB groups regarding the qRT-PCR technique was performed using Mann-Whitney U test.

Data were analyzed quantitatively by using SPSS version 22 for Windows program (Version 22, IBM, USA) and differences were considered significant at $P<0.05$.

CHAPTER IV
RESULTS

4.1 Microbiological analysis

To identify bacterial analysis, the samples of the non-ligature and ligature induced conditions from L group (n=3, l=6) at 0, 7, and 21 DPL were compared.

4.1.1 Bacterial strains and Total bacterial count

The results showed 11 bacterial strains by aerobic condition. At 0 DPL showed the isolation of *Corynebacterium* spp. (CUVDCR-6,7,8), *Staphylococcus gallinarum* (CUVDCR-1,2,3), *Staphylococcus sciuri*, *Staphylococccus xylosus*, and *Pasteurella canis* (CUVDCR-2). At 7 DPL found the *Corynebacterium* spp. (CUVDCR-1,2), *Enterococcus avium*, *Escherichia coli* (CUVDCR-1,2,3), and *Proteus mirabilis*. At 21 DPL isolated the *Acinetobacter baumannii* complex, *Budvicia aquatica*, *Corynebacterium* spp. (CUVDCR-3,4,5), *Kocuria kristinae*, and *Pasteurella canis* (CUVDCR-2). Corynebacterium was the most predominate (8/24) followed by Staphylococcal bacteria (5/24). Moreover, the number of bacteria at 7 DPL (^{a}) found the significant higher than 0 and 21 DPL ($P<0.05$) as seen in Table 3. However, there was no statistical difference between 0 and 21 DPL ($P\geq0.05$).

Table 3 Total bacterial count isolated from the first molars of male Wistar rats.

Day	Total bacterial count (mean ± SE)
0	$2.3 \times 10^5 \pm 8.5 \times 10^4$
7	$1.4 \times 10^6 \pm 2.9 \times 10^{5a}$
21	$1.3 \times 10^5 \pm 1.9 \times 10^4$

^{a} .- Data represents mean ± SE (n=3, l=6); values followed by superscript letter in the row is significantly different (P<0.05).

4.1.2 Disc diffusion assay

Results from the disc diffusion assay are shown in Table 4. The bubaline fibrin glue had a potential antimicrobial activity against the 6 bacterial strains (6/11), this being assessed by the presence or absence of inhibition zones. Activity against the

Gram positive bacteria *Corynebacterium* spp., *Kocuria kristinae* and against Gram negative bacteria *Acinetobacter baumannii* complex, *Budvicia aquatica*, *Escherichia coli* and *Pasteurella canis* was observed. However, it did not show antimicrobial activity against the Gram positive bacteria *Enterococcus avium*, Staphylococcal species and against the Gram negative bacteria *Proteus mirabilis*.

Table 4 Antimicrobial activity of the bubaline fibrin glue against bacteria isolated from oral cavity of the experimental rats determined by the disc diffusion method.

	Inhibitory zone		
Microorganisms	Bubaline fibrin glue (mm)	Enrofloxacin (mm)	Ceftiofur (mm)
1. *Acinetobacter baumannii complex*	8.5	27 (S)	17 (I)
2. *Budvicia aquatica*	22	50 (S)	32 (S)
3. *Corynebacterium* spp. (CUVDCR-1)	0	28 (S)	24.5 (S)
4. *Corynebacterium* spp. (CUVDCR-2)	22	41 (S)	38 (S)
5. *Corynebacterium* spp. (CUVDCR-3)	23	0	41 (S)
6. *Corynebacterium* spp. (CUVDCR-4)	21	42.5 (S)	33.5 (S)
7. *Corynebacterium* spp. (CUVDCR-5)	24	45 (S)	35 (S)
8. *Corynebacterium* spp. (CUVDCR-6)	20.5	40.5 (S)	38.5 (S)

9. *Corynebacterium* spp. (CUVDCR-7)	20.5	42.5 (S)	30 (S)
10. *Corynebacterium* spp. (CUVDCR-8)	23.5	41.5 (S)	40 (S)
11. *Enterococcus avium*	0	29.5 (S)	29 (S)
12. *Escherichia coli* (CUVDCR-1)	0	29.5 (S)	20.5 (I)
13. *Escherichia coli* (CUVDCR-2)	9	30 (S)	20.5 (I)
14. *Escherichia coli* (CUVDCR-3)	6	31.5 (S)	26.5 (S)
15. *Kocuria kristinae*	10	33.5 (S)	30 (S)
16. *Pasteurella canis* (CUVDCR-1)	24	50 (S)	38 (S)
17. *Pasteurella canis* (CUVDCR-2)	22	42 (S)	40 (S)
18. *Pasteurella* spp.	20	42 (S)	36 (S)
19. *Proteus mirabilis*	0	28.5 (S)	28 (S)
20. *Staphylococcus gallinarum* (CUVDCR-1)	0	24.5 (S)	23 (S)
21. *Staphylococcus gallinarum* (CUVDCR-2)	0	24.5 (S)	22.5 (S)
22. *Staphylococcus galiinarum* (CUVDCR-3)	0	24 (S)	17.5 (I)
23. *Staphylococcus scuiri*	0	24 (S)	23.5 (S)
24. *Staphylococcus xylosus*	0	25.5 (S)	23 (S)

S: Susceptible, I: Intermediate, R: Resistant

4.2 Clinical examination

4.2.1 Plaque Index (PI)

PI was increased up to 7 DPL and slightly declined after removing the ligature (8 to 21 DPL) in L group. The plaque was slightly increased from 7 to 21 DPL and showed significant different at 21 DPL in LB group compared with L group. However, the data revealed that at 7 DPL, PI was increased in the ligation groups compared with the non-ligation group (Table 5).

Table 5 Plaque Index (PI) assessed by using score 0–3 according to Zhang et al. (2013).

Group	Time of data collection				
	1 DPL	7 DPL	8 DPL	14 DPL	21 DPL
C	ND	ND	ND	ND	0.00±0.00
L	0.00±0.00	0.67±0.21	0.33±0.21	0.17±0.17	0.00±0.00
LB	0.00±0.00	0.50±0.22	0.00±0.00	0.70±0.21	0.40±0.16[a]

ND = Not Done

[a].- Data represents mean ± SE; values followed by superscript letter in the row is significantly different ($P<0.05$).

4.2.2 Gingival Index (GI)

During ligation (1 to 7 DPL), an increase in GI was seen in both L and LB groups. GI gradually decreased in both L and LB groups after removing the ligature (8 to 21 DPL). L group showed the lower value at 8–21 DPL compared with LB group. GI presented a statistically difference between ligation (L and LB) and non-ligation (C) groups at 21 DPL. However, there was no difference in statistical analysis between L and LB groups at any time-points (Table 6).

Table 6 Gingival Index (GI) assessed by using score 0–3 according to Zhang et al. (2013).

Group	Time of data collection				
	1 DPL	7 DPL	8 DPL	14 DPL	21 DPL
C	ND	ND	ND	ND	0.00±0.00[a]
L	0.00±0.00	1.00±0.26	0.67±0.21	0.67±0.21	0.50±0.22
LB	0.38±0.18	1.50±0.22	0.38±0.18	1.10±0.18	0.90±0.10

ND = Not Done

[a].- Data represents mean ± SE; values followed by superscript letter in the row is significantly different ($P<0.05$).

4.2.3 Mobility Index (MI)

There was no significant difference in the MI among three groups at any of the different time-points (Table 7).

Table 7 Mobility Index (MI) assessed by using score 0–3 according to Kerry et al. (1982) and Xu and Wei (2006).

Group	Time of data collection (MI)				
	1 DPL	7 DPL	8 DPL	14 DPL	21 DPL
C	ND	ND	ND	ND	0.00±0.00
L	0.00±0.00	1.00±0.63	0.00±0.00	0.00±0.00	0.00±0.00
LB	0.00±0.00	0.00±0.00	0.00±0.00	0.10±0.10	0.00±0.00

ND = Not Done

4.3 Histological analysis

4.3.1 Histological bone loss

The results showed that the ligation can increase histological bone loss in both L and LB groups during and after removing the ligature as seen in Table 8. Histological bone loss presented a statistically difference between non-ligation group (C group) and ligation groups (L and LB groups) at 21 DPL. L group showed the higher value compared with LB group at 1 to 14 DPL. Moreover, the results presented a statistically difference between L and LB groups after removing the ligature (14 DPL).

Table 8 Histological bone loss assessed by calculation the distance (mm) between cemento–enamel junction (CEJ) and alveolar bone crest (BC).

