The Effect of Bovine Thrombin, Thrombin Receptor Agonist Peptide-6 and Chitosan on Activating Platelet-Rich Plasma

Weihui Chen[1], Meng Lu[1], Chengyong Wang[1], Jie Long[2] and Joo L. Ong[3,*]

[1]*Department of Oral Surgery, Union Hospital, Fujian Medical University, Fuzhou, Fujian 350001, P.R. China*

[2]*Department of Oral & Maxillofacial Surgery, West China College of Stomatology, Sichuan University, Chengdu 610041, P.R. China*

[3]*Department of Biomedical Engineering, University of Texas at San Antonio, San Antonio, TX 78249, USA*

Abstract: In this study, the platelet-rich plasma (PRP) activation by bovine thrombin, thrombin receptor agonist peptide-6 (TRAP), and chitosan to become platelet-rich gel (PRG) was examined. In addition to measuring *in vitro* clotting times and clot retraction over time, platelet-derived growth factor (PDGF) and transforming growth factor β-1 (TGF-β1) generated by clot formation were also assayed. Rapid polymerization of PRP was observed when activated with bovine thrombin, followed by TRAP and chitosan. Bovine thrombin caused considerable clot retraction at 1, 2, 4 hours, but the clot retraction with different activators was similar at 24 hours. Although levels of TGF-β1 were similar for all activators used, greater concentration of PDGF was detected from PRG after TRAP treatment. It was concluded activating PRP with TRAP or chitosan was a safe alternative to bovine thrombin, resulting in excellent working time, significantly less clot retraction, and more growth factors compared to the currently available activator.

Keywords: Platelet-rich plasma, bovine thrombin, chitosan, TRAP, growth factors.

INTRODUCTION

The popular use of platelet-rich gel (PRG) as an adjunct to bone grafting procedures in oral and maxillofacial surgery has been increasing since its introduction by Whitman *et al*. in 1997 [1]. PRP has been suggested to promote bone reparation and the enhancement of wound healing by increasing availability and release of numerous growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), transforming growth factor-β2 (TGF-β2), insulin-like growth factor (IGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) [2-5]. These observations have been reported in a large number of experimental studies and clinical applications [6-12].

The preparation of PRG requires concentrating platelets through centrifugation and subsequent polymerization to form a semisolid gel. At present, all methods of PRP gelation require the use calcium and bovine thrombin as activators [13]. The use of bovine thrombin has unfortunately been associated with the development of antibodies to clotting factors V and XI, which usually results in the risk of potential life-threatening coagulopathies [14, 15]. Consequently, there is a growing interest in identifying alternative agents for PRP clotting. As such, the purpose of this study was to examine the activation of PRP by different activators, namely bovine thrombin, thrombin receptor agonist peptide-6 (TRAP) and chitosan. *In vitro* clotting times and clot retraction over time were used as indicators of PRP activation. Additionally, levels of PDGF and TGF-β1 generated by clot formation were also assayed in this study.

MATERIALS AND METHODS

Animal Model

Five male New Zealand white rabbits, each with a weight of approximately 2.5 Kg, were used in this experiment. Prior to experimentation, the animal protocol was evaluated and approved by the animal research committee at Fujian Medical University to ensure that the policies, standards, and guidelines for the proper use, care, handling, and treatment of animals were observed. The health status of all animals was determined to be normal at the onset of the study.

Preparation of Activators

Three solutions of activators are prepared in this study. The A1 solution was prepared by adding 10 KU of bovine thrombin (Sigma-Aldrich Corp., St. Louis, MO, USA) to 10 mL of 10% $CaCl_2$ solution (Sigma-Aldrich Corp., St. Louis, MO, USA), followed by mixing. The A2 solution was prepared by adding 40 mg chitosan (Sigma-Aldrich Corp., St. Louis, MO, USA) and 200mg antiscorbic acid (Sigma-Aldrich Corp., St.

