บทวิทยาการ
Original Article

A Histomorphometrical Comparative Study between the Use of Platelet Rich Plasma and e-PTFE Membrane in Bone Regeneration of Canine Mandible : A Pilot Study

การศึกษาทางฮิสโตมอร์โฟเมตริกเปรียบเทียบระหว่างการใช้เพลทเลทริชพลาสมาและแผ่นอีพีทีเอฟอีในกระบวนการสร้างกระดูกในขากรรไกรสุนัข : การศึกษานำร่อง

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Abstract

Platelet rich plasma (PRP) has been used as an accelerator in bone healing in implant dentistry with conflicting results. Aim of this study was to compare bone regeneration rate histomorphometrically, between the use of PRP and e- PTFE membrane in artificial defects of the canine mandible at different periods of healing. Four standardized artificial defects were prepared at the lower border of mandibles of ten dogs. One defect was filled with PRP, one was covered with e-PTFE membrane (Goretex®), one was filled with PRP and covered with e- PTFE membrane, and one defect served as control. Collagen (Tissue vlies®) was added to each defect.

บทคัดย่อ

มีการนำเพลทเลทริชพลาสมามาใช้เป็นตัวเร่งกระบวนการหายของกระดูกในทางทันตกรรมรากเทียม แต่ยังมีรายงานผลการศึกษาที่ขัดแย้งกัน การศึกษานี้มีวัตถุประสงค์ในการเปรียบเทียบอัตราของกระบวนการซ่อมสร้างกระดูกด้วยวิธีฮิสโตมอร์โฟเมตริก ระหว่างการใช้เพลทเลทริชพลาสมาและแผ่นอีพีทีเอฟอี ในรอยโรคที่สร้างขึ้นในขากรรไกรล่างของสุนัข ณ เวลาของการหายของแผลระยะต่างๆ โดยเตรียมรอยโรคขนาดเท่ากันสี่บริเวณที่ขอบล่างของขากรรไกรสุนัขจำนวนสิบตัว โดยที่รอยโรคที่ 1 ใส่เพลทเลทริชพลาสมา รอยโรคที่ 2 ปิดด้วยแผ่นอีพีทีเอฟอี (Goretex®) รอยโรคที่ 3 ใส่เพลทเลทริชพลาสมาและปิดทับด้วยแผ่นอีพีทีเอฟอี ส่วนรอยโรคที่ 4 เป็นตัวควบคุม โดยในทุกรอยโรคใส่คอลลาเจน (Tissue vlies®) ไว้ นำตัวอย่าง

Samples were taken from two animals at 2, 4, 6, 8 and 12 weeks. Representative bone specimens were fixed in 10 % buffered formalin prior to histological preparation of ground sections. Digital images of sections were analyzed histomorphometrically for newly formed bone. Results were analysed statistically. Results at different healing periods showed that addition of PRP did not improve healing of bone significantly (p=0.53). The use of e-PTFE membrane improved bone healing after two weeks significantly (p<0.01) relative to control, PRP alone and e-PTFE membrane and PRP.

Keywords: platelet rich plasma, e-PTFE membrane, experimental study, bone regeneration

จากสุนัขมาศึกษาที่เวลา 2,4,6,8 และ 12 สัปดาห์ สัปดาห์ละสองตัว แซ่ตัวอย่างกระดูกไว้ในบัฟเฟอร์ฟอร์มาลินความเข้มข้นร้อยละ 10 ก่อนการเตรียมทำกราวน์เซคชัน วิเคราะห์ภาพทางดิจิทัลของเนื้อเยื่อด้วยวิธีฮิสโตมอร์โฟเมตริกเพื่อให้เห็นกระดูกที่สร้างใหม่ นำผลที่ได้มาวิเคราะห์ทางสถิติ พบว่า ณ เวลาของการหายของแผลระยะต่างๆ นั้น เพลทเลทริชพลาสมาไม่ได้ช่วยในการหายของกระดูกอย่างมีนัยสำคัญทางสถิติที่ p=0.53 ส่วนการใช้แผ่นอีพีทีเอฟอีช่วยในการหายของกระดูกที่ 2 สัปดาห์อย่างมีนัยสำคัญทางสถิติที่ p<0.01 เมื่อเปรียบเทียบกับกลุ่มควบคุม กลุ่มที่ใส่แต่เพลทเลทริชพลาสมา และกลุ่มที่ใส่ทั้งแผ่นอีพีทีเอฟอีและเพลทเลทริชพลาสมา

