Sinus floor elevation applied tissue-engineered bone
Comparative study between mesenchymal stem cells/platelet-rich plasma (PRP) and autogenous bone with PRP complexes in rabbits

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Abstract: In the present study, we compared bone regeneration ability in sinus floor elevation between a tissue engineering method using mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP), and a promising new method using particulate cancellous bone and marrow (PCBM) and PRP. Bilateral sinus floor elevation procedures were performed in 18 adult Japanese white rabbits. MSCs/PRP or PCBM/PRP complexes were grafted to each maxillary sinus in the same rabbits. The MSCs were isolated from rabbit iliac crest marrow, and PRP was obtained from peripheral blood. PCBM were collected from the rabbit iliac crest and mixed with PRP. The animals were sacrificed at 2, 4, and 8 weeks after transplantation, and the bone formation ability of each implant was evaluated histologically and histometrically. According to the histological observations, both sites (MSCs/PRP and PCBM/PRP) showed well newly formed bone and neovascularization at 2 and 4 weeks. However, at 8 weeks, the lamellar bone was observed to be occupied by fatty marrow in large areas in both sites. There was no significant difference in bone volume or augmented height between MSCs/PRP and PCBM/PRP groups each week, but there were significant differences in bone volume and augmented height between 2 and 8 weeks in PCBM/PRP or MSCs/PRP groups and in bone volume between 4 and 8 weeks in the PCBM/PRP group (P<0.05). These results suggest that the MSCs/PRP complex may well be used for bone regeneration in sinus floor elevation, compared with the PCBM/PRP complex.

The dental restoration of the posterior maxilla using osseointegrated implants is one of the most problematic in implant dentistry. Patients with edentulous posterior maxillae often present with atrophy of the alveolar ridge and pneumatization of the maxillary sinuses following teeth loss. This situation limits the volume of bone available for implant placement. These clinical problems might be improved partly by utilizing sinus floor elevation procedures with autogenous bone grafts, which are considered as an ideal graft material and the gold standard to which other graft materials are compared. The bone grafts harvested from the iliac crest also have shown excellent survival with implants loaded and functional (Kent & Block 1989). However, the bone grafts have to injure the normality organization besides the operation field, and it involves morbidity (Boyne et al. 1980; Tatum 1986; Younger & Chapman 1989; Lundgren et al. 1996). Other autogenous approaches such as intraoral bone grafts generally result in less morbidity, but the bone volume offers less bone availability than the iliac crest as a donor site. Nowadays, bone substitutes such as hydroxyapatite, β-tricalcium phosphate ceramics, or coral scaffolds (Kadiyara et al. 1997b; Petite et al. 2000) are used to provide alternatives to
autogenous bone for the improvement of the bone volume. But in human specimens, there were problems where graft particles were still present after 4 years in the sinus floor elevation (Leonardis & Pecosa 1999; Piatelli et al. 1999). So it has been said that an ideal bone substitute should have the following characteristics: it should be biocompatible and be replaced by newly formed bone, and it should have osteoinductive properties [Jensen et al. 1996].

Langer & Vacanti (1993) have called the process involving the morphogenesis of new tissues formed from isolated cells and biocompatible polymers and growth factors ‘tissue engineering’. This form of bone regeneration by autogenous cell transplantation is one of the most promising treatment concepts being developed, since it eliminates the problems of donor site morbidity for autologous grafts, the immunogenicity of allogenic grafts, and loosening of alloplastic implants [Vacanti 1988; Vacanti et al. 1988]. In this study, we used mesenchymal stem cells (MSCs) as the isolated cells, and platelet-rich plasma (PRP) as the growth factors and scaffold. MSCs have been thought to be multipotent cells that can replicate as undifferentiated cells and that have the potential to differentiate into lineages of mesenchymal tissue including bone, cartilage, fat, tendon, muscle, and marrow stroma [Caplan 1991; Owen & Friedenstein 1998; Pittenger et al. 1999], and have received widespread attention because of their potential utility in tissue engineering applications. PRP is believed to result in early consolidation and graft mineralization in approximately half the time that it would take using an autogenous graft alone [Canalis 1981; Cho et al. 1995; Marx et al. 1998; Anitua 1999] and promote a 15–30% increase in the trabecular bone density [Marx et al. 1998].

So we have attempted to regenerate bone in a sinus floor elevation as for bone grafts with minimal invasiveness and good plasticity, and to provide a clinical alternative to autogenous bone grafts using MSCs/PRP complexes. Further, there are no reports that sinus floor elevation applied MSCs/PRP complexes and there have been comparative studies with autogenous bone grafts/PRP complexes.