*Address correspondence to this author at the Department of Biomedical Engineering, University of Texas at San Antonio, San Antonio, TX 78249, USA; Fax: 210-458-7007; E-mail: anson.ong@utsa.edu

E-ISSN: 2311-1755/14

Louis, MO, USA) to 10 mL of 10% $CaCl_2$ solution, followed by mixing. The last solution (A3 solution) was prepared by adding TRAP (Sigma-Aldrich Corp., St. Louis, MO, USA) to 10 mL of 10% $CaCl_2$ solution, followed by mixing. The final concentration of TRAP in the A3 solution after mixing was 0.6 µmol/ml.

Preparation of PRP

In this study, PRP was prepared under strict aseptic manipulation using the twice centrifugation method. Briefly, 20 mL of New Zealand white rabbit blood from the jugular vein was collected into 20 mL tubes containing 2.0 mL anticoagulation agent, ACD-A. The ACD-A solution contained 22.0 g/L trisodium citrate, 8.0 g/L citric acid, and 24.5 g/L dextrose. The tubes were then spun at 200 g for 10 minutes. This was followed by the transferring of plasma and buffy coat layer to 10-mL tubes. The 10-ml tubes were then spun at 1200 g for 10 minutes. At the end of the centrifugation, the upper three-quarter of the preparation was designated as the platelet-poor plasma (PPP) region and the lower one-quarter of the preparation was designated as the PRP region. The PRP in the PRP region was resuspended and used for this study. Platelet counts were performed on both whole blood and PRP.

Preparation of PRG

0.5 mL of PRP aliquots were dispensed into 24-well microtiter plates pre-coated with 1% bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO, USA). This is then divided into 3 groups, with A1 group having the addition of 0.1 mL bovine thrombin/calcium chloride solution, A2 group having the addition of 0.05 mL chitosan/calcium chloride, and A3 group having the addition of 0.1 mL TRAP/calcium chloride solution. With a sample size (n) of 5 per group, the mixture was allowed to solidify at room temperature. Clotting times were monitored by visualization. Clot retraction was determined by measuring the clot diameter at 1, 2, 4, and 24 hours.

Growth Factor Release from PRG

The temporal release of growth factors was examined as a function of time and mode of PRG preparation. Specifically, the PRP samples clotted with thrombin, TRAP and chitosan were allowed to gel, and all samples were incubated at 37°C in a humidified environment. Growth factor released was assayed after 24 hours incubation.

Quantification of Growth Factor in PRG Preparations

TGF- β1 and PDGF were assayed using diagnostic kits from R&D Systems (Minneapolis, MN, USA). Both assays use a sandwich enzyme immunoassay technique.

The TGF-β1 assay used a microplate which was coated with TGF-β receptor Ⅱ. Preparation and dilution of samples and standards were performed as directed by the manufacturer. Duplicates of 100 µL aliquots were applied to the microtiter plate, and enzyme conjugated polyclonal antibody to TGF-β1 was added. The plate was covered and incubated at 37°C for 1 hour, followed by washing. After washing, substrate was added and the plate was incubated for 15 minutes at 37°C. After incubation, a 50 µL stop solution was added and the plate was read using a BioTEX ELx800 universal microplate reader (BioTek, Houston, TX, USA) at 450nm. A standard curve was generated and the concentration of TGF-β1 levels of each sample was determined. The total amount of growth factor was also calculated based on the amount of supernatant obtained after clot retraction.

PDGF level was determined with a similar enzyme immunoassay technique. This assay uses a pre-coated microtiter plate with a monoclonal antibody to PDGF. The samples and standards were prepared as recommended by the manufacturer. Briefly, a conjugated antibody to PDGF was added to the wells and incubated at 37°C for 1 hour, followed by washing. After washing, substrate was added to the wells and the plate was incubated for 15 minutes at 37°C. After incubation, the reaction was stopped and the reaction was read using a BioTEX ELx800 universal microplate reader (BioTek, Houston, TX, USA) at 450 nm. From the standard curve, the concentration and total yield of PDGF were determined.

