Autologous Platelet-Rich Plasma as a Local Accelerator of the Bone Regeneration in Dog Mandible: A Descriptive Histology

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Abstract

The purpose of the study was to investigate the bone healing process in artificial bone defects when used platelet-rich plasma (PRP) as a local accelerating factor. PRP was prepared from fresh blood by centrifugation method. The concentration of platelet in PRP is about 3 times of normal platelet count. The average baseline platelet count and the average PRP platelet count is 146595 and 518900 respectively. These values confirmed the platelet sequestration ability of the process and quantified the average concentration as 356.16 % of baseline platelet counts. The study was performed in 10 experimental healthy dogs. The experimental and control defects are made at the lower border of the mandible in the same side extraorally under general anesthesia. Every two experimental animal were sacrificed at 2 weeks, 4 weeks, 6 weeks, 8 weeks and 12 weeks. The specimens were obtained and fixed in 10% formalin prior to histological preparation using Giemsa staining to evaluate the new bone formation. The results showed that the new bone formation may be found significantly better in PRP group at the early healing period (2 weeks and 4 weeks).

Introduction

Platelet-rich plasma (PRP) has been used in many fields of medicine such as orthopedics, neurosurgery, oral, and maxillofacial surgery etc. in order to accelerate the healing of both soft tissue and bone. Fracture of the mandible of domestic dogs is one of the most frequent cases in veterinary clinic. Normally, the treatment of those fractures is ligature wire to immobilize the mandible at least 6 weeks until the bone healing process is completed. The utilizing of autologous PRP may be an adjunct treatment modality not only to accelerate the healing process but also to enhance the bone regeneration. This helps shorten the immobilization time and minimize some complications of the conventional method. In some rare cases, such as bone tumor or metabolic bone diseases, PRP is also useful to accelerate and promote the bone formation process.

Platelet-Rich Plasma (PRP). Besides aggregation function, platelets produce and release multiple growth and differentiation factors that play the important role in stimulation and regulation the wound healing (Wartiovaara et al., 1998). These factors are platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF). The two important growth factors which are involved in wound healing both soft and hard tissues are PDGF and TGF-β.
Platelet-Derived Growth Factor was first found in platelet. It is stored in the alpha granules (Antoniades, 1981), it can also be found in other cells such as macrophages (Rappolee et al., 1998), endothelial cells (Sitaras et al., 1987), monocytes and fibroblasts (Antoniades et al., 1991), as well as in bone matrix (Hauschka et al., 1986). PDGF is a polypeptide with 30000 Daltons molecular weight, it remains stable under heat up to 100 °C and has a cationic nature (Antoniades et al., 1991). It has a dimetric structure formed by 2 amino acid chains named A and B (Antoniades et al., 1991).

Nash et al. (1994) and Vikjaer et al. (1997) showed that the application of exogenous PDGF enhance osteogenic differentiation and bone repair in fracture models (Nash et al., 1994), as well as critical size of calvaria defects (Vikjaer et al., 1997). PDGF also enhances the periodontal repair and regeneration (Park et al., 1995). In an animal model study, it is shown that PDGF promote the periodontal regeneration without significant ankylosis or root resorption (Lynch et al., 1991). Howell et al. (1997) showed that the application of PDGF resulted in a significant promotion in bone regeneration.

Transforming growth factor-β (TGF-β) was first isolated from transformed tissues (sarcomas) (Burgers, 1989). There are 2 types of TGF: alpha and beta. TGF-β has molecular weight of 25,000 Daltons, formed by two 12,500 Daltons subunits linked together by disulphur bridges (Assoian et al., 1983; Gentrella et al., 1986). This factor has 3 different structures TGF-β1, TGF-β2, and TGF-β3. The β1 is found abundantly in platelets, lymphocytes, and neutrophils, while β2 is found mainly in bone extracts, platelets, lymphocytes, and neutrophils. Type β1 and β2 are 72% similar. Type β3 is a heterodimer formed of a single chain of TGF-β1 and a single chain of TGF-β2. These factors favor bone formation by enlarging the rate of stem cell proliferation. Another suggested role is the inhibition of osteoclast formation and these bone formation (Anitua, 1999).