In this study, we compared the bone regeneration ability in sinus floor elevation with the MSCs/PRP groups and particulate cancellous bone and marrow (PCBM)/PRP groups. This research will be useful as a preliminary study for dental implant installation and as an improved method for maxillary sinus augmentation with minimal invasiveness.

**Material and methods**

**Rabbit animal model and sinus augmentation procedure**

This study used 18 adult female Japanese white rabbits that weighed 3.1–3.5 kg. A skin incision was made in the cheek a few millimeters above the inferior border of the incisive bone and maxilla. The subcutaneous tissue and the muscles were dissected to expose the maxillary periosteum, which was incised and elevated dorsally. A round diamond bur was used to draw the outline of the trap door (5 mm × 5 mm with the distal vertical osteotomy line 2 mm medial from the first molar) in the lateral antral wall of the maxilla. The trap door was removed, and the antral membrane was elevated carefully to avoid perforation. Exposure of the maxillary sinus was performed bilaterally, and the MSCs/PRP complex was placed into the left maxillary sinus, and the PCBM/PRP complex was placed into the right one. The periosteum and skin flap were replaced, sutured, and allowed to heal (Fig. 1a).

**MSCs isolation and cultivation, PRP, PRP gel preparation, and MSCs/PCBM/PRP admixture preparation**

The PRP and the gel preparation were carried out according to the same method developed by Yamada et al. (2004a). At first, approximately 30 ml whole blood was drawn from the Japanese white rabbits into centrifuge tubes. The blood was first centrifuged in a standard laboratory centrifuge machine, Himac CT (Hitachi Koki, Japan), for 5 min at 203 g. Subsequently, the yellow plasma (containing the buffy coat, which contained the platelets and leukocytes) was taken up into a neutral monovette with a long cannula. A second centrifugation at 1050 g for 5 min was performed to combine the platelets into a single pellet and the plasma supernatant, which was platelet-poor plasma (PPP) and contained relatively few cells, was removed. The resulting pellet of platelets, the buffy coat/plasma fraction (PPR), was resuspended in the residual 3 ml of plasma and used in the platelet gel. We evaluated the whole blood, PRP, PPP, and blood platelets. The platelet counts in the PRP and PPP were measured in Beckman Coulter GEN’S™, and Beckman Coulter STKS ERE™ [Beckman Coulter, Fullerton, CA, USA]. Platelet counts yielded a mean value of 227,000, with a range of 214,000–250,000. The PRP mean platelet count was 864,000 with a range of 834,000–892,000. These values confirmed the platelet sequestration ability of the process, which shows that the concentration was 383% above the baseline platelet counts. The PRP was stored at room temperature in a conventional shaker until use. Bovine thrombin in powder form (10,000 U) was dissolved in 10 ml 10% calcium chloride in a separate sterile cup. Next, 600 μl PRP was aspirated into a 1 ml syringe, while in a second 1 ml syringe 100 μl of the thrombin/calcium chloride mixture was aspirated. Here the cells resuspended directly into PRP. The two syringes were connected with a T connector and the plungers of the syringes were pushed and pulled alternatively, allowing the air bubble to transverse the two syringes. Within 5–30 s, the contents assumed a gel-like consistency as the thrombin affected the polymerization of the fibrin to produce an insoluble gel.

The MSCs were isolated from the left iliac crest marrow aspirates (10 ml) according to the previously reported method [Kadiyara et al. 1997a, 1997b]. Briefly, the basal medium, low-glucose Dulbecco’s modified Eagles medium, and growth supplements (50 ml of mesenchymal cell growth supplement, 10% fetal bovine serum, 10 ml of 200 mM L-glutamine, and 0.5 ml of penicillin-streptomycin mixture containing 25 μg of penicillin and 25 μg streptomycin) were purchased from Cambrex™ Inc. (Walkersville, MD, USA). Three supplements for inducing osteogenesis, Dexamethasone (Dex), sodium 2-glycerophosphate (2-GP), and L-ascorbic acid 2-phosphate (AsAP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The cells were incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO2. In culture, the MSCs trypsinized and were used for
implanting. Then, 600 μl PRP and MSCs (1 × 10⁷ cells/ml) were aspirated into a 1 ml syringe, while in another 1 ml syringe 100 μl of the thrombin/calcium chloride mixture was aspirated. Also, the graft volumes were standardized using a mold (5 mm × 5 mm × 5 mm), and the gel was implanted into the maxillary sinus.