Statistical Analyses

Data were statistically analyzed using the Multiway Analysis of Variance (ANOVA). *P-values* of <0.05 were considered statistically significant.

RESULTS

Platelet counts performed on both whole blood and PRP indicated that the PRP groups contain 4.64 times number of platelets when compared to whole blood. When bovine thrombin was added to the PRP, rapid clotting was observed, with complete polymerization

occurring at an average of 3.24 minutes (Figure **1**). The addition of 0.6 µmol/ml TRAP to the PRP resulted the solidification of the clot at an average of 9.56 minutes, whereas the addition of chitosan to the PRP resulted in clot solidification at an average of 10.96 minutes. Statistical differences of clotting times were observed between the three activator groups (P<0.01).

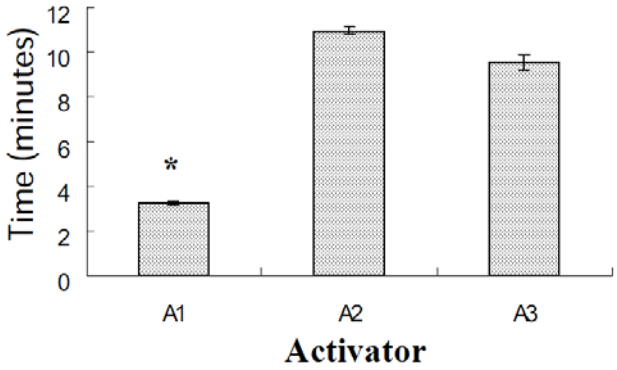

Figure 1: Clotting time of three different activators. Group A1 = bovine thrombin, Group A2 = Chitosan, and Group A3 = TRAP. Asterisk (*) indicates significant difference between A2 and A3 group at P < 0.01.

It was also observed from Figure **2** that all activators studied induced some degrees of clot retraction over time. Bovine thrombin was observed to cause considerable clot retraction, with an average PRG diameter of 0.96 cm, 0.8 cm and 0.66 cm after 1, 2, and 4 hours of adding the bovine thrombin to the PRP, respectively (Figure **3**). At 1, 2, and 4 hours after the addition of chitosan to the PRP, the average PRG diameter was 1.36 cm, 0.98 cm and 0.87 cm, respectively. An average PRG diameter of 1.50 cm, 1.15 cm and 1.01 cm was observed at 1, 2, and 4 hours after adding TRAP to the PRP, respectively. Statistical analysis indicated significant difference in clot retraction between the use of bovine thrombin and TRAP as activators (P<0.05) for all three time period studied. Additionally, statistical difference was also observed in clot retraction between the use of bovine thrombin and chitosan as activators (P<0.05) for all three time periods studied. From Figures **2** and **3**, the diameter of PRG in thrombin group, chitosan group and TRAP group was 0.56 cm, 0.56 cm and 0.69 cm respectively at 24 hours, with no statistical difference observed between the three groups (P>0.05).

The results of the TGF-β1 and PDGF released after 24 hours incubation are shown in Figures **4** to **7**. It was observed from this study that the concentration and total yield of TGF-β1 (Figures **4** and **6**) was similar regardless of the activator of PRG preparation (P>0.05), whereas the concentration and total yield of PDGF (Figures **5** and **7**) were significantly different between the bovine thrombin and the TRAP group (P<0.01), and between the bovine thrombin and the chitosan group (P<0.05). The concentration and total yield of PDGF is statistically highest in the TRAP group and statistically lowest in the thrombin group.