คำไขรหัส: เพลทเลทริชพลาสมา แผ่นอีพีทีเอฟอี การศึกษาเชิงทดลอง กระบวนการสร้างกระดูก

Introduction

Platelet rich plasma (PRP) is composed of three main important growth factors: Platelet derived growth factor (PDGF), transforming growth factor ß1 (TGF-ß1), and transforming growth factors ß2 (TGF-ß2), which play an important role in the stimulation and regulation of wound healing.[1] PDGF was first discovered in alpha granules of platelets.[2] It can also be found in endothelial cells[3], monocytes and fibroblasts[4], macrophages[5], as well as in bone matrix.[6] There are approximately 0.06 ng of PDGF per one million platelets.[7] PDGF enhances osteogenic differentiation and it has been shown to play a role in bone repair in the fracture model[8] and results showed in a significant promotion of bone regeneration.[9]

TGF-ß has 3 different structures; TGF-ß1, TGF-ß2, and TGF-ß3. TGF-ß1 is found abundantly in platelets, lymphocytes, and neutrophils.[10] TGF-ß2 is found mainly in bone extract but it is also found in platelets, lymphocytes, and neutrophils. TGF- ß1 and TGF- ß2 are similar in up to 72%. Both factors promote bone formation by increasing the rate of stem cell proliferation and inhibiting osteoclast function.[11] They seem to work through chemotaxis and mitogenesis of osteoblast precursors, and have the ability to stimulate osteoblast deposition of collagen matrix of soft tissue and bone healing.[12] With autologous blood, PRP is obtained by sequestering and concentrating platelets by gradient density centrifugation. This technique produced a concentration of human platelets of 338% and identified PDGF and TGF-ß within them.[13]

Clinically, PRP was used in many fields of medicine including orthopedics, oral and maxillofacial surgery, and implant dentistry. In one of the early studies, addition of PRP to bone grafts evidenced a radiographic maturation rate of 1.62 times that of grafts without PRP.[13] A number of further studies showed improved bone regeneration

using PRP for alveolar ridge and sinus augmentation and grafting[14-15], distraction osteogenesis[16] and in combination with dental implants.[17] Similarly, animal studies supported these findings.[18] In contrast, other studies showed that the addition of PRP did not enhance quality or quantity of new bone formation compared to that in guided bone regeneration (GBR) studies without PRP.[19]

The use of e- PTFE membranes for GBR is well established, particularly in association with dental implants.[20] Also, animal studies showed that implants placed into fresh extraction sockets and covered with e-PTFE membranes show significant amounts of newly formed bone when compared with those that have not been covered with e-PTFE membranes.[21]

The purpose of this study was to compare histomorphometrically bone regeneration in artificial defects between the use of PRP and e-PTFE membranes.

Materials and Methods

Ten systemically healthy dogs (5 males and 5 females, average age 1.70±0.71 years) were recruited for the study. The experimental design and the use of laboratory animals were approved by the ethical committee of the Faculty of Veterinary Medicine, Chiang Mai University. The permission criteria were based on regulations of the National Research Council, Thailand.

Pyrantel pamoates (Antiminth®, Pfizer) at a dose of 5 mg/kg were administered orally as antihelminthic drug. In addition, rabies vaccine (Rabigen Mono®, Virbac Laboratories, France) and multivalent vaccine (Vanguard 5/L®, Norden Laboratories, Nebraska, USA.) were subcutaneously administered.