Group	Time of data collection				
	1 DPL	7 DPL	8 DPL	14 DPL	21 DPL
C	ND	ND	ND	ND	795.48±57.94[a]
L	948.12±43.66	1098.73±182.88	1171.18±88.95	1346.33±89.51[a]	1271.56±54.63
LB	1026.72±93.58	1179.10±70.37	1049.40±53.99	1001.31±88.72	1312.06±48.02

ND = Not Done

[a].- Data represents mean ± SE ; values followed by superscript letters in each row are significantly different (P<0.05).

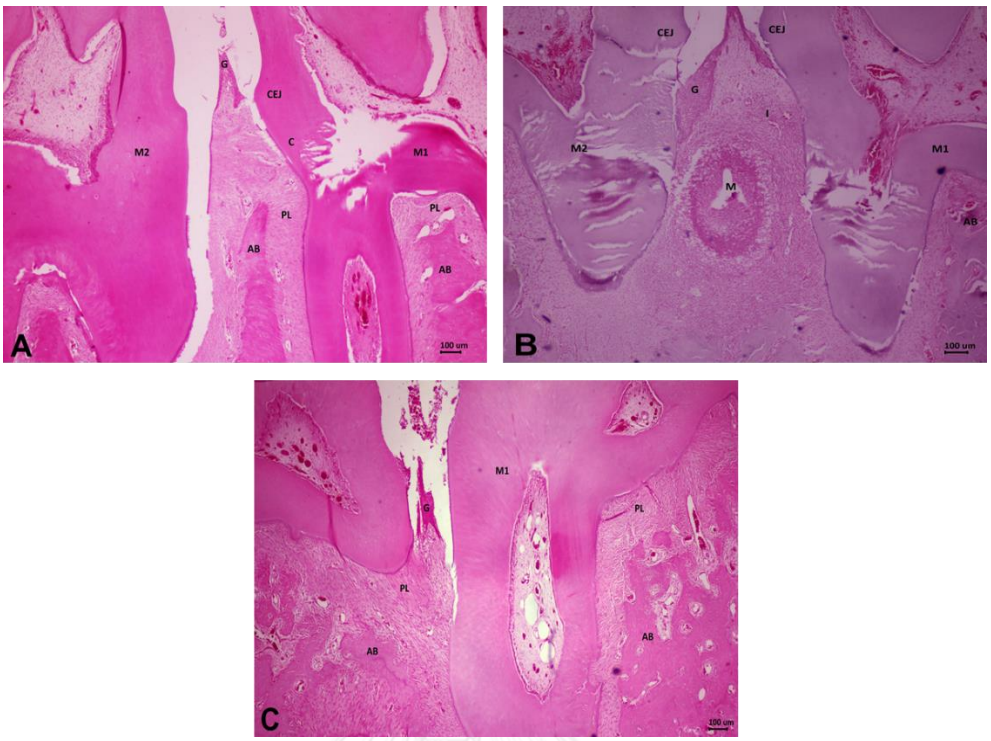

Figure 13 Histological observation of the mandibular first molar tooth at 14 DPL. (H&E stain; magnification x4. Scale bars = 100 μm)

(A) Histological observations of control (C) group showing normal periodontal tissue structure.

(B) Periodontium of rat subjected to ligation without treatment (L) group showing severe chronic localized granulomatous periodontitis and severe bone destruction.

(C) Periodontium of rat subjected to ligation with bubaline fibrin glue treatment (LB) group showing inflammatory cell infiltration with mild alveolar bone destruction.

Abbreviations: AB = Alveolar Bone; C =Cementum; CEJ = Cemento–Enamel Junction; F = Fibrocytes; G = Gingiva; I = inflammatory cells; M = Microoganisms; M_1 = Molar$_1$; M_2 = Molar$_2$; PL = Periodontal Ligament; RBC = Red Blood Cells

4.3.2 Histological attachment loss

The results showed that the ligation can increase histological attachment loss in both L and LB group during and after removing the ligature as seen in Table 9. An increase of histological attachment loss was observed from time to time in L group (1 to 21 DPL). An increase was also shown during ligation (1 to 7 DPL) but a significantly lower value was shown in LB when compared with L group after removing the ligature (8 to 14 DPL). Moreover, the results presented the difference in statistical analysis between non-ligation (C group) and ligation groups (L and LB groups) at 21 DPL.

Table 9 Histological attachment loss assessed by calculation the distance (mm) between cemento–enamel junction (CEJ) and periodontal ligament (PL).

Group	Time of data collection				
	1 DPL	7 DPL	8 DPL	14 DPL	21 DPL
C	ND	ND	ND	ND	190.65±52.35[a]
L	442.86±117.36	597.08±94.26	775.41±120.37[a]	1014.95±123.57[a]	889.98±65.40[b]
LB	514.70±67.67	674.31±50.83	385.14±41.34	371.03±41.56	488.13±70.88[c]

ND = Not Done

[a,b,c] .- Data represents mean ± SE ; values followed by different superscript letters in each row are significantly different ($P<0.05$).

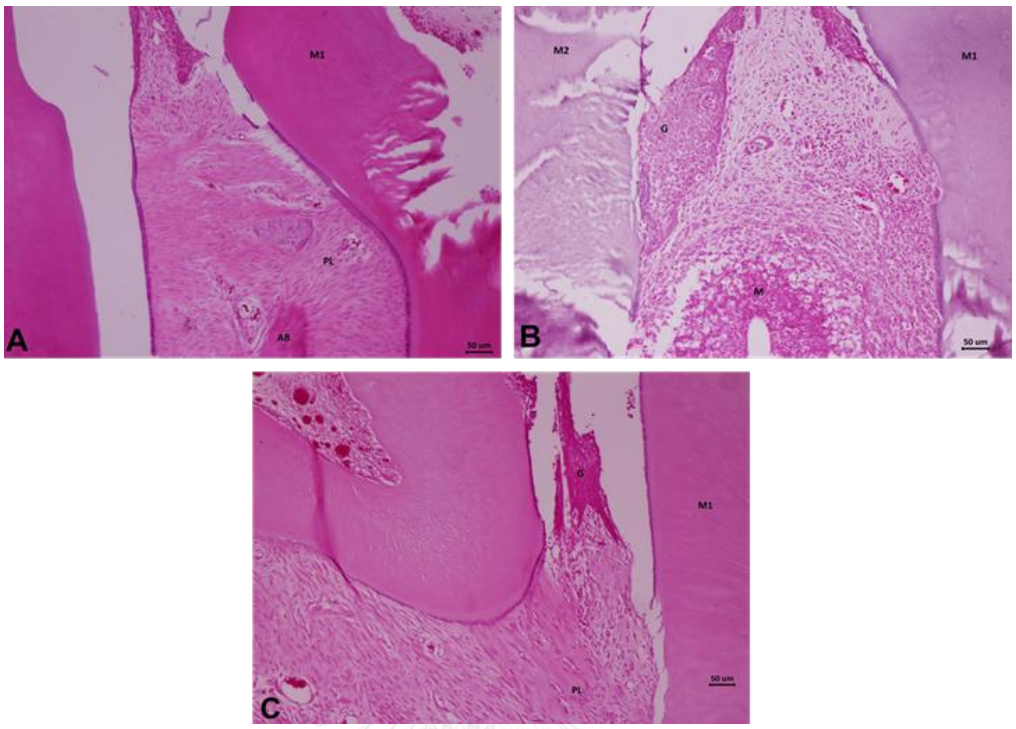

Figure 14 Histological observation of the periodontal ligament area at 14 DPL. (H&E stain; magnification x10. Scale bars = 50 μm)

(A) Histological observations of control (C) group showing normal periodontal ligament.

(B) Periodontium of rat subjected to ligation without treatment (L) group showing severe chronic localized granulomatous periodontitis and severe periodontal ligament destruction.

(C) Periodontium of rat subjected to ligation with bubaline fibrin glue treatment (LB) group showing inflammatory cells infiltrate in gingival epithelium and mild periodontal ligament destruction.

4.3.3 Inflammation score

The results showed that the ligation can increase inflammation score in both L and LB groups during and after removing the ligature as seen in Table 10. L group obtained approximately 36-75% inflammation cells, fibroblasts, and fibrocytes of the fields during ligation (7 DPL). Nevertheless, the score gradually decreased after removing the ligature until 21 DPL. An increase of inflammation score in LB group during ligation was also shown (7 DPL) but a significantly lower value after removing the ligature when compared with L group (14 to 21 DPL). Moreover, there was no statistical difference between C and LB groups at 21 DPL.