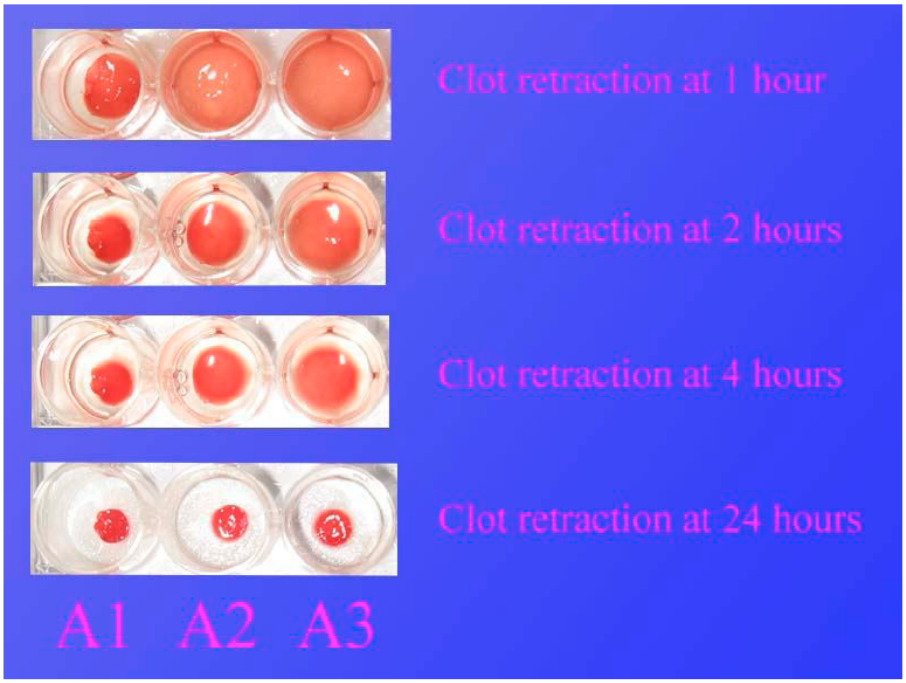

Figure 2: Clot retraction of PRG activated by different activators. Group A1 = bovine thrombin, Group A2 = Chitosan, and Group A3 = TRAP.

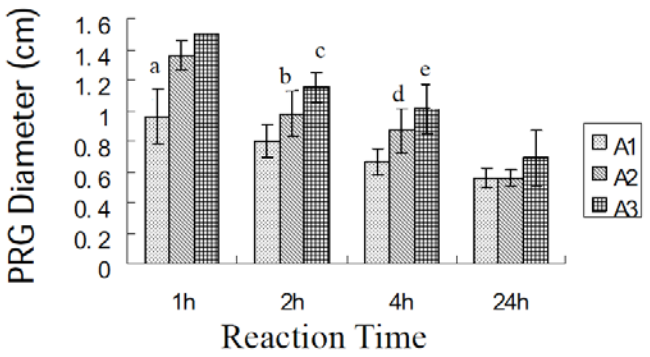

Figure 3: Clot retraction of 3 different activators. Group A1 = bovine thrombin, Group A2 = Chitosan, and Group A3 = TRAP. At 1 hr, A1 group was statistically different from A2 and A3 groups (P<0.01). At 2 hr and 4 hr, A1, A2, and A3 were statistically different from each other (P<0.05). No significant difference was observed at 24 hours.

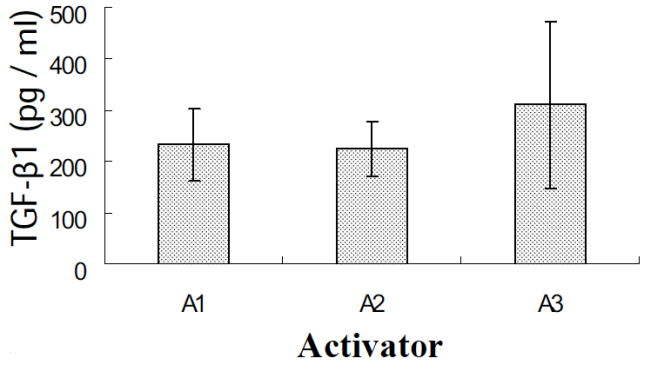

Figure 4: Concentration of TGF-β1 released by PRG. Group A1 = bovine thrombin, Group A2 = Chitosan, and Group A3 = TRAP. No significant difference between A1, A2, and A3 groups.