Ten ml of whole fresh blood from each dog was obtained at the time of surgical intervention. The blood was transferred in a tube containing 1.4 ml of citrate phosphate dextrose (CPD) as an anticoagulant. The rest of venous blood was used to determine the animalís basic platelet count. To produce PRP extracts, 8.5 ml of citrated blood was centrifuged in a standard laboratory centrifuge for 10 minutes at 2,400 rpm. Subsequently, plasma (containing the platelets) was taken up in a neutral tube. To combine the platelets into a single pellet, a second centrifugation step was performed with a second tube for 15 minutes at 3,600 rpm. The plasma supernatant (containing relatively few cells) was then reduced to approximately 0.4 ml. The pellet of platelets was resuspended in the residual 0.4 ml of plasma using a conventional shaker and was transferred to an Eppendorf tube for later analysis of platelet count.[22]

At the lower borders of mandibles skin incisions were made extraorally and periosteal flaps were elevated. Four standard defects with a diameter of 5 mm width and 8 mm depth were prepared using a trephine drill. One defect served as control, one defect was filled with 100 µl of PRP, one defect was covered with e-PTFE membrane (Goretex®, Gore), and the last defect was filled with 100 µl of PRP and covered with an e-PTFE membrane. All four defects were stabilized with collagen (Tissue Vlies, Resorba Wund-versorgung GmbH & Co. KG, Nürnberg, Germany) to hold blood clots and PRP in place. For sutures an absorbable suture material was used (Dexon 3-0®). Ibuprofen and doxycycline (Vibramycin®, Pfizer) were administered orally as analgesic and antibiotic for 3 days postoperatively. Soft canned food was fed for 7 days post-operatively. Two dogs each were sacrificed at 2, 4, 6, 8 and 12 weeks.

To euthanize the animals, pentobarbital sodium (Nembutal®, Sanofi, Animal Health, Thailand) at a dose of 25 mg/kg was injected intravenously until the animals were anaesthesized without pain or distress. Skin incision at ventral

aspect of the neck was made. The common carotid artery was identified and then ligated at the anterior part. The common carotid artery was cut incompletely caudally to the ligation point. The blood was then released from the whole body. A fixative of 10% formalin was injected into the body through the common carotid artery until excessive formalin drained from nostrils.

Left quadrants of mandibles with the experimental and control sites were collected. Radiographs of bone specimens were taken to investigate the regeneration of new bone. Specimens were fixed in buffered (neutral) 10 % formalin. Small blocks containing one experimental site each were prepared. Specimens were dehydrated in ascending grades of alcohol and embedded in light curing resin (Technovit 7200 VLC+BPO, Kulzer & Co., Germany). Further processing was done using the Exact Cutting and Grinding Equipment (Exact Apparatebau, Norderstedt, Germany). The blocks were cut along the vertical axis of the mandibles and reduced to a thickness of 30 µm. Subsequently, the undecalcified specimens were stained with Giemsa stain.[23]

For histomorphometric analysis digital photographs of each slide were taken. A public domain Java image processing program (National Institute of Health (NIH), version 1.24) was used to perform a quantitative analysis of the newly formed bone. Measurements were in units square pixels, which can be calibrated into the appropriate metric units using a stage micrometer or a standard metric scale. The square pixel units were changed to percent of new bone formation and the mean values for new bone regeneration were calculated. The amount of newly formed bone at 2, 4, 6, 8 and 12 weeks of experimental and control sites was then analyzed using SPSS (version 10) computer software. The Student independent T-test was used for significant differences ($p < 0.05$).

Results

Radiographic images of mandibular specimens of different healing periods are shown in Figs. 1-3. Figure 1 shows the surgical defects at the lower border of the mandible at 2 weeks postoperatively. The first and third defects represent the site of PRP application and control, respectively. The second and fourth defects represent sites of ePTFE membrane application and PRP-ePTFE application. The latter two defects show the radiopaque screws used to fix the membranes. No signs of bone healing are observed (Fig.1).

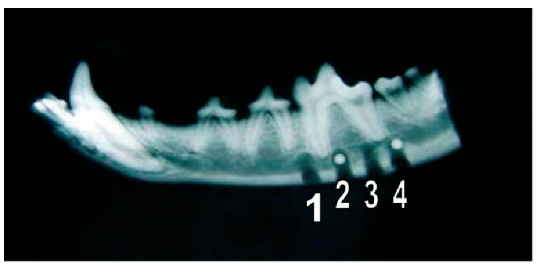

Figure 1 *Radiograph showing mandibular specimen after 2 weeks. The four defects are characterized by sharp outines. (1=PRP, 2=ePTFE membrane, 3=control, 4=ePTFE membrane+PRP).*

Figure 2 shows a specimen at 6 weeks period. The sharp outlines of all defects seen after 2 weeks have disappeared. The entrances of the defects appear rounded. Radiographically, no difference in radiopacity is seen when comparing the four defects (Fig. 2). Figure 3 shows the defects in a mandible after 12 weeks. The lower border of the mandible still shows some ‘cupping’ of the areas of the former defects. Radiographically, no differences in bone density of the four defects are seen.