Platelet rich plasma (PRP) has been investigated as an abundant source of PDGF and TGF-β. With autologous blood, PRP 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-β within them (Marx et al., 1998).

Clinically, PRP was used in many areas of medicine especially in orthopedic, oral and maxillofacial surgery, and implantology. Marx et al. (1998) had shown that the addition of PRP to bone grafts evidenced a radiographic maturation rate 1.62 to 2.16 times faster than grafts without PRP. As assessed by histomorphometry, there was also a greater bone density in grafts in which PRP was added (Marx et al., 1998). Anitua (1999) reported that reinforcing growth factor concentration through the application of PRP in the wound improved soft tissue repair and
bone regeneration (Park et al., 1995). Kassolis et al. (2000) suggested that ridge augmentation and sinus grafting with freeze-dried bone allograft in combination with PRP provide a viable therapeutic alternative for implant placement (Kassolis et al., 2000).

However the cases reported from Shanaman et al. (2001) showed that the addition of PRP did not appear to enhance the quality or quantity of new bone formation over that reported in comparable guided bone regeneration (GBR) studies without PRP.

Recently, a study from Zechner et al. (2001) has shown that when used dental implants with PRP, the histomorphometrical evaluation showed a significant higher percentage of bone-implant contact during the first 6 weeks (Zechner et al., 2001). Okazaki et al. (2001) has also shown that sinus floor augmentation using a combination of β-Tricalciumphosphate (β-TCP) and PRP cause a more rapid resorption of β-TCP in a maxillary sinus. PRP would have a potential to enhance bone formation and improve the healing of the graft in a maxillary sinus (Okazaki et al., 2001).

The purpose of the study is to describe histologically healing process when used the PRP as an accelerator for the bone regeneration compare to normal bone healing.

**Materials and Methods**

**Animal Selection.** Ten experimental healthy dogs (5 males and 5 females, age range 1-5 years) are recruited for the study. Exclusion criteria are animals with systemic illnesses, compromised immune systems, pregnant and / or lactating dogs.

**Ethical Consideration.** The experimental design and the use of laboratory animal had been approved by ethical committee of faculty of Veterinary Medicine, Chiangmai University.

**Presurgical Therapy.** Pyrantel pamoates 5 mg/kg are administered orally as an antihelminthic drug. Rabies vaccines and multivalent vaccines (Vanguard 5 L®) are administered subcutaneously as the vaccination program.

**Blood Collection.** 10 ml of whole blood from each dog is obtained from cephalic vein. The blood is then filled in a tube containing 1.4 ml of citrate phosphate dextrose as an anticoagulant. The rest of venous blood is used to determine the animal’s platelet count.

**PRP Preparation.** To produce PRP extracts, 8.5 ml of citrated blood is centrifuged in a standard laboratory centrifuge for 10 minutes at 2400 rpm. Subsequently, the yellow plasma (containing the platelets) is taken up into a neutral monovette with a long cannula, using an additional air-intake cannula. To combine the platelets into a single pellet, a second centrifugation step is performed with this second monovette for 15 minutes at 3600 rpm.
The plasma supernatant (containing relatively few cells) is then reduced to approximately 0.4 ml (by taking up with a second neutral monovette, a long cannula, and an air intake cannula). The pellet of platelets is resuspended in the residual 0.4 ml of plasma using a conventional shaker and transfer to an Eppendorf tube for later analysis of platelet count (Gernot et al., 2001).