Table 10 Inflammation score assessed by using score 0–3 according to Yücel et al. (2003).

Group	Time of data collection				
	1 DPL	7 DPL	8 DPL	14 DPL	21 DPL
C	ND	ND	ND	ND	0.00±0.000
L	1.33±0.67[a]	1.67±0.21	1.33±0.21	1.20±0.37[a]	0.83±0.31[a]
LB	0.3±0.15	1.25±0.25	1.57±0.20	0.22±0.15	0.20±0.13

ND = Not Done

[a,] .- Data represents mean ± SE ; values followed by superscript letters in each row are significantly different ($P<0.05$).

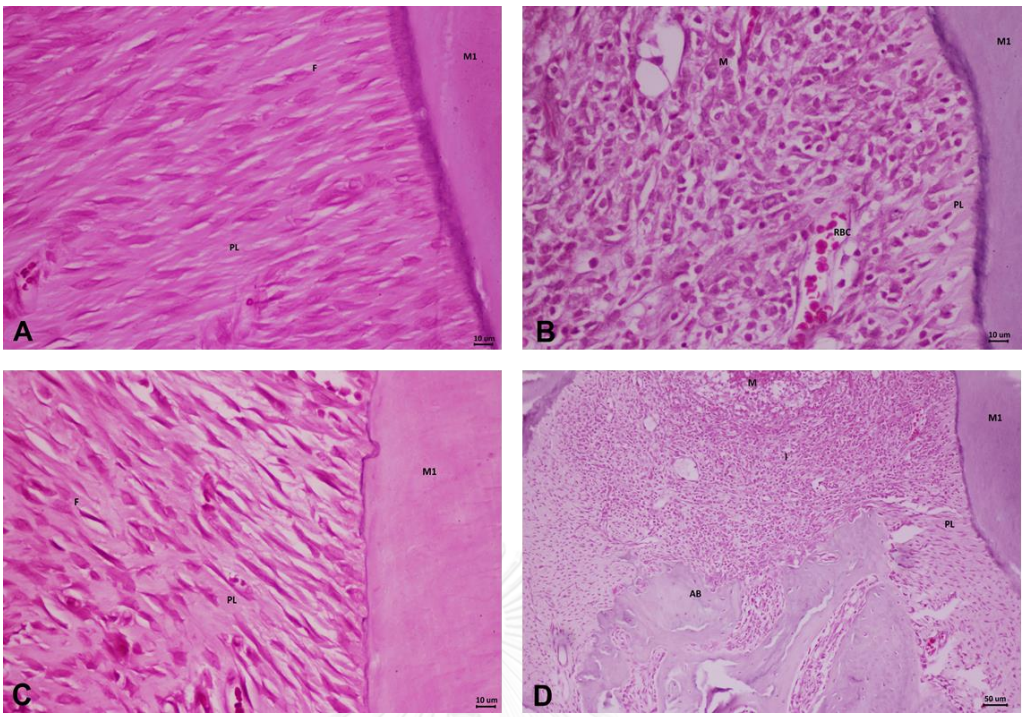

Figure 15 Histological observation of the periodontal ligament at 14 DPL. (H&E stain; magnification x40 (A–C), magnification x10 (D). Scale bars = 10 μm (A–C), Scale bars = 50 μm (D))

(A) Histological observations of control (C) group showing fibroblasts and fibrocytes of the normal periodontal ligament.

(B,D) Periodontium of rat subjected to ligation without treatment (L) group showing an infiltrative of mononuclear cells and severe alveolar bone destruction.

(C) Periodontium of rat subjected to ligation with bubaline fibrin glue treatment (LB) group showing mild inflammatory cells infiltrate and the accumulation of fibrocytes in periodontal ligament.

4.4 Cytokines

The relative expression of pro- and anti-inflammatory cytokines was analyzed by the $\log_{10}(2^{-\Delta\Delta ct})$ method (Rao et al., 2013) using β-actin (House-keeping gene) as normalizer and a pooled gingival tissue from normal control rats (C group) as a negative control.

4.4.1 Gingival IL-1β

The up-regulation of gene expression of Gingival IL-1β was observed during every time-point of data collection in both L and LB groups. After removing ligature, the gene expression tend to increase folds change (8 to 21 DPL) and the highest folds change was observed in L group at 21 DPL. LB group showed a higher value during the ligation (1-7 DPL). However, after remove ligation the expression showed a lower value when compared with L group (8 to 21 DPL) (Figure 16).

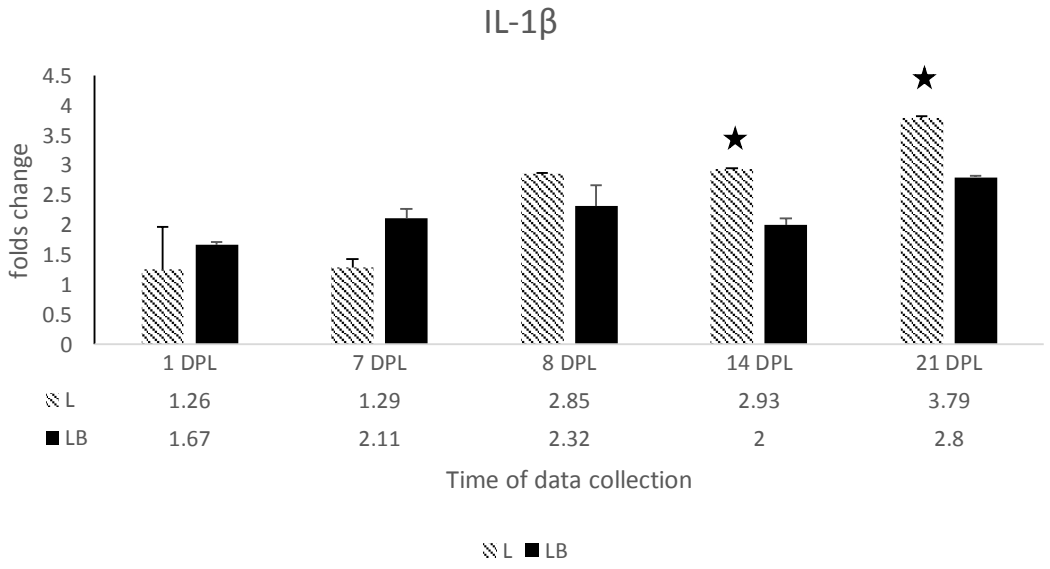

Figure 16 Expression of Gingival IL-1β. Values obtained in the experimental groups (L and LB) by $\log_{10}(2^{-\Delta\Delta Ct})$ method. Asterisk represents statistically significant differences ($P<0.05$).

4.4.2 Gingival TNF-α

The up-regulation of gene expression of Gingival TNF-α was observed during every time-point of data collection in both L and LB groups. After removing ligature, the gene expression tend to increase folds change (8 to 21 DPL) and the highest folds change was observed in L group at 14 DPL. LB group showed a higher value during the ligation (1 to 7 DPL). However, after remove ligation the expression showed a lower value when compared with L group (14 to 21 DPL) (figure 17).

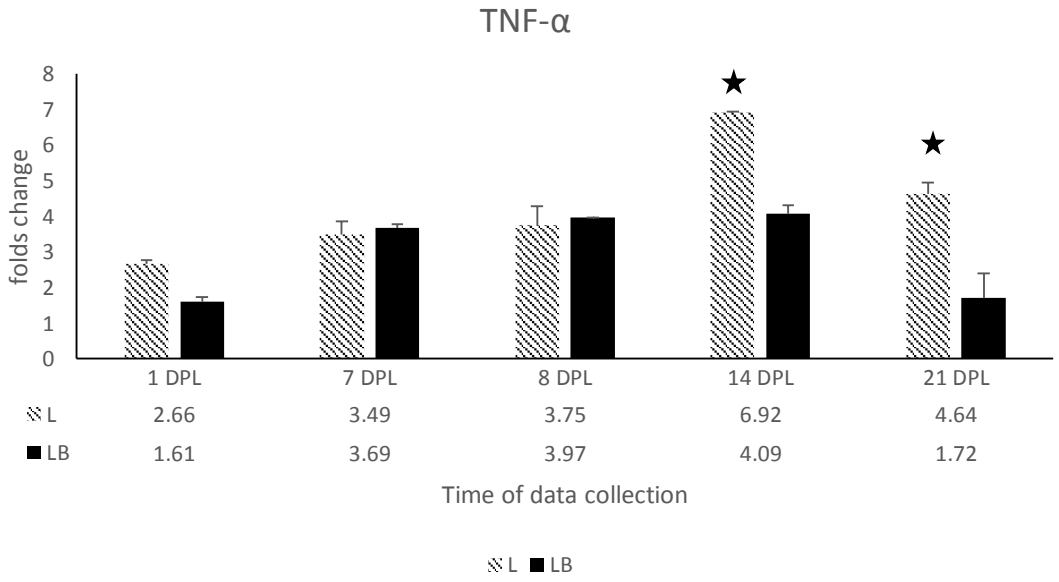

Figure 17 Expression of Gingival TNF-α. Values obtained in the experimental groups (L and LB) by $\log_{10}(2^{-\Delta\Delta Ct})$ method. Asterisk represents statistically significant differences ($P<0.05$).