Baseline platelet counts performed for each dog yielded a range of 102,000 to 195,750 while PRP platelet count ranged from 201,000 to 711,100. These values confirmed the platelet

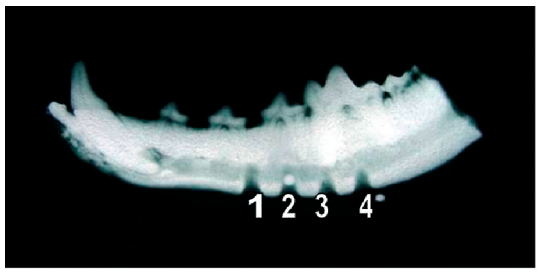

Figure 2 *Radiograph showing mandibular specimen after 6 weeks. The surgical defects appear rounded at the entrances at the lower border of the mandible. Initial radiopacity is seen in the deeper parts of the defects.*

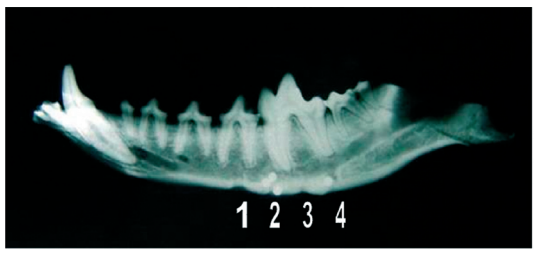

Figure 3 *Radiograph showing mandibular specimen after 12 weeks. The defects show an identical radiodensity. The lower border of the mandible shows some 'cupping' in the areas of the former surgical defects. Several screws to fix the membranes are seen in the areas of defect 2 and 4.*

sequestration ability of the process and quantified the average concentration as 356.16 % of baseline platelet counts (Table 1).

Histologic findings after two weeks showed the sharp outlines of the surgical defects in the compact bone of the lower border of the mandible. The defects traversed the entire compact bone and ended in spongious bone shortly before coming into contact with the mandibular nerve, artery and vein. The cavities contained the loose structures of the collagen sponge with some bony detritus and erythrocytes. Inflammatory infiltrates were not seen in any of the defects (Fig. 4).

Table 1 *The platelet count of fresh blood and platelet rich plasma*

Experimental dogs	Baseling platelet count	PRP platelet count	Percent of increase (%)
1	102,000	201,000	197.05
2	183,000	598,000	326.77
3	159,900	630,500	394.03
4	153,400	520,000	338.98
5	195,750	598,000	305.49
6	153,400	545,400	355.54
7	139,100	520,000	373.83
8	114,400	711,100	621.59
9	140,000	513,000	366.42
10	125,000	352,000	281.60
$\bar{x}$ ± S.D.			356.16±108.99

PRP=platelet rich plasma, $\bar{x}$±SD=mean % of increase ± standard deviation

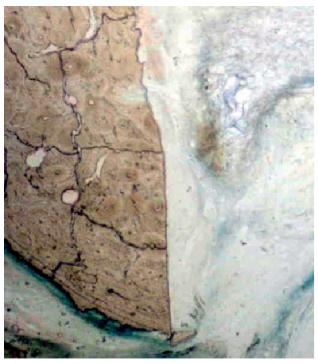

Figure 4 *Histological ground section at weeks 2 (PRP, defect 1). The shap outline of the defect is clearly seen. At the entrance of defect bone particles stemming from the surgical preparation are seen. The collagen network adjacent to the wall of the defect (Giemsa stain, x 40)*

Histologic findings after 4 weeks showed neo-angiogenesis in a delicate connective tissue matrix, particularly adjacent to spongious bone but also along the walls of the defects. Remnants of collagen sponges were still to be observed. At the entrances of the defects adjacent to periosteum islands of osteoid with respective osteoblasts could be observed.

Histologic findings after 6 and 8 weeks did not differ to a large extent in any of the 4 defects.