**Surgical Therapy.** Atropine sulfate (0.04 mg/kg) and xylazine hydrochloride (1 mg/kg) were administered intramuscularly as sedative agent. Thiopental sodium (12.5 mg/kg) was then administered intravenously 15 minutes later in order to induce the anesthesia. The anesthesia was maintained by halothane through endotracheal intubation. The chosen experimental area were lower border of mandible at 2nd, 3rd and 4th permanent premolar and 1st permanent molar area. The radiographs were taken to certify that there are none of deciduous teeth in the experimental areas. To prepare the experimental sites, left side of mandibular area was clipped and scrubbed with tincture iodine, follow by wiping with 70% isopropyl alcohol. Skin incision was made extraorally at the edge of the mandible at 2nd, 3rd and 4th permanent premolar and 1st permanent molar area. Periosteal flaps were elevated and two artificial defects with 5 mm diameter and 8 mm depth were made by using trephine drill, one defect served as control and the other one was adding with 100 μL of PRP. Both artificial defects were filled with collagen (Tissue Vlies, Baxter AG) in order to hold blood clot and PRP in the defects. The sutures were made with absorbable suture material (Dexon® 3-0). Ibuprofen and doxycycline were administered as postoperative analgesic and antibiotic orally for 3 and 7 days respectively. Soft canned food were fed to the experimental dogs for 7 days postoperatively. Every two dogs were sacrificed at 2 weeks, 4 weeks, 6 weeks, 8 weeks and 12 weeks. The experimental areas were blocked and fixed in 10 % formalin subject to ground sectioning process for histological evaluation. Giemsa staining were used for obvious vision of new bone formation. The histological sections were examined under microscope for histological description.

**Method of Euthanasia.** To euthanize the animals, pentobarbitonal sodium at dose 25 mg/kg were injected intravenously until the animals were anaesthetized without pain or distress. Skin incision at ventral of the neck was made caudally. Common carotid artery was identified and then ligated at anterior part. Common carotid artery was incompletely cut caudal to the ligation area. The blood is then released from the whole body. Fixative formalin was injected into the body through common carotid artery until excessive formalin is drained from nostrils. Conventional suture was ligated caudal to the cutting area to prevent pouring back of excessive formalin. Left quadrants of mandibles consisted of PRP and control sites were collected by using bone saw. The rest of the euthanized animals were donated to department of veterinary pre-clinic, faculty of veterinary medicine, Chiangmai university, as specimens for veterinary student course.
The healing process at 2 weeks, 4 weeks, 6 weeks, 8 weeks and 12 weeks of both PRP and control groups will be histologically examined under microscope to describe the bone healing process especially the woven bone formation.

**Results**

*Radiography.* Retained deciduous teeth at experimental sites are not presented in all 10 dogs.

*Platelet Count Study.* Platelet counts performed on each experimental dog yielded a mean platelet count value of 146595, with a range of 102000 to 195750. The average PRP platelet count was 518900, with a range of 201000 to 711100. These values confirmed the platelet sequestration ability of the process and quantified the average concentration as 356.16% of baseline platelet counts (Table 1).

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

<table>
<thead>
<tr>
<th>Experimental dogs</th>
<th>Baseline platelet count</th>
<th>PRP platelet count</th>
<th>Percent of increased platelet</th>
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<tbody>
<tr>
<td>1</td>
<td>102000</td>
<td>201000</td>
<td>197.05</td>
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<tr>
<td>2</td>
<td>183000</td>
<td>598000</td>
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<td>4</td>
<td>153400</td>
<td>520000</td>
<td>338.98</td>
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<tr>
<td>5</td>
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<td>598000</td>
<td>305.49</td>
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<tr>
<td>6</td>
<td>153400</td>
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<tr>
<td>8</td>
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<td>711100</td>
<td>621.59</td>
</tr>
<tr>
<td>9</td>
<td>140000</td>
<td>513000</td>
<td>366.42</td>
</tr>
<tr>
<td>10</td>
<td>125000</td>
<td>352000</td>
<td>281.60</td>
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</tbody>
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\[ \bar{x}_1 = 146595 \quad \bar{x}_2 = 518900 \quad \bar{x}_3 = 356.16 \]

*Histological Evaluation.* This study presents the healing process and bone regeneration of both control and PRP groups at different periods. In all animals the healing process of both extraoral soft tissue and bone defect progressed uneventfully. None of the animals showed changes in behavior nor signs of pain as well as for general infections. Neither wound infections nor fistulas were present.
Specimens at 2 weeks

**PRP group.** All defects revealed new bone formation slightly starting from the internal walls of the defect. Neither inflammatory cells nor bacteria were found around the artificial defects. Collagen was still present in the artificial defects. Extraoral soft tissue tends to cover the entrance of the defect.