4.4.3 Gingival IL-10

The gene expressions of IL-10 were up-regulated and down-regulated. After removing the ligature, the gene expression showed a decreasing folds change in L group (14 to 21 DPL). Data revealed that LB group had the greater value of folds change compared with L group during the induction period (1 to 7 DPL). Moreover, the results presented the significant different between L and LB groups after removing ligature (8 to 14 DPL) (Figure 18).

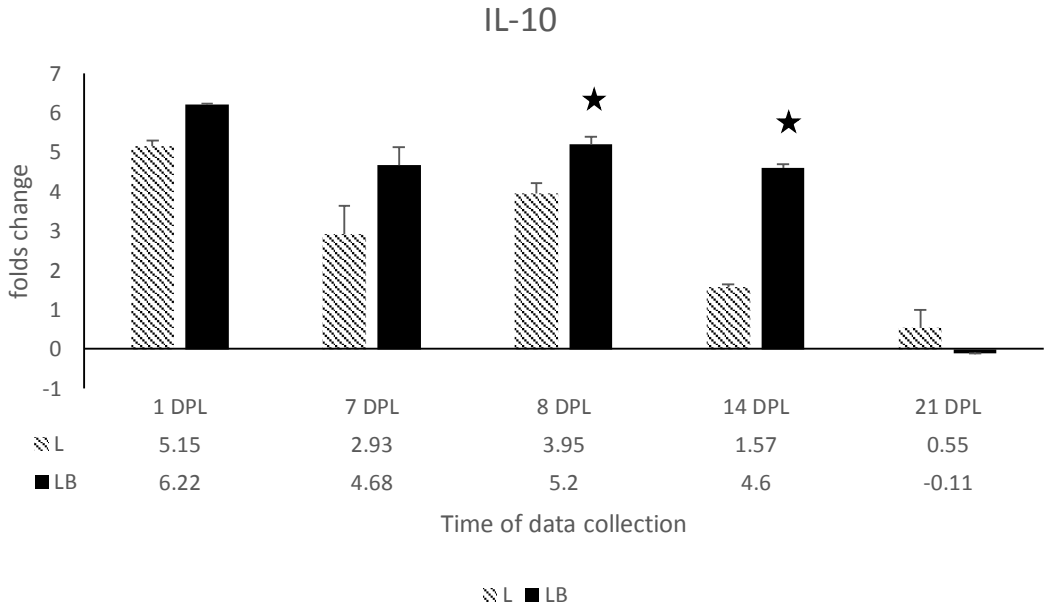

Figure 18 Expression of Gingival IL-10. Values obtained in the experimental groups (L and LB) by $\log_{10}(2^{-\Delta\Delta Ct})$ method. Asterisk represents statistically significant differences ($P \leq 0.05$).

4.4.4 Gingival PDGF-A

The up-regulation of gene expression of Gingival PDGF-A was observed during every time-point of data collection in both L and LB groups. After remove ligation, the gene expression showed a decreasing folds change (14 to 21 DPL) and the highest folds change was observed in L group at 8 DPL. LB group showed a higher value during the ligation (1 to 7 DPL). Moreover, after remove ligation the expression showed a superior value compared with L group (14 to 21 DPL) (Figure 19).

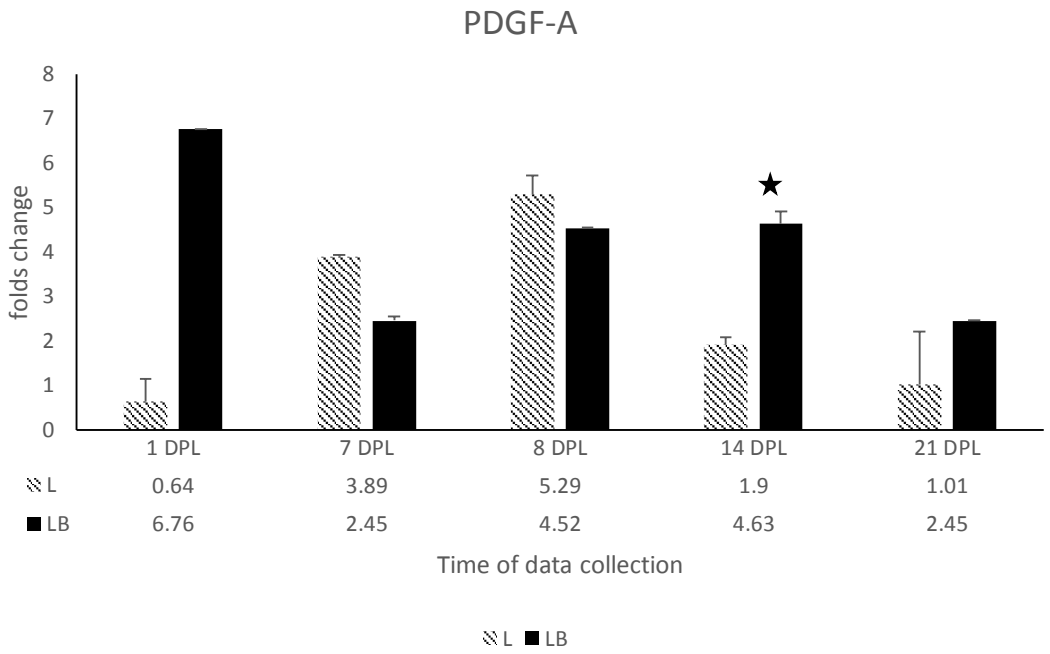

Figure 19 Expression of Gingival PDGF-A. Values obtained in the experimental groups (L and LB) by $\log_{10}(2^{-\Delta\Delta Ct})$ method. Asterisk represents statistically significant difference ($P<0.05$).

4.4.5 Gingival TGF-β_1

The up-regulation of gene expression of Gingival TGF-β_1 was observed during every time-point of data collection in both L and LB groups. After removing ligature, the gene expression showed a decreasing folds change in L group (8 to 21 DPL) compared with LB group. Moreover, the results presented the significant different between L and LB groups at 21 DPL. The highest folds change was observed in LB group at 1 DPL (figure 20).

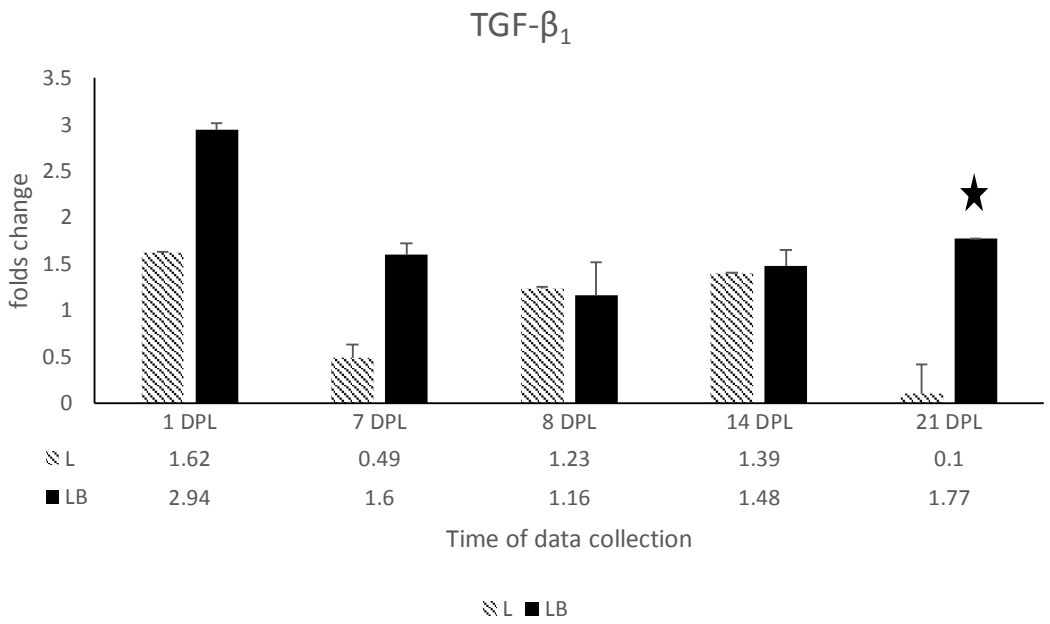

Figure 20 Expression of Gingival TGF-β_1. Values obtained in the experimental groups (L and LB) by $\log_{10}(2^{-\Delta\Delta Ct})$ method. Asterisk represents statistically significant difference ($P<0.05$).