PCBM was harvested from the right iliac crest of the animal from which the PCBM/PRP complex was made by mixing with PRP. The graft volumes were standardized using a mold (5 mm × 5 mm × 5 mm) [Fig. 1a]. The groups were implanted into the maxillary sinus area.

Histological observation
The rabbits were sacrificed at 2, 4, or 8 weeks after the transplantation. The maxillae were dissected and cut into smaller blocks that included the nasal and sinus cavities. Implants were fixed in buffered 10% formaldehyde and decalcified, embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin [Fig. 1b]. They were analyzed each six sections per operative site.

Histomorphometric analysis
Each image of the calcified specimens at the rostrocaudal midpoint of the antral wall was copied on a color reversal film, digitized as a 256 × 256 array of 8-bit density values, and transferred to a microcomputer for analysis [NIH Image, version 1.61, National Institutes of Health]. The augmented area was defined as the area that was enclosed within the bone labels in the residual maxilla and nasal wall, the sinus membrane, and the antral wall. The volume of total bone (newly formed bone and grafted bone) in the augmented area and augmented height (the maximal height of the augmented space) were quantified using this computer-based image-analysis system.

Statistical analysis
Group means and standard deviations were calculated for each measured parameter. The data were compared using the paired, two-tailed Student’s *t*-test for the total bone between the PCBM/PRP and MSCs/PRP groups. A *P*-value of < 0.05 indicated statistical significance.

Results
**Histological observation of the MSCs/PRP and PCBM/PRP groups**
The elevated sinus membrane had no perforation and maintained the form of the ciliated epithelium and serous glands in all animals at each week.

At 2 weeks after transplantation, the PCBM/PRP site showed a trabeculae of newly formed bone that were composed of woven bone around the grafted bone. Most of the lacunae around the osteocytes were large and all trabeculae were embedded in fibrovascular tissue. In the MSCs/PRP site, newly formed trabeculae were observed with abundant osteocyte, cuboidal osteoblasts, fibrovascular tissue, and many blood vessels [Fig. 2a–d].

At 4 weeks after transplantation in the PCBM/PRP and MSCs/PRP sites, the trabeculae were more mature than at 2 weeks; lamellar bone structure was apparent, woven bones decreased, and the bone lacuna became narrow in comparison with 2 weeks [Fig. 3a–d].

At 8 weeks in both sites, cortical bone formation was observed under the elevated membrane and at the lateral sinus wall. Trabeculae with clear lamellar structures were embedded in the fatty marrow in both sites [Fig. 4a–d].
Histomorphometric analysis

Bone volume

The volumes of total bone PCBM/PRP and MSCs/PRP groups were \(35 \pm 5.2\%\) and \(29.1 \pm 4.4\%\) at 2 weeks, \(28.6 \pm 3.4\%\) and \(24.1 \pm 3.6\%\) at 4 weeks, and \(20.6 \pm 4\%\) and \(20.9 \pm 4.1\%\) after 8 weeks, respectively. There was no difference in augmented height between MSCs/PRP and PCBM/PRP groups each week. But there were significant differences in augmented height between 2 and 8 weeks in PCBM/PRP or MSCs/PRP groups \(\text{[P}\text{<0.05]}\) (Fig. 5a).

Augmented height

The augmented heights of PCBM/PRP and MSCs/PRP groups were \(2.1 \pm 0.5\) and \(1.7 \pm 0.2\) mm at 2 weeks, \(1.7 \pm 0.6\) and \(1.5 \pm 0.3\) mm at 4 weeks, and \(1.3 \pm 0.4\) and \(1.3 \pm 0.3\) mm after 8 weeks, respectively. There was no difference in augmented height between MSCs/PRP and PCBM/PRP groups each week. But there were significant differences in augmented height between 2 and 8 weeks in PCBM/PRP or MSCs/PRP groups \(\text{[P}\text{<0.05]}\) (Fig. 5b).

Discussion

Generally, atrophy of the alveolar ridge is because of tooth loss, and autogenous bone grafts are used for the bone regeneration, with dental implants utilized for alveolus absorbing of the upper jaw bone (Boyne et al. 1980; Tatum 1986; Lundgren et al. 1996). In posterior maxilla restoration using osseointegrated implants, sinus floor elevation is often also undertaken by an autogenous bone graft.