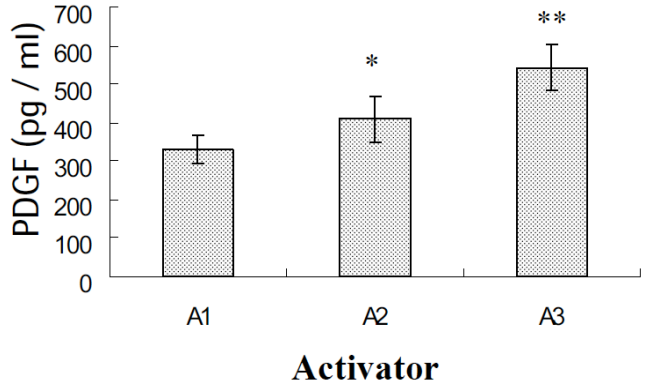

Figure 5: Concentration of PDGF released by PRG. Group A1 = bovine thrombin, Group A2 = Chitosan, and Group A3 = TRAP. Significant differences (indicated by asterisks (* and **) between the 3 groups were observed.

DISCUSSION

At present, all preparation of PRG requires the use of calcium and bovine thrombin as activators. Thrombin

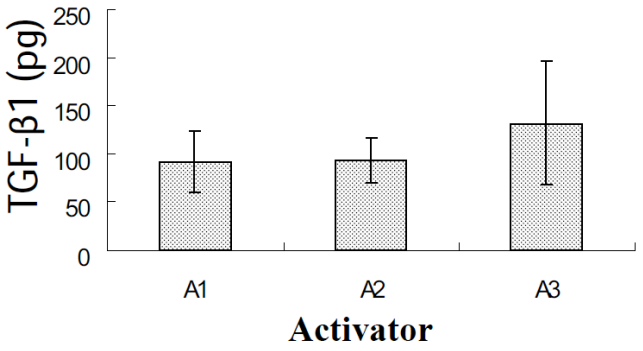

Figure 6: Total amount of TGF-β1 released by PRG, with no significant difference observed between the 3 groups. Group A1 = bovine thrombin, Group A2 = Chitosan, and Group A3 = TRAP.

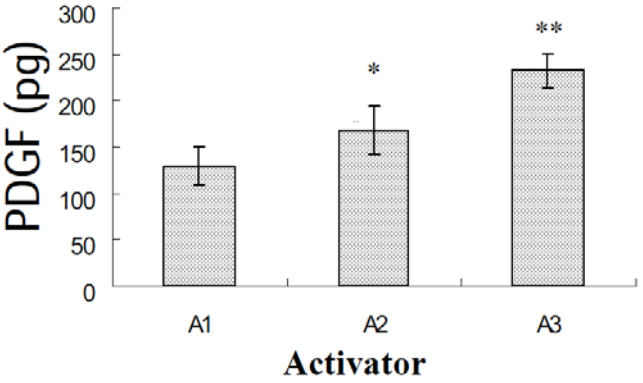

Figure 7: Total amount of PDGF released by PRG. Group A1 = bovine thrombin, Group A2 = Chitosan, and Group A3 = TRAP. Significant differences (indicated by asterisks (* and **) between the 3 groups were observed.

signaling of platelets is known to be mediated by a G protein– coupled protease-activated receptor (PAR). The PAR is activated after thrombin binding and subsequent cleavage of the amino-terminal end of the receptor [16]. This new amino terminus thus acts as a tethered ligand and binds intramolecularly to the body of the PAR, resulting in a transmembrane signal [17].