CHAPTER V
DISCUSSION

Various techniques have been used to induce periodontitis. For instance, inoculation of pathogenic bacteria, diet intake and ligature induction have been performed in experimental animals (Hart et al., 2004; Abe and Hajishengallis, 2013). In our study, we chose the ligature induction technique to achieve a specific microbiological profile and presence of local specific inflammation. Multifilament sutures enhance the accumulation of microorganisms in periodontal pocket leading to an infiltration of inflammatory cells that results in periodontitis.

The bacterial strains and total count from the oral cavity of the experimental rats at different times belonged to various strains and were presented in different amounts. Those animals in which the silk ligation around the first molar had been left 7 days obtained a higher total bacterial count compared with those with no ligation. Results confirmed that the major cause of periodontitis is bacterial infection. In our study, we found that Corynebacterium was the most predominate bacteria (8/24) followed by Staphylococcal bacteria (5/24). According to Isogai et al. (1985) the predominate microbiota isolated from the gingival crevice of Wistar Kyoto rats over first three months were Streptococcal, Staphylococcal, Enterococcal bacteria (Gram positive aerobic or facultative cocci) and Lactobacillus and Corynebacterium (Gram positive aerobic or facultative rods). In our study, we detected the Gram positive bacteria serve as the dominant microbiota at the first day before the ligature induction (0 DPL). After the progression of the disease, we also isolated the Gram negative bacteria in accordance with a previous study (Riggio et al., 2011). Moreover, focusing on anaerobic bacteria is essential for investigating those that are periodontopathic. Oz and Puleo (2011) reported that there was presence of *Porphyromonas gingivalis* in rats with induced periodontitis which presented horizontal bone loss. Further research with anaerobic bacteria in periodontitis animal models should be carried out to clearly understand the mechanism of periodontitis.

In the current study the bubaline fibrin glue showed an inhibitory effect against oral bacteria on disc diffusion assay in the experimental rats, suggesting the active ingredients in bubaline fibrin glue, fibrinogen and thrombin, have antimicrobial activity. According to Lahoda et al. (2006) the commercial fibrin glue could significantly reduce the presence of microorganisms on partial thickness burn wounds in vivo. The antimicrobial activity of GHR28 released from the β-chain of fibrinogen plays a role in inflammation and wound healing (Påhlman et al., 2013). On contrary, the human-derived fibrin glue had no antibacterial activity in vitro (Yuksel et al., 2014). Our study showed a different result, which might be due to the different sources of fibrin glue used. Indeed, in our study bubaline blood had a higher level of fibrinogen (1,400 mg/dl) even stored in -80°c over 3 months compared to human fibrinogen reported by Thomazini-Santos et al. (1998).

Various authors have reported the duration of ligature-induction in experimental rats. Some of them described that the destruction of alveolar bone at a microscopic level began at day 4 and increased to the maximum level at day 7 after the induction of periodontitis with nylon (3-0) thread ligature (Bezerra et al., 2002). In order to measure it morphometrically, Ozdemir et al. (2012) and Toker et al. (2009) reported the significant bone loss at day 11 after ligating with silk (4-0). Similarly, De Lima et al. (2000) reported alveolar bone loss by macroscopic examination after the ligation with by nylon (0) on the right second upper molars at day 3, the maximum loss being observed at day 7 but decrease of loss at day 14. Other studies detected the clinical signs of periodontitis by the change of color of gingiva at day 3 after ligation and found a significant accumulation of inflammatory cells at day 14 (Ionel et al., 2015). This differs from the study by Kuhr et al. (2004), who investigated for a longer period of ligatured induction of periodontitis in experimental rats. However, the presence of inflammation was first already detected at day 3 after ligation (Ionel et al., 2015). In our study the chosen duration of plaque accumulation was based on the information found in the available literatures regarding the appropriated period for cytokine detection. Therefore, we hypothesized that a period of 7 days might be enough for the determination of the pathogenesis of periodontitis by means of

macroscopic and microscopic examination. Indeed, we detected the differences in the morphological changes between the non-ligation group and the ligation group at 7 days after ligation (DPL), according to previous studies (De Lima et al., 2000; Bezerra et al., 2002)

In the present study, we studied the effects of the bubaline fibrin glue on the plaque index, the gingivitis index and the mobility index in experimentally induced periodontitis rats. Our morphological study demonstrated that the bubaline fibrin glue significantly decreased the plaque and the gingival indexes in a short-time period (the day after removing the ligatures) compared with the sham-ligated control. However, it had no significant decrease of those in a long-term period (14 and 21 DPL). No differences regarding the mobility index were found. It is possible that incompletely degraded bubaline fibrin glue remaining on the periodontal pocket might have been seen as plaque accumulation because it takes over 10–14 days to be degraded itself by macrophages and fibroblasts. However, fibrin glue made from natural bioactive agents has several advantages because it is biodegradable and biocompatible. This is different from synthetic agents, which have been associated with the induction of inflammation and fibrosis. Moreover, the morphological evaluation by scoring with naked eyes might not be a useful method for detection periodontitis in rats due to the limitation of the small dental size of rats. Cheng et al. (2010) and Lu et al. (2013) suggested that micro-computed tomography (Micro-CT) is an appropriate tool for detecting morphological changes.

The destruction of alveolar bone and ligament was evaluated by histopathological techniques and results revealed that the distance between the bone crest or the alveolar ligament and cemento–enamel junction was higher in ligated rats than in non-ligated rats, which is in accordance with the result of a previous study by Semenoff et al. (2008).

In order to investigate the effects of the bubaline fibrin glue by means of the histological examination, the grade of loss of periodontal ligament and alveolar bone was assessed. It was very surprising that the bubaline fibrin glue in periodontal pocket significantly decreases the loss of attachment at every time of the data collection

when compared with the untreated group. In addition, we detected a significant difference in the loss of bone between the treated and untreated groups at 7 days after glue application (14 DPL). Given the anti-inflammatory properties from Bβ15-42 of fibrin fragments (Jennewein et al., 2011) it is possible that fibrin glue might contribute to the attachment of periodontal ligament and the promotion of osteogenesis similarly as described in the previous study (Dogan et al., 1992). However, we did not detect the significant different in the loss of bone at the day after glue application (8 DPL), which can be explained by the fact that the alteration of the soft tissue is faster than hard tissue in line with the normal wound healing of periodontal structures. Therefore, we detected the loss of periodontal ligament earlier than the loss of alveolar bone.

The expression of pro-inflammatory cytokines in healthy periodontal tissues was detected but at a lower level than in inflamed tissues due to the existence of the stationary state (Jacob and Nath, 2013).

In terms of gene expression, this is the first study using the bubaline fibrin glue in the treatment of periodontitis. Our results revealed that the expression of pro-inflammatory cytokines (TNF-α and IL-1β) in the treatment group tended to decrease with time compared with the untreated group. For TNF-α, we could not detect the decline of expression on the day after removing the ligature and applying the intervention (8 DPL). However, we detected the lowest up-regulated expression was recorded at 21 DPL. Our results are in accordance with those of Stiller-Timor et al. (2012), who found that the levels of TNF-α and IL-6 in serum of patients treated with commercial fibrin glue after tonsillectomy were low. Moreover, our results showed the maximum up-regulation at day 7 after the inflammatory stimuli removal (14 DPL) in the untreated group. Data confirmed that there is continuously progressive inflammation response without inflammatory stimuli, which was in contrast to the finding of Jacob and Nath (2013). These authors reported that pro-inflammatory cytokines were extensively expressed within 3 hours after inflammatory response and maintained at the level at day 3 before declining to a similar level of that of the untreated group after 7 days. In a different study it was found that levels of TNF-α and PGE_2 in rats with periodontitis were significant higher than those of normal rats

after 6 weeks of the ligature induction (Liao et al., 2014). It is very surprising that our results showed a decreased expression of IL-1β after the ligature removal and the bubaline fibrin glue application in every time-points when compared with the untreated group. Data showed the lowest value at 7 days after the inflammatory stimuli removal (14 DPL).