Autogenous bone graft is considered the ideal graft to satisfy the following criteria: (1) low risk of infection, (2) low antigenicity, (3) the ability to produce bone by osteoinduction and osteoconduction, and (4) easy correction \(\text{[Block & Kent 1997]}\). However, the preferred autogenous material causes specific problems such as its limited supply, attendant donor-site morbidity, and the occasional unsuitability for the proposed reconstruction because of poor tissue quality, or the extremely difficulty in shaping the graft (Laurie et al. 1984; Summers & Eisenstein 1984; Younger & Chapman 1989). Therefore, in this study we use tissue engineering technology with minimal invasiveness for bone regeneration using MSCs as the autogenous cell and PRP as growth factors and scaffold. It is one of the most promising treatment concepts being developed, since it may be possible to eliminate the problems of donor site morbidity for autologous grafts (Vacanti 1988; Vacanti et al. 1988). The PRP also contains growth factors such as platelet-derived growth factor, transforming growth factors-\(\alpha\), and insulin growth factor PRP is also reported to promote wound healing, bone formation, and has attracted attention for use with autogenous bone (PCBM) in clinical cases (Marx et al. 1998; Wiltfang et al. 2003). MSCs have been thought to be multipotent cells that can replicate as undifferentiated cells, including bone, cartilage, fat, tendon, muscle, and marrow stroma, and have received widespread attention because...
of their potential utility in tissue engineering applications (Yoshikawa et al. 1996, 1998; Pittenger et al. 1999; Noshi et al. 2001; Yamada et al. 2004a, 2004b). Artificial bone utilizing MSCs and PRP-like gel, which had a low immune rejection response from autogenous blood, was reported in a study using the mandible of the dog (Yamada et al. 2004c). However, the study did not investigate spaces such as the maxillary sinus area for dental implant installation. Thus, we examined the ability of sinus floor elevation using tissue engineering method with MSCs/PRP complexes, and the promising PCBM/PRP complexes.

According to the histological analysis, woven bone was observed in both sites (MSCs/PRP and PCBM/PRP sites) at the early stage of 2 weeks after the transplant, and the maturity of the newly formed bone was observed at 4 weeks time dependently. However, 8 weeks after grafting, most newly formed or transplant bones were absorbed in both sites and its fatty marrow was found in the sinus floor area, and blood vessel structure was hardly observed. Therefore, it would be difficult to support implants in the posterior maxilla at 8 weeks, because it was a bone volume of around 20% with all that fatty tissue. The results of Watanabe et al. (1999) or Wada et al. (2001), which our experimental model is based upon, also showed fatty marrow in the entire the maxillary sinus at 8 weeks after the transplant when a block autogenous bone or PCBM without PRP is transplanted in the maxillary sinus floor of the rabbit. The fact that fatty marrow was formed might be because of the fatty marrow characteristic of the given animal. It is probable that the adipocyte cells were in a space where the general hematopoiesis function declined and therefore thrived when hematopoiesis cells decreased, and was not promoted by a physiological condition such as the addition of time. Conversely, when hematopoiesis cells increase, the necessary space for adipocyte cells is reduced (Tavasori & Crosby 1970, Tavassoli 1976a, 1976b). In this experiment, we observed new bone formation with newly abundant blood vessels in the early stages (2 and 4 weeks) in both groups (Figs 2 and 3), after which, the blood vessel space might be in the adipocyte cells. This stage may be a beneficial period to load cells compared with an earlier period prior to the remodeling of the bone. This procedure may be applicable to dental implants, for example, one-stage procedures that combine both dental implant installation and the bone graft, and in fact this method was successful in clinical situations (Ueda et al. 2003; Yamada et al. 2004a, 2004b), because new bone formation and active remodeling were observed when the bone was mechanically stimulated (Lanyon & Rubin 1984; Rubin & Lanyon 1987), such as by micromovement between 50 and 150 μm that starts bone formation (Szmukler-Moncler et al. 1998, 2000).