However, the use of bovine thrombin has unfortunately been associated with the risk of potential life-threatening coagulopathies [14, 15]. Consequently, there is a growing interest in identifying alternative activators for PRG preparation. In a study by E-Chin Shen *et al*., it was suggested that chitosan could be used to enhance platelet adhesion and aggregation [18]. This study also reported that activated human platelets after chitosan stimulation resulted in the release of growth factors, thereby suggesting chitosan to be an appropriate substitute for bovine thrombin in PRG preparation. In Landesberg *et al*. study where two methods of preparing PRG and the levels of PDGF and TGF-β1 in each preparation were evaluated, it was

reported that both methods of preparation could be performed in less than 30 minutes, and that the levels of PDGF and TGF-β1 were similar regardless of the method used for the initiation of clot formation [19]. In a subsequent Landesberg *et al*. study where the use of TRAP and bovine thrombin in the preparation of PRG, it was reported that polymerization of PRP was most rapid when thrombin was added, followed by TRAP/Allogro, TRAP/BioGlass, TRAP/BioOss, and TRAP alone [20]. Additionally, the Landesberg *et al*. study also reported considerable clot retraction (43%) with the addition of thrombin, whereas the addition of TRAP alone resulted in only 15% clot retraction [20]. TRAP is a synthetic hexapeptide that activates the thrombin receptor independent of receptor cleavage. It corresponds to amino acids 42 to 47 of the thrombin receptor and mimics the effects of thrombin [17, 21].

In this study, the potential of TRAP and chitosan to mimic the effects of thrombin was evaluated. TRAP and chitosan are relatively inexpensive as alternatives to thrombin for the preparation of PRG. Findings from this study are in agreement with other studies, showing that TRAP and chitosan resulted in longer working time and larger clot diameters when compared with thrombin [19, 20]. Additionally, it is also important to note that both TRAP and chitosan have greater biological security when compared to bovine thrombin.

The PRG capacity to enhance bone defect repair has been reported in a large number of experimental studies and clinical applications [22-26]. Although the exact mechanism has not been fully understood, it is postulated that the stimulation of bone healing by PRG is due to the increased concentration of relevant growth factors, including PDGF, TGF-B1, VEGF, and IGF. Review of related existing researches and achievements indicated that the presence of PDGF promoted the mitosis of bone marrow stromal cells, stimulated the proliferation of capillaries, and excited the chemotaxis of monocyte-macrophage cells [2]. TGF-β1 was reported to stimulate the chemotaxis and mitosis of osteoprogenitor cells and osteogenic cells, accelerate the synthesis of collagen fibers, and inhibit the formation of osteoclasts [27]. The main contribution of IGF was suggested to stimulate proliferation and differentiation of osteoblasts, promote generation of cartilage and bone matrix, while the presence VEGF was suggested to play an important role on the wound healing and angiogenesis [28].

In our study, PRP was prepared through a twice centrifugation method. Findings from this study indicated a 4.64 times more platelets in PRP when compared to whole blood, thereby satisfying the definition of PRP introduced by Marx [29, 30]. According to what is commonly accepted, it is always more desirable to have more growth factors released from PRG. In comparing the effect of TRAP and chitosan to thrombin, this study indicated that the addition of TRAP and chitosan resulted in extended PDGF release necessary for bone regeneration. These observations suggested that the alternative use of TRAP or chitosan to activate platelet in the preparation of PRG, and they are more superior platelet activator to accelerate tissue repair compared to thrombin. Similar levels of TGF-β1 were also observed in this study, regardless of the activator used. Possible explanations for this phenomenon are the small sample size used in this study, higher standard deviation, and the lower sensitivity of TGF-β1 diagnostic kit for rabbit plasma.

CONCLUSION

It was concluded from this study that the use of TRAP or chitosan may be a safe alternative to bovine thrombin for activating PRG formation. The use of TRAP or chitosan resulted in an excellent working time, significantly less clot retraction and more growth factors released when compared to the currently available activator.