Woven bone appeared at the lateral walls of the defects, particularly in the areas of the entrances to these. There was an obvious tendency to close the defects by bone formation originating from the walls of the defects and their respective entrances. Histologically, no marked differences between defects with PRP or membranes could be seen. Remnants of the collagen sponges were still found, mainly adjacent to the mandibular nerve and vessels. This area appeared to be particularly weak in bone regeneration (Fig. 5).

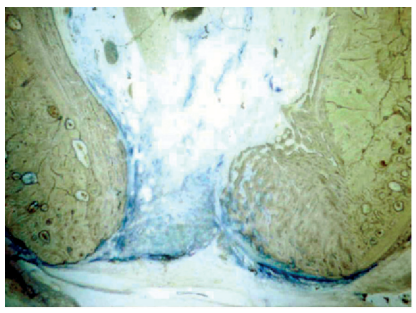

Figure 5 *Histological ground section at weeks 8 (PRP, defect 1). Woven bone formation is seen adjacent to the walls of the defect and particularly at the entrance to the former surgical defect. Collagen formation is seen between the newly formed bone. The center of the defect shows a less dense collagen matrix and no indication of osteogenesis (Giemsa stain, x 25).*

Histologic findings after 12 weeks showed almost complete healing of the bony defects. The entrances to the defects were closed especially those, which had been covered by e-PTFE membranes (Fig. 6, 7). None of the defects, however, showed complete bony healing. Most of the bone that had been formed was woven bone. Even at weeks 12, remnants of collagen sponges were to be seen. From the descriptive point of histology no differences in bone structure and

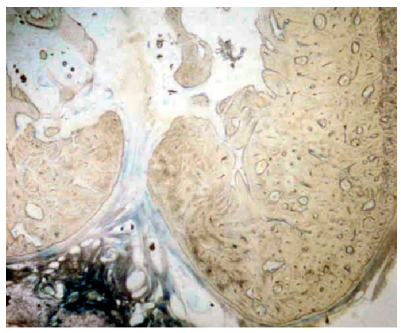

Figure 6 *Histological ground section at 12 weeks. (PRP, defect 1). Almost complete closure of the surgical defect by woven bone apposition. Some islands of bone formation are to be seen in the center of the defect. (Giemsa stain, x 25).*

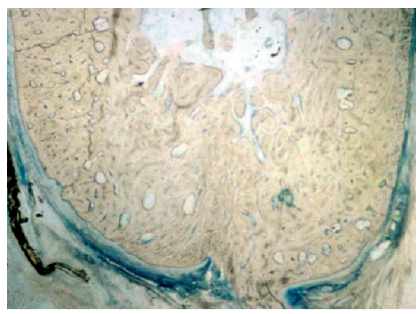

Figure 7 *Histological ground section at 12 weeks. (PRP+ePTFE membrane). The defect has been closed at the lower border of the mandible. Dense, compressed collagen fibrs are seen contour of the mandible, representing the periosteal membrane. Parts of the e-PTEF membrane are seen at the left border of the print (Giemsa stain, x 30).*

healing patterns could be observed among the four variants of surgical defects.

The bone regeneration rate in each experimental group is described in Table 2. Statistic results (paired T-test) are shown in Table 3. At week 2 bone regeneration of the experimental e-PTFE

membrane group and PRP - e-PTFE membrane group showed significantly better results than PRP group and control group ($p<0.01$). No significant bone regeneration was found when the PRP group was compared with the control group ($p=0.53$). At weeks 4 and 6 bone regeneration in all groups was not significantly different ($p=0.14$). At weeks 8 and 12 of the healing period, e-PTFE membranes seemed to promote bone healing in all defects.