The patterns of TNF-α and IL-1β expression were similar. TNF-α and IL-1β, produced by macrophages and monocytes, are considered to be two of the most important inflammatory cytokines involved in the pathogenesis of periodontal disease. The effects of TNF-α and IL-1β are induction of inflammation by stimulation of other inflammatory mediators, tissue destruction by matrix metalloproteinase induction, enhancement of bone resorption by stimulating osteoclast activity and by interfering with bone formation (Graves and Cochran, 2003).

Even though the mechanism of action of bubaline fibrin glue is unclear, it might have a similar mechanism to that of the commercial fibrin glue. The sealing function and anti-inflammatory effect from the fibrinogen and the thrombin of the bubaline fibrin glue could decrease the release of pro-inflammatory cytokines when a mechanical trauma was applied (Jennewein et al., 2011; Stiller-Timor et al., 2012). Bubaline fibrin glue's components is based on the concentrated of bubaline fibrinogen and a high effective thrombin. The combination of these two components formed a cross-linked fibrin clot and so it has effective wound healing properties. Michel and Harmand (1990) suggested that the fibrin glue containing thrombin and calcium could stimulate fibroblast proliferation and collagen synthesis in the wound healing process in healthy humans. Some literatures also used the commercial fibrin glue as an adhesive agent to promote wound healing after the extraction of the first molar in rats (Yücel et al., 2003) and as a hemostatic agent during a dental surgery in the patient with a bleeding disorder (Suwannuraks et al., 1999).

Our study revealed high levels of up-regulation of PDGF-A and TGF-β_1 at 7 and 14 days after the ligatures removal (14 and 21 DPL) in the treated group compared with the control group. It has been suggested that the wound healing and the anti-inflammatory properties of fibrin glue accelerate the expression of PDGF- A and TGF-

β. This is related to the collagen synthesis in the remodeling phase of the inflammatory response (Sporn and Roberts, 1990). Moreover, we detected the high level of up-regulation of IL-10 at days 1 to 7, while the highest down-regulation was detected at 14 days after removing the ligatures (21 DPL). It could be explained by the protective role of IL-10 on limiting the disease from turning gingivitis to periodontitis. IL-10 might be responsible for the acute phase of the inflammatory process. The expression of IL-10 in periodontitis was lower than in healthy gingiva (Goutoudi et al., 2004). IL-10 is an inflammatory inhibitor known as immunosuppressive cytokines, which is produced from macrophages, T-helper cells and B-cells. It was associated with inhibition of the synthesis of IL-1, TNF-α and the reduction of bone resorption. Moreover, due to a protective function it can limit the progressive of disease from gingivitis to chronic periodontitis, which implies the connective tissue destruction.

TGF-β and PDGF-A act as anti-inflammatory cytokines by stimulating bone proliferation and inhibiting bone resorption (Antoszewska et al., 2010). Indeed, the presence of TGF-β is associated with the progression of periodontal diseases. In relation to this, El-Sharkawy et al. (2007) showed that platelet rich plasma (PRP) had a significantly higher level of TGF-β and PDGF-A expressions compared to whole blood.

Our bubaline fibrin glue preparation protocol was modified from Thorn et al. (2004) who discovered that the autologous fibrin had a concentration of fibrinogen and of growth factor (PDGF) 12 and 8 times, respectively, higher than Platelet Rich Plasma (PRP). From the number of studies on its use, PRP is enriched with many growth factors and anti-inflammatory cytokines such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), fibroblast growth factor-basic (FGF-b), and epidermal growth factor (EGF) (Anitua et al., 2007; El-Sharkawy et al., 2007). These growth factors do not only stimulate fibroblasts and collagen fibers but also promote the proliferation of the periodontal ligament. PRP is also associated with the promotion of bone healing (Fennis et al., 2002) and their combination with thrombin in calcium chloride (also called platelet-rich gel) has antimicrobial activity in an in vitro study (Bielecki et al., 2007). This indicates that platelet play an important role in the antimicrobial defense response of the host.

In conclusion, this study demonstrates that fibrin glue from buffalo blood has the potential on prevention and treatment of periodontitis, by promoting the periodontal attachment and the osteogenesis activity, and reducing the inflammatory response by decreasing TNF-α and IL-1β, which play a crucial role in periodontal healing in rats with induced periodontitis. The novel fibrin glue can enhance wound healing by increasing level of PDGF-A, TGF-β_1 and IL-10. Moreover, it has anti-microbial activity *in vitro*. This might explain some of the most relevant effects attributed to the bubaline fibrin glue in experimental rats. The bubaline fibrin glue might be an alternative material to be applied in order to prevent the progress of the periodontal diseases. Clinical studies are needed to elucidate further the mechanisms of periodontal healing by the novel bubaline fibrin glue.

REFERENCES

Abe T and Hajishengallis G 2013. Optimization of the ligature-induced periodontitis model in mice. J Immunol Methods. 394(1): 49-54.

Abiraman S, Varma H, Umashankar P and John A 2002. Fibrin glue as an osteoinductive protein in a mouse model. Biomaterials. 23(14): 3023-3031.

Akıncıbay H, Şenel S and Yetkin Ay Z 2007. Application of chitosan gel in the treatment of chronic periodontitis. J Biomed Mater Res B Appl Biomater. 80(2): 290-296.

Aksoy H, Apikoğlu-Rabus Ş, Uras F, Savcı N, Alatlı FC, Erseven G and Emekli N 2009. Evaluation of the effect of fibrin glue prepared from single-donor plasma on wound healing in rats. Hacettepe University J Fac Pharm. 29(2): 83-93.

Albuquerque C, Morinha F, Requicha J, Martins T, Dias I, Guedes-Pinto H, Bastos E and Viegas C 2012. Canine periodontitis: the dog as an important model for periodontal studies. Vet J. 191(3): 299-305.

AlJehani YA 2014. Risk factors of periodontal disease: Review of the literature. Int J Dent. 2014.

Anitua E, Sánchez M, Orive G and Andía I 2007. The potential impact of the preparation rich in growth factors (PRGF) in different medical fields. Biomaterials. 28(31): 4551-4560.

Antoszewska J, Raftowicz-Wójcik K, Kawala B and Matthews-Brzozowska T 2010. Biological factors involved in implant-anchored orthodontics and in prosthetic-implant therapy: a literature review. Arch Immunol Ther Exp. 58(5): 379-383.

Bezerra M, Brito G, Ribeiro R and Rocha F 2002. Low-dose doxycycline prevents inflammatory bone resorption in rats. Braz J Med Biol Res. 35(5): 613-616.

Bielecki T, Gazdzik T, Arendt J, Szczepanski T, Krol W and Wielkoszynski T 2007. Antibacterial effect of autologous platelet gel enriched with growth factors and other active substances An in vitro study. J Bone Joint Surg Br. 89(3): 417-420.

Bösch P, Lintner F, Arbes H and Brand G 1980. Experimental investigations of the effect of the fibrin adhesive on the Kiel heterologous bone graft. Arch Orthop Trauma Surg. 96(3): 177-185.

Caffesse R, Smith B, Castelli W and Nasjleti C 1988. New attachment achieved by guided tissue regeneration in beagle dogs. J Periodontol. 59(9): 589-594.

Cheng WC, Huang RY, Chiang CY, Chen JK, Liu CH, Chu CL and Fu E 2010. Ameliorative effect of quercetin on the destruction caused by experimental periodontitis in rats. J Periodontal Res. 45(6): 788-795.

Dantas AM, Mohn CE, Burdet B, Zubilete MZ, Mandalunis PM, Elverdin JC and Fernández-Solari J 2012. Ethanol consumption enhances periodontal inflammatory markers in rats. Arch Oral Biol. 57(9): 1211-1217.

De Lima V, Bezerra MM, De Menezes Alencar VB, Daniel Portela Vidal F, Da Rocha FAC, De Castro Brito GA and De Albuquerque Ribeiro R 2000. Effects of chlorpromazine on alveolar bone loss in experimental periodontal disease in rats. Eur J Oral Sci. 108(2): 123-129.

de Pablo P, Chapple IL, Buckley CD and Dietrich T 2009. Periodontitis in systemic rheumatic diseases. Nat Rev Rheumatol. 5(4): 218-224.

Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR, Sacco RL and Papapanou PN 2005. Periodontal microbiota and carotid intima-media thickness the oral infections and vascular disease epidemiology study (INVEST). Circulation. 111(5): 576-582.