ACKNOWLEDGEMENTS

This work was supported by Program for New Century Excellent Talents in Fujian Province University (NCETFJ-0715) and National natural Science Foundation of China (NSFC 30300172).

REFERENCES

[1] Whitman DH, Berry RL, Green DM. Platelet gel: an autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. J Oral Maxillofac Surg 1997; 55: 1294-9. http://dx.doi.org/10.1016/S0278-2391(97)90187-7

[2] Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998; 85: 638-46. http://dx.doi.org/10.1016/S1079-2104(98)90029-4

[3] Sanchez AR, Sheridan PJ, Kupp LI. Is platelet-rich plasma the perfect enhancement factor? A current review. Int J Oral Maxillofac Implants 2003; 18: 93-103.

[4] Weibrich G, Kleis WK, Hafner G. Growth factor levels in the platelet-rich plasma produced by 2 different methods: curasan-type PRP kit *vs.* PCCS PRP system. Int J Oral Maxillofac Implants 2002; 17: 184-90.

[5] Carlson NE, Roach RB Jr. Platelet-rich plasma: clinical applications in dentistry. J Am Dent Assoc 2002; 133: 1383-6. http://dx.doi.org/10.14219/jada.archive.2002.0054

[6] Ueda M, Yamada Y, Ozawa R, Okazaki Y. Clinical case reports of injectable tissue-engineered bone for alveolar augmentation with simultaneous implant placement. Int J Periodontics Restorative Dent 2005; 25: 129-37.

[7] Sammartino G, Tia M, Marenzi G, di Lauro AE, D'Agostino E, Claudio PP. Use of autologous platelet-rich plasma (PRP) in periodontal defect treatment after extraction of impacted mandibular third molars. J Oral Maxillofac Surg 2005; 63: 766-70. http://dx.doi.org/10.1016/j.joms.2005.02.010

[8] Belli E, Longo B, Balestra FM. Autogenous platelet-rich plasma in combination with bovine-derived hydroxyapatite xenograft for treatment of a cystic lesion of the jaw. J Craniofac Surg 2005; 16: 978-80. http://dx.doi.org/10.1097/01.scs.0000183469.93084.f3

[9] Steigmann M, Garg AK. A comparative study of bilateral sinus lifts performed with platelet-rich plasma alone *vs.* alloplastic graft material reconstituted with blood. Implant Dent 2005; 14: 261-6. http://dx.doi.org/10.1097/01.id.0000177412.84225.05

[10] Anitua E. The use of plasma-rich growth factors (PRGF) in oral surgery. Pract Proced Aesthet Dent 2001; 13: 487-93.

[11] Okuda K, Kawase T, Momose M, Murata M, Saito Y, Suzuki H. Platelet-rich plasma contains high levels of platelet-derived growth factor and transforming growth factor-beta and modulates the proliferation of periodontally related cells *in vitro*. J Periodontol 2003; 74: 849-57. http://dx.doi.org/10.1902/jop.2003.74.6.849

[12] Tischler M. Platelet rich plasma, the use of autologous growth factors to enhance bone and soft tissue grafts. N Y State Dent J 2002; 68: 22-4.

[13] Jakse N, Tangl S, Gilli R, *et al*. Influence of PRP on autogenous sinus grafts: An experimental study on sheep. Clin Oral Implant Res 2003; 14: 578-83. http://dx.doi.org/10.1034/j.1600-0501.2003.00928.x

[14] Ortel TL, Mercer MC, Thames EH, Moore KD, Lawson JH. Immunologic impact and clinical outcomes after surgical exposure to bovine thrombin. Ann Surg 2001; 233: 88-96. http://dx.doi.org/10.1097/00000658-200101000-00014

[15] Landesberg R, Moses M, Karpatkin M. Risks of using platelet rich plasma gel. J Oral Maxillofac Surg 1998; 56: 1116-7. http://dx.doi.org/10.1016/S0278-2391(98)90286-5

[16] Coughlin SR. Protease-activated receptors and platelet function. Thromb Haemost 1999; 82: 353-6.