Table 2 *Mean % of new bone regeneration at different periods of experiments*

Period (weeks	CON (%±S.D.)	MB (%±S.D.)	PRP (%±S.D.)	MBPRP (%±S.D.)
2	7.37±0.99	13.56±6.02	8.86±2.01	12.51±4.74
4	17.27±6.70	18.15±6.29	18.24±5.93	14.39±3.55
6	16.02±5.48	13.57±3.00	16.01±2.07	16.26±6.72
8	13.82±3.68	20.77±6.88	13.11±3.11	23.93±9.41
12	18.80±6.09	20.24±5.75	15.11±2.77	26.82±13.73

CON=control, MB=e-PTFE membrane, PRP=platelet rich plasma, MBPRP=e-PTFE membrane plus platelet rich plasma (Pair T-test) $p<0.05$

Table 3 *Comparative results of bone regeneration rate*

Period (weeks)	PRP/ CON	PRP/ MB	PRP/ MBPRP	MB/ CON	MB/ MBPRP	MBPRP/ CON
2	NS	MB**	MBPRP**	MB**	NS	MBPRP**
4	NS	NS	NS	NS	NS	NS
6	NS	NS	NS	NS	NS	NS
8	NS	MB*	MBPRP*	MB*	NS	MBPRP*
12	NS	MB*	MBPRP*	NS	NS	NS

NS=non significant, CON=control, MB=e-PTFE membrane, PRP=platelet rich plasma, MBPRP=e-PTFE membrane plus platelet rich plasma, *=$p<0.05$, ** = $p<0.01$ (Pair T-test) $p<0.01$

Discussion

The PRP preparation method in this study was performed following the method described by Weibrich and coworkers.[22] This method is comparable to the one described by Marx and coworkers.[13] While the mean PRP concentration was 785,000 µl in the study by Marx *et al.*[13], it was 923,000 µl in the latter study.[22] Generally, PRP concentration should reach 338% compared to the normal platelet count in whole blood.[13] In the present study fresh PRP was prepared at the time of the surgical experiment and our mean PRP concentration reached 356%, a concentration, which should be sufficient to have an influence on bone regeneration rate.

Generally, the healing processes in all dogs were uneventful. Of interest was that there was a statistically significant increase of bone regeneration rate in all defects in which e-PTFE membranes were used. However, this increase was only 5 to 6% compared to that of other experimental sites. Whether or not this increase has an effect clinically, is questionable. Similarly, a sheep model also showed an increase of bone regeneration in only 3% to 5% in sinus grafts when PRP was added.[24] The authors concluded that their results showed a regenerative capacity of PRP of quite low potency. Also, in a rabbit model no statistically significant effect of PRP on bone formation could be found.[25] Aghaloo *et al.* (2002) also showed that there was no benefit of PRP when used in rabbit cranial defects compared with untreated defects.[26] Farrell and coworkers[27] found no enhanced bone regeneration when inferior border mandibular defects in dogs were treated with PRP (with or without barrier), a finding, which was confirmed with the present study.

In this context it is of interest that Arpornmaeklong *et al*. demonstrated direct effects of PRP on osteoprogenitor cells without additional effects of systemic factors.[28] A high concentration of PRP inhibited osteogenic differentiation and increased cell proliferation. Thus, enhancing effects of PRP on bone regeneration as reported in some clinical studies were not supported by this study.

Generally, a positive clinical effect of PRP in bone grafting might be the result of formation of

an autologous fibrin gel improving the adherence of the autograft particles and thus minimizing the risk of dislocations and displacement.[28] In this study, without adding thrombin and calcium chloride, which transforms PRP into a gel form, PRP was prepared in liquid form to avoid effects of other substances and to see the real effect of PRP. Thus, bovine collagen was used as a hemostasis material in each experimental defect in order to hold both PRP and the blood clot in place. Positive results were reported both in clinical and experimental studies.

Bone density improved when PRP was placed in extraction sockets.[11, 29] Other studies[17, 30] also showed a slight increase of initial osseointegration when PRP was used before implant placement in experimental studies. The effect of PRP on bone grafts in a goat animal model[18] also resulted in superior healing results of the PRP group. To date it is not quite clear why the results both of experimental and clinical studies vary so much. A number of factors including animal species, the preparation of PRP as well as local bone conditions have to be considered.

The use of e-PTFE barrier in guided bone regeneration is well established. In this study, e-PTFE membranes promoted bone regeneration at weeks 2, 8 and 12. However, the regeneration rate was only approximately 5% better than without membrane, although there was a statistical significance ($P< 0.05$). The clinical impact of this rate of newly formed bone is doubtable.

In conclusion the present study showed that PRP did not promote bone healing significantly in dogs. Further experimental and clinical studies are needed to clarify whether the addition of PRP for bone regeneration has sufficient effects to be routinely applied.

Acknowledgement

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