According to histomorphometric analysis, bone volume and augmented height showed peaks as early as 2 weeks and decreased over time after transplant in this study (Fig. 5a, b), whereas Wada et al. (2001) reported that newly formed bone
volume reaches a peak at 4 weeks and the volume showed the same value as our value at 8 weeks in transplanted PCBM without PRP to the same sinus floor elevation model. Thus, the early peak in the present study might be because of a bone-promoting effect by PRP, which is known to enhance the formation of new bone and accelerate wound healing (Marx et al. 1998). The use of PRP might provide optimal conditions for more rapid and effective bone regeneration of the grafted bone or MSCs. On the other hand, the bone absorption rates in MSCs/PRP or PCBM/PRP groups at 4 weeks from 2 weeks showed about 5% or 6.4%, respectively. This might be related to the process of bone formation such as the MSCs/PRP group induced bone tissue formation, which then MSCs self-organized according to the surrounding environment, although osteogenesis was induced from the surroundings of the grafted bone in the PCBM/PRP group.

In summary, the MSCs/PRP complex had well-formed newly formed bone and neovascularization, compared with the PCBM/PRP complex. The findings of this experimental study indicate that the use of a mixture of MSCs /PRP complex yielded good results in osteogenesis and bone volume comparable with that achieved by autogenous bone, and PCBM in sinus floor elevation. Therefore, this application of this promising new sinus floor elevation method for dental implants with tissue engineering technology deserves further study.

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Résumé

La possibilité de régénérer l’os dans l’épaisseur du sinus a été comparée entre une méthode de travail du tissu en utilisant des cellules souches primitives mesenchymateuses (MSC) et du plasma riche en plaquettes (PRP) et une nouvelle méthode utilisant de l’os cortical et spongieux (PCBM) et PRP. L’épaisseur bilatérale des sinus a été effectuée chez 18 lapins blancs japonais adultes. Les complexes MSC/PRP ou PCBM/PRP ont été placés dans chaque sinus de ces lapins. Les MSC étaient isolés à partir de la moelle de la crête iliaque du lapin et le PRP était obtenu du sang périphérique. Le PCBM étaient collectés de la crête iliaque du lapin et mélangés au PRP. Les animaux ont été sacrifiés deux, quatre et huit semaines après la transplantation et l’habilité à former de l’os de chaque implant a été évaluée tant histologiquement que histométriquement. Suivant les observations histologiques, les deux sites montrent une néo-osseosdefermentation après deux et quatre semaines. Cependant, après huit semaines, l’os lamellaire était occupé par une moelle grasse dans des grandes aires des deux sites. Il n’y avait aucune différence significative dans le volume osseux ou la hauteur d’épaissement entre les deux groupes, mais il y avait des différences significatives dans le volume osseux et la hauteur d’épaissement entre deux et huit semaines dans les deux groupes et dans le volume osseux entre les semaines 4 et 8 dans le groupe PCBM/PRP (p<0.05). Le complexe MSC/PRP peut aussi bien être utilisé pour la régénération osseuse dans l’épaisseur sinusale en comparaison avec le complexe PCBM/PRP.

Zusammenfassung


Resumen

En el presente estudio, hemos comparado la habilidad de regeneración en la elevación del seno maxilar entre un método de ingeniería tisular usando células madre mesenquimales (MSCs) y plasma rico en plaquetas (PRP), y un prometedor nuevo método usando hueso esponjoso particulado y médula (PCBM) y PRP. Se llevaron a cabo procedimientos de elevación del seno bilateralmente en 18 conejos blancos japoneses jóvenes. Se injertaron complejos MSCs/PRP o PCBM/PRP en cada seno maxilar en el mismo conejo. Se aislaron las MSCs de la médula de la cresta ilíaca del conejo, y el PRP se obtuvo de la sangre periférica. El PCBM se recolectó de la cresta ilíaca y se mezcló con el PRP. Los animales se sacrificaron a las 2, 4 y 8 semanas tras el trasplante, y se evaluó la habilidad de formación de hueso de cada implante histológicamente e histométricamente. De acuerdo con las observaciones histológicas, ambos lados (MSCs/PRP y PCBM/PRP) mostraron hueso neoformado y neovascularización a las semanas 2 y 4. De todos modos, a las 8 semanas, el hueso lamelar se observó ocupado por medula grasa en amplias áreas de ambos lados. No hubo una diferencia significativa en el volumen óseo en la altura aumentada entre los grupos MSCs/PRP y PCBM/PRP alrededor de cada semana, pero si hubo diferencias significativas en el volumen óseo y altura aumentada entre las semanas 2 y 8 en los grupos PCBM/PRP o MSCs/PRP y en el volumen óseo entre las semanas 4 y 8 en el grupo PCBM/PRP (p<0.05). Estos resultados sugieren que el complexe MSCs/PRP puede muy bien ser usado para regeneración ósea en elevación del seno, comparado con el complejo PCBM/PRP.

References