[17] Coughlin SR. Protease-activated receptors in vascular biology. Thromb Haemost 2001; 86: 298-307.

[18] Shen EC, Chou TC, Gau CH, Tu HP, Chen YT, Fu E. Releasing growth factors from activated human platelets after chitosan stimulation: a possible bio-material for platelet-rich plasma preparation. Clin Oral Implants Res 2006; 17: 572-8. http://dx.doi.org/10.1111/j.1600-0501.2004.01241.x

[19] Landesberg R, Roy M, Glickman RS. Quantification of growth factor levels using a simplified method of platelet-rich plasma gel preparation. J Oral Maxillofac Surg 2000; 58: 297-300. http://dx.doi.org/10.1016/S0278-2391(00)90058-2

[20] Landesberg R, Burke A, Pinsky D, Katz R, Vo J, Eisig SB, Lu HH. Activation of platelet-rich plasma using thrombin receptor agonist peptide. J Oral Maxillofac Surg 2005; 63: 529-35. http://dx.doi.org/10.1016/j.joms.2004.12.007

[21] Andersen Henrik, Greenberg DL, Fujikawa Kazuo. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. Proc Natl Acad Sci USA 1999; 96: 11189-93. http://dx.doi.org/10.1073/pnas.96.20.11189

[22] Torres J, Tamimi F, Tresguerres IF, Alkhraisat MH, Khraisat A, Blanco L. Effect of combining platelet-rich plasma with anorganic bovine bone on vertical bone regeneration: early healing assessment in rabbit calvariae. Int J Oral Maxillofac Implants 2010; 25: 123-9.

[23] Camarini ET, Zanoni JN, Leite PC, Boos FB. Use of biomaterials with or without platelet-rich plasma in postextraction sites: a microscopic study in dogs. Int J Oral Maxillofac Implants 2009; 24: 432-8.

[24] Shi B, Zhou Y, Wang YN, Cheng XR. Alveolar ridge preservation prior to implant placement with surgical-grade calcium sulfate and platelet-rich plasma: a pilot study in a canine model. Int J Oral Maxillofac Implants 2007; 22: 656-65.

[25] Torres J, Tresguerres I, Tamimi F, Clemente C, Niembro E, Blanco L. Influence of platelet-rich plasma on bone regeneration: a histomorphometric study in rabbit calvaria. Int J Oral Maxillofac Implants 2007; 22: 563-8.

[26] Aghaloo TL, Le AD, Freymiller EG, Avera S, Shimizu K, Nishimura RD. Immunohistochemical analysis of cortical and cancellous bone after radiation and the effect of platelet-rich plasma on autogenous bone grafting. Int J Oral Maxillofac Implants 2006; 21: 535-42.

[27] Carano RA, Filvaroff EH. Angiogenesis and bone repair. Drug Discov Today 2003; 8: 980-9. http://dx.doi.org/10.1016/S1359-6446(03)02866-6

[28] Keramaris NC, Calori GM, Nikolaou VS, Schemitsch EH, Giannoudis PV. Fracture vascularity and bone healing: a systematic review of the role of VEGF. Injury 2008; 39 (Suppl 2): S45-57. http://dx.doi.org/10.1016/S0020-1383(08)70015-9

[29] Marx RE. Discussion: Quantification of growth factor levels using a simplified method of platelet-rich plasma gel preparation. J Oral Maxillofac Surg 2000; 58: 300-1. http://dx.doi.org/10.1016/S0278-2391(00)90059-4

[30] Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? Implant Dent 2001; 10: 225-8. http://dx.doi.org/10.1097/00008505-200110000-00002

Received on 12-03-2014 Accepted on 17-06-2014 Published on 27-11-2014

DOI: http://dx.doi.org/10.12970/2311-1755.2014.02.02.3