Dogan A, Taner L, Oygur T and Balos K 1992. Effects of fibrin adhesive material (Tissucol) application on furcation defects in dogs. J Nihon Univ Sch Dent. 34(1): 34-41.

El-Sharkawy H, Kantarci A, Deady J, Hasturk H, Liu H, Alshahat M and Van Dyke TE 2007. Platelet-rich plasma: growth factors and pro-and anti-inflammatory properties. J Periodontol. 78(4): 661-669.

Endo Y, Tomofuji T, Ekuni D, Azuma T, Irie K, Kasuyama K and Morita M 2013. Preventive effects of trehalose on osteoclast differentiation in rat periodontitis model. J Clin Periodontol. 40(1): 33-40.

Evans LA and Morey AF 2006. Current applications of fibrin sealant in urologic surgery. International braz j urol. 32(2): 131-141.

Everts PA, Devilee RJ, Oosterbos CJ, Mahoney CB, Schattenkerk ME, Knape JT and van Zundert A 2007. Autologous platelet gel and fibrin sealant enhance the efficacy of total knee arthroplasty: improved range of motion, decreased length of stay

and a reduced incidence of arthrofibrosis. Knee Surg Sports Traumatol Arthrosc. 15(7): 888-894.

Fennis J, Stoelinga P and Jansen J 2002. Mandibular reconstruction: a clinical and radiographic animal study on the use of autogenous scaffolds and platelet-rich plasma. Int J Oral Maxillofac Surg. 31(3): 281-286.

Gammon RR, Avery N and Mintz PD 1997. Fibrin sealant: an evaluation of methods of production and the role of the blood bank. J Long Term Eff Med Implants. 8(2): 103-116.

Glickman LT, Glickman NW, Moore GE, Lund EM, Lantz GC and Pressler BM 2011. Association between chronic azotemic kidney disease and the severity of periodontal disease in dogs. Prev Vet Med. 99(2): 193-200.

Gorrel C, Andersson S and Verhaert L 2013. Veterinary Dentistry for the General Practitioner2: Veterinary Dentistry for the General Practitioner. In: Elsevier Health Sciences.

Goutoudi P, Diza E and Arvanitidou M 2004. Effect of periodontal therapy on crevicular fluid interleukin-1β and interleukin-10 levels in chronic periodontitis. J Dent. 32(7): 511-520.

Graves D and Cochran D 2003. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol. 74(3): 391-401.

Gross CW, Gallagher R, Schlosser RJ, Burks SG, Flanagan HL, Mintz PD, Avery NL, Mayers SL and Spotnitz WD 2001. Autologous fibrin sealant reduces pain after tonsillectomy. Laryngoscope. 111(2): 259-263.

Häkkinen L, Uitto V-J and Larjava H 2000. Cell biology of gingival wound healing. Periodontol 2000. 24(1): 127-152.

Hamp SE, Olsson SE, Farsø-Madsen K, Viklands P and Fornell J 1984. A macroscopic and radiologic investigation of dental diseases of the dog. Vet Radiol Ultrasound. 25(2): 86-92.

Hart G, Shaffer D, Akilesh S, Brown A, Moran L, Roopenian D and Baker P 2004. Quantitative gene expression profiling implicates genes for susceptibility and resistance to alveolar bone loss. Infect Immun. 72(8): 4471-4479.

Holmstrom SE, Fitch PF and Eisner ER 2004. Veterinary dental techniques for the small animal practitioner. In: Elsevier Health Sciences.

Ionel A, Lucaciu O, Moga M, Buhatel D, Ilea A, Tabaran F, Catoi C, Berce C, Toader S and Campian R 2015. Periodontal disease induced in Wistar rats-experimental study. HVM Bioflux. 7(2): 90-95.

Isogai E, Isogai H, Sawada H, Kaneko H and Ito N 1985. Microbial ecology of plaque in rats with naturally occurring gingivitis. Infect Immun. 48(2): 520-527.

Jacob SP and Nath S 2013. Rat gingival model for testing drugs influencing inflammation. IeJSME. 7(2): 8-16.

Jathal B, Trivedi A and Bhavsar N 2008. Use of fibrin glue in periodontal flap surgery. J Indian Soc Periodontol. 12(1): 21.

Jennewein C, Tran N, Paulus P, Ellinghaus P, Eble JA, Zacharowski K and Zacharowski K 2011. Novel aspects of fibrin (ogen) fragments in the course of inflammation. Mol Med. 17: 568-573.

Johnston TP, Mondal P, Pal D, MacGee S, Stromberg AJ and Alur H 2011. Canine periodontal disease control using a clindamycin hydrochloride gel. J Vet Dent. 28(4).

Jones JR 2013. Review of bioactive glass: from Hench to hybrids. Acta Biomater. 9(1): 4457-4486.

Kerry G, Morrison E, Ramfjord S, Hill R, Caffesse R, Nissle R and Appleberry E 1982. Effect of Periodontal Treatment on Tooth Mobility. J Periodontol. 53(10): 635-638.

Kuhr A, Popa-Wagner A, Schmoll H, Schwahn C and Kocher T 2004. Observations on experimental marginal periodontitis in rats. J Periodontal Res. 39(2): 101-106.

Lahoda L, Wang S and Vogt P 2006. A mixture of antimicrobial peptides and fibrin glue in treatment of partial-thickness burn wounds. Chirurg. 77(3): 251-256.

Liao C-H, Fei W, Shen Z-H, Yin M-P and Lu C 2014. Expression and distribution of TNF-α and PGE 2 of periodontal tissues in rat periodontitis model. Asian Pac J Trop Med. 7(5): 412-416.

Lu S-H, Huang R-Y and Chou T-C 2013. Magnolol ameliorates ligature-induced periodontitis in rats and osteoclastogenesis: in vivo and in vitro study. Evid. Based Complement. Alternat. Med. 2013.

Marcelli C, Yates AJ and Mundy GR 1990. In vivo effects of human recombinant transforming growth factor β on bone turnover in normal mice. J Bone Miner Res. 5(10): 1087-1096.

Marini E, Valdinucci F, Silvestrini G, Moretti S, Carlesimo M, Poggio C and Bonucci E 1994. Morphological investigations on bone formation in hydroxyapatite-fibrin implants in human maxillary and mandibular bone. Cells and Materials. 4(3): 231-246.

Michel D and Harmand M-F 1990. Fibrin seal in wound healing: effect of thrombin and [Ca 2+] on human skin fibroblast growth and collagen production. J Dermatol Sci. 1(5): 325-333.

Milasin J, Pucar A, Kenney BE, Stefanovic D, Sopta J, Jakoba NN and Lekovic V 2011. Periodontal Inflammation as Risk Factor for Pancreatic Diseases. In: INTECH Open Access Publisher.

Mintz PD, Mayers L, Avery N, Flanagan HL, Burks SG and Spotnitz WD 2001. Fibrin sealant: clinical use and the development of the University of Virginia Tissue Adhesive Center. Ann Clin Lab Sci. 31(1): 108-118.

Mutignani M, Iacopini F, Dokas S, Larghi A, Familiari P, Tringali A and Costamagna G 2006. Successful endoscopic closure of a lateral duodenal perforation at ERCP with fibrin glue. Gastrointest Endosc. 63(4): 725-727.

Narakas A 1988. The use of fibrin glue in repair of peripheral nerves. Orthop Clin North Am. 19(1): 187-199.

Nemec A, Petelin M and Pavlica Z 2013. Systemic inflammatory host response to periodontopathogenic bacteria in the oral cavity: from experimental to clinical studies.

Nieto JO, MSCED M, MFELAC M, Suárez JV, MSCG M and González ÁPR 2009. Utility of fibrin glue in therapeutic endoscopy. Rev Col Gastroenterol. vol.24 no.3 Bogotá July/Sept. 2009.

Nishiyama SAB, Senhorinho GNA, Gioso MA and Avila-Campos MJ 2007. Detection of putative periodontal pathogens in subgingival specimens of dogs. Braz. J. Microbiol. 38(1): 23-28.

Nociti FH, Cesco de Toledo R, Machado MA, Stefani CM, Line SR and Gonçalves RB 2001. Clinical and microbiological evaluation of ligature-induced peri-implantitis and periodontitis in dogs. Clin Oral Implants Res. 12(4): 295-300.

Oz HS and Puleo DA 2011. Animal models for periodontal disease. BioMed Research International. 2011.

Ozdemir H, Kara M, Erciyas K, Ozer H and Ay S 2012. Preventive effects of thymoquinone in a rat periodontitis model: a morphometric and histopathological study. J Periodontal Res. 47(1): 74-80.