**Control Group.** New bone formation of control defects at 2 weeks was not present. Inflammatory cells and bacteria were not found around the artificial defects. Collagen was also still present in the artificial defects as shown in PRP defects at the same period. Angiogenesis was slightly presented in the defect. Extraoral soft tissue tends to cover the entrance of the defect.

Specimens at 4 weeks

**PRP group.** All defects revealed more condensed new bone formation compared to the specimens obtained after 2 weeks. Woven bone of PRP group at 4 weeks was obviously seen in artificial defect. The new bone formation was found not only in the internal walls of the defect but also under the periosteum. Neither inflammatory cells nor bacteria were found around the artificial defects.

**Control group.** The budding of new capillaries into the defect (angiogenesis), a primary part of all wound healing, was still presented in control group at 4 weeks. All defects revealed new bone formation slightly starting from the internal walls of the defect. Woven bone of control group at 4 weeks was obviously less than in artificial defect of PRP group. New woven bone formation began at the walls of the bone defects. Inflammatory cells and bacteria were not found around the artificial defects.

Specimens at 6 weeks

**PRP group.** New bone formation in the defect of PRP group at 6 weeks seemed to be not different when compared to the control defect at the same period. The woven bone was found not only in the internal walls of the defect but also under the periosteum. Neither inflammatory cells nor bacteria were found around the artificial defects. The entrance of the defect was covered by the extraoral soft tissue.

**Control group.** The new bone formation was found not only in the internal walls of the defect but also under the periosteum. Connective tissue was still present in the control defect but was not found in the PRP defect. Control defect revealed a layer of fibrous tissue and woven bone formation similar to PRP defect at the same period. Extraoral soft tissue was present around the entrance of the defect.
Specimens at 8 weeks

**PRP group and control group.** Neither inflammatory cells nor bacteria were found around the artificial defects. Descriptively, the new bone formation of PRP and control groups seemed to be not different. All defects revealed more condensed bone compared to the specimens obtained at the former periods. Newly formed bone beneath as well as below both the periosteum and the entrance of the defect revealed a vascularized and more condensed bone. The entrance of the defect was covered by the extraoral soft tissue and periosteum.

Specimens at 12 weeks

**PRP group and control group.** The healing pattern of PRP and control groups revealed the similar finding. Descriptively, the new bone formation of these two groups seemed to be not differed. The maturation of the new bone are seen in both experimental defects and control defects. The entrance of the defect of both PRP and control group was covered by not only the extraoral soft tissue but also the newly formed bone. The artificial defects were completely filled by new bone formation. Inflammatory cells and bacteria were not found around the artificial defects.

The healing process and new bone formation at 2 weeks, 4 weeks, 6 weeks, 8 weeks and 12 weeks of both PRP and control groups are shown histologically in figure 1.
Figure 1 Histological findings of the bone healing at different periods in PRP and control groups.
Discussion

The average platelet count of the PRP in this study is 518900 or 356.16% compared to the average baseline of platelet count in fresh blood. The platelet counts of PRP from 8 experimental dogs are reached at least 300% when compared to the fresh blood, which was enough growth factors according to the suggestion of Marx et al. (1998). But the platelet counts of PRP from two experimental dogs are not reached 300%. It may be because of the technical errors. Collagen are filled in both experimental and control defects to ensure that blood clot and PRP will be stayed in the defects. The results showed that at the 2 week healing period and 4 week healing period; the woven bone are found in PRP group quite prominently when compared with control group. However, at 8 and 12 week healing period, there are no significant different finding in the bone healing of both group. It seems that, at the beginning of the bone healing period in this study, PRP may accelerate new bone formation than those without. The further investigation such as histomorphometry in bone formation to compare the new bone formation rate is needed to prove that whether PRP accelerate the new bone formation in early healing period.

This study also confirmed that the PRP preparation method from Gernot et al. (2001) can reach more than 300% platelet compare to the baseline of fresh blood platelet. This method are practical and easy to performed and less cost when used the commercial PRP preparation machine.

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References