Påhlman LI, Mörgelin M, Kasetty G, Olin AI, Schmidtchen A and Herwald H 2013. Antimicrobial activity of fibrinogen and fibrinogen-derived peptides–a novel link between coagulation and innate immunity. Thromb Haemost. 109(5): 930-939.

Panda A, Kumar S, Kumar A, Bansal R and Bhartiya S 2009. Fibrin glue in ophthalmology. Indian J Ophthalmol. . 57(5): 371.

Pandit N, Malik R and Philips D 2011. Tissue engineering: a new vista in periodontal regeneration. J Indian Soc Periodontol. 15(4): 328.

Peddle GD, Drobatz KJ, Harvey CE, Adams A and Sleeper MM 2009. Association of periodontal disease, oral procedures, and other clinical findings with bacterial endocarditis in dogs. J Am Vet Med Assoc. 234(1): 100-107.

Popova C and Mlachkova A 2010. Gingival tissue IL-1β and PGE2 levels in patients with chronic periodontitis after additional therapy with non-steroidal anti-inflammatory drugs. Journal of IMAB. Annual Proceeding (Scientific Papers) (vol. 16, book 4): 27-30.

Rao X, Huang X, Zhou Z and Lin X 2013. An improvement of the 2ˆ (–delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostat Bioinforma Biomath. 3(3): 71.

Reynolds MA, Aichelmann-Reidy ME, Branch-Mays GL and Gunsolley JC 2003. The efficacy of bone replacement grafts in the treatment of periodontal osseous defects. A systematic review. Ann Periodontol. 8(1): 227-265.

Riggio MP, Lennon A, Taylor DJ and Bennett D 2011. Molecular identification of bacteria associated with canine periodontal disease. Vet Microbiol. 150(3): 394-400.

Saini R, Saini S and Saini SR 2010. Periodontitis: A risk for delivery of premature labor and low-birth-weight infants. J Nat Sci Biol Med. 1(1): 40.

Sato S, Fonseca MJV, Ciampo JOD, Jabor JR and Pedrazzi V 2008. Metronidazole-containing gel for the treatment of periodontitis: an in vivo evaluation. Braz Oral Res. 22(2): 145-150.

Schwach-Abdellaoui K, Vivien-Castioni N and Gurny R 2000. Local delivery of antimicrobial agents for the treatment of periodontal diseases. Eur J Pharm Biopharm. 50(1): 83-99.

Semenoff TADV, Semenoff-Segundo A, Bosco ÁF, Nagata MJH, Garcia VG and Biasoli ER 2008. Histometric analysis of ligature-induced periodontitis in rats: a comparison of histological section planes. J Appl Oral Sci. 16(4): 251-256.

Sigusch BW, Pfitzner A, Albrecht V and Glockmann E 2005. Efficacy of photodynamic therapy on inflammatory signs and two selected periodontopathogenic species in a beagle dog model. J Periodontol. 76(7): 1100-1105.

Silver FH, Wang M-C and Pins GD 1995. Preparation and use of fibrin glue in surgery. Biomaterials. 16(12): 891-903.

Skalerič U, Kramar B, Petelin M, Pavllia Z and Wahl SM 1997. Changes in TGF-β1 levels in gingiva, crevicular fluid and serum associated with periodontal inflammation in humans and dogs. Eur J Oral Sci. 105(2): 136-142.

Soffer E, Ouhayoun JP and Anagnostou F 2003. Fibrin sealants and platelet preparations in bone and periodontal healing. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 95(5): 521-528.

Sporn MB and Roberts AB 1990. The Transforming Growth Factor-Betas: Past, Present, and Future. Ann N Y Acad Sci. 593(1): 1-6.

Stiller-Timor L, Goldbart A, Segal N, Amash A, Huleihel M, Leiberman A, Tal A, Holcberg G and Puterman M 2012. Circulating cytokines in patients undergoing tonsillectomy with fibrin glue. Int J Pediatr Otorhinolaryngol. 76(3): 419-422.

Suwannuraks M, Chuansumrit A and Sriudomporn N 1999. The use of fibrin glue as an operative sealant in dental extraction in bleeding disorder patients. Haemophilia. 5(2): 106-108.

Takahashi K, Takashiba S, Nagai A, Takigawa M, Myoukai F, Kurihara H and Murayama Y 1994. Assessment of Interleukin-6 in the Pathogenesis of Periodontal Disease*. J Periodontol. 65(2): 147-153.

Tasdemir C, Samdanci ET, Turtay MG, Firat C, Oguzturk H and Ozdemir H 2011. Histopathological effects of fibrin glue on penile fracture in a rat model. Clin Invest Med. 34(6): 336-340.

Taylor GW, Burt BA, Becker MP, Genco RJ, Shlossman M, Knowler WC and Pettitt DJ 1996. Severe periodontitis and risk for poor glycemic control in patients with non-insulin-dependent diabetes mellitus. J Periodontol. 67(10s): 1085-1093.

Thomazini-Santos I, Giannini M, Toscano E, Machado P, Lima C and Barraviera B 1998. The evaluation of clotting time in bovine thrombin, Reptilase®, and thrombin-like fraction of Crotalus durissus terrificus venom using bovine, equine, ovine, bubaline and human cryoprecipitates. J Venom Anim Toxins. 4(2): 120-136.

Thorn J, Sørensen H, Weis-Fogh U and Andersen M 2004. Autologous fibrin glue with growth factors in reconstructive maxillofacial surgery. Int J Oral Maxillofac Surg. 33(1): 95-100.

Toker H, Ozdemir H, Eren K, Ozer H and Sahin G 2009. N-acetylcysteine, a thiol antioxidant, decreases alveolar bone loss in experimental periodontitis in rats. J Periodontal Res. 80(4): 672-678.

Toriumi D and O'Grady K 1994. Surgical tissue adhesives in otolaryngology-head and neck surgery. Otolaryngol Clin North Am. 27(1): 203-209.

Van der Ham A, Kort W, Weijma I, Ingh V, Den H and Jeekel J 1991. Effect of fibrin sealant on the healing colonic anastomosis in the rat. Br J Surg. 78(1): 49-53.

Wohlsen T, Bates J, Vesey G, Robinson W and Katouli M 2006. Evaluation of the methods for enumerating coliform bacteria from water samples using precise reference standards. Lett Appl Microbiol. 42(4): 350-356.

Xiong X, Buekens P, Fraser W, Beck J and Offenbacher S 2006. Periodontal disease and adverse pregnancy outcomes: a systematic review. BJOG. 113(2): 135-143.

Xu Y and Wei W 2006. A comparative study of systemic subantimicrobial and topical treatment of minocycline in experimental periodontitis of rats. Arch Oral Biol. 51(9): 794-803.

Yücel EA, Oral O, Olgaç V and Oral CK 2003. Effects of fibrin glue on wound healing in oral cavity. J Dent. 31(8): 569-575.

Yuksel E, Akata F, Yuksel N, Tufan HA and Mert BD 2014. Human-derived fibrin glue: in vitro antibacterial effects and antibiotic permeation. Turk J Ophthalmol 44(5): 347-351.

Zhang G, Kong Y, Gao Y-Q, Lv J, Wu X and Tan Y-H 2013. Periodontitis in a High-altitude Hypoxic Environment Through Rat Model Simulation. Asian J Anim Vet Adv. 8(4).

APPENDIX

LIST OF ABBREVIATIONS

°C = degree Celcius

x *g* = times-gravity

β-actin gene = Beta-actin gene

µg = microgram

µl = microliter

cDNA = Complementary Deoxyribonucleic Acid

CFU = Colony-forming unit

CUVDCR = Chulalongkorn University Veterinary Dental Clinic Rats

DPL = days post ligation

g = grams

h = hour

IL-1β = Interleukin-1β

l = lesion

min = minute

ml = milliliter

mm = millimeter

mM = millimoles

n = number

ng = nanogram

nM = nanomoles

PCR = Polymerase Chain Reaction

PDGF = Platelet-Derived Growth Factor

PRP = Platelet Rich Plasma

qPCR = Quantitative (Real-time) Polymerase Chain Reaction

qRT-PCR = Quantitative Reverse Transcription Polymerase Chain Reaction

RNA = Ribonucleic Acid

rpm = revolutions per minute

RT-PCR = Reverse Transcriptase Polymerase Chain Reaction

sec = second

TGF-β = Transforming Growth Factor-β

TNF-α = Tumor Necrosis Factor-α

VEGF = Vascular Endothelial Growth Factor

VITA

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